

(86) Date de dépôt PCT/PCT Filing Date: 2011/06/02

(87) Date publication PCT/PCT Publication Date: 2011/12/08

(45) Date de délivrance/Issue Date: 2021/02/09

(85) Entrée phase nationale/National Entry: 2012/11/28

(86) N° demande PCT/PCT Application No.: US 2011/038889

(87) N° publication PCT/PCT Publication No.: 2011/153323

(30) **Priorités/Priorities:** 2010/06/02 (US61/350,829);
2010/08/06 (US61/371,481); 2010/10/29 (US61/408,271)

(51) Cl.Int./Int.Cl. *C12N 15/113*(2010.01),
A61K 31/713(2006.01), *A61P 1/16*(2006.01),
C07H 21/02(2006.01), *C12N 15/12*(2006.01),
C12N 15/18(2006.01)

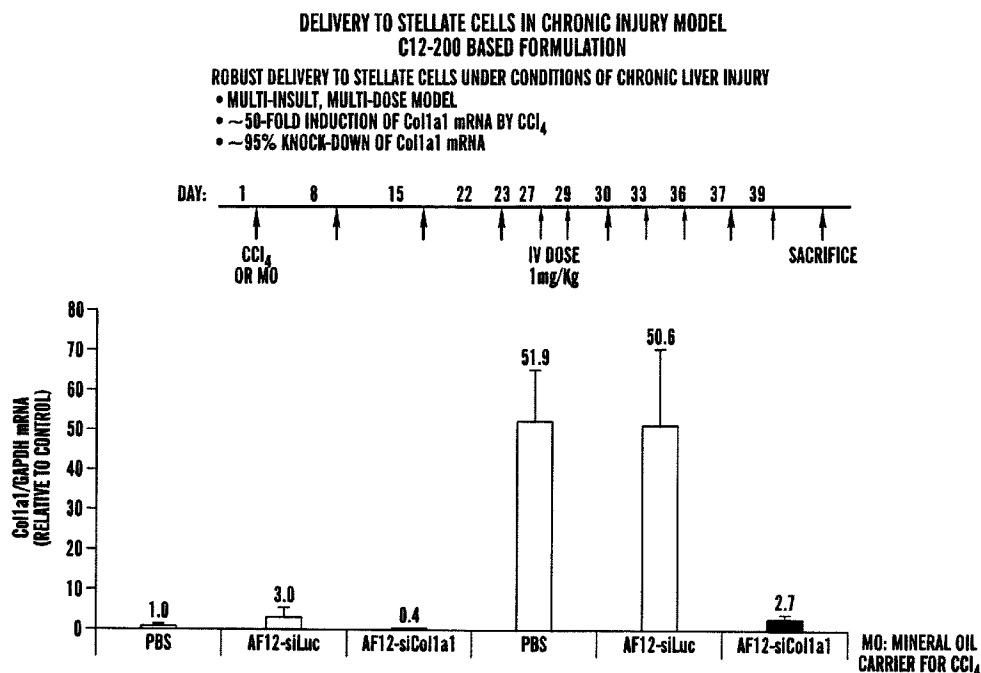
(72) **Inventeurs/Inventors:**
HINKLE, GREGORY, US;
KOTELIANSKI, VICTOR, US;
BETTENCOURT, BRIAN, US;
SEHGAL, ALFICA, US;
NOVOBRANTSEVA, TATIANA, US

(73) Propriétaire/Owner:
ALNYLAM PHARMACEUTICALS, INC., US

(74) Agent: GOWLING WLG (CANADA) LLP

(54) Titre : COMPOSITIONS ET METHODES POUR TRAITER UNE FIBROSE HEPATIQUE

(54) Title: COMPOSITIONS AND METHODS DIRECTED TO TREATING LIVER FIBROSIS



(57) **Abrégé/Abstract:**

The invention relates to double-stranded ribonucleic acid (dsRNA) compositions targeting the COL1A1, TGF- β , and SMAD2/3 genes, and methods of using such dsRNA compositions to inhibit expression of COL1A1, TGF- β , and SMAD2/3.

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

CORRECTED VERSION

(19) World Intellectual Property
Organization
International Bureau



(10) International Publication Number
WO 2011/153323 A9

(43) International Publication Date
8 December 2011 (08.12.2011)

- (51) International Patent Classification:
C07H 21/00 (2006.01) *A61K 31/7088* (2006.01)
C12N 15/113 (2010.01)
- (21) International Application Number:
PCT/US2011/038889
- (22) International Filing Date:
2 June 2011 (02.06.2011)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
61/350,829 2 June 2010 (02.06.2010) US
61/371,481 6 August 2010 (06.08.2010) US
61/408,271 29 October 2010 (29.10.2010) US
- (71) Applicant (for all designated States except US):
ALNYLAM PHARMACEUTICALS, INC. [—/US]; 300
Third Street, Third Floor, Cambridge, MA 02142 (US).

Third Floor, Cambridge, MA 02142 (US). **SEHGAL, Alfica** [IN/US]; 300 Third Street, Third Floor, Cambridge, MA 02142 (US). **NOVOBRANTSEVA, Tatiana** [US/US]; 300 Third Street, Third Floor, Cambridge, MA 02142 (US).

(74) Agents: **RESNICK, David** et al.; Nixon Peabody LLP, 100 Summer Street, Boston, MA 02110 (US).

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

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,

[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS DIRECTED TO TREATING LIVER FIBROSIS

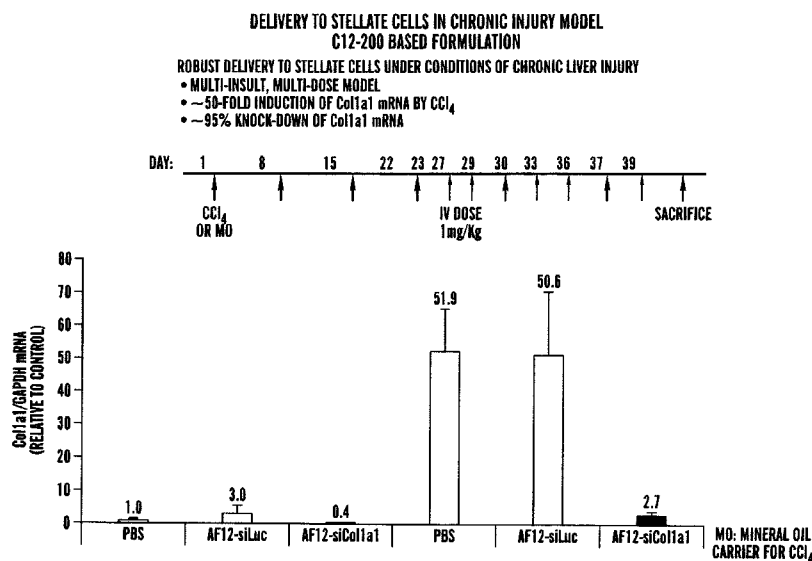


FIG. 13

(57) Abstract: The invention relates to double-stranded ribonucleic acid (dsRNA) compositions targeting the COL1A1, TGF- β , and SMAD2/3 genes, and methods of using such dsRNA compositions to inhibit expression of COL1A1, TGF- β , and SMAD2/3.

WO 2011/153323 A9

MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, ML, MR, NE, SN, TD, TG).

(88) Date of publication of the international search report:
22 March 2012

(48) Date of publication of this corrected version:
24 May 2012

Declarations under Rule 4.17:

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

(15) Information about Corrections:
see Notice of 24 May 2012

Previous Correction:
see Notice of 12 April 2012

Published:

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

COMPOSITIONS AND METHODS DIRECTED TO TREATING LIVER FIBROSIS

SEQUENCE LISTING

[0001] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web. Said ASCII copy, created on June 2, 2011, is named 51058PCT.txt and is 864,110 bytes in size.

FIELD OF THE INVENTION

[0002] The invention relates to the specific inhibition of the expression of the collagen 1A1 (COL1A1), transforming growth factor beta 1 (TGF β) and SMAD homolog 2 and 3 (SMAD2/3) genes in the liver, and the inhibition of hepatic fibrosis. In particular, the inhibition is directed at the genes expression in hepatic stellate cells.

BACKGROUND OF THE INVENTION

[0003] In the United States, chronic liver disease is the 12th leading cause of disease-related death, with approximately 28,000 people dying each year from chronic liver disease. In the US and Europe, liver cirrhosis is the most common cause of non-neoplastic death among hepatobiliary and digestive diseases. Furthermore, the fourth leading cause of death in urban American males is alcoholic liver disease. Chronic liver disease is characterized by a process of progressive destruction and regeneration of liver cells that leads to hepatic fibrosis and cirrhosis.

[0004] Hepatic fibrosis is a process that occurs when the liver is damaged. Such damage can be the result of viral activity (*e.g.*, chronic hepatitis types B or C) or other liver infections (*e.g.*, parasites, bacteria); exposure to chemicals (*e.g.*, pharmaceuticals, recreational drugs, excessive alcohol, exposure to pollutants); autoimmune processes (*e.g.*, autoimmune hepatitis); metabolic disorders (*e.g.*, lipid, glycogen, cholesterol or metal storage disorders); or cancer growth (primary or secondary liver cancer).

[0005] The principal cell type responsible for liver fibrosis is the hepatic stellate cell (HSC), a resident perisinusoidal cell that takes up vitamin A from circulation and stores it.

[0006] In the liver, fibrosis is not only the result of necrosis, collapse and scar formation but also the result of derangements in the synthesis and degradation of matrix by injured mesenchymal cells that synthesize the various components of the extracellular matrix (ECM). Hepatic fibrosis is a gradual process of increased secretion and decreased degradation of extracellular materials. It is believed that the process is initiated by damage to hepatic cells. Necrotic hepatic cells present after injury evoke a reaction

of inflammatory cells which secrete mediators including cytokines that stimulate matrix producing cells. More particularly, the damage to hepatic cells leads to the activation and secretion of multiple cellular factors from Kupffer cells, the macrophages which line the liver sinusoids. These factors, along with the cellular factors secreted by damaged hepatic cells, thrombocytes, and endothelial cells of the hepatic sinusoid become activators of HSCs. Upon activation, the HSCs differentiate to proliferative, fibrogenic and contractile myofibroblasts, and, via self-secretion and parasecretion, the HSCs proliferate and synthesize a massive amount of ECM materials, which gradually accumulate and form fibrous masses in the liver. The myofibroblasts secrete procollagen, which accumulates as insoluble collagen after its terminal domains are cleaved by procollagen peptides, causing fibrosis. The collagen-specific chaperone, heat shock protein 47 (HSP47), facilitates collagen secretion by ensuring proper triple-helix formation of procollagen in the endoplasmic reticulum and has also been implicated in translational regulation of procollagen synthesis.

[0007] The ECM materials are of the following categories: collagens (type I, III, IV, V, VI, VII), glycoprotein (laminin, fibronectin, entactin, undulin, and elastin) and proteoglycans (chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate and heparin). These ECM materials interact with cell surface receptors and macromolecules such as growth factors, collagens, fibronectin, laminin etc. Fibrosis develops after repeated and persistent injury that overcomes the ability of the liver to degrade the ECM matrix via degrading enzymes produced by fibroblasts, neutrophils and macrophages. The excessive ECM materials built up in the liver lead to derangement of the hepatic architecture and portal hypertension, and can produce the irreversible and detrimental rearrangement of the liver circulation known as cirrhosis. Cirrhosis is characterized by replacement of liver tissue by fibrous, scar tissue and regenerative nodules. These regenerative nodules are the result of a process in which damaged tissue tries to regenerate and this leads to the loss of liver function. Therefore, fibrosis is both a sign of liver damage and a major contributor to liver failure via progressive cirrhosis of the liver. Since fibrosis is a common development in a variety of chronic liver diseases, treatment and/or prevention of liver fibrosis is of great importance.

SUMMARY OF THE INVENTION

[0008] Described herein are compositions and methods that effect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of a collagen 1A1 (COL1A1), transforming growth factor beta 1 (TGF β) and/or SMAD homolog 2 and 3 (SMAD2/3) genes, in a cell or mammal. Also described are compositions and methods for treating and/or preventing liver fibrosis caused by the overexpression of a COL1A1 gene that occurs as a result of damage to liver cells, including, but not limited to, chronic liver disease. Liver fibrosis is a result of a viral or other infection, an autoimmune disorder, a bile duct obstruction, metabolic disorders, alcohol abuse, primary biliary cirrhosis, non-alcoholic steatohepatitis (NASH), exposure to chemicals, and cancer among others.

[0009] The embodiments of compositions and methods for treating and/or preventing chronic liver disease and hepatic fibrosis are based, at least in part, on the premise that if at least one of the

extracellular matrix (ECM) materials that are produced in excess and are contributory to fibrosis during liver damage, including damage occurring under chronic liver injury conditions, can be reduced, then fibrosis in the liver can be, at a minimum, reduced if not prevented. The compositions and methods are based on RNA interference of gene expression of relevant ECM genes and also of the signaling molecules, e.g., TGF- β and SMAD2/3, which are involved in the activation of hepatic stellate cells (HSCs), which are the major producers of ECM materials during the fibrosis process.

[0010] As used herein, the term “iRNA” refers to an agent that contains RNA as that term is defined herein, and which mediates the targeted cleavage of an RNA transcript via an RNA-induced silencing complex (RISC) pathway. In one embodiment, an iRNA as described herein inhibits COL1A1, TGF β or SMAD2/3 gene expression in a cell or mammal.

[0011] The iRNAs included in the compositions featured herein 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 a COL1A1, TGF β or SMAD2/3 gene.

[0012] In one embodiment, an iRNA for inhibiting expression of a COL1A1, TGF β or SMAD2/3 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 COL1A1, TGF β or SMAD2/3 and the region of complementarity is 30 nucleotides or less, and at least 15 nucleotides in length. Generally, the iRNA is 19 to 24, e.g., 19 to 21 nucleotides in length. In some embodiments the iRNA is from about 15 to about 25 nucleotides in length, and in other embodiments the iRNA is from about 25 to about 30 nucleotides in length. The iRNA, upon contacting with a cell expressing COL1A1, TGF β and/or SMAD2/3, inhibits the expression of a COL1A1, TGF β or SMAD2/3 gene respectively 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 COL1A1iRNA, TGF β iRNA or SMAD2/3iRNA is formulated in a stable nucleic acid lipid particle (SNALP).

[0013] In one embodiment, an iRNA featured herein includes a first sequence of a dsRNA that is selected from the group consisting of the sense sequences of Tables 3-7 and a second sequence that is selected from the group consisting of the corresponding antisense sequences of Tables 3-7. The iRNA molecules featured herein can include naturally occurring nucleotides or can include at least one modified nucleotide, including, but not limited to a 2'-O-methyl modified nucleotide, a nucleotide having a 5'-phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative. Alternatively, the modified nucleotide can be chosen from the group of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide. Generally, such a modified sequence will be based on a first sequence of iRNA selected from

the group consisting of the sense sequences of Tables 3-7, and a second sequence selected from the group consisting of the corresponding antisense sequences of Tables 3-7.

[0014] In one embodiment, an iRNA as described herein targets a COL1A1 RNA transcript, and in another embodiment, the iRNA targets a TGF β RNA transcript. In yet another embodiment, the iRNA targets a SMAD2 transcript. In yet another embodiment, the iRNA targets a SMAD3 transcript.

[0015] In some embodiments of the aspects described herein, the iRNA is a dsRNA which comprises the RNA sequence pair (AD-21349-b1) of SEQ ID NO: 53 (41100-b1D) and SEQ ID NO: 54 (41101-b1D), the RNA sequence pair (AD-22138) of SEQ ID NO: 69 (42861) and SEQ ID NO: 70 (42862), the RNA sequence pair (AD-22149) of SEQ ID NO: 91 (42883) and SEQ ID NO: 92 (42884) or the RNA sequence pair (AD-20916-b1) of SEQ ID NO: 140 (40322-b1) and SEQ ID NO: 141 (40323-b1).

[0016] In one embodiment, an iRNA featured in the invention targets a non-coding region of a COL1A1, TGF β or SMAD2/3 RNA transcript, such as the 5' or 3' untranslated region.

[0017] In one aspect, embodiments of the invention provide a cell containing at least one of the iRNAs featured in the invention. The cell is generally a mammalian cell, such as a human cell. In one preferred embodiment, the cell is a hepatic cell. In a more preferred embodiment, the cell is a HSC.

[0018] In another aspect, embodiments of the invention provide a pharmaceutical composition for inhibiting the expression of a COL1A1, TGF β and/or SMAD2/3 gene in an organism, preferably a human subject. The composition typically includes one or more of the iRNAs described herein and a pharmaceutically acceptable carrier or delivery vehicle. In one embodiment, the composition is used for treating liver disease, including, but not limited to, a chronic liver disease. In one embodiment, the composition is used for treating hepatic fibrosis. In some embodiments, the liver disease or hepatic fibrosis is a result of a viral or other infection, an autoimmune disorder, a bile duct obstruction, metabolic disorders, alcohol abuse, primary biliary cirrhosis, NASH, exposure to chemicals, or cancer.

[0019] In another embodiment, the pharmaceutical composition is formulated for administration in 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 five years, or ten years, or longer, including the remaining lifetime of a subject, *e.g.*, for a subject having a chronic liver disease or condition.

[0020] In another embodiment, a composition containing an iRNA featured described herein, *e.g.*, a dsRNA targeting COL1A1, is administered with a non-iRNA therapeutic agent, such as an agent known to treat the underlying cause of the liver disease or hepatic fibrosis, *e.g.*, a viral or other infection, an autoimmune disorder, a bile duct obstruction, metabolic disorders, alcohol abuse, primary biliary cirrhosis, NASH, exposure to chemicals, and cancer. In another embodiment, a composition containing an iRNA featured described herein, *e.g.*, a dsRNA targeting COL1A1, is administered along with a non-iRNA therapeutic regimen, such as an anti-hepatitis C virus agent. For example, an iRNA featured in the invention can be administered along with an interferon alpha for treatment of hepatitis C infection.

[0021] In another embodiment, a COL1A1 iRNA, TGF β iRNA and/or SMAD2/3 iRNA is administered to a patient, and then the non-iRNA agent or therapeutic regimen is administered to the patient (or vice versa). In another embodiment, a COL1A1 iRNA, TGF β iRNA and/or SMAD2/3 iRNA and the non-iRNA therapeutic agent or therapeutic regimen are administered at the same time. In one embodiment, the therapeutic agent is, for example, an interferon alpha for the treatment of hepatitis C infection. In another embodiment, a combination of more than one iRNA is administered to the patient. For example, a combination of COL1A1 iRNA and TGF β iRNA, a combination of COL1A1 iRNA and SMAD2/3 iRNA or a combination of COL1A1 iRNA, TGF β iRNA and SMAD2/3 iRNA.

[0022] In another aspect, provided herein is a method for inhibiting the expression of a COL1A1, a TGF β , and/or a SMAD2/3 gene in a cell by performing the following steps:

- (a) introducing into the cell a double-stranded ribonucleic acid (dsRNA), wherein the dsRNA includes at least two sequences that are complementary to each other. The dsRNA has a sense strand having a first sequence and an antisense strand having a second sequence; the antisense strand has a region of complementarity that is substantially complementary to at least a part of an mRNA encoding COL1A1, TGF β , and/or a SMAD2/3 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 COL1A1, TGF β , and/or a SMAD2/3 inhibits expression of a COL1A1, TGF β , or SMAD2/3 gene respectively by at least 10%, preferably at least 20%, at least 30%, at least 40% or more; and
- (b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of the COL1A1, TGF β , or SMAD2/3 gene, thereby inhibiting expression of a COL1A1, TGF β , or SMAD2/3 gene respectively in the cell.

[0023] In another embodiment, the method is for inhibiting target gene expression in a liver cell, preferably in a hepatic stellate cell.

[0024] In other aspects, provided herein are methods for treating, preventing, reversing or managing liver disease or hepatic fibrosis mediated by, in part, COL1A1 overexpression. In one embodiment, the method includes administering to a patient in need of such treatment, prevention, reversal or management a therapeutically or prophylactically effective amount of one or more of the iRNAs featured herein. In some embodiments, the patient has a chronic liver disease caused by a viral or other infection, an autoimmune disorder, a bile duct obstruction, metabolic disorders, alcohol abuse, primary biliary cirrhosis, NASH, exposure to chemicals, or cancer. In another embodiment, administration of the iRNA targeting COL1A1, TGF β , or SMAD2/3 alleviates or relieves the severity of at least one symptom of a liver disease or hepatic fibrosis, in the patient, such as high serum albumin and/or jaundice.

[0025] In one aspect, the invention provides a vector for inhibiting the expression of a COL1A1, TGF β , and/or a SMAD2/3 gene in a cell. In one embodiment, the vector includes at least one regulatory

sequence operably linked to a nucleotide sequence that encodes at least one strand of an iRNA as described herein.

[0026] In another aspect, the invention provides a cell containing a vector for inhibiting the expression of a COL1A1, TGF β , and/or a SMAD2/3 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.

[0027] In yet another aspect, the invention provides a composition containing a COL1A1iRNA, in combination with a second iRNA targeting a second gene that is involved in signaling pathway leading to the excess deposition of collagen extracellularly in the liver. For example, the second gene can be the gene encoding TGF β , SMAD2 or SMAD3.

[0028] 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.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] **FIGURE 1** shows the efficacy of single dose collagen1A1 siRNA in NIH-3T3 cells *in vitro*. siRNA AD-21349 (SEQ ID NO: 53 and SEQ ID NO: 54), has an IC₅₀ of ~65 pM on the production of COL1A1 mRNA.

[0030] **FIGURE 2** demonstrates a treatment regimen of a CCl₄ mouse model in which the AF09 and AF12-based delivery of siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54) was evaluated.

[0031] **FIGURE 3** demonstrates efficacy of COL1A1 knock-down in a CCl₄ mouse model using AF09 and AF12-based delivery of siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54).

[0032] **FIGURE 4** demonstrates a treatment regimen of a CCl₄ mouse model in which the dosage response of the AF09 and AF12-based delivery of siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54) was evaluated.

[0033] **FIGURE 5** demonstrates dose dependent efficacy of COL1A1 knock-down in a CCl₄ mouse model using the AF12-based delivery of siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54).

[0034] **FIGURE 6** shows a treatment regimen of a CCl₄ mouse model in which AF11-based delivery of siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54) was evaluated.

[0035] **FIGURE 7** demonstrates efficacy of COL1A1 knock-down in a CCl₄ mouse model using AF11-based delivery of siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54).

[0036] **FIGURE 8** demonstrates efficacy of ACTA2 knock-down in a CCl₄ mouse model using AF12-based delivery of siACTA2.

[0037] **FIGURE 9** shows efficacy of a single dose TGF β siRNA (10nM) in NIH-3T3 cells *in vitro*. The TGF β siRNAs AD22149 (SEQ ID NO: 91 and SEQ ID NO: 92) and AD22138 (SEQ ID NO: 69 and SEQ ID NO: 70) have an IC₅₀ of ~20 pM and ~70 pM respectively on TGF β expression.

[0038] **FIGURE 10** shows efficacy of a single dose SMAD2 siRNA (10nM) in NIH-3T3 cells *in vitro*. The siRNA AD-20916 (SEQ ID NO: 140 and SEQ ID NO: 141) has an IC₅₀ of ~75 pM on SMAD2 expression.

[0039] **FIGURE 11** shows efficacy of SMAD2 knock-down in a CCl₄ mouse model using lipid nanoparticle-based delivery of siSMAD2 AD-20916 (SEQ ID NO: 140 and SEQ ID NO: 141).

[0040] **FIGURE 12** shows effects of SMAD2 knock-down on the expression of COL1A1 in a CCl₄ mouse model using lipid nanoparticle-based delivery of siSMAD2 AD-20916 (SEQ ID NO: 140 and SEQ ID NO: 141). SMAD2/3 siRNA leads to decrease in Col1a1 levels (~50-60% from Luc siRNA injected animals) and the data demonstrates that SMADs are involved in TGF β signaling in fibrosis development

[0041] **FIGURE 13** shows efficacy of COL1A1 knockdown in a chronic liver injury model using a lipid nanoparticle-based formulation of siCOL1A1.

[0042] **FIGURE 14** shows efficacy of delivery to stellate cells with alpha-smooth muscle actin (α -SMA) in a CCl₄ mouse model using AF12-based delivery of siACTA2.

[0043] **FIGURES 15A-15C** demonstrate Col1a1 knockdown is mediated by RNAi. **FIGURE 15A** shows a scheme for 5'RACE assay. **FIGURE 15B** shows animals injected with a lipid nanoparticle-siCol1a1 formulation and sacrificed after 24 hours. **FIGURE 15C** shows an agarose gel showing DNA bands for nested PCR.

[0044] **FIGURE 16** demonstrates experimental design and efficacy of COL1A1 knock-down in a bile duct ligation mouse model using AF12-based delivery of siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54).

[0045] **FIGURE 17** shows relative Col1a1 expression in bile duct ligated animals.

[0046] **FIGURE 18** demonstrates efficacy of COL1A1 knock-down in a bile duct ligation mouse model using siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54).

[0047] **FIGURE 19** demonstrates experimental design and efficacy of COL1A1 knock-down in a TAA mouse model of liver injury using siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54).

DETAILED DESCRIPTION OF THE INVENTION

[0048] Aspects and embodiments described herein relate to compositions and methods for the treatment and/or prevention of liver disease and hepatic fibrosis, including, but not limited to, hepatic fibrosis associated with chronic liver disease. The embodiments exploit the premise that if at least one of the extracellular matrix (ECM) materials that are produced in excess and are contributory to hepatic fibrosis during liver damage can be reduced, then fibrosis of the liver can be, at a minimum, reduced, if not prevented. The compositions and methods described herein are based on RNA interference with gene expression of relevant ECM genes and also those of signaling molecules, e. g. TGF- β and SMAD2/3, that are involved in the activation of hepatic stellate cells (HSCs) during the fibrotic process.

[0049] Double-stranded RNA molecules (dsRNA) have been shown to block gene expression in a highly conserved regulatory mechanism known as RNA interference (RNAi). WO 99/32619 (Fire *et al.*)

disclosed the use of a dsRNA of at least 25 nucleotides in length to inhibit the expression of genes in *Caenorhabditis elegans*. dsRNA has also been shown to degrade target RNA in other organisms, including plants (see, *e.g.*, WO 99/53050, Waterhouse *et al.*; and WO 99/61631, Heifetz *et al.*), *Drosophila* (see, *e.g.*, Yang, D., *et al.*, Curr. Biol. (2000) 10:1191-1200), and mammals (see WO 00/44895, Limmer; and DE 101 00 586.5, Kreutzer *et al.*). This natural mechanism has now become the focus for the development of a new class of pharmaceutical agents for treating disorders that are caused by the aberrant or unwanted regulation of a gene.

[0050] One such ECM constituent that contributes to fibrosis in the liver, during chronic liver disease conditions, is collagen 1A1 (COL1A1). As described herein, the inventors show that lipid nanoparticle (LNP) mediated delivery of siRNAs to HSCs *in vivo* significantly reduced COL1A1 expression in activated HSCs in a carbon tetrachloride (CCl₄) induced mouse liver injury model (FIGURES 2-3). Following CCl₄ treatment, siRNAs formulated in LNPs comprising the cationic lipid C12-200 (PNAS, 2010, 107(2):1864) were administered via intravenous injection. Silencing of the expression of the HSC-specific target, COL1A1, demonstrated effective siRNA delivery to HSCs. The COL1A1 knock-down was dose dependent with an IC₅₀ of approximately 0.1 mg/kg (FIGURES 4-5). The inventors further surprisingly showed that effective and significant reduction of COL1A1 expression can also be achieved in a mouse model of chronic liver disease, in which mice were subjected to multiple (X6) liver insults over a 39 day period, using the COL1A1 siRNA formulations described herein (FIGURE 13).

[0051] Signaling molecules involved in the activation of HSCs during the fibrotic process include, but are not limited to, TGF- β and SMAD2/3. The inventors demonstrate that LNP-mediated delivery of siRNAs targeting specifically TGF- β and SMAD2/3 significantly reduced the expression of TGF- β and SMAD2/3 *in vitro* (FIGURES 9-10). The inventors further demonstrate that LNP-mediated delivery of siRNAs targeting specifically SMAD2/3 in the liver significantly reduced the expression of SMAD2/3 and COL1A1 in the CCl₄ induced mouse liver injury model (FIGURES 11-12).

[0052] Fibrosis is a common pathological feature of many chronic organ failure diseases, including chronic liver disease. Necrotic cells after injury evoke reaction of inflammatory cells which secrete mediators such as cytokines which stimulate matrix producing cells. Essentially, damaged liver cells activate HSCs that secrete materials (*e.g.*, collagens) into the matrix outside the cells. Inappropriate collagen deposition in the ECM is the hallmark of fibrosis and it occurs in the late stage of pathogenesis. Stopping fibrosis can stabilize liver function, delaying time to transplant or providing a survival benefit.

[0053] The biology of fibrosis is very well understood. HSC play a central role in the pathogenesis of liver fibrosis, transdifferentiating in chronic liver disease from “quiescent” HSC to fibrogenic myofibroblasts. It is a TGF- β driven process, and SMAD2 and SMAD3 function as TGF β signaling intermediates. TGF- β , acting both directly and indirectly, is a critical mediator of this process. One approach of treating and/or preventing liver fibrosis is to inhibit HSC proliferation and/or promote HSC apoptosis. Another is to inhibit the HSCs from producing and secreting the ECM components such as collagen to the hepatic ECM that forms the fibrotic mass. COL1A1 is normally present at very low levels

in liver but is up-regulated and produced by activated HSCs after insult to the liver. RNAi inhibition of expression of COL1A1, TGF β , TGF β -signaling intermediates, such as SMAD2 and SMAD 3, and other proteins/factors that contribute to COL1A1 overexpression can be clinically relevant in treating hepatic fibrosis, regardless of etiology.

[0054] Accordingly, aspects and embodiments described herein include iRNAs and methods of using them for inhibiting the expression of a COL1A1, TGF β and/or SMAD2/3 gene in a cell or a mammal where the iRNA targets a COL1A1, TGF β and/or SMAD2/3 gene respectively. Also provided herein are compositions and methods for treating and/or preventing liver fibrosis by inhibiting the expression of a COL1A1, TGF β and/or SMAD2/3 gene. iRNA directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi).

[0055] In one embodiment of the aspects, provided herein is a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of COL1A1, wherein the dsRNA comprises a sense strand and an antisense strand, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO: 53 and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the corresponding antisense nucleotide sequence of SEQ ID NO: 54.

[0056] In another embodiment of the aspects, provided herein is a dsRNA for inhibiting expression of TGF β , wherein the dsRNA comprises a sense strand and an antisense strand, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO: 69 and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the corresponding antisense nucleotide sequence of SEQ ID NO: 70.

[0057] In another embodiment of the aspects, provided herein is a dsRNA for inhibiting expression of TGF β , wherein the dsRNA comprises a sense strand and an antisense strand, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO: 91 and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the corresponding antisense nucleotide sequence of SEQ ID NO: 92.

[0058] In another embodiment of the aspects, provided herein is a dsRNA for inhibiting expression of SMAD2/3, wherein the dsRNA comprises a sense strand and an antisense strand, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO: 140 and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the corresponding antisense nucleotide sequence of SEQ ID NO: 141.

[0059] In some embodiments of the aspects described herein, the iRNA is a dsRNA which comprises the RNA sequence pair (AD-21349-b1) of SEQ ID NO: 53 (41100-b1D) and SEQ ID NO: 54 (41101-b1D), the RNA sequence pair (AD-22138) of SEQ ID NO: 69 (42861) and SEQ ID NO: 70

(42862), the RNA sequence pair (AD-22149) of SEQ ID NO: 91 (42883) and SEQ ID NO: 92 (42884) and/or the RNA sequence pair (AD-20916-b1) of SEQ ID NO: 140 (40322-b1) and SEQ ID NO: 141 (40323-b1).

[0060] In some embodiments, the sequences for the iRNA comprise sequences identified by the sequence identifiers found in Tables 3-7. Tables 3-7 comprise sequence identifiers (SEQ ID NOs) for sense and corresponding antisense strands. Each row of Table 3 (SEQ ID NO: 1-SEQ ID NO: 62) identifies two separate iRNAs, *i.e.*, two separate pairs of sense strands (odd number sequence identifiers of SEQ ID NO: 1-SEQ ID NO: 62) and corresponding antisense strands (even number sequence identifiers of SEQ ID NO: 1-SEQ ID NO: 62), targeting COL1A1 for use in the compositions and methods described herein. Similarly, each row of Table 4 (SEQ ID NO: 63-SEQ ID NO: 130) identifies two separate iRNAs, *i.e.*, two separate pairs of sense strands (odd number sequence identifiers of SEQ ID NO: 63-SEQ ID NO: 130) and corresponding antisense strands (even number sequence identifiers of SEQ ID NO: 63-SEQ ID NO: 130), targeting TGF- β for use in the compositions and methods described herein. Each row of Table 5 (SEQ ID NO: 132-SEQ ID NO: 167) identifies two separate iRNAs, *i.e.*, two separate pairs of sense strands (even number sequence identifiers of SEQ ID NO: 132-SEQ ID NO: 167) and corresponding antisense strands (odd number sequence identifiers of SEQ ID NO: 132-SEQ ID NO: 167), targeting SMAD2/3 for use in the compositions and methods described herein. Each row of Table 6 (SEQ ID NO: 185-SEQ ID NO: 2792) identifies two separate iRNAs, *i.e.*, two separate pairs of sense strands (odd number sequence identifiers of SEQ ID NO: 185-SEQ ID NO: 2792) and corresponding antisense strands (even number sequence identifiers of SEQ ID NO: 185-SEQ ID NO: 2792) targeting COL1A1 for use in the compositions and methods described herein. Each row of Table 7 (SEQ ID NO: 2793-SEQ ID NO: 2860) identifies two separate iRNAs, *i.e.*, two separate pairs of sense strands (even number sequence identifiers of SEQ ID NO: 2793-SEQ ID NO: 2860) and corresponding antisense strands (odd number sequence identifiers of SEQ ID NO: 2793-SEQ ID NO: 2860) targeting SMAD2/3 for use in the compositions and methods described herein. Accordingly, in some embodiments, the iRNA is a dsRNA which comprises an RNA sequence pair (“sense and corresponding antisense duplex”) or “sense strand and corresponding antisense strand” as identified by sequence identifiers (SEQ ID NO) listed in Tables 3-7 and provided herein.

[0061] The iRNAs of the compositions featured herein comprise an RNA strand (the antisense strand) having a region which is 30 nucleotides or less in length, *i.e.*, 15-30 nucleotides in length, generally 19-24 nucleotides in length, which region is substantially complementary to at least part of an mRNA transcript of a COL1A1, TGF β and/or SMAD2/3 gene. The use of these iRNAs enables the targeted degradation of mRNAs of genes that are implicated in the fibrotic process associated with COL1A1 expression or overexpression in the liver. Very low dosages of COL1A1, TGF β and/or SMAD2/3 iRNAs in particular can specifically and efficiently mediate RNAi, resulting in significant inhibition of expression of a COL1A1, TGF β and/or SMAD2/3 gene respectively. Using cell-based assays and *in vivo* model systems, the present inventors have demonstrated that iRNAs targeting

COL1A1, TGF β and/or SMAD2/3 can specifically and efficiently mediate RNAi, resulting in significant inhibition of expression of a respective gene. The inventors have also surprisingly discovered that iRNAs targeting COL1A1 can be used to mediate significant reduction of COL1A1 expression under conditions of repeated or chronic liver injury. Also the inventors demonstrate that SMAD2/3 RNAi mediated inhibition of SMAD2/3 expression indirectly led to the inhibition of COL1A1 expression. Thus, methods and compositions including these iRNAs are useful for treating and/or preventing liver disease and/or liver fibrosis by directly or indirectly down regulating COL1A1 production. The following detailed description discloses how to make and use compositions containing iRNAs to inhibit the expression of a COL1A1, TGF β and/or SMAD2/3 gene, as well as compositions and methods for treating and/or preventing, and possibly reversing, liver disease characterized by liver fibrosis caused by the expression or overexpression of COL1A1.

[0062] Embodiments of compositions featured herein also include an iRNA having an antisense strand having a region of complementarity which is 30 nucleotides or less in length, generally 19-24 nucleotides in length, and is substantially complementary to at least part of an RNA transcript of a COL1A1, TGF β or SMAD2/3 gene.

[0063] Embodiments of the pharmaceutical compositions featured herein include an iRNA having an antisense strand comprising a region which is 30 nucleotides or less in length, generally 19-24 nucleotides in length, which region is substantially complementary to at least part of an RNA transcript of a COL1A1, TGF β and/or SMAD2/3 gene, together with a pharmaceutically acceptable carrier.

[0064] Accordingly, in some aspects, pharmaceutical compositions containing a COL1A1 iRNA, a TGF β iRNA and/or a SMAD2/3 iRNA and a pharmaceutically acceptable carrier, methods of using the compositions to inhibit expression of a COL1A1, a TGF β , a SMAD2 and/or a SMAD3 gene, and methods of using the pharmaceutical compositions to treat and/or prevent possibly reverse liver fibrosis caused by the overexpression of a COL1A1 gene are featured in the invention.

[0065] In one embodiment, the pharmaceutical composition comprises more than one iRNA type, i. e. iRNAs targeting more than one gene target. For example, the pharmaceutical composition comprises a combination of COL1A1 iRNA and TGF β iRNA, a combination of COL1A1 iRNA and SMAD2/3 iRNA or a combination of COL1A1 iRNA, TGF β iRNA and SMAD2/3 iRNA.

[0066] In one embodiment, the iRNA is delivered using a vitamin-A-coupled liposome. The HSCs in the liver take up vitamin A from circulation and store it. The Vitamin-A-coupled liposomes function to specifically target the iRNA to the Vitamin A-sequestering HSC.

[0067] In one embodiment, the lipid nanoparticle (LNP)-formulation for delivery of iRNA described herein are coupled to vitamin A in order to target the LNP-encapsulated iRNA to the vitamin A-sequestering HSC.

[0068] In some embodiments, the pharmaceutical compositions and/or treatment therapies described herein further comprise an additional iRNA targeting a collagen-specific chaperone, heat shock protein 47 (HNP47). Sato, Y. *et al.*, (2008, Nature Biotechnology, 26:431-442) showed that inhibiting expression

of HSP47 with an siRNA-bearing vitamin A-coupled liposome almost completely resolved liver fibrosis and prolonged survival in rat treated with lethal dimethylnitrosamine.

I. Definitions

[0069] 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.

[0070] As used herein, the terms “chronic liver disease” or “chronic liver condition” refer to those diseases in which the liver is subjected to repeated insults and/or injuries over a long-term period, resulting in a process of progressive destruction and regeneration of the liver parenchyma leading to fibrosis and cirrhosis. Chronic liver disease can occur as a consequence of, or is associated with, any of a variety of etiologies, including, but not limited to, viral causes (*e.g.*, Hepatitis B, Hepatitis C, Cytomegalovirus (CMV), Epstein Barr Virus (EBV)); repeated use of toxic substances and/or drugs (*e.g.*, alcoholic liver disease, amiodarone, methotrexate, nitrofurantoin); metabolic/genetic causes/conditions (*e.g.*, non-alcoholic fatty liver disease, haemochromatosis, Wilson's disease); and autoimmune disorders (*e.g.*, autoimmune chronic hepatitis; primary biliary cirrhosis; primary sclerosing cholangitis). A liver disease or condition is considered to be ‘chronic,’ as used herein, when the condition lasts or continues for at least one month, though, in some preferred embodiments, ‘chronic’ can be defined as a period of 12 weeks or greater (*e.g.*, such as the remaining lifetime of a subject).

[0071] As used herein, the term “fibrosis” refers to the formation of fibrous tissue as a reparative or reactive process, rather than as a normal constituent of an organ or tissue. Fibrosis is characterized by fibroblast accumulation and collagen deposition in excess of normal deposition in any particular tissue. Fibrosis occurs as the result of inflammation, irritation, or healing. As used herein the term “fibrosis” is used synonymously with “fibroblast accumulation and collagen deposition”. Fibroblasts are connective tissue cells, which are dispersed in connective tissue throughout the body. Fibroblasts secrete a nonrigid ECM containing type I and/or type III collagen. In response to an injury to a tissue, nearby fibroblasts migrate into the wound, proliferate, and produce large amounts of collagenous extracellular matrix. Collagen is a fibrous protein rich in glycine and proline that is a major component of the extracellular matrix and connective tissue, cartilage, and bone. Collagen molecules are triple-stranded helical structures called α -chains, which are wound around each other in a ropelike helix. Collagen exists in several forms or types; of these, type I, the most common, is found in skin, tendon, and bone; and type III is found in skin, blood vessels, and internal organs.

[0072] As used herein, the term “fibrotic process” refers to the temporal and progressive deposition of fibrous tissue during fibrosis.

[0073] As used herein, the terms “hepatic fibrosis” refers to the fibrosis present and/or occurring in the liver. The term “hepatic fibrosis” and “liver fibrosis” are used interchangeably.

[0074] As used herein, the term “cirrhosis” refers to a late stage of hepatic fibrosis where the liver experiences loss of functional liver cells. Normal liver tissue is replaced with fibrous tissue resulting in

widespread distortion of normal hepatic architecture. A major characteristic is regenerative nodules surrounded by dense fibrotic tissue. The overgrowth of fibrosis scar tissue inhibits the liver's proper functioning. Cirrhosis is usually considered irreversible.

[0075] “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 can 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 can base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine can be replaced in the nucleotide sequences of dsRNA featured herein by a nucleotide containing, for example, inosine. In another example, adenine and cytosine anywhere in the oligonucleotide can be replaced with guanine and uracil, respectively to form G-U Wobble base pairing with the target mRNA. Sequences containing such replacement moieties are suitable for the compositions and methods described herein.

[0076] As used herein, the term “iRNA” refers to an agent that contains RNA as that term is defined herein, and which mediates the targeted cleavage of an RNA transcript via an RNA-induced silencing complex (RISC) pathway. In one embodiment, an iRNA as described herein inhibits the expression of COL1A1, TGF β , or SMAD2/3 gene in a cell.

[0077] As used herein, “target sequence” refers to a contiguous portion of the nucleotide sequence of an mRNA molecule formed during the transcription of a COL1A1, a TGF β , a SMAD2 or a SMAD3 gene, including messenger (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.

[0078] 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.

[0079] 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 can include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C for 12-16 hours followed by washing. Other conditions, such as physiologically relevant conditions as can be encountered inside an organism, can apply. The skilled person will be able to determine the set of conditions most appropriate for a test of complementarity of two sequences in accordance with the ultimate application of the hybridized nucleotides.

[0080] Complementary sequences within an iRNA, *e.g.*, within a dsRNA as described herein, include base-pairing of the oligonucleotide or polynucleotide comprising a first nucleotide sequence to an oligonucleotide or polynucleotide comprising a second nucleotide sequence over the entire length of one or both nucleotide sequences. Such sequences can be referred to as “fully complementary” with respect to each other herein. However, where a first sequence is referred to as “substantially complementary” with respect to a second sequence herein, the two sequences can be fully complementary, or they can form one or more, but generally not more than 5, 4, 3 or 2 mismatched base pairs upon hybridization for a duplex up to 30 base pairs (bp), while retaining the ability to hybridize under the conditions most relevant to their ultimate application, *e.g.*, inhibition of gene expression via a RISC pathway. However, where two oligonucleotides are designed to form, upon hybridization, one or more single stranded overhangs, such overhangs shall not be regarded as mismatches with regard to the determination of complementarity. For example, a dsRNA comprising one oligonucleotide 21 nucleotides in length and another oligonucleotide 23 nucleotides in length, wherein the longer oligonucleotide comprises a sequence of 21 nucleotides that is fully complementary to the shorter oligonucleotide, can yet be referred to as “fully complementary” for the purposes described herein.

[0081] “Complementary” sequences, as used herein, can also include, or be formed entirely from, non-Watson-Crick base pairs and/or base pairs formed from non-natural and modified nucleotides, in as far as the above requirements with respect to their ability to hybridize are fulfilled. Such non-Watson-Crick base pairs includes, but are not limited to, G:U Wobble or Hoogsteen base pairing.

[0082] The terms “complementary,” “fully complementary” and “substantially complementary” herein can be used with respect to the base matching between the sense strand and the antisense strand of a dsRNA, or between the antisense strand of an iRNA agent and a target sequence, as will be understood from the context of their use.

[0083] As used herein, a polynucleotide that is “substantially complementary to at least part of” an mRNA refers to a polynucleotide that is substantially complementary to a contiguous portion of the mRNA of interest (*e.g.*, an mRNA encoding COL1A1). For example, a polynucleotide is complementary to at least a part of a COL1A1mRNA if the sequence is substantially complementary to a non-interrupted portion of an mRNA encoding COL1A1.

[0084] The term “double-stranded RNA” or “dsRNA,” as used herein refers to an iRNA that includes an RNA molecule or complex of molecules having a hybridized duplex region that comprises

two anti-parallel and substantially complementary nucleic acid strands, which will be referred to as having “sense” and “antisense” orientations with respect to a target RNA. The duplex region can be of any length that permits specific degradation of a desired target RNA through a RISC pathway, but will typically range from 9 to 36 base pairs (bp) in length, *e.g.*, 15-30 bp in length. Considering a duplex between 9 and 36 bp 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 bp, 15-26 bp, 15-23 bp, 15-22 bp, 15-21 bp, 15-20 bp, 15-19 bp, 15-18 bp, 15-17 bp, 18-30 bp, 18-26 bp, 18-23 bp, 18-22 bp, 18-21 bp, 18-20 bp, 19-30 bp, 19-26 bp, 19-23 bp, 19-22 bp, 19-21 bp, 19-20 bp, 20-30 bp, 20-26 bp, 20-25 bp, 20-24 bp, 20-23 bp, 20-22 bp, 20-21 bp, 21-30 bp, 21-26 bp, 21-25 bp, 21-24 bp, 21-23 bp, or 21-22 bp. dsRNAs generated in the cell by processing with Dicer and similar enzymes are generally in the range of 19-22 bp 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.

[0085] The skilled artisan will recognize that the term “RNA molecule” or “ribonucleic acid molecule” encompasses not only RNA molecules as expressed or found in nature, but also analogs and derivatives of RNA comprising one or more ribonucleotide/ribonucleoside analogs or derivatives as described herein or as known in the art. Strictly speaking, a “ribonucleoside” includes a nucleoside base and a ribose sugar, and a “ribonucleotide” is a ribonucleoside with one, two or three phosphate moieties. However, the terms “ribonucleoside” and “ribonucleotide” can be considered to be equivalent as used herein. The RNA can be modified in the nucleobase structure or in the ribose-phosphate backbone structure, *e.g.*, as described herein below. However, the molecules comprising ribonucleoside analogs or derivatives must retain the ability to form a duplex. As non-limiting examples, an RNA molecule can also include at least one modified ribonucleoside including but not limited to a 2'-O-methyl modified nucleoside, a nucleoside comprising a 5' phosphorothioate group, a terminal nucleoside linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group, a locked nucleoside, an abasic nucleoside, a 2'-deoxy-2'-fluoro modified nucleoside, a 2'-amino-modified nucleoside, 2'-alkyl-modified nucleoside, morpholino nucleoside, a phosphoramidate or a non-natural base comprising nucleoside, or

any combination thereof. Alternatively, an RNA molecule can comprise at least two modified ribonucleosides, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20 or more, up to the entire length of the dsRNA molecule. The modifications need not be the same for each of such a plurality of modified ribonucleosides in an RNA molecule. In one embodiment, modified RNAs contemplated for use in methods and compositions described herein are peptide nucleic acids (PNAs) that have the ability to form the required duplex structure and that permit or mediate the specific degradation of a target RNA via a RISC pathway.

[0086] In one aspect, a modified ribonucleoside includes a deoxyribonucleoside. In such an instance, an iRNA agent can comprise one or more deoxynucleosides, including, for example, a deoxynucleoside overhang(s), or one or more deoxynucleosides within the double stranded portion of a dsRNA. However, it is self evident that under no circumstances is a double stranded DNA molecule encompassed by the term “iRNA.”

[0087] In one aspect, an RNA interference agent includes a single stranded RNA that interacts with a target RNA sequence to direct the cleavage of the target RNA. Without wishing to be bound by theory, long double stranded RNA introduced into plants and invertebrate cells is broken down into siRNA by a Type III endonuclease known as Dicer (Sharp *et al.*, Genes Dev. 2001, 15:485). Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs (Bernstein, *et al.*, (2001) Nature 409:363). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, *et al.*, (2001) Cell 107:309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleaves the target to induce silencing (Elbashir, *et al.*, (2001) Genes Dev. 15:188). Thus, in one aspect the invention relates to a single stranded RNA that promotes the formation of a RISC complex to effect silencing of the target gene.

[0088] 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) can 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.

[0089] 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.

[0090] 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.

[0091] 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 can 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.

[0092] 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.

[0093] As used herein, in one embodiment, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a vesicle of lipids coating a reduced aqueous interior comprising a nucleic acid such as an iRNA or a plasmid from which an iRNA is transcribed. SNALPs are described, *e.g.*, in U.S. Patent Application Publication Nos. 20060240093, 20070135372, and in International Application No. WO2009082817. Examples of "SNALP" formulations are described elsewhere herein.

[0094] "Introducing into a cell," when referring to an iRNA, means facilitating or effecting uptake or absorption into the cell, as is understood by those skilled in the art. Absorption or uptake of an iRNA can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. The meaning of this term is not limited to cells *in vitro*; an iRNA can also be "introduced into a cell," wherein the cell is part of a living organism. In such an instance, introduction into the cell will include the delivery to the organism. For example, for *in vivo* delivery, iRNA can be injected into a tissue site or administered systemically. *In vivo* delivery can also be by a beta-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.

In vitro introduction into a cell includes methods known in the art such as electroporation and lipofection. Further approaches are described herein below or are known in the art.

[0095] As used herein, the phrase "inhibit the expression of," refers to at least partial "reduction" of COL1A1, TGF β or SMAD2/3 gene expression in a cell treated with an iRNA composition as described herein compared to the expression of COL1A1, TGF β or SMAD2/3 respectively in an untreated cell.

[0096] The terms "silence," "inhibit the expression of," "down-regulate the expression of," "suppress the expression of," and the like, in so far as they refer to a COL1A1, TGF β and/or SMAD2/3

gene, herein refer to the at least partial suppression of the expression of the COL1A1, TGF β and/or SMAD2/3 gene, as manifested by a reduction of the amount of COL1A1, TGF β and/or SMAD2/3 mRNA respectively which can be isolated from or detected in a first cell or group of cells in which the COL1A1, TGF β and/or SMAD2/3 gene is transcribed and which has or have been treated such that the expression of the COL1A1, TGF β and/or SMAD2/3 gene is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has or have not been so treated (control cells). The degree of inhibition is usually expressed in terms of

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

[0097] Alternatively, the degree of inhibition can be given in terms of a reduction of a parameter that is functionally linked to COL1A1, TGF β or SMAD2/3 gene expression, *e.g.*, the amount of protein encoded by the COL1A1, TGF β or SMAD2/3 gene respectively, or the number of cells displaying a certain phenotype, *e.g.*, lack of or decreased cytokine production. In principle, COL1A1, TGF β and/or SMAD2/3 gene silencing can be determined in any cell expressing COL1A1, TGF β and/or SMAD2/3 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 COL1A1, TGF β and/or SMAD2/3 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.

[0098] For example, in certain instances, expression of the COL1A1, TGF β and/or SMAD2/3 gene is suppressed by at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% by administration of an iRNA featured herein. In some embodiments, the COL1A1, TGF β and/or SMAD2/3 gene is suppressed by at least about 60%, 70%, or 80% by administration of an iRNA featured herein. In some embodiments, the COL1A1, TGF β and/or SMAD2/3 gene is suppressed by at least about 85%, 90%, 95%, 98%, 99%, or more by administration of an iRNA as described herein.

[0099] As used herein in the context of COL1A1, TGF β and/or SMAD2/3 expression, the terms “treat,” “treatment,” and the like, refer to relief from or alleviation of liver fibrosis mediated by COL1A1 overexpression. In some embodiment, the terms “treat,” “treatment,” and the like mean to relieve or alleviate at least one symptom associated with liver fibrosis, or to slow or reverse the fibrotic process.

[00100] By “lower” in the context of a liver function, or liver fibrosis biomarker or a liver fibrosis symptom is meant a statistically significant decrease in such level. The decrease can be, for example, at least 10%, at least 20%, at least 30%, at least 40% or more, and is preferably down to a level accepted as within the range of normal for an individual without liver failure, a chronic liver disease, liver fibrosis, or cirrhosis.

[00101] 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 liver fibrosis, which is mediated by COL1A1 overexpression. The specific amount that is therapeutically effective can be readily determined by an ordinary medical practitioner, and can vary

depending on factors known in the art, such as, for example, the patient's history and age, the stage of liver disease or liver fibrosis, and the administration of other agents that indirectly inhibit the COL1A1 overexpression and other agents that treat the underlying causes leading to chronic liver injury and fibrosis.

[00102] As used herein, a "pharmaceutical composition" comprises a pharmacologically effective amount of an iRNA and a pharmaceutically acceptable carrier. As used herein, "pharmacologically effective amount," "therapeutically effective amount" or simply "effective amount" refers to that amount of an iRNA effective to produce the intended pharmacological, therapeutic or preventive result. For example, if a given clinical treatment is considered effective when there is at least a 10% reduction in a measurable parameter associated with a disease or disorder, a therapeutically effective amount of a drug for the treatment of that disease or disorder is the amount necessary to effect at least a 10% reduction in that parameter. For example, a therapeutically effective amount of an iRNA targeting a COL1A1 gene can reduce COL1A1 protein levels by at least 10%.

[00103] 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 can include starch and gelatin, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If desired, the tablets can 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.

[00104] As used herein, a therapeutic that "prevents" a fibrosis is a composition that, in a statistical sample, reduces the occurrence of fibrosis in the treated sample relative to an untreated control sample, or delays the onset or reduces the severity of one or more symptoms of the disorder or condition relative to the untreated control sample.

[00105] An "infection" as used herein refers to a disease or condition attributable to the presence in a host of a foreign organism or agent that reproduces within the host. Infections typically involve breach of a normal mucosal or other tissue barrier by an infectious organism or agent. A subject that has an infection is a subject having objectively measurable infectious organisms or agents present in the subject's body.

[00106] As used herein, a "subject" is a mammal, e. g. a dog, horse, cat, and other non-human primates. In a preferred embodiment, a subject is a human.

[00107] As used herein, the term "LNPXX", wherein the "XX" are numerals, is also referred to as "AFX" herein. For example, LNP09 is also referred to AF09 and LNP12 is also known as or referred to as AF12.

[00108] As used herein, the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not.

[00109] As used herein, the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[00110] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

II. Double-stranded ribonucleic acid (dsRNA)

[00111] Described herein are iRNA agents that inhibit the expression of the COL1A1, TGF β and/or SMAD2/3 genes. In one embodiment, the iRNA agent includes double-stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of a COL1A1, TGF β and/or SMAD2/3 gene respectively in a cell or mammal, *e.g.*, in a human having a liver disease characterized by hepatic fibrosis, 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 a COL1A1, TGF β or SMAD2/3 gene, 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 COL1A1, TGF β or SMAD2/3 gene respectively, inhibits the expression of the COL1A1, TGF β and/or SMAD2/3 gene respectively 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. Expression of a COL1A1 gene in cell culture, such as in COS cells, HeLa cells, primary hepatocytes, HepG2 cells, primary cultured HSCs or in a biological sample from a subject can be assayed by measuring COL1A1 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 flowcytometric techniques.

[00112] A dsRNA includes two RNA strands that are complementary to hybridize to form a duplex structure under conditions in which the dsRNA will be used. One strand of a dsRNA (the antisense strand) includes a region of complementarity that is substantially complementary, and generally fully complementary, to a target sequence. The target sequence can be derived from the sequence of an mRNA formed during the expression of a COL1A1, TGF β or SMAD2/3 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 bp can, under some circumstances, mediate RNAi-directed RNA cleavage. Most often a target will be at least 15 nucleotides in length, preferably 15-30 nucleotides in length.

[00113] 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 bp. Thus, in one embodiment, to the extent that it becomes processed to a functional duplex of *e.g.*, 15-30 bps that targets a desired RNA for cleavage, an RNA molecule or complex of RNA molecules having a duplex region greater than 30 bp 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 COL1A1, TGF β and/or SMAD2/3 expression is not generated in the target cell by cleavage of a larger dsRNA.

[00114] A dsRNA as described herein can further include one or more single-stranded nucleotide overhangs. The dsRNA can be synthesized by standard methods known in the art as further discussed below, *e.g.*, by use of an automated DNA synthesizer, such as are commercially available from, for example, Biosearch, Applied Biosystems, Inc.

[00115] In one embodiment, a COL1A1 gene is a human COL1A1 gene. In another embodiment the COL1A1 gene is a mouse or a rat COL1A1 gene. In one embodiment, an iRNA cross react with human and/or mouse and rat COL1A1 sequence. In specific embodiments, the first sequence is a sense strand of a dsRNA that includes a sense sequence from Table 3 and Table 6, and the second sequence is selected from the group consisting of the corresponding antisense sequences of Table 3 and Table 6. Alternative dsRNA agents that target elsewhere in the target sequence provided in Table 3 and Table 6 can readily be determined using the target sequence and the flanking COL1A1 sequence.

[00116] In one embodiment, a TGF β gene is a human TGF β gene. In another embodiment the TGF β gene is a mouse or a rat TGF β gene. In specific embodiments, the first sequence is a sense strand of a dsRNA that includes a sense sequence from Table 4 and Table 7, and the second sequence is selected from the group consisting of the corresponding antisense sequences of Table 4 and Table 7. Alternative dsRNA agents that target elsewhere in the target sequence provided in Table 4 and Table 7 can readily be determined using the target sequence and the flanking TGF β sequence. In one embodiment, an iRNA cross react with human and/or mouse and rat TGF β sequence.

[00117] In one embodiment, a SMAD2/3 gene is a human SMAD2/3 gene. In another embodiment the SMAD2/3 gene is a mouse or a rat SMAD2/3 gene. In specific embodiments, the first sequence is a sense strand of a dsRNA that includes a sense sequence from Table 5, and the second sequence is selected from the group consisting of the corresponding antisense sequences of Table 5. Alternative dsRNA agents that target elsewhere in the target sequence provided in Table 5 can readily be determined using the target sequence and the flanking SMAD2/3 sequence. In one embodiment, an iRNA cross react with human and/or mouse and rat SMAD2/3 sequence.

[00118] In one aspect, a dsRNA will include at least two nucleotide sequences, a sense and an anti-sense sequence, whereby the sense strand is selected from the groups of sequences provided in Tables 3-7, and the corresponding antisense strand of the sense strand selected from Table 3-7. In this aspect, one of the two sequences is complementary to the other of the two sequences, with one of the sequences being substantially complementary to a sequence of an mRNA generated in the expression of a COL1A1, TGF β and/or SMAD2/3 gene. As such, in this aspect, a dsRNA will include two oligonucleotides, where one oligonucleotide is described as the sense strand in Table 3-7, and the second oligonucleotide is described as the corresponding antisense strand of the sense strand from Tables 3-7. 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.

[00119] 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 identified by sequence identifiers provided in Tables 3-7, dsRNAs described herein can include at least one strand of a length of minimally 21 nt. It can be reasonably expected that shorter duplexes having one of the sequences of Tables 3-7 minus only a few nucleotides on one or both ends can be similarly effective as compared to the dsRNAs described above. Hence, dsRNAs having a partial sequence of at least 15, 16, 17, 18, 19, 20, or more contiguous nucleotides from one of the sequences of Tables 3-7, and differing in their ability to inhibit the expression of a COL1A1, TGF β and/or SMAD2/3 gene by not more than 5, 10, 15, 20, 25, or 30 % inhibition from a dsRNA comprising the full sequence, are contemplated according to the invention.

[00120] In addition, the RNAs provided in Tables 3-7 identify a site in a COL1A1, TGF β and/or SMAD2/3 transcript that is susceptible to RISC-mediated cleavage. As such, the present invention further features iRNAs that target within one of such sequences. As used herein, an iRNA is said to target within a particular site of an RNA transcript if the iRNA promotes cleavage of the transcript anywhere within that particular site. Such an iRNA will generally include at least 15 contiguous nucleotides from one of the sequences provided in Tables 3-7 coupled to additional nucleotide sequences

taken from the region contiguous to the selected sequence in a COL1A1, TGF β and/or SMAD2/3 gene respectively.

[00121] 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 can 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, by sequence identifier in Tables 3-7 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.

[00122] Further, it is contemplated that for any sequence identified by sequence identifier, *e.g.*, in Tables 3-7, further optimization could 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.

[00123] An iRNA as described herein can contain one or more mismatches to the target sequence. In one embodiment, an iRNA as described herein contains no more than 3 mismatches. If the antisense strand of the iRNA contains mismatches to a target sequence, it is preferable that the area of mismatch not be located in the center of the region of complementarity. If the antisense strand of the iRNA contains mismatches to the target sequence, it is preferable that the mismatch be restricted to be within the last 5 nucleotides from either the 5' or 3' end of the region of complementarity. For example, for a 23

nucleotide iRNA agent RNA strand which is complementary to a region of a COL1A1, TGF β and/or SMAD2/3 gene, the RNA strand generally does not contain any mismatch within the central 13 nucleotides. The methods described herein or methods known in the art can be used to determine whether an iRNA containing a mismatch to a target sequence is effective in inhibiting the expression of a COL1A1, TGF β and/or SMAD2/3 gene respectively. Consideration of the efficacy of iRNAs with mismatches in inhibiting expression of a COL1A1, TGF β and/or SMAD2/3 gene is important, especially if the particular region of complementarity in a COL1A1, TGF β and/or SMAD2/3 gene is known to have polymorphic sequence variation within the population.

[00124] 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 can 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.

Modifications include, for example, (a) end modifications, *e.g.*, 5' end modifications (phosphorylation, conjugation, inverted linkages, etc.) 3' end modifications (conjugation, DNA nucleotides, inverted linkages, etc.), (b) base modifications, *e.g.*, replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners, removal of bases (abasic nucleotides), or conjugated bases, (c) sugar modifications (*e.g.*, at the 2' position or 4' position) or replacement of the sugar, as well as (d) backbone modifications, including modification or replacement of the phosphodiester linkages. Specific examples of RNA compounds useful in the embodiments described herein include, but are not limited to RNAs containing modified backbones or no natural internucleoside linkages. RNAs having modified backbones include, among others, those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified RNAs that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. In particular embodiments, the modified RNA will have a phosphorus atom in its internucleoside backbone.

[00125] 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.

[00126] 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.

[00127] 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 CH₂ component parts.

[00128] 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.

[00129] In other embodiments, suitable RNA mimetics suitable are contemplated for use in iRNAs, in which 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.

Further teaching of PNA compounds can be found, for example, in Nielsen *et al.*, Science, 1991, 254, 1497-1500.

[00130] Some embodiments featured in the invention include RNAs with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH₂--NH--CH₂--, --CH₂--N(CH₃)--O--CH₂--[known as a methylene (methylimino) or MMI backbone], --CH₂--O--N(CH₃)--CH₂--, --CH₂--N(CH₃)--N(CH₃)--CH₂-- and --N(CH₃)--CH₂--CH₂--[wherein the native phosphodiester backbone is represented as --O--P--O--CH₂--] 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.

[00131] Modified RNAs can also contain one or more substituted sugar moieties. The iRNAs, *e.g.*, dsRNAs, featured herein can include one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl can be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Exemplary suitable modifications include O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. In other embodiments, dsRNAs include one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, 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--CH₂CH₂OCH₃, 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(CH₂)₂ON(CH₃)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--CH₂--O--CH₂--N(CH₃)₂, also described in examples herein below.

[00132] Other modifications include 2'-methoxy (2'-OCH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications can also be made at other positions on the RNA of an iRNA, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. iRNAs can 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.

[00133] An iRNA can also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-

thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo, particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 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, those 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 O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (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.

[00134] 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, and U.S. Pat. No. 5,750,692.

[00135] The RNA of an iRNA can also be modified to include one or more locked nucleic acids (LNA). A locked nucleic acid is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting the 2' and 4' carbons. This structure effectively "locks" the ribose in the 3'-endo structural conformation. The addition of locked nucleic acids to siRNAs has been shown to increase siRNA stability in serum, and to reduce off-target effects (Elmen, J. *et al.*, (2005) *Nucleic Acids Research* 33(1):439-447; Mook, OR. *et al.*, (2007) *Mol Canc Ther* 6(3):833-843; Grunweller, A. *et al.*, (2003) *Nucleic Acids Research* 31(12):3185-3193).

[00136] Representative U.S. Patents that teach the preparation of locked nucleic acid nucleotides include, but are not limited to, the following: U.S. Pat. Nos. 6,268,490; 6,670,461; 6,794,499; 6,998,484; 7,053,207; 7,084,125; and 7,399,845.

[00137] Another modification of the RNA of an iRNA featured in the invention involves chemically linking to the RNA one or more ligands, moieties or conjugates that enhance the activity, cellular distribution 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. Acad. Sci. USA*, 1989, 86: 6553-6556), cholic acid (Manoharan *et al.*, *Biorg. Med. Chem. Lett.*, 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. Lett., 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).

[00138] In one embodiment, a ligand alters the distribution, targeting or lifetime of an iRNA agent into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, *e.g.*, molecule, cell or cell type, compartment, *e.g.*, a cellular or organ compartment, tissue, organ or region of the body, as, *e.g.*, compared to a species absent such a ligand. Preferred ligands will not take part in duplex pairing in a duplexed nucleic acid.

[00139] 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 can 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-glycolid) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HIMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazene. 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 alpha helical peptide.

[00140] 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-glucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, vitamin A, biotin, or an RGD peptide or RGD peptide mimetic.

[00141] 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.

[00142] 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 hepatic stellate cell. Ligands can 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 TGFβ receptor.

[00143] 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.

[00144] In some embodiments, a ligand attached to an iRNA as described herein acts as a pharmacokinetic (PK) modulator. As used herein, a "PK modulator" refers to a pharmacokinetic modulator. PK modulators include lipophiles, bile acids, steroids, phospholipid analogues, peptides, protein binding agents, PEG, vitamins etc. Exemplary PK modulators include, but are not limited to, cholesterol, fatty acids, cholic acid, lithocholic acid, dialkylglycerides, diacylglyceride, phospholipids, sphingolipids, naproxen, ibuprofen, vitamin E, biotin etc. Oligonucleotides that comprise a number of phosphorothioate linkages are also known to bind to serum protein, thus short oligonucleotides, *e.g.*, oligonucleotides of about 5 bases, 10 bases, 15 bases or 20 bases, comprising multiple of phosphorothioate linkages in the 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.

Lipid Conjugates

[00145] In one embodiment, the ligand or conjugate is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, *e.g.*, human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, *e.g.*, a non-kidney target tissue of the body. For example, the target tissue can be the liver, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used.

A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, *e.g.*, HSA.

[00146] A lipid based ligand can be used to modulate, *e.g.*, control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

[00147] In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue.

However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

[00148] In another preferred embodiment, the lipid based ligand binds HSA weakly or not at all, such that the conjugate will be preferably distributed to the kidney. Other moieties that target to kidney cells can also be used in place of or in addition to the lipid based ligand.

[00149] 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 target cells such as HSC in the liver. Also included are HSA and low density lipoprotein (LDL).

Cell Permeation Peptide and Agents

[00150] In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopodia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

[00151] 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.

[00152] 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: 168). An RFGF analogue (*e.g.*, amino acid sequence AAII.PVII.AAP (SEQ ID NO: 169)) 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 (GRKKRRQRRPPQ (SEQ ID NO: 170)) and the *Drosophila Antennapedia* protein (RQIKIWFQNRRMKWKK (SEQ ID NO: 171)) 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). Examples of a peptide or peptidomimetic tethered to a dsRNA agent via an incorporated monomer unit for cell targeting purposes is 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.

[00153] An RGD peptide moiety can be used to target a tumor cell, such as an endothelial tumor cell or a breast cancer tumor cell (Zitzmann *et al.*, Cancer Res., 62:5139-43, 2002). An RGD peptide can facilitate targeting of an dsRNA agent to tumors of a variety of other tissues, including the lung, kidney, spleen, or liver (Aoki *et al.*, Cancer Gene Therapy 8:783-787, 2001). Preferably, the RGD peptide will facilitate targeting of an iRNA agent to the kidney. The RGD peptide can be linear or cyclic, and can be modified, *e.g.*, glycosylated or methylated to facilitate targeting to specific tissues. For example, a glycosylated RGD peptide can deliver a iRNA agent to a tumor cell expressing $\alpha_v\beta_3$ (Haubner *et al.*, Jour. Nucl. Med., 42:326-336, 2001).

[00154] Peptides that target markers enriched in proliferating cells can be used, *e.g.*, RGD containing peptides and peptidomimetics can target cancer cells, in particular cells that exhibit an $\alpha_v\beta_3$ integrin. Thus, one could use RGD peptides, cyclic peptides containing RGD, RGD peptides that include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the $\alpha_v\beta_3$ integrin ligand. Generally, such ligands can be used to control proliferating cells and angiogenesis.

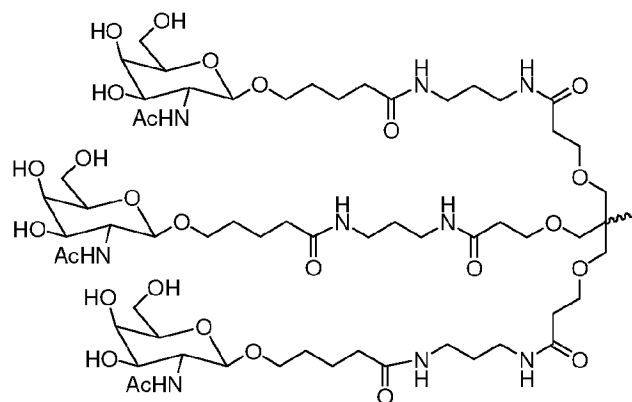
[00155] A “cell permeation peptide” is capable of permeating a cell, *e.g.*, a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, a α -helical linear peptide (*e.g.*, IJL-37 or Ceropin P1), a disulfide 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

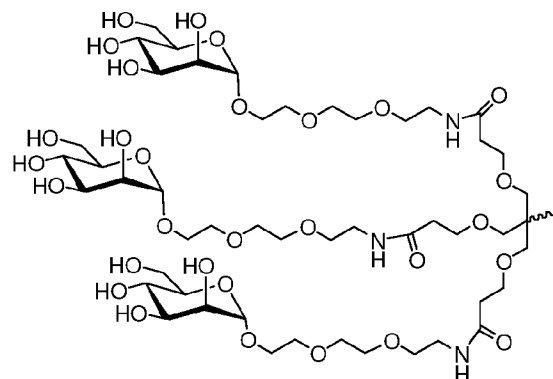
[00156] In some embodiments, the iRNA oligonucleotides described herein further comprise carbohydrate conjugates. The carbohydrate conjugates are advantageous for the *in vivo* delivery of nucleic acids, as well as compositions suitable for *in vivo* therapeutic use, as described herein. As used herein, “carbohydrate” refers to a compound which is either a carbohydrate per se made up of one or

more monosaccharide units having at least 6 carbon atoms (which 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 4-9 monosaccharide units), and polysaccharides such as starches, glycogen, cellulose and polysaccharide gums. Specific monosaccharides include C₅ and above (preferably C₅-C₈) sugars; di- and trisaccharides include sugars having two or three monosaccharide units (preferably C₅-C₈).

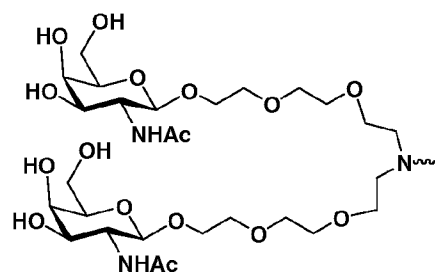
[00157] In one embodiment, the carbohydrate conjugate is selected from the group consisting of:



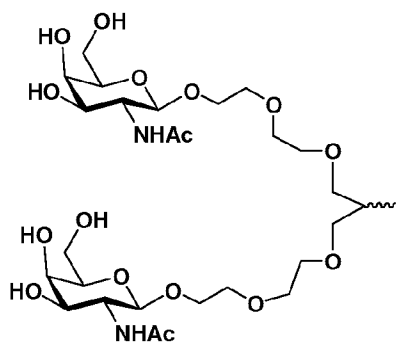
Formula II,



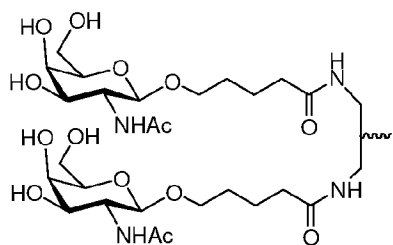
Formula III,



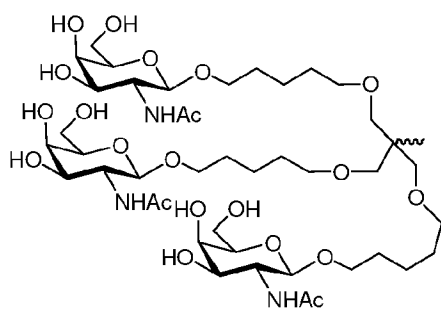
Formula IV,



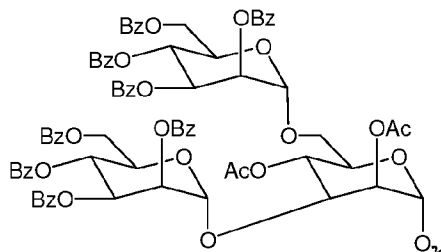
Formula V,



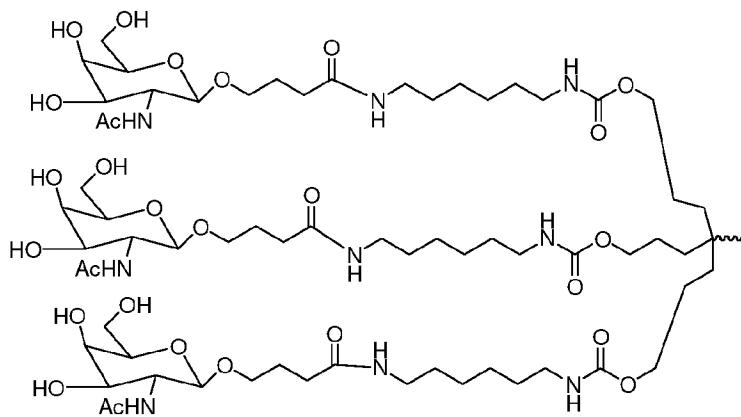
Formula VI,



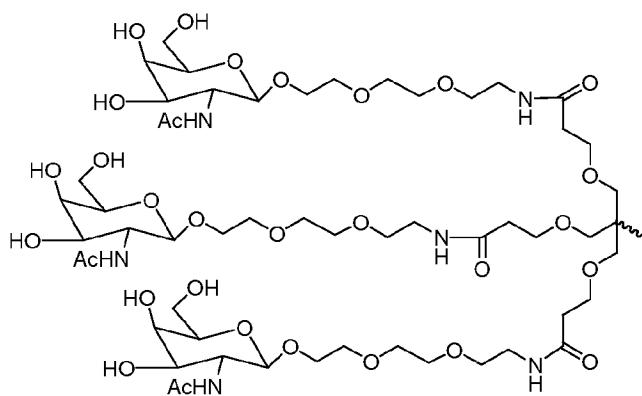
Formula VII,



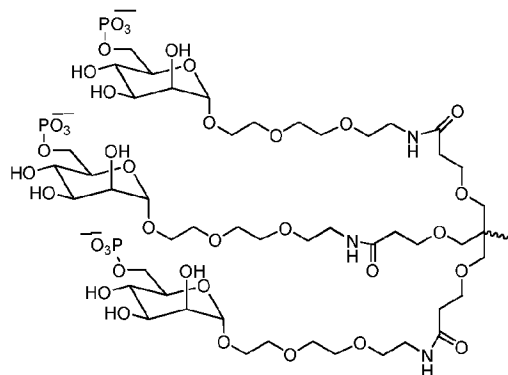
Formula VIII,



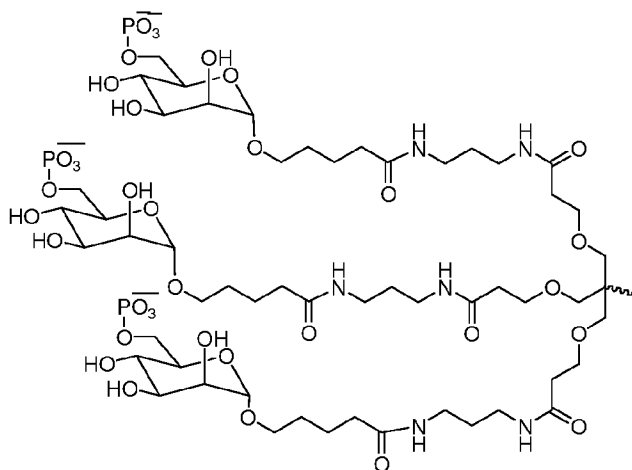
Formula IX,



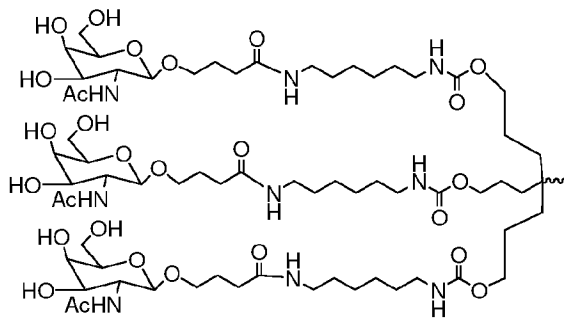
Formula X,



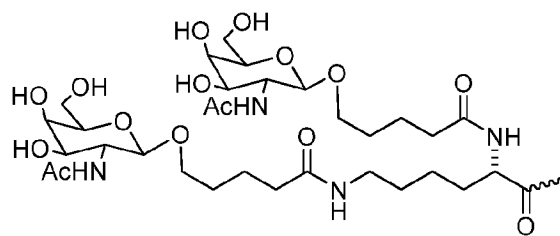
Formula XI,



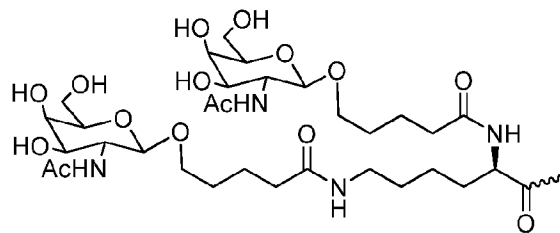
Formula XII,



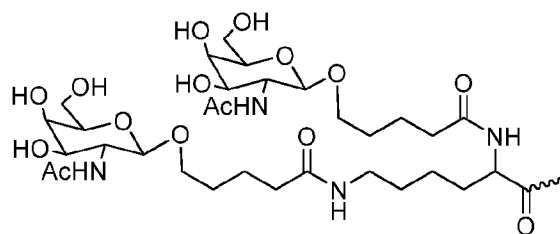
Formula XIII,



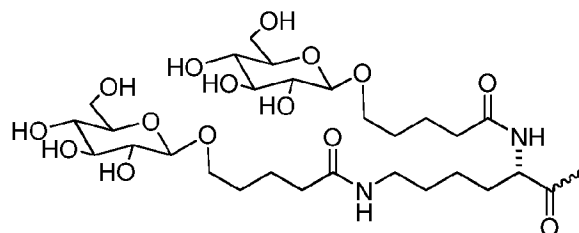
Formula XIV,



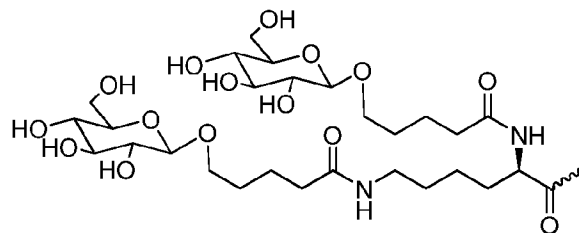
Formula XV,



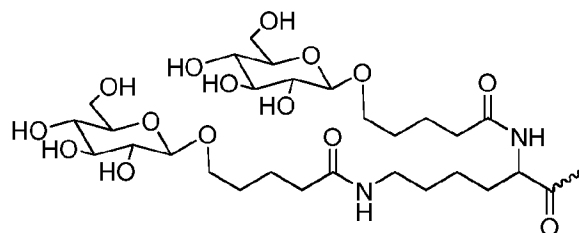
Formula XVI,



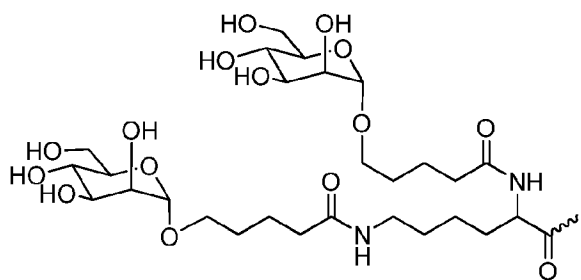
Formula XVII,



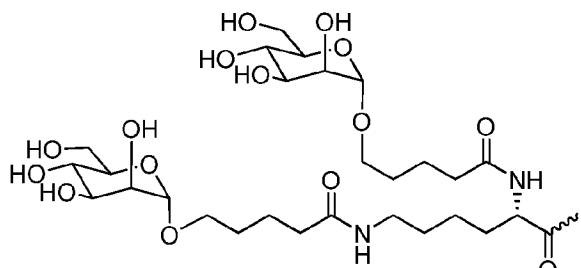
Formula XVIII,



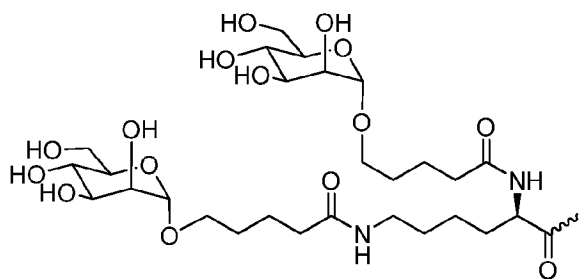
Formula XIX,



Formula XX,

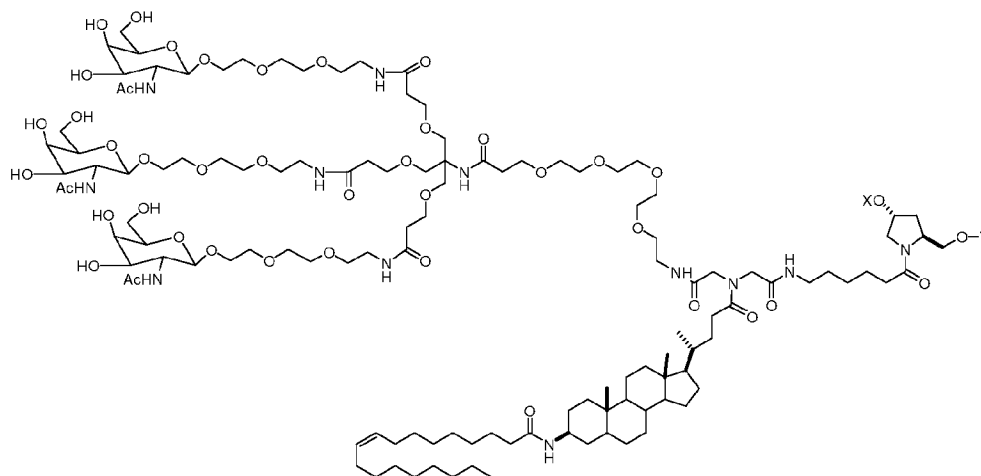


Formula XXI,

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

XXII.

[00158] 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.

[00159] In some embodiments, the carbohydrate conjugate further comprises other ligand such as, but not limited to, PK modulator, endosomolytic ligand, and cell permeation peptide.

Linkers

[0021] In some embodiments, the conjugates described herein can be attached to the iRNA oligonucleotide with various linkers that can be cleavable or non cleavable.

[0022] The term "linker" or "linking group" means an organic moiety that connects two parts of a compound. Linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NR^8 , $\text{C}(\text{O})$, $\text{C}(\text{O})\text{NH}$, 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, heterocyclalkyl, heterocyclalkenyl, heterocyclalkynyl, aryl, heteroaryl, heterocycl, cycloalkyl, cycloalkenyl, alkylarylalkyl, alkylarylalkenyl, alkylarylalkynyl, alkenylarylalkyl, alkenylarylalkenyl, alkenylarylalkynyl, alkynylarylalkyl, alkynylarylalkenyl, alkylheteroarylalkyl, alkylheteroarylalkenyl, alkylheteroarylalkynyl, alkenylheteroarylalkyl, alkenylheteroarylalkenyl, alkenylheteroarylalkynyl, alkynylheteroarylalkyl, alkynylheteroarylalkenyl, alkynylheteroarylalkynyl, alkylheterocyclalkyl, alkylheterocyclalkenyl, alkylheterocyclalkynyl, alkenylheterocyclalkyl, alkenylheterocyclalkenyl, alkenylheterocyclalkynyl, alkynylheterocyclalkyl, alkynylheterocyclalkenyl, alkynylheterocyclalkynyl, alkylaryl, alkenylaryl, alkynylaryl, alkylheteroaryl, alkenylheteroaryl, alkynylheteroaryl, which one or more methylenes can be interrupted or terminated by O, S, $\text{S}(\text{O})$, SO_2 , $\text{N}(\text{R}^8)$, $\text{C}(\text{O})$, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocyclic; where R^8 is hydrogen, acyl, aliphatic or substituted aliphatic. In one embodiment, the linker is between 1-24 atoms, preferably 4-24 atoms, preferably 6-18 atoms, more preferably 8-18 atoms, and most preferably 8-16 atoms.

[0023] A cleavable linking group is one which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding together. In a preferred embodiment, the cleavable linking group is cleaved at least 10 times or more, preferably at least 100 times faster in the target cell or under a first reference condition (which can, *e.g.*, be selected to mimic or represent intracellular conditions) than in the blood of a subject, or under a second reference condition (which can, *e.g.*, be selected to mimic or represent conditions found in the blood or serum).

[0024] 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.

[0025] A cleavable linkage group, such as a disulfide bond can be susceptible to pH. The pH of human serum is 7.4, while the average intracellular pH is slightly lower, ranging from about 7.1-7.3. Endosomes have a more acidic pH, in the range of 5.5-6.0, and lysosomes have an even more acidic pH

at around 5.0. Some linkers will have a cleavable linking group that is cleaved at a preferred pH, thereby releasing the cationic lipid from the ligand inside the cell, or into the desired compartment of the cell.

[0026] A linker can include a cleavable linking group that is cleavable by a particular enzyme. The type of cleavable linking group incorporated into a linker can depend on the cell to be targeted. For example, liver targeting ligands can be linked to the cationic lipids through a linker that includes an ester group. Liver cells are rich in esterases, and therefore the linker will be cleaved more efficiently in liver cells than in cell types that are not esterase-rich. Other cell-types rich in esterases include cells of the lung, renal cortex, and testis.

[0027] Linkers that contain peptide bonds can be used when targeting cell types rich in peptidases, such as liver cells and synoviocytes.

[0028] 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 2, 4, 10 or 100 times faster in the cell (or under *in vitro* conditions selected to mimic intracellular conditions) as compared to blood or serum (or under *in vitro* conditions selected to mimic extracellular conditions).

Redox cleavable linking groups

[0029] One class of cleavable linking groups are redox cleavable linking groups that are cleaved upon reduction or oxidation. An example of reductively cleavable linking group is a disulphide linking group (-S-S-). To determine if a candidate cleavable linking group is a suitable "reductively cleavable linking group," or for example is suitable for use with a particular iRNA moiety and particular targeting agent one can look to methods described herein. For example, a candidate can be evaluated by incubation with dithiothreitol (DTT), or other reducing agent using reagents known in the art, which mimic the rate of cleavage which would be observed in a cell, *e.g.*, a target cell. The candidates can also be evaluated under conditions which are selected to mimic blood or serum conditions. In a preferred embodiment, candidate compounds are cleaved by at most 10% in the blood. In preferred embodiments, useful candidate compounds are degraded at least 2, 4, 10 or 100 times faster in the cell (or under *in vitro* conditions selected to mimic intracellular conditions) as compared to blood (or under *in vitro* conditions selected to mimic extracellular conditions). The rate of cleavage of candidate compounds can be determined using standard enzyme kinetics assays under conditions chosen to mimic intracellular media and compared to conditions chosen to mimic extracellular media.

Phosphate-based cleavable linking groups

[0030] Phosphate-based cleavable linking groups are cleaved by agents that degrade or hydrolyze the phosphate group. An example of an agent that cleaves phosphate groups in cells are enzymes such as phosphatases in cells. Examples of phosphate-based linking groups are -O-P(O)(ORk)-O-, -O-P(S)(ORk)-O-, -O-P(S)(SRk)-O-, -S-P(O)(ORk)-O-, -O-P(O)(ORk)-S-, -S-P(O)(ORk)-S-, -O-P(S)(ORk)-S-, -S-P(S)(ORk)-O-, -O-P(O)(Rk)-O-, -O-P(S)(Rk)-O-, -S-P(O)(Rk)-O-, -S-P(S)(Rk)-O-, -S-P(O)(Rk)-S-, -O-P(S)(Rk)-S-. Preferred embodiments are -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(II)-O-, -O-P(S)(II)-O-, -S-P(O)(II)-O-, -S-P(S)(II)-O-, -S-P(O)(II)-S-, -O-P(S)(II)-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

[0031] Acid cleavable linking groups are linking groups that are cleaved under acidic conditions. In preferred embodiments acid cleavable linking groups are cleaved in an acidic environment with a pII of about 6.5 or lower (*e.g.*, about 6.0, 5.5, 5.0, or lower), or by agents such as enzymes that can act as a general acid. In a cell, specific low pH organelles, such as endosomes and lysosomes can provide a cleaving environment for acid cleavable linking groups. Examples of acid cleavable linking groups include but are not limited to hydrazones, esters, and esters of amino acids. Acid cleavable groups can have the general formula -C=NN-, C(O)O, or -OC(O). A preferred embodiment is when the carbon attached to the oxygen of the ester (the alkoxy group) is an aryl group, substituted alkyl group, or tertiary alkyl group such as dimethyl pentyl or t-butyl. These candidates can be evaluated using methods analogous to those described above.

Ester-based linking groups

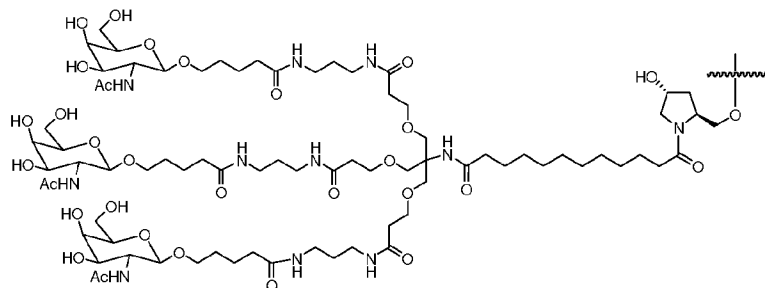
[0032] Ester-based cleavable linking groups are cleaved by enzymes such as esterases and amidases in cells. Examples of ester-based cleavable linking groups include but are not limited to esters of alkylene, alkenylene and alkynylene groups. Ester cleavable linking groups have the general formula -C(O)O-, or -OC(O)-. These candidates can be evaluated using methods analogous to those described above.

Peptide-based cleaving groups

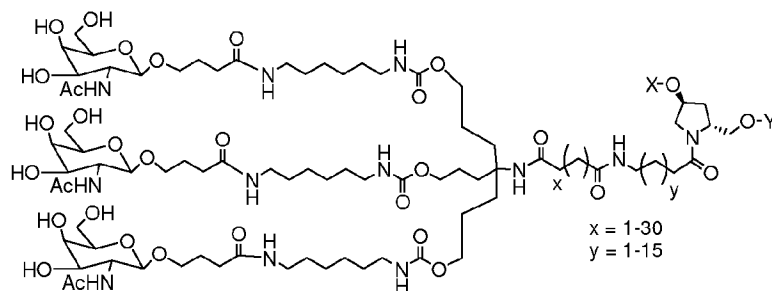
[0033] Peptide-based cleavable linking groups are cleaved by enzymes such as peptidases and proteases in cells. Peptide-based cleavable linking groups are peptide bonds formed between amino acids to yield oligopeptides (*e.g.*, dipeptides, tripeptides etc.) and polypeptides. Peptide-based cleavable groups do not include the amide group (-C(O)NH-). The amide group can be formed between any alkylene, alkenylene or alkynylene. A peptide bond is a special type of amide bond formed between amino acids to yield peptides and proteins. The peptide based cleavage group is generally limited to the peptide bond (*i.e.*, the amide bond) formed between amino acids yielding peptides and proteins and does not include the entire amide functional group. Peptide-based cleavable linking groups have the general formula -NHCHR^AC(O)NHCHR^BC(O)- (SEQ ID NO: 876), where R^A and R^B are the R groups of the

two adjacent amino acids. These candidates can be evaluated using methods analogous to those described above.

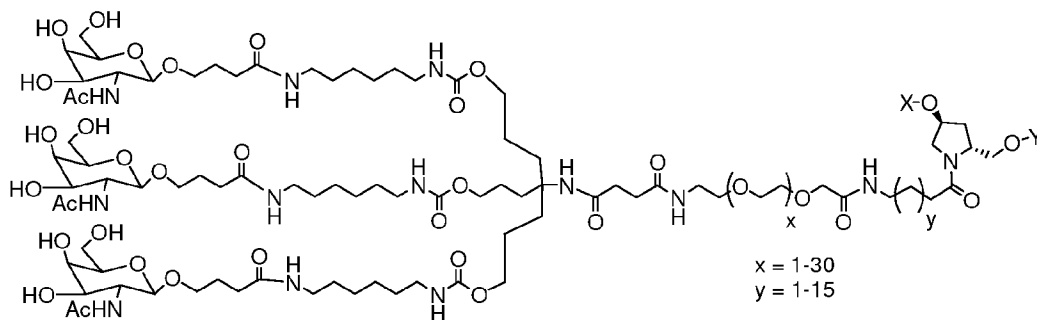
[0034] Representative carbohydrate conjugates with linkers include, but are not limited to,



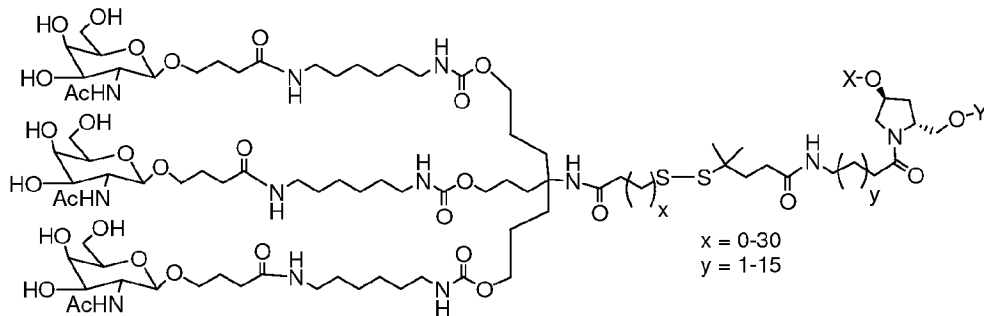
(Formula XXIV),



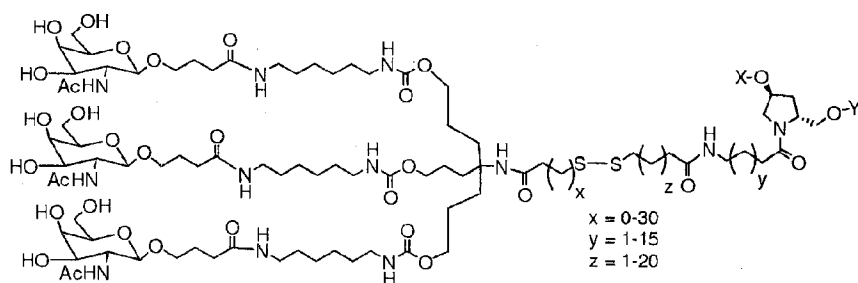
(Formula XXV),



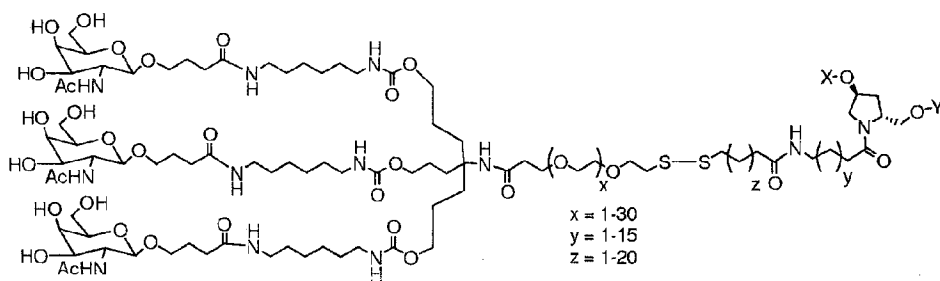
(Formula XXVI),



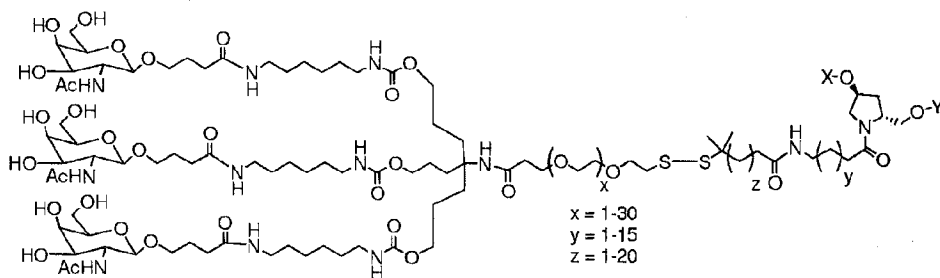
(Formula XXVII),



(Formula XXVIII),



(Formula XXIX), and



(Formula XXX),

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

[00160] 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.

[00161] It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications can be incorporated in a single compound or even at a single nucleoside within an iRNA. The present invention also includes iRNA compounds that are chimeric compounds. "Chimeric" iRNA compounds or "chimeras," in the context of this invention, are iRNA compounds, preferably dsRNAs, which contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of a dsRNA compound. These iRNAs typically contain at least one region wherein the RNA is modified so as to confer upon the iRNA increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the iRNA can 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.

[00162] 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; Lctsingcr *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 can 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

[00163] 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

[00164] 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).

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 can degrade the agent, and permits a lower total dose of the iRNA molecule to be administered. Several studies have shown successful knockdown of gene products when an iRNA is administered locally. For example, intraocular delivery of a VEGF dsRNA by intravitreal injection in cynomolgus monkeys (Tolentino, MJ., et al (2004) Retina 24:132-138) and subretinal injections in mice (Reich, SJ., et al (2003) Mol. Vis. 9:210-216) were both shown to prevent neovascularization in an experimental model of age-related macular degeneration. In addition, direct intratumoral injection of a dsRNA in mice reduces tumor volume (Pille, J., et al (2005) Mol. Ther. 11:267-274) and can prolong survival of tumor-bearing mice (Kim, WJ., et al (2006) Mol. Ther. 14:343-350; Li, S., et al (2007) Mol. Ther. 15:515-523). RNA interference has also shown success with local delivery to the CNS by direct injection (Dorn, G., et al. (2004) Nucleic Acids 32:e49; Tan, PH., et al (2005) Gene Ther. 12:59-66; Makimura, H., et al (2002) BMC Neurosci. 3:18; Shishkina, GT., et al (2004) Neuroscience 129:521-528; Thakker, ER., et al (2004) Proc. Natl. Acad. Sci. U.S.A. 101:17270-17275; Akaneya, Y., et al (2005) J. Neurophysiol. 93:594-602) and to the lungs by intranasal administration (Howard, KA., et al (2006) Mol. Ther. 14:476-484; Zhang, X., et al (2004) J. Biol. Chem. 279:10677-10684; Bitko, V., et al (2005) Nat. Med. 11:50-55). For administering an iRNA systemically for the treatment of a disease, the RNA can be modified or alternatively delivered using a drug delivery system; both methods act to prevent the rapid degradation of the dsRNA by endo- and exo-nucleases *in vivo*. Modification of the RNA or the pharmaceutical carrier can also permit targeting of the iRNA composition to the target tissue and avoid undesirable off-target effects. iRNA molecules can be modified by chemical conjugation to lipophilic groups such as cholesterol to enhance cellular uptake and prevent degradation. For example, an iRNA directed against ApoB conjugated to a lipophilic cholesterol moiety was injected systemically into mice and resulted in knockdown of apoB mRNA in both the liver and

jejunum (Soutschek, J., et al (2004) *Nature* 432:173-178). Conjugation of an iRNA to an aptamer has been shown to inhibit tumor growth and mediate tumor regression in a mouse model of prostate cancer (McNamara, JO., et al (2006) *Nat. Biotechnol.* 24:1005-1015). In an alternative embodiment, the iRNA can be delivered using drug delivery systems such as a nanoparticle, a dendrimer, a polymer, 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).

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.

Vector encoded dsRNAs

[00165] In another aspect, iRNA targeting the COL1A1, TGF β and/or SMAD2/3 gene can be expressed from transcription units inserted into DNA or RNA vectors (see, *e.g.*, Couture, A, *et al.*, *TIG.*, 1996, 12:5-10; Skillern, A., *et al.*, International PCT Publication No. WO 00/22113, Conrad, PCT Publication No. WO 00/22114, and Conrad, U.S. Pat. No. 6,054,299). Expression can be transient (on the order of hours to weeks) or sustained (weeks to months or longer), depending upon the specific construct used and the target tissue or cell type. These transgenes can be introduced as a linear construct, a circular plasmid, or a viral vector, which can be an integrating or non-integrating vector. The transgene can also be constructed to permit it to be inherited as an extrachromosomal plasmid (Gassmann, *et al.*, *Proc. Natl. Acad. Sci. USA*, 1995, 92:1292).

[00166] 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 inverted repeat polynucleotides joined by a linker polynucleotide sequence such that the dsRNA has a stem and loop structure.

[00167] iRNA expression vectors are generally DNA plasmids or viral vectors. Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can be used to produce recombinant constructs for the expression of an iRNA as described herein. Eukaryotic cell expression vectors are well known in the art and are available from a number of commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired nucleic acid segment. Delivery of iRNA expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that allows for introduction into a desired target cell.

[00168] iRNA expression plasmids can be transfected into target cells as a complex with cationic lipid carriers (*e.g.*, Oligofectamine) or non-cationic lipid-based carriers (*e.g.*, Transit-TKO™). Multiple lipid transfections for iRNA-mediated knockdowns targeting different regions of a target RNA over a period of a week or more are also contemplated by the invention. Successful introduction of vectors into host cells can be monitored using various known methods. For example, transient transfection can be signaled with a reporter, such as a fluorescent marker, such as Green Fluorescent Protein (GFP). Stable transfection of cells *ex vivo* can be ensured using markers that provide the transfected cell with resistance to specific environmental factors (*e.g.*, antibiotics and drugs), such as hygromycin B resistance.

[00169] 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) SV 40 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 can 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.

[00170] 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.

[00171] 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-beta-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.

[00172] 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.

[00173] 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.

[00174] 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.

[00175] Another preferred viral vector is a pox virus such as a vaccinia virus, for example an attenuated vaccinia such as Modified Virus Ankara (MVA) or NYVAC, an avipox such as fowl pox or canary pox.

[00176] 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.

[00177] 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

[00178] In one embodiment, provided herein are pharmaceutical compositions containing an iRNA and a pharmaceutically acceptable carrier. The pharmaceutical composition containing the iRNA is useful for treating hepatic fibrosis related to the overexpression a COL1A1 gene. Such pharmaceutical compositions are formulated based on the mode of delivery. One example is compositions that are formulated for systemic administration via parenteral delivery, *e.g.*, by intravenous (IV) delivery. Another example is compositions that are formulated for direct delivery into the brain parenchyma, *e.g.*, by infusion into the brain, such as by continuous pump infusion.

[00179] The pharmaceutical compositions featured herein are administered in dosages sufficient to inhibit expression of COL1A1, TGF β and/or SMAD2/3 genes. In general, a suitable dose of iRNA will be in the range of 0.01 to 200.0 milligrams per kilogram body weight of the recipient per day, generally in the range of 1 to 50 mg per kilogram body weight per day. For example, the dsRNA can be administered at 0.05 mg/kg, 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 3 mg/kg, 10 mg/kg, 20 mg/kg, 30 mg/kg, 40 mg/kg, or 50 mg/kg per single dose. The pharmaceutical composition can be administered once daily or the iRNA can 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 could be used with the agents of the present invention. In this embodiment, the dosage unit contains a corresponding multiple of the daily dose.

[00180] The effect of a single dose on COL1A1, TGF β and/or SMAD2/3 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.

[00181] The skilled artisan will appreciate that certain factors can 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.

[00182] A number of mouse models for the study of liver fibrosis of various causes are available, for example, as carbon tetrachloride (CCl₄), dimethylnitrosamine and thioacetamide-treated mouse models. Such models can be used for *in vivo* testing of iRNA, as well as for determining a therapeutically effective dose.

[00183] 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 can be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration can be 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.

[00184] The iRNA can be delivered in a manner to target a particular tissue, such as the liver (*e.g.*, the HSCs of the liver).

[00185] Pharmaceutical compositions and formulations for topical administration can 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 can be necessary or desirable. Coated condoms, gloves and the like can 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, distearoylphosphatidyl choline) negative (*e.g.*, dimyristoylphosphatidyl glycerol DMPG) and cationic (*e.g.*, dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). iRNAs featured in the invention can be encapsulated within liposomes or can form complexes thereto, in particular to cationic liposomes. Alternatively, iRNAs can 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, dicaprato, tricaprato, monoolein, dilaurin, glyceryl 1-monocaprato, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C₁₋₂₀ 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.

Liposomal formulations

[00186] 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.

[00187] 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*.

[00188] 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.

[00189] 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.

[00190] 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 can act.

[00191] 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.

[00192] 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

[00193] 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 pII 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).

[00194] 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).

[00195] 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.

[00196] 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).

[00197] 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 NovasomeTM I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and NovasomeTM II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporine A into different layers of the skin (Hu *et al.* S.T.P.Pharm. Sci., 1994, 4(6):466).

[00198] 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).

[00199] 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.*).

[00200] 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, 2C₁₂15G, 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.*, DSPF-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Pat. Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Pat. No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Pat. No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Pat. No. 5,540,935 (Miyazaki *et al.*) and U.S. Pat. No. 5,556,948 (Tagawa *et al.*) describes PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

[00201] 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 can 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.

[00202] Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes can 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.

[00203] 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).

[00204] 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.

[00205] 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.

[00206] 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.

[00207] 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.

[00208] 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

[00209] In one embodiment, a COL1A1, TGF β and SMAD2/3 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.

[00210] 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.

[00211] The cationic lipid can be, for example, N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleoyloxypropylamine (DODMA), 1,2-DiLinoleoyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-Dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-Dilinoleoxy-3-(dimethylamino)acetoxyp propane (DLin-DAC), 1,2-Dilinoleoxy-3-morpholinopropane (DLin-MA), 1,2-Dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-Dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-Linoleoyl-2-linoleoyloxy-3-dimethylaminopropane (DLin-2-DMA), 1,2-Dilinoleoxyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-Dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-Dilinoleoxyloxy-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-Dilinoleoxyloxy-3-(2-N,N-

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

[00212] 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,

[00213] 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.

[00214] The non-cationic lipid can 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), palmitoyl-oleoylphosphatidylcholine (POPC), palmitoyl-oleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-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-phosphatidylethanolamine (SOPE), cholesterol, or a mixture thereof. The non-cationic lipid can 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.

[00215] The conjugated lipid that inhibits aggregation of particles can be, for example, a polyethyleneglycol (PEG)-lipid including, without limitation, a PEG-diacylglycerol (DAG), a PEG-dialkylxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or a mixture thereof. The PEG-DAA conjugate can be, for example, a PEG-dilauryloxypropyl (C_{12}), a PEG-dimyristyloxypropyl (C_{14}), a PEG-dipalmitoxypropyl (C_{16}), or a PEG-distearoxypropyl (C_{18}). The conjugated lipid that prevents aggregation of particles can be from 0 mol % to about 20 mol % or about 2 mol % of the total lipid present in the particle.

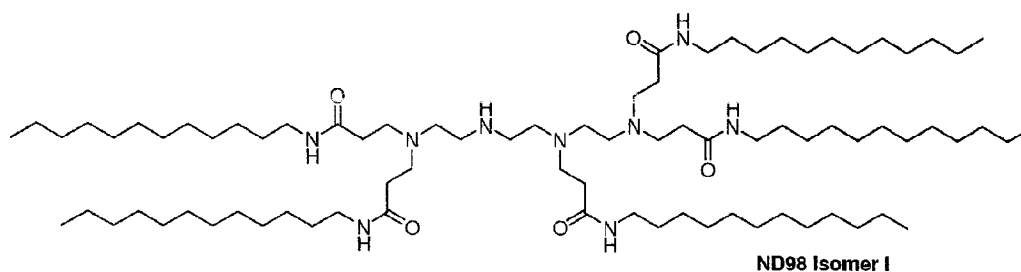
[00216] In some embodiments, the nucleic acid-lipid particle further includes cholesterol at, *e.g.*, about 10 mol % to about 60 mol % or about 48 mol % of the total lipid present in the particle.

LNP01

[00217] In one embodiment, the lipidoid ND98-4HCl (MW 1487) (see U.S. Patent Application No. 12/056,230, filed 3/26/2008),

Cholesterol (Sigma-Aldrich),

and PEG-Ceramide C16 (Avanti Polar Lipids) can be used to prepare lipid-dsRNA nanoparticles (*i.e.*, LNP01 particles). Stock solutions of each in ethanol can be prepared as follows: ND98, 133 mg/ml; Cholesterol, 25 mg/ml, PEG-Ceramide C16, 100 mg/ml. The ND98, Cholesterol, and PEG-Ceramide C16 stock solutions can then be combined in a, *e.g.*, 42:48:10 molar ratio. The combined lipid solution can be mixed with aqueous dsRNA (*e.g.*, in sodium acetate pH 5) such that the final ethanol concentration is about 35-45% and the final sodium acetate concentration is about 100-300 mM. Lipid-dsRNA nanoparticles typically form spontaneously upon mixing. Depending on the desired particle size distribution, the resultant nanoparticle mixture can be extruded through a polycarbonate membrane (*e.g.*, 100 nm cut-off) using, for example, a thermobarrel extruder, such as Lipex Extruder (Northern Lipids, Inc). In some cases, the extrusion step can be omitted. Ethanol removal and simultaneous buffer exchange can be accomplished by, for example, dialysis or tangential flow filtration. Buffer can be exchanged with, for example, phosphate buffered saline (PBS) at about pH 7, *e.g.*, about pH 6.9, about pH 7.0, about pH 7.1, about pH 7.2, about pH 7.3, or about pH 7.4.



Formula I

[00218] LNP01 formulations are described, *e.g.*, in International Application Publication No. WO 2008/042973,

[00219] Additional exemplary lipid-dsRNA formulations are as follows:

	Cationic Lipid	cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate Lipid:siRNA ratio
SNALP-1	1,2-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)ethylazanediyldidodecan-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-dimyristoyl 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)

[00220] SNALP (1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)) comprising formulations are described in International Publication No. WO2009/127060, filed April 15, 2009.

[00221] 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.

[00222] 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.

[00223] ALNY-100 comprising formulations are described, *e.g.*, International patent application number PCT/US09/63933, filed on November 10, 2009.

[00224] 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.

Synthesis of cationic lipids

[00225] Any of the compounds, *e.g.*, cationic lipids and the like, used in the nucleic acid-lipid particles of the invention can be prepared by known organic synthesis techniques, including the methods described in more detail in the Examples. All substituents are as defined below unless indicated otherwise.

[00226] "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.

[00227] "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.

[00228] “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-butylnyl, 2-butylnyl, 1-pentylnyl, 2-pentylnyl, 3-methyl-1 butynyl, and the like.

[00229] “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.

[00230] “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 can be optionally oxidized, and the nitrogen heteroatom can be optionally quaternized, including bicyclic rings in which any of the above heterocycles are fused to a benzene ring. The heterocycle can be attached via any heteroatom or carbon atom. Heterocycles include heteroaryls as defined below. Heterocycles include morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, and the like.

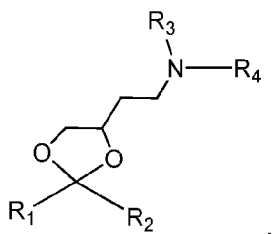
[00231] The terms “optionally substituted alkyl”, “optionally substituted alkenyl”, “optionally substituted alkynyl”, “optionally substituted acyl”, and “optionally substituted heterocycle” means that, when substituted, at least one hydrogen atom is replaced with a substituent. In the case of an oxo 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^xSO₂R^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 can 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^xSO₂R^y, -C(=O)R^x, -C(=O)OR^x, -C(=O)NR^xR^y, -SO_nR^x and -SO_nNR^xR^y.

[00232] “Halogen” means fluoro, chloro, bromo and iodo.

[00233] In some embodiments, the methods of the invention can require the use of protecting groups. Protecting group methodology is well known to those skilled in the art (see, for example, Protective Groups in Organic Synthesis, Green, T.W. *et al.*, Wiley-Interscience, New York City, 1999). Briefly, protecting groups within the context of this invention are any group that reduces or eliminates unwanted reactivity of a functional group. A protecting group can be added to a functional group to mask its reactivity during certain reactions and then removed to reveal the original functional group. In some embodiments an “alcohol protecting group” is used. An “alcohol protecting group” is any group which decreases or eliminates unwanted reactivity of an alcohol functional group. Protecting groups can be added and removed using techniques well known in the art.

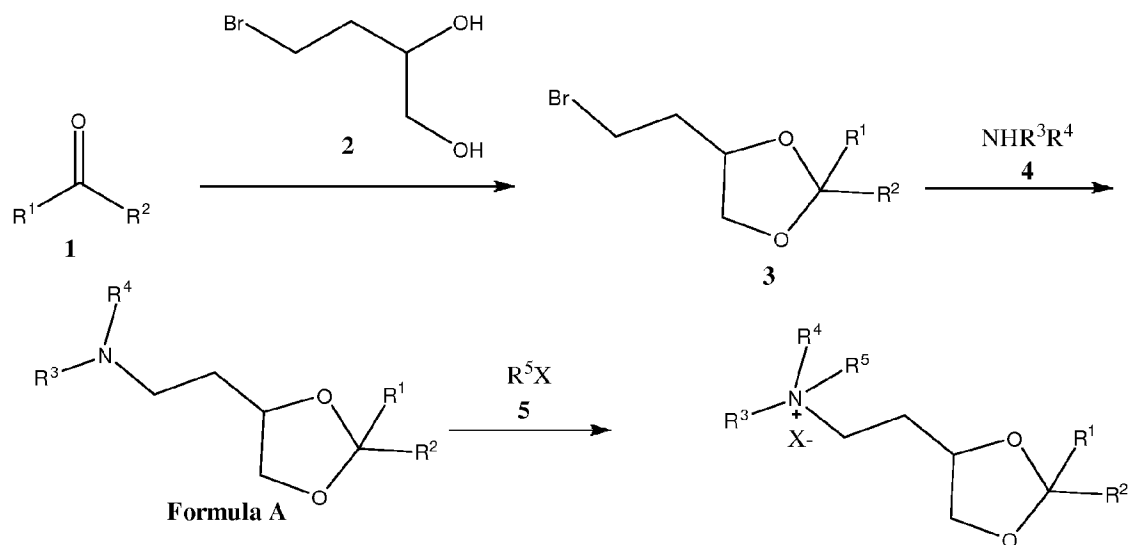
Synthesis of Formula A

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



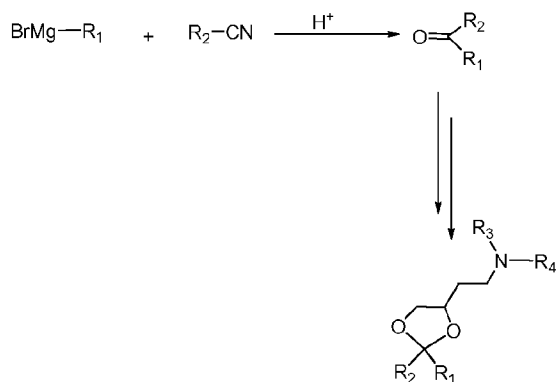
where R₁ and R₂ are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R₃ and R₄ are independently lower alkyl or R₃ and R₄ can be taken together to form an optionally substituted heterocyclic ring. In some embodiments, the cationic lipid is XTC (2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane).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



[00234] Alternatively, the ketone 1 starting material can be prepared according to Scheme 2.

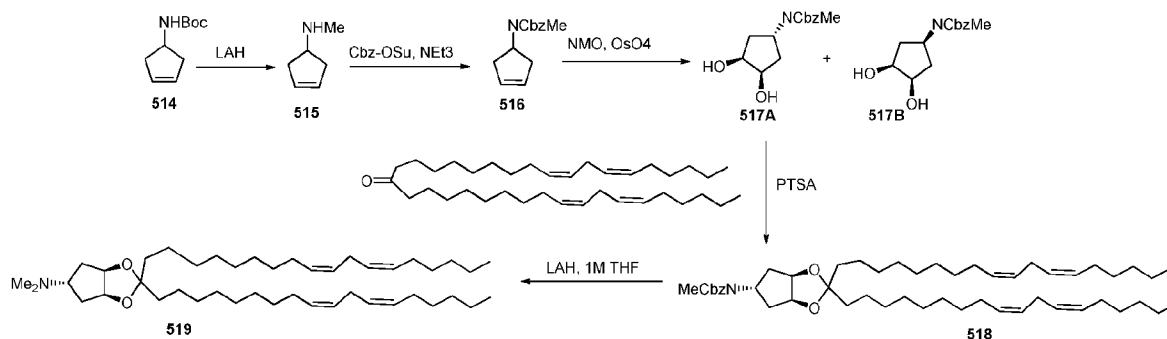
Grignard reagent 6 and cyanide 7 can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of 6 and 7 yields ketone 1. Conversion of ketone 1 to the corresponding lipids of formula A is as described in Scheme 1.

Synthesis of MC3

[00235] 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

[00236] Synthesis of ketal 519 [ALNY-100] was performed using the following scheme 3:



Synthesis of 515

[00237] 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 °C 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 °C 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

[00238] 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 °C 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

[00239] 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 anhyd. 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

[00240] 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

[00241] 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

[00242] 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 δ = 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, 22.6, 14.1; Electrospray MS (+ve): Molecular weight for C₄₄H₈₀NO₂ (M + H)⁺ Calc. 654.6, Found 654.6.

[00243] 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.

[00244] 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 minitabets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders can 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 enhancer 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, glucolic 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 can 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, pullulans, 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(isohexylcyanoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(D,L-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.

[00245] Compositions and formulations for parenteral, intraparenchymal (into the brain), intrathecal, intraventricular or intrahepatic administration can include sterile aqueous solutions which can also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

[00246] Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions can be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids. Particularly preferred are formulations that target the liver when treating hepatic disorders such as hepatic carcinoma.

[00247] The pharmaceutical formulations of the present invention, which can conveniently be presented in unit dosage form, can 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.

[00248] The compositions of the present invention can be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention can also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions can further contain

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

Additional Formulations

Emulsions

[00249] The compositions of the present invention can be prepared and formulated as emulsions. Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μ m 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 can 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 can contain additional components in addition to the dispersed phases, and the active drug which can 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 can also be present in emulsions as needed. Pharmaceutical emulsions can 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.

[00250] 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 can 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 can be incorporated into either phase of the emulsion. Emulsifiers can 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).

[00251] 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 can 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).

[00252] 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.

[00253] A large variety of non-emulsifying materials is 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).

[00254] 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.

[00255] Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that can 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 can 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.

[00256] 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.

[00257] In one embodiment of the present invention, the compositions of iRNAs and nucleic acids are formulated as microemulsions. A microemulsion can 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).

[00258] 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.

[00259] 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 can, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase can 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 can 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.

[00260] 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 can form spontaneously when their components are brought together at ambient temperature. This can 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.

[00261] Microemulsions of the present invention can 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 can 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

[00262] 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 can 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.

[00263] Penetration enhancers can 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.

[00264] 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).

[00265] 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, dicaprinate, tricaprinate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one,

acylcarnitines, acylcholines, C₁₋₂₀ 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).

[00266] 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), glucolic acid (sodium glucolate), 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).

[00267] Chelating Agent. 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 beta-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).

[00268] Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of iRNAs through the alimentary mucosa

(see *e.g.*, Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33). This class of penetration enhancers includes, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, J. Pharm. Pharmacol., 1987, 39, 621-626).

[00269] Agents that enhance uptake of iRNAs at the cellular level can 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), XtremeGENE 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; Marscille, France), EcoTransfect (OZ Biosciences; Marscille, France), TransPass® 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.

[00270] Other agents can 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

[00271] 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

[00272] 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 can 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.*).

[00273] 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.

[00274] Formulations for topical administration of nucleic acids can 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 can 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.

[00275] 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

[00276] The compositions of the present invention can additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions can contain additional, compatible, pharmaceutically-active materials such as,

for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or can 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.

[00277] Aqueous suspensions can contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension can also contain stabilizers.

[00278] In some embodiments, pharmaceutical compositions featured in the invention include (a) one or more iRNA compounds and (b) one or more other agents which function by a non-RNAi mechanism. Examples of such other agents include but are not limited one or more recombinant cytokines (*e.g.*, IL6, IFN- γ , and TNF), PPAR gamma ligands, and corticosteroids. Basically, the other non-RNAi agents are targeted at the underlying causes of the liver fibrosis and/or aimed at alleviating the symptoms of the condition. Therapeutics for the underlying causes of liver fibrosis are known in the art. Some of such therapeutics and strategies are shown in Table 2.

[00279] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds that exhibit high therapeutic indices are preferred.

[00280] The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of compositions featured herein lies generally within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the methods featured in the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range of the compound or, when appropriate, of the polypeptide product of a target sequence (*e.g.*, achieving a decreased concentration of the polypeptide) that includes the IC₅₀ (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

[00281] In addition to their administration, as discussed above, the iRNAs featured herein can be administered in combination with other known agents effective in treatment of hepatic fibrosis mediated by COL1A1 overexpression. 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.

IV. The iRNA target genes: COL1A1, TGF β , and SMAD2/3

[00282] The *Homo sapiens* collagen, type I, alpha 1 (COL1A1) is also known as collagen alpha-1(I) chain; alpha-1 type I collagen; pro-alpha-1 collagen type 1; collagen alpha 1 chain type I; collagen of skin, tendon and bone, alpha-1 chain; OI4; and COL1A1.

[00283] The COL1A1 gene encodes the pro-alpha1 chains of type I collagen whose triple helix comprises two alpha1 chains and one alpha2 chain. Type I collagen is a fibril-forming collagen found in most connective tissues and is abundant in bone, cornea, dermis and tendon. Mutations in this gene are associated with osteogenesis imperfecta types I-IV, Ehlers-Danlos syndrome type VIIA, Ehlers-Danlos syndrome Classical type, Caffey Disease and idiopathic osteoporosis. Reciprocal translocations between chromosomes 17 and 22, where this gene and the gene for platelet-derived growth factor beta are located, are associated with a particular type of skin tumor called dermatofibrosarcoma protuberans, resulting from unregulated expression of the growth factor. Two transcripts, resulting from the use of alternate polyadenylation signals, have been identified for this gene. The human COL1A1 gene is conserved in chimpanzee, dog, cow, mouse, rat, and zebrafish.

[00284] The GENBANK™ Accession No. for the human COL1A1 mRNA is NM_000088.3 and the sequence is provided herein as SEQ ID NO: 172.

[00285] The GENBANK™ Accession No. for the mouse COL1A1 mRNA is NM_007742.3 and the sequence is provided herein as SEQ ID NO: 173.

[00286] The *Homo sapiens* transforming growth factor, beta 1 (TGF β 1) is also known as TGF-beta-1, TGF-beta 1 protein, latency-associated peptide, CED, LAP, DPD1, TGFb and TGFbeta. This gene encodes a member of the transforming growth factor beta (TGF β) family of cytokines, which are multifunctional peptides that regulate proliferation, differentiation, adhesion, migration, and other functions in many cell types. Many cells have TGF β receptors, and the protein positively and negatively regulates many other growth factors. The secreted protein is cleaved into a latency-associated peptide (LAP) and a mature TGF β 1 peptide, and is found in either a latent form composed of a TGF β 1 homodimer, a LAP homodimer, and a latent TGF β 1-binding protein, or in an active form composed of a TGF β 1 homodimer. The mature peptide can also form heterodimers with other TGF β family members. The human TGF β 1 gene is conserved in chimpanzee, dog, mouse, rat, and zebrafish.

[00287] The GENBANK™ Accession No. for the human TGF β 1 mRNA is 1.NM_000660.4 and the sequence is provided herein as SEQ ID NO: 174.

[00288] The Genbank™ Accession No. for the mouse TGF β 1 mRNA is 1.NM_011577.1; GI:6755774 and the sequence is provided herein as SEQ ID NO: 175.

[00289] The *Homo sapiens* SMAD family member 2 (SMAD2) is also known as “mothers against decapentaplegic homolog 2”, MAD homolog 2, Mad protein homolog, Mad-related protein 2, mother against DPP homolog 2, mothers against DPP homolog 2, Sma- and Mad-related protein 2, JV18;

MADH2; MADR2; JV18-1; hMAD-2; hSMAD2; MGC22139; MGC34440; and SMAD2. SMAD proteins are signal transducers and transcriptional modulators that mediate multiple signaling pathways. SMAD2 is recruited to the TGF-beta receptors through its interaction with the SMAD anchor for receptor activation (SARA) protein. In response to TGF-beta signal, SMAD2 is phosphorylated by the TGF-beta receptors. The phosphorylation induces the dissociation of this protein with SARA and the association with the family member SMAD4. The association with SMAD4 is important for the translocation of this protein into the nucleus, where it binds to target promoters and forms a transcription repressor complex with other cofactors. SMAD2 can also be phosphorylated by activin type 1 receptor kinase, and mediates the signal from the activin. Alternatively spliced transcript variants encoding the same protein have been observed. The human SMAD2 gene is conserved in chimpanzee, dog, cow, mouse, rat, chicken, and zebrafish.

[00290] There are three known isoforms of the human SMAD2 transcripts. The GENBANK™ Accession Nos. for these three human SMAD2 mRNAs are NM_001003652.2, NM_001135937.1 and NM_005901.4 and the sequences are provided herein as SEQ ID NO: 176, SEQ ID NO: 177, and SEQ ID NO: 178, respectively.

[00291] There is one known transcript for the mouse SMAD2. The GENBANK™ Accession Nos. for the mouse SMAD2 mRNAs is NM_010754.4 and the sequence is provided herein as SEQ ID NO: 179.

[00292] The *Homo sapiens* SMAD family member 3 (SMAD3) is also known as “mothers against decapentaplegic homolog 3”, MADH3, hMAD-3, hSMAD3, MAD homolog 3, mad protein homolog, mothers against DPP homolog 3, SMA- and MAD-related protein 3, mad homolog JV15-2, HSPC193, HsT17436, MGC60396, DKFZp586N0721, and DKFZp686J10186.

[00293] The protein encoded by this gene belongs to the SMAD, a family of proteins similar to the gene products of the *Drosophila* gene 'mothers against decapentaplegic' (Mad) and the *C. elegans* gene Sma. SMAD3 mediates the signal of the transforming growth factor (TGF)-beta, and thus regulates multiple cellular processes, such as cell proliferation, apoptosis, and differentiation. SMAD3 functions as a transcriptional modulator activated by transforming growth factor-beta and is thought to play a role in the regulation of carcinogenesis. The human SMAD3 gene is conserved in chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, fruit fly, and mosquito.

[00294] There are at least four isoforms of the SMAD3 transcripts. The GENBANK™ Accession Nos. for the four human SMAD3 mRNAs are NM_001145102.1, NM_001145103.1, NM_001145104.1, and NM_005902.3 and the sequences are provided herein as SEQ ID NO: 180, SEQ ID NO: 181, SEQ ID NO: 182, and SEQ ID NO: 183, respectively.

[00295] There is one known transcript for the mouse SMAD3. The GENBANK™ Accession Nos. for the mouse SMAD3 mRNAs is NM_016769.4 and the sequence is provided herein as SEQ ID NO: 184.

V. The liver, liver disease, fibrosis and cirrhosis

[00296] Fibrosis is characterized by the formation of an abnormal amount of fibrous connective tissue in an organ or part as the result of inflammation, irritation, or healing. Fibrotic disorders, include, but are not limited to, systemic sclerosis (SSc), idiopathic pulmonary fibrosis (IPF), interstitial lung

disease (ILD), cirrhosis of the liver, nephrogenic systemic fibrosis (NSF), pneumoconiosis (chronic respiratory disease caused by inhaling metallic or mineral particles), fasciitis, scleromyxedema, retroperitoneal fibrosis, pulmonary fibrosis, liver fibrosis, chronic graft versus host disease and chronic allograft rejection and cystic fibrosis, are characterized by abnormal and excessive deposition of collagen and other extracellular matrix (ECM) components in various tissues. Active fibrosis also can occur in rheumatoid arthritis, lupus, autoimmune diseases, Lyme disease, asthma, idiopathic pulmonary fibrosis, chronic pulmonary fibrosis, uterine fibrosis, and ovarian fibrosis. Although their etiology is quite diverse, the presence of ECM-producing fibroblasts displaying an activated phenotype in the affected tissues is typical of all fibrotic diseases. Fibroblast activation is characterized by a marked increase in the transcriptional activity of the genes encoding type I and type III collagens and fibronectin, initiation of the expression of alpha-smooth muscle actin (α -SMA), and the reduction of ECM degradative activities. Activated fibroblasts display contractile properties resulting from the expression of stress fibers containing α -SMA, and their pro-fibrotic activation is part of a complex set of molecular and biochemical changes that are conserved for multiple sequential passages *in vitro*.

[00297] In the body, the liver is the most complex and metabolically active organ and it performs over 500 vital functions. It is also the major detoxification organ in the body. The liver regulates most chemical levels in the blood and secretes bile, which helps carry away waste products from the liver. All the blood leaving the stomach and intestines passes through the liver. The liver processes this blood and breaks down the nutrients and other components into forms that are become available for use for the rest of the body. Some of the important functions are as follows: (1) provides immunity against infection; (2) manufactures most of the important proteins in the body, and also cholesterol and lipoproteins in which all body fats are carried; (3) clears the blood of most chemicals, drugs and alcohol; (4) excretes bile into the intestine. Bile is vital for digestion of fats, and also serves to carry body wastes; (5) regulates clotting of blood by manufacturing vital proteins; and (6) converts and stores extra sugar (glucose) in the form of starch (glycogen) which can be used in times of starvation. In addition, the liver has an enormous reserve function capacity. All liver functions remain normal even if 70% of it is removed. The liver is also the only organ in the body which can regenerate itself after large portions of it are removed.

[00298] Fibrosis of the liver occurs when an abnormal amount of fibrous connective tissue is produced as the result of inflammation, irritation, or healing caused by an underlying acute or chronic liver condition. Hepatitis is an inflammation of the liver that results in liver cell damage and destruction. Hepatitis can be acute, *i.e.*, acute liver disease, or chronic, *i.e.*, chronic liver disease. Patients who do not recover fully from acute hepatitis can develop chronic hepatitis, as the liver continues to sustain more damage and inflammation. Hepatitis is considered chronic if symptoms persist longer than three months to six months. Inflammation/irritation/healing in a chronic liver condition can be due to a viral (*e.g.* hepatitis A, B, C, D, E or G) or other infection (*Entamoeba histolytica*, malaria parasite *Plasmodium* sp., roundworm *Trichinella spiralis*, the Chinese liver fluke, *Clonorchis sinensis*, and fluke of the genus *Schistosoma*); autoimmune hepatitis; bile duct obstruction or biliary diseases due to biliary atresia, a congenital disorder, primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC); metabolic

disorders (*e.g.*, hemochromatosis—too much iron; Wilson's Disease—too much copper), fat accumulation due to alcohol abuse or the idiopathic non- NASH, which is often associated with diabetes and obesity; and exposure to toxic chemicals, *e.g.*, overdose of drugs such as acetaminophen, paracetamol, dry cleaning chemicals, and some wild mushrooms; and liver cancer. Approximately 90 percent of patients who develop liver tumors suffer from cirrhosis. Types of liver cancer include: hepatocellular carcinoma, fibrolamellar carcinoma, secondary liver cancer, and cholangiocarcinoma (bile duct tumor). Other causes of liver fibrosis include obstruction of outflow of blood from the liver (*i.e.*, Budd-Chiari syndrome), Crigler-Najjar syndrome, Gilbert syndrome, Dubin-Johnson syndrome, hyperbilirubinemia, heart and blood vessel disturbances, alpha1-antitrypsin deficiency, high blood galactose levels, high blood tyrosine levels at birth, glycogen storage disease, diabetes and malnutrition.

[00299] The process that initiates the progression towards chronic liver disease and fibrosis of the liver starts with an acute hepatitis. If acute hepatitis is not resolved, chronic hepatitis occurs and sets the stage for chronic liver disease characterized by fibrosis, and in some cases, cirrhosis. As an illustration, alcohol-induced liver disease caused by excessive consumption of alcohol starts with the fatty liver (Stage I). Fatty liver is excessive accumulation of fat inside the liver cells. This is the most common alcohol-induced liver disorder. The liver is enlarged, causing upper abdominal discomfort on the right side. In stage II, alcoholic hepatitis occurs which is an acute inflammation of the liver, accompanied by the destruction of individual liver cells and scarring. Symptoms can include fever, jaundice, an increased white blood cell count, an enlarged, tender liver, and spider-like veins in the skin. Stage III is alcoholic cirrhosis, characterized by the destruction of normal liver tissue, leaving non-functioning scar tissue. Symptoms can include those of alcoholic hepatitis, in addition to portal hypertension (leading to blood vomiting), enlarged spleen, ascites, excessive bleeding (due to poor clotting), kidney failure, confusion, or liver cancer.

[00300] Cirrhosis of the liver is a chronic disease of the liver characterized by the replacement of normal tissue with fibrous tissue and the loss of functional liver cells. The general architecture of liver cirrhosis is regenerative nodules surrounded by dense fibrotic tissue. The overgrowth of fibrosis scar tissue inhibits the liver's proper functioning. Liver fibrosis usually results in cirrhosis and is a late stage of hepatic fibrosis that has resulted in widespread distortion of normal hepatic architecture. The causes of cirrhosis are the same as those of fibrosis. Cirrhosis is usually considered irreversible. This irreversible scarring of the liver can be life-threatening. In an advanced stage, 80-90% liver can be damaged and replaced with scar tissue. Symptoms of cirrhosis vary, depending on severity. Mild cirrhosis can not exhibit any symptoms at all. Initial symptoms of liver cirrhosis can include general symptoms such as tiredness, lethargy, yellowness of eyes and urine (mild jaundice), swollen feet, excessive itching and anemia (low hemoglobin). In more advanced stages, the patient can have severe life threatening complications such as blood vomiting, bloated stomach due to water (ascites) in the abdomen which can develop serious infection, mental deterioration and coma, deep jaundice and kidney impairment. In addition, the patient can have a bleeding tendency due to low levels of the liver protein prothrombin, and low platelet count both of which are vital for normal clotting of blood.

[00301] Fibrosis and cirrhosis of the liver can be assessed and diagnosed by medical procedures known to one skilled in the art. The medical procedures include but are not limited to specific laboratory tests, liver function tests, liver biopsy, Doppler ultrasonography, CT and/or MR imaging and cholangiography (x-rays of the bile ducts).

[00302] Specific laboratory tests, liver and function tests include non-invasive diagnostic procedures comprising a series of blood tests which can often determine whether or not the liver is functioning properly. These tests can also distinguish between acute and chronic liver disorders and between hepatitis and cholestasis. The most commonly performed blood tests include: (1) serum bilirubin test - elevated levels of bilirubin often indicate an obstruction of bile flow or a defect in the processing of bile by the liver-bilirubin is produced by the liver and is excreted in the bile; (2) serum albumin test - below-normal levels of albumin, are associated with many chronic liver disorders; (3) serum alkaline phosphatase test - elevated levels of alkaline phosphatase, an enzyme found in the bile, usually indicate an obstruction of bile flow, liver injury, or certain cancers; (4) serum aminotransferases (transaminases) - this enzyme is released from damaged liver cells; (5) prothrombin time (PTT) test - this test measures the time it takes for blood to clot. Blood clotting requires vitamin K and a protein made by the liver. Liver cell damage and bile flow obstruction can both interfere with proper blood clotting; (7) alanine transaminase (ALT) test - this enzyme is released from damaged liver cells; (8) aspartate transaminase (AST) test - this enzyme is released from damaged liver, heart, muscle, or brain cells; (9) gamma-glutamyl transpeptidase test - this enzyme is produced by the liver, pancreas, and kidneys and released into the blood when these organs are injured; (10) lactic dehydrogenase test - this enzyme is released when organs such as the liver, heart, lung, or brain are injured; (11) 5-nucleotidase test - this enzyme is released by the liver when the liver is injured due to bile duct obstruction or impaired bile flow; (12) alpha-fetoprotein test - this protein is produced by the fetal liver and testes, indicating hepatitis or cancer when hepatitis is experienced in adults; (13) mitochondrial antibodies test - the presence of these antibodies can indicate primary biliary cirrhosis, chronic active hepatitis, and certain other autoimmune disorders. In some embodiments, biomarkers of fibrosis are used to assess and diagnose liver fibrosis and/or cirrhosis. Biomarkers of liver fibrosis are known in the art, for example, see Table 9. Test results may be normal or may detect nonspecific abnormalities due to complications of cirrhosis or alcoholism. ALT and AST levels are often modestly elevated. Alkaline phosphatase and γ -glutamyl transpeptidase (GGT) are often normal; elevated levels indicate cholestasis or biliary obstruction. Bilirubin is usually normal but increases when cirrhosis progresses, particularly in primary biliary cirrhosis (see below). Decreased serum albumin and a prolonged PT directly reflect impaired hepatic synthesis—usually an end-stage event. Albumin can also be low when nutrition is poor. Serum globulin increases in cirrhosis and in most liver disorders with an inflammatory component. Anemia is common and usually normocytic with a high RBC distribution width. Anemia is often multifactorial: microcytic from chronic GI bleeding; macrocytic from folate nutritional deficiency or hemolysis (especially in alcohol abuse) and hypersplenism. CBC can also detect leucopenia, thrombocytopenia, or pancytopenia.

[00303] Liver biopsy is currently considered the gold standard for the accurate diagnosis of liver fibrosis and cirrhosis, or for ruling out any coexisting liver disease, staging and grading the severity of liver disease, treatment decisions, patient and provider reassurance, and as a benchmark for gauging future disease progression. A diagnosis biopsy is a very important and helpful test in the diagnosis of numerous diseases that affect the liver and bile ducts. In most cases, this allows establishment of a very specific diagnosis and also permit staging and grading of the condition. Several staging and grading scales are known to in the art, for example, see Table 8 (F. Grünhage and F. Lammert, Chapter 20: Assessment of hepatic fibrosis in chronic viral hepatitis, in *Hepatology: a clinical textbook*. Eds. Mauss, Berg, Rockstroh, Sarrazin and Wedemeyer, 2nd ed. 2010) and the Child-Turcotte-Pugh Scoring System (Merck Medical Manuals). A monitoring liver biopsy can help the doctor monitor the effectiveness of therapy that the patient is receiving for a disease that affects the liver. There are many different types of liver biopsies, such as the percutaneous (via a needle through the skin), transjugular (through the blood vessels), laparoscopic (via small abdomen incisions), fine needle aspiration and open surgery liver biopsy. During the procedure, small pieces of liver tissue are removed in order to be sent to a laboratory for histological examination.

[00304] Other non-invasive techniques for diagnosis and staging liver fibrosis are also used. For example, liver fibrosis markers: α -2-macroglobulin(a-MA), transferrin, apolipoproteinA1, hyaluronic acid (HA), laminin, N-terminal procollagen III(PIIINP), 7S collagen IV (7S-IV), total bilirubin, indirect bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase(AST), AST/ALT, g-glutamyl transpeptidase(GGT), alkaline phosphatase(ALP), albumin, albumin/globulin, blood urea nitrogen(BUN), creatinine(Cr), triglyceride, cholesterol, and high density lipoprotein and low density lipoprotein. Lun-Gen Lu *et al.* (World J. Gastroenterology, 2003, 9(11):2574-2578) measured over 20 non-invasive parameters of 200 patients with chronic liver disease and attempted to correlate inflammatory activity, as determined by the levels of the non-invasive parameters, with fibrosis of consecutive liver biopsies from these patients in order to stage and grade liver fibrosis without having to perform the gold standard liver biopsy. In pathological diagnosis, Stage 1 and Stage 2 indicated mild fibrosis, Stage 3 and Stage 4 indicated severe fibrosis. Lun-Gen Lu *et al.* found that there was a close correlation between liver fibrosis and inflammatory activity. AST, GGT, albumin, albumin/globulin, ALP, AFP, hyaluronic acid, N-terminal procollagen III(P III NP), collagen type IV(Col IV), tissue inhibitors of metalloproteinases-1(TIMP-1), alpha-2-macroglobulin, natural killer cells(NK), some parameters of Doppler ultrasonography, CT and/or MR imaging were all related to the degree of inflammatory activity. GGT, albumin, albumin/globulin, ALP, AFP, hyaluronic acid, Col IV, TIMP-1, alpha-2- macroglobulin, transforming growth factor-beta 1(TGF β 1), NK, some parameters of Doppler ultrasonography, CT and/or MR imaging were all related to the staging of fibrosis. It was found that the level of TGF β and TIMP-1 increased more significantly in inflammation and early stage of fibrosis, indicating that they could reflect the changes of liver inflammation and fibrosis. GGT, albumin, albumin/globulin, ALP, AFP, HA, 7S-IV, and α 2-MA had a positive correlation with liver fibrosis. In this study, PIIINP, laminin, transferrin, and apoproteinA1 which were known to have significant diagnostic value, were not confirmed.

[00305] Treatment of liver disease and consequent liver fibrosis includes, but is not limited to, treating the primary cause of the inflammation or irritation in the liver and controlling the inflammation (see Table 2). In addition to the dsRNA-mediated approaches described herein, treatment can also include one/more of the following: Antiviral Agents - when liver disease or liver fibrosis is caused by hepatitis B or C, inflammation of the liver can be stopped with the injectable antiviral drug interferon-alpha. In addition, in Hepatitis B, oral anti-viral agents such as lamivudine or adefovir can be used, while in Hepatitis C, ribavirin can be used. Corticosteroids - corticosteroids can be used to treat chronic liver disease caused by an autoimmune disorder. However, even when inflammation is suppressed, it is possible that scarring of the liver may continue. Discontinuation of certain drugs - when chronic hepatitis is caused by certain drugs, discontinuing those drugs usually clears up any symptoms. Discontinuance of alcohol - while this is essential for recovery in alcohol induced chronic liver disease, it is also highly advisable in Hepatitis C and other chronic diseases of the liver. Examples of other therapies include those disclosed in Table 2. In addition, traditional Chinese medicine such as Qinggan Capsule, ginkgo leaf, Ruangan, salvia, bupleurum and white peony can be used in combination with the therapeutic methods and compositions for the treatment, prevention and/or management of liver fibrosis.

VI. Methods for inhibiting expression of a COL1A1, TGF β , and/or SMAD2/3 gene

[00306] In one aspect, provided herein is a method of inhibiting COL1A1 expression in a cell, the method comprising: (a) introducing into the cell a dsRNA that targets a COL1A1, TGF β or SMAD2/3 gene in the cell; and (b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of a COL1A1, TGF β or SMAD2/3 gene respectively, thereby inhibiting expression of the COL1A1 gene in the cell. In the embodiments where the dsRNA targets TGF β and/or SMAD2/3 gene expression, the expression of the COL1A1 gene in the cell is indirectly inhibited by way of reduced signaling from TGF β which stimulates the cell to overexpress COL1A1. The inhibition is not by way of degradation of the mRNA transcript of the COL1A1 gene. Reduction in gene expression can be assessed by any methods known in the art and by methods, e. g. qRT-PCR, described herein.

[00307] In one embodiment, the cell is a mammalian cell, preferably a human cell. In another embodiment, the cell is a mammalian liver cell. In a preferred embodiment, the cell is a HSC.

[00308] In one embodiment, more than one dsRNA is introduced into the cell. In one embodiment, the dsRNAs targeting COL1A1, TGF β and/or SMAD2/3 gene are introduced to the cell, *i.e.* a combination of dsRNA are introduced into the cell and more than one gene is targeted. For example, a combination of COL1A1iRNA and TGF β iRNA, a combination of COL1A1iRNA and SMAD2/3iRNA or a combination of COL1A1iRNA, TGF β iRNA and SMAD2/3iRNA.

[00309] In one embodiment, the dsRNA is introduced to the cell, preferably, in a liposome, *e.g.* a LNP-formulated liposome known in the art and/or described herein. In one embodiment, the LNP is formulated to target a specific cell such as a HSC. For example, the LNP-formulated liposome can be

coupled to Vitamin A in order to target the LNP-encapsulated dsRNA to the vitamin A-sequestering HSC.

[00310] In one embodiment, the COL1A1 expression is inhibited by at least, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or even 100%. In the embodiments where TGF β and/or SMAD2/3 genes expression are also inhibited by dsRNAs, their respectively expressions are inhibited by at least 30%, in addition to a decrease in COL1A1 expression.

In one aspect, provided herein is a method for inhibiting the expression of a COL1A1 gene in a mammal, the method comprising (a) administering to the mammal a composition comprising a dsRNA that targets a COL1A1, TGF β , or SMAD2/3 gene in a cell of the mammal; and (b) maintaining the mammal of step (a) for a time sufficient to obtain degradation of the mRNA transcript of a COL1A1, TGF β , or SMAD2/3 gene respectively, thereby inhibiting expression of the COL1A1 gene in the cell. In the embodiments where the dsRNA targets TGF β and/or SMAD2/3 gene expression, the expression of COL1A1 in the cell is indirectly inhibited by way of reduced signaling from TGF β which stimulates the cell to overexpress COL1A1; the inhibition is not by way of degradation of the mRNA transcript of the COL1A1 gene. Reduction in gene expression can be assessed by any methods known in the art and by methods, e. g. qRT-PCR, described herein. In one embodiment, a puncture liver biopsy sample serves as the tissue material for monitoring the reduction in the target gene expression.

[00311] In one embodiment, the method includes administering a composition featured herein to the mammal such that expression of the target COL1A1 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.

[00312] Preferably, the iRNAs useful for the methods and compositions featured herein specifically target RNAs (primary or processed) of the target COL1A1, TGF β , or SMAD2/3 gene. Compositions and methods for inhibiting the expression of these genes using iRNAs can be prepared and performed as described elsewhere herein.

[00313] In some embodiments of the aspects described herein, the methods and compositions featured herein comprise more than one iRNA such that more than one iRNA is administered to the mammal and more than one gene is targeted in the mammal. For example, a combination of COL1A1iRNA and TGF β iRNA, a combination of COL1A1iRNA and SMAD2/3iRNA or a combination of COL1A1iRNA, TGF β iRNA and SMAD2/3iRNA.

[00314] Accordingly, in some embodiments of the aspects described herein, the methods and the composition described herein comprises iRNAs targeting a COL1A1 gene and a TGF β gene. In these methods, both the COL1A1 and TGF β gene expressions are inhibited. In another embodiment, the methods and the composition described herein comprises iRNAs targeting a COL1A1 gene and a SMAD2/3 gene. In these methods, both the COL1A1 and SMAD2/3 gene expressions are inhibited. In yet another embodiment, the methods and the composition described herein comprises iRNAs targeting a

COL1A1 gene, a TGF β gene and a SMAD2/3 gene. In these methods, all three the COL1A1, SMAD2/3 and TGF β gene expressions are inhibited.

[00315] In one embodiment of the aspects described herein, 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 COL1A1, TGF β , or SMAD2/3 gene of the mammal to be treated. When the organism to be treated is a mammal such as a human, the composition can 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.

VII. Methods for treating liver disease and liver fibrosis characterized by the overexpression of a COL1A1 gene

[00316] Aspects and embodiments of the invention relate in particular to the use of an iRNA targeting COL1A1 gene expression and compositions containing at least one iRNA for the treatment of a COL1A1-mediated liver fibrosis. Embodiments of the invention also relate to the use of an iRNA targeting the molecules in the signaling pathway that promote COL1A1 gene expression, *e.g.* TGF β and SMAD2/3. For example, a composition containing an iRNA targeting a COL1A1 gene is used for treating and/or preventing liver fibrosis or a composition containing an iRNA targeting a TGF β and/or SMAD2/3 gene is used for treating and/or preventing liver fibrosis

[00317] In one embodiment, provided herein is a method of treating and/or preventing hepatic fibrosis in a subject in need of such treatment, the method comprising administering to the subject a therapeutically effective amount of the iRNA that targets a COL1A1, TGF β , or SMAD2/3 gene.

[00318] In one embodiment, the method of treating and/or preventing hepatic fibrosis comprises (a) administering to the subject a composition comprising a iRNA that targets a COL1A1, TGF β , or SMAD2/3 gene in a cell of the mammal; and (b) maintaining the subject of step (a) for a time sufficient to obtain degradation of the mRNA transcript of a COL1A1, TGF β , or SMAD2/3 gene respectively, thereby inhibiting or reducing expression of the COL1A1 gene in the cell. In the embodiments where the iRNA targets TGF β and/or SMAD2/3 gene expression, the expression of COL1A1 in the cell is indirectly reduced by way of reduced signaling from TGF β which stimulates the cell to over express COL1A1; the reduction of COL1A1 expression is not by way of degradation of the mRNA transcript of the COL1A1 gene. Reduction in gene expression can be assessed by any methods known in the art and by methods, *e.g.* qRT-PCR, described herein. In one embodiment, a puncture liver biopsy sample serves as the tissue material for monitoring the reduction in the target gene expression.

[00319] In one embodiment, the liver fibrosis is a result of a viral or other infection, an autoimmune disorder, a bile duct obstruction, metabolic disorders, alcoholic abuse, primary biliary cirrhosis, NASH,

exposure to chemicals or cancer. In other embodiments, the liver fibrosis is the results of other indications described herein.

[00320] In one embodiment, more than one dsRNA is administered to the subject, and as a result thereof, more than one gene is targeted. For example, a combination of COL1A1 iRNA and TGF β iRNA, a combination of COL1A1 iRNA and SMAD2/3 iRNA or a combination of COL1A1 iRNA, TGF β iRNA and SMAD2/3 iRNA.

[00321] In one embodiment, the method of treating and/or preventing hepatic fibrosis described herein comprises iRNAs targeting a COL1A1 gene and a TGF β gene. In this embodiment, both the COL1A1 and TGF β gene expressions are inhibited. In another embodiment, the method of treating and/or preventing hepatic fibrosis described herein comprises iRNAs targeting a COL1A1 gene and a SMAD2/3 gene. In this embodiment, both the COL1A1 and SMAD2/3 gene expression are inhibited. In yet another embodiment, the method of treating and/or preventing hepatic fibrosis described herein comprises iRNAs targeting a COL1A1 gene, a TGF β gene and a SMAD2/3 gene. In this embodiment, all three of the COL1A1, SMAD2/3 and TGF β gene expressions are inhibited.

[00322] In one embodiment, iRNAs targeting a COL1A1 gene, a TGF β gene, and a SMAD2/3 gene are administered.

[00323] In one embodiment, the iRNA is administered at a concentration of 0.01 mg/kg-5 mg/kg bodyweight of the subject.

[00324] In one embodiment, the method further relates to the use of an iRNA or a pharmaceutical composition thereof, *e.g.*, for treating and/or preventing chronic liver disease or hepatic fibrosis, in combination with other pharmaceuticals and/or other therapeutic methods, *e.g.*, with known pharmaceuticals and/or known therapeutic methods, for example, those which are currently employed for treating the underlying causes of the chronic liver disease or hepatic fibrosis. For example, the iRNA or pharmaceutical composition thereof can also be administered in conjunction with one or more additional anti-hepatic cancer treatments, such as biological chemotherapy and radiation. Accordingly, a treatment can include, for example, chemotherapy (for example, chlorambucil, prednisone, prednisolone, vincristine, cytarabine, clofarabine, farnesyl transferase inhibitors, decitabine, inhibitors of MDR1), rituximab, interferon- α , anthracycline drugs (such as daunorubicin or idarubicin), L-asparaginase, doxorubicin, cyclophosphamide, doxorubicin, bleomycin, fludarabine, etoposide, pentostatin, or cladribine), bone marrow transplant, stem cell transplant, anti-metabolite drugs (methotrexate and 6-mercaptopurine), or any combination thereof.

[00325] Chemotherapy is the treatment of cancer with drugs that can destroy cancer cells. In current usage, the term "chemotherapy" usually refers to cytotoxic drugs which affect rapidly dividing cells in general, in contrast with targeted therapy. Chemotherapy drugs interfere with cell division in various possible ways, *e.g.* with the duplication of DNA or the separation of newly formed chromosomes. Most forms of chemotherapy target all rapidly dividing cells and are not specific to cancer cells, although some degree of specificity may come from the inability of many cancer cells to repair DNA damage, while

normal cells generally can. Most chemotherapy regimens are given in combination. Exemplary chemotherapeutic agents include, but are not limited to, 5-FU Enhancer, 9-AC, AG2037, AG3340, Aggrecanase Inhibitor, Aminoglutethimide, Amsacrine (m-AMSA), Asparaginase, Azacitidine, Batimastat (BB94), BAY 12-9566, BCH-4556, Bis-Naphtalimide, Busulfan, Capecitabine, Carboplatin, Carmustaine+Polifepir Osan, cdk4/cdk2 inhibitors, Chlorombucil, CI-994, Cisplatin, Cladribine, CS-682, Cytarabine HCl, D2163, Dactinomycin, Daunorubicin HCl, DepoCyt, Dexifosamide, Docetaxel, Dolastain, Doxifluridine, Doxorubicin, DX8951f, E 7070, EGFR, Epirubicin, Erythropoietin, Estramustine phosphate sodium, Etoposide (VP16-213), Farnesyl Transferase Inhibitor, FK 317, Flavopiridol, Floxuridine, Fludarabine, Fluorouracil (5-FU), Flutamide, Fragyline, Gemcitabine, Hexamethylmelamine (HMM), Hydroxyurea (hydroxycarbamide), Ifosfamide, Interferon Alfa-2a, Interferon Alfa-2b, Interleukin-2, Irinotecan, ISI 641, Krestin, Lemonal DP 2202, Leuprolide acetate (LHRH-releasing factor analogue), Levamisole, LiGLA (lithium-gamma linolenate), Iodine Seeds, Lometexol, Lomustine (CCNU), Marimistat, Mechlorethamine HCl (nitrogen mustard), Megestrol acetate, Meglamine GLA, Mercaptopurine, Mesna, Mitoguazone (methyl-GAG; methyl glyoxal bis-guanylhydrazone; MGBG), Mitotane (o,p'-DDD), Mitoxantrone, Mitoxantrone HCl, MMI 270, MMP, MTA/LY 231514, Octreotide, ODN 698, OK-432, Oral Platinum, Oral Taxoid, Paclitaxel (TAXOL.RTM.), PARP Inhibitors, PD 183805, Pentostatin (2' deoxycoryformycin), PKC 412, Plicamycin, Procarbazine HCl, PSC 833, Raltitrexed, RAS Farnesyl Transferase Inhibitor, RAS Oncogene Inhibitor, Semustine (methyl-CCNU), Streptozocin, Suramin, Tamoxifen citrate, Taxane Analog, Temozolomide, Teniposide (VM-26), Thioguanine, Thiotepa, Topotecan, Tyrosine Kinase, UFT (Tegafur/Uracil), Valrubicin, Vinblastine sulfate, Vindesine sulfate, VX-710, VX-853, YM 116, ZD 0101, ZD 0473/Anormed, ZD 1839, ZD 9331.

[00326] Efficacy of treatment of liver fibrosis or amelioration of liver fibrosis can be assessed, for example by periodic monitoring liver fibrosis markers: α -2-macroglobulin(a-MA), transferrin, apolipoproteinA1, hyaluronic acid (HA), laminin, N-terminal procollagen III(PIIINP), 7S collagen IV (7S-IV), total bilirubin, indirect bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase(ALT), ALT/ALT, g-glutamyl transpeptidase(GGT), alkaline phosphatase(ALP), albumin, albumin/globulin, blood urea nitrogen(BUN), creatinine(Cr), triglyceride, cholesterol, high density lipoprotein and low density lipoprotein and liver puncture biopsy. Liver fibrosis markers can be measured and/or liver puncture biopsy can be performed before treatment (initial readings) and subsequently (later readings) during the treatment regimen. Comparisons of the later readings with the initial readings provide a physician indication of whether the treatment is effective. 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 COL1A1 or pharmaceutical composition thereof, "effective against" a hepatic fibrosis condition indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as a improvement of symptoms, a cure, a reduction in disease load, reduction in tumor mass or cell numbers, extension of life, improvement in quality of life,

or other effect generally recognized as positive by medical doctors familiar with treating liver fibrosis and the related causes.

[00327] A treatment or preventive effect is evident when there is a statistically significant improvement in one or more parameter markers of hepatic fibrosis status, or by a failure to worsen or to develop symptoms where they would otherwise be anticipated. As an example, a favorable change of at least 10% in a measurable parameter of disease, and preferably at least 20%, 30%, 40%, 50% or more can be indicative of effective treatment, for example, a lower level of liver enzyme activity in the serum or an increase in serum albumin indicating an improvement in liver's ability to synthesize albumin. Efficacy for a given iRNA drug or formulation of that drug can also be judged using an experimental animal model of liver fibrosis as known in the art, *e.g.* a mouse model described herein. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant reduction in a marker, symptom or extracellular collagen as viewed histologically is observed.

[00328] Infection by viruses and parasites can cause inflammation and hepatic fibrosis. Some examples are the Hepadnaviridae (Hepatitis A and B viruses); Hepatitis D virus, Hepatitis E virus, and unclassified viruses (*e.g.*, the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=enterally transmitted; class 2=parenterally transmitted (*i.e.*, Hepatitis C); Norwalk and related viruses, and astroviruses). Exemplary parasites include, but are not limited to: *Entamoeba histolytica*; the malaria parasite *Plasmodium* species (*Plasmodium falciparum*, *P. malariae*, *P. ovale*, *P. vivax*), the nematode *Trichinella spiralis*, the trematods *Clonorchis sinensis*, *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum* and any combination thereof.

[00329] In other embodiments, administration of an iRNA targeting COL1A1, TGF β and/or SMAD2/3 is performed in combination with an anti-viral medicament or agent. Exemplary antiviral agents useful for the methods described herein include, but are not limited to, immunoglobulins, amantadine, interferon, nucleoside analogues, and protease inhibitors. Specific examples of antiviral agents include but are not limited to Acemannan; Acyclovir; Acyclovir Sodium; Adefovir; Alovudine; Alvircept Sudotox; Amantadine Hydrochloride; Aranotin; Arildone; Ateviridine Mesylate; Avidine; Cidofovir; Cipamfylline; Cytarabine Hydrochloride; Delavirdine Mesylate; Desciclovir; Didanosine; Disoxaril; Edoxudine; Enviroxime; Enviroxime; Famciclovir; Famotidine Hydrochloride; Fiacitabine; Fialuridine; Fosarilate; Foscamet Sodium; Fosfonet Sodium; Ganciclovir; Ganciclovir Sodium; Idoxuridine; Kethoxal; Lamivudine; Lobucavir; Memotidine Hydrochloride; Methisazone; Nevirapine; Penciclovir; Pirodavin; Ribavirin; Rimantadine Hydrochloride; Saquinavir Mesylate; Somantadine Hydrochloride; Sorivudine; Statolon; Stavudine; Tilorone Hydrochloride; Trifluridine; Valacyclovir Hydrochloride; Vidarabine; Vidarabine Phosphate; Vidarabine Sodium Phosphate; Viroxime; Zalcitabine; Zidovudine; and Zidovudine.

[00330] In further embodiments, administration of an iRNA targeting COL1A, TGF β and/or SMAD2/3 is administered in combination with an anti-parasitic medicament or agent. An "antiparasitic medicament" refers to an agent that kills or inhibits the growth or function of infective parasites.

Examples of antiparasitic medicaments, also referred to as parasiticides, useful for the methods described herein include, but are not limited to, albendazole, amphotericin B, benznidazole, bithionol, chloroquine HCl, chloroquine phosphate, clindamycin, dehydroemetine, diethylcarbamazine, diloxanide furoate, doxycycline, eflornithine, furazolidone, glucocorticoids, halofantrine, iodoquinol, ivermectin, mebendazole, mefloquine, meglumine antimoniate, melarsoprol, metrifonate, metronidazole, niclosamide, nifurtimox, oxamniquine, paromomycin, pentamidine isethionate, piperazine, praziquantel, primaquine phosphate, proguanil, pyrantel pamoate, pyrimethamine-sulfonamides, pyrimethamine-sulfadoxine, quinacrine HCl, quinine sulfate, quinidine gluconate, spiramycin, stibogluconate sodium (sodium antimony gluconate), suramin, tetracycline, thiabendazole, timidazole, trimethoprim-sulfamethoxazole, and tryparsamide, some of which are used alone or in combination with others.

[00331] The iRNA and an additional therapeutic agent can be administered in combination in the same composition, *e.g.*, parenterally, or the additional therapeutic agent can be administered as part of a separate composition or by another method described herein.

[00332] In one embodiment, the iRNA is administered with a non-iRNA therapeutic agent, such as an agent known to treat the underlying cause of the liver disease or hepatic fibrosis, *e.g.*, a viral or other infection, an autoimmune disorder, a bile duct obstruction, metabolic disorders, alcohol abuse, primary biliary cirrhosis, NASH, exposure to chemicals, and liver cancer. Some examples of non-iRNA therapeutics are shown in Table 2. In another embodiment, the iRNA featured described herein, *e.g.*, a dsRNA targeting COL1A1, TGF β and/or SMAD2/3, is administered along with a non-iRNA therapeutic agent, such as an anti-hepatitis C virus agent. For example, an iRNA featured herein can be administered along with an interferon alpha for treatment of hepatitis C infection.

[00333] In one embodiment, the iRNA is COL1A1iRNA and it is administered in combination with a second iRNA targeting a second gene that is involved in signaling pathway leading to the excess deposition of collagen extracellularly in the liver. For example, the second gene can be the gene encoding TGF β , SMAD2 or SMAD3.

[00334] In one embodiment, the iRNA is COL1A1iRNA, TGF β iRNA or SMAD2/3iRNA it is administered in combination with a second iRNA targeting a second gene that is involved in maturation of the pro-collagen produced by the activated HSCs, the collagen-specific chaperone, HSP47.

[00335] Patients can be administered a therapeutic amount of iRNA, such as 0.01 mg/kg, 0.05 mg/kg, 0.1 mg/kg, 0.5 mg/kg, 1.0 mg/kg, 1.5 mg/kg, 2.0 mg/kg, or 2.5 mg/kg dsRNA. The iRNA can be administered by intravenous infusion over a period of time, such as over a 5 minute, 10 minute, 15 minute, 20 minute, or 25 minute period. The administration is repeated, for example, on a regular basis, such as biweekly (*i.e.*, every two weeks) for one month, two months, three months, four months or longer. After an initial treatment regimen, the treatments can be administered on a less frequent basis. For example, after administration biweekly for three months, administration can be repeated once per month, for six months or a year or longer. Administration of the iRNA can reduce COL1A1 levels, *e.g.*, in a cell, tissue, *e.g.* liver tissue 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.

[00336] Before administration of a full dose of the iRNA, patients can be administered a smaller dose, such as a 5% infusion reaction, and monitored for adverse effects, such as an allergic reaction, or for elevated lipid levels or blood pressure. In another example, the patient can be monitored for unwanted immunostimulatory effects, such as increased cytokine (e.g., TNF-alpha or IFN-alpha) levels.

[00337] Owing to the inhibitory effects on COL1A1 expression, a composition according to the invention or a pharmaceutical composition prepared therefrom can enhance the quality of life and prolong survival in that irreversible liver damage can be prevented.

[00338] 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.

In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

EXAMPLES

Example 1: Interference RNA (iRNA) synthesis

Source of reagents

[00339] Where the source of a reagent is not specifically given herein, such reagent can be obtained from any supplier of reagents for molecular biology at a quality/purity standard for application in molecular biology.

Oligonucleotide Synthesis

[00340] Applicants have used several different methods to generate the iRNA molecules described herein. This Example describes one approach that has been used. The ordinarily skilled artisan can use any method known in the art to prepare iRNAs as described herein.

[00341] Oligonucleotides are synthesized on an AKTA oligopilot 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-butyl dimethylsilyl-adenosine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-O-dimethoxytrityl-N4-acetyl-2'-t-butyl dimethylsilyl-cytidine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-O-dimethoxytrityl-N2-isobutryl-2'-t-butyl dimethylsilyl-guanosine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, and 5'-O-dimethoxytrityl-2'-t-butyl dimethylsilyl-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'-fluoro-cytidine-3'-O-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite and 5'-O-dimethoxytrityl-2'-fluoro-

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 (CH₃CN) 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.

[00342] 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 CH₃CN 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)

[00343] 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)

[00344] 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

[00345] 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

[00346] 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% CH₃CN (buffer A) and 20 mM sodium phosphate (pH 8.5) in 10% CH₃CN, 1M NaBr (buffer B). Fractions containing full-length oligonucleotides are pooled, desalted, and lyophilized. Approximately 0.15 OD of desalted oligonucleotides are diluted in water to 150 μ L and then pipetted into special vials for CGE and LC/MS analysis. Compounds are then analyzed by LC-ESMS and CGE.

iRNA preparation

[00347] For the general preparation of iRNA, equimolar amounts of sense and antisense strand are heated in 1xPBS at 95°C for 5 min and slowly cooled to room temperature. Integrity of the duplex is confirmed by HPLC analysis.

[00348] Nucleic acid sequences are represented below using standard nomenclature, and specifically the abbreviations of Table 1.

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

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

Example 2: COL1A1, TGF β , SMAD2/3 siRNAs Design and Synthesis

Transcripts

[00349] Oligonucleotide designs were carried out to identify siRNAs targeting the genes encoding the human collagen1A1 molecule (COL1A1), the human transforming growth factor beta 1 (TGF β), the SMAD family member 2 and 3, and the orthologous sequences from mice (*Mus musculus*) and rat (*Rattus norvegicus*). The design process used the human COL1A1 mRNA NM_000088.3 (SEQ ID NO: 172), the mouse COL1A1 mRNA NM_007742.3 (SEQ ID NO: 173), the human TGF β mRNA NM_000660.4 (SEQ ID NO: 174), the mouse TGF β mRNA NM_011577.1 (SEQ ID NO: 175), the four

human SMAD3 mRNAs NM_001145102.1 (SEQ ID NO: 180), NM_001145103.1 (SEQ ID NO: 181), NM_001145104.1 (SEQ ID NO: 182), and NM_005902.3 (SEQ ID NO: 183), the mouse SMAD3 mRNAs NM_016769.4 (SEQ ID NO: 184), the three human SMAD2 mRNAs are NM_001003652.2 (SEQ ID NO: 176), NM_001135937.1 (SEQ ID NO: 177), and NM_005901.4 (SEQ ID NO: 178), and the mouse SMAD2 mRNAs is NM_010754.4. All sequences were obtained from the NCBI Refseq collection.

[00350] Two sets of oligos were designed: a human-specific set of oligos with 100% identity to human COL1A1, but less than 100% identity in mouse or rat, and a second set of siRNAs with 100% identity to the human, non-human primate, mouse and both rat COL1A1 transcripts. A set of oligos was designed with 100% identity to mouse SMAD2 (NM_010754) and mouse SMAD3 (NM_016769). For TGF β , the oligo set was designed with 100% identity to human and mouse sequences.

siRNA Design and Specificity Prediction

[00351] The specificity of the 19mer oligo sets was predicted from each sequence. The COL1A1, TGF β AND SMAD2/3 siRNAs were used in a comprehensive search against their respective human, or mouse and rat transcriptomes (defined as NM_ and XM_ records within the NCBI Refseq set) using the FASTA algorithm. The Python script 'offtargetFasta.py' was then used to parse the alignments and 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. The off-target score is calculated as follows: mismatches between the oligo and the transcript are given penalties. A mismatch in the seed region in positions 2-9 of the oligo is given a penalty of 2.8; mismatches in the putative cleavage sites 10 and 11 are given a penalty of 1.2, and all other mismatches a penalty of 1. The off-target score for each oligo-transcript pair is then calculated by summing the mismatch penalties. The lowest off-target score from all the oligo-transcript pairs is then determined and used in subsequent sorting of oligos. 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 moderate specific. In picking which oligos to synthesize, we sorted from high to low by the off-target score of the antisense strand and took the best (lowest off-target score) oligo pairs.

Synthesis of COL1A1, TGF β and SMAD2/3 RNA Sequences

[00352] COL1A1, TGF β AND SMAD2/3 iRNA sequences were synthesized on a MerMade 192 synthesizer at 1 μ mol scale.

[00353] For all the 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

Synthesis, Cleavage and deprotection

[00354] The synthesis of COL1A1, TGF β and SMAD2/3 RNA sequences used solid supported oligonucleotide synthesis using phosphoramidite chemistry.

[00355] The synthesis of the above sequences was performed at 1 μ m 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.

[00356] The synthesized sequences were cleaved and deprotected in 96 well plates, using methylamine in the first step and fluoride reagent in the second step. The crude sequences were precipitated using acetone: ethanol (80:20) mix and the pellet were re-suspended in 0.02M 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

[00357] COL1A1, TGF β and SMAD2/3 RNA sequences were purified on AKTA explorer purification system using Source 15Q column. A column temperature of 65C was maintained during purification. 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 purified sequences were desalted on a Sephadex G25 column using AKTA purifier. The desalted COL1A1, TGF β and SMAD2/3 sequences were analyzed for concentration (by UV measurement at A260) and purity (by ion exchange HPLC). The single strands were then submitted for annealing.

EXAMPLE 3: *In vitro* screening of COL1A1 siRNA in NIH3T3 cells

Cell culture and transfections

[00358] NIH3T3 cells were grown to near confluence at 37°C in an atmosphere of 5% CO₂ in DMEM (INVITROGEN™) supplemented with 10% FBS before being released from the plate by trypsinization. Reverse transfection was carried out by adding 5 μ l of Opti-MEM to 5 μ l of siRNA duplexes per well into a 96-well plate along with 10 μ l of Opti-MEM plus 0.2 μ l of Lipofectamine RNAiMax per well (INVITROGEN™, Carlsbad CA, cat # 13778-150) and incubated at room temperature for 15 minutes. Cells were added to the transfection mix and incubated for 24 hours prior to RNA purification. Experiments were performed at 10nM final duplex concentration for single dose screens with each of the COL1A1 duplexes. A subset of 10 duplexes that showed robust silencing in the 10nM single dose screens were assayed over a range of concentrations from 10nM to 10fM using serial dilutions to determine their IC₅₀. Selected AD 21349 (SEQ ID NO: 53 and SEQ ID NO: 54) has an IC₅₀ of 65pM. The siRNA tested were cross-reactive for rodent, NHP, and human (See FIGURE 1).

Total RNA isolation using MagMAX-96 Total RNA Isolation Kit (Applied Biosystem, Forer City CA, part #: AM1830)

[00359] Cells were harvested and lysed in 140 μ l of Lysis/Binding Solution then mixed for 1 minute at 850 rpm using and Eppendorf Thermomixer (the mixing speed was the same throughout the process). Twenty micro liters of magnetic beads and Lysis/Binding Enhancer mixture were added into cell-lysate

and mixed for 5 minutes. Magnetic beads were captured using magnetic stand and the supernatant was removed without disturbing the beads. After removing supernatant, magnetic beads were washed with Wash Solution 1 (isopropanol added) and mixed for 1 minute. Beads were capture again and supernatant removed. Beads were then washed with 150µl Wash Solution 2 (Ethanol added), captured and supernatant was removed. 50µl of DNase mixture (MagMax™ turbo DNase Buffer and Turbo DNase) was then added to the beads and they were mixed for 10 to 15 minutes. After mixing, 100µl of RNA Rebinding Solution was added and mixed for 3 minutes. Supernatant was removed and magnetic beads were washed again with 150µl Wash Solution 2 and mixed for 1 minute and supernatant was removed completely. The magnetic beads were mixed for 2 minutes to dry before RNA was eluted with 50µl of water.

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

[00360] A master mix of 2µl 10X Buffer, 0.8µl 25X dNTPs, 2µl Random primers, 1µl Reverse Transcriptase, 1µl RNase inhibitor and 3.2µl of H₂O per reaction were added into 10µl total RNA. cDNA was generated using a MJ Research or 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

[00361] 2µl of cDNA were added to a master mix containing 0.5µl GAPDH TaqMan Probe (Applied Biosystems Cat # 4326317F), 0.5µl COL1A1, TGFβ or SMAD2/3 TaqMan probe and 5µl Roche Probes Master Mix (Roche Cat # 04887301001) in a total of 10µl per well in a LightCycler 480 384 well plate (Roche cat # 0472974001). Real time PCR was done in a LightCycler 480 Real Time PCR machine (Roche). Each duplex was tested in at least two independent transfections. Each transfection was assayed by qPCR in duplicate.

[00362] Real time data were analyzed using the $\Delta\Delta C_t$ method. Each sample was normalized to GAPDH expression and knockdown was assessed relative to cells transfected with the non-targeting duplex AD-1955. IC₅₀s were defined using a 4 parameter fit model in XLfit. As shown in FIGURE 1, the efficacy of single doses of collagen1A1 siRNA in NIH-3T3 cells was examined in vitro. siRNA AD-21349 (SEQ ID NO: 53 and SEQ ID NO: 54), has an IC₅₀ of ~65 pM on the production of COL1A1 mRNA.

EXAMPLE 4: Carbon tetrachloride (CCl₄)-induced mouse liver fibrosis models

[00363] In general, liver injury can be caused by many factors, for example, toxic chemicals such as carbon tetrachloride (CCl₄), dimethylnitrosamine and thioacetamide; damage due to immunological response *in vivo*, e. g. to heterologous serum and due to pathogens such as schistosomes and viruses; ligation of the common bile duct that leads to biliary fibrosis; and alcohol abuse. Examples of alcoholic liver injury animal models are the baboon placed on ethanol diet and the Tsukamoto/French model in rats.

[00364] The experimental mouse model use herein is one wherein liver injury is induced with the chemical CCl₄. This mouse model is a well-investigated chemical induced model of liver fibrosis.

Hepatic pathological changes observed in CCl₄-treated mice include necrosis, inflammation, and fibrosis, along with increased serum alanine aminotransferase activity. Several microarray studies indicated CCl₄-induced up-regulation of genes involved in liver fibrogenesis, *e.g.* procollagen type 1A, which correlated well with prolong fibrotic changes in the liver (Jiang Y., *et al.*, 2004, *Toxicol. Sci.*, 79:404-410). Oral gavage of Balb/c animals with CCl₄ 1.75ml/kg in mineral oil (MO), was administered on days 1 & 8. At 24 hours after the initial dose of CCl₄, the serum liver enzyme levels were measured. At 10 or 11 days, the animals were sacrificed and the histology of the livers was stained with Sirius red to determine the level of extracellular collagen.

[00365] The level of collagen expression was significantly increased, as were the two liver function biomarker enzymes, alanine transaminase (ALT) and aspartate transaminase (AST), within 24 of the initial dose of CCl₄.

[00366] Similarly, the CCl₄-treated mouse livers showed increased staining for extracellular collagen by way of Sirius red staining compared to control mineral oil treated animals.

EXAMPLE 5: *In vivo* testing of COL1A1 siRNA in CCl₄ mouse model

[00367] To evaluate the AF09 and AF12-based delivery of siCOL1A1 for COL1A1 knock-down in the CCl₄ mouse model, liver injury in Balb/c animals was induced using CCl₄ by oral gavage (2X) on Day1 and Day 8 at a final dose of 1.75ml/kg. Control animals were given mineral oil only. Subsequently, on Day 9, the mice were injected intravenously with the AF09 and AF12 formulated siRNAs and then killed at about 40 hours post siRNA injection on Day 11. Control siRNA used was siLUC (AD1955). The dosage of both siCOL1A1 (AD21349) (SEQ ID NO: 53 and SEQ ID NO: 54) and siLUC (AD1955) was 3mg/kg. The COL1A1 and GAPDH mRNA levels in liver were analyzed using Taqman-QPCR. A total of 10-15 animals were used per group per condition. The COL1A1 mRNA level was normalized to that of the GAPDH mRNA level.

FIGURE 2 illustrates the CCl₄/siRNA treatment regimen and FIGURE 3 summarizes the results obtained. Both the AF09 and AF12 formulated siCOL1A1 (AD21349) (SEQ ID NO: 53 and SEQ ID NO: 54) significantly reduced the level of the COL1A1 mRNA level compared to the control siLUC siRNA in CCl₄-treated mice. In addition, COL1A1 knockdown with AF12-21349 (SEQ ID NO: 53 and SEQ ID NO: 54) was better than AF09-21349 at 3mg/Kg.

[00368] To evaluate the dosage response of the AF12-based delivery siCOL1A1 in the CCl₄-treated mouse model, liver injury in Balb/c animals was induced using CCl₄ by oral gavage (2X) on Day 1 and Day 8 at a final dose of 1.75ml/kg. Control animals were given mineral oil only. On Day 9, the mice were injected intravenously with the AF12 formulated siRNA at various dosages and then killed at about 40 hours post siRNA injection on Day 11. Control siRNA used was AF12_LUC (AD1955). The dosages of both AF12_COL1A1 (AD21349) (SEQ ID NO: 53 and SEQ ID NO: 54) and siLUC (AD1955) ranged from 0.1mg/kg to 3mg/kg. The COL1A1 and GAPDH mRNA levels in liver were analyzed using Taqman-QPCR. A total of 10 animals were used per group per dosage and 5 animals were used for the mineral oil control group. The COL1A1 mRNA level was normalized to that of the GAPDH mRNA level.

[00369] FIGURE 4 illustrates the dosage response in the CCl₄/siRNA treatment regimen and FIGURE 5 summarizes the results obtained. The AF12_COL1A1 (AD21349) (SEQ ID NO: 53 and SEQ ID NO: 54) formulation at dosages of 3 mg/kg, 1mg/kg, and 0.5mg/kg significantly reduced the level of the COL1A1 mRNA level as compared to the control siLUC siRNA in CCl₄ treated mice. The ED₅₀ was ~ 0.1 mg/kg for COL1A1 knockdown with AF12-AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54). The data was normalized to AF12_Luc.

[00370] To evaluate the AF11-based delivery of siCOL1A1 in the mouse model, liver injury in Balb/c animals was induced using CCl₄ by oral gavage (2X) on Day1 and Day 7 at a final dose of 1.75ml/kg. Control animals were given mineral oil only. On Day 8, the mice were injected intravenously with the AF11 formulated siRNAs at various doses and then killed at about 40 hours post siRNA injection on Day 10. Control siRNA used was siLUC (AD1955). The dosage of both siCOL1A1 (AD21349) (SEQ ID NO: 53 and SEQ ID NO: 54) and siLUC (AD1955) was 3mg/kg. The COL1A1 and GAPDH mRNA levels in liver were analyzed using Taqman™-QPCR. A total of 10 animals were used per group per dosage, 5 animals for the mineral oil control group. The COL1A1 mRNA level was normalized to that of the GAPDH mRNA level.

[00371] FIGURE 6 illustrates the CCl₄ /AF11-SiRNA treatment regimen and FIGURE 7 summarizes the results obtained. The AF11_COL1A1 (AD21349) (SEQ ID NO: 53 and SEQ ID NO: 54) formulation at dosage of 3 mg/kg significantly reduced the level of the COL1A1 mRNA level as compared to the control siLUC siRNA in CCl₄-treated mice. Approximately 65% COL1A1 mRNA knockdown was achieved with AF11-21349 formulation. The data was normalized to AF12_Luc.

[00372] Hepatic stellate cells (HSC) are major contributors to the abnormal matrix deposition in during liver fibrosis. These cells undergo transdifferentiation from a “quiescent” HSc into “activated” fibrogenic myofibroblasts both in chronic liver disease. The transdifferentiation is characterized by an increase in proliferation, changes in morphology with expression of α -smooth muscle actin (α -SMA), and the deposition of extracellular matrix proteins, fibrillar collagen (Friedman *et al.*, 1992, Hepatology, 15:234-243; Friedman, 2000, J. Biol. Chem., 275:2247-2250). Uemura M. *et al.*, (2005, Mol. Biol. Cell, 16:4214-4224) demonstrated that SMAD3 overexpression in primary rat HSC and treating primary rat HSC with TGF β led to increased focal adhesion and α -SMA organization. This indicates that TGF β , signalling via SMAD3, plays an important role in the morphological and functional maturation of the “activated” HSC or myofibroblasts.

[00373] To confirm that the AF-based (i. e. AF09, AF11, AF12-based formulation) delivery method did deliver the siRNA to the “activated” HSC or myofibroblasts in the CCl₄-treated mice, the knock-down of α -SMA, which is also overexpressed in the “activated” HSC, was determined using AF09_ACTA2. α -SMA was selected as a target because it is present in activated myofibroblasts, and vascular smooth muscle cells but is absent in hepatocytes. FIGURE 8 showed ACTA2 expression knock-down in the liver cells of the CCl₄-treated mice. This data confirmed the delivery of AF09-ACTA2 to HSC in the CCl₄-treated mice.

EXAMPLE 6: *In vitro* screening of TGF β siRNA in NIH3T3 cells

NIH3T3 cells were grown, cultured and treated with siRNA as described in Example 3 except the siRNAs applied were targeted to the transforming growth factor beta 1 (TGF β) gene. Experiments were performed at 10nM final duplex concentration for single dose screens with each of the TGF β duplexes (FIGURE 9).

A subset of 10 duplexes that showed robust silencing in the 10nM single dose screens was assayed over a range of concentrations from 10nM to 10fM using serial dilutions to determine their IC₅₀. Selected siRNA AD22149 (SEQ ID NO: 91 and SEQ ID NO: 92) and AD22138 (SEQ ID NO: 69 and SEQ ID NO: 70) have an IC₅₀ of ~20 pM and ~70 pM respectively on the mRNA of TGF β . The siRNA tested were cross-reactive for rodent, NHP, and human.

EXAMPLE 7: *In vitro* screening of SMAD2 siRNA in NIH3T3 cells

[00374] NIH3T3 cells were grown, cultured and treated with siRNA as described in Example 3 except the siRNAs applied were targeted to the SMAD family member 2 and 3 (SMAD2 and SMAD3) genes. Experiments were performed at 10nM final duplex concentration for single dose screens with each of the SMAD2/3 duplexes (FIGURE 10).

[00375] A subset of 10 duplexes that showed robust silencing in the 10nM single dose screens was assayed over a range of concentrations from 10nM to 10fM using serial dilutions to determine their IC₅₀. Selected siRNA AD-20916 (SEQ ID NO: 140 and SEQ ID NO: 141) has an IC₅₀ of ~75 pM on the mRNA of SMAD2.

EXAMPLE 8: *In vivo* testing of SMAD2 siRNA in CCl₄ mouse model

[00376] Because SMADs are involved in the TGF β signaling in fibrosis development, it was examined whether if knocking down SMAD expression would affect COL1A1 expression in the liver injury mouse model. To evaluate the LNP-based delivery of siSMAD2 AD-20916 (SEQ ID NO: 140 and SEQ ID NO: 141) for SMAD2 knock-down in the CCl₄ mouse model, liver injury in Balb/c animals was induced using CCl₄ by oral gavage (2X) on Day 0 at a dose of 1.75ml/kg. Control animals were given mineral oil only. On Day 2, the mice were injected intravenously with the LNP-formulated siRNAs and then killed on Day 4 post CCl₄ induction. Control siRNA was siLUC (AD1955). The dosage of both siSMAD2/3 AD-20916 (SEQ ID NO: 140 and SEQ ID NO: 141) and siLUC (AD1955) was 3mg/kg. The SMAD2 and GAPDH mRNA levels in liver were analyzed using Taqman-QPCR. A total of 10 animals were used per group per condition. The SMAD2 mRNA level was normalized to that of the GAPDH mRNA level.

[00377] FIGURE 11 summarizes the results obtained. The siSMAD2/3 AD-20916 (SEQ ID NO: 140 and SEQ ID NO: 141) significantly reduced the level of the SMAD2/3 mRNA level as compared to the control siLUC siRNA in the CCl₄-treated mice. The reduction in SMAD2 expression was approximately 80%.

[00378] To evaluate the LNP-based delivery of siSMAD2 AD-20916 (SEQ ID NO: 140 and SEQ ID NO: 141) for SMAD2 knock-down and its effect on COL1A1 expression in the CCl₄ mouse model, liver

injury in Balb/c animals was induced using CCl₄ by oral gavage (2X) on Day 0 at a dose of 1.75ml/kg. Control animals were given mineral oil only. On Day 2, the mice were injected intravenously with the LNP- formulated siRNAs and then killed on Day 4 post CCl₄ induction. Control siRNA was siLUC (AD1955). The dosage of both siSMAD2/3 AD-20916 (SEQ ID NO: 140 and SEQ ID NO: 141) and siLUC (AD1955) was 3mg/Kg. The SMAD2, COL1A1 and GAPDH mRNA levels in liver were analyzed using Taqman-QPCR. A total of 10 animals were used per group per condition. The SMAD2 mRNA level was normalized to that of the GAPDH mRNA level.

[00379] FIGURE 12 summarizes the results obtained. SMAD2 knock-down using the LNP-based delivery of siSMAD2 AD-20916 led to a decrease in the expression of COL1A1 in the CCl₄-treated mice. The decrease was about 50-60% relative to the Luc siRNA injected animals.

EXAMPLE 9: In vivo demonstration of COL1A1 siRNA inhibition in a mouse model of chronic liver disease

[00380] Chronic liver disease is caused by repeated insults to the liver, resulting in a progressive process of liver destruction and regeneration, that ultimately leads to liver fibrosis and cirrhosis. To evaluate whether effective inhibition of COL1A1 can be mediated under such repeated insults to the liver, and whether such treatment is effective in reducing fibrosis under such circumstances, a mouse model of chronic liver injury was established involving multiple-insults, and the effects of repeated administration of siRNA against COL1A1 for COL1A1 knock-down were determined. In this mouse model of chronic liver injury, liver injury in Balb/c animals was induced using CCl₄ by oral gavage on Day 1, Day 8, Day 15, Day 22, Day 29, and Day 36 at a final dose of 1.75ml/kg. Control animals were given mineral oil only on these same days. On Day 23, Day 27, Day 30, Day 33, and Day 37 the mice were injected intravenously with the AF09 and AF12 formulated COL1A1 siRNAs and then killed at about 40-48 hours post the final siRNA injection, *i.e.*, on Day 39. Control siRNA used was siLUC (AD1955). The dosage of both siCOL1A1 (AD21349) (SEQ ID NO: 53 and SEQ ID NO: 54) and siLUC (AD1955) was 1 mg/kg. The COL1A1 and GAPDH mRNA levels in liver were analyzed using Taqman-QPCR. A total of 10 animals were used per group per condition. The COL1A1 mRNA level was normalized to that of the GAPDH mRNA level.

[00381] FIGURE 13 summarizes the results obtained. Surprisingly, COL1A1 knock-down using the C12-based delivery of siCOL1A1 (AD21349) (SEQ ID NO: 53 and SEQ ID NO: 54) led to a significant and unexpected decrease in the expression of COL1A1 in mice receiving multiple (X6) liver insults over a 39 day period. The decrease was about 95% relative to the Luc siRNA injected animals.

EXAMPLE 10: In vivo demonstration of COL1A1 siRNA inhibition in mouse models of chronic liver disease (CCl₄ Model, TAA Model, and Bile Duct Ligation Model)

[00382] The CCl₄ model leads to liver damage that is severely shifted to the pericentral region only, and thus is a good model of liver damage to the pericentral region of the liver.

[00383] The TAA model leads to uniform liver damage (*i.e.*, pericentral and periportal). The TAA model is slower than CCl₄, and thus is considered to more closely mimic liver injury developing in the human population.

[00384] The bile duct-ligated model is mediated by ligation or cannulation of bile-duct. This model effects the periductal regions and is a good model for biliary fibrosis in humans. In contrast to the other two models, the bile duct ligation model results in continued and progressive injury, *i.e.*, chronic liver disease.

[00385] FIGURE 16 demonstrates experimental design and efficacy of COL1A1 knock-down in a bile duct ligation mouse model using AF12-based delivery of siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54).

[00386] FIGURE 17 shows relative Col1a1 expression in bile duct ligated animals.

[00387] FIGURE 18 demonstrates efficacy of COL1A1 knock-down in a bile duct ligation mouse model using siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54).

[00388] FIGURE 19 demonstrates experimental design and efficacy of COL1A1 knock-down in a TAA mouse model of liver injury using siCOL1A1 AD21349 (SEQ ID NO: 53 and SEQ ID NO: 54).

Table 2. Exemplary diseases in which fibrosis can be reduced by treating the underlying disorder

Disease	Therapy
Hepatitis B	Lamivudine, others
Hepatitis C	Interferon alpha
Autoimmune hepatitis	Corticosteroids
Bile duct obstruction	Surgical decompression
Hemochromatosis	Iron depletion
Alcoholic hepatitis ^b	Corticosteroids
Primary biliary cirrhosis ^b	Ursodeoxycholic acid, MTX
Non-alcoholic steatohepatitis ^c	PPAR gamma ligands

Table 3: COL1A1 siRNA Duplexes

Sense and Corresponding Antisense Duplexes				Sense and Corresponding Antisense Duplexes			
Sense Strand		Corresponding Antisense Strand		Sense Strand		Corresponding Antisense Strand	
SEQ ID NO: 1	sense	SEQ ID NO: 2	antisense	SEQ ID NO: 3	sense	SEQ ID NO: 4	antisense
SEQ ID NO: 5	sense	SEQ ID NO: 6	antisense	SEQ ID NO: 7	sense	SEQ ID NO: 8	antisense
SEQ ID NO: 9	sense	SEQ ID NO: 10	antisense	SEQ ID NO: 11	sense	SEQ ID NO: 12	antisense
SEQ ID NO: 13	sense	SEQ ID NO: 14	antisense	SEQ ID NO: 15	sense	SEQ ID NO: 16	antisense
SEQ ID NO: 17	sense	SEQ ID NO: 18	antisense	SEQ ID NO: 19	sense	SEQ ID NO: 20	antisense
SEQ ID NO: 21	sense	SEQ ID NO: 22	antisense	SEQ ID NO: 23	sense	SEQ ID NO: 24	antisense
SEQ ID NO: 25	sense	SEQ ID NO: 26	antisense	SEQ ID NO: 27	sense	SEQ ID NO: 28	antisense
SEQ ID NO: 29	sense	SEQ ID NO: 30	antisense	SEQ ID NO: 31	sense	SEQ ID NO: 32	antisense
SEQ ID NO: 33	sense	SEQ ID NO: 34	antisense	SEQ ID NO: 35	sense	SEQ ID NO: 36	antisense
SEQ ID NO: 37	sense	SEQ ID NO: 38	antisense	SEQ ID NO: 39	sense	SEQ ID NO: 40	antisense
SEQ ID NO: 41	sense	SEQ ID NO: 42	antisense	SEQ ID NO: 43	sense	SEQ ID NO: 44	antisense
SEQ ID NO: 45	sense	SEQ ID NO: 46	antisense	SEQ ID NO: 47	sense	SEQ ID NO: 48	antisense
SEQ ID NO: 49	sense	SEQ ID NO: 50	antisense	SEQ ID NO: 51	sense	SEQ ID NO: 52	antisense
SEQ ID NO: 53	sense	SEQ ID NO: 54	antisense	SEQ ID NO: 55	sense	SEQ ID NO: 56	antisense
SEQ ID NO: 57	sense	SEQ ID NO: 58	antisense	SEQ ID NO: 59	sense	SEQ ID NO: 60	antisense
SEQ ID NO: 61	sense	SEQ ID NO: 62	antisense				

Table 4. TGF- β siRNA duplexes

Sense and Corresponding Antisense Duplexes				Sense and Corresponding Antisense Duplexes			
Sense Strand		Corresponding Antisense Strand		Sense Strand		Corresponding Antisense Strand	
SEQ ID NO: 63	sense	SEQ ID NO: 64	antisense	SEQ ID NO: 65	sense	SEQ ID NO: 66	antisense
SEQ ID NO: 67	sense	SEQ ID NO: 68	antisense	SEQ ID NO: 69	sense	SEQ ID NO: 70	antisense
SEQ ID NO: 71	sense	SEQ ID NO: 72	antisense	SEQ ID NO: 73	sense	SEQ ID NO: 74	antisense
SEQ ID NO: 75	sense	SEQ ID NO: 76	antisense	SEQ ID NO: 77	sense	SEQ ID NO: 78	antisense
SEQ ID NO: 79	sense	SEQ ID NO: 80	antisense	SEQ ID NO: 81	sense	SEQ ID NO: 82	antisense
SEQ ID NO: 83	sense	SEQ ID NO: 84	antisense	SEQ ID NO: 85	sense	SEQ ID NO: 86	antisense
SEQ ID NO: 87	sense	SEQ ID NO: 88	antisense	SEQ ID NO: 89	sense	SEQ ID NO: 90	antisense
SEQ ID NO: 91	sense	SEQ ID NO: 92	antisense	SEQ ID NO: 93	sense	SEQ ID NO: 94	antisense
SEQ ID NO: 95	sense	SEQ ID NO: 96	antisense	SEQ ID NO: 97	sense	SEQ ID NO: 98	antisense
SEQ ID NO: 99	sense	SEQ ID NO: 100	antisense	SEQ ID NO: 101	sense	SEQ ID NO: 102	antisense
SEQ ID NO: 103	sense	SEQ ID NO: 104	antisense	SEQ ID NO: 105	sense	SEQ ID NO: 106	antisense
SEQ ID NO: 107	sense	SEQ ID NO: 108	antisense	SEQ ID NO: 109	sense	SEQ ID NO: 110	antisense
SEQ ID NO: 111	sense	SEQ ID NO: 112	antisense	SEQ ID NO: 113	sense	SEQ ID NO: 114	antisense
SEQ ID NO: 115	sense	SEQ ID NO: 116	antisense	SEQ ID NO: 117	sense	SEQ ID NO: 118	antisense
SEQ ID NO: 119	sense	SEQ ID NO: 120	antisense	SEQ ID NO: 121	sense	SEQ ID NO: 122	antisense
SEQ ID NO: 123	sense	SEQ ID NO: 124	antisense	SEQ ID NO: 125	sense	SEQ ID NO: 126	antisense
SEQ ID NO: 127	sense	SEQ ID NO: 128	antisense	SEQ ID NO: 129	sense	SEQ ID NO: 130	antisense

Table 5: SMAD2/3 siRNA duplexes

Sense and Corresponding Antisense Duplexes				Sense and Corresponding Antisense Duplexes			
Sense Strand		Corresponding Antisense Strand		Sense Strand		Corresponding Antisense Strand	
SEQ ID NO: 132	sense	SEQ ID NO: 133	antisense	SEQ ID NO: 134	sense	SEQ ID NO: 135	antisense
SEQ ID NO: 136	sense	SEQ ID NO: 137	antisense	SEQ ID NO: 138	sense	SEQ ID NO: 139	antisense
SEQ ID NO: 140	sense	SEQ ID NO: 141	antisense	SEQ ID NO: 142	sense	SEQ ID NO: 143	antisense
SEQ ID NO: 144	sense	SEQ ID NO: 145	antisense	SEQ ID NO: 146	sense	SEQ ID NO: 147	antisense
SEQ ID NO: 148	sense	SEQ ID NO: 149	antisense	SEQ ID NO: 150	sense	SEQ ID NO: 151	antisense
SEQ ID NO: 152	sense	SEQ ID NO: 153	antisense	SEQ ID NO: 154	sense	SEQ ID NO: 155	antisense
SEQ ID NO: 156	sense	SEQ ID NO: 157	antisense	SEQ ID NO: 158	sense	SEQ ID NO: 159	antisense
SEQ ID NO: 160	sense	SEQ ID NO: 161	antisense	SEQ ID NO: 162	sense	SEQ ID NO: 163	antisense
SEQ ID NO: 164	sense	SEQ ID NO: 165	antisense	SEQ ID NO: 166	sense	SEQ ID NO: 167	antisense

Table 6: Human COL1A1 iRNA Sequences Designed on the Basis of the Human *COL1A1* Transcript NM_000088.3 (SEQ ID NO: 172) that are Cross-Reactive with Mouse *Col1a1* Transcript NM_007742.3 (SEQ ID NO: 173)

Sense and Corresponding Antisense Duplexes				Sense and Corresponding Antisense Duplexes			
Sense Strand		Corresponding Antisense Strand		Sense Strand		Corresponding Antisense Strand	
SEQ ID NO: 185	sense	SEQ ID NO: 186	antisense	SEQ ID NO: 187	sense	SEQ ID NO: 188	antisense
SEQ ID NO: 189	sense	SEQ ID NO: 190	antisense	SEQ ID NO: 191	sense	SEQ ID NO: 192	antisense
SEQ ID NO: 193	sense	SEQ ID NO: 194	antisense	SEQ ID NO: 195	sense	SEQ ID NO: 196	antisense
SEQ ID NO: 197	sense	SEQ ID NO: 198	antisense	SEQ ID NO: 199	sense	SEQ ID NO: 200	antisense
SEQ ID NO: 201	sense	SEQ ID NO: 202	antisense	SEQ ID NO: 203	sense	SEQ ID NO: 204	antisense
SEQ ID NO: 205	sense	SEQ ID NO: 206	antisense	SEQ ID NO: 207	sense	SEQ ID NO: 208	antisense
SEQ ID NO: 209	sense	SEQ ID NO: 210	antisense	SEQ ID NO: 211	sense	SEQ ID NO: 212	antisense
SEQ ID NO: 213	sense	SEQ ID NO: 214	antisense	SEQ ID NO: 215	sense	SEQ ID NO: 216	antisense
SEQ ID NO: 217	sense	SEQ ID NO: 218	antisense	SEQ ID NO: 219	sense	SEQ ID NO: 220	antisense
SEQ ID NO: 221	sense	SEQ ID NO: 222	antisense	SEQ ID NO: 223	sense	SEQ ID NO: 224	antisense
SEQ ID NO: 225	sense	SEQ ID NO: 226	antisense	SEQ ID NO: 227	sense	SEQ ID NO: 228	antisense
SEQ ID NO: 229	sense	SEQ ID NO: 230	antisense	SEQ ID NO: 231	sense	SEQ ID NO: 232	antisense
SEQ ID NO: 233	sense	SEQ ID NO: 234	antisense	SEQ ID NO: 235	sense	SEQ ID NO: 236	antisense
SEQ ID NO: 237	sense	SEQ ID NO: 238	antisense	SEQ ID NO: 239	sense	SEQ ID NO: 240	antisense
SEQ ID NO: 241	sense	SEQ ID NO: 242	antisense	SEQ ID NO: 243	sense	SEQ ID NO: 244	antisense
SEQ ID NO: 245	sense	SEQ ID NO: 246	antisense	SEQ ID NO: 247	sense	SEQ ID NO: 248	antisense
SEQ ID NO: 249	sense	SEQ ID NO: 250	antisense	SEQ ID NO: 251	sense	SEQ ID NO: 252	antisense
SEQ ID NO: 253	sense	SEQ ID NO: 254	antisense	SEQ ID NO: 255	sense	SEQ ID NO: 256	antisense
SEQ ID NO: 257	sense	SEQ ID NO: 258	antisense	SEQ ID NO: 259	sense	SEQ ID NO: 260	antisense
SEQ ID NO: 261	sense	SEQ ID NO: 262	antisense	SEQ ID NO: 263	sense	SEQ ID NO: 264	antisense
SEQ ID NO: 265	sense	SEQ ID NO: 266	antisense	SEQ ID NO: 267	sense	SEQ ID NO: 268	antisense
SEQ ID NO: 269	sense	SEQ ID NO: 270	antisense	SEQ ID NO: 271	sense	SEQ ID NO: 272	antisense
SEQ ID NO: 273	sense	SEQ ID NO: 274	antisense	SEQ ID NO: 275	sense	SEQ ID NO: 276	antisense
SEQ ID NO: 277	sense	SEQ ID NO: 278	antisense	SEQ ID NO: 279	sense	SEQ ID NO: 280	antisense
SEQ ID NO: 281	sense	SEQ ID NO: 282	antisense	SEQ ID NO: 283	sense	SEQ ID NO: 284	antisense
SEQ ID NO: 285	sense	SEQ ID NO: 286	antisense	SEQ ID NO: 287	sense	SEQ ID NO: 288	antisense
SEQ ID NO: 289	sense	SEQ ID NO: 290	antisense	SEQ ID NO: 291	sense	SEQ ID NO: 292	antisense
SEQ ID NO: 293	sense	SEQ ID NO: 294	antisense	SEQ ID NO: 295	sense	SEQ ID NO: 296	antisense
SEQ ID NO: 297	sense	SEQ ID NO: 298	antisense	SEQ ID NO: 299	sense	SEQ ID NO: 300	antisense
SEQ ID NO: 301	sense	SEQ ID NO: 302	antisense	SEQ ID NO: 303	sense	SEQ ID NO: 304	antisense
SEQ ID NO: 305	sense	SEQ ID NO: 306	antisense	SEQ ID NO: 307	sense	SEQ ID NO: 308	antisense
SEQ ID NO: 309	sense	SEQ ID NO: 310	antisense	SEQ ID NO: 311	sense	SEQ ID NO: 312	antisense
SEQ ID NO: 313	sense	SEQ ID NO: 314	antisense	SEQ ID NO: 315	sense	SEQ ID NO: 316	antisense
SEQ ID NO: 317	sense	SEQ ID NO: 318	antisense	SEQ ID NO: 319	sense	SEQ ID NO: 320	antisense
SEQ ID NO: 321	sense	SEQ ID NO: 322	antisense	SEQ ID NO: 323	sense	SEQ ID NO: 324	antisense
SEQ ID NO: 325	sense	SEQ ID NO: 326	antisense	SEQ ID NO: 327	sense	SEQ ID NO: 328	antisense
SEQ ID NO: 329	sense	SEQ ID NO: 330	antisense	SEQ ID NO: 331	sense	SEQ ID NO: 332	antisense
SEQ ID NO: 333	sense	SEQ ID NO: 334	antisense	SEQ ID NO: 335	sense	SEQ ID NO: 336	antisense
SEQ ID NO: 337	sense	SEQ ID NO: 338	antisense	SEQ ID NO: 339	sense	SEQ ID NO: 340	antisense
SEQ ID NO: 341	sense	SEQ ID NO: 342	antisense	SEQ ID NO: 343	sense	SEQ ID NO: 344	antisense
SEQ ID NO: 345	sense	SEQ ID NO: 346	antisense	SEQ ID NO: 347	sense	SEQ ID NO: 348	antisense
SEQ ID NO: 349	sense	SEQ ID NO: 350	antisense	SEQ ID NO: 351	sense	SEQ ID NO: 352	antisense
SEQ ID NO: 353	sense	SEQ ID NO: 354	antisense	SEQ ID NO: 355	sense	SEQ ID NO: 356	antisense
SEQ ID NO: 357	sense	SEQ ID NO: 358	antisense	SEQ ID NO: 359	sense	SEQ ID NO: 360	antisense
SEQ ID NO: 361	sense	SEQ ID NO: 362	antisense	SEQ ID NO: 363	sense	SEQ ID NO: 364	antisense
SEQ ID NO: 365	sense	SEQ ID NO: 366	antisense	SEQ ID NO: 367	sense	SEQ ID NO: 368	antisense
SEQ ID NO: 369	sense	SEQ ID NO: 370	antisense	SEQ ID NO: 371	sense	SEQ ID NO: 372	antisense
SEQ ID NO: 373	sense	SEQ ID NO: 374	antisense	SEQ ID NO: 375	sense	SEQ ID NO: 376	antisense
SEQ ID NO: 377	sense	SEQ ID NO: 378	antisense	SEQ ID NO: 379	sense	SEQ ID NO: 380	antisense
SEQ ID NO: 381	sense	SEQ ID NO: 382	antisense	SEQ ID NO: 383	sense	SEQ ID NO: 384	antisense
SEQ ID NO: 385	sense	SEQ ID NO: 386	antisense	SEQ ID NO: 387	sense	SEQ ID NO: 388	antisense

[illegible]

[illegible]

101

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

Table 7: Transforming growth factor beta iRNA sequences

Sense and Corresponding Antisense Duplexes							
Sense Strand		Corresponding Antisense Strand		Sense Strand		Corresponding Antisense Strand	
SEQ ID NO: 2793	sense	SEQ ID NO: 2794	antisense	SEQ ID NO: 2795	sense	SEQ ID NO: 2796	antisense
SEQ ID NO: 2797	sense	SEQ ID NO: 2798	antisense	SEQ ID NO: 2799	sense	SEQ ID NO: 2800	antisense
SEQ ID NO: 2801	sense	SEQ ID NO: 2802	antisense	SEQ ID NO: 2803	sense	SEQ ID NO: 2804	antisense
SEQ ID NO: 2805	sense	SEQ ID NO: 2806	antisense	SEQ ID NO: 2807	sense	SEQ ID NO: 2808	antisense
SEQ ID NO: 2809	sense	SEQ ID NO: 2810	antisense	SEQ ID NO: 2811	sense	SEQ ID NO: 2812	antisense
SEQ ID NO: 2813	sense	SEQ ID NO: 2814	antisense	SEQ ID NO: 2815	sense	SEQ ID NO: 2816	antisense
SEQ ID NO: 2817	sense	SEQ ID NO: 2818	antisense	SEQ ID NO: 2819	sense	SEQ ID NO: 2820	antisense
SEQ ID NO: 2821	sense	SEQ ID NO: 2822	antisense	SEQ ID NO: 2823	sense	SEQ ID NO: 2824	antisense
SEQ ID NO: 2825	sense	SEQ ID NO: 2826	antisense	SEQ ID NO: 2827	sense	SEQ ID NO: 2828	antisense
SEQ ID NO: 2829	sense	SEQ ID NO: 2830	antisense	SEQ ID NO: 2831	sense	SEQ ID NO: 2832	antisense

SEQ ID NO: 2833	sense	SEQ ID NO: 2834	antisense	SEQ ID NO: 2835	sense	SEQ ID NO: 2836	antisense
SEQ ID NO: 2837	sense	SEQ ID NO: 2838	antisense	SEQ ID NO: 2839	sense	SEQ ID NO: 2840	antisense
SEQ ID NO: 2841	sense	SEQ ID NO: 2842	antisense	SEQ ID NO: 2843	sense	SEQ ID NO: 2844	antisense
SEQ ID NO: 2845	sense	SEQ ID NO: 2846	antisense	SEQ ID NO: 2847	sense	SEQ ID NO: 2848	antisense
SEQ ID NO: 2849	sense	SEQ ID NO: 2850	antisense	SEQ ID NO: 2851	sense	SEQ ID NO: 2852	antisense
SEQ ID NO: 2853	sense	SEQ ID NO: 2854	antisense	SEQ ID NO: 2855	sense	SEQ ID NO: 2856	antisense
SEQ ID NO: 2857	sense	SEQ ID NO: 2858	antisense	SEQ ID NO: 2859	sense	SEQ ID NO: 2860	antisense

Table 8. Commonly used liver fibrosis staging scores.

Staging System	Fibrosis stages	Remark	
METAVIR Score	F0, F1, F2, F3, F4	Best evaluated in HCV fibrosis	(The French METAVIR Cooperative Study Group 1994)
Knodel Score	F0, F1, F3, F4	No intermediate stage	(Knodel 1981)
Desmet & Scheuer	Analogous to METAVIR	Recommended by German guidelines for the assessment of liver fibrosis	(Desmet 1994; Schirmacher 2004)
Batts & Ludwig	Similar to METAVIR		(Batts 1995)
Ishak Score	F0, F1, F2, F3, F4, F5, F6		(Ishak 1995)

Batts KP, Ludwig J. Chronic hepatitis. An update on terminology and reporting. *Am J Surg Pathol* 1995;19(12):1409-17.

Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994, 19(6):1513-20.

Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, *et al.* Histological grading and staging of chronic hepatitis. *J Hepatol* 1995, 22(6):696-9.

Knodel RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, Wollman J. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981, 1(5):431-5.

Schirmacher P, Fleig WE, Dienes HP. (Biopsy diagnosis of chronic hepatitis). *Z Gastroenterol* 2004, 42(2):175-85.

Table 9. Common biomarkers of liver

Index	Markers	Calculation	Interpretation	PPV/NPV (%)
Direct surrogate markers				
MP3	PIIINP, MMP-1	$0.5901(\log \text{PIIINP}[\text{ng/ml}]) - 0.1749(\log \text{MMP-1}[\text{ng/ml}])$	$<0.3 \approx \text{F0-2}$ $>0.4 \approx \text{F3-4}$ $<0.3 \approx \text{F0-1}$ $>0.4 \approx \text{F2-4}$	NPV=95 PPV=66 NPV=75 PPV=91
ELF	PIIINP, HA	Proprietary	>0.102 Scheuer 3-4 <0.102 Scheuer 0-2	PPV = 35 NPV = 92
Indirect surrogate markers				
Forns	Age, pti, γ GT, cholesterol	$7.811 - 3.131 \times \ln(\text{pti}) + 0.781 \times \ln(\gamma\text{GT}) + 3.467 \times \ln(\text{age}) - 0.014 (\text{cholesterol})$	$>6.9 \approx \text{Scheuer 2-4}$ $<4.2 \approx \text{Scheuer 0-1}$	PPV = 66 NPV = 96
APRI	AST, pti	$([\text{AST}/\text{ULN}]/\text{pti} [\times 109/\text{l}]) \times 100$	$>1.5 \approx \text{Ishak 3-6}$ $\leq 0.5 \approx \text{Ishak 0-2}$	PPV = 91 NPV = 90
Fibrotest Fibrosure	Haptoglobin, $\alpha 2$ -MC, apo-A1, γ GT, bilirubin, γ -globulin	Proprietary	$0.75-1.00 \approx \text{F4}$ $0.73-0.74 \approx \text{F3-F4}$ $0.59-0.72 \approx \text{F3}$ $0.49-0.58 \approx \text{F2}$ $0.32-0.48 \approx \text{F1-F2}$ $0.28-0.31 \approx \text{F1}$ $0.22-0.27 \approx \text{F0-F1}$ $0.00-0.21 \approx \text{F0}$	PPV = 78 PPV = 76 PPV = 76 PPV = 67 PPV/NPV = 61/85 NPV = 91 NPV = 92 NPV = 94
Fibroindex	Pti, AST, γ GT	$1.738 - 0.064 (\text{pti} [\times 104/\text{mm}^3]) + 0.005 (\text{AST} [\text{IU/L}]) + 0.463 \times (\gamma\text{GT}[\text{g/dl}])$	$\leq 1.25 \approx \text{F0-F1}$ $\geq 2.25 \approx \text{F2-F3}$	NPV = 61.7 PPV = 90
Testa	Pti, spleen diameter	Pti count/spleen diameter	$>1750 \approx \text{Ishak } \leq 2$ $\leq 1750 \approx \text{Ishak } > 2$	NPV = 79 PPV = 78.9c
Fibrosis probability index	AST, cholesterol, past alcohol intake, HOMA, age	$E^*/1 + e^*$, where $E^* = -10.929 + (1.827 \times \ln(\text{AST})) + (0.081 \times \text{age}) + (0.768 \times [\text{past alcohol use graded as 0-2}]) + (0.385 \times \text{HOMA})$	$<0.2 \approx \text{F0-F1}$ $\geq 0.8 \approx \text{F2-F4}$	NPV = 77.4 PPV = 87
FIB-4	Pti, AST, ALT, age	$(\text{Age} \times \text{AST})/(\text{pti count} \times \text{ALT}^{1/2})$	$<1.45 \approx \text{Ishak } < 4-6$ $>3.25 \approx \text{Ishak } \geq 4-6$	NPV = 90 PPV = 65
Bonancini	ALT, AST, INR, pti	Sum (range 0-11) of (pti score) + (ALT/AST score) + (INR score) pti ($\times 109/\text{l}$): $>340 = 0$; $280-339 = 1$; $220-279 = 2$; $160-219 = 3$; $100-159 = 4$; $40-89 = 5$; $<40 = 6$ ALT/AST ratio: $>1.7 = 0$; $1.2-1.7 = 1$; $0.6-1.19 = 2$; $<0.6 = 3$ INR: $1.4 = 2$	$>8 \approx \text{Knodel 3-4}$	PPV = 92.9
Pohl	AST, ALT, pti	Positive if: $\text{AST}/\text{ALT} \geq 1$ and platelet count $<150 \times 109/\text{l}$	Positive $\approx \text{F3-F4}$	PPV = 93
Shet Park	AST, ALT, AST, ALT	AST/ALT AST/ALT	$\geq 1 \approx \text{Scheuer 4}$ $\geq 1 \approx \text{Scheuer 4}$	PPV = 100 PPV = 73.7
Age-Platelet	Pti, age	Age score + pti score (0-10 possible score) age: $<30 = 0$; $30-39 = 1$; $40-49 = 2$; $50-59 = 3$; $60-69 = 4$; $\geq 70 = 5$. Pti ($\times 109/\text{l}$): $\geq 225 = 0$; $200-224 = 1$; $175-199 = 2$; $150-174 = 3$; $125-149 = 4$; $<125 = 5$	$\geq 6 \approx \text{F2-F4}$	PPV = 95
Combined direct and indirect surrogate markers				
SHASTA	HA, AST, albumin	$-3.84 + 1.70 (1 \text{ if HA } 41-85 \text{ ng/ml, 0 otherwise}) + 3.28 (1 \text{ if HA } >85 \text{ ng/ml, 0 otherwise}) + 1.58 (1 \text{ if albumin } <3.5 \text{ g/dl, 0 otherwise}) + 1.78 (1 \text{ if AST } >60 \text{ IU/l, 0 otherwise})$	$>0.8 \approx \text{Ishak } \geq 3$ $<0.3 \approx \text{Ishak } \leq 2$	PPV = 100 NPV = 94
FM	pti, PI, AST, HA, $\alpha 2$ -MC, gender, age	$-0.007 \text{ pti (G/L)} - 0.049 \text{ PI (\%)} + 0.012 \text{ AST (IU/l)} + 0.005 \text{ } \alpha 2\text{-MC (mg/dl)} + 0.021 \text{ HA (g/l)} - 0.270 \text{ urea (mmol/l)} + 0.027 \text{ age (years)} + 3.718$	$\geq \text{F2}$	PPV = 85.3/96.6
Hepascore	HA, $\alpha 2$ -MC, γ GT, age, gender	$y/1 + y$, where $y = \exp[-4.185918 - (0.0249 \times \text{age}) + 0.7464 \times \text{sex}] + (1.0039 \times \alpha 2\text{-MC}) + (0.0302 \times \text{HA}) + (0.0691 \times \text{bilirubin}) - (0.0012 \times \gamma\text{GT})$	$\geq 0.5 \approx \text{F2-F4}$ $<0.5 \approx \text{F0-F1}$	PPV = 88 NPV = 98
FSII	HA, $\alpha 2$ -MC, TIMP-1	Proprietary	$\geq 42 \approx \text{F2-F4}$ $<40 \approx \text{F0-F1}$	PPV = 77.4 NPV = 78

What is claimed is:

1. A double-stranded ribonucleic acid (dsRNA) for inhibiting expression of COL1A1, wherein said dsRNA comprises a sense strand and an antisense strand, wherein the sense strand consists of SEQ ID NO: 53 and the antisense strand consists of SEQ ID NO: 54.
2. A double-stranded ribonucleic acid (dsRNA) for inhibiting expression of COL1A1, wherein said dsRNA comprises a sense strand and an antisense strand,

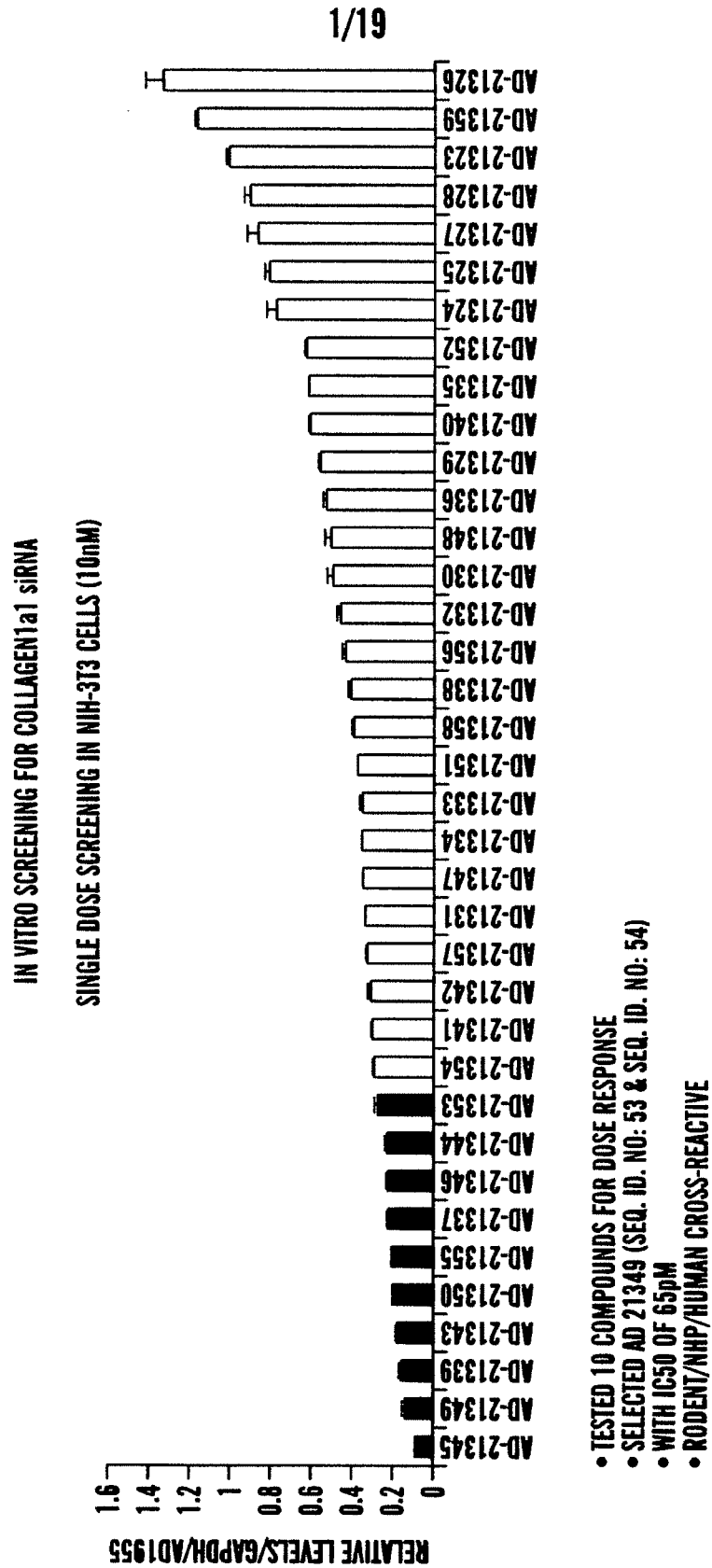
the sense strand consisting of a sequence selected from the group consisting of SEQ ID NOs: 1-62 and 185-2792 having odd number sequence identifiers, and

the antisense strand comprising a region of complementarity to a COL1A1 RNA transcript, the region of complementarity selected from the antisense sequences of SEQ ID NOs: 1-62 and 185-2792 having even number sequence identifiers, and

wherein the sense strand and the region of complementarity are substantially complementary to each other.
3. The dsRNA according to claim 1 or 2, wherein said dsRNA comprises at least one modified nucleotide.
4. The dsRNA of claim 3, wherein at least one of said modified nucleotides is chosen from the group consisting of: a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group, a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide.
5. The dsRNA according to claim 2, wherein the region of complementarity is 19 to 21 nucleotides in length.
6. The dsRNA according to any one of claims 2 to 5, wherein each strand is no more than 30 nucleotides in length.

7. The dsRNA according to any one of claims 2 to 6, wherein at least one strand comprises a 3' overhang of at least 1 nucleotide.
8. The dsRNA according to any one of claims 1 to 7, further comprising a ligand.
9. The dsRNA of claim 8, wherein the ligand is conjugated to the 3' end of the sense strand of the dsRNA.
10. A cell containing the dsRNA according to claim 1 or 2.
11. A pharmaceutical composition for inhibiting expression of a COL1A1 gene comprising the dsRNA of claim 1 or 2 and a pharmaceutically acceptable carrier.
12. The pharmaceutical composition according to claim 11, further comprising a lipid formulation.
13. The pharmaceutical composition of claim 12, wherein the lipid formulation is a SNALP, or XTC formulation.
14. An *in vitro* method of inhibiting COL1A1 expression in a cell, the method comprising:
 - a. introducing into the cell the dsRNA according to claim 1 or 2; and
 - b. maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of a COL1A1 gene, thereby inhibiting expression of the COL1A1 gene in the cell.
15. The method of claim 14, wherein the cell is a hepatic stellate cell.
16. The method of claim 14 or 15, wherein the COL1A1 expression is inhibited by at least 30%.
17. Use of the dsRNA according to claim 1 or 2 for inhibiting COL1A1 expression in a cell, wherein the dsRNA is for introduction into the cell which is maintained for a time sufficient to obtain degradation of the mRNA transcript of a COL1A1 gene.
18. The use of claim 17, wherein the cell is a hepatic stellate cell.
19. The use of claim 17 or 18, wherein the COL1A1 expression is inhibited by at least 30%.

20. Use of the dsRNA according to claim 1 or 2 or the pharmaceutical composition according to any one of claims 11 to 13 in the manufacture of a medicament for treating liver fibrosis in a human in need of such treatment.
21. Use of the dsRNA according to claim 1 or 2 or the pharmaceutical composition according to any one of claims 11 to 13 for treating liver fibrosis in a human in need of such treatment.
22. The use of claim 20 or 21, wherein the dsRNA is for administration at a concentration of 0.01 mg/kg-5 mg/kg bodyweight of the subject.
23. The dsRNA according to claim 1 or 2 or the pharmaceutical composition according to any one of claims 11 to 13 for use in the treatment of liver fibrosis.
24. 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 COL1A1, wherein said dsRNA is 30 base pairs or less in length, wherein the region of complementarity is selected from the antisense sequences of SEQ ID NOs: 1-62 and 185-2792 having even number sequence identifiers, and wherein said dsRNA targets said mRNA for cleavage.
25. The vector according to claim 24, wherein the region of complementarity is 19 to 21 nucleotides in length.
26. A cell comprising the vector according to claim 24 or 25.
27. Use of a therapeutically effective amount of the dsRNA according to claim 1 or 2 or the pharmaceutical composition according to any one of claims 11 to 13 for treating a chronic liver disease characterized by liver fibrosis in a human in need of such treatment.
28. The dsRNA according to claim 1 or 2 or the pharmaceutical composition according to any one of claims 11 to 13 for use in the treatment of chronic liver disease characterized by liver fibrosis.
29. Use of a therapeutically effective amount of the dsRNA according to claim 1 or 2 or the pharmaceutical composition according to any one of claims 11 to 13 in the manufacture of a medicament for treating a chronic liver disease characterized by liver fibrosis in a human in need of such treatment.

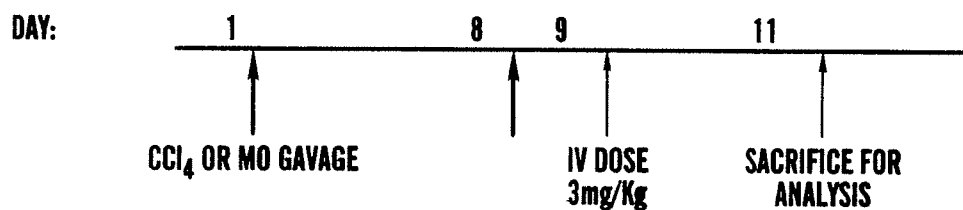
**FIG. 1**

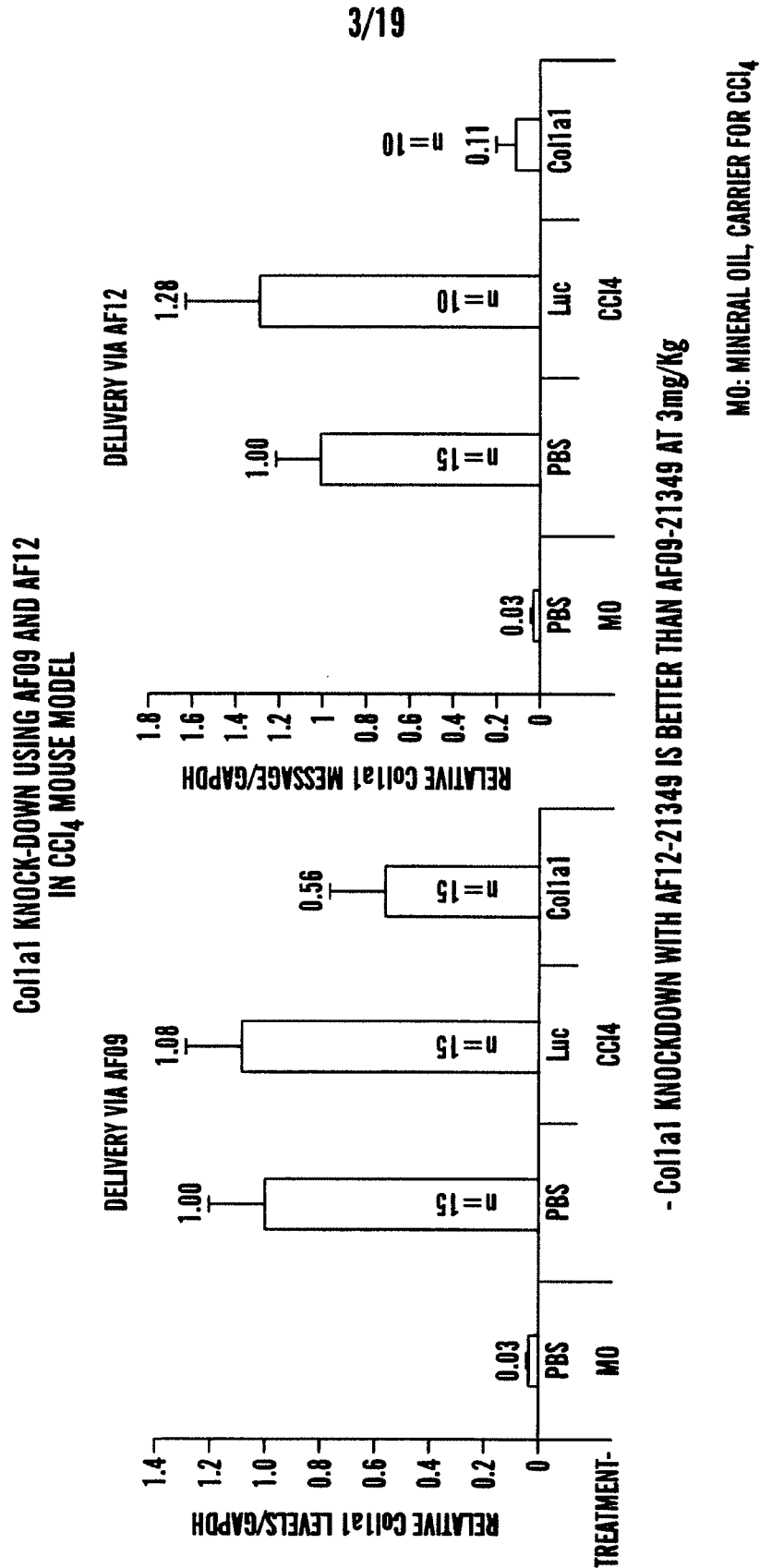
2/19

EVALUATION OF AF09 & AF12 FOR Col1a1 KNOCK-DOWN IN CCl₄ MOUSE MODEL**— EXPERIMENT:**

- INDUCE LIVER INJURY USING CCl₄ BY ORAL GAVAGE (2X) ON DAY1 & DAY8
 - FINAL DOSE 1.75ml/Kg
- IV INJECT MICE WITH FORMULATED siRNAs ON DAY9
- SACRIFICE AT 40 HOURS POST siRNA INJECTION ON DAY11
- ANALYZE Col1a1 AND GAPDH mRNA LEVELS IN LIVER USING TAQMAN-QPCR

- siCol1a1 (AD21349), siLUC (AD1955) DOSE: 3mg/Kg
- N=10-15

***FIG. 2***

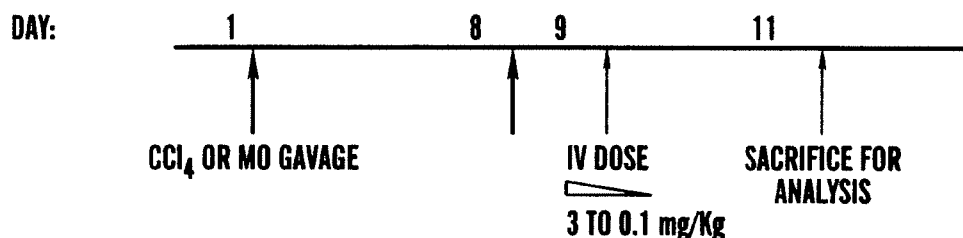
**FIG. 3**

4/19

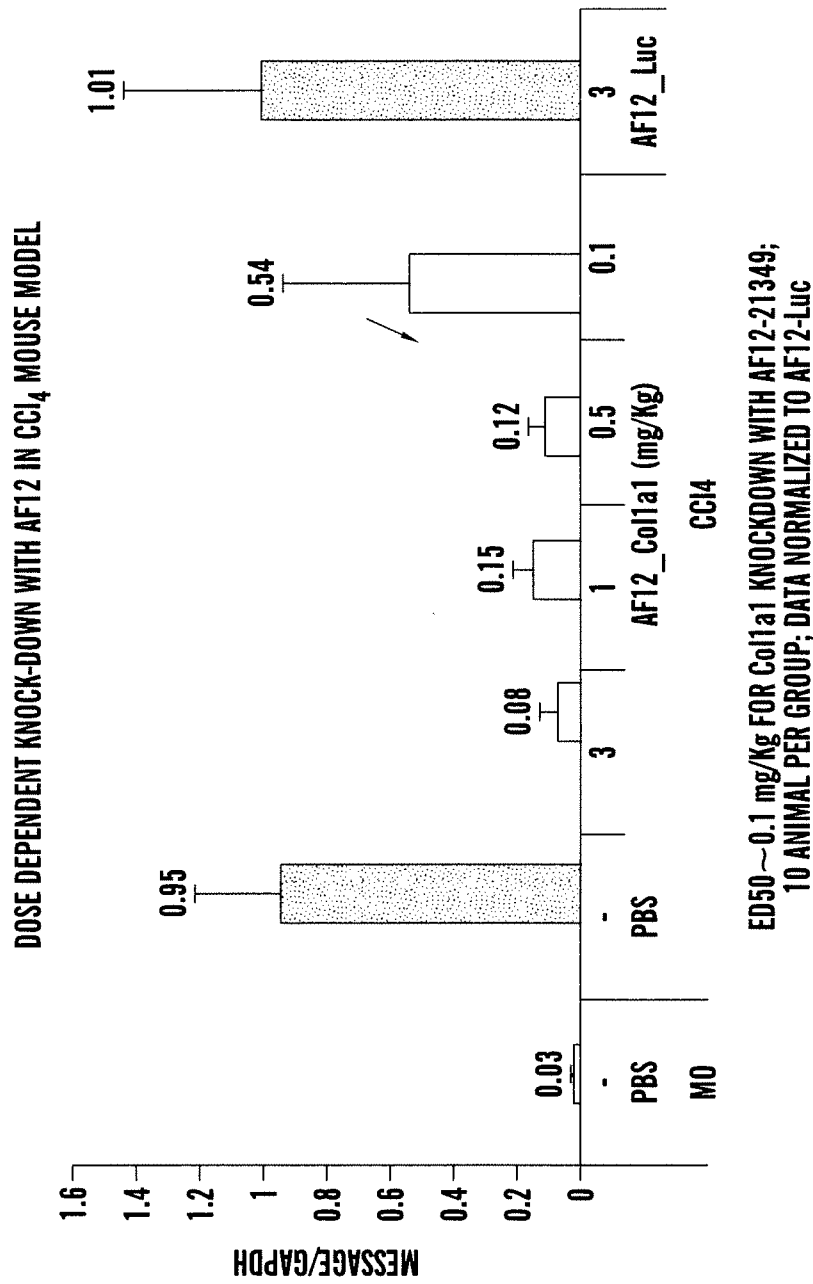
DOSE RESPONSE FOR Col1a1 KNOCK-DOWN WITH AF12 IN CCl₄ MOUSE MODEL**— EXPERIMENT:**

- INDUCE LIVER INJURY USING CCl₄ BY ORAL GAVAGE (2X) ON DAY1 & DAY8
 - FINAL DOSE 1.75ml/Kg
- IV INJECT MICE WITH FORMULATED siRNAs ON DAY9
- SACRIFICE AT 40 HOURS POST siRNA INJECTION ON DAY11
- ANALYZE Col1a1 AND GAPDH mRNA LEVELS IN LIVER USING TAQMAN-QPCR

- siCol1a1 (AD21349) DOSE: 0.1, 0.5, 1, 3mg/Kg
- siLUC (AD1955) DOSE: 3mg/Kg
- 10 ANIMALS PER EXPERIMENTAL GROUP (5 FOR MINERAL OIL)

***FIG. 4***

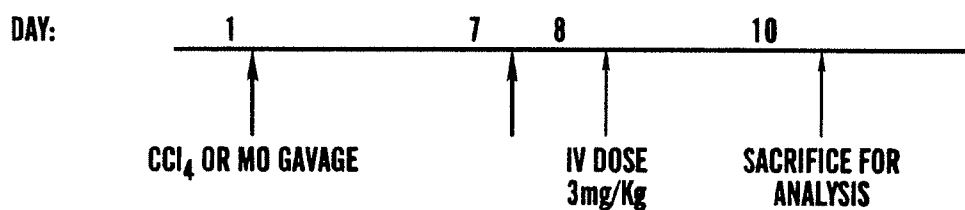
5/19

**FIG. 5**

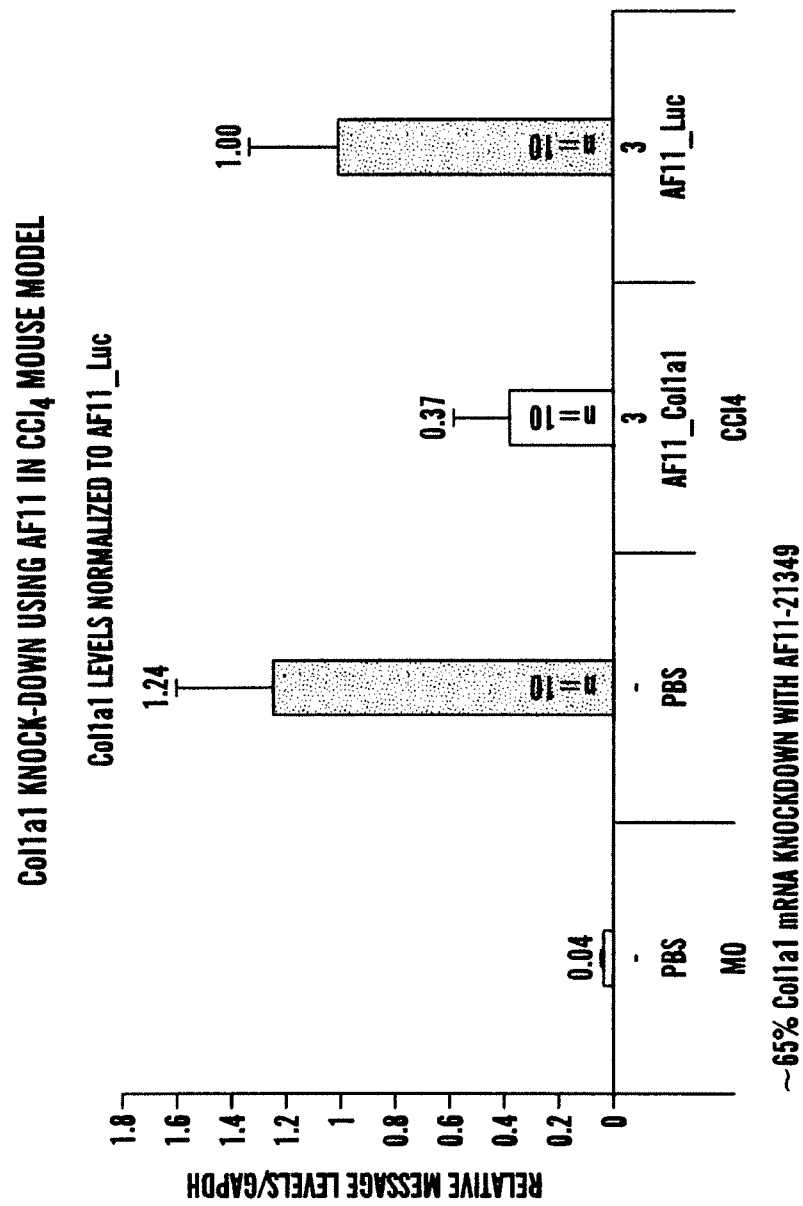
6/19

EVALUATION OF AF-11 FOR Col1a1 KNOCK-DOWN IN CCl₄ MOUSE MODEL**— EXPERIMENT:**

- INDUCE LIVER INJURY USING CCl₄ BY ORAL GAVAGE (2X) ON DAY1 & DAY7
 - FINAL DOSE 1.75ml/Kg
 - IV INJECT MICE WITH FORMULATED siRNAs ON DAY8
 - SACRIFICE AT 40 HOURS POST siRNA INJECTION ON DAY10
 - ANALYZE Col1a1 AND GAPDH mRNA LEVELS IN LIVER USING TAQMAN-QPCR
- siCol1a1 (AD21349), siLUC (AD1955) DOSE: 3mg/Kg
- STARTED WITH 10 ANIMALS PER EXPERIMENTAL GROUP (5 FOR MINERAL OIL)

**FIG. 6**

7/19

**FIG. 7**

CONFIRMATION FOR STELLATE CELL DELIVERY
USING AF09_Acta2

- Acta2: SMOOTH MUSCLE ACTIN
- ABSENT IN HEPATOCYTES
 - PRESENT IN ACTIVATED FIBROBLASTS AND VASCULAR SMOOTH MUSCLE CELLS

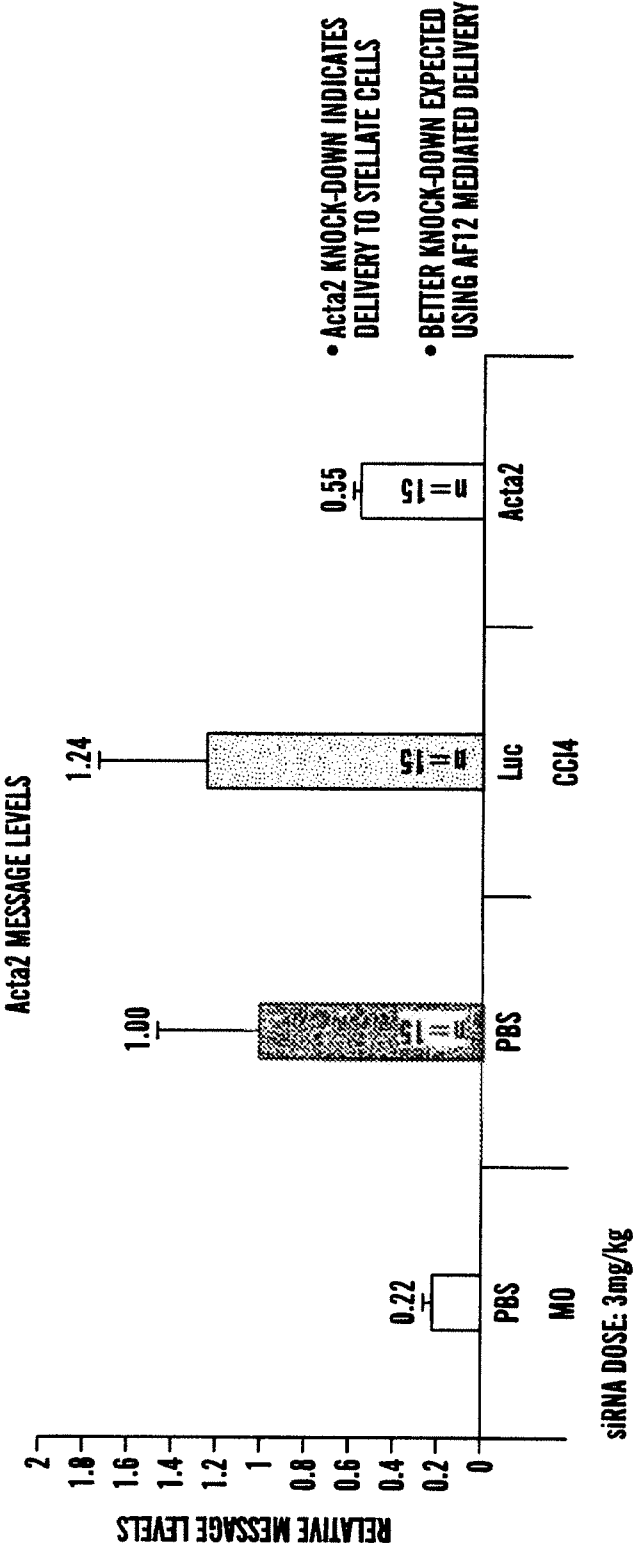
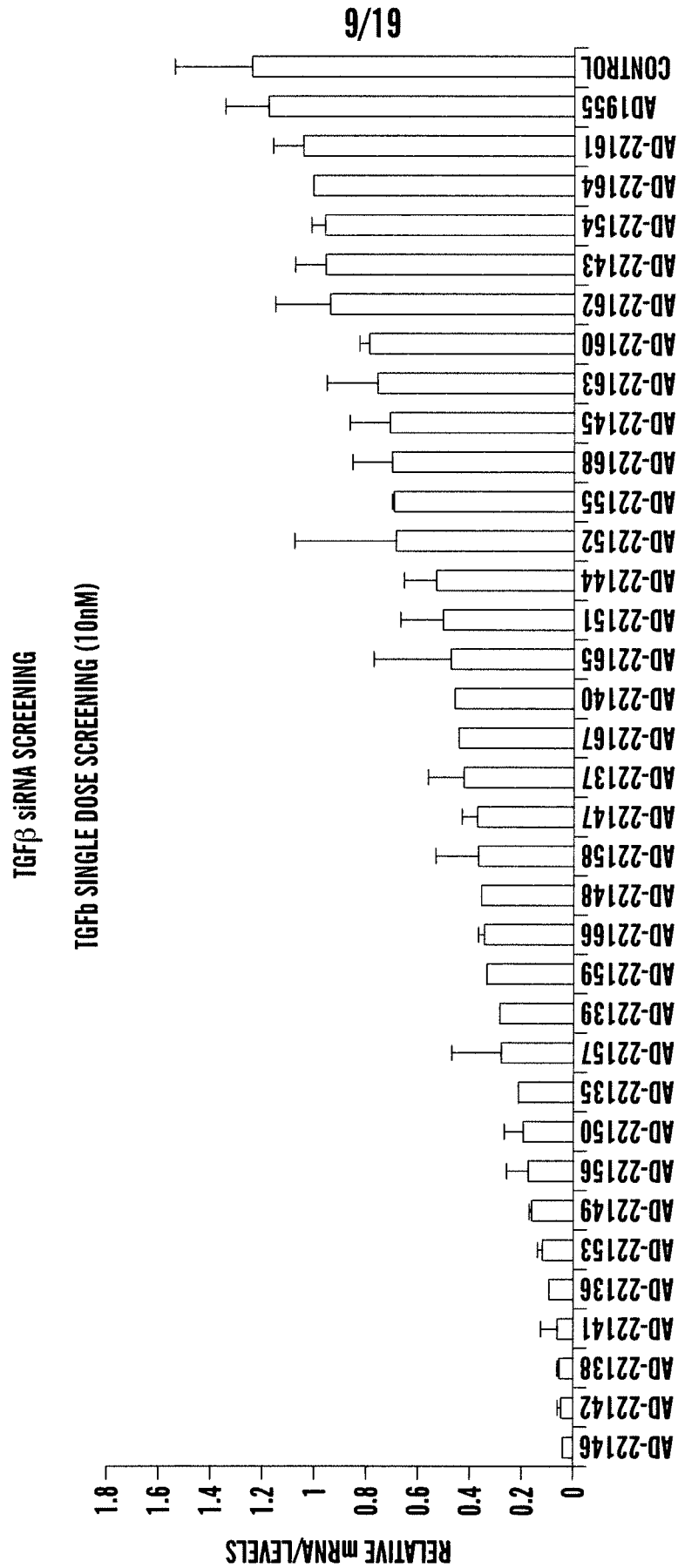
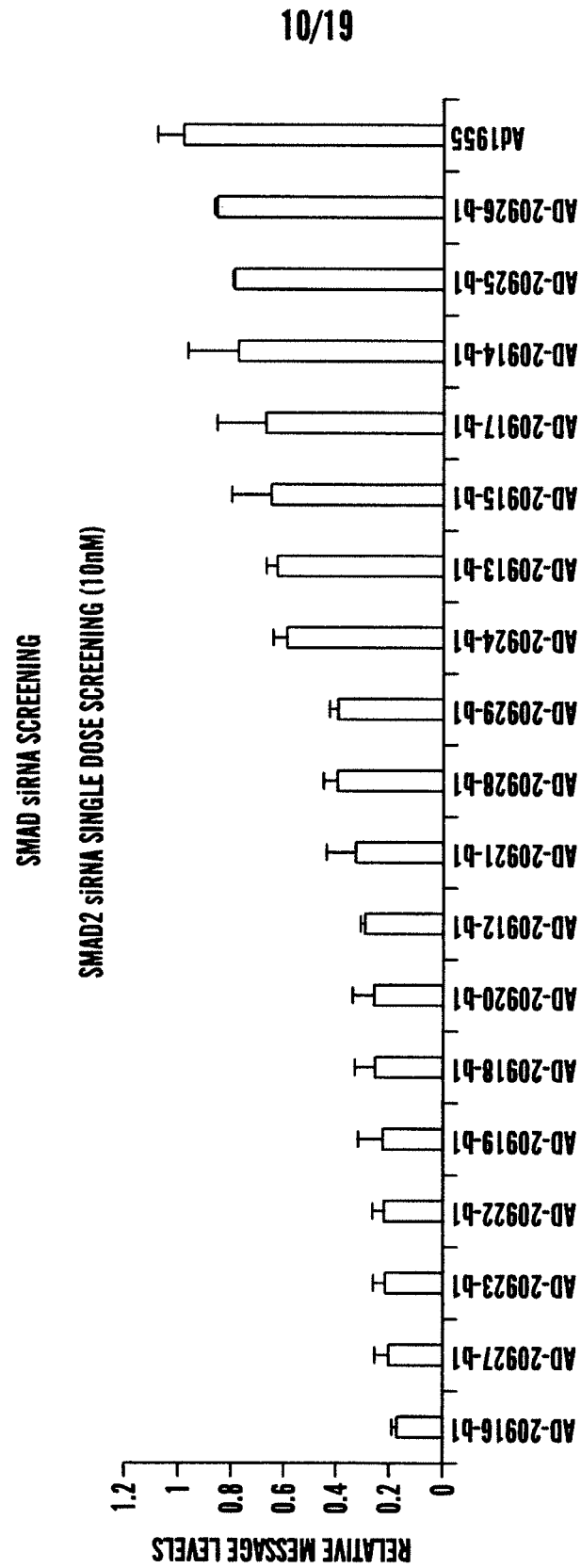
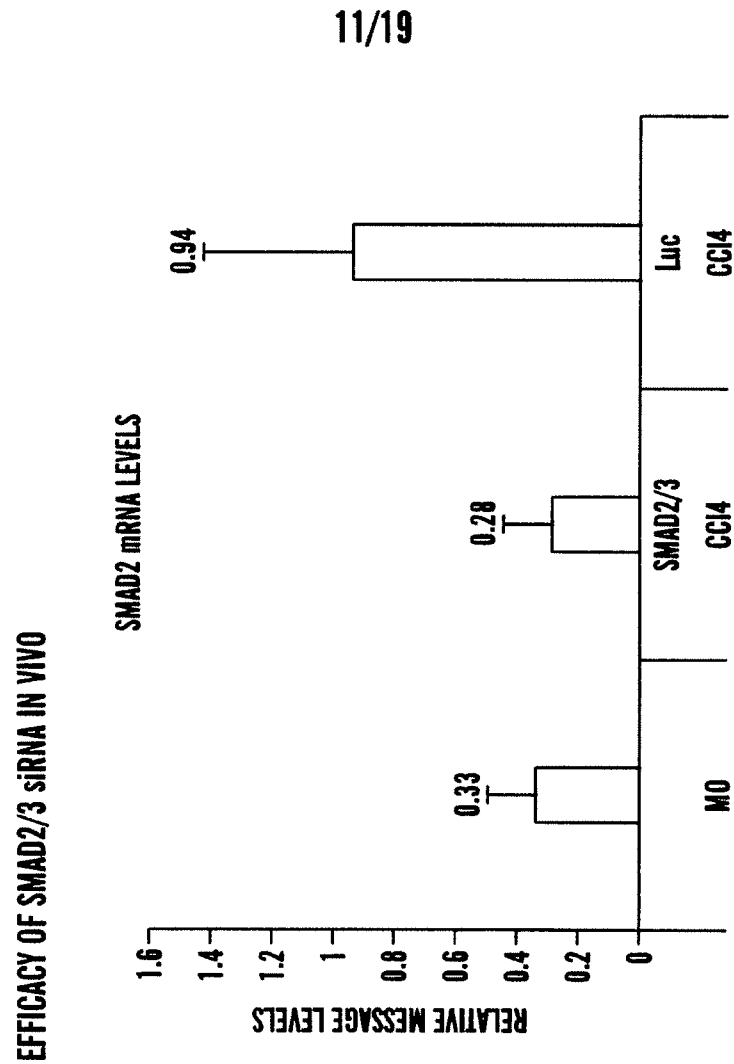


FIG. 8

**FIG. 9**

**FIG. 10**

**FIG. 11**

- EXPERIMENT**
- INDUCE LIVER INJURY USING CCl₄ DAY0; n = 10
 - IV INJECT MICE WITH FORMULATED siRNA AT 3mg/kg ON DAY2
 - SACRIFICE ON DAY4
 - ANALYZE mRNA LEVELS IN LIVER USING TAQMAN-QPCR
- SMAD2/3 siRNA DECREASE SMAD mRNA LEVELS BY 80% AFTER CCl₄ INJURY

12/19

EFFICACY OF SMAD2/3 siRNA ON COL1A1 EXPRESSION IN VIVO

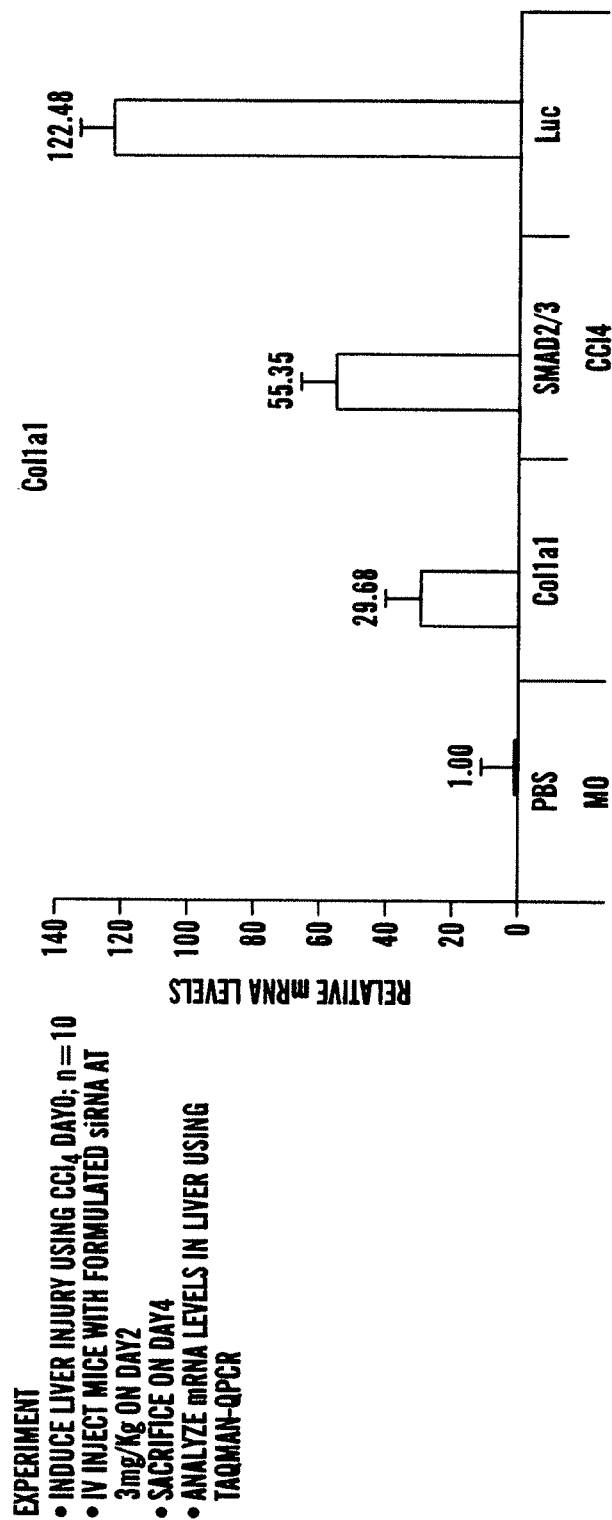
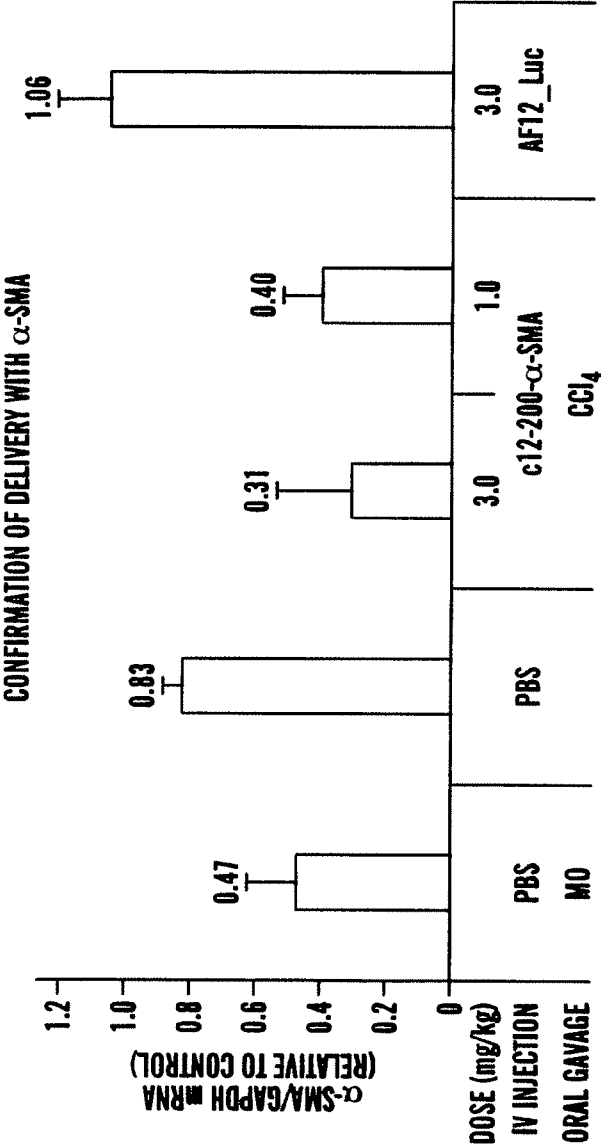


FIG. 12



CONFIRM DELIVERY TO STELLATE CELLS WITH ALPHA-SMOOTH MUSCLE ACTIN (α -SMA). α -SMA IS ABSENT IN HEPATOCYTES; IT IS PRESENT IN ACTIVATED STELLATE CELLS AND VASCULAR SMOOTH MUSCLE CELLS. CCl₄ TREATMENT INDUCES α -SMA. FORMULATED siRNA AGAINST α -SMA DECREASES α -SMA LEVELS BY 70% AS AGAINST THE CONTROL Luc siRNA.

FIG. 14

Col1a1 KNOCKDOWN IS MEDIATED BY RNAi

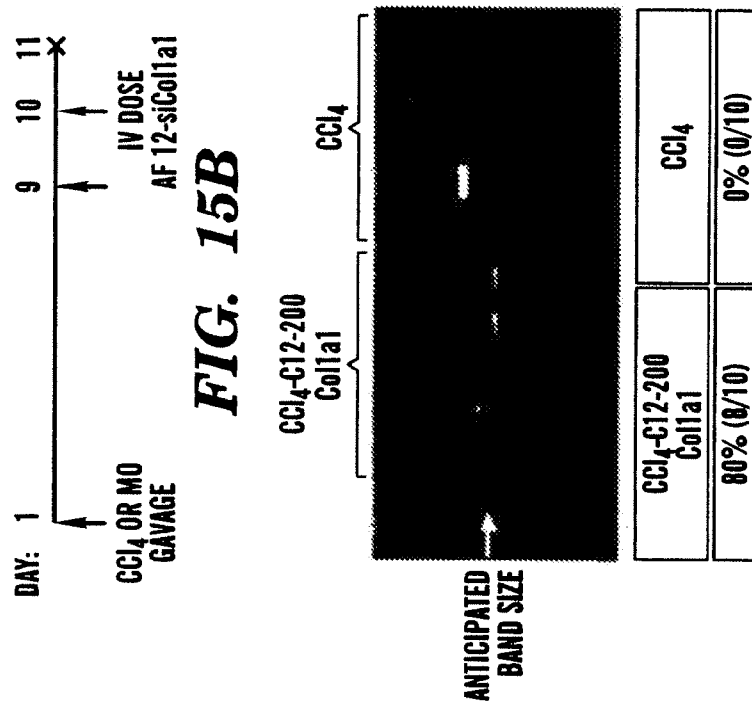


FIG. 15B

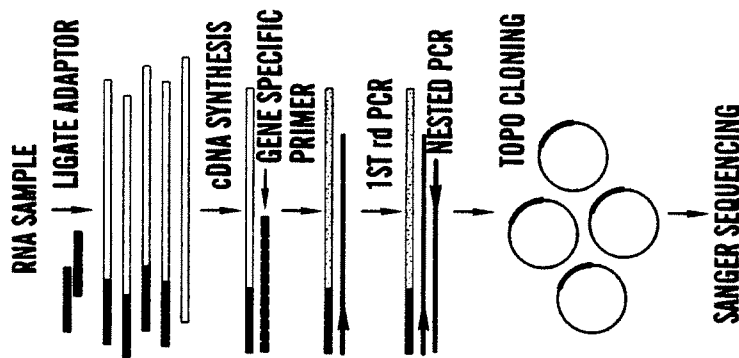


FIG. 15A

Col1a1 KNOCKDOWN IS MEDIATED BY RNAi. 10 animals were given CCl₄ BY ORAL GAVAGE. (A) SCHEME FOR 5'RACE ASSAY. TOTAL LIVER mRNA WAS PROCESSED USING GENERACER KIT (INVITROGEN) FOR cDNA SYNTHESIS AND 5'RACE TO ANALYZE THE CLEAVED col1a1. THE PCR PRODUCTS WERE CLONED INTO TA VECTORS AND SEQUENCED. (B) RESULTS WERE INJECTED WITH C12-200-siCol1a1 AND SACRIFICED AFTER 24 HOURS. (C) AGAROSE GEL SHOWING DNA BANDS FOR NESTED PCR. 80% OF THE CLONES FROM C12-200-Col1a1 GROUP SHOWED EXPECTED CLEAVAGE PRODUCT (AT POSITION 9 OF THE siRNA SEQUENCE).

FIG. 15C

AF12-Col1a1 IN BILE DUCT LIGATED ANIMALS

EXPERIMENT:

- BILE DUCT LIGATED Balb/c MALE MICE FROM CHARLES RIVERS
- IV INJECT MICE WITH FORMULATED siRNAs ON DAYS 5, 7, 11
- SACRIFICE 7 HOURS AFTER LAST INJECTION ON DAY11
- ANALYZE mRNA LEVELS IN LIVER USING TAQMAN-QPCR

— siCol1a1 (AD21349) (SEQ ID NO: 53 AND SEQ ID NO: 54), siLUC (AD1955) DOSE: 0.5 mg/Kg

STUDY DESIGN

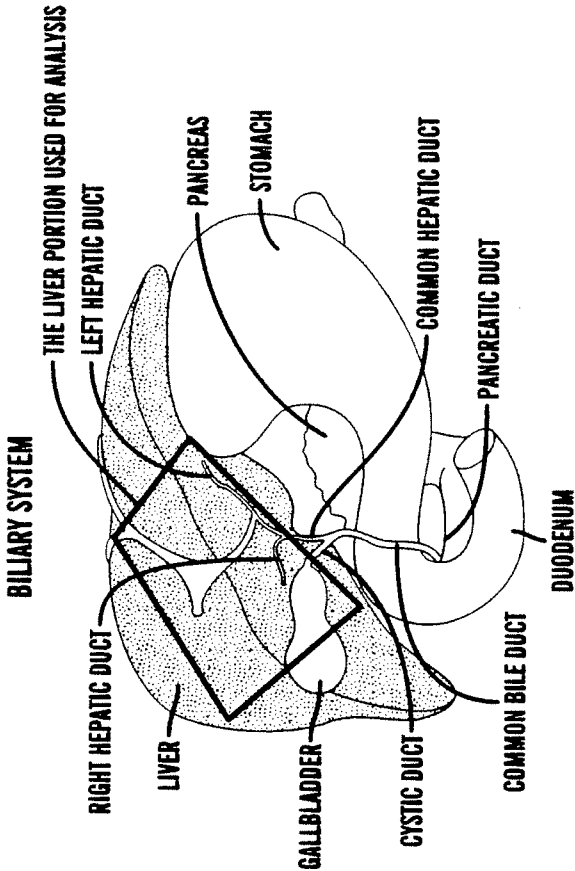
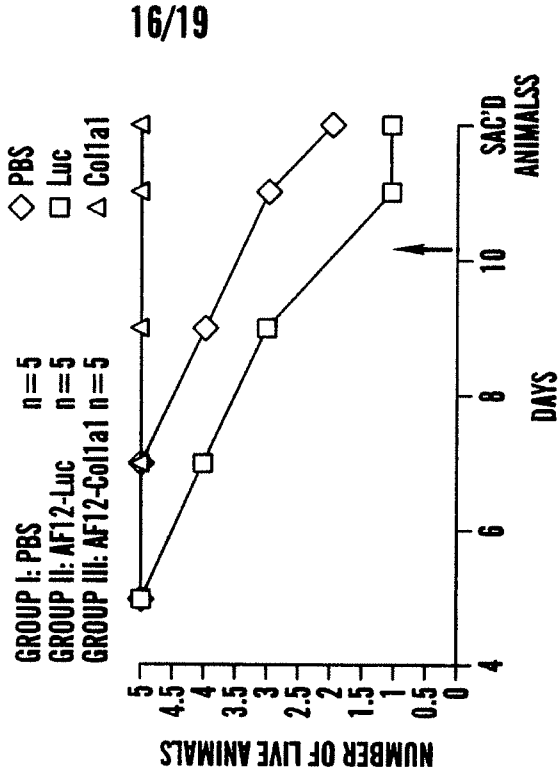
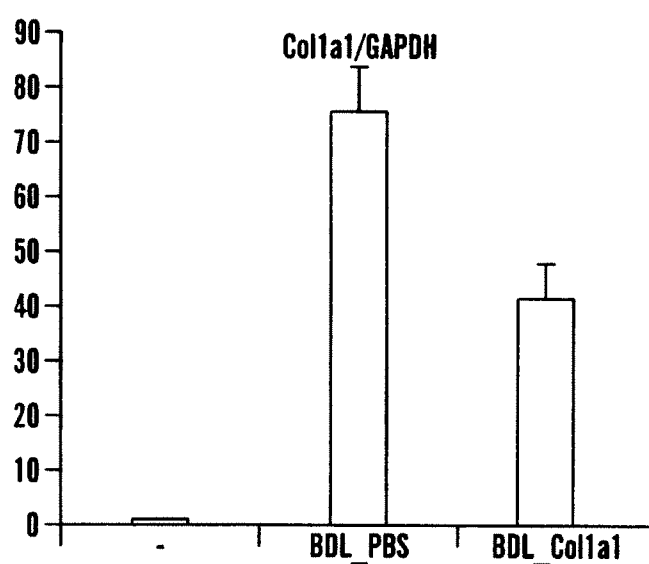


FIG. 16

17/19

RELATIVE Col1a1 EXPRESSION IN THE BILE-DUCT LIGATED ANIMALS



- ~50% REDUCTION IN Col1a1 mRNA LEVELS
- INCREASED SURVIVAL OF Col1a1 TREATED ANIMALS

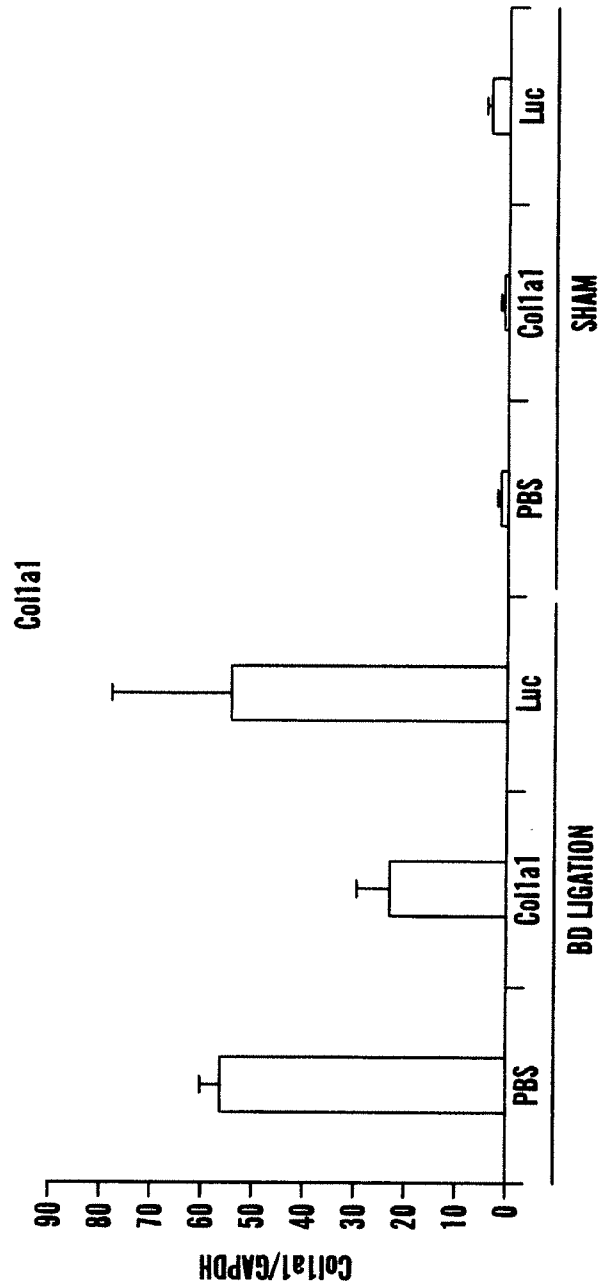
FIG. 17

18/19

EXPERIMENT:

- » BILE DUCT LIGATED Balb/c MALE MICE FROM CHARLES RIVERS
- » IV INJECT MICE WITH FORMULATED siRNAs ON DAYS 4, 7, 10
- » SACRIFICE 24 HOURS AFTER LAST INJECTION ON DAY11
- » ANALYZE mRNA LEVELS IN LIVER USING TAQMAN-QPCR

- siCol1a1 (AD21349), siLUC (AD1955) (SEQ ID NO: 53 AND SEQ ID NO: 54) DOSE: 0.5 mg/kg
- ~60% Col1a1 KNOCK-DOWN IN BILE-DUCT LIGATED ANIMALS

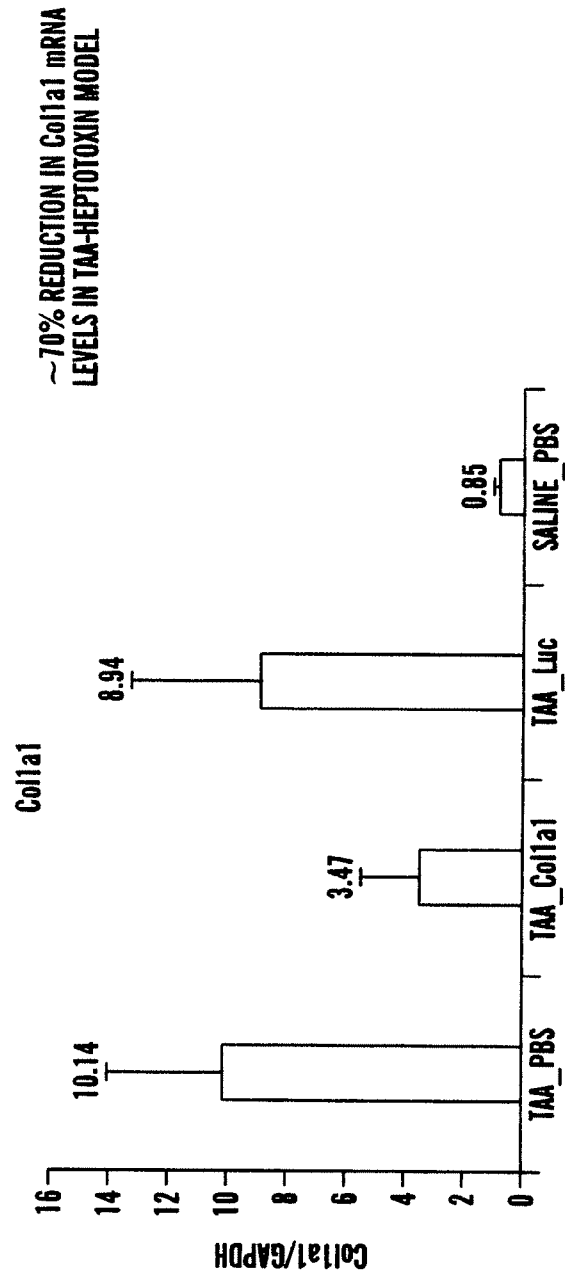
**FIG. 18**

19/19

EXPERIMENT:

- INDUCE LIVER INJURY USING TAA BY INTRAPERITONEAL INJECTIONS: DAY 1, 4, 8
— FINAL DOSE 200mg/Kg
- IV INJECT MICE WITH FORMULATED siRNAs ON DAY 9
- SACRIFICE AT 72 HOURS POST siRNA INJECTION ON DAY 12
- ANALYZE mRNA LEVELS IN LIVER USING TAQMAN-QPCR

— siCol1a1 (AD21349) (SEQ ID NO: 53 AND SEQ ID NO: 54), siLUC (AD1955) FINAL DOSE: 0.5mg/Kg

**FIG. 19**

**DELIVERY TO STELLATE CELLS IN CHRONIC INJURY MODEL
C12-200 BASED FORMULATION**

ROBUST DELIVERY TO STELLATE CELLS UNDER CONDITIONS OF CHRONIC LIVER INJURY

- MULTI-INSULT, MULTI-DOSE MODEL
- ~50-FOLD INDUCTION OF Col1a1 mRNA BY CCl₄
- ~95% KNOCK-DOWN OF Col1a1 mRNA

