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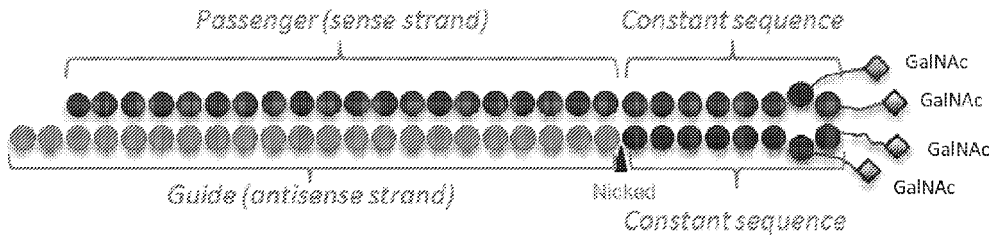
(54) Title: COMPOSITIONS AND METHODS FOR INHIBITING HMGB1 EXPRESSION

FIG. 2



(57) Abstract: This disclosure relates to oligonucleotides, compositions and methods useful for reducing HMGB1 expression, particularly in hepatocytes. Disclosed oligonucleotides for the reduction of HMGB1 expression may be double-stranded or single-stranded, and may be modified for improved characteristics such as stronger resistance to nucleases and lower immunogenicity. Disclosed oligonucleotides for the reduction of HMGB1 expression may also be designed to include targeting ligands to target a particular cell or organ, such as the hepatocytes of the liver, and may be used to treat liver fibrosis and related conditions.

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COMPOSITIONS AND METHODS FOR INHIBITING HMGB1 EXPRESSION

RELATED APPLICATIONS

[0001] This application claims the benefit of the filing date under 35 U.S.C. §119 of United States Provisional Application Serial Number 62/526,971, filed June 29, 2017, and entitled "COMPOSITIONS AND METHODS FOR INHIBITING HMGB1 EXPRESSION", the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present application relates to oligonucleotides and uses thereof, particularly uses relating to the treatment of conditions involving fibrosis.

REFERENCE TO THE SEQUENCE LISTING

[0003] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled D0800.70002WO00-SEQ.txt created on June 28, 2018 which is 85 kilobytes in size. The information in electronic format of the sequence listing is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0004] Tissue fibrosis is a condition characterized by an abnormal accumulation of extracellular matrix and inflammatory factors that result in scarring and promote chronic organ injury. In liver, fibrosis is a multi-cellular response to hepatic injury that can lead to cirrhosis and hepatocellular cancer. The response is often triggered by liver injury associated with conditions such as alcohol abuse, viral hepatitis, metabolic diseases, and liver diseases, such as a cholestatic liver disease, nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). Studies have implicated high mobility group box 1 (HMGB1) protein as having a pro-fibrotic role in liver fibrosis. (*See*, *e.g.*, Li L-C, *et al.*, Emerging role of HMGB1 in fibrotic diseases, J. Cell. Mol. Med. Vol 18, No 12, 2014 pp. 2331-2339) HMGB1 is a nuclear protein released from injured cells that functions as a proinflammatory mediator and has been shown to recruit hepatic stellate cells and liver endothelial cells to sites of liver injury. (Seo, YS, *et al.*, HMGB1 recruits hepatic stellate cells and liver endothelial cells to sites of ethanol-induced parenchymal cell injury, Am J Physiol Gastrointest Liver Physiol 305:

G838–G848, 2013.) Hepatic stellate cells are believed to play a central role in the progression of liver fibrosis through their transformation into proliferative myofibroblastic cells that promote fibrogenic activity in the liver. (*See*, Kao YH, et al. High-mobility group box 1 protein activates hepatic stellate cells in vitro. Transplant Proc. 2008; 40: 2704–5)

BRIEF SUMMARY OF THE INVENTION

[0005] Aspects of the disclosure relate to compositions and methods for treating fibrosis (e.g., liver fibrosis) in a subject. In some embodiments, potent RNAi oligonucleotides have been developed for selectively inhibiting HMGB1 expression. Accordingly, in some embodiments, RNAi oligonucleotides provided herein are useful for reducing HMGB1 expression, particularly in hepatocytes, and thereby decreasing or preventing fibrosis (see, *e.g.*, Examples 2-4; FIGS. 14A-C, 17A-D, 19, and 22-27). In some embodiments, RNAi oligonucleotides incorporating nicked tetraloop structures are conjugated with GalNAc moieties to facilitate delivery to liver hepatocytes (through interactions with asialoglycoprotein receptor, which is primarily expressed on the surface of hepatocytes) to inhibit HMGB1 expression for the treatment of liver fibrosis. In some embodiments, methods are provided herein involving the use of RNAi oligonucleotides for treating subjects having or suspected of having liver conditions such as, for example, cholestatic liver disease, nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). In further embodiments, the disclosure is based on an identification of key regions (referred to as hotspots) of HMGB1 mRNA that are particularly amenable to targeting using oligonucleotide-based approaches. Accordingly, in some embodiments, oligonucleotides have been developed that target these mRNA hotspots to inhibit HMGB1 expression to treat fibrosis.

[0006] One aspect of the present disclosure provides oligonucleotides for reducing expression of HMGB1. In some embodiments, the oligonucleotides comprise an antisense strand comprising a sequence as set forth in any one of SEQ ID NOs: 97-192, 273-362, and 366-370. In some embodiments, the antisense strand consists of a sequence as set forth in any one of SEQ ID NOs: 97-192, 273-362, and 366-370. In some embodiments, the antisense strand comprises, or consists of, a sequence as set forth in any one of SEQ ID NOs: 273-362, and 366-370. In some embodiments, the antisense strand comprises, or consists of, a sequence as set forth in SEQ ID NO: 286, 367, 369, or 370. In some embodiments, the oligonucleotides further comprise a sense strand that comprises a sequence as set forth in any one of SEQ ID NOs: 1-96, 193-272, and 363-365. In some embodiments, the sense strand consists of a sequence as set forth in any one of SEQ ID NOs: 1-96, 193-272, and 363-365. In some

embodiments, the sense strand comprises, or consists of, a sequence as set forth in any one of SEQ ID NOs: 193-272 and 363-365. In some embodiments, the sense strand comprises, or consists of, a sequence as set forth in SEQ ID NO: 204, 211, 364, 365.

[0007] One aspect of the present disclosure provides oligonucleotides for reducing expression of HMGB1, in which the oligonucleotides comprise an antisense strand of 15 to 30 nucleotides in length. In some embodiments, the antisense strand has a region of complementarity to a target sequence of HMGB1 as set forth in any one of SEQ ID NOs: 374-381. In some embodiments, the region of complementarity is at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, or at least 22 contiguous nucleotides in length. In some embodiments, the region of complementarity is fully complementary to the target sequence of HMGB1. In some embodiments, the region of complementarity to HMGB1 is at least 19 contiguous nucleotides in length.

[0008] In some embodiments, the antisense strand is 19 to 27 nucleotides in length. In some embodiments, the antisense strand is 21 to 27 nucleotides in length. In some embodiments, the oligonucleotide further comprises a sense strand of 15 to 40 nucleotides in length, in which the sense strand forms a duplex region with the antisense strand. In some embodiments, the sense strand is 19 to 40 nucleotides in length. In some embodiments, the antisense strand is 27 nucleotides in length and the sense strand is 25 nucleotides in length. In some embodiments, the duplex region is at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, or at least 21 nucleotides in length. In some embodiments, the antisense strand and sense strand form a duplex region of 25 nucleotides in length.

[0009] In some embodiments, an oligonucleotide comprises an antisense strand and a sense strand that are each in a range of 21 to 23 nucleotides in length. In some embodiments, an oligonucleotide comprises a duplex structure in a range of 19 to 21 nucleotides in length. In some embodiments, an oligonucleotide comprises a 3′-overhang sequence of one or more nucleotides in length, in which the 3′-overhang sequence is present on the antisense strand, the sense strand, or the antisense strand and sense strand. In some embodiments, an oligonucleotide further comprises a 3′-overhang sequence on the antisense strand of two nucleotides in length. In some embodiments, an oligonucleotide comprises a 3′-overhang sequence of two nucleotides in length, in which the 3′-overhang sequence is present on the antisense strand, and in which the sense strand is 21 nucleotides in length and the antisense strand is 23 nucleotides in length, such that the sense strand and antisense strand form a duplex of 21 nucleotides in length.

[00010] In some embodiments, the sense strand comprises a sequence as set forth in

any one of SEQ ID NOs: 1-96, 193-272, and 363-365. In some embodiments, the sense strand consists of a sequence as set forth in any one of SEQ ID NOs: 1-96, 193-272, and 363-365. In some embodiments, the antisense strand comprises a sequence as set forth in any one of SEQ ID NOs: 97-192, 273-362, and 366-370. In some embodiments, the antisense strand consists of a sequence as set forth in any one of SEQ ID NOs: 97-192, 273-362, and 366-370.

[00011] In some embodiments, the sense strand comprises at its 3′-end a stem-loop set forth as: S1-L-S2, in which S1 is complementary to S2, and in which L forms a loop between S1 and S2 of 3 to 5 nucleotides in length.

[00012] Another aspect of the present disclosure provides an oligonucleotide for reducing expression of HMGB1, the oligonucleotide comprising an antisense strand and a sense strand, in which the antisense strand is 21 to 27 nucleotides in length and has a region of complementarity to HMGB1, in which the sense strand comprises at its 3′-end a stem-loop set forth as: S1-L-S2, in which S1 is complementary to S2, and in which L forms a loop between S1 and S2 of 3 to 5 nucleotides in length, and in which the antisense strand and the sense strand form a duplex structure of at least 19 nucleotides in length but are not covalently linked (see, e.g., FIG. 2) . In some embodiments, the region of complementarity is fully complementary to at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, or at least 21 contiguous nucleotides of HMGB1 mRNA. In some embodiments, L is a tetraloop. In some embodiments, L is 4 nucleotides in length. In some embodiments, L comprises a sequence set forth as GAAA.

[00013] In some embodiments, an oligonucleotide comprises at least one modified nucleotide. In some embodiments, the modified nucleotide comprises a 2′-modification. In some embodiments, the 2′-modification is a modification selected from: 2′-aminoethyl, 2′-fluoro, 2′-O-methyl, 2′-O-methoxyethyl, and 2′-deoxy-2′-fluoro-β-d-arabinonucleic acid. In some embodiments, all of the nucleotides of an oligonucleotide are modified.

[00014] In some embodiments, an oligonucleotide comprises at least one modified internucleotide linkage. In some embodiments, the at least one modified internucleotide linkage is a phosphorothioate linkage. In some embodiments, the 4′-carbon of the sugar of the 5′-nucleotide of the antisense strand comprises a phosphate analog. In some embodiments, the phosphate analog is oxymethylphosphonate, vinylphosphonate, or malonylphosphonate.

[00015] In some embodiments, at least one nucleotide of an oligonucleotide is conjugated to one or more targeting ligands. In some embodiments, each targeting ligand comprises a carbohydrate, amino sugar, cholesterol, polypeptide, or lipid. In some embodiments, each targeting ligand comprises a N-acetylgalactosamine (GalNAc) moiety. In

some embodiments, the GalNac moiety is a monovalent GalNAc moiety, a bivalent GalNAc moiety, a trivalent GalNAc moiety, or a tetravalent GalNAc moiety. In some embodiments, up to 4 nucleotides of L of the stem-loop are each conjugated to a monovalent GalNAc moiety. In some embodiments, the targeting ligand comprises an aptamer.

[00016] Another aspect of the present disclosure provides a composition comprising an oligonucleotide of the present disclosure and an excipient. Another aspect of the present disclosure provides a method comprising administering a composition of the present disclosure to a subject. In some embodiments, the method results in a decreased level or prevention of liver fibrosis. In some embodiments, expression of HMGB1 protein is reduced by administering to the subject an oligonucleotide disclosed herein. In some embodiments, a subject has cholestatic or autoimmune liver disease.

[00017] Another aspect of the present disclosure provides a method of treating a subject having or at risk of having liver fibrosis. In some embodiments, methods provided herein comprise administering to the subject an oligonucleotide that reduces expression of HMGB1. In some embodiments, the subject has cholestatic or autoimmune liver disease. In some embodiments, the subject has nonalcoholic steatohepatitis (NASH). In some embodiments, the oligonucleotide administered to the subject is an RNAi oligonucleotide.

[00018] In some embodiments, an oligonucleotide is administered prior to exposure of a subject to a hepatotoxic agent. In some embodiments, an oligonucleotide is administered subsequent to exposure of a subject to a hepatotoxic agent. In some embodiments, the oligonucleotide is administered simultaneously with a subject's exposure to a hepatotoxic agent.

[00019] Another aspect of the present disclosure provides a method of treating a subject having or at risk of having nonalcoholic steatohepatitis (NASH). In some embodiments, themethod comprises administering to the subject an oligonucleotide that reduces expression of HMGB1 in the subject.

[00020] In some embodiments, administration of an oligonucleotide disclosed herein results in a reduction in liver HMGB1 levels. In some embodiments, administration of an oligonucleotide disclosed herein results in a reduction in serum HMGB1 levels.

[00021] Another aspect of the present disclosure provides an oligonucleotide for reducing expression of HMGB1, in whicholigonucleotide comprises a sense strand of 15 to 50 nucleotides in length and an antisense strand of 15 to 30 nucleotides in length, in which the sense strand forms a duplex region with the antisense strand, in which the sense strand comprises a sequence as set forth in any one of SEQ ID NO: 1-96, and in which the antisense

strand comprises a complementary sequence selected from SEQ ID NO: 97-192. In some embodiments, the sense strand consists of a sequence as set forth in any one of SEQ ID NO: 1-96. In some embodiments, the antisense strand consists of a complementary sequence selected from SEQ ID NO: 97-192.

[00022] Another aspect of the present disclosure provides an oligonucleotide for reducing expression of HMGB1, in which oligonucleotide comprising a sense strand of 15 to 50 nucleotides in length and an antisense strand of 15 to 30 nucleotides in length, in which the sense strand forms a duplex region with the antisense strand, in which the sense strand comprises a sequence as set forth in any one of SEQ ID NO: 193-272 or 363-365 and in which the antisense strand comprises a complementary sequence selected from SEQ ID NO: 273-362 or 366-370. In some embodiments, the sense strand consists of a sequence as set forth in any one of SEQ ID NO: 193-272 or 363-365. In some embodiments, the antisense strand consists of a complementary sequence selected from SEQ ID NO: 273-362 or 366-370.

[00023] Another aspect of the present disclosure provide an oligonucleotide for reducing expression of HMGB1, in which the oligonucleotide comprises a sense strand of 15 to 50 nucleotides in length and an antisense strand of 15 to 30 nucleotides in length, in which the sense strand forms a duplex region with the antisense strand, in which the sense strand comprises a sequence as set forth in any one of SEQ ID NO: 204, 211, 364, 365 and in which the antisense strand comprises a complementary sequence selected from SEQ ID NO: 286, 367, 369, 370. In some embodiments, the sense strand comprises a sequence as set forth in SEQ ID NO: 204, and the antisense strand comprises a sequences as set for in SEQ ID NO: 286. In some embodiments, the sense strand comprises a sequence as set forth in SEQ ID NO: 211, andthe antisense strand comprises a sequences as set for in SEQ ID NO: 367. In some embodiments, the sense strand comprises a sequence as set forth in SEQ ID NO: 364, and the antisense strand comprises a sequences as set for in SEQ ID NO: 369. In some embodiments, the sense strand comprises a sequence as set forth in SEQ ID NO: 365, and the antisense strand comprises a sequences as set for in SEQ ID NO: 370. In some embodiments, the sense strand consists of a sequence as set forth in SEQ ID NO: 204, and the antisense strand consists of a sequences as set for in SEQ ID NO: 286. In some embodiments, the sense strand consists of a sequence as set forth in SEQ ID NO: 211, and the antisense strand consists of a sequences as set for in SEQ ID NO: 367. In some embodiments, the sense strand consists of a sequence as set forth in SEQ ID NO: 364, and the antisense strand consists of a sequences as set for in SEQ ID NO: 369. In some embodiments, the sense strand consists of a sequence as set forth in SEQ ID NO: 365, and the antisense strand consists of a sequences as set for in SEQ ID NO: 370.

[00024] Another aspect of the present disclosure provide an oligonucleotide for reducing expression of HMGB1, in which the oligonucleotide comprises a pair of sense and antisense strands selected from a row of Table 7. In some embodiments, an oligonucleotide provided herein comprises at least one modified nucleotide. In some embodiments, the modified nucleotide comprises a 2'-modification. In some embodiments, the 2'-modification is a modification selected from: 2'-aminoethyl, 2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl, and 2'-deoxy-2'-fluoro-β-d-arabinonucleic acid. In some embodiments, all nucleotides in the oligonucleotide are modified. In some embodiments, the oligonucleotide comprises at least one modified internucleotide linkage. In some embodiments, the at least one modified internucleotide linkage is a phosphorothioate linkage. In some embodiments, the 4'-carbon of the sugar of the 5'-nucleotide of the antisense strand comprises a phosphate analog. In some embodiments, the phosphate analog is oxymethylphosphonate, vinylphosphonate, or malonylphosphonate. In some embodiments, at least one nucleotide of the oligonucleotide is conjugated to one or more targeting ligands. In some embodiments, each targeting ligand comprises a carbohydrate, amino sugar, cholesterol, polypeptide or lipid. In some embodiments, each targeting ligand comprises a N-acetylgalactosamine (GalNAc) moiety. In some embodiments, the GalNac moiety is a monovalent GalNAc moiety, a bivalent GalNAc moiety, a trivalent GalNAc moiety, or a tetravalent GalNAc moiety. In some embodiments, up to 4 nucleotides of L of the stem-loop are each conjugated to a monovalent GalNAc moiety.

BRIEF DESCRIPTION OF THE DRAWINGS

[00025] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate certain embodiments, and together with the written description, serve to provide non-limiting examples of certain aspects of the compositions and methods disclosed herein.

[00026] FIG. 1 is a flowchart depicting the experimental design used to select compounds for testing in cell and animal models and to develop double-stranded oligonucleotides for reducing expression of HMGB1. SAR: Structure-Activity Relationship.

[00027] FIG. 2 is a schematic showing a non-limiting example of a double-stranded oligonucleotide with a nicked tetraloop structure that has been conjugated to four GalNAc moieties (diamonds on the right side of the molecule).

[00028] FIGs. 3A-3D are a series of graphs showing the percent of HMGB1 mRNA remaining after a phase 1 screen of 96 HMGB1 oligonucleotides in Hepa1-6 cells. "Mm HMBG1 533-646" indicates the position of the 5' RT-PCR site and "Mm HMBG1 1541-1675"

indicates the position of the 3' RT-PCR site on the mouse mRNA.

[00029] FIG. 4 is a graph showing the percentage of mRNA remaining after HMGB1 oligonucleotide phase 2 screening of 96 HMGB1 oligonucleotides at three different concentrations (1 nM, 0.1nM and 0.01nM) in Hepa1-6 cells (3' assay).

[00030] FIG. 5 is a graph showing the results of screening in Hepa1-6 cells using HMGB1 oligonucleotides of different base sequences in the nicked tetraloop structure, adapted to different modification patterns and an unmodified control.

[00031] FIG. 6 is a graph showing the IC_{50} results for six HMGB1 oligonucleotides selected from dose response curve screening in Hepa1-6 cells.

[00032] FIG. 7 is a graph showing the amount of HMGB1 mRNA relative to the amount HPRT mRNA remaining in the liver 72 hours after subcutaneous administration of 10 mg/kg (mpk) GalNAc-conjugated HMGB1 oligonucleotide in C57BL/6.

[00033] FIG. 8 is a graph showing an *in vivo* activity evaluation of three HMGB1 oligonucleotides in a nicked tetraloop structure. LNP formulated oligonucleotides were administered intravenously to mice at three different dosages (0.3 mg/kg, 1 mg/kg and 3 mg/kg). The data show the amount of HMGB1 mRNA remaining at 24 hours after administration normalized to a PBS control treatment.

[00034] FIG. 9 is a graph showing an *in vivo* activity evaluation of GalNAc-conjugated HMGB1 oligonucleotides in a nicked tetraloop structure. Two different oligonucleotide sequences were tested with four different oligonucleotide modification patterns (denoted as M49, M54, M55, M56, which contain different arrangements of 2'-fluoro and 2'-O-methyl modified nucleotides, phophorothiate linkages, and included a phosphate analog positioned at the 5′ terminal nucleotide of their antisense strands). Oligonucleotides were subcutaneously administered to mice at two different dosages (3 mg/kg and 10 mg/kg). The data show the amount of HMGB1 mRNA remaining at 24 hours after administration normalized to a PBS control treatment.

[00035] FIG. 10 is a graph showing an *in vivo* activity evaluation of GalNAc-conjugated HMGB1 oligonucleotides in a nicked tetraloop structure. Different oligonucleotide sequences were tested in four different oligonucleotide modification patterns (denoted as M49, M51, M53, M57). Oligonucleotides were subcutaneously administered to mice at 3 mg/kg. The data show the amount of HMGB1 mRNA remaining at 72 hours after administration normalized to a PBS control treatment.

[00036] FIG. 11 is a graph showing an *in vivo* activity evaluation of GalNAc-conjugated HMGB1 oligonucleotides in a nicked tetraloop structure. Two different

oligonucleotide sequences were tested in different modification patterns (denoted as M29, M49, and M50). The HMGB1 oligonucleotides were designed to bind to conserved sequences identified by the algorithm in the human, rhesus, and mouse sequences ("triple common" sequences). The data show the amount of HMGB1 mRNA remaining at day 5 (normalized to a PBS control treatment) after a 1 mg/kg subcutaneous dose.

[00037] FIG. 12 is a schematic showing the flowchart of an experimental model for APAP-induced liver injury with C57BL/6 male mice (10 to 11 weeks). In this model, mice received an intraperitoneal injection of 350 or 250 mg/kg APAP after a fasting period of 12 hours. Blood and/or tissue samples were collected at 6, 24, and 48 hours.

[00038] FIGs. 13A-13B are a set of graphs showing the percentage of HMGB1 mRNA (normalized to Hprt1 mRNA) remaining in mice after a 350 mg/kg APAP injection and treatment with GalNAc-conjugated HMGB1 oligonucleotides, LNP formulated HMGB1 oligonucleotides, or a PBS control. FIG. 13A shows liver HMGB1 expression and FIG. 16B shows serum HMGB1 expression. Saline-injected animals were used as controls. The HMGB1 oligonucleotide used in FIGs. 13A-13B is S212-AS296-M49.

[00039] FIG. 14A-14C are a series of graphs showing AST, ALT, and serum miR122 expression post HMGB1 treatment in mice after a 350 mg/kg APAP injection and treatment with GalNAc-conjugated HMGB1 oligonucleotides, LNP formulated HMGB1 oligonucleotides, or a PBS control. Saline-injected animals were used as controls. The HMGB1 oligonucleotide used in FIGs. 14A-14C is S212-AS296-M49.

[00040] FIG. 15 is a schematic showing the flowchart of an experimental model for APAP-induced liver injury. In this model, animals were treated with either five doses of a GalNAc-conjugated HMGB1 oligonucleotides at 5 mg/kg or two doses of LNP formulated HMGB1 oligonucleotides at 1.5 mg/kg. Following a two-week period of administration of the oligonucleotides and a fasting period of 12 hours, mice received an intraperotineal (I.P.) injection of 350 mg/kg APAP in 0.9% NaCl. One day (24 hours) after administration of the APAP injection, tissue and/or blood samples were harvested.

[00041] FIGs. 16A-16B are graphs showing the percentage of HMGB1 mRNA remaining in the liver or serum of mice after a 350 mg/kg APAP injection and treatment with GalNAc-conjugated HMGB1 oligonucleotides, LNP formulated HMGB1 oligonucleotides, or a PBS control. FIG. 16A shows liver HMGB1 expression and FIG. 16B shows serum HMGB1 expression. Saline-injected animals were used as controls. The HMGB1 oligonucleotide used in FIGs. 16A-16B is S212-AS296-M56.

[00042] FIGs. 17A-17D are a series of graphs showing ALT (FIG. 17A), AST (FIG.

20B), LDH (FIG. 20C), and miR122 expression (FIG. 17D) in 350 mg/kg APAP treated animals after treatment with GalNAc-conjugated HMGB1 oligonucleotides, LNP formulated HMGB1 oligonucleotides, or a PBS control. Saline-injected animals were used as controls. The HMGB1 oligonucleotide used in FIGs. 17A-17D is S212-AS296-M56.

[00043] FIG. 18 is a schematic showing the flowchart of a carbon tetrachloride (CCl_4) model for anti-fibrotic activity with GalNAc-conjugated HMGB1 oligonucleotide dosing regimens.

[00044] FIG. 19 is a graph of Sirius Red relative mean intensity levels in a CCl_4 model for anti-fibrotic activity after treatment with a subcutaneous 5 mg/kg dose of GalNAc-conjugated HMGB1 oligonucleotides every 5 or 7 weeks or a PBS control. The HMGB1 oligonucleotide used in FIG. 19 is S212-AS296-M49.

[00045] FIG. 20 is a graph of alpha-smooth muscle actin relative mean intensity levels in a CCl_4 model for anti-fibrotic activity after treatment with a subcutaneous 5 mg/kg dose of GalNAc-conjugated HMGB1 oligonucleotides every 5 or 7 weeks or a PBS control. The HMGB1 oligonucleotide used in FIG. 20 is S212-AS296-M49.

[00046] FIG. 21 is a graph of liver weight to body weight ratios in a CCl_4 model for anti-fibrotic activity after treatment with a subcutaneous 5 mg/kg dose of GalNAc-conjugated HMGB1 oligonucleotides every 5 or 7 weeks or a PBS control. The HMGB1 oligonucleotide used in FIG. 21 is S212-AS296-M49.

[00047] FIG. 22 is a graph of F4/80+ area in a CCl_4 model for anti-fibrotic activity after treatment with a subcutaneous 5 mg/kg dose of GalNAc-conjugated HMGB1 oligonucleotides every 5 or 7 weeks or a PBS control. The HMGB1 oligonucleotide used in FIG. 22 is S212-AS296-M49.

[00048] FIG. 23 is a graph of Sirius Red relative mean intensity levels in a CCl_4 model for anti-fibrotic activity 43 days after treatment with a 5 mg/kg subcutaneous dose GalNAc-conjugated HMGB1 oligonucleotides in a tetraloop structure or a 2.5 mg/kg intravenous dose of LNP-formulated oligonucleotide in a tetraloop structure. PBS-treated animals and animals untreated with CCl_4 were used as controls. The HMGB1 oligonucleotide used in FIG. 23 is S212-AS296-M49.

[00049] FIG. 24 is a schematic showing the flowchart for a Concavalin A induced hepatitis model in mice alongside a chart showing a basic outline of the experimental design.

[00050] FIG. 25 is a series of pictures demonstrating the results of GalNAc-conjugated HMGB1 oligonucleotide treatment in a Concavalin A model of induced hepatitis in mice. Data from mice treated with PBS control are shown at left. Data from mice treated with a

subcutaneous dose of 10 mg/kg of GalNAc-conjugated HMGB1 oligonucleotides are shown at right. The HMGB1 oligonucleotide used in FIG. 25 is S212-AS296-M49.

[00051] FIGs. 26A-26D are a series of graphs demonstrating the results of subcutaneous GalNAc-conjugated HMGB1 oligonucleotide treatment on HMGB1 (FIGs. 26A and 26B), Col1a1 (FIG. 26C), and Vimentin (FIG. 26D) mRNA levels in mice on a choline-deficient amino acid-defined high-fat diet (CDAHFD) or control high-fat diet (HFD) in comparison to PBS control treatment. The HMGB1 oligonucleotide used in FIGs. 26A-26D is S194-AS274-M30.

[00052] FIG. 27 is a set of graphs demonstrating the results of subcutaneous GalNAc-conjugated HMGB1 oligonucleotide treatment on ALT and AST levels in mice on a choline-deficient amino acid-defined high-fat diet (CDAHFD) or control high-fat diet (HFD) in comparison to PBS control treatment. The HMGB1 oligonucleotide used in FIG. 27 is S194-AS274-M30.

[00053] FIG. 28 is a graph showing an in vivo activity evaluation of eight GalNAc-conjugated HMGB1 oligonucleotides with 3 different modification patterns on day 5 following 1mg/kg of subcutaneous administration. The percentage of remaining HMGB1 mRNA in the liver 5 days after administration of the oligonucleotides, normalized to the remaining HMGB1 mRNA levels in mice treated with PBS, is shown. The results show that all tested HMGB1 oligonucleotides with different chemical modification patterns are potent in knocking down HMGB1 in mice hepatocytes.

[00054] FIGs. 29A-29B are graphs showing an in vivo activity evaluation of eight GalNAc-conjugated HMGB1 oligonucleotides at three different dosages (1, 0.5, or 0.25 mg/kg). The percent remaining HMGB1 mRNA in the liver 5 days after administration of the oligonucleotides, normalized to the remaining HMGB1 mRNA levels in mice treated with PBS, was evaluated in either a 5′ qPCR reaction (FIG. 29 A) or a 3′ qPCR reaction (FIG. 29B). The results show that the potency of all tested HMGB1 oligonucleotides are dose-dependent.

[00055] FIGs. 30A-30D are graphs showing an in vivo activity evaluation of three GalNAc-conjugated HMGB1 oligonucleotides at 4-different time points. The data show the amount of HMGB1 mRNA remaining (normalized to the remaining HMGB1 mRNA levels in mice treated with PBS) on day 7, 14, 21, or 28 after administration of the oligonucleotides. FIGs. 30A and 30B show the results of 5' qPCR reactions. FIGs. 30C and 30D show the results of 3' qPCR reactions. The results showed that all tested GalNAc-conjugated HMGB1 oligonucleotides were potent in knocking down HMGB1 3 weeks after injection.

[00056] FIGs. 31A-31B are graphs showing an in vivo activity evaluation of four

GalNAc-conjugated HMGB1 oligonucleotides at three different dosages (1, 0.5, or 0.25 mg/kg). The percentage of remaining HMGB1 mRNA in the liver 5 days after administration of the oligonucleotides, normalized to the remaining HMGB1 mRNA levels in mice treated with PBS, was evaluated in either a 5′ qPCR reaction (FIG. 31 A) or a 3′ qPCR reaction (FIG. 31B). All tested HMGB1 oligonucleotide inhibitors showed an ED_{50} (effective dose for 50% of recipient receiving the drug) of about 0.5 to 1.0 mg/kg.

[00057] FIGs. 32A-32B are graphs showing an in vivo activity evaluation of four GalNAc-conjugated HMGB1 oligonucleotides 21 days after subcutaneous administration. The percent percentage of remaining HMGB1 mRNA in the liver 21 days after administration of the oligonucleotides, normalized to the remaining HMGB1 mRNA levels in mice treated with PBS, was evaluated in either a 5′ qPCR reaction (FIG. 32 A) or a 3′ qPCR reaction (FIG. 32B). The results showed that all tested GalNAc-conjugated HMGB1 oligonucleotides retained potency in knockdown HMGB1 3 weeks post injection.

[00058] FIGs. 33A-33G are IC50 curves demonstrating uptake and activity of the 2 GalNAc-conjugates HMGB1 oligonucleotides in primary monkey hepatocytes. The IC50 curves were normalized to mock treatment. Results for RhHMGB1 5' qPCR reactions are shown in FIGs. 33A-33C), and results for 3' qPCR reactions are shown in FIGs. 33D-33F. A GalNAc- conjugate LDHA oligonucleotide was used as assay control (FIGs. 33C, 33F, and 33G). The level of remaining RhLDHA mRNA was measured by a LDHA specific primer in qPCR assay (FIG. 33G).

[00059] FIGs. 34A-34G are IC50 curves demonstrating uptake and activity of the 2 GalNAc-conjugates HMGB1 oligonucleotides in primary human hepatocytes. The IC50 curves were normalized to mock treatment. Results for hsHMGB1 5' qPCR reactions are shown in FIGs. 34A-34C), and results for 3' qPCR reactions are shown in FIGs. 34D-34F. A GalNAc- conjugate LDHA oligonucleotide was used as assay control (FIGs. 34C, 34F, and 34G). The level of remaining HsLDHA mRNA was measured by a LDHA specific primer in qPCR assay (FIG. 34G).

DETAILED DESCRIPTION OF THE INVENTION

[00060] According to some aspects, the disclosure provides oligonucleotides targeting HMGB1 mRNA that are effective for reducing HMGB1 expression in cells, particularly liver cells (e.g., hepatocytes) for the treatment of liver fibrosis. Accordingly, in related aspects, the disclosure provided methods of treating fibrosis that involve selectively reducing HMGB1 gene expression in liver. In certain embodiments, HMGB1 targeting

oligonucleotides provided herein are designed for delivery to selected cells of target tissues (*e.g.*, liver hepatocytes) to treat fibrosis in those tissues.

[00061] Further aspects of the disclosure, including a description of defined terms, are provided below.

I. Definitions

[00062] **Approximately:** As used herein, the term "approximately" or "about," as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term "approximately" or "about" refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[00063] **Administering**: As used herein, the terms "administering" or "administration" means to provide a substance (*e.g.*, an oligonucleotide) to a subject in a manner that is pharmacologically useful (*e.g.*, to treat a condition in the subject).

[00064] **Asialoglycoprotein receptor (ASGPR)**: As used herein, the term "Asialoglycoprotein receptor" or "ASGPR" refers to a bipartite C-type lectin formed by a major 48 kDa (ASGPR-1) and minor 40 kDa subunit (ASGPR-2). ASGPR is primarily expressed on the sinusoidal surface of hepatocyte cells, and has a major role in binding, internalization, and subsequent clearance of circulating glycoproteins that contain terminal galactose or N-acetylgalactosamine residues (asialoglycoproteins).

[00065] **Attenuates**: As used herein, the term "attenuates" means reduces or effectively halts. As a non-limiting example, one or more of the treatments provided herein may reduce or effectively halt the onset or progression of liver fibrosis or liver inflammation in a subject. This attenuation may be exemplified by, for example, a decrease in one or more aspects (*e.g.*, symptoms, tissue characteristics, and cellular, inflammatory or immunological activity, *etc.*) of liver fibrosis or liver inflammation, no detectable progression (worsening) of one or more aspects of liver fibrosis or liver inflammation, or no detectable aspects of liver fibrosis or liver inflammation in a subject when they might otherwise be expected.

[00066] **Complementary:** As used herein, the term "complementary" refers to a structural relationship between two nucleotides (*e.g.*, on two opposing nucleic acids or on opposing regions of a single nucleic acid strand) that permits the two nucleotides to form base pairs with one another. For example, a purine nucleotide of one nucleic acid that is

complementary to a pyrimidine nucleotide of an opposing nucleic acid may base pair together by forming hydrogen bonds with one another. In some embodiments, complementary nucleotides can base pair in the Watson-Crick manner or in any other manner that allows for the formation of stable duplexes. In some embodiments, two nucleic acids may have regions of multiple nucleotides that are complementary with each other so as to form regions of complementarity, as described herein.

[00067] **Deoxyribonucleotide:** As used herein, the term "deoxyribonucleotide" refers to a nucleotide having a hydrogen in place of a hydroxyl at the 2' position of its pentose sugar as compared with a ribonucleotide. A modified deoxyribonucleotide is a deoxyribonucleotide having one or more modifications or substitutions of atoms other than at the 2' position, including modifications or substitutions in or of the sugar, phosphate group or base.

[00068] **Double-stranded oligonucleotide:** As used herein, the term "double-stranded oligonucleotide" refers to an oligonucleotide that is substantially in a duplex form. In some embodiments, the complementary base-pairing of duplex region(s) of a double-stranded oligonucleotide is formed between of antiparallel sequences of nucleotides of covalently separate nucleic acid strands. In some embodiments, complementary base-pairing of duplex region(s) of a double-stranded oligonucleotide is formed between antiparallel sequences of nucleotides of nucleic acid strands that are covalently linked. In some embodiments, complementary base-pairing of duplex region(s) of a double-stranded oligonucleotide is formed from single nucleic acid strand that is folded (*e.g.*, via a hairpin) to provide complementary antiparallel sequences of nucleotides that base pair together. In some embodiments, a double-stranded oligonucleotide comprises two covalently separate nucleic acid strands that are fully duplexed with one another. However, in some embodiments, a double-stranded oligonucleotide comprises two covalently separate nucleic acid strands that are partially duplexed, *e.g.*, having overhangs at one or both ends. In some embodiments, a double-stranded oligonucleotide comprises antiparallel sequence of nucleotides that are partially complementary, and thus, may have one or more mismatches, which may include internal mismatches or end mismatches.

[00069] **Duplex:** As used herein, the term "duplex," in reference to nucleic acids (*e.g.*, oligonucleotides), refers to a structure formed through complementary base pairing of two antiparallel sequences of nucleotides.

[00070] **Excipient:** As used herein, the term "excipient" refers to a non-therapeutic agent that may be included in a composition, for example, to provide or contribute to a desired consistency or stabilizing effect.

[00071] **Hepatocyte**: As used herein, the term "hepatocyte" or "hepatocytes" refers to cells of the parenchymal tissues of the liver. These cells make up approximately 70-85% of the liver's mass and manufacture serum albumin, fibrinogen, and the prothrombin group of clotting factors (except for Factors 3 and 4). Markers for hepatocyte lineage cells may include, but are not limited to: transthyretin (Ttr), glutamine synthetase (Glul), hepatocyte nuclear factor 1a (Hnf1a), and hepatocyte nuclear factor 4a (Hnf4a). Markers for mature hepatocytes may include, but are not limited to: cytochrome P450 (Cyp3a11), fumarylacetoacetate hydrolase (Fah), glucose 6-phosphate (G6p), albumin (Alb), and OC2-2F8. See, *e.g.*, Huch *et al.*, (2013), Nature, 494(7436): 247-250, the contents of which relating to hepatocyte markers is incorporated herein by reference.

[00072] **Hepatotoxic agent**: As used herein, a "hepatotoxic agent" is a chemical compound, virus, or other substance that is itself toxic to the liver or can be processed to form a metabolite that is toxic to the liver. Hepatotoxic agents may include, but are not limited to, carbon tetrachloride (CCl_4), acetaminophen (paracetamol), vinyl chloride, arsenic, chloroform, nonsteroidal anti-inflammatory drugs (such as aspirin and phenylbutazone).

[00073] **Liver inflammation**: As used herein, the term "liver inflammation" or "hepatitis" refers to a physical condition in which the liver becomes swollen, dysfunctional, and/or painful, especially as a result of injury or infection, as may be caused by exposure to a hepatotoxic agent. Symptoms may include jaundice (yellowing of the skin or eyes), fatigue, weakness, nausea, vomiting, appetite reduction, and weight loss. Liver inflammation, if left untreated, may progress to fibrosis, cirrhosis, liver failure, or liver cancer.

[00074] **Liver fibrosis**: As used herein, the term "liver fibrosis" or "fibrosis of the liver" refers to an excessive accumulation in the liver of extracellular matrix proteins, which could include collagens (I, III, and IV), fibronectin, undulin, elastin, laminin, hyaluronan, and proteoglycans resulting from inflammation and liver cell death. Liver fibrosis, if left untreated, may progress to cirrhosis, liver failure, or liver cancer.

[00075] **Loop:** As used herein the term, "loop" refers to a unpaired region of a nucleic acid (*e.g.*, oligonucleotide) that is flanked by two antiparallel regions of the nucleic acid that are sufficiently complementary to one another, such that under appropriate hybridization conditions (*e.g.*, in a phosphate buffer, in a cells), the two antiparallel regions, which flank the unpaired region, hybridize to form a duplex (referred to as a "stem").

[00076] **Modified Internucleotide Linkage:** As used herein, the term "modified internucleotide linkage" refers to a internucleotide linkage having one or more chemical modifications compared with a reference internucleotide linkage comprising a phosphodiester

bond. In some embodiments, a modified nucleotide is a non-naturally occurring linkage. Typically, a modified internucleotide linkage confers one or more desirable properties to a nucleic acid in which the modified internucleotide linkage is present. For example, a modified nucleotide may improve thermal stability, resistance to degradation, nuclease resistance, solubility, bioavailability, bioactivity, reduced immunogenicity, *etc.*

[00077] **Modified Nucleotide:** As used herein, the term "modified nucleotide" refers to a nucleotide having one or more chemical modifications compared with a corresponding reference nucleotide selected from: adenine ribonucleotide, guanine ribonucleotide, cytosine ribonucleotide, uracil ribonucleotide, adenine deoxyribonucleotide, guanine deoxyribonucleotide, cytosine deoxyribonucleotide and thymidine deoxyribonucleotide. In some embodiments, a modified nucleotide is a non-naturally occurring nucleotide. In some embodiments, a modified nucleotide has one or more chemical modification in its sugar, nucleobase and/or phosphate group. In some embodiments, a modified nucleotide has one or more chemical moieties conjugated to a corresponding reference nucleotide. Typically, a modified nucleotide confers one or more desirable properties to a nucleic acid in which the modified nucleotide is present. For example, a modified nucleotide may improve thermal stability, resistance to degradation, nuclease resistance, solubility, bioavailability, bioactivity, reduced immunogenicity, *etc.*

[00078] **Nicked Tetraloop Structure**: A "nicked tetraloop structure" is a structure of a RNAi oligonucleotide characterized by the presense of separate sense (passenger) and antisense (guide) strands, in which the sense strand has a region of complementarity with the antisense strand, and in which at least one of the strands, generally the sense strand, has a tetraloop configured to stabilize an adjacent stem region formed within the at least one strand.

[00079] **Oligonucleotide:** As used herein, the term "oligonucleotide" refers to a short nucleic acid, *e.g.*, of less than 100 nucleotides in length. An oligonucleotide may be single-stranded or double-stranded. An oligonucleotide may or may not have duplex regions. As a set of non-limiting examples, an oligonucleotide may be, but is not limited to, a small interfering RNA (siRNA), microRNA (miRNA), short hairpin RNA (shRNA), dicer substrate interfering RNA (dsiRNA), antisense oligonucleotide, short siRNA, or single-stranded siRNA. In some embodiments, a double-stranded oligonucleotide is an RNAi oligonucleotide.

[00080] **Overhang:** As used herein, the term "overhang" refers to terminal non-base pairing nucleotide(s) resulting from one strand or region extending beyond the terminus of a complementary strand with which the one strand or region forms a duplex. In some embodiments, an overhang comprises one or more unpaired nucleotides extending from a

duplex region at the 5′ terminus or 3′ terminus of a double-stranded oligonucleotide. In certain embodiments, the overhang is a 3′ or 5′ overhang on the antisense strand or sense strand of a double-stranded oligonucleotides.

[00081] **Phosphate analog:** As used herein, the term "phosphate analog" refers to a chemical moiety that mimics the electrostatic and/or steric properties of a phosphate group. In some embodiments, a phosphate analog is positioned at the 5′ terminal nucleotide of an oligonucleotide in place of a 5'-phosphate, which is often susceptible to enzymatic removal. In some embodiments, a 5′ phosphate analogs contain a phosphatase-resistant linkage. Examples of phosphate analogs include 5′ phosphonates, such as 5′ methylenephosphonate (5′-MP) and 5′-(E)-vinylphosphonate (5′-VP). In some embodiments, an oligonucleotide has a phosphate analog at a 4'-carbon position of the sugar (referred to as a "4'-phosphate analog") at a 5'-terminal nucleotide. An example of a 4'-phosphate analog is oxymethylphosphonate, in which the oxygen atom of the oxymethyl group is bound to the sugar moiety (*e.g.*, at its 4'-carbon) or analog thereof. See, for example, U.S. Provisional Application numbers 62/383,207, filed on September 2, 2016, and 62/393,401, filed on September 12, 2016, the contents of each of which relating to phosphate analogs are incorporated herein by reference. Other modifications have been developed for the 5′ end of oligonucleotides (see, *e.g.*, WO 2011/133871; U.S. Patent No. 8,927,513; and Prakash *et al.* (2015), Nucleic Acids Res., 43(6):2993-3011, the contents of each of which relating to phosphate analogs are incorporated herein by reference).

[00082] **Reduced expression:** As used herein, the term "reduced expression" of a gene refers to a decrease in the amount of RNA transcript or protein encoded by the gene and/or a decrease in the amount of activity of the gene in a cell or subject, as compared to an appropriate reference cell or subject. For example, the act of treating a cell with a double-stranded oligonucleotide (*e.g.*, one having an antisense strand that is complementary to HMGB1 mRNA sequence) may result in a decrease in the amount of RNA transcript, protein and/or activity (*e.g.*, encoded by the HMGB1 gene) compared to a cell that is not treated with the double-stranded oligonucleotide. Similarly, "reducing expression" as used herein refers to an act that results in reduced expression of a gene (*e.g.*, HMGB1).

[00083] **Region of Complementarity:** As used herein, the term "region of complementary" refers to a sequence of nucleotides of a nucleic acid (*e.g.*, a double-stranded oligonucleotide) that is sufficiently complementary to an antiparallel sequence of nucleotides to permit hybridization between the two sequences of nucleotides under appropriate hybridization conditions, *e.g.*, in a phosphate buffer, in a cell, *etc.*

[00084] **Ribonucleotide:** As used herein, the term "ribonucleotide" refers to a nucleotide having a ribose as its pentose sugar, which contains a hydroxyl group at its 2' position. A modified ribonucleotide is a ribonucleotide having one or more modifications or substitutions of atoms other than at the 2' position, including modifications or substitutions in or of the ribose, phosphate group or base.

[00085] **RNAi Oligonucleotide:** As used herein, the term "RNAi oligonucleotide" refers to either (a) a double stranded oligonucleotide having a sense strand (passenger) and antisense strand (guide), in which the antisense strand or part of the antisense strand is used by the Argonaute 2 (Ago2) endonuclease in the cleavage of a target mRNA or (b) a single stranded oligonucleotide having a single antisense strand, where that antisense strand (or part of that antisense strand) is used by the Ago2 endonuclease in the cleavage of a target mRNA.

[00086] **Strand:** As used herein, the term "strand" refers to a single contiguous sequence of nucleotides linked together through internucleotide linkages (*e.g.*, phosphodiester linkages, phosphorothioate linkages). In some embodiments, a strand has two free ends, *e.g.*, a 5'-end and a 3'-end.

[00087] **Subject:** As used herein, the term "subject" means any mammal, including mice, rabbits, and humans. In one embodiment, the subject is a human or non-human primate. The terms "individual" or "patient" may be used interchangeably with "subject."

[00088] **Synthetic:** As used herein, the term "synthetic" refers to a nucleic acid or other molecule that is artificially synthesized (*e.g.*, using a machine (*e.g.*, a solid state nucleic acid synthesizer)) or that is otherwise not derived from a natural source (*e.g.*, a cell or organism) that normally produces the molecule.

[00089] **Targeting ligand**: As used herein, the term "targeting ligand" refers to a molecule (*e.g.*, a carbohydrate, amino sugar, cholesterol, polypeptide or lipid) that selectively binds to a cognate molecule (*e.g.*, a receptor) of a tissue or cell of interest and that is conjugatable to another substance for purposes of targeting the other substance to the tissue or cell of interest. For example, in some embodiments, a targeting ligand may be conjugated to an oligonucleotide for purposes of targeting the oligonucleotide to a specific tissue or cell of interest. In some embodiments, a targeting ligand selectively binds to a cell surface receptor. Accordingly, in some embodiments, a targeting ligand when conjugated to an oligonucleotide facilitates delivery of the oligonucleotide into a particular cell through selective binding to a receptor expressed on the surface of the cell and endosomal internalization by the cell of the complex comprising the oligonucleotide, targeting ligand and receptor. In some embodiments,

a targeting ligand is conjugated to an oligonucleotide via a linker that is cleaved following or during cellular internalization such that the oligonucleotide is released from the targeting ligand in the cell.

[00090] **Tetraloop**: As used herein, the term "tetraloop" refers to a loop that increases stability of an adjacent duplex formed by hybridization of flanking sequences of nucleotides. The increase in stability is detectable as an increase in melting temperature (Tm) of an adjacent stem duplex that is higher than the Tm of the adjacent stem duplex expected, on average, from a set of loops of comparable length consisting of randomly selected sequences of nucleotides. For example, a tetraloop can confer a melting temperature of at least 50° C, at least 55° C., at least 56° C, at least 58° C, at least 60° C, at least 65° C or at least 75° C in 10 mM $NaHPO_4$ to a hairpin comprising a duplex of at least 2 base pairs in length. In some embodiments, a tetraloop may stabilize a base pair in an adjacent stem duplex by stacking interactions. In addition, interactions among the nucleotides in a tetraloop include but are not limited to non-Watson-Crick base pairing, stacking interactions, hydrogen bonding, and contact interactions (Cheong *et al.*, Nature 1990 Aug. 16; 346(6285):680-2; Heus and Pardi, Science 1991 Jul. 12; 253(5016):191-4). In some embodiments, a tetraloop comprises or consists of 3 to 6 nucleotides, and is typically 4 to 5 nucleotides. In certain embodiments, a tetraloop comprises or consists of three, four, five, or six nucleotides, which may or may not be modified (*e.g.*, which may or may not be conjugated to a targeting moiety). In one embodiment, a tetraloop consists of four nucleotides. Any nucleotide may be used in the tetraloop and standard IUPAC-IUB symbols for such nucleotides may be used as described in Cornish-Bowden (1985) Nucl. Acids Res. 13: 3021-3030. For example, the letter "N" may be used to mean that any base may be in that position, the letter "R" may be used to show that A (adenine) or G (guanine) may be in that position, and "B" may be used to show that C (cytosine), G (guanine), or T (thymine) may be in that position. Examples of tetraloops include the UNCG family of tetraloops (*e.g.*, UUCG), the GNRA family of tetraloops (*e.g.*, GAAA), and the CUUG tetraloop. (Woese *et al.*, Proc Natl Acad Sci USA. 1990 November; 87(21):8467-71; Antao *et al.*, Nucleic Acids Res. 1991 Nov. 11; 19(21):5901-5). Examples of DNA tetraloops include the d(GNNA) family of tetraloops (*e.g.*, d(GTTA), the d(GNRA)) family of tetraloops, the d(GNAB) family of tetraloops, the d(CNNG) family of tetraloops, and the d(TNCG) family of tetraloops (*e.g.*, d(TTCG)). See, for example: Nakano *et al.* Biochemistry, 41 (48), 14281-14292, 2002. SHINJI *et al.* Nippon Kagakkai Koen Yokoshu VOL. 78th; NO. 2; PAGE. 731 (2000), which are incorporated by reference herein for their relevant disclosures. In some embodiments, the tetraloop is contained within a nicked tetraloop structure.

[00091] **Treat**: As used herein, the term "treat" refers to the act of providing care to a subject in need thereof, *e.g.*, through the administration a therapeutic agent (*e.g.*, an oligonucleotide) to the subject, for purposes of improving the health and/or well-being of the subject with respect to an existing condition (*e.g.*, a disease, disorder) or to prevent or decrease the likelihood of the occurrence of a condition. In some embodiments, treatment involves reducing the frequency or severity of at least one sign, symptom or contributing factor of a condition (*e.g.*, disease, disorder) experienced by a subject.

II. Oligonucleotide-based inhibitors of HMGB1 expression

i. HMGB1 Hotspots

[00092] In some embodiments, oligonucleotide-based inhibitors of HMGB1 expression are provided herein that can be used to achieve a therapeutic benefit. Through examination of the HMGB1 mRNA, including mRNAs of multiple different species (human, rhesus monkey, and mouse (*see, e.g.*, Example 1) and *in vitro* and *in vivo* testing, it has been discovered that certain regions of HMGB1 mRNA are hotspots for targeting because they are more amenable than others to oligonucleotide-based inhibition. In some embodiments, a hotspot region of HMGB1 comprises, or consists of, a sequence as forth in any one of SEQ ID NO:374-381. These regions of HMGB1 mRNA may be targeted using oligonucleotides as discussed herein for purposes of inhibiting HMGB1 mRNA expression.

[00093] Accordingly, in some embodiments, oligonucleotides provided herein are designed so as to have regions of complementarity to HMGB1 mRNA (e.g., within a hotspot of HMGB1 mRNA) for purposes of targeting the mRNA in cells and inhibiting its expression. The region of complementary is generally of a suitable length and base content to enable annealling of the oligonucleotide (or a strand thereof) to HMGB1 mRNA for purposes of inhibiting its expression. In some embodiments, the region of complementarity is at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19 or at least 20 nucleotides in length. In some embodiments, an oligonucleotide provided herein has a region of complementarity to HMGB1 that is in the range of 12 to 30 (*e.g.*, 12 to 30, 12 to 22, 15 to 25, 17 to 21, 18 to 27, 19 to 27, or 15 to 30) nucleotides in length. In some embodiments, an oligonucleotide provided herein has a region of complementarity to HMGB1 that is 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides in length.

[00094] In some embodiments, an oligonucleotide disclosed herein comprises a region of complementarity (*e.g.*, on an antisense strand of a double-stranded oligonucleotide)

that is at least partially complementary to a sequence as set forth in any one of SEQ ID NO: 1-96. In some embodiments, an oligonucleotide disclosed herein comprises a region of complementarity (*e.g.*, on an antisense strand of a double-stranded oligonucleotide) that is fully complementary to a sequence as set forth in any one of SEQ ID NO: 1-96. In some embodiments, a region of complementarity of an oligonucleotide (*e.g.*, on an antisense strand of a double-stranded oligonucleotide) is complementary to a contiguous sequence of nucleotides of a sequence as set forth in any one of SEQ ID NOs: 1-96,193-272, and 363-365that is in the range of 10 to 25 nucleotides (*e.g.*, 10 to 25, 12 to 22, 15 to 25, 17 to 21, 18 to 25, 19-25, or 15 to 30 nucleotides) in length. In some embodiments, a region of complementarity of an oligonucleotide (*e.g.*, on an antisense strand of a double-stranded oligonucleotide) is complementary to a contiguous sequence of nucleotides of a sequence as set forth in any one of SEQ ID NOs: 1-96, 193-272, and 363-365 that is 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25contiguous nucleotides in length.

[00095] In some embodiments, a region of complementarity of an oligonucleotide that is complementary to contiguous nucleotides of a sequence as set forth in any one of SEQ ID NOs: 1-96, 193-272, and 363-365 spans the entire length of an antisense strand. In some embodiments, a region of complementarity of an oligonucleotide that is complementary to contiguous nucleotides of a sequence as set forth in any one of SEQ ID NOs:1-96, 193-272, and 363-365spans a portion of the entire length of an antisense strand. In some embodiments, an oligonucleotide disclosed herein comprises a region of complementarity (*e.g.*, on an antisense strand of a double-stranded oligonucleotide) that is at least partially (e.g., fully) complementary to a contiguous stretch of nucleotides spanning nucleotides 1-19 of a sequence as set forth in any one of SEQ ID NOs: 193-272, 193-272, and 363-365.

[00096] In some embodiments, a region of complementarity to HMGB1 may have one or more mismatches compared with a corresponding sequence of HMGB1 mRNA. A region of complementarity on an oligonucleotide may have up to 1, up to 2, up to 3, up to 4, up to 5, *etc.* mismatches provided that it maintains the ability to form complementary base pairs with HMGB1 mRNA under appropriate hybridization conditions. Alternatively, a region of complementarity on an oligonucleotide may have no more than 1, no more than 2, no more than 3, no more than 4, or no more than 5 mismatches provided that it maintains the ability to form complementary base pairs with HMGB1 mRNA under appropriate hybridization conditions. In some embodiments, if there are more than one mismatches in a region of complementarity, they may be positioned consecutively (*e.g.*, 2, 3, 4, or more in a row), or interspersed throughout the region of complementarity provided that the oligonucleotide

maintains the ability to form complementary base pairs with HMGB1 mRNA under appropriate hybridization conditions.

ii. Types of Oligonucleotides

[00097] There are a variety of structures of oligonucleotides that are useful for targeting HMGB1 in the methods of the present disclosure, including RNAi, antisense, miRNA, *etc*. Any of the structures described herein or elsewhere may be used as a framework to incorporate or target a sequence described herein (*e.g.*, a hotpot sequence of HMBG1 such as those illustrated in SEQ ID NOs: 374-381).

[00098] In some embodiments, oligonucleotides for reducing the expression of HMGB1 expression engage RNA interference (RNAi) pathways upstream or downstream of dicer involvement. For example, RNAi oligonucleotides have been developed with each strand having sizes of 19-25 nucleotides with at least one 3' overhang of 1 to 5 nucleotides (see, *e.g.*, U.S. Patent No. 8,372,968). Longer oligonucleotides have also been developed that are processed by Dicer to generate active RNAi products (see, *e.g.*, U.S. Patent No. 8,883,996). Further work produced extended double-stranded oligonucleotides where at least one end of at least one strand is extended beyond a duplex targeting region, including structures where one of the strands includes a thermodynamically-stabilizing tetraloop structure (see, *e.g.*, U.S. Patent Nos. 8,513,207 and 8,927,705, as well as WO2010033225, which are incorporated by reference herein for their disclosure of these oligonucleotides). Such structures may include single-stranded extensions (on one or both sides of the molecule) as well as double-stranded extensions.

[00099] In some embodiments, oligonucleotides provided herein are designed to engage in the RNA interference pathway downstream of the involvement of dicer (*e.g.*, dicer cleavage). Such oligonucleotides may have an overhang (*e.g.*, of 1, 2, or 3 nucleotides in length) in the 3' end of the sense strand. Such oligonucleotides (*e.g.*, siRNAs) may comprise a 21 nucleotide guide strand that is antisense to a target RNA and a complementary passenger strand, in which both strands anneal to form a 19-bp duplex and 2 nucleotide overhangs at either or both 3' ends. Longer oligonucleotide designs are also available including oligonucleotides having a guide strand of 23 nucleotides and a passenger strand of 21 nucleotides, where there is a blunt end on the right side of the molecule (3′-end of passenger strand/5′-end of guide strand) and a two nucleotide 3′-guide strand overhang on the left side of the molecule (5′-end of the passenger strand/3′-end of the guide strand). In such molecules, there is a 21 base pair duplex region. See, for example, US9012138, US9012621, and

US9193753, each of which are incorporated herein for their relevant disclosures.

[000100] In some embodiments, oligonucleotides as disclosed herein may comprise sense and antisense strands that are both in the range of 17 to 26 (*e.g.*, 17 to 26, 20 to 25, or 21-23) nucleotides in length. In some embodiments, an oligonucleotide as disclosed herein comprises a sense and antisense strand that are both in the range of 19-21 nucleotide in length. In some embodiments, the sense and antisense strands are of equal length. In some embodiments, an oligonucleotide comprises sense and antisense strands, such that there is a 3'-overhang on either the sense strand or the antisense strand, or both the sense and antisense strand. In some embodiments, for oligonucleotides that have sense and antisense strands that are both in the range of 21-23 nucleotides in length, a 3' overhang on the sense, antisense, or both sense and antisense strands is 1 or 2 nucleotides in length. In some embodiments, the oligonucleotide has a guide strand of 23 nucleotides and a passenger strand of 21 nucleotides, where there is a blunt end on the right side of the molecule (3′-end of passenger strand/5′-end of guide strand) and a two nucleotide 3′-guide strand overhang on the left side of the molecule (5′-end of the passenger strand/3′-end of the guide strand). In such molecules, there is a 21 base pair duplex region.

Other oligonucleotides designs for use with the compositions and methods disclosed herein include: 16-mer siRNAs (see, *e.g.*, Nucleic Acids in Chemistry and Biology. Blackburn (ed.), Royal Society of Chemistry, 2006), shRNAs (*e.g.*, having 19 bp or shorter stems; see, *e.g.*, Moore *et al.* Methods Mol. Biol. 2010; 629:141-158), blunt siRNAs (*e.g.*, of 19 bps in length; see: *e.g.*, Kraynack and Baker, RNA Vol. 12, p163-176 (2006)), asymmetrical siRNAs (aiRNA; see, *e.g.*, Sun *et al.*, Nat. Biotechnol. 26, 1379–1382 (2008)), asymmetric shorter-duplex siRNA (see, *e.g.*, Chang *et al.*, Mol Ther. 2009 Apr; 17(4): 725-32), fork siRNAs (see, *e.g.*, Hohjoh, FEBS Letters, Vol 557, issues 1-3; Jan 2004, p 193-198), single-stranded siRNAs (Elsner; Nature Biotechnology 30, 1063 (2012)), dumbbell-shaped circular siRNAs (see, *e.g.*, Abe *et al.* J Am Chem Soc 129: 15108-15109 (2007)), and small internally segmented interfering RNA (sisiRNA; see, *e.g.*, Bramsen *et al.*, Nucleic Acids Res. 2007 Sep; 35(17): 5886–5897). Each of the foregoing references is incorporated by reference in its entirety for the related disclosures therein. Further non-limiting examples of an oligonucleotide structures that may be used in some embodiments to reduce or inhibit the expression of HMGB1 are microRNA (miRNA), short hairpin RNA (shRNA), and short siRNA (see, *e.g.*, Hamilton *et al.*, Embo J., 2002, 21(17): 4671-4679; see also U.S. Application No. 20090099115).

[000101] Still, in some embodiments, an oligonucleotide for reducing HMGB1 expression as described herein is single-stranded. Such structures may include, but are not

limited to single-stranded RNAi molecules. Recent efforts have demonstrated the activity of single-stranded RNAi molecules (see, *e.g.*, Matsui *et al.* (May 2016), Molecular Therapy, Vol. 24(5), 946-955). However, in some embodiments, oligonucleotides provided herein are antisense oligonucleotides (ASOs). An antisense oligonucleotide is a single-stranded oligonucleotide that has a nucleobase sequence which, when written in the 5′ to 3′ direction, comprises the reverse complement of a targeted segment of a particular nucleic acid and is suitably modified (e.g., as a gapmer) so as to induce RNaseH mediated cleavage of its target RNA in cells or (e.g., as a mixmer) so as to inhibit translation of the target mRNA in cells. Antisense oligonucleotides for use in the instant disclosure may be modified in any suitable manner known in the art including, for example, as shown in U.S. Patent No. 9,567,587, which is incorporated by reference herein for its disclosure regarding modification of antisense oligonucleotides (including, *e.g.*, length, sugar moieties of the nucleobase (pyrimidine, purine), and alterations of the heterocyclic portion of the nucleobase). F urther, antisense molecules have been used for decades to reduce expression of specific target genes (see, *e.g.*, Bennett *et al.*; Pharmacology of Antisense Drugs, Annual Review of Pharmacology and Toxicology, Vol. 57: 81-105).

iii. Double-Stranded Oligonucleotides

[000102] Double-stranded oligonucleotides for targeting HMGB1 expression (e.g., via the RNAi pathway) generally have a sense strand and an antisense strand that form a duplex with one another. In some embodiments, the sense and antisense strands are not covalently linked. However, in some embodiments, the sense and antisense strands are covalently linked. In some embodiments, a duplex formed between a sense and antisense strand is at least 15 (*e.g.*, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, or at least 21) nucleotides in length. In some embodiments, a duplex formed between a sense and antisense strand is in the range of 15-30 nucleotides in length (*e.g.*, 15 to 30, 15 to 27, 15 to 22, 18 to 22, 18 to 25, 18 to 27, 18 to 30, or 21 to 30 nucleotides in length). In some embodiments, a duplex formed between a sense and antisense strand is 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. In some embodiments a duplex formed between a sense and antisense strand does not span the entire length of the sense strand and/or antisense strand. In some embodiments, a duplex between a sense and antisense strand spans the entire length of either the sense or antisense strands. In certain embodiments, a duplex between a sense and antisense strand spans the entire length of both the sense strand and the antisense strand.

[000103] In some embodiments, an oligonucleotide provided herein comprises a sense strand having a sequence as set forth in any one of SEQ ID NO: 1-96 and an antisense strand comprising a complementary sequence selected from SEQ ID NO: 97-192, as is arranged Table 7 (*e.g.*, a sense strand comprising a sequence as set forth in SEQ ID NO: 1 and an antisense strand comprising a sequence as set forth in SEQ ID NO: 97). In some embodiments, an oligonucleotide provided herein comprises a sense strand comprising a sequence as set forth in any one of SEQ ID NO: 193-272 and 363-365 and an antisense strand comprising a complementary sequence selected from SEQ ID NO: 273-362 and 366-370, as is also arranged Table 7 (*e.g.*, a sense strand comprising a sequence as set forth in SEQ ID NO: 193 and an antisense strand comprising a sequence as set forth in SEQ ID NO: 273). It should be appreciated that, in some embodiments, sequences presented in the sequence listing may be referred to in describing the structure of an oligonucleotide or other nucleic acid. In such embodiments, the actual oligonucleotide or other nucleic acid may have one or more alternative nucleotides (e.g., an RNA counterpart of a DNA nucleotide or a DNA counterpart of an RNA nucleotide) and/or one or more modified nucleotides and/or one or more modified internucleotide linkages and/or one or more other modification compared with the specified sequence while retaining essentially same or similar complementary properties as the specified sequence.

[000104] In some embodiments, a double-stranded oligonucleotide comprises a 25 nucleotide sense strand and a 27 nucleotide antisense strand that when acted upon by a dicer enzyme results in an antisense strand that is incorporated into the mature RISC. In some embodiments, a sense strand of an oligonucleotide is longer than 27 nucleotides (*e.g.*, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 nucleotides). In some embodiments, a sense strand of an oligonucleotide is longer than 25 nucleotides (*e.g.*, 26, 27, 28, 29 or 30 nucleotides). The length of a duplex formed between a sense and antisense strand of an oligonucleotide may be 12 to 30 nucleotides (*e.g.*, 12 to 30, 12 to 27, 15 to 25, 18 to 30 or 19 to 30 nucleotides) in length. In some embodiments, the length of a duplex formed between a sense and antisense strand of an oligonucleotide is at least 12 nucleotides long (*e.g.*, at least 12, at least 15, at least 20, or at least 25 nucleotides long). In some embodiments, the length of a duplex formed between a sense and antisense strand of an oligonucleotide is 12, 13,14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

[000105] In some embodiments, oligonucleotides provided herein have one 5'end that is thermodynamically less stable compared to the other 5' end. In some embodiments, an asymmetry oligonucleotide is provided that includes a blunt end at the 3' end of a sense strand

and an overhang at the 3' end of an antisense strand. In some embodiments, a 3' overhang on an antisense strand is 1-8 nucleotides in length (*e.g.*, 1, 2, 3, 4, 5, 6, 7 or 8 nucleotides in length). Typically, an oligonucleotide for RNAi has a two nucleotide overhang on the 3' end of the antisense (guide) strand. However, other overhangs are possible. In some embodiments, an overhang is a 3′ overhang comprising a length of between one and six nucleotides, optionally one to five, one to four, one to three, one to two, two to six, two to five, two to four, two to three, three to six, three to five, three to four, four to six, four to five, five to six nucleotides, or one, two, three, four, five or six nucleotides. However, in some embodiments, the overhang is a 5′ overhang comprising a length of between one and six nucleotides, optionally one to five, one to four, one to three, one to two, two to six, two to five, two to four, two to three, three to six, three to five, three to four, four to six, four to five, five to six nucleotides, or one, two, three, four, five or six nucleotides.

[000106] In some embodiments, two terminal nucleotides on the 3' end of an antisense strand are modified. In some embodiments, the two terminal nucleotides on the 3' end of the antisense strand are complementary with the target. In some embodiments, the two terminal nucleotides on the 3' end of the antisense strand are not complementary with the target. In some embodiments, two terminal nucleotides on each 3' end of an oligonucleotide in the nicked tetraloop structure are GG. Typically, one or both of the two terminal GG nucleotides on each 3' end of an oligonucleotide is not complementary with the target.

[000107] In some embodiments, there is one or more (*e.g.*, 1, 2, 3, 4, 5) mismatch between a sense and antisense strand. If there is more than one mismatch between a sense and antisense strand, they may be positioned consecutively (*e.g.*, 2, 3 or more in a row), or interspersed throughout the region of complementarity. In some embodiments, the 3'-terminus of the sense strand contains one or more mismatches. In one embodiment, two mismatches are incorporated at the 3' terminus of the sense strand. In some embodiments, base mismatches or destabilization of segments at the 3'-end of the sense strand of the oligonucleotide improved the potency of synthetic duplexes in RNAi, possibly through facilitating processing by Dicer.

a. Antisense Strands

[000108] In some embodiments, an antisense strand of an oligonucleotide may be referred to as a "guide strand." For example, if an antisense strand can engage with RNA-induced silencing complex (RISC) and bind to an Argonaut protein, or engage with or bind to one or more similar factors, and direct silencing of a target gene, it may be referred to as a

guide strand. In some embodiments a sense strand complementary with a guide strand may be referred to as a "passenger strand."

[000109] In some embodiments, an oligonucleotide provided herein comprises an antisense strand that is up to 50 nucleotides in length (*e.g.*, up to 30, up to 27, up to 25, up to 21, or up to 19 nucleotides in length). In some embodiments, an oligonucleotide provided herein comprises an antisense strand is at least 12 nucleotides in length (*e.g.*, at least 12, at least 15, at least 19, at least 21, at least 25, or at least 27 nucleotides in length). In some embodiments, an antisense strand of an oligonucleotide disclosed herein is in the range of 12 to 50 or 12 to 30 (*e.g.*, 12 to 30, 11 to 27, 11 to 25, 15 to 21, 15 to 27, 17 to 21, 17 to 25, 19 to 27, or 19 to 30) nucleotides in length. In some embodiments, an antisense strand of any one of the oligonucleotides disclosed herein is 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides in length.

[000110] In some embodiments an oligonucleotide disclosed herein comprises an antisense strand comprising a sequence as set forth in any one of SEQ ID NO: 97-192, 273-362, or 366-370. In some embodiments, an oligonucleotide comprises an antisense strand comprising at least 12 (*e.g.*, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, or at least 23) contiguous nucleotides of a sequence as set forth in any one of SEQ ID NO: 97-192, 273-362, or 366-370. In some embodiments, an oligonucleotide comprises an antisense strand comprising a contiguous sequence of nucleotides that is in the range of 12 to 30 nucleotides (*e.g.*, 12 to 27, 12 to 22, 15 to 25, 17 to 21, 18 to 27, 19 to 27, or 15 to 27 nucleotides) in length of any of the sequences as set forth in any one of SEQ ID NO: 97-192, 273-362, or 366-370. In some embodiments, an oligonucleotide comprises an antisense strand comprising a contiguous sequence of nucleotides of a sequence as set forth in any one of SEQ ID NO: 97-192, 273-362, or 366-370 that is 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, or 27contiguous nucleotides in length. In some embodiments, an oligonucleotide comprises an antisense strand that consists of a sequence as set forth in any one of SEQ ID NO: 97-192, 273-362, or 366-370.

b. Sense Strands

[000111] In some embodiments, a double-stranded oligonucleotide may have a sense strand of up to 40 nucleotides in length (*e.g.*, up to 40, up to 35, up to 30, up to 27, up to 25, up to 21, up to 19 up to 17, or up to 12 nucleotides in length). In some embodiments, an

oligonucleotide may have a sense strand of at least 12 nucleotides in length (*e.g.*, at least 12, at least 15, at least 19, at least 21, at least 25, at least 27, at least 30, at least 35, or at least 38 nucleotides in length). In some embodiments, an oligonucleotide may have a sense strand in a range of 12 to 50 (*e.g.*, 12 to 40, 12 to 36, 12 to 32, 12 to 28, 15 to 40, 15 to 36, 15 to 32, 15 to 28, 17 to 21, 17 to 25, 19 to 27, 19 to 30, 20 to 40, 22 to 40, 25 to 40, or 32 to 40) nucleotides in length. In some embodiments, an oligonucleotide may have a sense strand of 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides in length. In some embodiments, a sense strand of an oligonucleotide is longer than 27 nucleotides (*e.g.*, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 nucleotides). In some embodiments, a sense strand of an oligonucleotide is longer than 25 nucleotides (*e.g.*, 26, 27, 28, 29 or 30 nucleotides).

[000112] In some embodiments, an oligonucleotide disclosed herein comprises a sense strand sequence as set forth in in any one of SEQ ID NO: 1-96, 193-272, or 363-365. In some embodiments, an oligonucleotide has a sense strand that comprises at least 12 (*e.g.*, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, or at least 23) contiguous nucleotides of a sequence as set forth in in any one of SEQ ID NO: 1-96, 193-272, or 363-365. In some embodiments, an oligonucleotide has a sense strand that comprises a contiguous sequence of nucleotides that is in the range of 7 to 36 nucleotides (*e.g.*, 12 to 30, 12 to 27, 12 to 22, 15 to 25, 17 to 21, 18 to 27, 19-27, 20-36, or 15 to 36 nucleotides) in length of any of the sequences as set forth in any one of SEQ ID NO: 1-96, 193-272, or 363-365. In some embodiments, an oligonucleotide has a sense strand that comprises a contiguous sequence of nucleotides of a sequence as set forth in any one of SEQ ID NO: 1-96, 193-272, or 363-365 that is 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 nucleotides in length. In some embodiments, an oligonucleotide has a sense strand that consists of a sequence as set forth in any one of SEQ ID NO: 1-96, 193-272, or 363-365.

[000113] In some embodiments, a sense strand comprises a stem-loop at its 3'-end. In some embodiments, a sense strand comprises a stem-loop at its 5'-end. In some embodiments, a strand comprising a stem loop is in the range of 2 to 66 nucleotides long (*e.g.*, 2 to 66, 10 to 52, 14 to 40, 2 to 30, 4 to 26, 8 to 22, 12 to 18, 10 to 22, 14 to 26, or 14 to 30 nucleotides long). In some embodiments, a strand comprising a stem loop is 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. In some embodiments, a stem comprises a duplex of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 nucleotides in length. In some embodiments, a stem-loop provides the molecule better

protection against degradation (*e.g.*, enzymatic degradation) and facilitates targeting characteristics for delivery to a target cell. For example, in some embodiments, a loop provides added nucleotides on which modification can be made without substantially affecting the gene expression inhibition activity of an oligonucleotide. In certain embodiments, an oligonucleotide is provided herein in which the sense strand comprises (*e.g.*, at its 3'-end) a stem-loop set forth as: S_1-L-S_2, in which S_1 is complementary to S_2, and in which L forms a loop between S_1 and S_2 of up to 10 nucleotides in length (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length).

[000114] In some embodiments, a loop (L) of a stem-loop is a tetraloop (*e.g.*, within a nicked tetraloop structure). A tetraloop may contain ribonucleotides, deoxyribonucleotides, modified nucleotides, and combinations thereof. Typically, a tetraloop has 4 to 5 nucleotides. However, in some embodiments, a tetraloop comprises or consists of 3 to 6 nucleotides, and typically consists of 4 to 5 nucleotides. In certain embodiments, a tetraloop comprises or consists of three, four, five, or six nucleotides.

[000115]

iv. Oligonucleotide Modifications

[000116] Oligonucleotides may be modified in various ways to improve or control specificity, stability, delivery, bioavailability, resistance from nuclease degradation, immunogenicity, base-paring properties, RNA distribution and cellular uptake and other features relevant to therapeutic or research use. See, *e.g.*, Bramsen *et al.*, Nucleic Acids Res., 2009, 37, 2867-2881; Bramsen and Kjems (Frontiers in Genetics, 3 (2012): 1-22). Accordingly, in some embodiments, oligonucleotides of the present disclosure may include one or more suitable modifications. In some embodiments, a modified nucleotide has a modification in its base (or nucleobase), the sugar (*e.g.*, ribose, deoxyribose), or the phosphate group.

[000117] The number of modifications on an oligonucleotide and the positions of those nucleotide modifications may influence the properties of an oligonucleotide. For example, oligonucleotides maybe be delivered *in vivo* by conjugating them to or encompassing them in a lipid nanoparticle (LNP) or similar carrier. However, when an oligonucleotide is not protected by an LNP or similar carrier, it may be advantageous for at least some of the its nucleotides to be modified. Accordingly, in certain embodiments of any of the oligonucleotides provided herein, all or substantially all of the nucleotides of an oligonucleotide are modified. In certain embodiments, more than half of the nucleotides are modified. In certain embodiments, less

than half of the nucleotides are modified. Typically, with naked delivery, every sugar is modified at the 2′-position. These modifications may be reversible or irreversible. In some embodiments, an oligonucleotide as disclosed herein has a number and type of modified nucleotides and sufficient to cause the desired characteristic (*e.g.*, protection from enzymatic degradation, capacity to target a desired cell after *in vivo* administration, and/or thermodynamic stability).

a. Sugar Modifications

[000118] In some embodiments, a modified sugar (also referred herein to a sugar analog) includes a modified deoxyribose or ribose moiety, *e.g.*, in which one or more modifications occur at the 2′, 3′, 4′, and/or 5′ carbon position of the sugar. In some embodiments, a modified sugar may also include non-natural alternative carbon structures such as those present in locked nucleic acids ("LNA") (see, *e.g.*, Koshkin *et al.* (1998), Tetrahedron 54, 3607-3630), unlocked nucleic acids ("UNA") (see, *e.g.*, Snead *et al.* (2013), Molecular Therapy – Nucleic Acids, 2, e103), and bridged nucleic acids ("BNA") (see, *e.g.*, Imanishi and Obika (2002), The Royal Society of Chemistry, Chem. Commun., 1653-1659). Koshkin *et al.*, Snead *et al.*, and Imanishi and Obika are incorporated by reference herein for their disclosures relating to sugar modifications.

[000119] In some embodiments, a nucleotide modification in a sugar comprises a 2'-modification. A 2'-modification may be 2'-aminoethyl, 2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl, and 2'-deoxy-2'-fluoro-β-d-arabinonucleic acid. Typically, the modification is 2'-fluoro, 2'-O-methyl, or 2'-O-methoxyethyl. In some embodiments a modification in a sugar comprises a modification of the sugar ring, which may comprise modification of one or more carbons of the sugar ring. For example, a modification of a sugar of a nucleotide may comprise a 2'-oxygen of a sugar is linked to a 1'-carbon or 4'-carbon of the sugar, or a 2'-oxygen is linked to the 1'-carbon or 4'-carbon via an ethylene or methylene bridge. In some embodiments, a modified nucleotide has an acyclic sugar that lacks a 2'-carbon to 3'-carbon bond. In some embodiments, a modified nucleotide has a thiol group, *e.g.*, in the 4' position of the sugar.

[000120] In some embodiments, the terminal 3'-end group (*e.g.*, a 3'-hydroxyl) with a phosphate group or other group, which can be used, for example, to attach linkers, adapters or labels or for the direct ligation of an oligonucleotide to another nucleic acid.

b. 5' Terminal Phophates

[000121] In some embodiments, 5'-terminal phosphate groups of oligonucleotides enhance the interaction with Argonaut 2. However, oligonucleotides comprising a 5'-phosphate group may be susceptible to degradation via phosphatases or other enzymes, which can limit their bioavailability *in vivo*. In some embodiments, oligonucleotides include analogs of 5' phosphates that are resistant to such degradation. In some embodiments, a phosphate analog may be oxymethylphosphonate, vinylphosphonate, or malonylphosphonate. In certain embodiments, the 5' end of an oligonucleotide strand is attached to chemical moiety that mimics the electrostatic and steric properties of a natural 5'-phosphate group ("phosphate mimic") (see, *e.g.,* Prakash *et al.* (2015), Nucleic Acids Res., Nucleic Acids Res. 2015 Mar 31; 43(6): 2993–3011, the contents of which relating to phosphate analogs are incorporated herein by reference). Many phosphate mimics have been developed that can be attached to the 5' end (see, *e.g.,* U.S. Patent No. 8,927,513, the contents of which relating to phosphate analogs are incorporated herein by reference). Other modifications have been developed for the 5' end of oligonucleotides (see, *e.g.,* WO 2011/133871, the contents of which relating to phosphate analogs are incorporated herein by reference). In certain embodiments, a hydroxyl group is attached to the 5' end of the oligonucleotide.

[000122] In some embodiments, an oligonucleotide has a phosphate analog at a 4'-carbon position of the sugar (referred to as a "4'-phosphate analog"). See, for example, U.S. Provisional Application numbers 62/383,207, entitled *4'-Phosphate Analogs and Oligonucleotides Comprising the Same*, filed on September 2, 2016, and 62/393,401, filed on September 12, 2016, entitled *4'-Phosphate Analogs and Oligonucleotides Comprising the Same*, the contents of each of which relating to phosphate analogs are incorporated herein by reference. In some embodiments, an oligonucleotide provided herein comprise a 4'-phosphate analog at a 5'-terminal nucleotide. In some embodiments, a phosphate analog is an oxymethylphosphonate, in which the oxygen atom of the oxymethyl group is bound to the sugar moiety (*e.g.*, at its 4'-carbon) or analog thereof. In other embodiments, a 4'-phosphate analog is a thiomethylphosphonate or an aminomethylphosphonate, in which the sulfur atom of the thiomethyl group or the nitrogen atom of the aminomethyl group is bound to the 4'-carbon of the sugar moiety or analog thereof. In certain embodiments, a 4'-phosphate analog is an oxymethylphosphonate. In some embodiments, an oxymethylphosphonate is represented by the formula $-O-CH_2-PO(OH)_2$ or -O-CH2-$PO(OR)_2$, in which R is independently selected from H, CH_3, an alkyl group, CH_2CH_2CN, $CH_2OCOC(CH_3)_3$, $CH_2OCH_2CH_2Si(CH_3)_3$, or a protecting group. In certain embodiments, the alkyl group is CH_2CH_3. More typically, R is independently selected from H, CH_3, or CH_2CH_3.

c. Modified Intranucleoside Linkages

[000123] In some embodiments, phosphate modifications or substitutions may result in an oligonucleotide comprises at least one (*e.g.*, at least 1, at least 2, at least 3 or at least 5) comprising a modified internucleotide linkage. In some embodiments, any one of the oligonucleotides disclosed herein comprises 1 to 10 (*e.g.*, 1 to 10, 2 to 8, 4 to 6, 3 to 10, 5 to 10, 1 to 5, 1 to 3 or 1 to 2) modified internucleotide linkages. In some embodiments, any one of the oligonucleotides disclosed herein comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 modified internucleotide linkages.

[000124] A modified internucleotide linkage may be a phosphorodithioate linkage, a phosphorothioate linkage, a phosphotriester linkage, a thionoalkylphosphonate linkage, a thionalkylphosphotriester linkage, a phosphoramidite linkage, a phosphonate linkage or a boranophosphate linkage. In some embodiments, at least one modified internucleotide linkage of any one of the oligonucleotides as disclosed herein is a phosphorothioate linkage.

d. Base modifications

[000125] In some embodiments, oligonucleotides provided herein have one or more modified nucleobases. In some embodiments, modified nucleobases (also referred to herein as base analogs) are linked at the 1′ position of a nucleotide sugar moiety. In certain embodiments, a modified nucleobase is a nitrogenous base. In certain embodiments, a modified nucleobase does not contain nitrogen atom. *See e.g.*, U.S. Published Patent Application No. 20080274462. In some embodiments, a modified nucleotide comprises a universal base. However, in certain embodiments, a modified nucleotide does not contain a nucleobase (abasic).

[000126] In some embodiments a universal base is a heterocyclic moiety located at the 1′ position of a nucleotide sugar moiety in a modified nucleotide, or the equivalent position in a nucleotide sugar moiety substitution, that, when present in a duplex, can be positioned opposite more than one type of base without substantially altering structure of the duplex. In some embodiments, compared to a reference single-stranded nucleic acid (*e.g.*, oligonucleotide) that is fully complementary to a target nucleic acid, a single-stranded nucleic acid containing a universal base forms a duplex with the target nucleic acid that has a lower Tm than a duplex formed with the complementary nucleic acid. However, in some embodiments, compared to a reference single-stranded nucleic acid in which the universal base has been replaced with a base to generate a single mismatch, the single-stranded nucleic acid

containing the universal base forms a duplex with the target nucleic acid that has a higher T_m than a duplex formed with the nucleic acid comprising the mismatched base.

[000127] Non-limiting examples of universal-binding nucleotides include inosine, 1-β-D-ribofuranosyl-5-nitroindole, and/or 1-β-D-ribofuranosyl-3-nitropyrrole (US Pat. Appl. Publ. No. 20070254362 to Quay *et al.*; Van Aerschot *et al.*, An acyclic 5-nitroindazole nucleoside analogue as ambiguous nucleoside. Nucleic Acids Res. 1995 Nov 11;23(21):4363-70; Loakes *et al.*, 3-Nitropyrrole and 5-nitroindole as universal bases in primers for DNA sequencing and PCR. Nucleic Acids Res. 1995 Jul 11;23(13):2361-6; Loakes and Brown, 5-Nitroindole as an universal base analogue. Nucleic Acids Res. 1994 Oct 11;22(20):4039-43. Each of the foregoing is incorporated by reference herein for their disclosures relating to base modifications).

e. Reversible Modifications

[000128] While certain modifications to protect an oligonucleotide from the *in vivo* environment before reaching target cells can be made, they can reduce the potency or activity of the oligonucleotide once it reaches the cytosol of the target cell. Reversible modifications can be made such that the molecule retains desirable properties outside of the cell, which are then removed upon entering the cytosolic environment of the cell. Reversible modification can be removed, for example, by the action of an intracellular enzyme or by the chemical conditions inside of a cell (*e.g.*, through reduction by intracellular glutathione).

[000129] In some embodiments, a reversibly modified nucleotide comprises a glutathione-sensitive moiety. Typically, nucleic acid molecules have been chemically modified with cyclic disulfide moieties to mask the negative charge created by the internucleotide diphosphate linkages and improve cellular uptake and nuclease resistance. *See* U.S. Published Application No. 2011/0294869 originally assigned to Traversa Therapeutics, Inc. ("Traversa"), PCT Publication No. WO 2015/188197 to Solstice Biologics, Ltd. ("Solstice"), Meade *et al.*, *Nature Biotechnology*, 2014,32:1256-1263 ("Meade"), PCT Publication No. WO 2014/088920 to Merck Sharp & Dohme Corp, each of which are incorporated by reference for their disclosures of such modifications. This reversible modification of the internucleotide diphosphate linkages is designed to be cleaved intracellularly by the reducing environment of the cytosol (*e.g.* glutathione). Earlier examples include neutralizing phosphotriester modifications that were reported to be cleavable inside cells (Dellinger *et al. J. Am. Chem. Soc.* 2003,125:940-950).

[000130] In some embodiments, such a reversible modification allows protection during

in vivo administration (*e.g.*, transit through the blood and/or lysosomal/endosomal compartments of a cell) where the oligonucleotide will be exposed to nucleases and other harsh environmental conditions (*e.g.*, pH). When released into the cytosol of a cell where the levels of glutathione are higher compared to extracellular space, the modification is reversed and the result is a cleaved oligonucleotide. Using reversible, glutathione sensitive moieties, it is possible to introduce sterically larger chemical groups into the oligonucleotide of interest as compared to the options available using irreversible chemical modifications. This is because these larger chemical groups will be removed in the cytosol and, therefore, should not interfere with the biological activity of the oligonucleotides inside the cytosol of a cell. As a result, these larger chemical groups can be engineered to confer various advantages to the nucleotide or oligonucleotide, such as nuclease resistance, lipophilicity, charge, thermal stability, specificity, and reduced immunogenicity. In some embodiments, the structure of the glutathione-sensitive moiety can be engineered to modify the kinetics of its release.

[000131] In some embodiments, a glutathione-sensitive moiety is attached to the sugar of the nucleotide. In some embodiments, a glutathione-sensitive moiety is attached to the 2'carbon of the sugar of a modified nucleotide. In some embodiments, the glutathione-sensitive moiety is located at the 5′-carbon of a sugar, particularly when the modified nucleotide is the 5′-terminal nucleotide of the oligonucleotide. In some embodiments, the glutathione-sensitive moiety is located at the 3′-carbon of sugar, particularly when the modified nucleotide is the 3′-terminal nucleotide of the oligonucleotide. In some embodiments, the glutathione-sensitive moiety comprises a sulfonyl group. *See*, *e.g.*, U.S. Prov. Appl. No. 62/378,635, entitled Compositions Comprising Reversibly Modified Oligonucleotides and Uses Thereof, which was filed on August 23, 2016, and the contents of which are incorporated by reference herein for its relevant disclosures.

v. Targeting Ligands

[000132] In some embodiments, it may be desirable to target the oligonucleotides of the disclosure to one or more cells or one or more organs. Such a strategy may help to avoid undesirable effects in other organs, or may avoid undue loss of the oligonucleotide to cells, tissue or organs that would not benefit for the oligonucleotide. Accordingly, in some embodiments, oligonucleotides disclosed herein may be modified to facilitate targeting of a particular tissue, cell or organ , *e.g.*, to facilitate delivery of the oligonucleotide to the liver. In certain embodiments, oligonucleotides disclosed herein may be modified to facilitate delivery of the oligonucleotide to the hepatocytes of the liver. In some embodiments, an

oligonucleotide comprises a nucleotide that is conjugated to one or more targeting ligand.

[000133] A targeting ligand may comprise a carbohydrate, amino sugar, cholesterol, peptide, polypeptide, protein or part of a protein (*e.g.*, an antibody or antibody fragment) or lipid. In some embodiments, a targeting ligand is an aptamer. For example, a targeting ligand may be an RGD peptide that is used to target tumor vasculature or glioma cells, CREKA peptide to target tumor vasculature or stoma, transferring, lactoferrin, or an aptamer to target transferrin receptors expressed on CNS vasculature, or an anti-EGFR antibody to target EGFR on glioma cells. In certain embodiments, the targeting ligand is one or more GalNAc moieties.

[000134] In some embodiments, 1 or more (*e.g.*, 1, 2, 3, 4, 5 or 6) nucleotides of an oligonucleotide are each conjugated to a separate targeting ligand. In some embodiments, 2 to 4 nucleotides of an oligonucleotide are each conjugated to a separate targeting ligand. In some embodiments, targeting ligands are conjugated to 2 to 4 nucleotides at either ends of the sense or antisense strand (*e.g.*, ligand are conjugated to a 2 to 4 nucleotide overhang or extension on the 5' or 3' end of the sense or antisense strand) such that the targeting ligands resemble bristles of a toothbrush and the oligonucleotide resembles a toothbrush. For example an oligonucleotide may comprise a stem-loop at either the 5' or 3' end of the sense strand and 1, 2, 3 or 4 nucleotides of the loop of the stem may be individually conjugated to a targeting ligand.

[000135] In some embodiments, it is desirable to target an oligonucleotide that reduces the expression of HMGB1 to the hepatocytes of the liver of the subject. Any suitable hepatocyte targeting moiety may be used for this purpose.

[000136] GalNAc is a high affinity ligand for asialoglycoprotein receptor (ASGPR), which is primarily expressed on the sinusoidal surface of hepatocyte cells and has a major role in binding, internalization, and subsequent clearance of circulating glycoproteins that contain terminal galactose or N-acetylgalactosamine residues (asialoglycoproteins). Conjugation (either indirect or direct) of GalNAc moieties to oligonucleotides of the instant disclosure may be used to target these oligonucleotides to the ASGPR expressed on these hepatocyte cells.

[000137] In some embodiments, an oligonucleotide of the instant disclosure is conjugated directly or indirectly to a monovalent GalNAc. In some embodiments, the oligonucleotide is conjugated directly or indirectly to more than one monovalent GalNAc (*i.e.*, is conjugated to 2, 3, or 4 monovalent GalNAc moieties, and is typically conjugated to 3 or 4 monovalent GalNAc moieties). In some embodiments, an oligonucleotide of the instant disclosure is conjugated to a one or more bivalent GalNAc, trivalent GalNAc, or tetravalent GalNAc moieties.

[000138] In some embodiments, 1 or more (*e.g.*, 1, 2, 3, 4, 5 or 6) nucleotides of an oligonucleotide are each conjugated to a GalNAc moiety. In some embodiments, 2 to 4 nucleotides of the loop (L) of the stem-loop are each conjugated to a separate GalNAc. In some embodiments, targeting ligands are conjugated to 2 to 4 nucleotides at either ends of the sense or antisense strand (*e.g.*, ligand are conjugated to a 2 to 4 nucleotide overhang or extension on the 5' or 3' end of the sense or antisense strand) such that the GalNAc moieties resemble bristles of a toothbrush and the oligonucleotide resembles a toothbrush. For example an oligonucleotide may comprise a stem-loop at either the 5' or 3' end of the sense strand and 1, 2, 3 or 4 nucleotides of the loop of the stem may be individually conjugated to a GalNAc moiety. In some embodiments, GalNAc moieties are conjugated to a nucleotide of the sense strand. For example, four GalNAc moieties can be conjugated to nucleotides in the tetraloop of the sense strand where each GalNAc moiety is conjugated to one nucleotide.

[000139] Any appropriate method or chemistry (*e.g.*, click chemistry) can be used to link a targeting ligand to a nucleotide. In some embodiments, a targeting ligand is conjugated to a nucleotide using a click linker. In some embodiments, an acetal-based linker is used to conjugate a targeting ligand to a nucleotide of any one of the oligonucleotides described herein. Acetal-based linkers are disclosed, for example, in International Patent Application Publication Number WO2016100401 A1, which published on June 23, 2016, and the contents of which relating to such linkers are incorporated herein by reference. In some embodiments, the linker is a labile linker. However, in other embodiments, the linker is fairly stable.

[000140] In some embodiments, a duplex extension (*e.g.*, of up to 3, 4, 5, or 6 base pairs in length) is provided between a targeting ligand (*e.g.*, a GalNAc moiety) and a double-stranded oligonucleotide.

III. Formulations

[000141] Various formulations have been developed to facilitate oligonucleotide use. For example, oligonucleotides can be delivered to a subject or a cellular environment using a formulation that minimizes degradation, facilitates delivery and/or uptake, or provides another beneficial property to the oligonucleotides in the formulation. In some embodiments, provided herein are compositions comprising oligonucleotides (*e.g.*, single-stranded or double-stranded oligonucleotides) to reduce the expression of HMGB1. Such compositions can be suitably formulated such that when administered to a subject, either into the immediate environment of a target cell or systemically, a sufficient portion of the oligonucleotides enter the cell to reduce HMGB1 expression. Any of a variety of suitable oligonucleotide formulations can be used to

deliver oligonucleotides for the reduction of HMGB1 as disclosed herein. In some embodiments, an oligonucleotide is formulated in buffer solutions such as phosphate buffered saline solutions, liposomes, micellar structures, and capsids.

[000142] Formulations of oligonucleotides with cationic lipids can be used to facilitate transfection of the oligonucleotides into cells. For example, cationic lipids, such as lipofectin, cationic glycerol derivatives, and polycationic molecules (*e.g.*, polylysine, can be used. Suitable lipids include Oligofectamine, Lipofectamine (Life Technologies), NC388 (Ribozyme Pharmaceuticals, Inc., Boulder, Colo.), or FuGene 6 (Roche) all of which can be used according to the manufacturer's instructions.

[000143] Accordingly, in some embodiments, a formulation comprises a lipid nanoparticle. In some embodiments, an excipient comprises a liposome, a lipid, a lipid complex, a microsphere, a microparticle, a nanosphere, or a nanoparticle, or may be otherwise formulated for administration to the cells, tissues, organs, or body of a subject in need thereof (see, *e.g.*, Remington: The Science and Practice of Pharmacy, 22nd edition, Pharmaceutical Press, 2013).

[000144] In some embodiments, formulations as disclosed herein comprise an excipient. In some embodiments, an excipient confers to a composition improved stability, improved absorption, improved solubility and/or therapeutic enhancement of the active ingredient. In some embodiments, an excipient is a buffering agent (*e.g.*, sodium citrate, sodium phosphate, a tris base, or sodium hydroxide) or a vehicle (*e.g.*, a buffered solution, petrolatum, dimethyl sulfoxide, or mineral oil). In some embodiments, an oligonucleotide is lyophilized for extending its shelf-life and then made into a solution before use (*e.g.*, administration to a subject). Accordingly, an excipient in a composition comprising any one of the oligonucleotides described herein may be a lyoprotectant (*e.g.*, mannitol, lactose, polyethylene glycol, or polyvinyl pyrolidone), or a or a collapse temperature modifier (*e.g.*, dextran, ficoll, or gelatin).

[000145] In some embodiments, a pharmaceutical composition is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration.

[000146] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor

EL.TM. (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Sterile injectable solutions can be prepared by incorporating the oligonucleotides in a required amount in a selected solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization.

[000147] In some embodiments, a composition may contain at least about 0.1% of the therapeutic agent (*e.g.*, an oligonucleotide for reducing HMGB1 expression) or more, although the percentage of the active ingredient(s) may be between about 1% 80% or more of the weight or volume of the total composition. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[000148] Even though a number of embodiments are directed to liver-targeted delivery of any of the oligonucleotides disclosed herein, targeting of other tissues is also contemplated.

IV. Methods of Use

i. Reducing HMGB1 Expression in Cells

[000149] In some embodiments, methods are provided for delivering to a cell an effective amount any one of oligonucleotides disclosed herein for purposes of reducing expression of HMGB1 in the cell. Methods provided herein are useful in any appropriate cell type. In some embodiments, a cell is any cell that expresses HMGB1 (*e.g.*, hepatocytes, macrophages, monocyte-derived cells, prostate cancer cells, cells of the brain, endocrine tissue, bone marrow, lymph nodes, lung, gall bladder, liver, duodenum, small intestine, pancreas, kidney, gastrointestinal tract, bladder, adipose and soft tissue and skin). In some embodiments, the cell is a primary cell that has been obtained from a subject and that may have undergone a limited number of a passages, such that the cell substantially maintains is natural phenotypic properties. In some embodiments, a cell to which the oligonucleotide is delivered is *ex vivo* or *in vitro* (*i.e.*, can be delivered to a cell in culture or to an organism in which the cell resides). In specific embodiments, methods are provided for delivering to a cell an effective amount any one of oligonucleotides disclosed herein for purposes of reducing expression of HMGB1 solely in hepatocytes.

[000150] In some embodiments, oligonucleotides disclosed herein can be introduced using appropriate nucleic acid delivery methods including injection of a solution containing the oligonucleotides, bombardment by particles covered by the oligonucleotides, exposing the cell or organism to a solution containing the oligonucleotides, or electroporation of cell membranes in the presence of the oligonucleotides. Other appropriate methods for delivering oligonucleotides to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, and cationic liposome transfection such as calcium phosphate, and others.

[000151] The consequences of inhibition can be confirmed by an appropriate assay to evaluate one or more properties of a cell or subject, or by biochemical techniques that evaluate molecules indicative of HMGB1 expression (*e.g.*, RNA, protein). In some embodiments, the extent to which an oligonucleotide provided herein reduces levels of expression of HMGB1 is evaluated by comparing expression levels (*e.g.*, mRNA or protein levels of HMGB1 to an appropriate control (*e.g.*, a level of HMGB1 expression in a cell or population of cells to which an oligonucleotide has not been delivered or to which a negative control has been delivered). In some embodiments, an appropriate control level of HMGB1 expression may be a predetermined level or value, such that a control level need not be measured every time. The predetermined level or value can take a variety of forms. In some embodiments, a predetermined level or value can be single cut-off value, such as a median or mean.

[000152] In some embodiments, administration of an oligonucleotide as described herein results in a reduction in the level of HMGB1 expression in a cell. In some embodiments, the reduction in levels of HMGB1 expression may be a reduction to 1% or lower, 5% or lower, 10% or lower, 15% or lower, 20% or lower, 25% or lower, 30% or lower, 35% or lower, 40% or lower, 45% or lower, 50% or lower, 55% or lower, 60% or lower, 70% or lower, 80% or lower, or 90% or lower compared with an appropriate control level of HMGB1. The appropriate control level may be a level of HMGB1 expression in a cell or population of cells that has not been contacted with an oligonucleotide as described herein. In some embodiments, the effect of delivery of an oligonucleotide to a cell according to a method disclosed herein is assessed after a finite period of time. For example, levels of HMGB1 may be analyzed in a cell at least 8 hours, 12 hours, 18 hours, 24 hours; or at least one, two, three, four, five, six, seven, or fourteen days after introduction of the oligonucleotide into the cell.

[000153] In some embodiments, an oligonucleotide is delivered in the form of a transgene that is engineered to express in a cell the oligonucleotides (*e.g.*, its sense and antisense strands). In some embodiments, an oligonucleotide is delivered using a transgene that is engineered to express any oligonucleotide disclosed herein. Transgenes may be

delivered using viral vectors (*e.g.*, adenovirus, retrovirus, vaccinia virus, poxvirus, adeno-associated virus or herpes simplex virus) or non-viral vectors (*e.g.*, plasmids or synthetic mRNAs). In some embodiments, transgenes can be injected directly to a subject.

ii. Treatment Methods

[000154] Aspects of the disclosure relate to methods for reducing HMGB1 expression in for attenuating the onset or progression of liver fibrosis in a subject. In some embodiments, the methods may comprise administering to a subject in need thereof an effective amount of any one of the oligonucleotides disclosed herein. Such treatments could be used, for example, to slow or halt any type of liver fibrosis. The present disclosure provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disease or disorder associated with liver fibrosis and/or liver inflammation.

[000155] In certain aspects, the disclosure provides a method for preventing in a subject, a disease or disorder as described herein by administering to the subject a therapeutic agent (*e.g.*, an oligonucleotide or vector or transgene encoding same). In some embodiments, the subject to be treated is a subject who will benefit therapeutically from a reduction in the amount of HMGB1 protein, *e.g.*, in the liver. Subjects at risk for the disease or disorder can be identified by, for example, one or a combination of diagnostic or prognostic assays known in the art (*e.g.*, identification of liver fibrosis and/or liver inflammation). Administration of a prophylactic agent can occur prior to the detection of or the manifestation of symptoms characteristic of the disease or disorder, such that the disease or disorder is prevented or, alternatively, delayed in its progression.

[000156] Methods described herein are typically involve administering to a subject in an effective amount of an oligonucleotide, that is, an amount capable of producing a desirable therapeutic result. A therapeutically acceptable amount may be an amount that is capable of treating a disease or disorder. The appropriate dosage for any one subject will depend on certain factors, including the subject's size, body surface area, age, the particular composition to be administered, the active ingredient(s) in the composition, time and route of administration, general health, and other drugs being administered concurrently.

[000157] In some embodiments, a subject is administered any one of the compositions disclosed herein either enterally (*e.g.*, orally, by gastric feeding tube, by duodenal feeding tube, via gastrostomy or rectally), parenterally (*e.g.*, subcutaneous injection, intravenous injection or infusion, intra-arterial injection or infusion, intraosseous infusion, intramuscular injection, intracerebral injection, intracerebroventricular injection, intrathecal), topically (*e.g.*,

epicutaneous, inhalational, via eye drops, or through a mucous membrane), or by direct injection into a target organ (*e.g.*, the liver of a subject). Typically, oligonucleotides disclosed herein are administered intravenously or subcutaneously.

[000158] As a non-limiting set of examples, the oligonucleotides of the instant disclosure would typically be administered quarterly (once every three months), bi-monthly (once every two months), monthly, or weekly. For example, the oligonucleotides may be administered every one, two, or three weeks. The oligonucleotides may be administered daily.

[000159] In some embodiments, the subject to be treated is a human or non-human primate or other mammalian subject. Other exemplary subjects include domesticated animals such as dogs and cats; livestock such as horses, cattle, pigs, sheep, goats, and chickens; and animals such as mice, rats, guinea pigs, and hamsters.

EXAMPLES

Example 1: Development of HMGB1 RNAi oligonucleotides using human and mouse cell-based assays

[000160] FIG. 1 shows a workflow using human and mouse-based assays to develop candidate RNAi oligonucleotides for inhibition of HMGB1 expression. First, a computer-based algorithm was used to generate candidate oligonucleotide sequences (25-27-mer) for HMGB1 inhibition. Cell-based assays and PCR assays were then employed for evaluation of candidate RNAi oligonucleotides for their ability to reduce HMBG1 expression.

[000161] The computer algorithm provided RNAi oligonucleotide sequences that were complementary to the human HMGB1 mRNA (SEQ ID NO: 371, Table 1), of which certain sequences were also complementary to the monkey HMGB1 mRNA (SEQ ID NO: 372, Table 1), and the mouse HMGB1 mRNA (SEQ ID NO: 373, Table 1).

Table 1. Sequences of human, rhesus monkey and mouse HMGB1 mRNA

Species	*GenBank RefSeq #*	*Sequence Identifier*
Human	NM_002128.5	SEQ ID NO: 371
Monkey	NM_001283356.1	SEQ ID NO: 372
Mouse	NM_010439.4	SEQ ID NO: 373

[000162] The algorithm produced hundreds of RNAi oligonucleotide sequences of which 96 oligonucleotides were identified bioinformatically as top candidates for experimental evaluation in a cell-based assay. In this assay, Hepa 1-6 (ATCC® CRL-1830™) hepatocyte

cells expressing HMGB1 were transfected with the oligonucleotides. Cells were maintained for a period of time following transfection and then levels of remaining HMGB1 mRNA were interrogated using TAQMAN®-based qPCR assays. Two qPCR assays, a 3' assay and a 5' assay were used. All 96 RNAi oligonucleotides were evaluating using the same modification pattern, designated M15, which contains a combination of ribonucleotides, deoxyribonucleotides and 2'-O-methyl modified nucleotides. The sequences of the oligonucleotides tested are provided in Table 2.

Table 2. Candidate oligonucleotide Sequences for Mouse Hepa 1-6 Cell-Based Assay

Hs	Rh	Mm	Sense SEQ ID NO	Corresponding Antisense SEQ ID NO
X	X	X	1-11; 24-43; 54-69; 96	97-107; 120-139; 150-165; 192
X	X		12-23; 53; 70; 71; 73-75; 89-93; 95	108-119; 149; 166; 167; 169-171; 185-189; 191
X			44-52	140-148

Hs: human, Rh: rhesus monkey, and Mm: mouse; the sense and antisense SEQ ID NO columns provide the sense strand and respective antisense strand that are hybridized to make each oligonucleotide. For example, sense strand with SEQ ID NO: 1 hybridizes with antisense strand with SEQ ID NO: 97; each of the oligonucleotides tested had the same modification pattern.

Hot Spots in HMGB1 mRNA

[000163] Data from the screen of the 96 candidate RNAi oligonucleotides is shown in FIGs. 3A-3D. Top performing oligonucleotides in the cell based assay that resulted in less than or equal to 25% mRNA remaining compared to negative controls were selected as hits for further experimental characterization. Oligonucleotides that were not found to inhibit HMGB1 expression were used as negative controls. House-keeping gene Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a normalizing gene for HMGB1 expression, as its expression is not altered by inhibiting the expression of HMGB1.

[000164] Hotspots in human HMGB1 mRNA were identified by examining the activity and locations of these oligonucleotides. A hotspot was identified as a stretch on the human HMGB1 mRNA sequence associated with at least two oligonucleotides resulting in mRNA

levels that were less than or equal to 25% in either assay compared with controls. Accordingly, the following hotspots within the human HMGB1 mRNA sequence were identified: 935-965; 1100-1130; 1160-1215; 1230-1270; 1470-1500; 1680-1710; 2190-2220; and 2280-2310.

[000165] The sequences of the hotspots are outlined in Table 3.

Table 3. Sequences of Hotspots

Hotspot Position	Sequence	SEQ ID NO:
935-965	TAAGATTTGTTTTTAAACTGTACAGTGTCTT	374
1100-1130	TTGGTGCACAGCACAAATTAGTTATATATGG	375
1160-1215	TCTGATGCAGCTTATACGAAATAATTGTTGTTCTGTTAACTGAATACCACTCTGTA	376
1230-1270	AAAAAAGTTGCAGCTGTTTTGTTGACATTCTGAATGCTTCT	377
1470-1500	TGAGATAGTTTTCATCCATAACTGAACATCC	378
1680-1710	TACCATGTAATGGCAGTTATATTTTGCAGTT	379
2190-2220	TTTACACGCTTTTGTGATGGAGTGCTGTTTT	380
2280-2310	AATACTGAACATCTGAGTCCTGGATGATACT	381

Dose Response Analysis

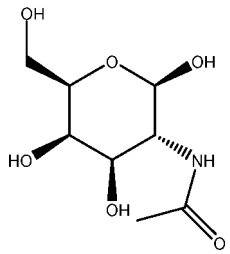
[000166] Twenty-four top performing RNAi oligonucleotides were selected for analysis in a secondary screen, taking into account gene location and sequence conservation between species,. In this secondary screen, the RNAi oligonucleotides were tested using the same assay as in the primary screen, but at three different concentrations (1 nM, 0.1nM and 0.01nM) (FIG.4). Those RNAi oligonucleotides showing activity at two more concentrations were then subjected to further analysis.

[000167] In this further analysis of the top candidates from the secondary screen, the sequences were incorporated into RNAi oligonucleotides in a nicked tetraloop structure format (a 36-mer passenger strand with a 22-mer guide strand). These RNAi oligonucleotides in the nicked tetraloop structure format were initially tested at 1nM for their ability to reduce HMGB1 mRNA expression. FIG. 5 shows data for RNAi oligonucleotides made from different base sequences with nicked tetraloop structures, each adapted to four different modification patterns, designated M28, M47, M48, and M52, which comprise in their sense

and antisense strands 2'-fluoro and 2'-O-methyl modified nucleotides in different arrangements and phosphorothioate and phosphodiester linkages, and include in their antisense strands a phosphate analog positioned at the 5′ terminal nucleotide, and an unmodified control, designated M46. The top RNAi oligonucleotides were further tested using a full dose response curve in Hepa1-6 cells in order to determine the half maximal inhibitory concentration (IC_{50}) for each compound (see FIG. 6).

In vivo murine experimentation

[000168] Data from the above *in vitro* experiments were assessed to identify tetraloops and modification patterns that would improve delivery properties while maintaining activity for reduction of HMGB1 expression in the mouse hepatocytes. Based on this analysis, select oligonucleotides were then conjugated to GalNAc moieties. Monovalent GalNAc moieties were conjugated to each nucleotide of the GAAA sequence in the tetraloop of the sense strand, as illustrated in FIG. 2. Conjugation was performed using a click linker. The GalNAc used was as shown below:



N-Acetyl-b-D-galactosamine (CAS#: 14131-60-3)

[000169] Groups of GalNAc-conjugated RNAi oligonucleotides with different modification patterns and nicked tetraloop structures were subcutaneously administered to C57BL/6 mice at 10 mg/kg. The percentage of HMGB1 mRNA as compared to HPRT control mRNA was measured at 72 hours (FIG. 7).

[000170] Three candidate GalNAc-conjugated oligonucleotides containing nicked tetraloop structures were tested again at three different doses (0.3 mg/kg, 1 mg/kg, and 3 mg/kg) for the amount of HMGB1 mRNA remaining at 24 hours after oligonucleotide administration compared to a PBS control using TAQMAN®-based qPCR assays (FIG. 8).

[000171] Two of the RNAi oligonucleotides were selected for further testing using different modification patterns at 3 mg/kg or 3 mg/kg and 10 mg/kg. Again, the percentage of HMGB1 mRNA remaining at 72 hours after oligonucleotide administration compared to a PBS

control was interrogated using TAQMAN®-based qPCR assays (FIG. 9 and FIG. 10, respectively). The oligonucleotides were tested in different modification patterns denoted as M49, M54, M55, M56, which comprise in their sense and antisense strands 2'-fluoro and 2'-O-methyl modified nucleotides in different arrangements and phosphorothioate and phosphodiester linkages, and include in their antisense strands a phosphate analog positioned at the 5′ terminal nucleotide.

[000172] The percentage of HMGB1 mRNA remaining at day 5 (as compared to a PBS control) after a 1 mg/kg subcutaneous dose of GalNAc-conjugated HMGB1 RNAi oligonucleotides containing nicked tetraloop structures was determined for oligonucleotides of the same sequence with different modifications (FIG. 11) as well as oligonucleotides of different sequence that retained the same modification pattern.

[000173] The triple-common sequences were also tested alongside sequences that were only identified in human HMGB1 gene ("human uniques") for activity in mice that were hydrodynamically injected with the RNAi oligonucleotides (HDI mice). Briefly, mice were intravenously administered 2 mL of human HMGB1 and NEOMYCIN plasmid--containing solution over seven seconds through the tail vein. 1 mg/kg RNAi oligonucleotide was subcutaneously injected into each mouse four days prior to HDI injection. The percentage of remaining HMGB1 mRNA was subsequently determined (normalized to NEO-168) using TAQMAN®-based qPCR assays. These results demonstrated that RNAi oligonucleotides complementary the triple common sequences selected from the previous screening downregulate HMGB1 expression more strongly than selected oligonucleotides that bound only the human unique sequences.

Example 2: Evaluation of Treatment in Models of Hepatotoxic Agents

[000174] Candidate RNAi oligonucleotides identified in Example 1 were further tested in models of APAP-induced (acetaminophen-induced) liver injury. Acetaminophen is known to be metabolized and form the active metabolite N-acetyl-p-benzoquinone imine (NAPQI). NAPQI binds to mitochondrial proteins to form NAPQI protein adducts, leading to hepatocyte necrosis.

Model 1 - APAP

[000175] Groups of 10 C57BL/6 male mice (10-11 weeks old) were fasted for 12 hours prior to administration of 250 or 350 mg/kg APAP in a 0.9 % NaCl intraperotineal injection. Blood and/or liver tissues were collected at 6, 24, and 48 hours. Endpoint measurements for

the study were quantification of liver damage (ALT, AST, and GLDH-*IDEXX* levels), profiling biomarkers (serum/liver HMGB1 levels and miR122 levels through ELISA, Western blots, and RT-qPCR), and liver staining (H&E, HMGB1, Ly6-B, and F4/80 staining). See schematic in FIG. 12.

[000176] FIGs. 13A and 13B show the percentage of HMGB1 remaining in the liver (normalized to Hprt1) and serum (mg/mL) of animals treated with 350 mg/kg APAP versus a saline control injection. HMGB1 mRNA levels were interrogated using TAQMAN®-based qPCR assays. GalNAc conjugated RNAi oligonucleotides containing nicked tetraloop structures and RNAi oligonucleotides provided in a lipid nanoparticle formulation (LNP formulated RNAi oligonucleotides) downregulated >80% *HMGB1* mRNA. However, serum HMGB1 was significantly reduced only in HMGB1-LNP treated animals. The HMGB1 oligonucleotide used in the experiments depicted in FIGs. 13A-13B is S212-AS296-M49.

[000177] Liver function tests (LFTs) were also performed in animals treated with 350 mg/kg APAP. These experiments also demonstrated a decrease in hepatotoxicity according to serum AST, ALT, and miR122 levels in both LNP formulated and GalNAc conjugated HMGB1 oligonucleotides containing nicked tetraloop structures with a larger decrease seen when using the LNP formulated RNAi oligonucleotides (see FIGs. 14A-14C). The HMGB1 oligonucleotide in the experiments depicted in FIGs. 14A-14C is S212-AS296-M49.

Model 2 - APAP

[000178] In a second study using a model for APAP toxicity, groups of 10 C57BL/6 male mice (10-11 weeks old) were fasted for 12 hours prior to administration of 350 mg/kg APAP in a 0.9 % NaCl intraperotineal injection. Animals were treated with 5 mg/kg of GalNAc-conjugated RNAi oligonucleotide containing nicked tetraloop structures or 1.5 mg/kg of LNP formulated RNAi oligonucleotide multiple times prior to APAP administration. Blood and/or liver tissues were collected 24 hours after APAP administration. Endpoint measurements for the study were quantification of liver damage (ALT, AST, and GLDH-*IDEXX* levels), profiling biomarkers (serum/liver HMGB1 levels and miR122 levels through ELISA and RT-qPCR), and liver staining (H&E and HMGB1 immunohistochemistry). See schematic shown in FIG. 15.

[000179] FIGs. 16A-16B show the percentage of HMGB1 remaining in the liver (normalized to Hprt1) and serum (mg/mL) of animals treated with 350 mg/kg APAP versus a saline control injection. HMGB1 mRNA levels were interrogated using TAQMAN®-based qPCR assays. GalNAc conjugated RNAi oligonucleotides containing nicked tetraloop

structuresand LNP formulated RNAi oligonucleotides downregulated >80% *HMGB1* mRNA in the liver. Serum HMGB1 was also significantly reduced using both GalNAc conjugated RNAi oligonucleotides containing nicked tetraloop structures and LNP formulated RNAi oligonucleotides. The HMGB1 oligonucleotide used in the experiments depicted in FIGs. 16A-16B is S212-AS296-M56.

[000180] Further, ALT, AST, LDH, and serum miR122 levels were measured in APAP-treated mice and demonstrated that administration of GalNAc conjugated or LNP formulated HMGB1 oligonucleotides inhibited liver damage (see FIGs. 17A-17D). The HMGB1 oligonucleotide used in the experiments depicted in FIGs. 17A-17D is S212-AS296-M56.

[000181] Collectively, the studies using APAP toxicity models demonstrated that HMGB1 oligonucleotide treated animals have decreased HMGB1 serum levels and liver injury when compared to control animals.

Model 3 – Carbon Tetrachloride

[000182] Groups of mice were administered 3 µl per gram of body weight carbon tetrachloride mixed with olive oil (CCl_4:olive oil at a 1:5 v/v ratio) or an olive oil control solution twice per week for six weeks through intraperotineal injection. As shown in Table 4 and FIG. 18, animals were randomized into the groups shown and either left untreated or were treated with GalNAc-conjugated HMGB1 oligonucleotides containing nicked tetraloop structures or PBS via subcutaneous (S.C.) or intravenous (I.V.) injection once per week for 5 or 6 wks according to study design.

Group	CCl_4	Treatment	Dose (mg/kg)	Volume (ml/kg)	Dosing start	Frequency and days of dosing	No. mice	Route
1	Olive oil	-	-	-	-	-	10	-
2	CCl_4	PBS	0	10	Day -4	qw x 6 weeks	10	S.C.
3	CCl_4	GalNAc-conjugated HMGB1 oligo	5	10	Day 11	qw x 5 weeks	10	S.C.

4	CCl_4	GalNAc-conjugated HMGB1 oligo	5	10	Day -4	qw x 6 weeks	10	S.C.
5	CCl_4	LNP HMGB1 oligo	2.5	10	Day -4	qw x 6 weeks	10	I.V.

Table 4: Group Designation and compound dosing schedule

[000183] Several parameters indicative of liver injury were examined in the mice at the end of the study. Significant reductions were seen in both fibrosis (using Sirius Red staining) and macrophage infiltration and activation (using immunohistochemistry analysis with an antibody specific for the F4/80 protein, which is a mouse macrophage marker) in both groups of HMGB1 RNAi oligonucleotide-treated animals when compared to PBS-treated animals. Significant reductions were also seen in stellate cell activation (measured through α-SMA staining) and hepatomegaly (measured using liver weight to body weight ratio) using the longer dosing regimen data shown in FIGs. 19-22). The HMGB1 RNAi oligonucleotides used in this study are identified as S212-AS296-M49, and have monovalent GalNac moieties conjugated at each of the four nucleotide positions of the GAAA loop.

[000184] Additionally, livers from animals treated as above with carbon tetrachloride were examined at 43 days post-injection using Sirius Red staining. These experiments revealed that administration of GalNAc-conjugated HMGB1 RNAi oligonucleotide containing a nicked tetraloop structure (either at a 5 mg/kg subcutaneous dose or a 2.5 mg/kg intravenous dose) significantly reduced the relative Sirius Red-positive area in liver samples, indicating that liver fibrosis in these animals was significantly reduced (FIG. 23). The HMGB1 oligonucleotide used in the experiments depicted in FIG. 23 is S212-AS296-M49.

Example 3: Evaluation of Treatment in Model of Autoimmune Liver Disease

[000185] Two groups of five C57BL/6 male mice (10-11 weeks old) were treated with 20 mg/kg of intravenous concavalin A six days after subcutaneous administration of either 10 mg/kg GalNAc-conjugated HMGB1 oligonucleotide containing a nicked tetraloop structure or PBS. One day following concavalin A administration, the animals were examined for ALT and AST serum levels as well as H&E staining of the liver (experimental schematic shown in FIG. 24). FIG. 25 demonstrates that animals were three times more likely to survive when treated with the GalNAc-conjugated HMGB1 RNAi oligonucleotide containing a nicked

tetraloop structure than with PBS. Further, surviving HMGB1 oligonucleotide-treated animals had less necrosis in H&E stained samples of liver than the PBS controls. The HMGB1 oligonucleotide used in the experiments depicted in FIG. 25 is S212-AS296-M49.

Example 4: Evaluation of Treatment in Model of Choline-Deficient Amino Acid-Defined High-Fat Diet (CDAHFD)

[000186] C57Bl/6 mice were fed either a control high-fat diet (CDHFD) or a choline deficient amino acid defined high fat diet (CDAHFD) and normal or sugar water. CDAHFD has a lower methionine content than CDHFD. As a result of the difference in methionine content, CDHFD is expected to generate steatosis, but is not expected to generate the NAFLD-like liver pathology seen in the CDAHFD diet. As shown in Tables 5 and 6, the animals were left untreated or were administered a PBS control or GalNAc-conjugated HMGB1 RNAi oligonucleotides containing nicked tetraloop structures at 5 mg/kg for 6 weeks (subcutaneous administration) starting six weeks after the mice were placed on the high fat diets.

Table 5: Treatment of animals with high fat control diet and normal or sugar water

Treatment	Diet	# mice	Necropsy week
-	CDHFD + normal water	5	6
-	CDHFD + normal water	5	9
PBS	CDHFD + normal water	5	12
GalNAc-conjugated HMGB1 oligo	CDHFD + normal water	5	12
-	CDHFD + sugar water	5	6
-	CDHFD + sugar water	5	9
PBS	CDHFD + sugar water	5	12
GalNAc-conjugated HMGB1 oligo	CDHFD + sugar water	5	12

Table 6: Treatment of animals with choline deficient amino acid defined high fat diet (CDAHFD) and normal or sugar water

Treatment	Diet	# mice	Necropsy week
-	CDAHFD + normal water	5	6
-	CDAHFD + normal water	5	9
PBS	CDAHFD + normal water	5	12

GalNAc-conjugated HMGB1 oligo	CDAHFD + normal water	5	12
-	CDAHFD + sugar water	5	6
-	CDAHFD + sugar water	5	9
PBS	CDAHFD + sugar water	5	12
GalNAc-conjugated HMGB1 oligo	CDAHFD + sugar water	5	12

[000187] HMGB1 mRNA was elevated in mice that consumed the choline deficient amino acid defined high fat diet (CDAHFD) in comparison to the CDHFD control high-fat diet (FIGs. 26A and 26B). Further, Collagen 1a1 (Col1a1) and Vimentin mRNA levels were elevated by CDAHFD >100x, relative to the CDHFD alone (FIGs. 26C and 26D, respectively). GalNAc-conjugated HMGB1 oligonucleotides containing nicked tetraloop structures demonstrated significant reduction on these markers after 6 weeks of treatment.

[000188] AST and ALT levels were also elevated in mice consuming CDAHFD, relative to mice consuming the control high fat diet (CDHFD) (FIG. 27). GalNAc-conjugated HMGB1 RNAi oligonucleotides demonstrated moderate reduction on these markers after 6 weeks of treatment. The HMGB1 oligonucleotide used in FIGs. 26A-26D and 27 is S194-AS274-M30. comprises in sense and antisense strands 2'-fluoro and 2'-O-methyl modified nucleotides in different arrangements and phosphorothioate and phosphodiester linkages, and include in its antisense strands a phosphate analog positioned at the 5′ terminal nucleotide.

Example 5. Testing the activity of additional HMGB1 oligonucleotides

[000189] All additional HMGB1 oligonucleotides used in this example were designed to bind to conserved sequences identified by the algorithm in the human, monkey (both rhesus), and mouse sequences ("triple common" sequences). The additional HMGB1 oligonucleotides were modified as described in Example 1 to contain tetraloops and adapt different modification patterns. The different modification patterns (e.g., designated M58, M59, M60, or M61) comprise in their sense and antisense strands 2'-fluoro and 2'-O-methyl modified nucleotides in different arrangements and phosphorothioate and phosphodiester linkages, and include in their antisense strands a phosphate analog positioned at the 5′ terminal nucleotide. The oligonucleotide inhibitors were also conjugated to GalNAc moieties. Three GalNAc moieties were conjugated to nucleotides in the tetraloop of the sense strand. Conjugation was performed using a click linker as described in Example 1. The GalNAc-conjugated HMGB1

oligonucleotides were then tested for their ability to reduce HMGB1 mRNA expression in mice and in vitro in cultured hepatocytes.

[000190] Eight different GalNAc-conjugated HMGB1 oligonucleotides (S204-AS286, S205-AS287, S210-AS366, S211-AS367, S363-AS368, S364-AS369, S212-AS296, and S365-AS370) with three different modification patterns (denoted M58, M59, and M60) were tested for their potency in knocking down HMGB1. One single dose (1 mg/kg) of indicated HMGB1 oligonucleotides with a specific modification pattern were injected subcutaneously to CD-1 mice. Mice were euthanized 5 days post injection and liver samples were obtained. RNA was extracted from the liver samples to evaluate HMGB1 mRNA levels by qPCR (normalized to HPRT1-F576, a housekeeping gene). The levels of remaining HMGB1 mRNA were interrogated using TAQMAN®-based qPCR assays. All tested HMGB1 oligonucleotides and modification patterns were potent in knocking down HMGB1, with modification patters M59 and M60 exhibiting higher potency (FIG. 28).

[000191] A confirmatory dose-response assay was also performed on six GalNAc-conjugated HMGB1 oligonucleotides (S204-AS286, S205-AS287, S211-AS367, S364-AS369, S212-AS296, and S365-AS370) with different modification patterns (M58, M59, or M60). A single dose (1, 0.5, or 0.25 mg/kg) of indicated GalNAc-conjugated HMGB1 oligonucleotides were administered to CD-1 mice subcutaneously. The mice were euthanized on day 5 after administration and liver samples were obtained. RNA was extracted to evaluate HMGB1 mRNA levels by qPCR (normalized to HPRT1-F576, a housekeeping gene). The levels of remaining HMGB1 mRNA were interrogated using TAQMAN®-based qPCR assays. PBS was used as negative control, and S204-AS286-M61 was used as positive control in this experiment. Almost all tested HMGB1 oligonucleotide inhibitors demonstrated dose-dependent potency (FIGs. 29A and 29B). Some of the tested HMGB1 oligonucleotide inhibitors showed improved potency compared to the S204-AS286-M61 control (FIGs. 29A and 29B). The confirmatory dose-response assay was repeated for S204-AS286-M59, S211-AS367-M59, S364-AS369-M60, and S365-AS370-M59. Similar results were obtained and all tested HMGB1 oligonucleotide inhibitors showed an ED_{50} of about 0.5 to 1.0 mg/kg, if HMGB1 expression level in non-hepatocytes is considered the expression baseline (FIGs. 31A and 31B).

[000192] Two GalNAc-conjugated HMGB1 oligonucleotides (S211-AS367-M59, S364-AS369-M60) with different modification patterns (denoted as M59 and M60, respectively) were tested in vivo in a duration study to evaluate their activity in inhibiting HMGB1 expression in mice. PBS was used as negative control, and S204-AS286-M58 was

used as positive control in this experiment. Mice were injected subcutaneously with a single dose (1 mg/kg) of indicated GalNAc-conjugated HMGB1 oligonucleotides. Mice were euthanized on days 7, 14, 21, and 28 post injection and liver samples were obtained. RNA was extracted to evaluate HMGB1 mRNA levels by qPCR (normalized to HPRT1-F576, a housekeeping gene). The qPCR was performed using two different primers specific to different regions in the HMGB1 mRNA. The qPCR performed using the primer at the 5' end relative to the other primer was designated "5' qPCR." Similarly, the qPCR performed using the primer at the 3' end relative to the other primer was designated "3' qPCR." The data showed that all tested HMGB1 oligonucleotides were potent in knockdown HMGB1 3 weeks after injection, as indicated by the reduced amount of HMGB1 mRNA remaining in mice liver at days 7, 14, 21, and 28 (normalized to a PBS control treatment) (FIGs. 30A and 30B).

[000193] All HMGB1 oligonucleotide inhibitors tested in FIGs. 31A and 31B (S204-AS286-M59, S211-AS367-M59, S364-AS369-M60, and S365-AS370-M59) were selected to be further tested in a 3-week duration study. In this study, a single dose (4 mg/kg) of the indicated HMGB1 oligonucleotide inhibitors were injected subcutaneously to CD-1mice. Mice were euthanized 21 days post injection and liver samples were obtained. RNA was extracted to evaluate HMGB1 mRNA levels by qPCR (normalized to HPRT1-F576 housekeeping gene). The levels of remaining HMGB1 mRNA were interrogated using TAQMAN®-based qPCR assays. The results showed that all tested HMGB1 oligonucleotide inhibitors retained potency in knocking down HMGB1 3 weeks post injection (FIGs. 32A and 32B).

[000194] Certain GalNAc-conjugated HMGB1 oligonucleotides were further tested using a full dose response curve in rhesus monkey or human hepatocytes in order to determine the half maximal inhibitory concentration (IC_{50}) for each inhibitor. Human primary hepatocytes (HPCH10+, Lot #1410214, , Cryopreserved Xenotech) or monkey primary hepatocyte (P2000.H15, Lot #1410285, Cryopreserved Xenotech) were thawed and plated at 50000 cells(human) or100000(monkey) per well in 96-well tissue culture plates in OptiPlate Hepatocyte Media(K8200). Plated hepatocytes were allowed to recover for 1-6 h at 37°C and 5% CO2 and subsequently maintained using OptiCulture Hepatocyte Media(K8300). Five-fold serially diluted (7-times) GalNAc-conjugated HMGB1 oligonucleotides (S204-AS286-M61 and S365-AS370-M59) were added to cells. The uptake of the oligonucleotides into the monkey or human primary hepatocyte cells was mediated by ASGPR receptor. Cells were incubated for 48h at 37°C and 5% CO2. RNA was extracted from cells using SV 96 Total RNA Isolation System(Promega) to evaluate remaining HMGB1 mRNA levels by qPCR

(normalized to RhPPIB or HsSFRS9 and HsPIO8, housekeeping genes). The levels of remaining HMGB1 mRNA were interrogated using TAQMAN®-based qPCR assays. A GalNAc-conjugate LDHA oligonucleotide was used as assay control (FIGs. 33C, 33F, 33G, 34C, 34F, 34G,) and the level of remaining LDHA mRNA was measured by RhLDHA-F887 qPCR assay(FIGs. 33G and 34G).

The results showed that the HMGB1 oligonucleotide inhibitors were potent (sub-nM IC_{50} values) in knocking down HMGB1 in primary mice hepatocytes, as demonstrated by the sub-nM to nM IC_{50} values (FIGs. 33A,33B, 33D, and 33E). Similar knock down potency was observed in in primary human hepatocytes, as demonstrated by sub-nM IC_{50} values (FIGs. 34A, 34B, 34D, and 34E).

Materials and Methods

Transfection

[000195] For the first screen, Lipofectamine RNAiMAX™ was used to complex the oligonucleotides for efficient transfection. Oligonucleotides, RNAiMAX and Opti-MEM were added to a plate and incubated at room temperature for 20 minutes prior to transfection. Media was aspirated from a flask of actively passaging cells and the cells are incubated at 37°C in the presence of trypsin for 3-5 minutes. After cells no longer adhered to the flask, cell growth media (lacking penicillin and streptomycin) was added to neutralize the trypsin and to suspend the cells. A 10 µL aliquot was removed and counted with a hemocytometer to quantify the cells on a per millimeter basis. For HeLa cells, 20,000 cells were seeded per well in 100 µL of media. The suspension was diluted with the known cell concentration to obtain the total volume required for the number of cells to be transfected. The diluted cell suspension was added to the 96 well transfection plates, which already contained the oligonucleotides in Opti-MEM. The transfection plates were then incubated for 24 hours at 37°C. After 24 hours of incubation, media was aspirated from each well. Cells were lysed using the lysis buffer from the Promega RNA Isolation kit. The lysis buffer was added to each well. The lysed cells were then transferred to the Corbett XtractorGENE (QIAxtractor) for RNA isolation or stored at -80°C.

[000196] For subsequent screens and experiments, *e.g.*, the secondary screen, Lipofectamine RNAiMAx was used to complex the oligonucleotides for reverse transfection. The complexes were made by mixing RNAiMAX and siRNAs in OptiMEM medium for 15 minutes. The transfection mixture was transferred to multi-well plates and cell suspension was added to the wells. After 24 hours incubation the cells were washed once with PBS and then

lysed using lysis buffer from the Promega SV96 kit. The RNA was purified using the SV96 plates in a vacuum manifold. Four microliters of the purified RNA was then heated at 65°C for 5 minutes and cooled to 4°C. The RNA was then used for reverse transcription using the High Capacity Reverse Transcription kit (Life Technologies) in a 10 microliter reaction. The cDNA was then diluted to 50 µl with nuclease free water and used for quantitative PCR with multiplexed 5'-endonuclease assays and SSoFast qPCR mastermix (Bio-Rad laboratories).

cDNA Synthesis

[000197] RNA was isolated from mammalian cells in tissue culture using the Corbett X-tractor Gene™ (QIAxtractor). A modified SuperScript II protocol was used to synthesize cDNA from the isolated RNA. Isolated RNA (approximately 5 ng/µL) was heated to 65°C for five minutes and incubated with dNPs, random hexamers, oligo dTs, and water. The mixture was cooled for 15 seconds. An "enzyme mix," consisting of water, 5X first strand buffer, DTT, SUPERase•In™ (an RNA inhibitor), and SuperScript II RTase was added to the mixture. The contents were heated to 42°C for one hour, then to 70°C for 15 minutes, and then cooled to 4°C using a thermocycler. The resulting cDNA was then subjected to SYBR®-based qPCR. The qPCR reactions were multiplexed, containing two 5' endonuclease assays per reaction.

qPCR Assays

[000198] Primer sets were initially screened using SYBR®-based qPCR. Assay specificity was verified by assessing melt curves as well as "minus RT" controls. Dilutions of cDNA template (10-fold serial dilutions from 20 ng and to 0.02 ng per reaction) from HeLa and Hepa1-6 cells are used to test human (Hs) and mouse (Mm) assays, respectively. qPCR assays were set up in 384-well plates, covered with MicroAmp film, and run on the 7900HT from Applied Biosystems. Reagent concentrations and cycling conditions included the following: 2x SYBR mix, 10 µM forward primer, 10 µM reverse primer, DD H_2O, and cDNA template up to a total volume of 10 µL.

[000199] In some cases, as noted, qPCR was performed using TAQMAN®-based qPCR assays. TAQMAN® probes target two different positions (5' and 3' to one another) within the coding region of the target mRNA (e.g., HMGB1) were generally used to provide additional confirmation of mRNA levels in the analysis.

Cloning

[000200] PCR amplicons that displayed a single melt-curve were ligated into the

pGEM®-T Easy vector kit from Promega according to the manufacturer's instructions. Following the manufacturer's protocol, JM109 High Efficiency cells were transformed with the newly ligated vectors. The cells were then plated on LB plates containing ampicillin and incubated at 37°C overnight for colony growth.

PCR Screening and Plasmid Mini-Prep

[000201] PCR was used to identify colonies of *E. coli* that had been transformed with a vector containing the ligated amplicon of interest. Vector-specific primers that flank the insert were used in the PCR reaction. All PCR products were then run on a 1% agarose gel and imaged by a transilluminator following staining. Gels were assessed qualitatively to determine which plasmids appeared to contain a ligated amplicon of the expected size (approximately 300 bp, including the amplicon and the flanking vector sequences specific to the primers used).

[000202] The colonies that were confirmed transformants by PCR screening were then incubated overnight in cultures consisting of 2 mL LB broth with ampicillin at 37°C with shaking. *E. coli* cells were then lysed, and the plasmids of interest were isolated using Promega's Mini-Prep kit. Plasmid concentration was determined by UV absorbance at 260 nm.

Plasmid Sequencing and Quantification

[000203] Purified plasmids were sequenced using the BigDye® Terminator sequencing kit. The vector-specific primer, T7, was used to give read lengths that span the insert. The following reagents were used in the sequencing reactions: water, 5X sequencing buffer, BigDye terminator mix, T7 primer, and plasmid (100 ng/µL) to a volume of 10 µL. The mixture was held at 96°C for one minute, then subjected to 15 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 1 minute, 15 seconds; 5 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 1 minute, 30 seconds; and 5 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 2 minutes. Dye termination reactions were then sequenced using Applied Biosystems' capillary electrophoresis sequencers.

[000204] Sequence-verified plasmids were then quantified. They were linearized using a single cutting restriction endonuclease. Linearity was confirmed using agarose gel electrophoresis. All plasmid dilutions were made in TE buffer (pH 7.5) with 100 µg of tRNA per mL buffer to reduce non-specific binding of plasmid to the polypropylene vials.

[000205] The linearized plasmids were then serially diluted from 1,000,000 to 01 copies per µL and subjected to qPCR. Assay efficiency was calculated and the assays were

deemed acceptable if the efficiency was in the range of 90-110%.

Multi-Plexing Assays

[000206] For each target, mRNA levels were quantified by two 5' nuclease assays. In general, several assays are screened for each target. The two assays selected displayed a combination of good efficiency, low limit of detection, and broad 5'→3' coverage of the gene of interest (GOI). Both assays against one GOI could be combined in one reaction when different fluorophores were used on the respective probes. Thus, the final step in assay validation was to determine the efficiency of the selected assays when they were combined in the same qPCR or "multi-plexed".

[000207] Linearized plasmids for both assays in 10-fold dilutions were combined and qPCR was performed. The efficiency of each assay was determined as described above. The accepted efficiency rate was 90-110%.

[000208] While validating multi-plexed reactions using linearized plasmid standards, C_q values for the target of interest were also assessed using cDNA as the template. For human or mouse targets, HeLa and Hepa1-6 cDNA were used, respectively. The cDNA, in this case, was derived from RNA isolated on the Corbett (~5 ng/µl in water) from untransfected cells. In this way, the observed C_q values from this sample cDNA were representative of the expected C_q values from a 96-well plate transfection. In cases where C_q values were greater than 30, other cell lines were sought that exhibit higher expression levels of the gene of interest. A library of total RNA isolated from via high-throughput methods on the Corbett from each human and mouse line was generated and used to screen for acceptable levels of target expression.

Description of oligonucleotide nomenclature

[000209] All oligonucleotides described herein are designated either SN_1-ASN_2-MN_3. The following designations apply:

- N_1: sequence identifier number of the sense strand sequence
- N_2: sequence identifier number of the antisense strand sequence
- N_3: reference number of modification pattern, in which each number represents a pattern of modified nucleotides in the oligonucleotide.

For example, S27-AS123-M15 represents an oligonucleotide with a sense sequence that is set forth by SEQ ID NO: 27, an antisense sequence that is set forth by SEQ ID NO: 123, and which is adapted to modification pattern number 15.

Table 7: HMGB1 RNAi Oligonucleotide Sequences

Sense (S) – Antisense (AS) Designation	Sense Sequence/mRNA seq	S SEQ ID NO	Antisense Sequence	AS SEQ ID NO
S1-AS97	AGAUUUGUUUUUAAACUG UACAGTG	1	CACUGUACAGUUUA AAAACAAAUCUUA	97
S2-AS98	GUACAGUGUCUUUUUUUG UAUAGTT	2	AACUAUACAAAAAA AGACACUGUACAG	98
S3-AS99	ACAGUGUCUUUUUUUGUA UAGUUAA	3	UUAACUAUACAAAA AAAGACACUGUAC	99
S4-AS100	AGUGUCUUUUUUUGUAUA GUUAACA	4	UGUUAACUAUACAA AAAAAGACACUGU	100
S5-AS101	GUGUCUUUUUUUGUAUAG UUAACAC	5	GUGUUAACUAUACA AAAAAAGACACUG	101
S6-AS102	GGUGGUAUUUUCAAUAGC CACUAAC	6	GUUAGUGGCUAUU GAAAAUACCACCAG	102
S7-AS103	GUAUUUUCAAUAGCCACU AACCUTG	7	CAAGGUUAGUGGCU AUUGAAAAUACCA	103
S8-AS104	GUGCACAGCACAAAUUAG UUAUATA	8	UAUAUAACUAAUU UGUGCUGUGCACCA	104
S9-AS105	GUUGUCUCUGAUGCAGCU UAUACGA	9	UCGUAUAAGCUGCA UCAGAGACAACUG	105
S10-AS106	UCUGAUGCAGCUUAUACG AAAUAAT	10	AUUAUUUCGUAUA AGCUGCAUCAGAGA	106
S11-AS107	CUGAUGCAGCUUAUACGA AAUAATT	11	AAUUAUUUCGUAU AAGCUGCAUCAGAG	107
S12-AS108	GAUGCAGCUUAUACGAAA UAAUUGT	12	ACAAUUAUUUCGUA UAAGCUGCAUCAG	108
S13-AS109	AUGCAGCUUAUACGAAAU AAUUGTT	13	AACAAUUAUUUCGU AUAAGCUGCAUCA	109
S14-AS110	GCAGCUUAUACGAAAUAA UUGUUGT	14	ACAACAAUUAUUUC GUAUAAGCUGCAU	110
S15-AS111	CAGCUUAUACGAAAUAAU UGUUGTT	15	AACAACAAUUAUUU CGUAUAAGCUGCA	111
S16-AS112	AGCUUAUACGAAAUAAUU GUUGUTC	16	GAACAACAAUUAUU UCGUAUAAGCUGC	112
S17-AS113	GCUUAUACGAAAUAAUUG UUGUUCT	17	AGAACAACAAUUAU UUCGUAUAAGCUG	113
S18-AS114	UACGAAAUAAUUGUUGUU CUGUUAA	18	UUAACAGAACAACA AUUAUUUCGUAUA	114
S19-AS115	ACGAAAUAAUUGUUGUUC UGUUAAC	19	GUUAACAGAACAAC AAUUAUUUCGUAU	115
S20-AS116	CGAAAUAAUUGUUGUUCU GUUAACT	20	AGUUAACAGAACAA CAAUUAUUUCGUA	116
S21-AS117	GAAAUAAUUGUUGUUCUG UUAACTG	21	CAGUUAACAGAACA ACAAUUAUUUCGU	117
S22-AS118	AAAUAAUUGUUGUUCUGU UAACUGA	22	UCAGUUAACAGAAC AACAAUUAUUUCG	118
S23-AS119	AAUAAUUGUUGUUCUGUU AACUGAA	23	UUCAGUUAACAGAA CAACAAUUAUUUC	119
S24-AS120	AUAAUUGUUGUUCUGUUA ACUGAAT	24	AUUCAGUUAACAGA ACAACAAUUAUUU	120

Sense (S) – Antisense (AS) Designation	Sense Sequence/mRNA seq	S SEQ ID NO	Antisense Sequence	AS SEQ ID NO
S25-AS121	UAAUUGUUGUUCUGUUAA CUGAATA	25	UAUUCAGUUAACAG AACAACAAUUAUU	121
S26-AS122	AAUUGUUGUUCUGUUAAC UGAAUAC	26	GUAUUCAGUUAACA GAACAACAAUUAU	122
S27-AS123	AUUGUUGUUCUGUUAACU GAAUACC	27	GGUAUUCAGUUAAC AGAACAACAAUUA	123
S28-AS124	UUGUUGUUCUGUUAACUG AAUACCA	28	UGGUAUUCAGUUA ACAGAACAACAAUU	124
S29-AS125	UGUUGUUCUGUUAACUGA AUACCAC	29	GUGGUAUUCAGUU AACAGAACAACAAU	125
S30-AS126	GUUGUUCUGUUAACUGAA UACCACT	30	AGUGGUAUUCAGU UAACAGAACAACAA	126
S31-AS127	UUGUUCUGUUAACUGAAU ACCACTC	31	GAGUGGUAUUCAG UUAACAGAACAACA	127
S32-AS128	GUUCUGUUAACUGAAUAC CACUCTG	32	CAGAGUGGUAUUCA GUUAACAGAACAA	128
S33-AS129	UGUUAACUGAAUACCACU CUGUAAT	33	AUUACAGAGUGGU AUUCAGUUAACAGA	129
S34-AS130	GUUAACUGAAUACCACUC UGUAATT	34	AAUUACAGAGUGG UAUUCAGUUAACAG	130
S35-AS131	UUAACUGAAUACCACUCU GUAAUTG	35	CAAUUACAGAGUGG UAUUCAGUUAACA	131
S36-AS132	UAACUGAAUACCACUCUG UAAUUGC	36	GCAAUUACAGAGUG GUAUUCAGUUAAC	132
S37-AS133	AACUGAAUACCACUCUGU AAUUGCA	37	UGCAAUUACAGAGU GGUAUUCAGUUAA	133
S38-AS134	ACUGAAUACCACUCUGUA AUUGCAA	38	UUGCAAUUACAGAG UGGUAUUCAGUUA	134
S39-AS135	CUGAAUACCACUCUGUAA UUGCAAA	39	UUUGCAAUUACAGA GUGGUAUUCAGUU	135
S40-AS136	UGAAUACCACUCUGUAAU UGCAAAA	40	UUUUGCAAUUACAG AGUGGUAUUCAGU	136
S41-AS137	GAAUACCACUCUGUAAUU GCAAAAA	41	UUUUUGCAAUUACA GAGUGGUAUUCAG	137
S42-AS138	AAUACCACUCUGUAAUUG CAAAAAA	42	UUUUUUGCAAUUAC AGAGUGGUAUUCA	138
S43-AS139	AUACCACUCUGUAAUUGC AAAAAAA	43	UUUUUUUGCAAUU ACAGAGUGGUAUUC	139
S44-AS140	AUGCAGCUUAUACGAAGA UAAUUGT	44	ACAAUUAUCUUCGU AUAAGCUGCAUCA	140
S45-AS141	UGCAGCUUAUACGAAGAU AAUUGTT	45	AACAAUUAUCUUCG UAUAAGCUGCAUC	141
S46-AS142	GCAGCUUAUACGAAGAUA AUUGUTG	46	CAACAAUUAUCUUC GUAUAAGCUGCAU	142
S47-AS143	CAGCUUAUACGAAGAUAA UUGUUGT	47	ACAACAAUUAUCUU CGUAUAAGCUGCA	143
S48-AS144	GCUUAUACGAAGAUAAUU GUUGUTC	48	GAACAACAAUUAUC UUCGUAUAAGCUG	144
S49-AS145	CUUAUACGAAGAUAAUUG UUGUUCT	49	AGAACAACAAUUAU CUUCGUAUAAGCU	145
S50-AS146	AUACGAAGAUAAUUGUUG UUCUGTT	50	AACAGAACAACAAU UAUCUUCGUAUAA	146

Sense (S) – Antisense (AS) Designation	Sense Sequence/mRNA seq	S SEQ ID NO	Antisense Sequence	AS SEQ ID NO
S51-AS147	ACGAAGAUAAUUGUUGUU CUGUUAA	51	UUAACAGAACAACA AUUAUCUUCGUAU	147
S52-AS148	CGAAGAUAAUUGUUGUUC UGUUAAC	52	GUUAACAGAACAAC AAUUAUCUUCGUA	148
S53-AS149	GCAGCUGUUUUGUUGACA UUCUGAA	53	UUCAGAAUGUCAAC AAAACAGCUGCAA	149
S54-AS150	GCUGUUUUGUUGACAUUC UGAAUGC	54	GCAUUCAGAAUGUC AACAAAACAGCUG	150
S55-AS151	CUGUUUUGUUGACAUUCU GAAUGCT	55	AGCAUUCAGAAUGU CAACAAAACAGCU	151
S56-AS152	GUUUUGUUGACAUUCUGA AUGCUTC	56	GAAGCAUUCAGAAU GUCAACAAAACAG	152
S57-AS153	GUUGACAUUCUGAAUGCU UCUAAGT	57	ACUUAGAAGCAUUC AGAAUGUCAACAA	153
S58-AS154	GACAUUCUGAAUGCUUCU AAGUAAA	58	UUUACUUAGAAGCA UUCAGAAUGUCAA	154
S59-AS155	ACAUUCUGAAUGCUUCUA AGUAAAT	59	AUUUACUUAGAAGC AUUCAGAAUGUCA	155
S60-AS156	AUUCUGAAUGCUUCUAAG UAAAUAC	60	GUAUUUACUUAGA AGCAUUCAGAAUGU	156
S61-AS157	UUCUGAAUGCUUCUAAGU AAAUACA	61	UGUAUUUACUUAG AAGCAUUCAGAAUG	157
S62-AS158	UCUGAAUGCUUCUAAGUA AAUACAA	62	UUGUAUUUACUUA GAAGCAUUCAGAAU	158
S63-AS159	CUGAAUGCUUCUAAGUAA AUACAAT	63	AUUGUAUUUACUU AGAAGCAUUCAGAA	159
S64-AS160	UGAAUGCUUCUAAGUAAA UACAATT	64	AAUUGUAUUUACU UAGAAGCAUUCAGA	160
S65-AS161	GAAUGCUUCUAAGUAAAU ACAAUTT	65	AAAUUGUAUUUAC UUAGAAGCAUUCAG	161
S66-AS162	AAUGCUUCUAAGUAAAUA CAAUUTT	66	AAAAUUGUAUUUA CUUAGAAGCAUUCA	162
S67-AS163	AUGCUUCUAAGUAAAUAC AAUUUTT	67	AAAAAUUGUAUUU ACUUAGAAGCAUUC	163
S68-AS164	UGCUUCUAAGUAAAUACA AUUUUTT	68	AAAAAAUUGUAUU UACUUAGAAGCAUU	164
S69-AS165	GUUGUCCUUUUCAUAGGU CUGAAAT	69	AUUUCAGACCUAUG AAAAGGACAACAA	165
S70-AS166	UGAGAUAGUUUUCAUCCA UAACUGA	70	UCAGUUAUGGAUG AAAACUAUCUCAAC	166
S71-AS167	GAGAUAGUUUUCAUCCAU AACUGAA	71	UUCAGUUAUGGAU GAAAACUAUCUCAA	167
S72-AS168	ACAUUUUCAUCCAUAGUU GAAGAAT	72	AUUCUUCAACUAUG GAUGAAAAUGUUA	168
S73-AS169	CAUUUACAAACUGAAGAG UAAUCAA	73	UUGAUUACUCUUCA GUUUGUAAAUGUA	169
S74-AS170	AUUUACAAACUGAAGAGU AAUCAAT	74	AUUGAUUACUCUUC AGUUUGUAAAUGU	170
S75-AS171	ACAAACUGAAGAGUAAUC AAUCUAC	75	GUAGAUUGAUUAC UCUUCAGUUUGUAA	171
S76-AS172	GUAAUGACAGUUAUAUUU UGCAGTT	76	AACUGCAAAAUAUA ACUGUCAUUACAC	172

Sense (S) – Antisense (AS) Designation	Sense Sequence/mRNA seq	S SEQ ID NO	Antisense Sequence	AS SEQ ID NO
S77-AS173	AAUGACAGUUAUAUUUUG CAGUUTC	77	GAAACUGCAAAAUA UAACUGUCAUUAC	173
S78-AS174	AAUACAAGACUGCUGUAC UAUUUGT	78	ACAAAUAGUACAGC AGUCUUGUAUUUU	174
S79-AS175	AUACAAGACUGCUGUACU AUUUGTT	79	AACAAAUAGUACAG CAGUCUUGUAUUU	175
S80-AS176	UACAAGACUGCUGUACUA UUUGUTG	80	CAACAAAUAGUACA GCAGUCUUGUAUU	176
S81-AS177	ACAAGACUGCUGUACUAU UUGUUGA	81	UCAACAAAUAGUAC AGCAGUCUUGUAU	177
S82-AS178	CAAGACUGCUGUACUAUU UGUUGAC	82	GUCAACAAAUAGUA CAGCAGUCUUGUA	178
S83-AS179	GACUGCUGUACUAUUUGU UGACCTT	83	AAGGUCAACAAAUA GUACAGCAGUCUU	179
S84-AS180	ACUGCUGUACUAUUUGUU GACCUTA	84	UAAGGUCAACAAAU AGUACAGCAGUCU	180
S85-AS181	GUGGUUUGUCCCUUUAUG AAUCAGA	85	UCUGAUUCAUAAAG GGACAAACCACAG	181
S86-AS182	CAAUACAUUUGCUUUUUC UUUAUAA	86	UUAUAAAGAAAAA GCAAAUGUAUUGG A	182
S87-AS183	AUACAUUUGCUUUUUCUU UAUAAAA	87	UUUUAUAAAGAAA AAGCAAAUGUAUU G	183
S88-AS184	UGAUGGAGUGCUGUUAUA UAAUUTT	88	AAAAUUAUAUAAC AGCACUCCAUCACA	184
S89-AS185	GAUGGAGUGCUGUUUUGU UAUAUAA	89	UUAUAUAACAAAAC AGCACUCCAUCAC	185
S90-AS186	AUGGAGUGCUGUUUUGUU AUAUAAT	90	AUUAUAUAACAAA ACAGCACUCCAUCA	186
S91-AS187	GGAGUGCUGUUUUGUUAU AUAAUTT	91	AAAUUAUAUAACA AAACAGCACUCCAU	187
S92-AS188	GAGUGCUGUUUUGUUAUA UAAUUTA	92	UAAAUUAUAUAAC AAAACAGCACUCCA	188
S93-AS189	CUGUUUUGUUAUAUAAUU UAGACTT	93	AAGUCUAAAUUAU AUAACAAAACAGCA	189
S94-AS190	CAUUUGCGUUGUUAAUGU AAUUUGA	94	UCAAAUUACAUUAA CAACGCAAAUGUA	190
S95-AS191	AUGUAAUUUCAGGAGGAA UACUGAA	95	UUCAGUAUUCCUCC UGAAAUUACAUAA	191
S96-AS192	GAGUCCUGGAUGAUACUA AUAAACT	96	AGUUUAUUAGUAU CAUCCAGGACUCAG	192
S193-AS273	GUACAGUGUCUUUUUUUG UAGCAGCCGAAAGGCUGC	193	UACAAAAAAAGACA CUGUACGG	273
S194-AS274	ACAGUGUCUUUUUUUGUA UAGCAGCCGAAAGGCUGC	194	UAUACAAAAAAAG ACACUGUGG	274
S195-AS275	UCUGAUGCAGCUUAUACG AAGCAGCCGAAAGGCUGC	195	UUCGUAUAAGCUGC AUCAGAGA	275
S195-AS361	UCUGAUGCAGCUUAUACG AAGCAGCCGAAAGGCUGC	195	UTCGUAUAAGCUGC AUCAGAGA	361
S196-AS276	CUGAUGCAGCUUAUACGA AAGCAGCCGAAAGGCUGC	196	UUUCGUAUAAGCUG CAUCAGAG	276

Sense (S) – Antisense (AS) Designation	Sense Sequence/mRNA seq	S SEQ ID NO	Antisense Sequence	AS SEQ ID NO
S196-AS277	CUGAUGCAGCUUAUACGA AAGCAGCCGAAAGGCUGC	196	UUUCGUAUAAGCUG CAUCAGGG	277
S196-AS278	CUGAUGCAGCUUAUACGA AAGCAGCCGAAAGGCUGC	196	UUCGUAUAAGCUGC AUCAGGG	278
S196-AS362	CUGAUGCAGCUUAUACGA AAGCAGCCGAAAGGCUGC	196	UTUCGUAUAAGCTG CATCAGAG	362
S197-AS279	AUGCAGCUUAUACGAAAU AAGCAGCCGAAAGGCUGC	197	UUAUUUCGUAUAA GCUGCAUGG	279
S198-AS280	GCAGCUUAUACGAAAUAA UAGCAGCCGAAAGGCUGC	198	UAUUAUUUCGUAU AAGCUGCGG	280
S199-AS281	CAGCUUAUACGAAAUAAU UAGCAGCCGAAAGGCUGC	199	UAAUUAUUUCGUA UAAGCUGGG	281
S200-AS282	GCUUAUACGAAAUAAUUG UAGCAGCCGAAAGGCUGC	200	UACAAUUAUUUCGU AUAAGCGG	282
S201-AS283	AAAUAAUUGUUGUUCUGU UAGCAGCCGAAAGGCUGC	201	UAACAGAACAACAA UUAUUUGG	283
S202-AS284	AAUAAUUGUUGUUCUGUU AAGCAGCCGAAAGGCUGC	202	UUAACAGAACAACA AUUAUUGG	284
S203-AS285	AUUGUUGUUCUGUUAACU GAGCAGCCGAAAGGCUGC	203	UCAGUUAACAGAAC AACAAUGG	285
S204-AS286	UUGUUGUUCUGUUAACUG AAGCAGCCGAAAGGCUGC	204	UUCAGUUAACAGAA CAACAAGG	286
S205-AS287	UGUUGUUCUGUUAACUGA AAGCAGCCGAAAGGCUGC	205	UUUCAGUUAACAGA ACAACAGG	287
S206-AS288	GUUGUUCUGUUAACUGAA UAGCAGCCGAAAGGCUGC	206	UAUUCAGUUAACAG AACAACGG	288
S207-AS289	AACUGAAUACCACUCUGU AAGCAGCCGAAAGGCUGC	207	UUACAGAGUGGUA UUCAGUUGG	289
S208-AS290	CUGAAUACCACUCUGUAA UAGCAGCCGAAAGGCUGC	208	UAUUACAGAGUGG UAUUCAGGG	290
S209-AS291	AAUACCACUCUGUAAUUG CAGCAGCCGAAAGGCUGC	209	UGCAAUUACAGAGU GGUAUUGG	291
S210-AS292	AUUCUGAAUGCUUCUAAG UAGCAGCCGAAAGGCUGC	210	UACUUAGAAGCAUU CAGAAUGU	292
S210-AS293	AUUCUGAAUGCUUCUAAG UAGCAGCCGAAAGGCUGC	210	UACUUAGAAGCATU CAGAAUGT	293
S210-AS366	AUUCUGAAUGCUUCUAAG UAGCAGCCGAAAGGCUGC	210	UACUUAGAAGCAUU CAGAAUGG	366
S211-AS294	UUCUGAAUGCUUCUAAGU AAGCAGCCGAAAGGCUGC	211	UTACUUAGAAGCAU UCAGAATG	294
S211-AS295	UUCUGAAUGCUUCUAAGU AAGCAGCCGAAAGGCUGC	211	UUACUUAGAAGCAU UCAGAAUG	295
S211-AS367	UUCUGAAUGCUUCUAAGU AAGCAGCCGAAAGGCUGC	211	UUACUUAGAAGCAU UCAGAAGG	367
S212-AS296	UGAAUGCUUCUAAGUAAA UAGCAGCCGAAAGGCUGC	212	UAUUUACUUAGAA GCAUUCAGG	296
S212-AS297	UGAAUGCUUCUAAGUAAA UAGCAGCCGAAAGGCUGC	212	UAUUUACUUAGAA GCAUUCAGA	297
S212-AS298	UGAAUGCUUCUAAGUAAA UAGCAGCCGAAAGGCUGC	212	AUUUACUUAGAAGC AUUCAGG	298
S212-AS299	UGAAUGCUUCUAAGUAAA UAGCAGCCGAAAGGCUGC	212	UAUUUACUUAGAA GCATUCAGA	299

Sense (S) – Antisense (AS) Designation	Sense Sequence/mRNA seq	S SEQ ID NO	Antisense Sequence	AS SEQ ID NO
S213-AS300	GAAUGCUUCUAAGUAAAU ACGCAGCCGAAAGGCUGC	213	GUAUUUACUUAGA AGCAUUCAG	300
S213-AS301	GAAUGCUUCUAAGUAAAU ACGCAGCCGAAAGGCUGC	213	GTAUUUACUUAGAA GCAUTCAG	301
S214-AS302	AAUGCUUCUAAGUAAAUA CAGCAGCCGAAAGGCUGC	214	UGUAUUUACUUAG AAGCAUUGG	302
S215-AS303	AUGCUUCUAAGUAAAUAC AAGCAGCCGAAAGGCUGC	215	UUGUAUUUACUUA GAAGCAUGG	303
S216-AS304	UGAGAUAGUUUUCAUCCA UAGCAGCCGAAAGGCUGC	216	UAUGGAUGAAAAC UAUCUCAGG	304
S217-AS305	GAGAUAGUUUUCAUCCAU AAGCAGCCGAAAGGCUGC	217	UUAUGGAUGAAAA CUAUCUCGG	305
S218-AS306	AUUUACAAACUGAAGAGU AAGCAGCCGAAAGGCUGC	218	UUACUCUUCAGUUU GUAAAUGG	306
S219-AS307	AAUACAAGACUGCCAUAU UAGCAGCCGAAAGGCUGC	219	UAAUAUGGCAGUCU UGUAUUGG	307
S220-AS308	AUACAAGACUGCCAUAUU AAGCAGCCGAAAGGCUGC	220	UUAAUAUGGCAGUC UUGUAUGG	308
S221-AS309	UACAAGACUGCCAUAUUA AAGCAGCCGAAAGGCUGC	221	UUUAAUAUGGCAG UCUUGUAGG	309
S222-AS310	CAAGACUGCCAUAUUAAA UAGCAGCCGAAAGGCUGC	222	UAUUUAAUAUGGC AGUCUUGGG	310
S223-AS311	AAGACUGCCAUAUUAAAU UAGCAGCCGAAAGGCUGC	223	UAAUUUAAUAUGG CAGUCUUGG	311
S224-AS312	AGACUGCCAUAUUAAAUU UAGCAGCCGAAAGGCUGC	224	UAAAUUUAAUAUG GCAGUCUGG	312
S225-AS313	GACUGCCAUAUUAAAUUU UAGCAGCCGAAAGGCUGC	225	UAAAAUUUAAUAU GGCAGUCGG	313
S226-AS314	ACUGCCAUAUUAAAUUUU UAGCAGCCGAAAGGCUGC	226	UAAAAAUUUAAUA UGGCAGUGG	314
S227-AS315	AGUAUGAAUUAUUCAAUU UAGCAGCCGAAAGGCUGC	227	UAAAUUGAAUAAU UCAUACUGG	315
S228-AS316	GUAUGAAUUAUUCAAUUU AAGCAGCCGAAAGGCUGC	228	UUAAAUUGAAUAA UUCAUACGG	316
S229-AS317	GAUGGAGUGCUGUUUUGU UAGCAGCCGAAAGGCUGC	229	UAACAAAACAGCAC UCCAUCGG	317
S230-AS318	AUGGAGUGCUGUUUUGUU AAGCAGCCGAAAGGCUGC	230	UUAACAAAACAGCA CUCCAUGG	318
S231-AS319	GGAGUGCUGUUUUGUUAU AAGCAGCCGAAAGGCUGC	231	UUAUAACAAAACAG CACUCCGG	319
S232-AS320	GAGUGCUGUUUUGUUAUA UAGCAGCCGAAAGGCUGC	232	UAUAUAACAAAACA GCACUCGG	320
S233-AS321	CUGUUUUGUUAUAUAAUU UAGCAGCCGAAAGGCUGC	233	UAAAUUAUAUAAC AAAACAGGG	321
S234-AS322	UGCAGAGGUUUUAAAUAC UAGCAGCCGAAAGGCUGC	234	UAGUAUUUAAAACC UCUGCAGG	322
S235-AS323	GCAGAGGUUUUAAAUACU AAGCAGCCGAAAGGCUGC	235	UUAGUAUUUAAAA CCUCUGCGG	323
S236-AS324	CAGAGGUUUUAAAUACUA GAGCAGCCGAAAGGCUGC	236	UCUAGUAUUUAAA ACCUCUGGG	324
S237-AS325	GAGGUUUUAAAUACUAGU UAGCAGCCGAAAGGCUGC	237	UAACUAGUAUUUA AAACCUCGG	325

Sense (S) – Antisense (AS) Designation	Sense Sequence/mRNA seq	S SEQ ID NO	Antisense Sequence	AS SEQ ID NO
S238-AS326	AGAUUUUGUUACAUAUUU UAGCAGCCGAAAGGCUGC	238	UAAAAUAUGUAAC AAAAUCUGG	326
S239-AS327	AUUUUGUUACAUAUUUUU AAGCAGCCGAAAGGCUGC	239	UUAAAAAUAUGUA ACAAAAUGG	327
S240-AS328	AAAAUACUCACUUUAUGC UAGCAGCCGAAAGGCUGC	240	UAGCAUAAAGUGA GUAUUUUGG	328
S241-AS329	AAAUACUCACUUUAUGCU UAGCAGCCGAAAGGCUGC	241	UAAGCAUAAAGUG AGUAUUUGG	329
S242-AS330	AAUACUCACUUUAUGCUU AAGCAGCCGAAAGGCUGC	242	UUAAGCAUAAAGU GAGUAUUGG	330
S243-AS331	AUAAAAGGUUUUGUCAAA CAGCAGCCGAAAGGCUGC	243	UGUUUGACAAAACC UUUUAUGG	331
S244-AS332	AGGUUUUGUCAAACAUUG CAGCAGCCGAAAGGCUGC	244	UGCAAUGUUUGACA AAACCUGG	332
S245-AS333	GGUUUUGUCAAACAUUGC AAGCAGCCGAAAGGCUGC	245	UUGCAAUGUUUGAC AAAACCGG	333
S246-AS334	GCAAGUAUUCGGUGCUAU AAGCAGCCGAAAGGCUGC	246	UUAUAGCACCGAAU ACUUGCGG	334
S247-AS335	AGAUGGAAGUUUCUACUG UAGCAGCCGAAAGGCUGC	247	UACAGUAGAAACUU CCAUCUGG	335
S248-AS336	AUGGAAGUUUCUACUGUA UAGCAGCCGAAAGGCUGC	248	UAUACAGUAGAAAC UUCCAUGG	336
S249-AS337	GGAAGUUUCUACUGUAUA GAGCAGCCGAAAGGCUGC	249	UCUAUACAGUAGAA ACUUCCGG	337
S250-AS338	GAAGUUUCUACUGUAUAG AAGCAGCCGAAAGGCUGC	250	UUCUAUACAGUAGA AACUUCGG	338
S251-AS339	AAGUUUCUACUGUAUAGA AAGCAGCCGAAAGGCUGC	251	UUUCUAUACAGUAG AAACUUGG	339
S252-AS340	AGUUUCUACUGUAUAGAA AAGCAGCCGAAAGGCUGC	252	UUUUCUAUACAGUA GAAACUGG	340
S253-AS341	CUACUGUAUAGAAAUCAC CAGCAGCCGAAAGGCUGC	253	UGGUGAUUUCUAU ACAGUAGGG	341
S254-AS342	CUGUAUAGAAAUCACCAU UAGCAGCCGAAAGGCUGC	254	UAAUGGUGAUUUC UAUACAGGG	342
S255-AS343	GUCAUGACAACUACCAUU UAGCAGCCGAAAGGCUGC	255	UAAAUGGUAGUUG UCAUGACGG	343
S256-AS344	GACAACUACCAUUUUUUU AAGCAGCCGAAAGGCUGC	256	UUAAAAAAAUGGU AGUUGUCGG	344
S257-AS345	AGUUGGAUGUCUAAAACU CAGCAGCCGAAAGGCUGC	257	UGAGUUUUAGACA UCCAACUGG	345
S258-AS346	GUUGGAUGUCUAAAACUC AAGCAGCCGAAAGGCUGC	258	UUGAGUUUUAGAC AUCCAACGG	346
S259-AS347	GAUGUCUAAAACUCAAGU AAGCAGCCGAAAGGCUGC	259	UUACUUGAGUUUU AGACAUCGG	347
S260-AS348	GAUAAGUGUAAAGCCUUG UAGCAGCCGAAAGGCUGC	260	UACAAGGCUUUACA CUUAUCGG	348
S261-AS349	AUAAGUGUAAAGCCUUGU AAGCAGCCGAAAGGCUGC	261	UUACAAGGCUUUAC ACUUAUGG	349
S262-AS350	GCCUUGUAACUGAAGAUG AAGCAGCCGAAAGGCUGC	262	UUCAUCUUCAGUUA CAAGGCGG	350
S263-AS351	GUGUAUAGAAACUAUUUU AAGCAGCCGAAAGGCUGC	263	UUAAAAUAGUUUC UAUACACGG	351

Sense (S) – Antisense (AS) Designation	Sense Sequence/mRNA seq	S SEQ ID NO	Antisense Sequence	AS SEQ ID NO
S264-AS352	AAAGACUUUGUUGACAUC AAGCAGCCGAAAGGCUGC	264	UUGAUGUCAACAAA GUCUUUGG	352
S265-AS353	GAAGAUGCUUUUUAAAAC UAGCAGCCGAAAGGCUGC	265	UAGUUUUAAAAAG CAUCUUCGG	353
S266-AS354	AAGAUGCUUUUUAAAACU AAGCAGCCGAAAGGCUGC	266	UUAGUUUUAAAAA GCAUCUUGG	354
S267-AS355	GAGCUAUUGCUGAUUAGU UAGCAGCCGAAAGGCUGC	267	UAACUAAUCAGCAA UAGCUCGG	355
S268-AS356	GUGUUGUUAAUGUUUGCU GAGCAGCCGAAAGGCUGC	268	UCAGCAAACAUUAA CAACACGG	356
S269-AS357	GUUGUUAAUGUUUGCUGU AAGCAGCCGAAAGGCUGC	269	UUACAGCAAACAUU AACAACGG	357
S270-AS358	UGUUAAUGUUUGCUGUAU UAGCAGCCGAAAGGCUGC	270	UAAUACAGCAAACA UUAACAGG	358
S271-AS359	GUUAAUGUUUGCUGUAUU UAGCAGCCGAAAGGCUGC	271	UAAAUACAGCAAAC AUUAACGG	359
S272-AS360	UUAAUGUUUGCUGUAUUU AAGCAGCCGAAAGGCUGC	272	UUAAAUACAGCAAA CAUUAAGG	360
S363-AS368	UCUGAAUGCUUCUAAGUA AAGCAGCCGAAAGGCUGC	363	UUUACUUAGAAGCA UUCAGAGG	368
S364-AS369	CUGAAUGCUUCUAAGUAA AAGCAGCCGAAAGGCUGC	364	UUUUACUUAGAAGC AUUCAGGG	369
S365-AS370	GAAUGCUUCUAAGUAAAU AAGCAGCCGAAAGGCUGC	365	UUAUUUACUUAGA AGCAUUCGG	370

[000210] The disclosure illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that are not specifically disclosed herein. Thus, for example, in each instance herein any of the terms “comprising”, “consisting essentially of”, and “consisting of” may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

[000211] In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that

the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

[000212] The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (*i.e.*, meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[000213] Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description.

[000214] The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

What is claimed is:

1. An oligonucleotide for reducing expression of HMGB1, the oligonucleotide comprising an antisense strand of 15 to 30 nucleotides in length, wherein the antisense strand has a region of complementarity to HMGB1 that is complementary to at least 15 contiguous nucleotides of a sequence as set forth in any one of SEQ ID NO: 374-381, 193-272, and 363-365.

2. The oligonucleotide of claim 1, wherein the antisense strand is 19 to 27 nucleotides in length.

3. The oligonucleotide of claim 1, wherein the antisense strand is 21 to 27 nucleotides in length.

4. The oligonucleotide of any one of claims 1 to 3, wherein the region of complementarity is complementary to at least 15 contiguous nucleotides of a sequence as set forth in any one of SEQ ID NO: 193-272 and 363-365.

5. The oligonucleotide of any one of claims 1 to 4, further comprising a sense strand of 15 to 50 nucleotides in length, wherein the sense strand forms a duplex region with the antisense strand.

6. The oligonucleotide of claim 5, wherein the sense strand is 19 to 50 nucleotides in length.

7. The oligonucleotide of claim 5 or 6, wherein the duplex region is at least 19 nucleotides in length.

8. The oligonucleotide of any one of claims 1 to 7, wherein the region of complementarity with HMGB1 is complementary to at least 19 contiguous nucleotides of a sequence as set forth in SEQ ID NO:374-381.

9. The oligonucleotide of any one of claims 1 to 8, wherein the region of complementarity with HMGB1 is complementary to at least 19 contiguous nucleotides of a sequence as set forth in any one of SEQ ID NO: 193-272 and 363-365.

10. The oligonucleotide of any one of claims 5 to 9, wherein the sense strand comprises a sequence as set forth in any one of SEQ ID NOs: 193-272 or 363-365.

11. The oligonucleotide of any one of claims 5 to 10, wherein the sense strand comprises a sequence as set forth in SEQ ID NO: 204, 211, 364, or 365.

12. The oligonucleotide of any one of claims 10, wherein the antisense strand comprises a sequence as set forth in any one of SEQ ID NOs: 273-362 or 366-370.

13. The oligonucleotide of any one of claims 11, wherein the antisense strand comprises a sequence as set forth in any one of SEQ ID NO: 286, 367, 369 or 370.

14. The oligonucleotide of any one of claims 5 to 13, wherein the sense strand comprises at its 3'-end a stem-loop set forth as: S_1-L-S_2, wherein S_1 is complementary to S_2, and wherein L forms a loop between S_1 and S_2 of 3 to 5 nucleotides in length.

15. An oligonucleotide for reducing expression of HMGB1, the oligonucleotide comprising an antisense strand and a sense strand,

wherein the antisense strand is 21 to 27 nucleotides in length and has a region of complementarity with HMGB1,

wherein the sense strand comprises at its 3'-end a stem-loop set forth as: S_1-L-S_2, wherein S_1 is complementary to S_2, and wherein L forms a loop between S_1 and S_2 of 3 to 5 nucleotides in length,

and wherein the antisense strand and the sense strand form a duplex structure of at least 19 nucleotides in length but are not covalently linked.

16. The oligonucleotide of claim 15, wherein the region of complementarity is complementary to at least 19 contiguous nucleotides of HMGB1 mRNA.

17. The oligonucleotide of any one of claims 14 to 16, wherein L is a tetraloop.

18. The oligonucleotide of any one of claims 14 to 17, wherein L is 4 nucleotides in length.

19. The oligonucleotide of any one of claims 14 to 18, wherein L comprises a sequence set forth as GAAA.

20. The oligonucleotide of any one of claims 5 to 13, wherein the antisense strand is 27 nucleotides in length and the sense strand is 25 nucleotides in length.

21. The oligonucleotide of claim 20, wherein the antisense strand and sense strand form a duplex region of 25 nucleotides in length.

22. The oligonucleotide of claim 21, further comprising a 3'-overhang sequence on the antisense strand of two nucleotides in length.

23. The oligonucleotide of any one of claims 5 to 13, wherein the oligonucleotide comprises an antisense strand and a sense strand that are each in a range of 21 to 23 nucleotides in length.

24. The oligonucleotide of claim 23, wherein the oligonucleotide comprises a duplex structure in a range of 19 to 21 nucleotides in length.

25. The oligonucleotide of claim 23 or 24, wherein the oligonucleotide comprises a 3'-overhang sequence of one or more nucleotides in length, wherein the 3'-overhang sequence is present on the antisense strand, the sense strand, or the antisense strand and sense strand.

26. The oligonucleotide of any one of the preceding claims, wherein the oligonucleotide comprises at least one modified nucleotide.

27. The oligonucleotide of claim 26, wherein the modified nucleotide comprises a 2'-modification.

28. The oligonucleotide of claim 27, wherein the 2'-modification is a modification selected from: 2'-aminoethyl, 2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl, and 2'-deoxy-2'-fluoro-β-d-arabinonucleic acid.

29. The oligonucleotide of any one of claims 26 to 28, wherein all of the nucleotides of the oligonucleotide are modified.

30. The oligonucleotide of any one of the preceding claims, wherein the oligonucleotide comprises at least one modified internucleotide linkage.

31. The oligonucleotide of claim 30, wherein the at least one modified internucleotide linkage is a phosphorothioate linkage.

32. The oligonucleotide of any one of the preceding claims, wherein the 4'-carbon of the sugar of the 5'-nucleotide of the antisense strand comprises a phosphate analog.

33. The oligonucleotide of claim 36, wherein the phosphate analog is oxymethylphosphonate, vinylphosphonate, or malonylphosphonate.

34. The oligonucleotide of any one of the preceding claims, wherein at least one nucleotide of the oligonucleotide is conjugated to one or more targeting ligands.

35. The oligonucleotide of claim 34, wherein each targeting ligand comprises a carbohydrate, amino sugar, cholesterol, polypeptide or lipid.

36. The oligonucleotide of claim 35, wherein each targeting ligand comprises a N-acetylgalactosamine (GalNAc) moiety.

37. The oligonucleotide of claim 36, wherein the GalNac moiety is a monovalent GalNAc moiety, a bivalent GalNAc moiety, a trivalent GalNAc moiety, or a tetravalent GalNAc moiety.

38. The oligonucleotide of any one of claims 14 to 17, wherein up to 4 nucleotides of L of the stem-loop are each conjugated to a monovalent GalNAc moiety.

39. The oligonucleotide of claim 34, wherein the targeting ligand comprises an aptamer.

40. A composition comprising an oligonucleotide of any one of the preceding claims and an excipient.

41. A method of delivering an oligonucleotide to a subject, the method comprising administering the composition of claim 40 to the subject.

42. The method of claim 41, wherein the subject has or is at risk of having liver fibrosis.

43. The method of any one of claims 41-42, wherein expression of HMGB1 protein is reduced by administering to the subject the oligonucleotide.

44. The method of claim 43, wherein the subject has cholestatic or autoimmune liver disease.

45. A method of treating a subject having or at risk of having liver fibrosis, the method comprising administering to the subject an oligonucleotide that reduces expression of HMGB1.

46. The method of claim 45, wherein the subject has cholestatic or autoimmune liver disease.

47. The method of claim 45, wherein the subject has nonalcoholic steatohepatitis (NASH).

48. The method of claim 45, wherein the oligonucleotide is administered prior to exposure of the subject to a hepatotoxic agent.

49. The method of claim 45, wherein the oligonucleotide is administered subsequent to exposure of the subject to a hepatotoxic agent.

50. The method of claim 45, wherein the oligonucleotide is administered simultaneously with the subject's exposure to a hepatotoxic agent.

51. A method of treating a subject having or at risk of having nonalcoholic steatohepatitis (NASH), the method comprising administering to the subject an RNAi oligonucleotide that reduces expression of HMGB1 in the subject.

52. The method of any one of claims 41-51, wherein the administration results in a reduction in liver HMGB1 levels.

53. The method of any one of claims 41-51, wherein the administration results in a reduction in serum HMGB1 levels.

54. An oligonucleotide for reducing expression of HMGB1, the oligonucleotide comprising a sense strand of 15 to 50 nucleotides in length and an antisense strand of 15 to 30 nucleotides in length, wherein the sense strand forms a duplex region with the antisense strand, wherein the sense strand comprises a sequence as set forth in any one of SEQ ID NO: 1-96, and wherein the antisense strand comprises a complementary sequence selected from SEQ ID NO: 97-192.

55. The oligonucleotide of claim 54, wherein the sense strand consists of a sequence as set forth in any one of SEQ ID NO: 1-96,

56. The oligonucleotide of claim 54 or 55, wherein the antisense strand consists of a complementary sequence selected from SEQ ID NO: 97-192.

57. An oligonucleotide for reducing expression of HMGB1, the oligonucleotide comprising a sense strand of 15 to 50 nucleotides in length and an antisense strand of 15 to 30 nucleotides in length, wherein the sense strand forms a duplex region with the antisense strand, wherein the sense strand comprises a sequence as set forth in any one of SEQ ID NO: 193-272 or 363-365 and wherein the antisense strand comprises a complementary sequence selected from SEQ ID NO: 273-362 or 366-370.

58. The oligonucleotide of claim 57, wherein the sense strand consists of a sequence as set forth in any one of SEQ ID NO: 193-272 or 363-365.

59. The oligonucleotide of claim 57 or 58, wherein the antisense strand consists of a complementary sequence selected from SEQ ID NO: 273-362 or 366-370.

60. An oligonucleotide for reducing expression of HMGB1, the oligonucleotide comprising a sense strand of 15 to 50 nucleotides in length and an antisense strand of 15 to 30 nucleotides in length, wherein the sense strand forms a duplex region with the antisense strand, wherein the sense strand comprises a sequence as set forth in any one of SEQ ID NO: 204, 211, 364, 365 and wherein the antisense strand comprises a complementary sequence selected from SEQ ID NO: 286, 367, 369, 370.

61. The oligonucleotide of claim 60, wherein the sense strand comprises a sequence as set forth in SEQ ID NO: 204, and wherein the antisense strand comprises a sequences as set for in SEQ ID NO: 286.

62. The oligonucleotide of claim 60, wherein the sense strand comprises a sequence as set forth in SEQ ID NO: 211, and wherein the antisense strand comprises a sequences as set for in SEQ ID NO: 367.

63. The oligonucleotide of claim 60, wherein the sense strand comprises a sequence as set forth in SEQ ID NO: 364, and wherein the antisense strand comprises a sequences as set for in SEQ ID NO: 369.

64. The oligonucleotide of claim 60, wherein the sense strand comprises a sequence as set forth in SEQ ID NO: 365, and wherein the antisense strand comprises a sequences as set for in SEQ ID NO: 370.

65. The oligonucleotide of claim 60, wherein the sense strand consists of a sequence as set forth in SEQ ID NO: 204, and wherein the antisense strand consists of a sequences as set for in SEQ ID NO: 286.

66. The oligonucleotide of claim 60, wherein the sense strand consists of a sequence as set forth in SEQ ID NO: 211, and wherein the antisense strand consists of a sequences as set for in SEQ ID NO: 367.

67. The oligonucleotide of claim 60, wherein the sense strand consists of a sequence as set forth in SEQ ID NO: 364, and wherein the antisense strand consists of a sequences as set for in SEQ ID NO: 369.

68. The oligonucleotide of claim 60, wherein the sense strand consists of a sequence as set forth in SEQ ID NO: 365, and wherein the antisense strand consists of a sequences as set for in SEQ ID NO: 370.

69. An oligonucleotide for reducing expression of HMGB1, the oligonucleotide comprising a pair of sense and antisense strands selected from a row of Table 7.

70. The oligonucleotide of any one of claims 54-69, wherein the oligonucleotide comprises at least one modified nucleotide.

71. The oligonucleotide of claim 70, wherein the modified nucleotide comprises a 2'-modification.

72. The oligonucleotide of claim 71, wherein the 2'-modification is a modification selected from: 2'-aminoethyl, 2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl, and 2'-deoxy-2'-fluoro-β-d-arabinonucleic acid.

73. The oligonucleotide of any one of claims 70-72, wherein all nucleotides in the oligonucleotide are modified.

74. The oligonucleotide of any one of claims 54-72, wherein the oligonucleotide comprises at least one modified internucleotide linkage.

75. The oligonucleotide of claim 74, wherein the at least one modified internucleotide linkage is a phosphorothioate linkage.

76. The oligonucleotide of any one of any one of claims 54-75, wherein the 4'-carbon of the sugar of the 5'-nucleotide of the antisense strand comprises a phosphate analog.

77. The oligonucleotide of claim 76, wherein the phosphate analog is oxymethylphosphonate, vinylphosphonate, or malonylphosphonate.

78. The oligonucleotide of any one of claims 54-77, wherein at least one nucleotide of the oligonucleotide is conjugated to one or more targeting ligands.

79. The oligonucleotide of claim 78, wherein each targeting ligand comprises a carbohydrate, amino sugar, cholesterol, polypeptide or lipid.

80. The oligonucleotide of claim 79, wherein each targeting ligand comprises a N-acetylgalactosamine (GalNAc) moiety.

81. The oligonucleotide of claim 80, wherein the GalNac moiety is a monovalent GalNAc moiety, a bivalent GalNAc moiety, a trivalent GalNAc moiety, or a tetravalent GalNAc moiety.

82. The oligonucleotide of claim 80 or claim 81, wherein up to 4 nucleotides of L of the stem-loop are each conjugated to a monovalent GalNAc moiety.

FIG. 1

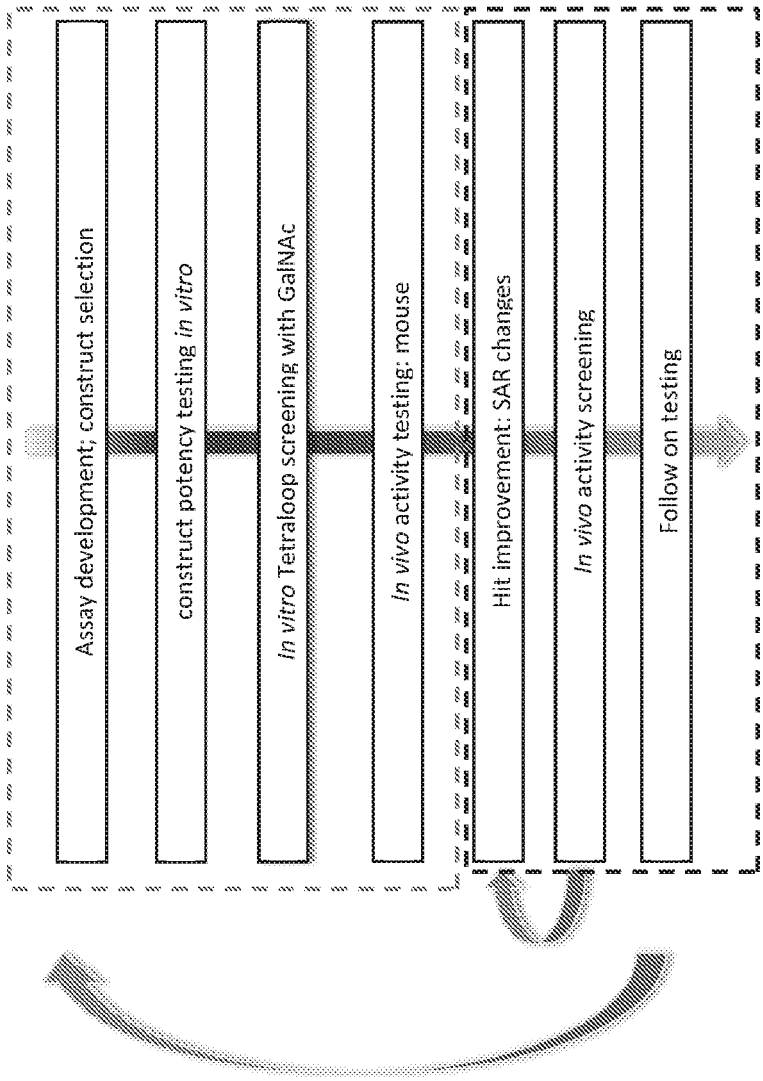

Assay development; construct selection
construct potency testing *in vitro*
In vitro Tetraloop screening with GalNAc
In vivo activity testing: mouse
Hit improvement: SAR changes
In vivo activity screening
Follow on testing

FIG. 2

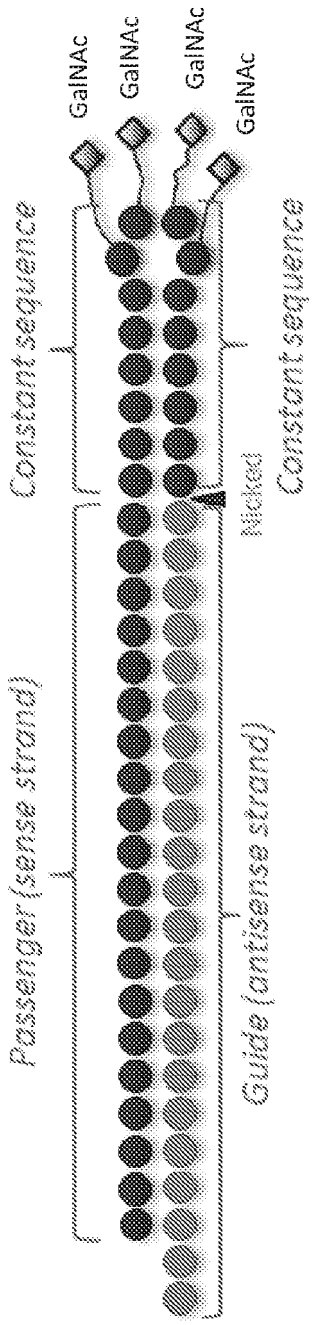

GalNAc
GalNAc
GalNAc
GalNAc
Constant sequence
Nicked
Constant sequence
Passenger (sense strand)
Guide (antisense strand)

FIG. 3A

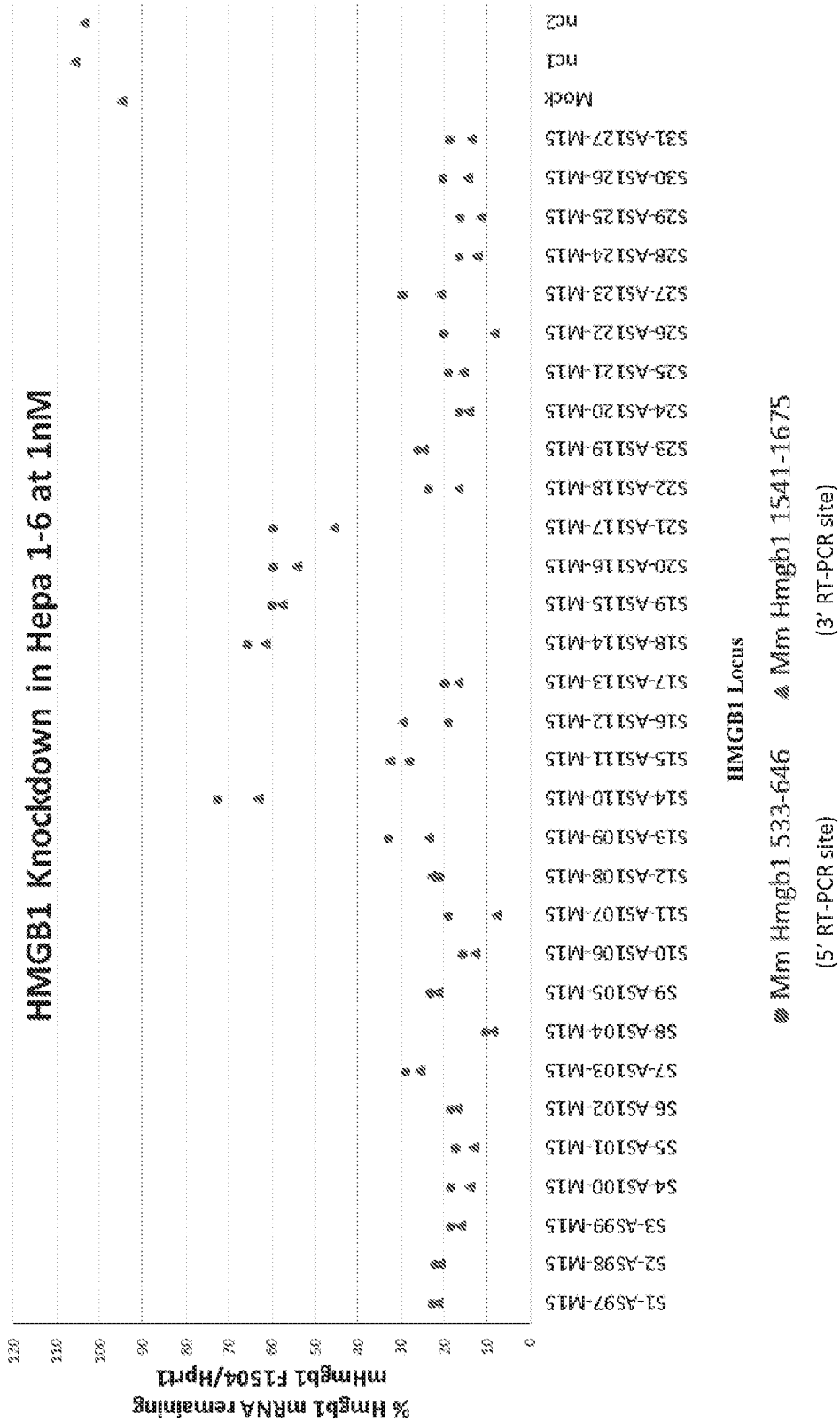

HMGB1 Knockdown in Hepa 1-6 at 1nM
% Hmgb1 mRNA remaining
mHmgb1 F1504/Hprt1
120
110
100
90
80
70
60
50
40
30
20
10
0
S1-AS97-M15
S2-AS98-M15
S3-AS99-M15
S4-AS100-M15
S5-AS101-M15
S6-AS102-M15
S7-AS103-M15
S8-AS104-M15
S9-AS105-M15
S10-AS106-M15
S11-AS107-M15
S12-AS108-M15
S13-AS109-M15
S14-AS110-M15
S15-AS111-M15
S16-AS112-M15
S17-AS113-M15
S18-AS114-M15
S19-AS115-M15
S20-AS116-M15
S21-AS117-M15
S22-AS118-M15
S23-AS119-M15
S24-AS120-M15
S25-AS121-M15
S26-AS122-M15
S27-AS123-M15
S28-AS124-M15
S29-AS125-M15
S30-AS126-M15
S31-AS127-M15
Mock
nc1
nc2
HMGB1 Locus
Mm Hmgb1 533-646
(5' RT-PCR site)
Mm Hmgb1 1541-1675
(3' RT-PCR site)

FIG. 3B

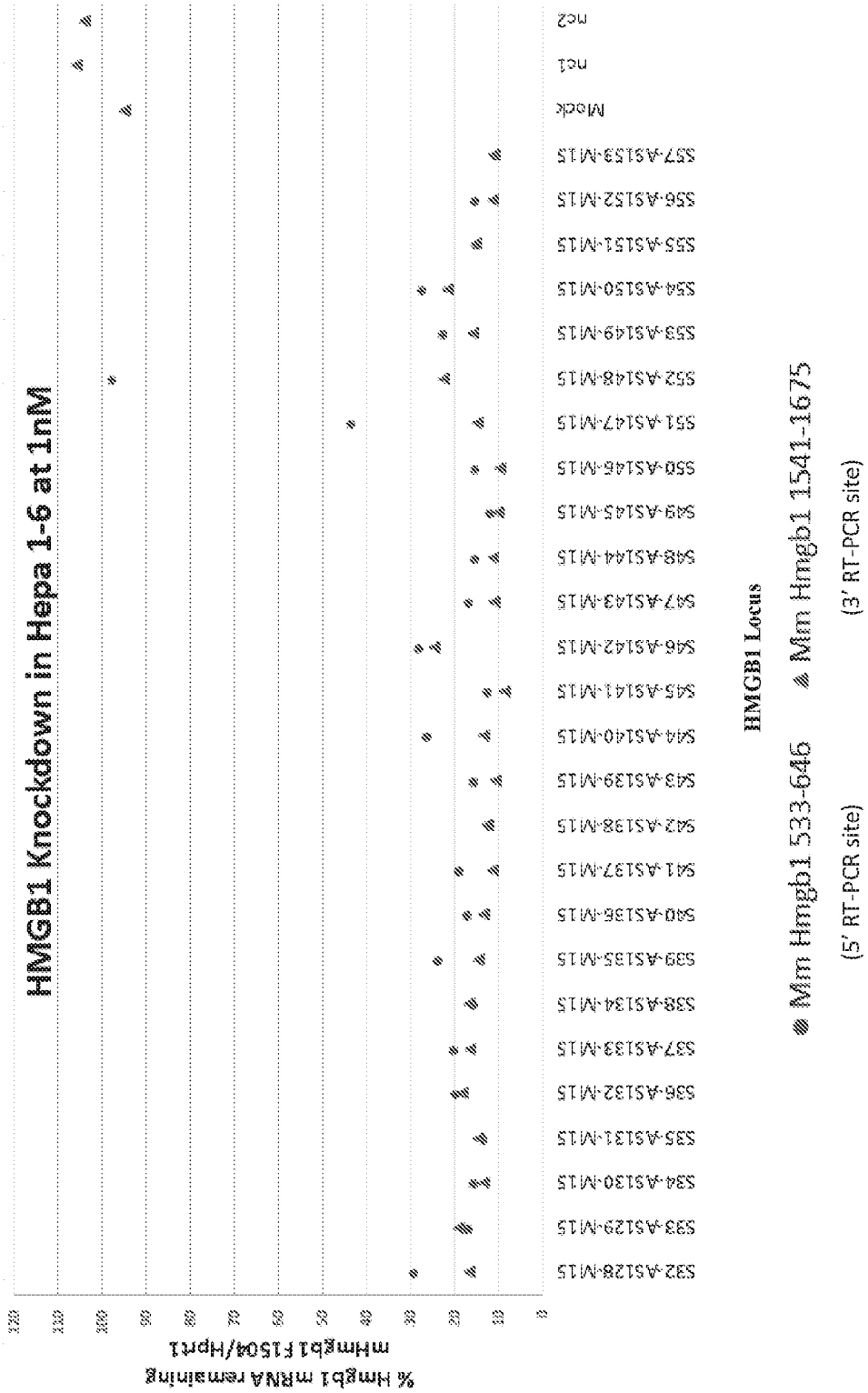

HMGB1 Knockdown in Hepa 1-6 at 1nM
% Hmgb1 mRNA remaining
mHmgb1 F1504/Hprt1
120
110
100
90
80
70
60
50
40
30
20
10
0
S32-AS128-M1S
S33-AS129-M1S
S34-AS130-M1S
S35-AS131-M1S
S36-AS132-M1S
S37-AS133-M1S
S38-AS134-M1S
S39-AS135-M1S
S40-AS136-M1S
S41-AS137-M1S
S42-AS138-M1S
S43-AS139-M1S
S44-AS140-M1S
S45-AS141-M1S
S46-AS142-M1S
S47-AS143-M1S
S48-AS144-M1S
S49-AS145-M1S
S50-AS146-M1S
S51-AS147-M1S
S52-AS148-M1S
S53-AS149-M1S
S54-AS150-M1S
S55-AS151-M1S
S56-AS152-M1S
S57-AS153-M1S
Mock
nc1
nc2
HMGB1 Locus
Mm Hmgb1 533-646
(5' RT-PCR site)
Mm Hmgb1 1541-1675
(3' RT-PCR site)

FIG. 3C

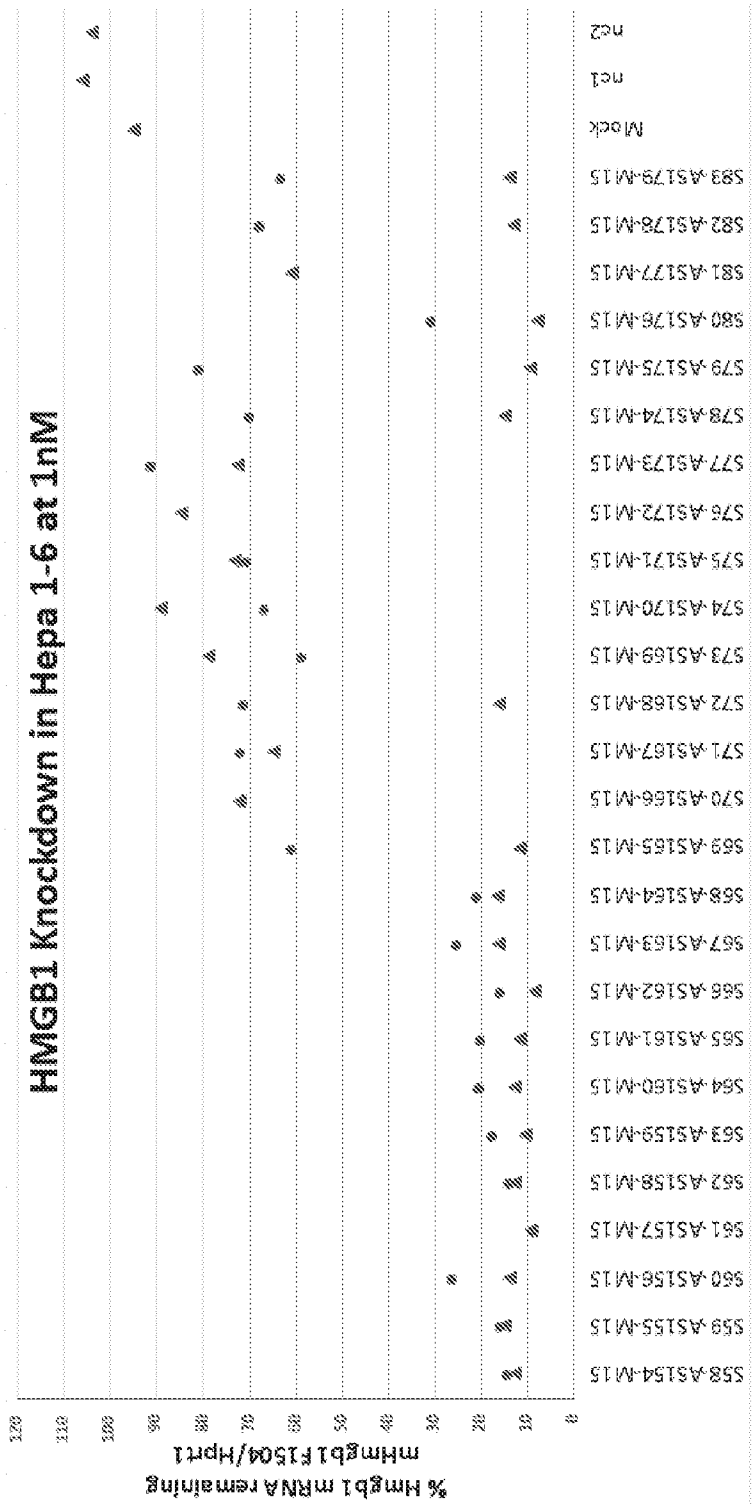

HMGB1 Knockdown in Hepa 1-6 at 1nM
% Hmgb1 mRNA remaining
mHmgb1 F1504/Hprt1
120
110
100
90
80
70
60
50
40
30
20
10
0
S58.AS154-M15
S59.AS155-M15
S60.AS156-M15
S61.AS157-M15
S62.AS158-M15
S63.AS159-M15
S64.AS160-M15
S65.AS161-M15
S66.AS162-M15
S67.AS163-M15
S68.AS164-M15
S69.AS165-M15
S70.AS166-M15
S71.AS167-M15
S72.AS168-M15
S73.AS169-M15
S74.AS170-M15
S75.AS171-M15
S76.AS172-M15
S77.AS173-M15
S78.AS174-M15
S79.AS175-M15
S80.AS176-M15
S81.AS177-M15
S82.AS178-M15
S83.AS179-M15
Mock
mc1
nc2
HMGB1 Locus
Mm Hmgb1 533-646
(5' RT-PCR site)
Mm Hmgb1 1541-1675
(3' RT-PCR site)

FIG. 3D

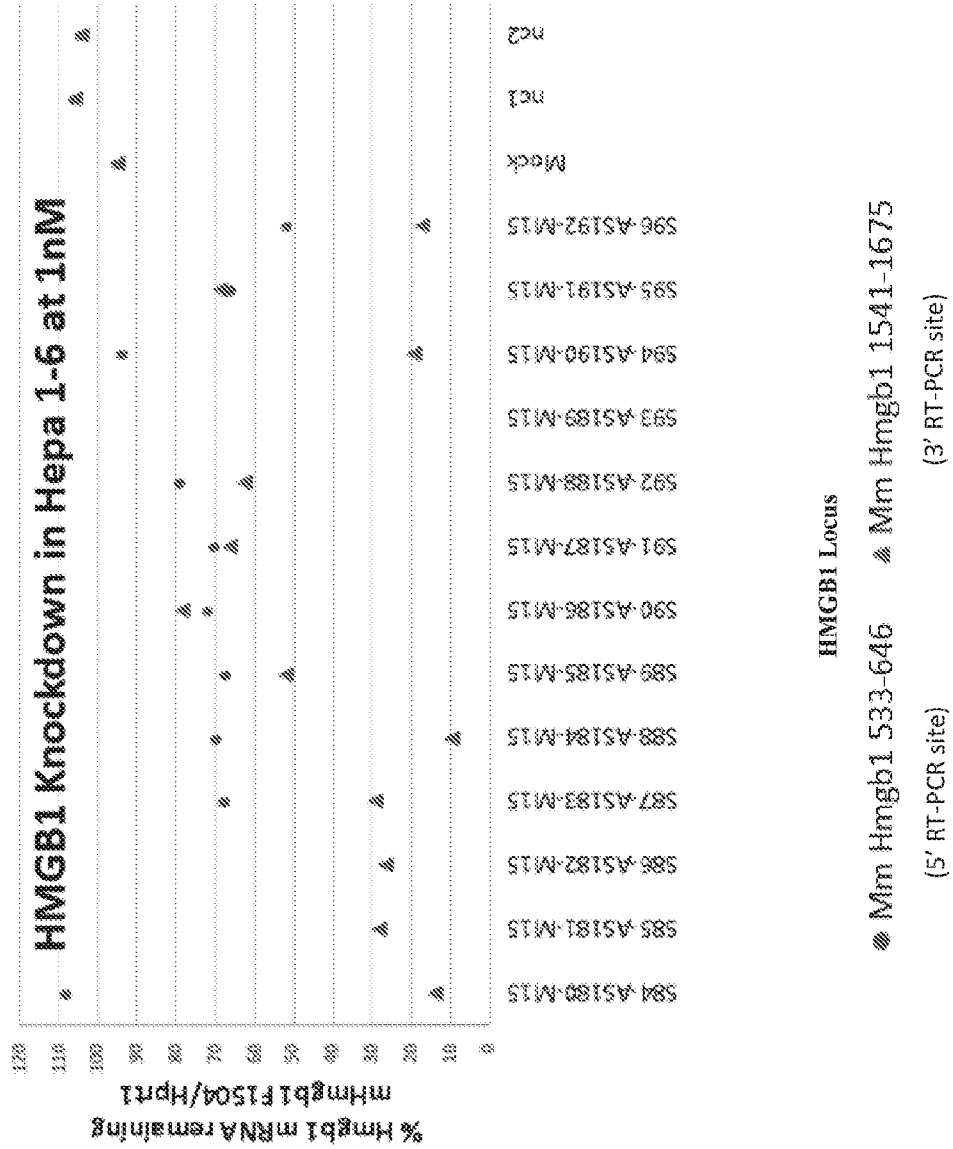

HMGB1 Knockdown in Hepa 1-6 at 1nM
% Hmgb1 mRNA remaining
mHmgb1 F150A/Hprt1
120
110
100
90
80
70
60
50
40
30
20
10
0
S84-AS180-M1S
S85-AS181-M1S
S86-AS182-M1S
S87-AS183-M1S
S88-AS184-M1S
S89-AS185-M1S
S90-AS186-M1S
S91-AS187-M1S
S92-AS188-M1S
S93-AS189-M1S
S94-AS190-M1S
S95-AS191-M1S
S96-AS192-M1S
Mock
nc1
nc2
HMGB1 Locus
Mm Hmgb1 533-646
(5' RT-PCR site)
Mm Hmgb1 1541-1675
(3' RT-PCR site)

FIG. 4

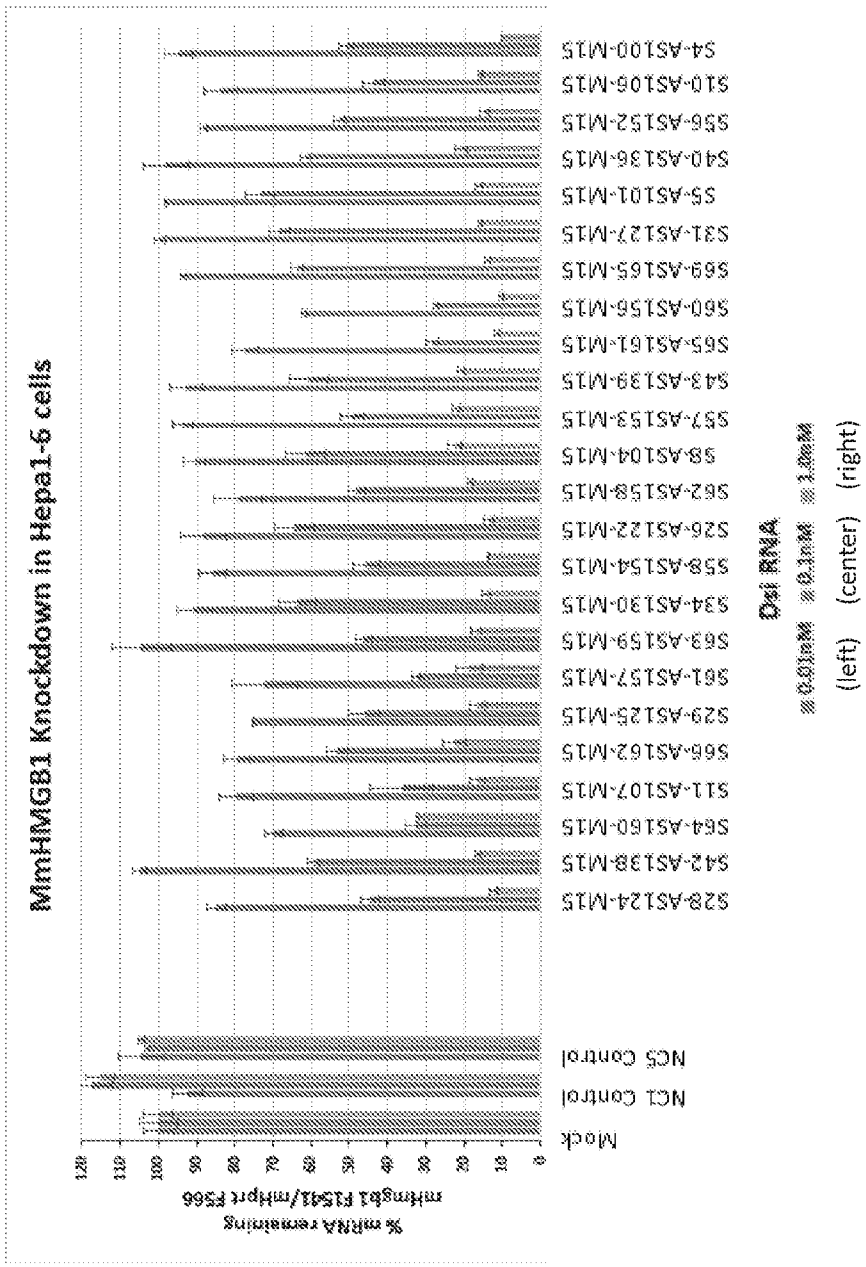

MmHMGB1 Knockdown in Hepa1-6 cells
% mRNA remaining
mHmgb1 F1541/mHprt F566
0
10
20
30
40
50
60
70
80
90
100
110
120
Mock
NC1 Control
NC5 Control
S28-AS124-M15
S42-AS138-M15
S64-AS160-M15
S11-AS107-M15
S66-AS162-M15
S29-AS125-M15
S61-AS157-M15
S63-AS159-M15
S34-AS130-M15
S58-AS154-M15
S26-AS122-M15
S62-AS158-M15
S8-AS104-M15
S57-AS153-M15
S43-AS139-M15
S65-AS161-M15
S60-AS156-M15
S69-AS165-M15
S31-AS127-M15
S5-AS101-M15
S40-AS136-M15
S56-AS152-M15
S10-AS106-M15
S4-AS100-M15
Osi RNA
0.01nM
0.1nM
1.0nM
(left) (center) (right)

FIG. 5

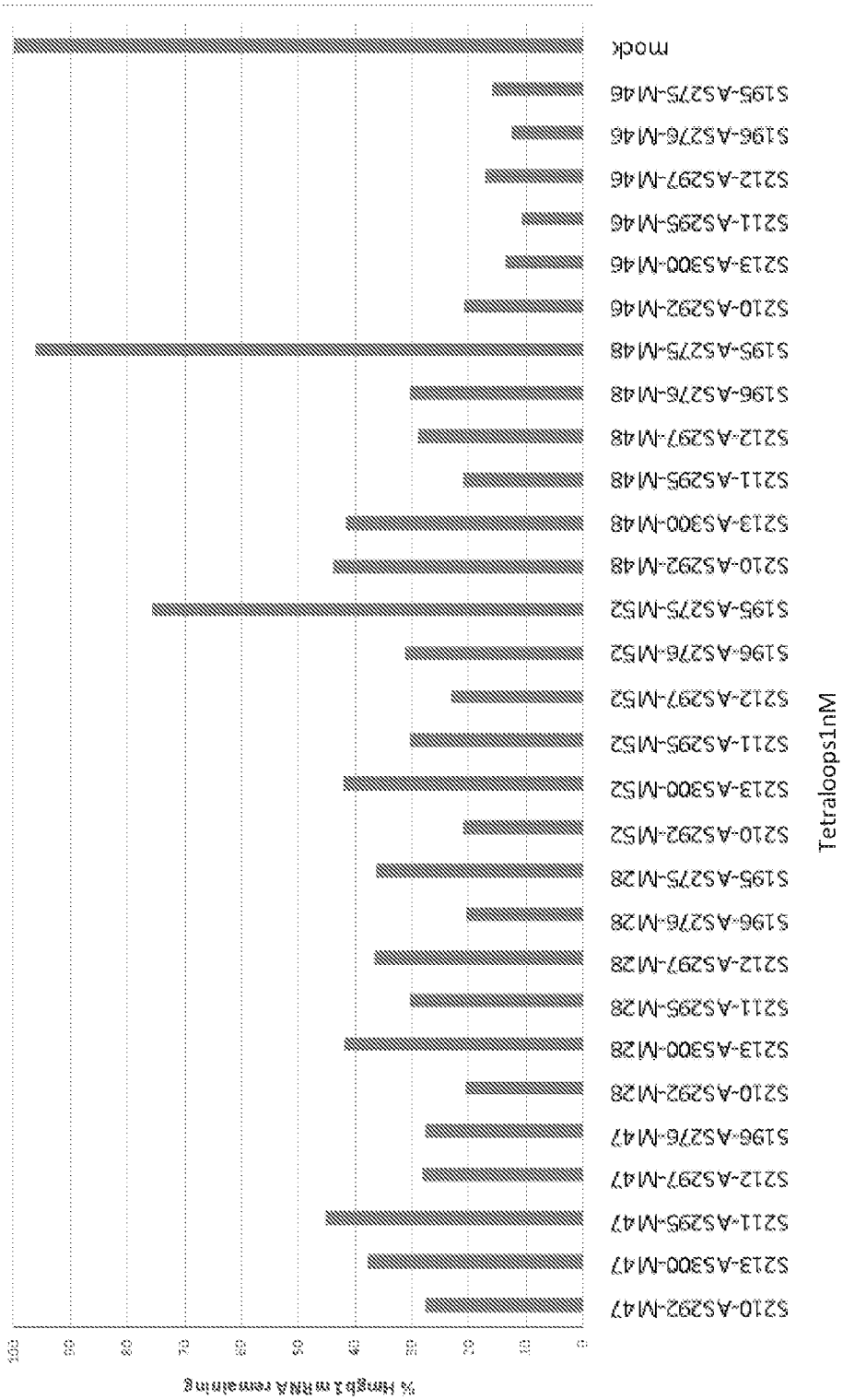

% Hmgb1 mRNA remaining
0
10
20
30
40
50
60
70
80
90
100
Tetraloops1nM
S210-AS292-M47
S213-AS300-M47
S211-AS295-M47
S212-AS297-M47
S196-AS276-M47
S210-AS292-M28
S213-AS300-M28
S211-AS295-M28
S212-AS297-M28
S196-AS276-M28
S195-AS275-M28
S210-AS292-M52
S213-AS300-M52
S211-AS295-M52
S212-AS297-M52
S196-AS276-M52
S195-AS275-M52
S210-AS292-M48
S213-AS300-M48
S211-AS295-M48
S212-AS297-M48
S196-AS276-M48
S195-AS275-M48
S210-AS292-M46
S213-AS300-M46
S211-AS295-M46
S212-AS297-M46
S196-AS276-M46
S195-AS275-M46
mock

FIG. 6

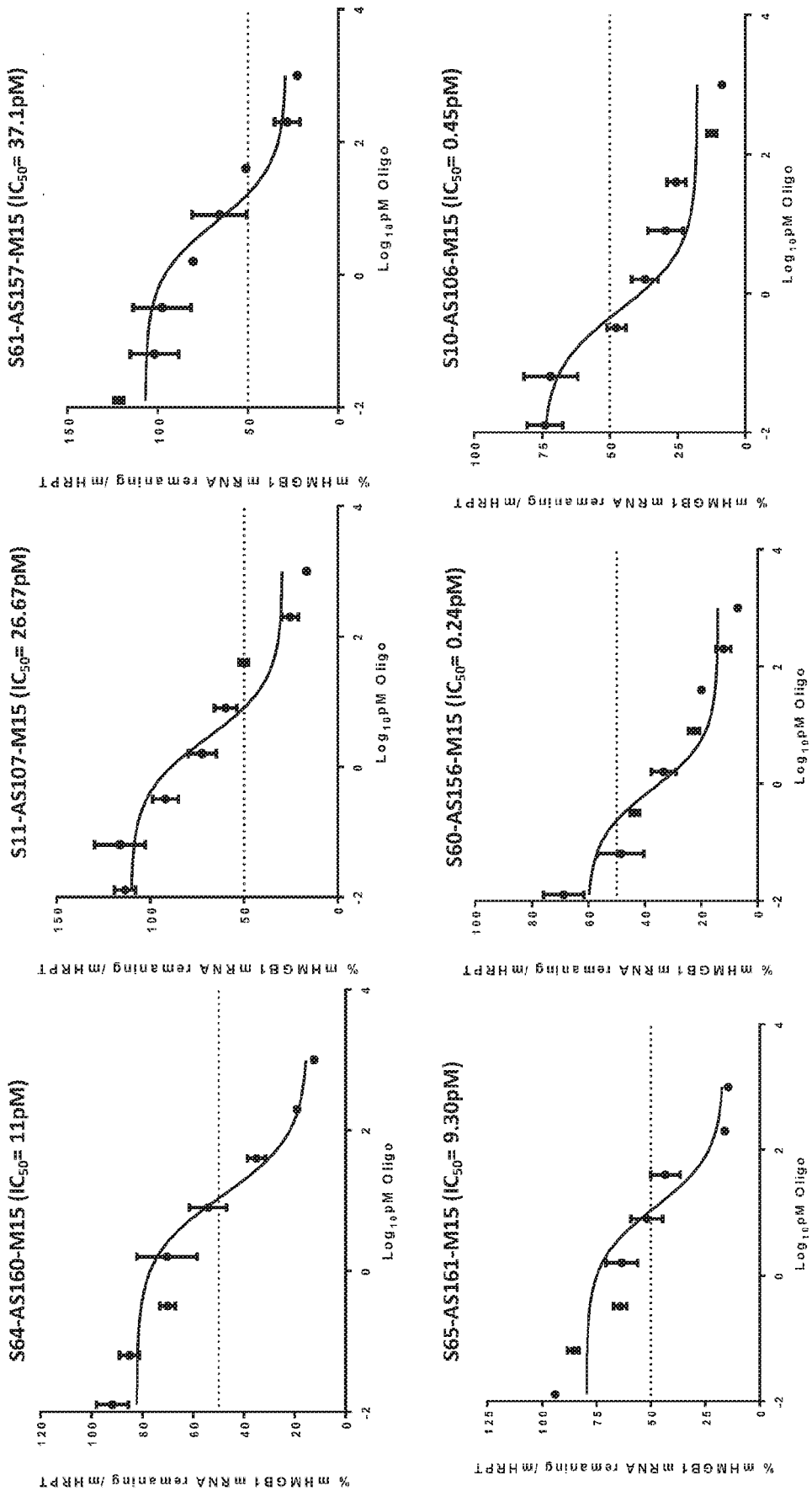

S61-AS157-M15 (IC_{50}= 37.1pM)
S10-AS106-M15 (IC_{50}= 0.45pM)
S11-AS107-M15 (IC_{50}= 26.67pM)
S60-AS156-M15 (IC_{50}= 0.24pM)
S64-AS160-M15 (IC_{50}= 11pM)
S65-AS161-M15 (IC_{50}= 9.30pM)
% mHMGB1 mRNA remaining /mHRPT
Log_{10}pM Oligo

FIG. 7

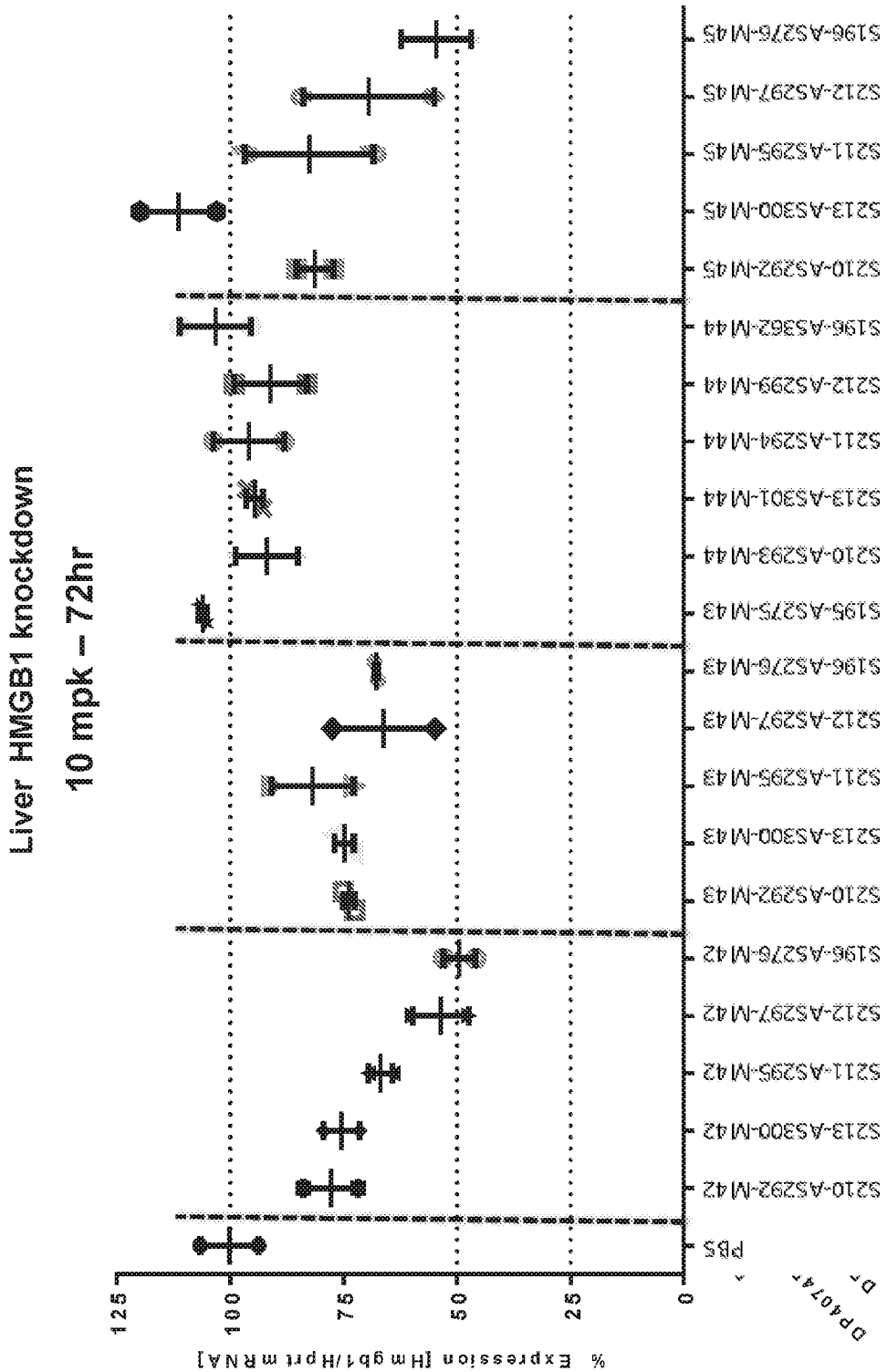

Liver HMGB1 knockdown
10 mpk – 72hr
% Expression [Hmgb1/Hprt mRNA]
125
100
75
50
25
0
PBS
S210-AS292-M42
S213-AS300-M42
S211-AS295-M42
S212-AS297-M42
S196-AS276-M42
S210-AS292-M43
S213-AS300-M43
S211-AS295-M43
S212-AS297-M43
S196-AS276-M43
S195-AS275-M43
S210-AS293-M44
S213-AS301-M44
S211-AS294-M44
S212-AS299-M44
S196-AS362-M44
S210-AS292-M45
S213-AS300-M45
S211-AS295-M45
S212-AS297-M45
S196-AS276-M45

FIG. 8

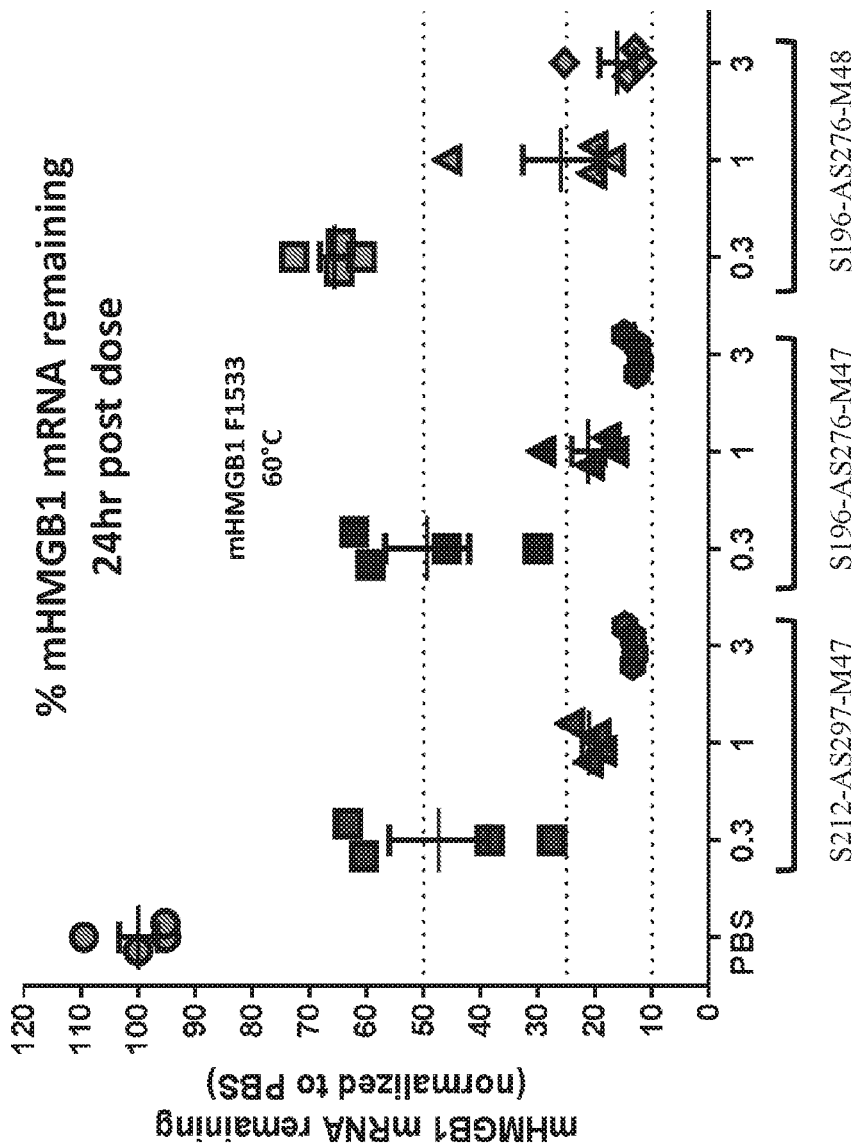

% mHMGB1 mRNA remaining
24hr post dose
mHMGB1 F1533
60°C
mHMGB1 mRNA remaining
(normalized to PBS)
120
110
100
90
80
70
60
50
40
30
20
10
0
PBS
0.3
1
3
0.3
1
3
0.3
1
3
S212-AS297-M47
S196-AS276-M47
S196-AS276-M48

FIG. 9

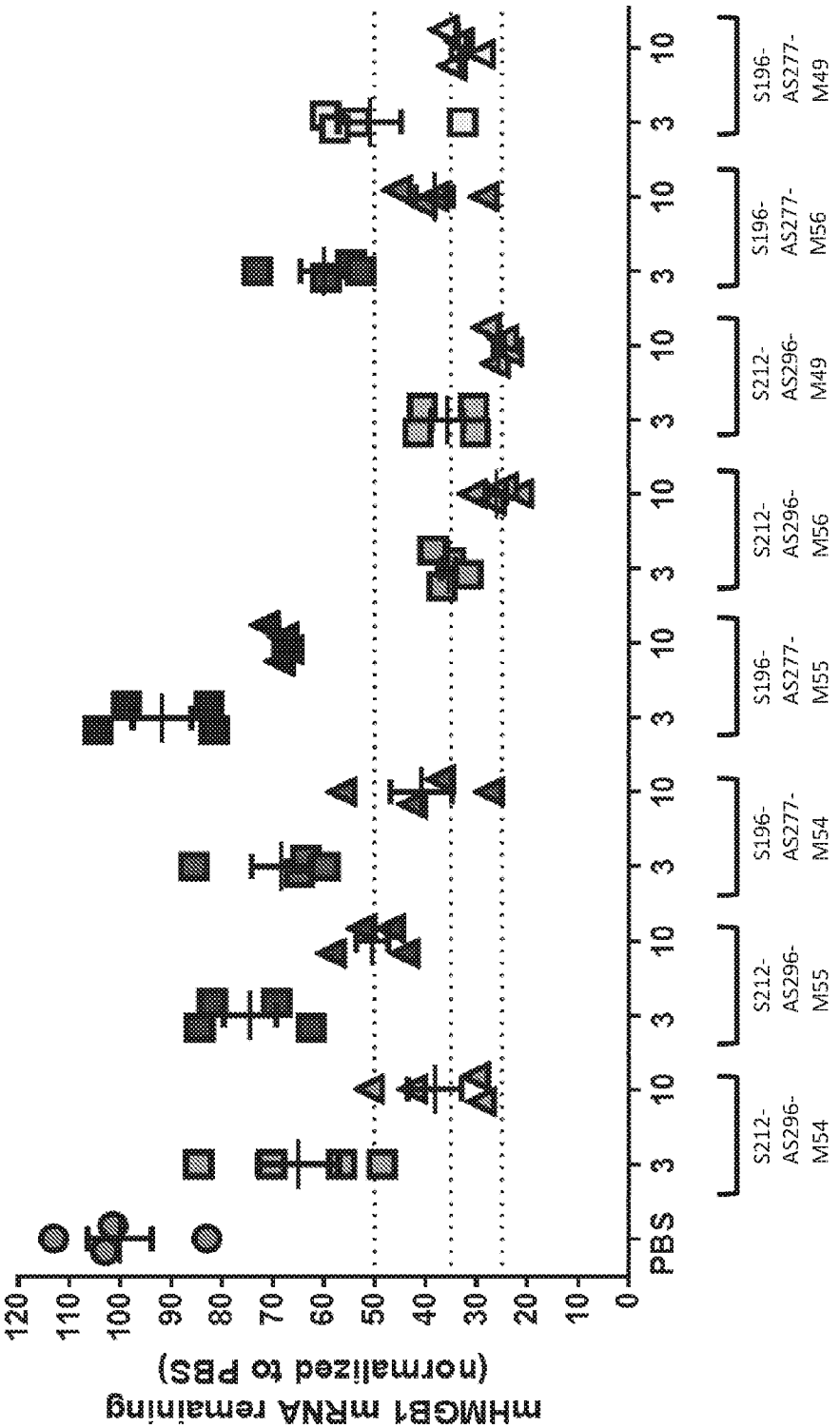

mHMGB1 mRNA remaining
(normalized to PBS)
120
110
100
90
80
70
60
50
40
30
20
10
0
PBS
3
10
3
10
3
10
3
10
3
10
3
10
3
10
3
10
3
10
S212-AS296-M54
S212-AS296-M55
S196-AS277-M54
S196-AS277-M55
S212-AS296-M56
S212-AS296-M49
S196-AS277-M56
S196-AS277-M49

FIG. 10

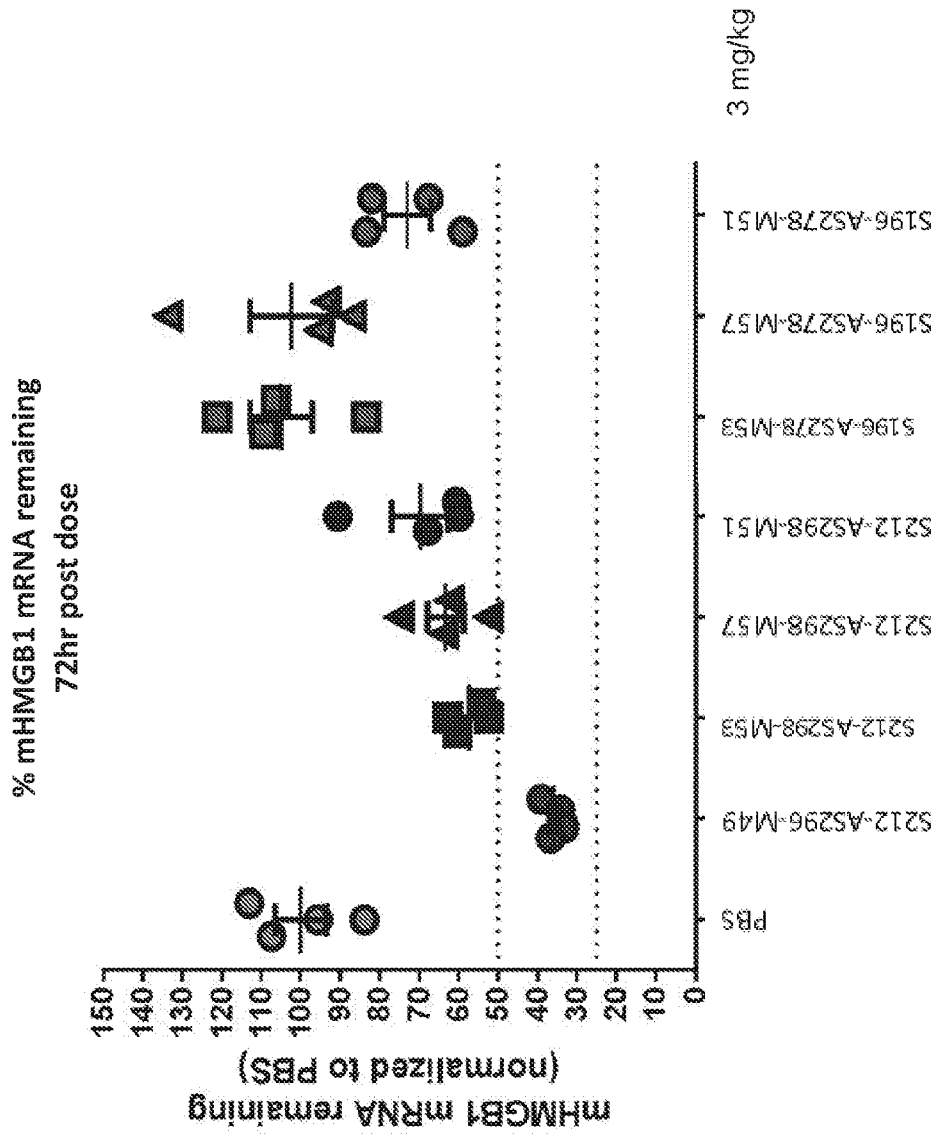

% mHMGB1 mRNA remaining
72hr post dose
mHMGB1 mRNA remaining
(normalized to PBS)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
PBS
S212-AS296-M49
S212-AS298-M53
S212-AS298-M57
S212-AS298-M51
S196-AS278-M53
S196-AS278-M57
S196-AS278-M51
3 mg/kg

FIG. 11

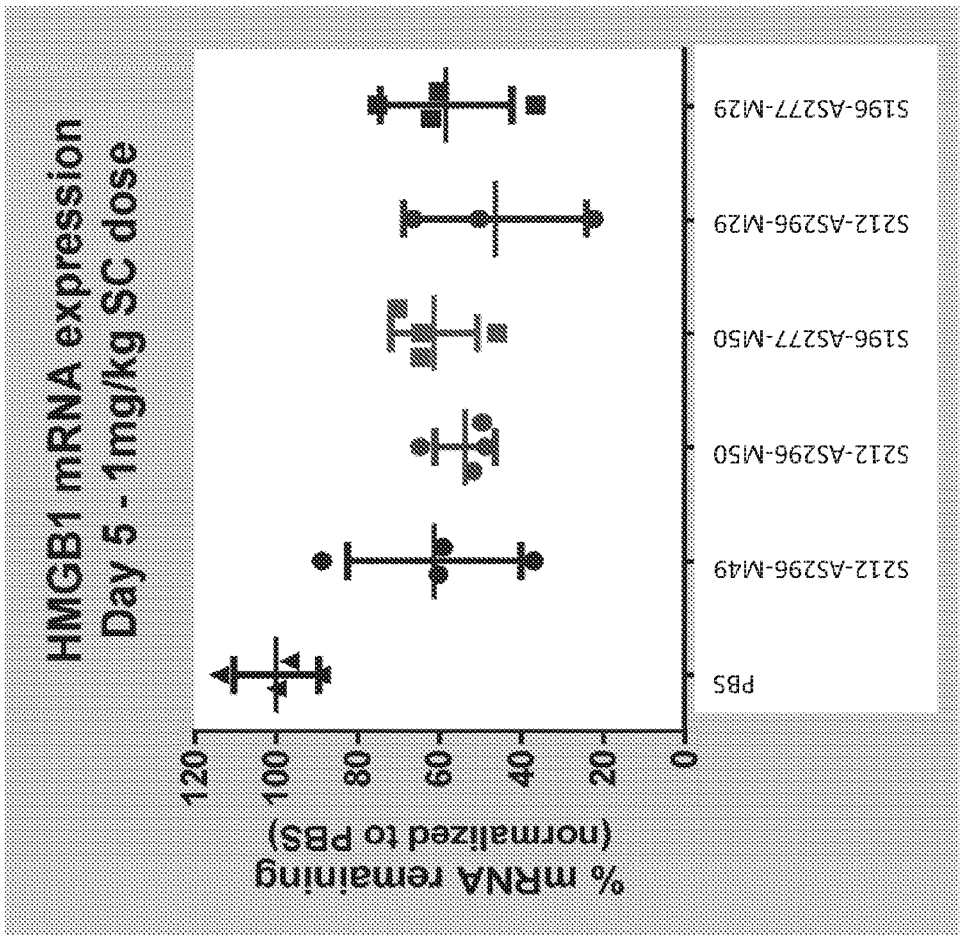

HMGB1 mRNA expression
Day 5 - 1mg/kg SC dose
% mRNA remaining
(normalized to PBS)
120
100
80
60
40
20
0
PBS
S212-AS296-M49
S212-AS296-M50
S196-AS277-M50
S212-AS296-M29
S196-AS277-M29

FIG. 12

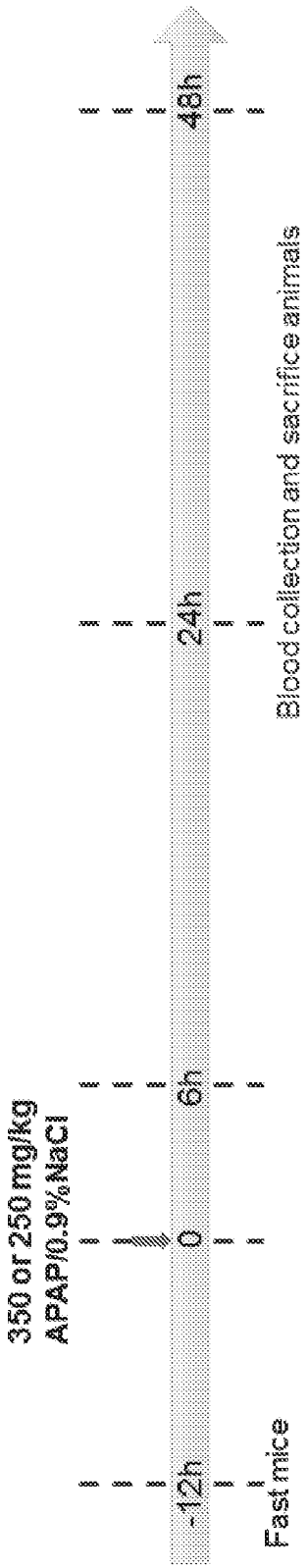

-12h
Fast mice
0
350 or 250 mg/kg
APAP/0.9%NaCl
6h
24h
48h
Blood collection and sacrifice animals

FIG. 13A

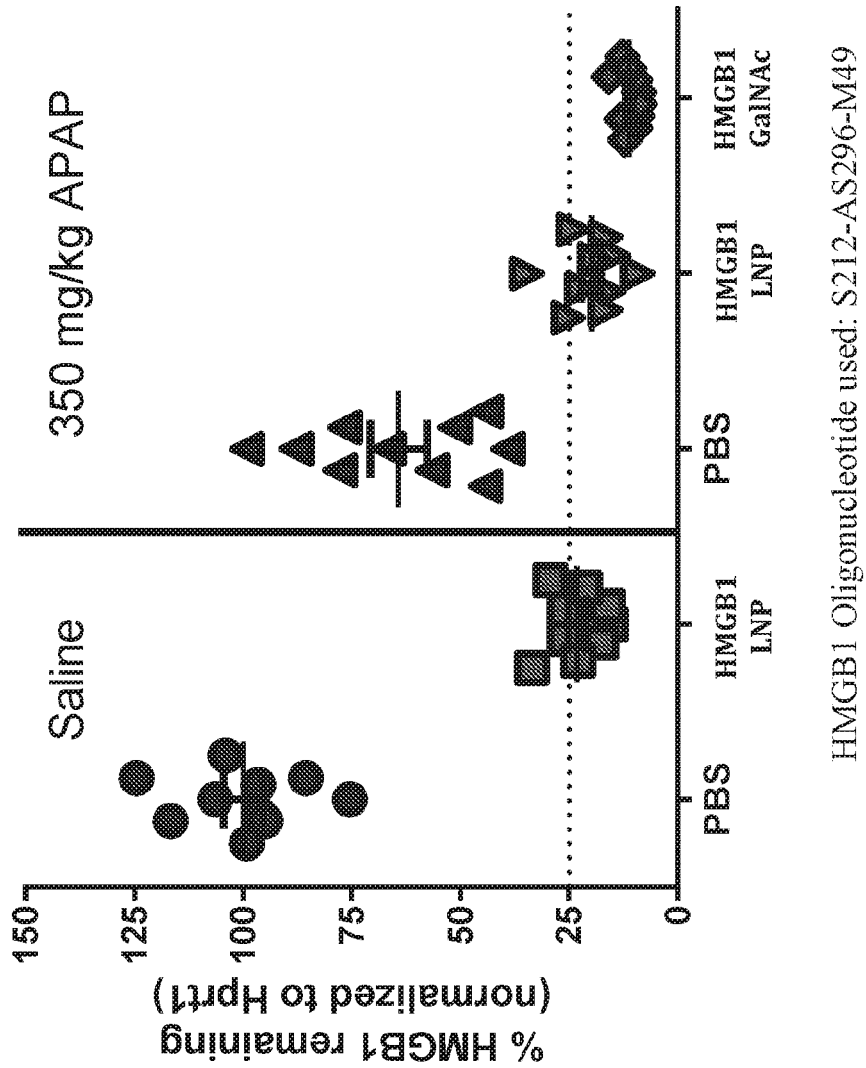

Liver HMGB1 Expression
Saline
350 mg/kg APAP
% HMGB1 remaining
(normalized to Hprt1)
150
125
100
75
50
25
0
PBS
HMGB1
LNP
PBS
HMGB1
LNP
HMGB1
GalNAc
HMGB1 Oligonucleotide used: S212-AS296-M49

FIG. 13B

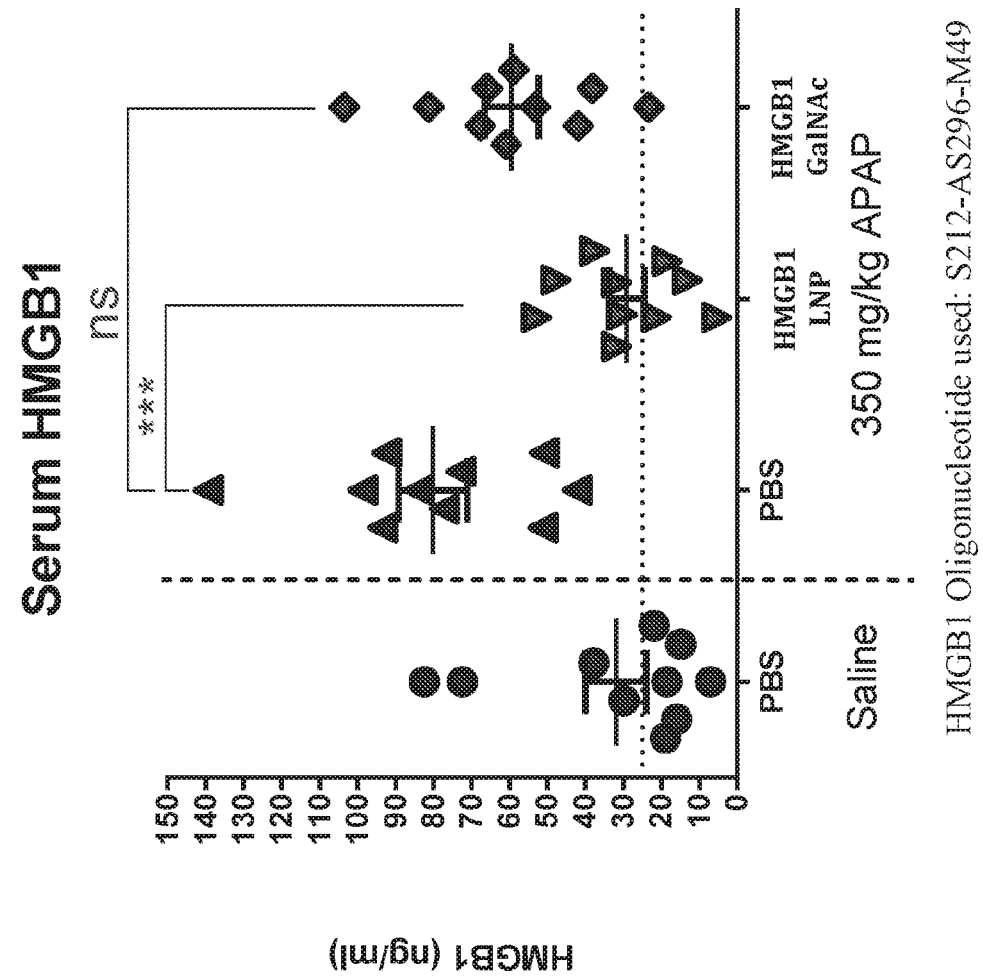

Serum HMGB1
ns
***
HMGB1 (ng/ml)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
PBS
PBS
HMGB1 LNP
HMGB1 GalNAc
Saline
350 mg/kg APAP
HMGB1 Oligonucleotide used: S212-AS296-M49

FIG. 14A

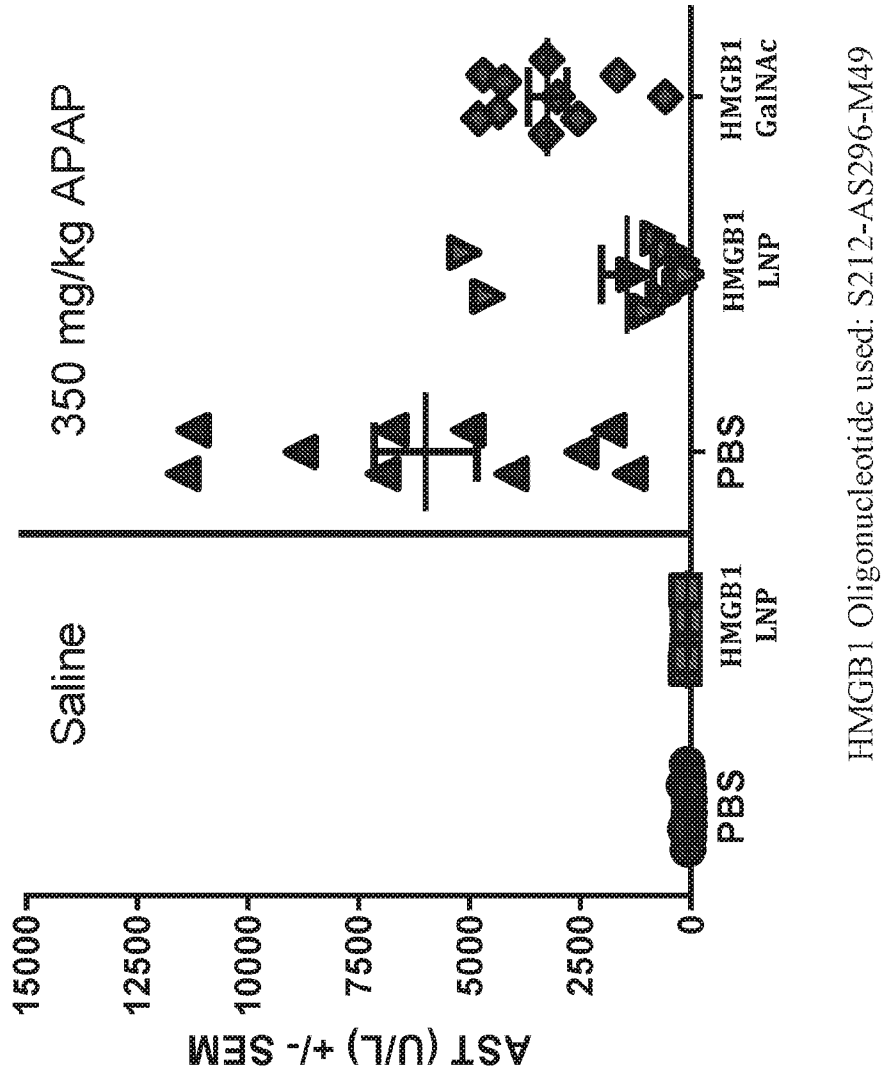
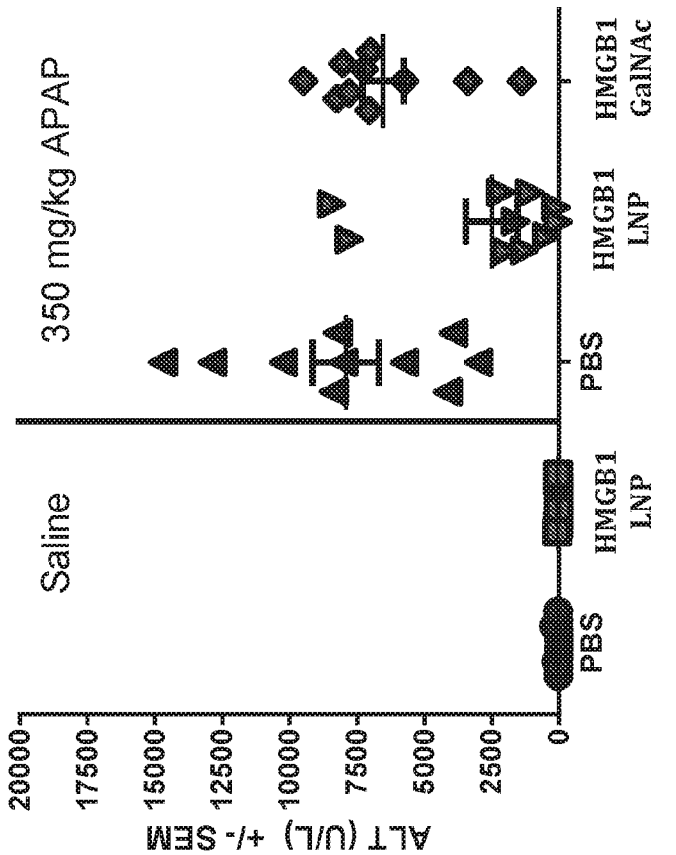
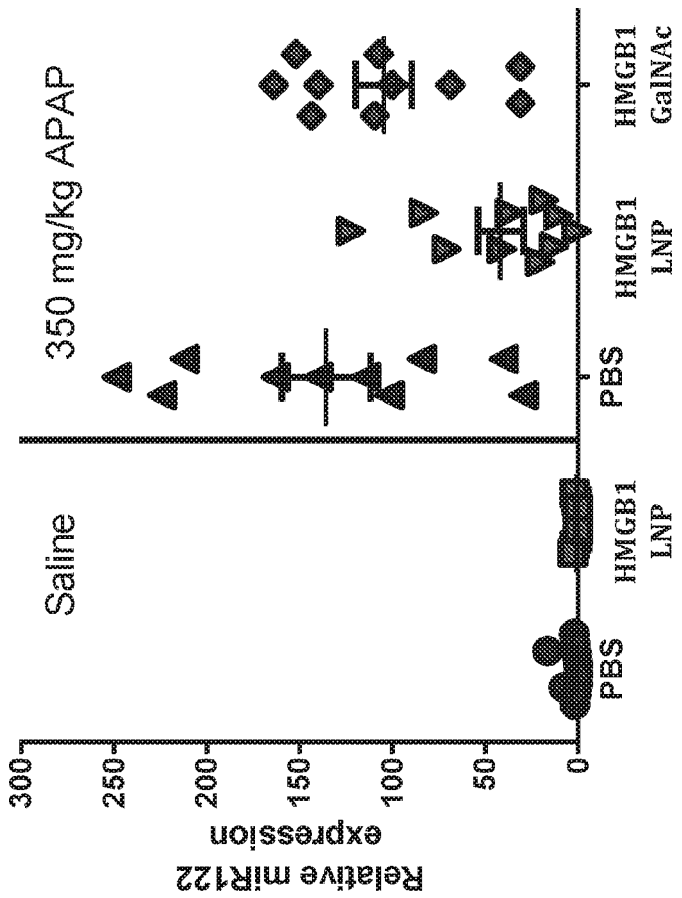

AST
Saline
350 mg/kg APAP
AST (U/L) +/- SEM
15000
12500
10000
7500
5000
2500
0
PBS
HMGB1 LNP
PBS
HMGB1 LNP
HMGB1 GalNAc
HMGB1 Oligonucleotide used: S212-AS296-M49

FIG. 14B



HMGB1 Oligonucleotide used: S212-AS296-M49

FIG. 14C



HMGB1 Oligonucleotide used: S212-AS296-M49

FIG. 15

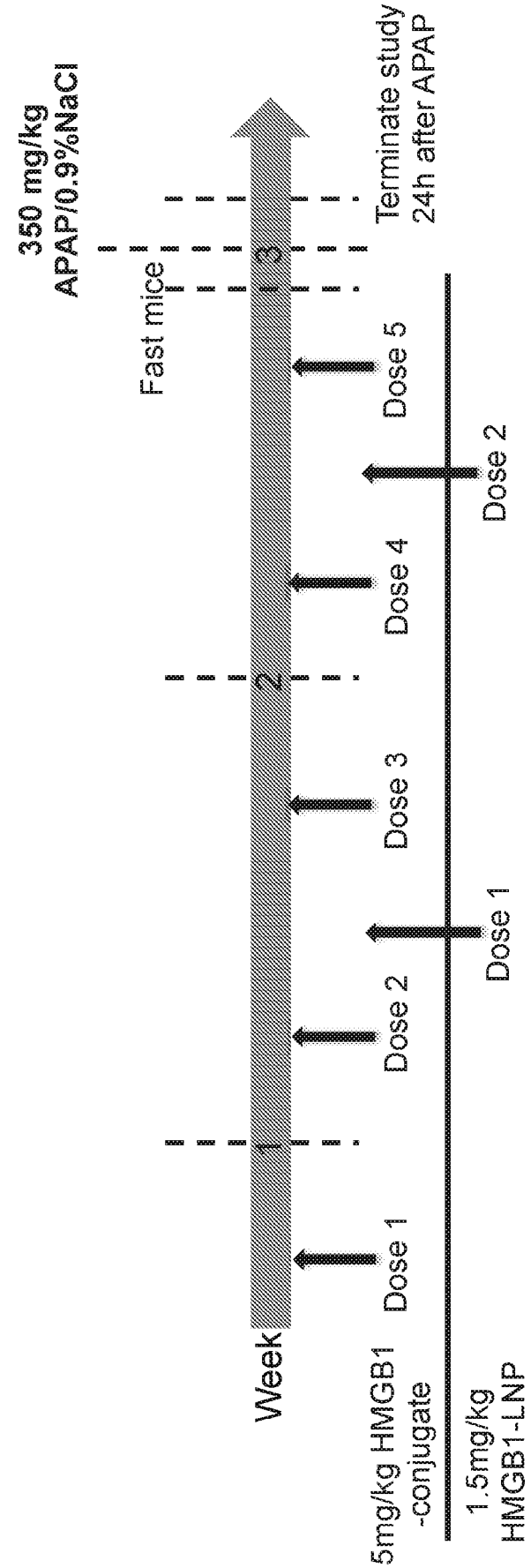
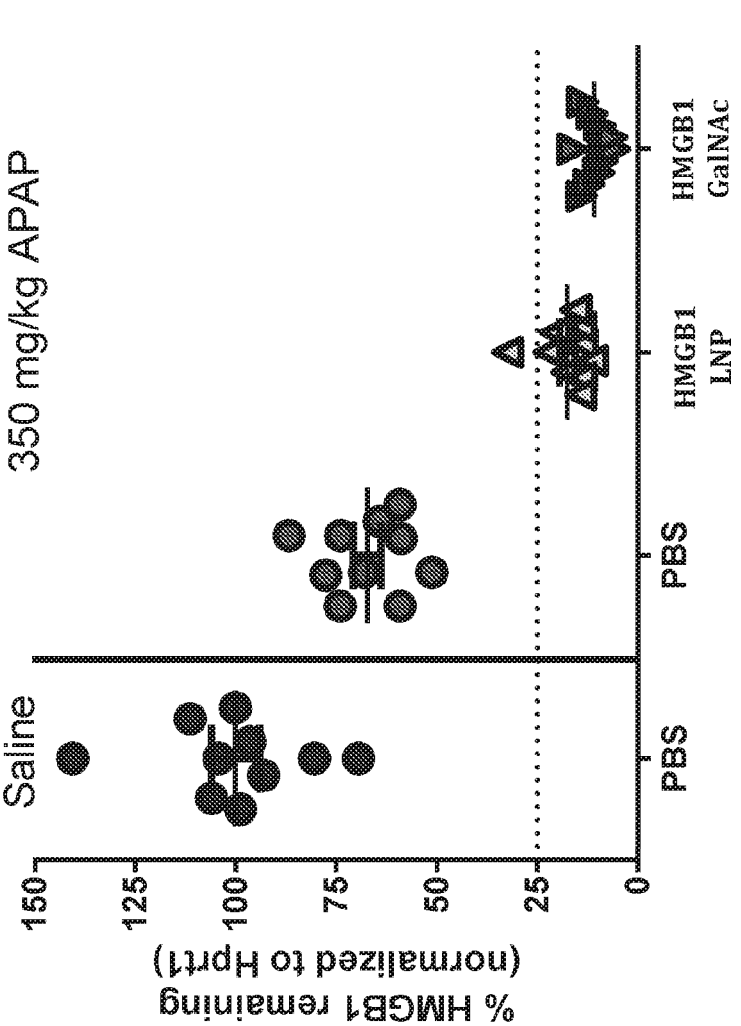
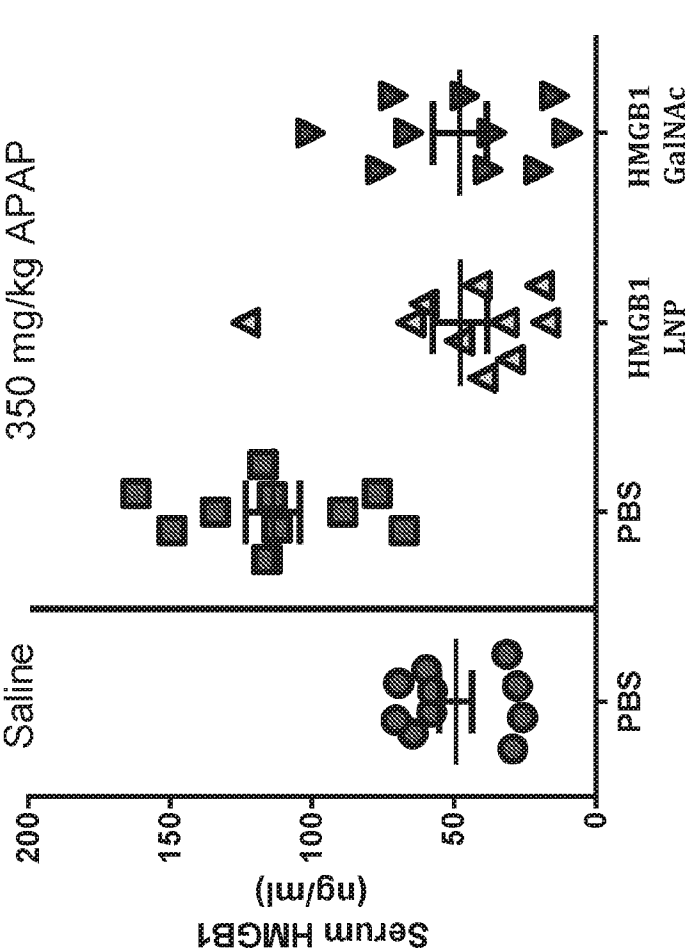

350 mg/kg
APAP/0.9%NaCl
Fast mice
Terminate study
24h after APAP
Week
1
2
3
Dose 1
Dose 2
Dose 3
Dose 4
Dose 5
5mg/kg HMGB1
-conjugate
1.5mg/kg
HMGB1-LNP

FIG. 16A



HMGB1 Oligonucleotide used: S212-AS296-M56

FIG. 16B



HMGB1 Oligonucleotide used: S212-AS296-M56

FIG. 17A

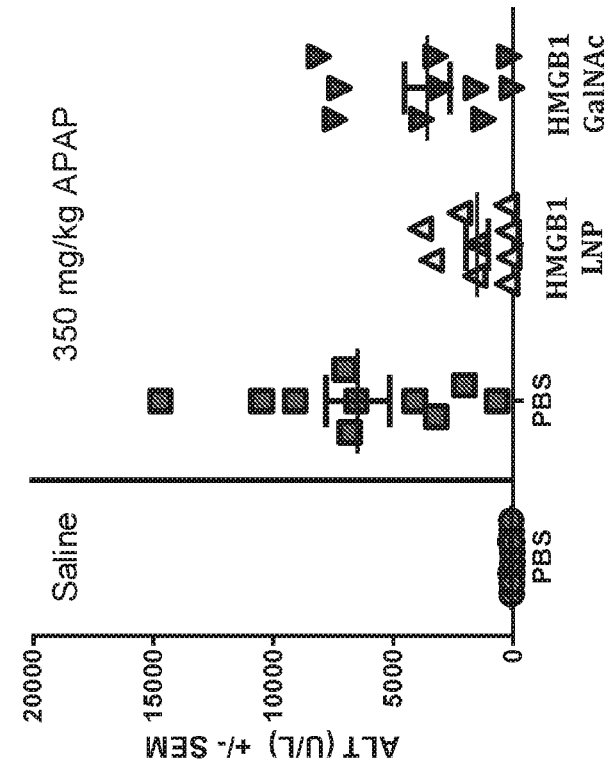


FIG. 17B

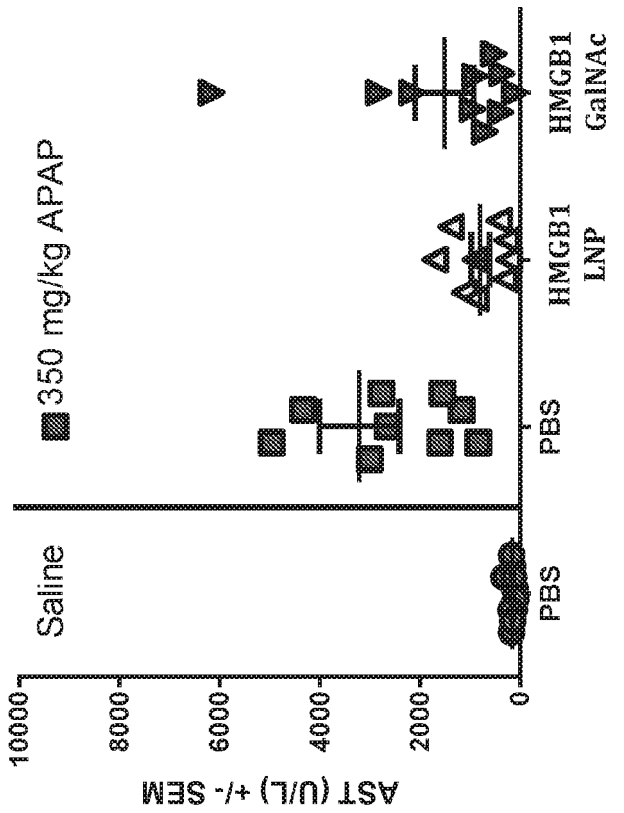


FIG. 17C

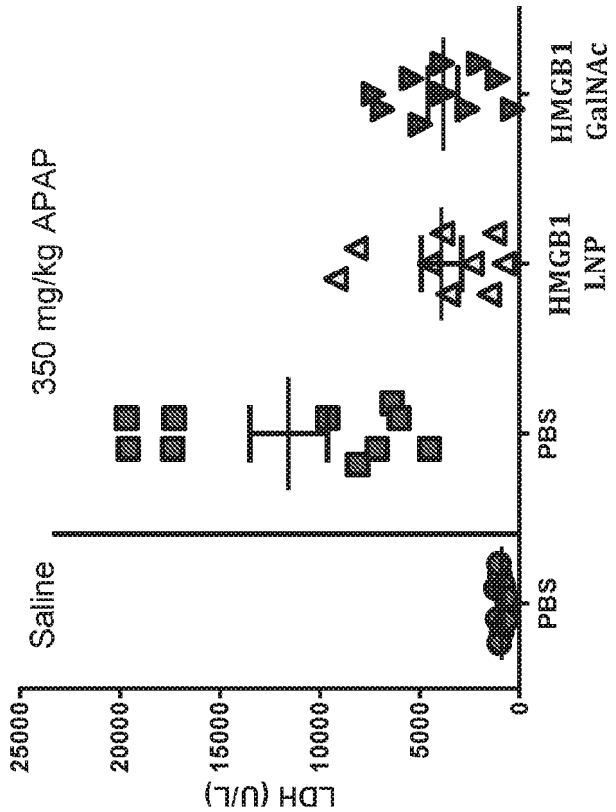


FIG. 17D

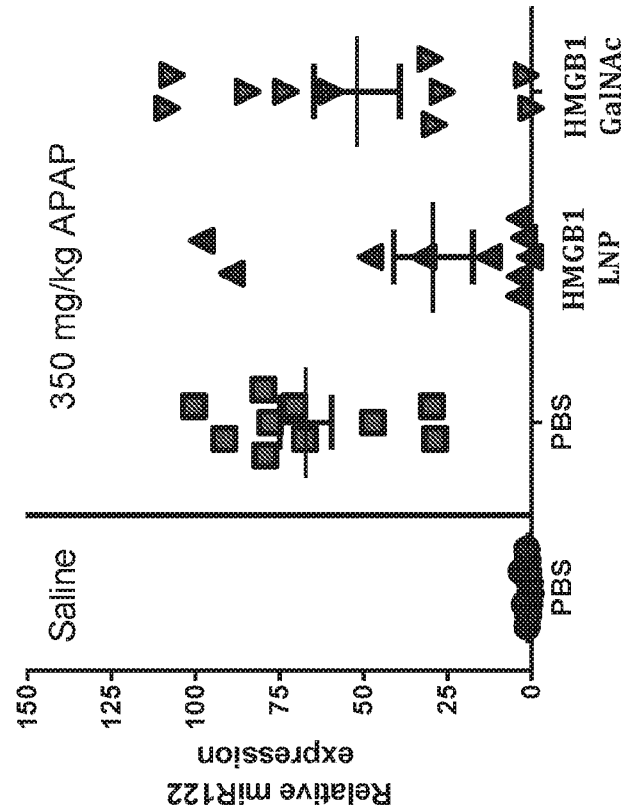


FIG. 18

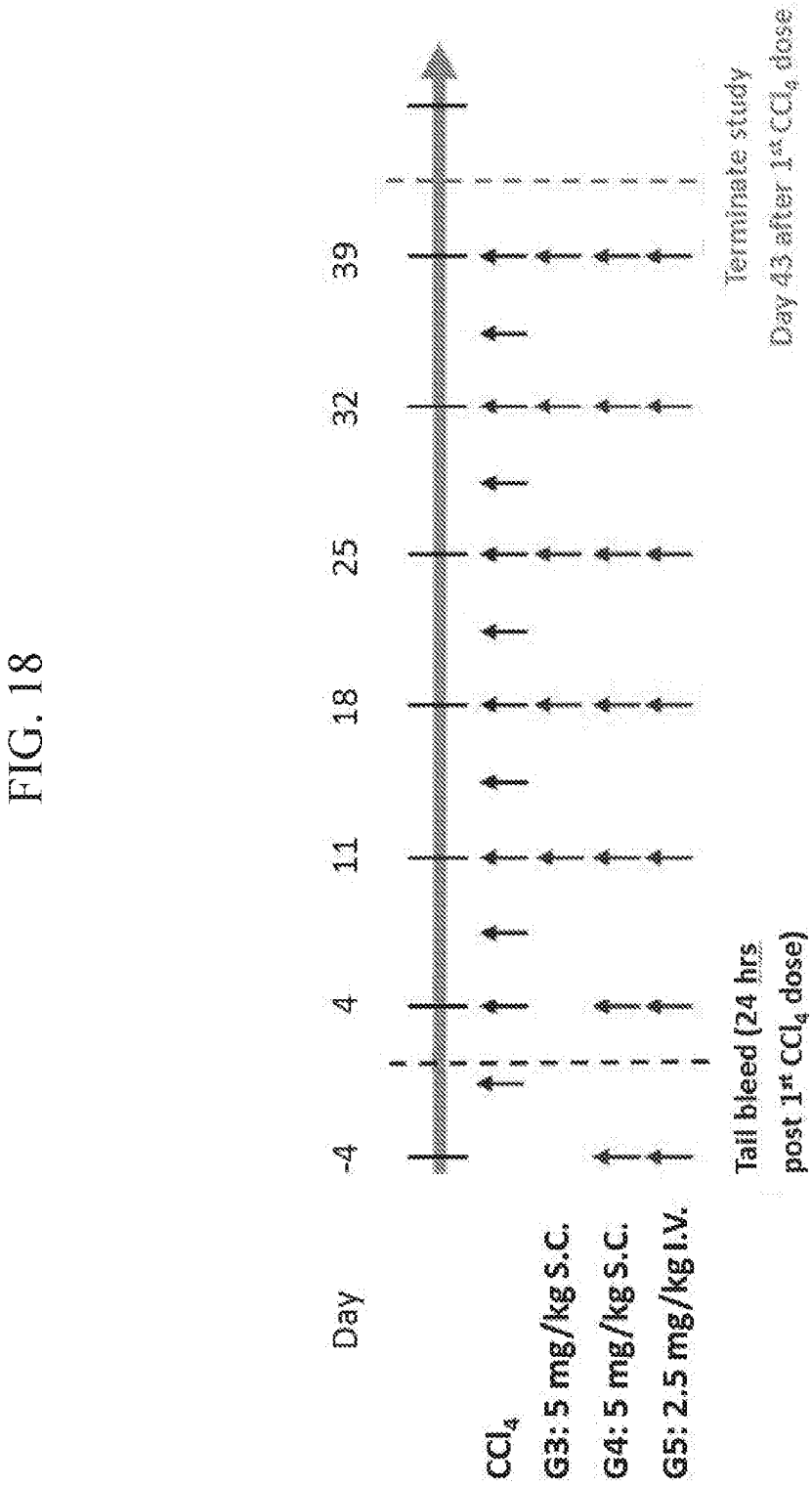

Day
-4
4
11
18
25
32
39
CCl_4
G3: 5 mg/kg S.C.
G4: 5 mg/kg S.C.
G5: 2.5 mg/kg I.V.
Tail bleed (24 hrs post 1st CCl_4 dose)
Terminate study
Day 43 after 1st CCl_4 dose

FIG. 19

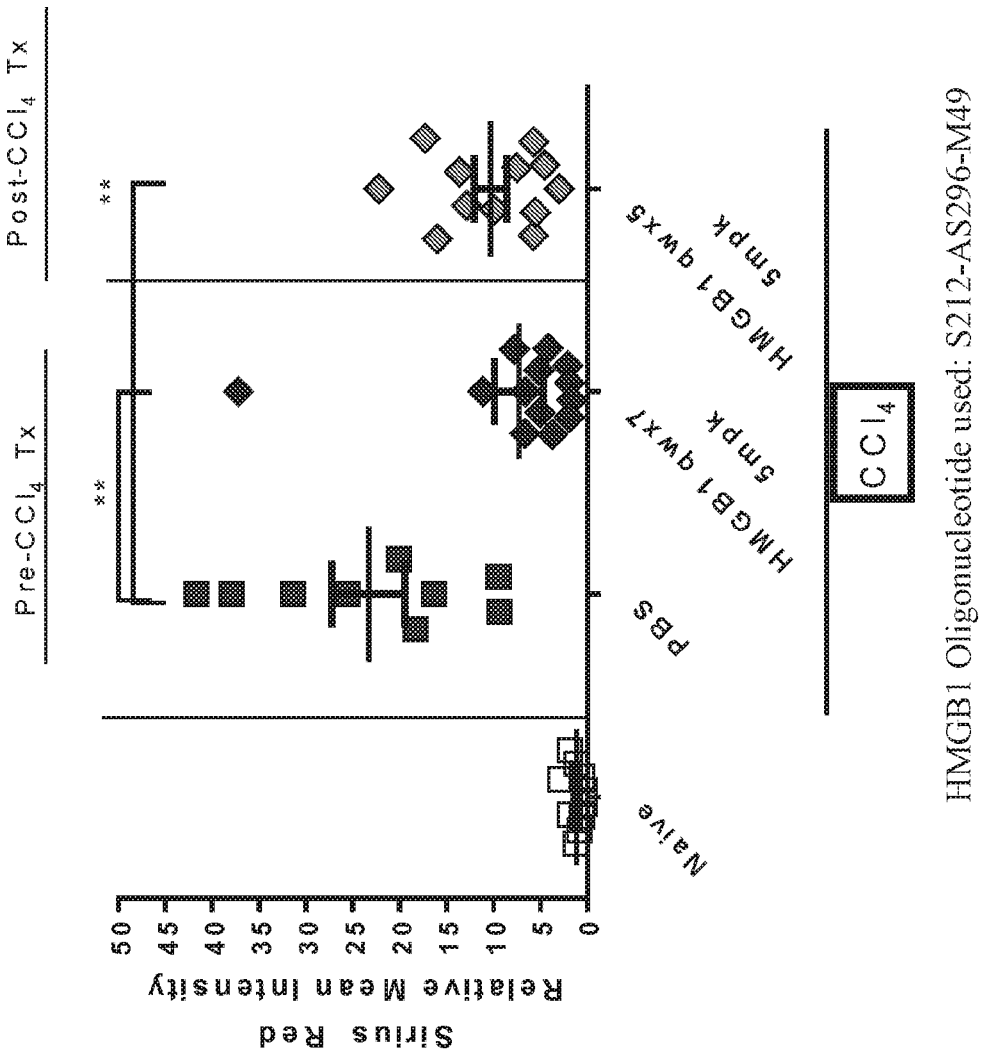
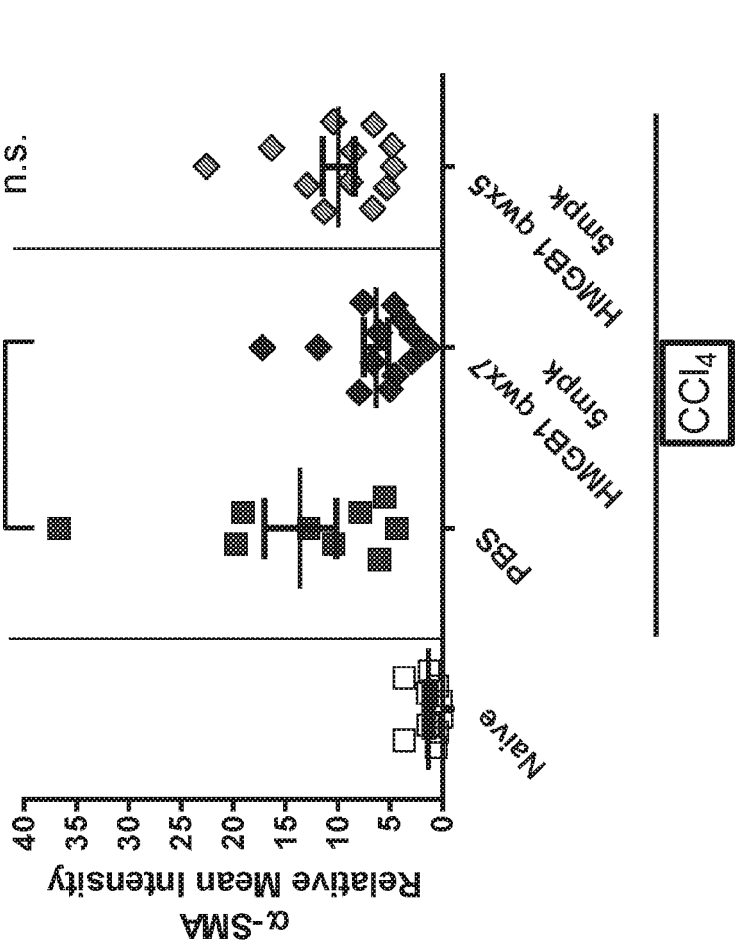

Pre-CCl4 Tx
Post-CCl4 Tx
**
**
Sirius Red
Relative Mean Intensity
50
45
40
35
30
25
20
15
10
5
0
Naive
PBS
HMGB1 qwx7
5mpk
HMGB1 qwx5
5mpk
CCl4
HMGB1 Oligonucleotide used: S212-AS296-M49

FIG. 20



HMGB1 Oligonucleotide used: S212-AS296-M49

FIG. 21

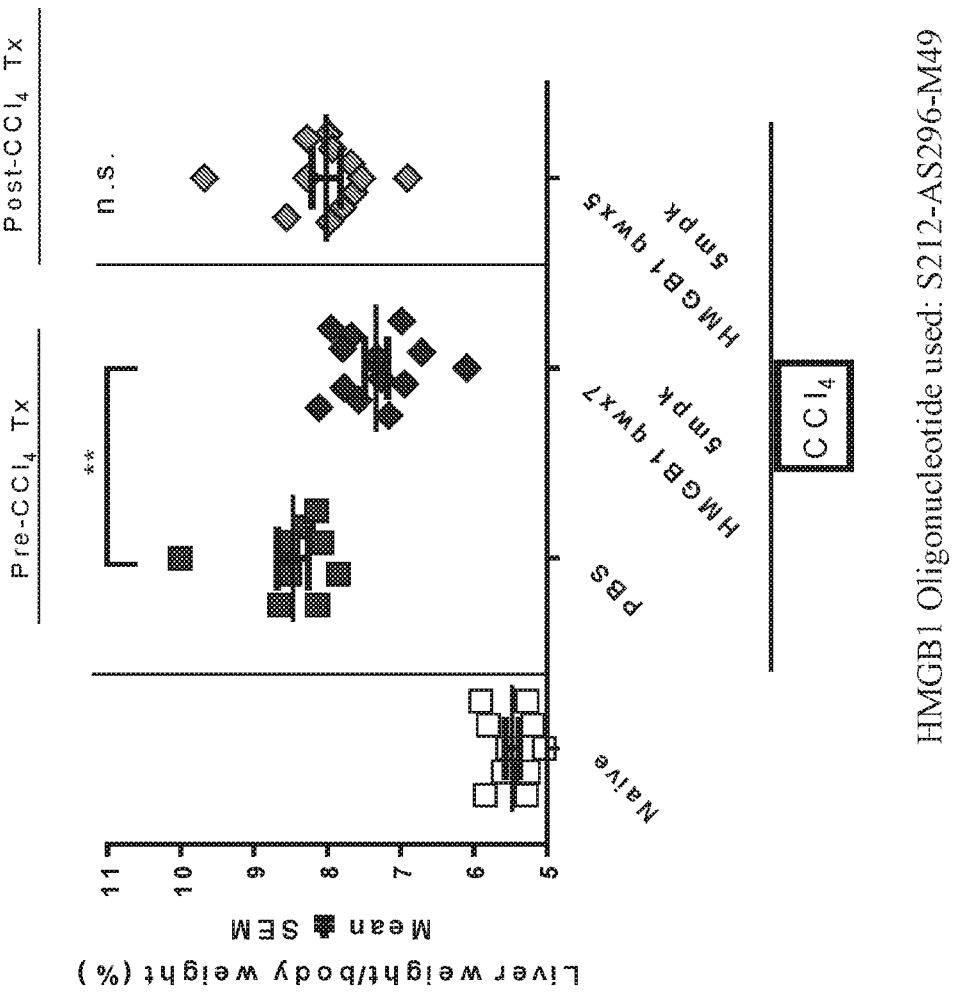

Liver weight/body weight (%)
Mean ± SEM
11
10
9
8
7
6
5
Naïve
PBS
HMGB1 qwx7 5mpk
HMGB1 qwx5 5mpk
CCl4
Pre-CCl4 Tx
Post-CCl4 Tx
**
n.s.
HMGB1 Oligonucleotide used: S212-AS296-M49

FIG. 22

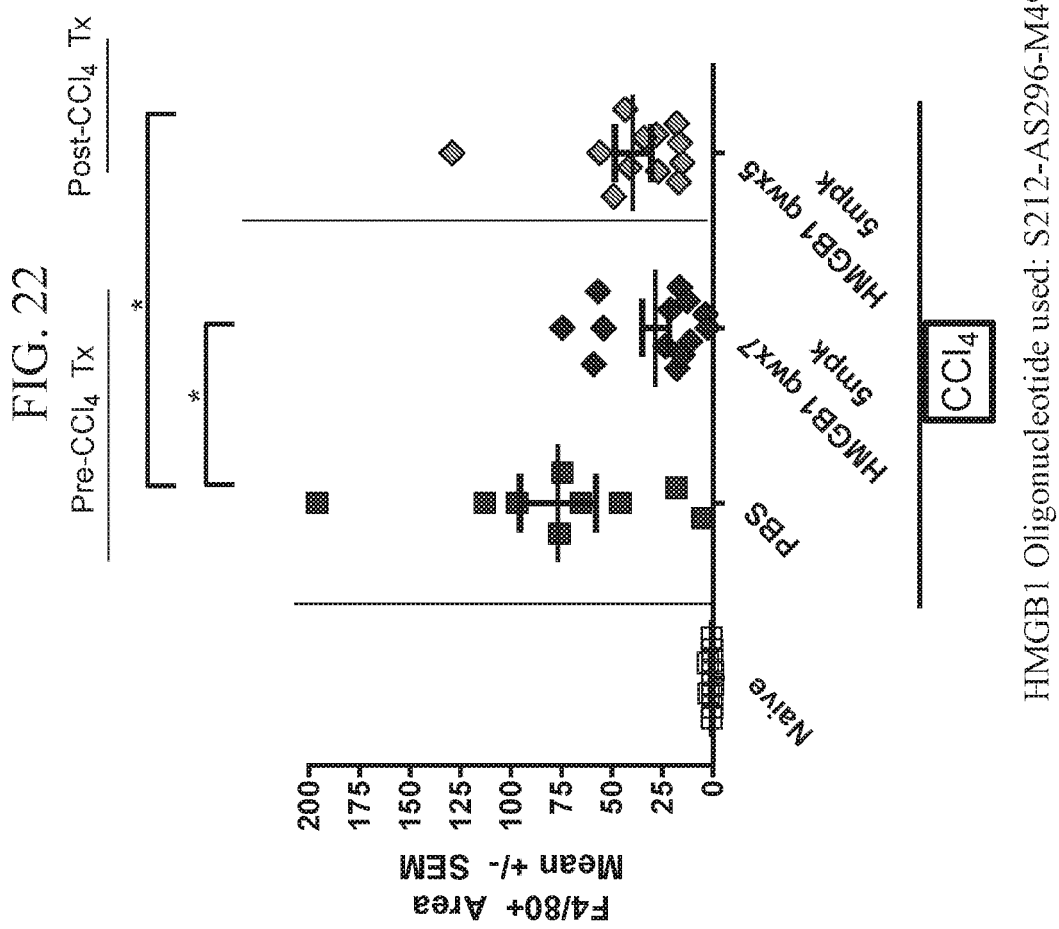
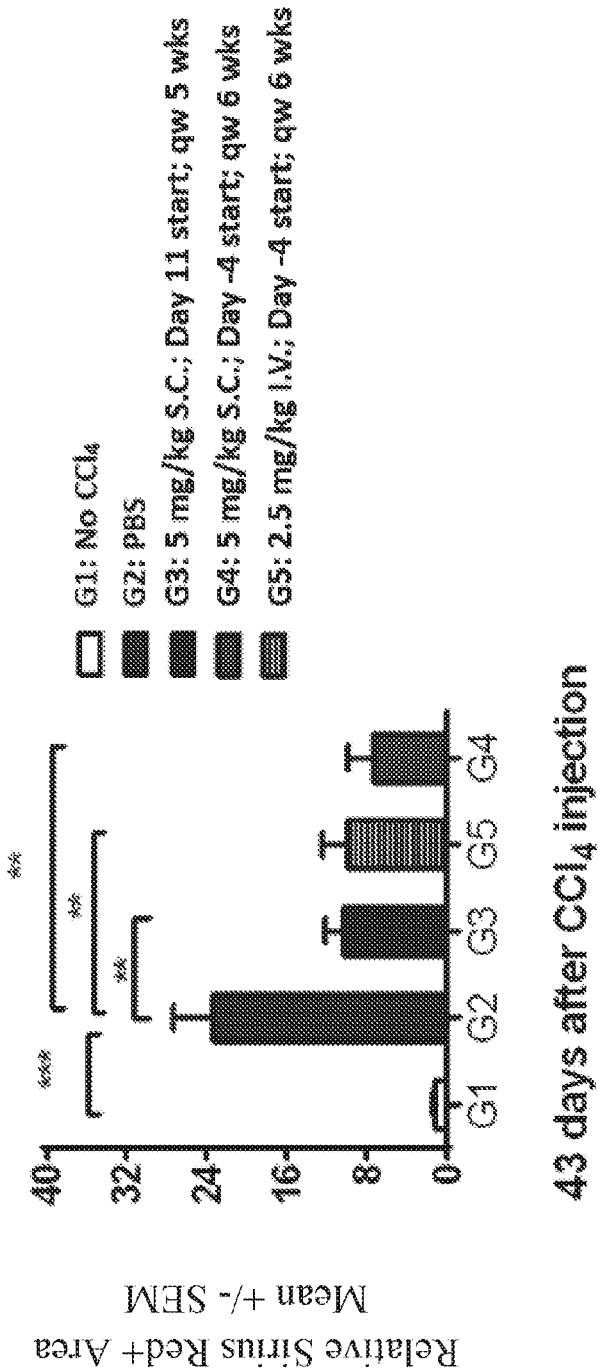

Pre-CCl4 Tx
Post-CCl4 Tx
*
*
F4/80+ Area
Mean +/- SEM
200
175
150
125
100
75
50
25
0
Naive
PBS
HMGB1 qwx7
5mpk
HMGB1 qwx5
5mpk
CCl4
HMGB1 Oligonucleotide used: S212-AS296-M49

FIG. 23



HMGB1 Oligonucleotide used: S212-AS296-M49

FIG. 24

Group	N	Con A (IV)	Treatment (SC) Day -5	Take down	Endpoints
A	5	20mg/kg	PBS	24hr	Blood: ALT, AST Liver: H&E mRNA
B	5	20mg/kg	HMGB1 (10mpk)	24hr	

FIG. 25

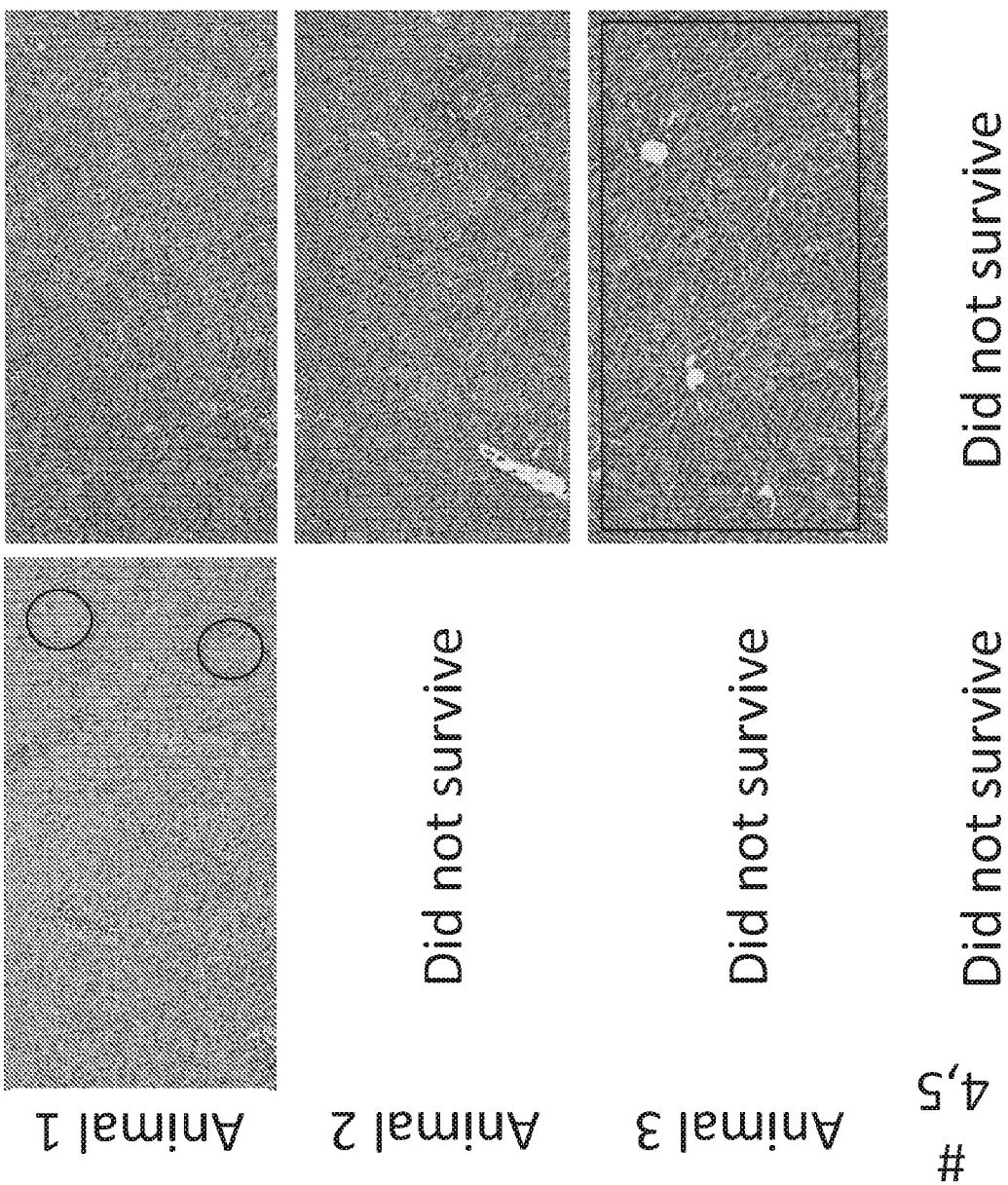
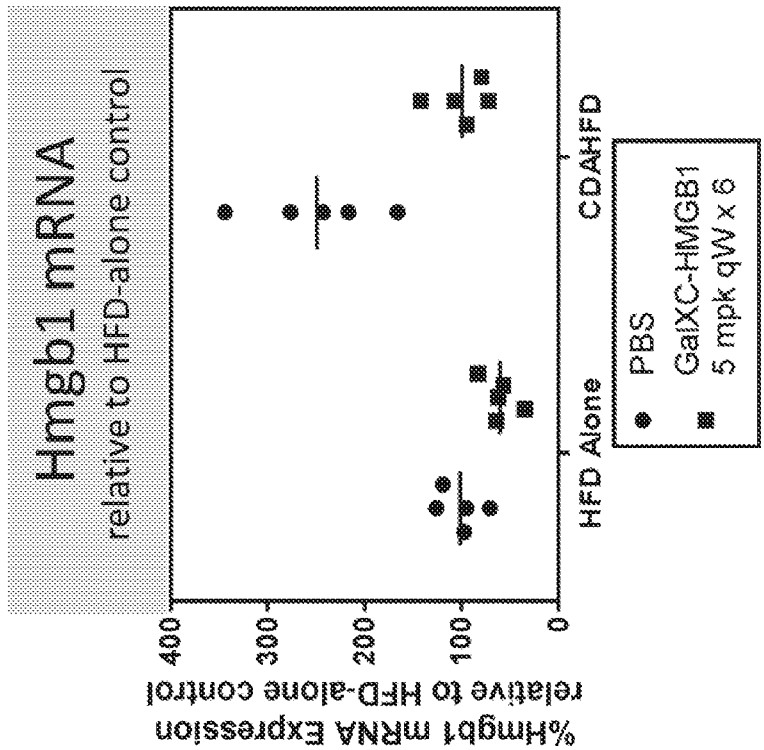
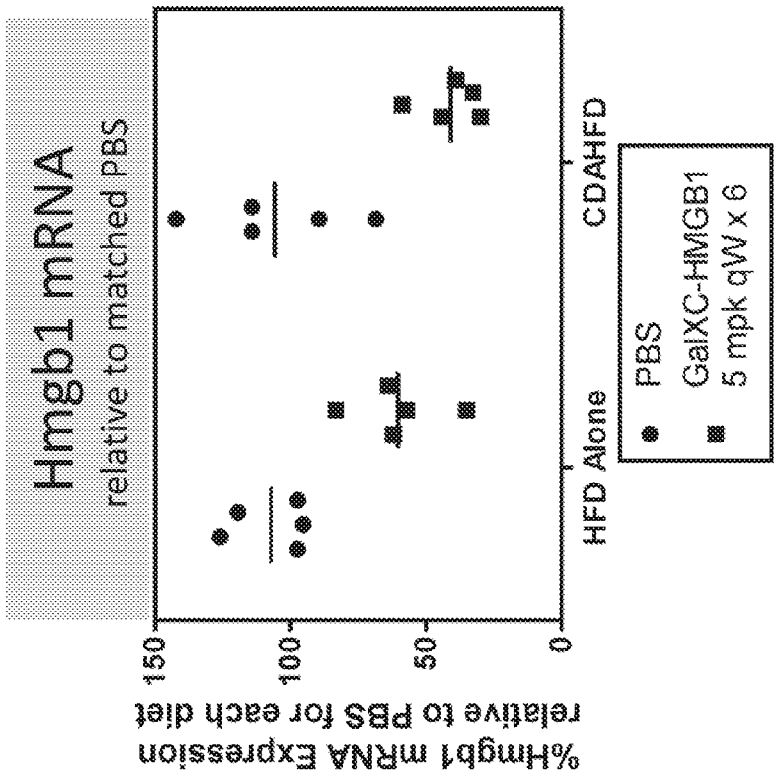

PBS
HMGB1 10mpk SC
Necrotic area
Healthy section
HMGB1 Oligonucleotide used:
S212-AS296-M49
Animal 1
Animal 2
Animal 3
#
4,5
Did not survive
Did not survive
Did not survive

FIG. 26A



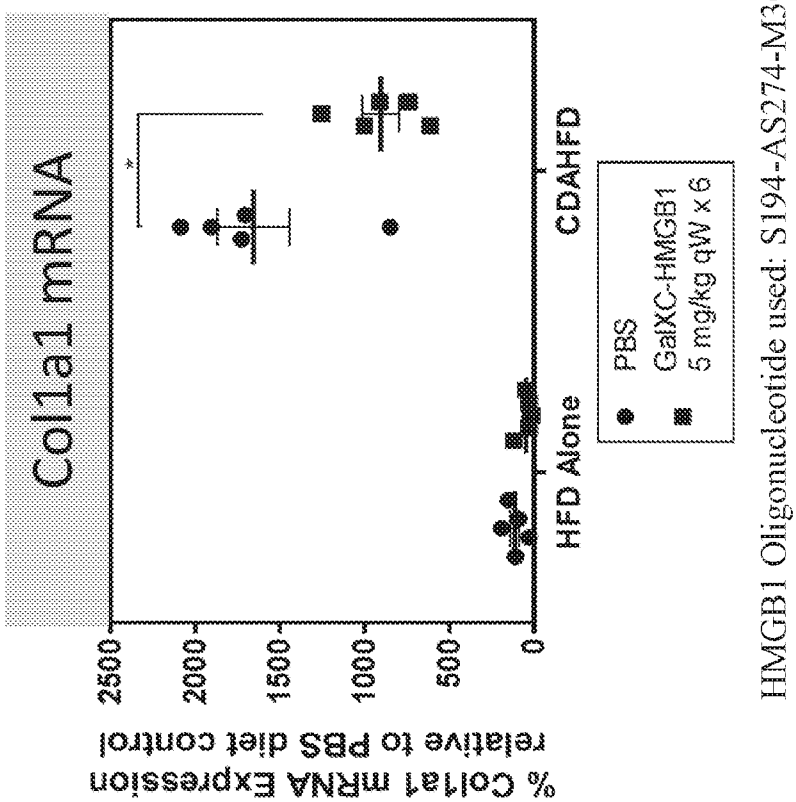
HMGB1 Oligonucleotide used: S194-AS274-M30

FIG. 26B



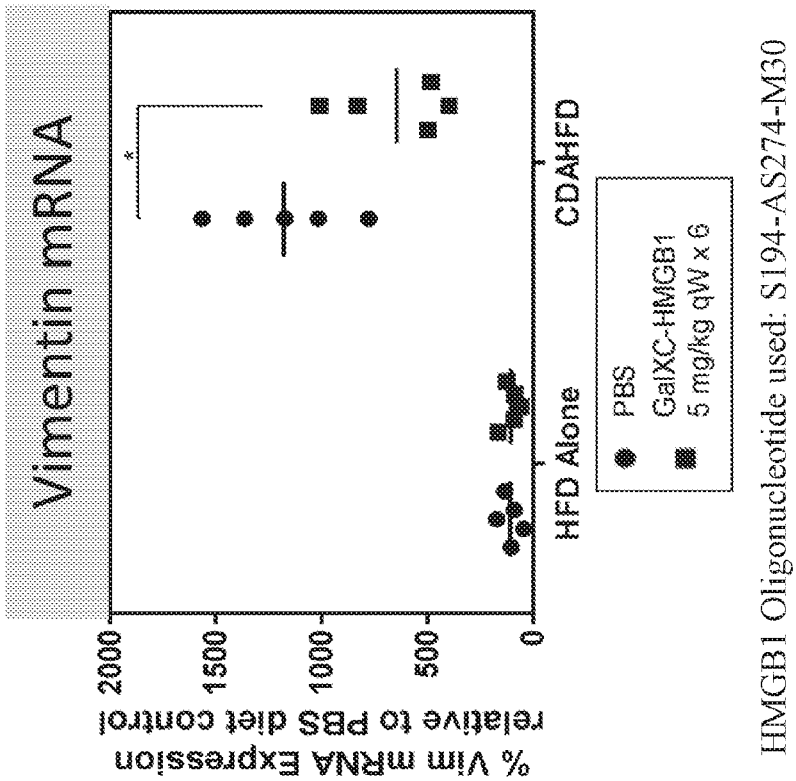
HMGB1 Oligonucleotide used: S194-AS274-M30

FIG. 26C



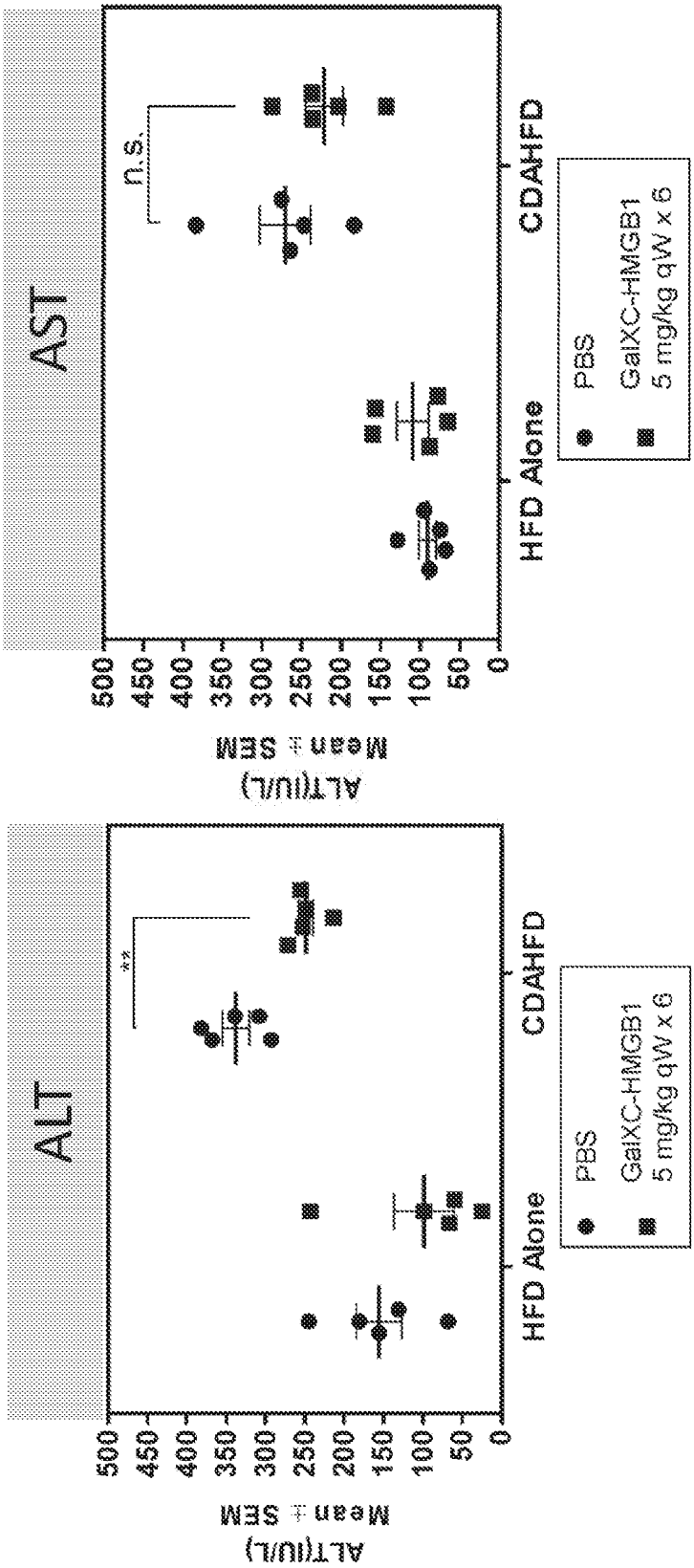
HMGB1 Oligonucleotide used: S194-AS274-M30

FIG. 26D



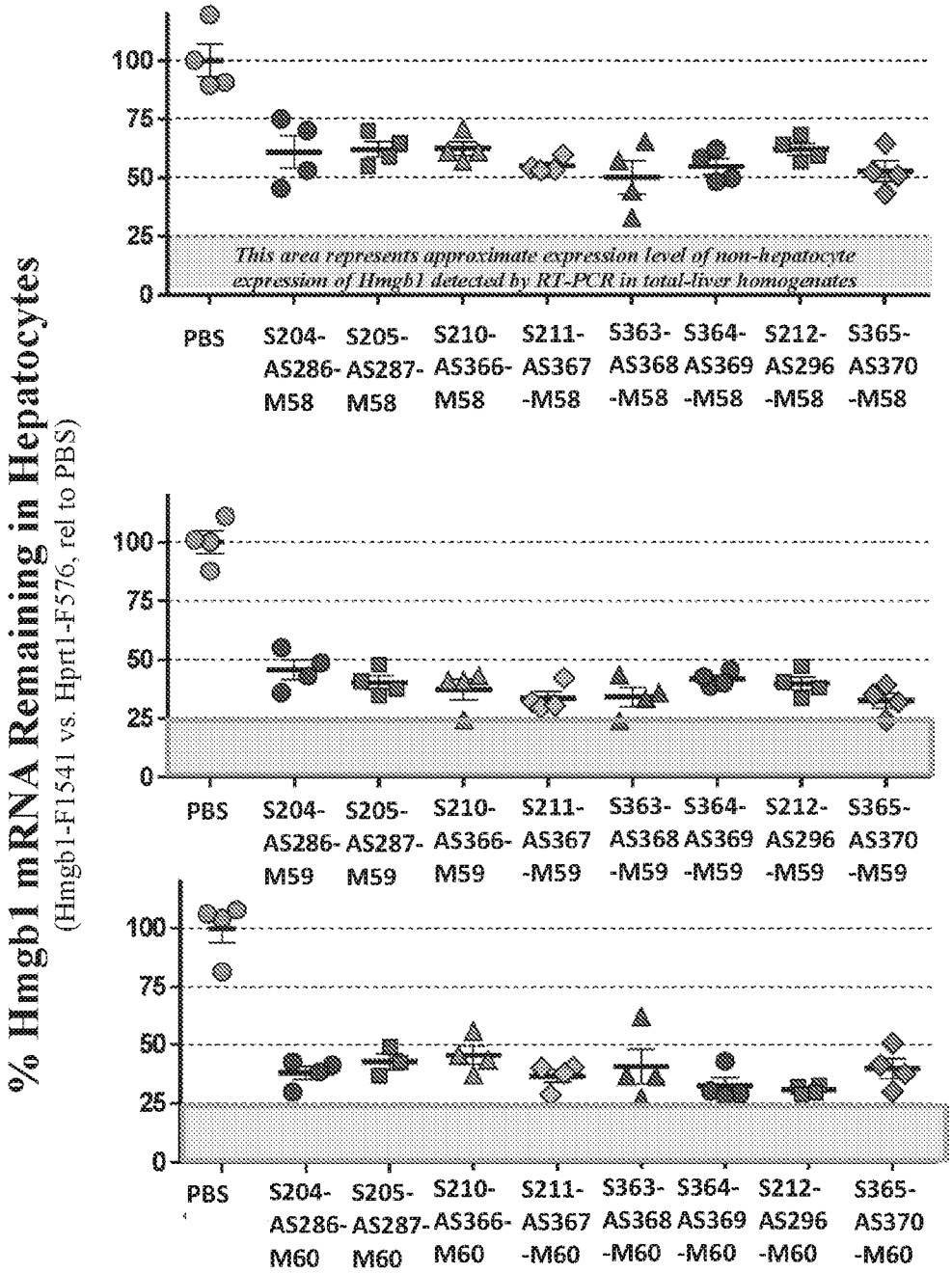
HMGB1 Oligonucleotide used: S194-AS274-M30

FIG. 27



HMGB1 Oligonucleotide used: S194-AS274-M30

FIG. 28



GalXC-HMGB1 Target Site

FIG. 29A

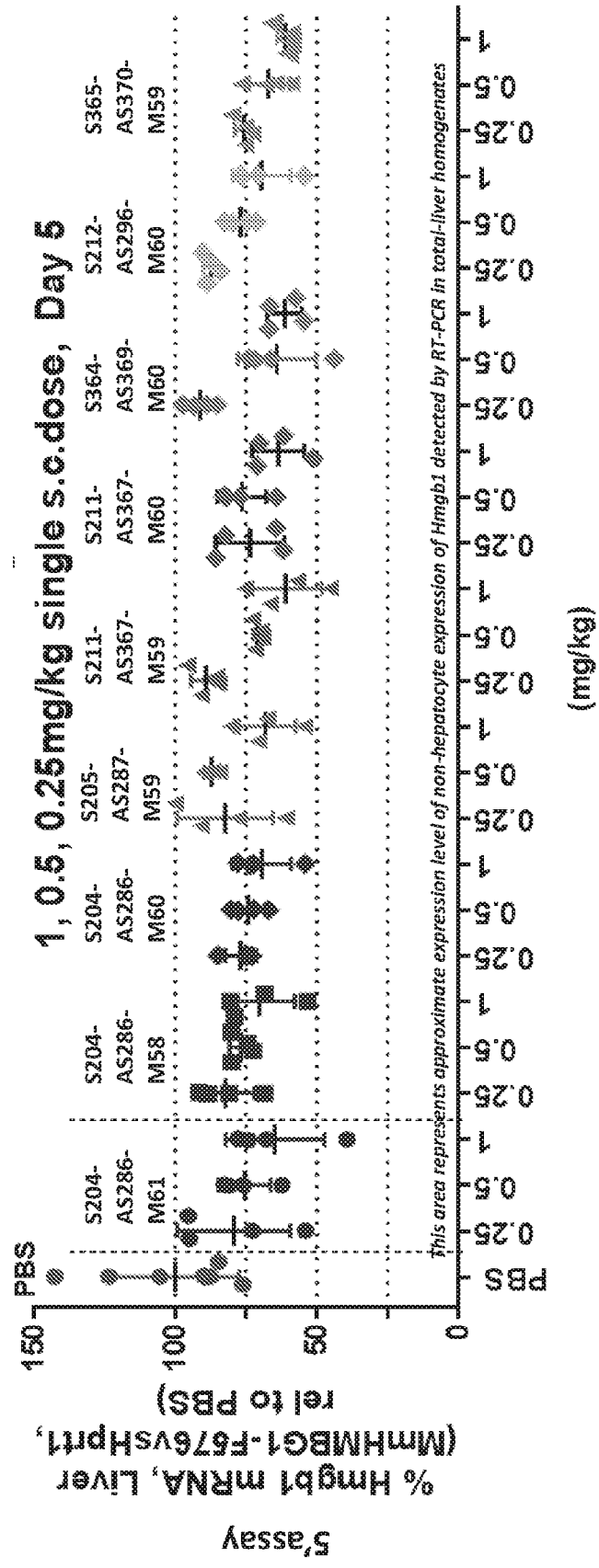

5'assay
% Hmgb1 mRNA, Liver
(MmHMBG1-F676vsHprt1, rel to PBS)
150
100
50
0
PBS
1, 0.5, 0.25mg/kg single s.c.dose, Day 5
S204-AS286-M61
S204-AS286-M58
S204-AS286-M60
S205-AS287-M59
S211-AS367-M59
S211-AS367-M60
S364-AS369-M60
S212-AS296-M60
S365-AS370-M59
this area represents approximate expression level of non-hepatocyte expression of Hmgb1 detected by RT-PCR in total-liver homogenates
(mg/kg)

FIG. 29B

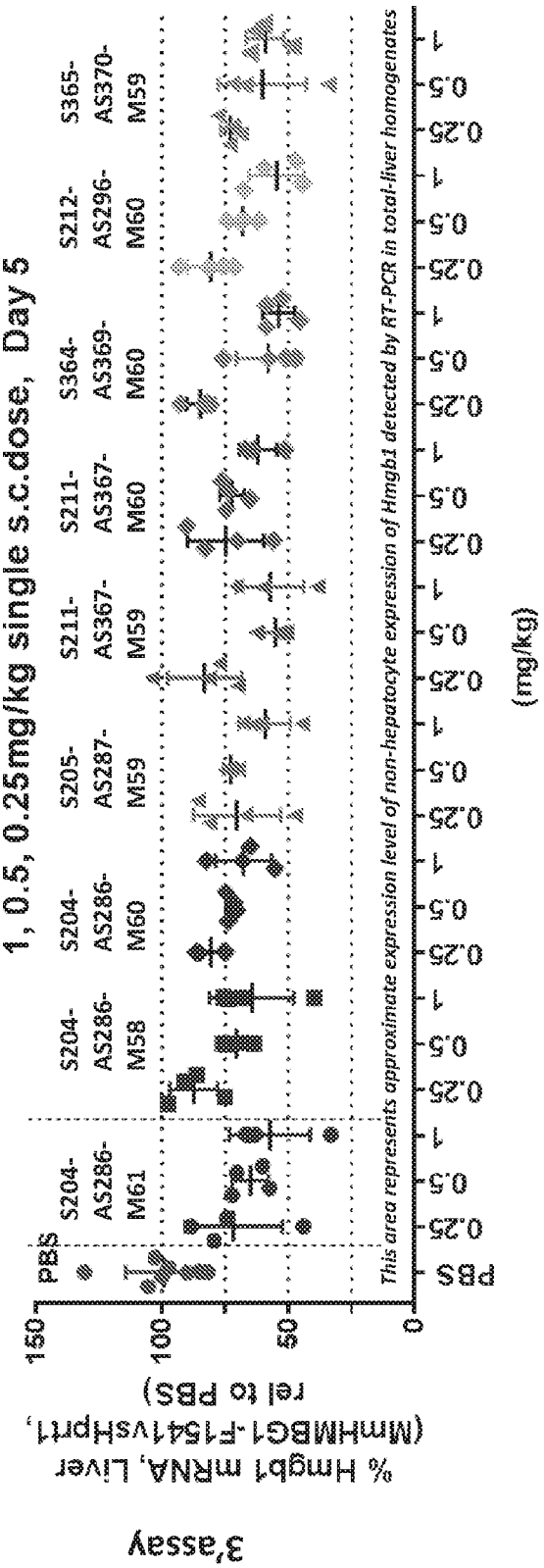

1, 0.5, 0.25mg/kg single s.c.dose, Day 5
3'assay
% Hmgb1 mRNA, Liver
(MmHMBG1-F1541vsHprt1,
rel to PBS)
150
100
50
0
PBS
S204-AS286-M61
S204-AS286-M58
S204-AS286-M60
S205-AS287-M59
S211-AS367-M59
S211-AS367-M60
S364-AS369-M60
S212-AS296-M60
S365-AS370-M59
0.25
0.5
1
This area represents approximate expression level of non-hepatocyte expression of Hmgb1 detected by RT-PCR in total-liver homogenates
(mg/kg)

FIG. 30A

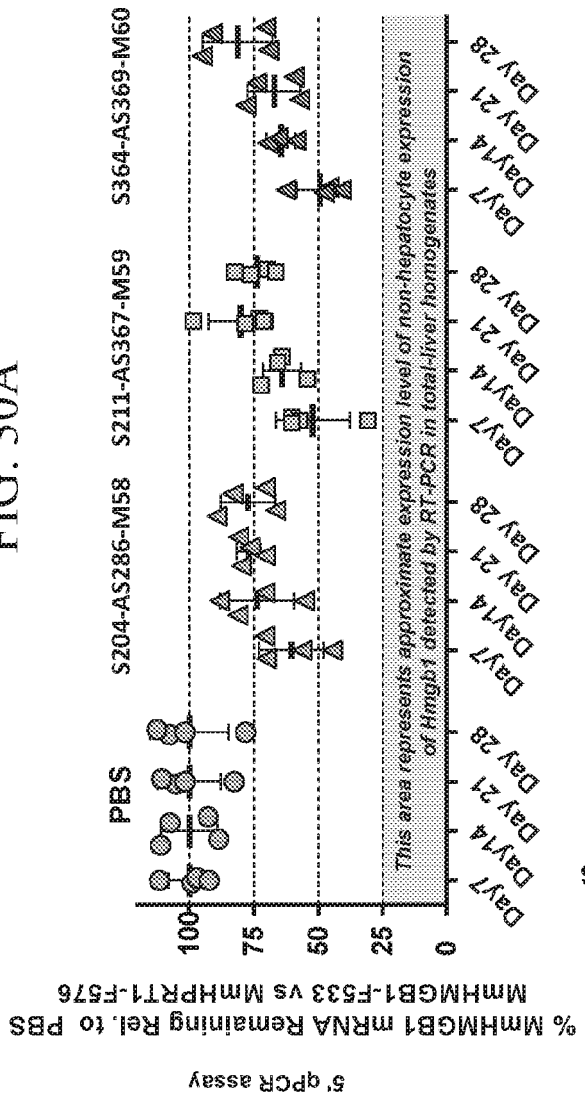


FIG. 30B

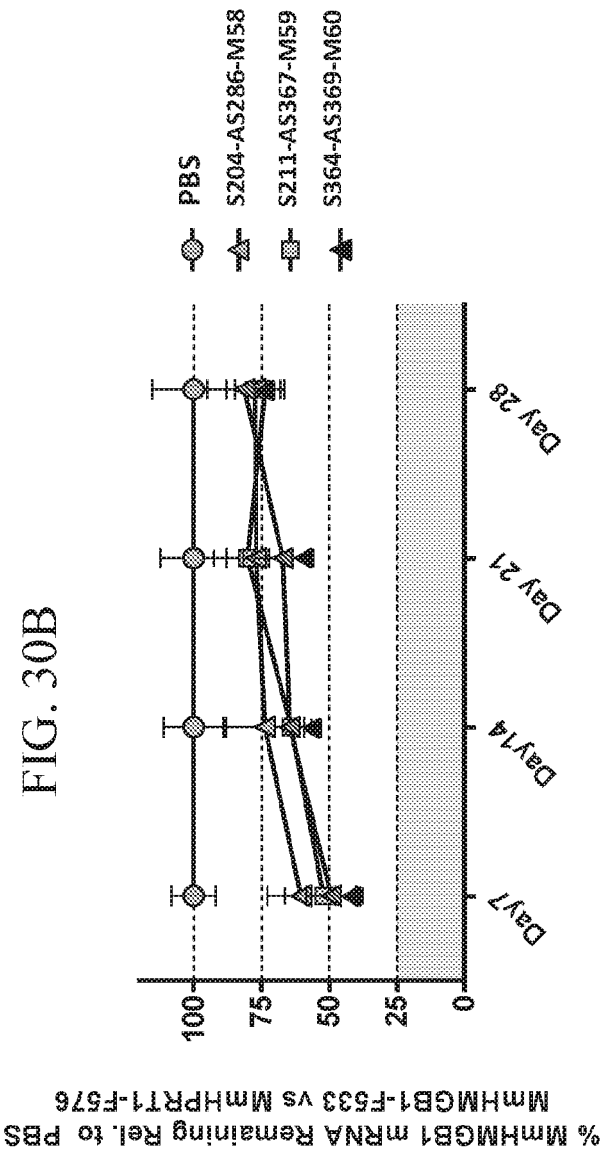


FIG. 30C

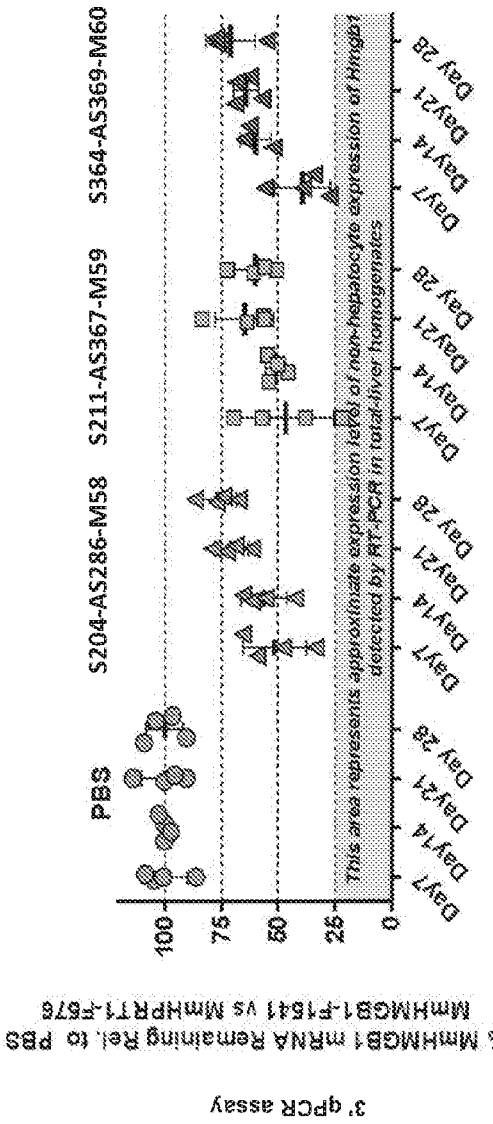


FIG. 30D

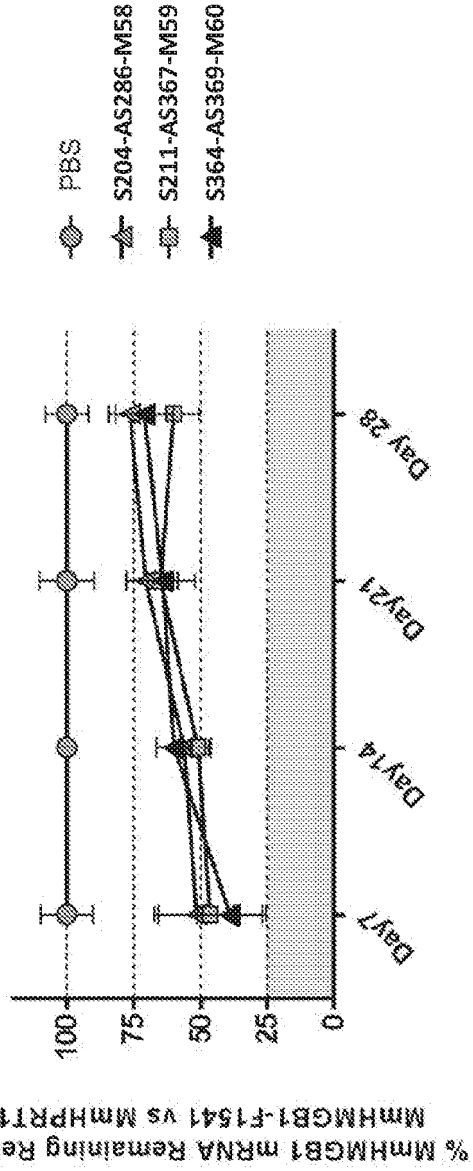


FIG. 31A

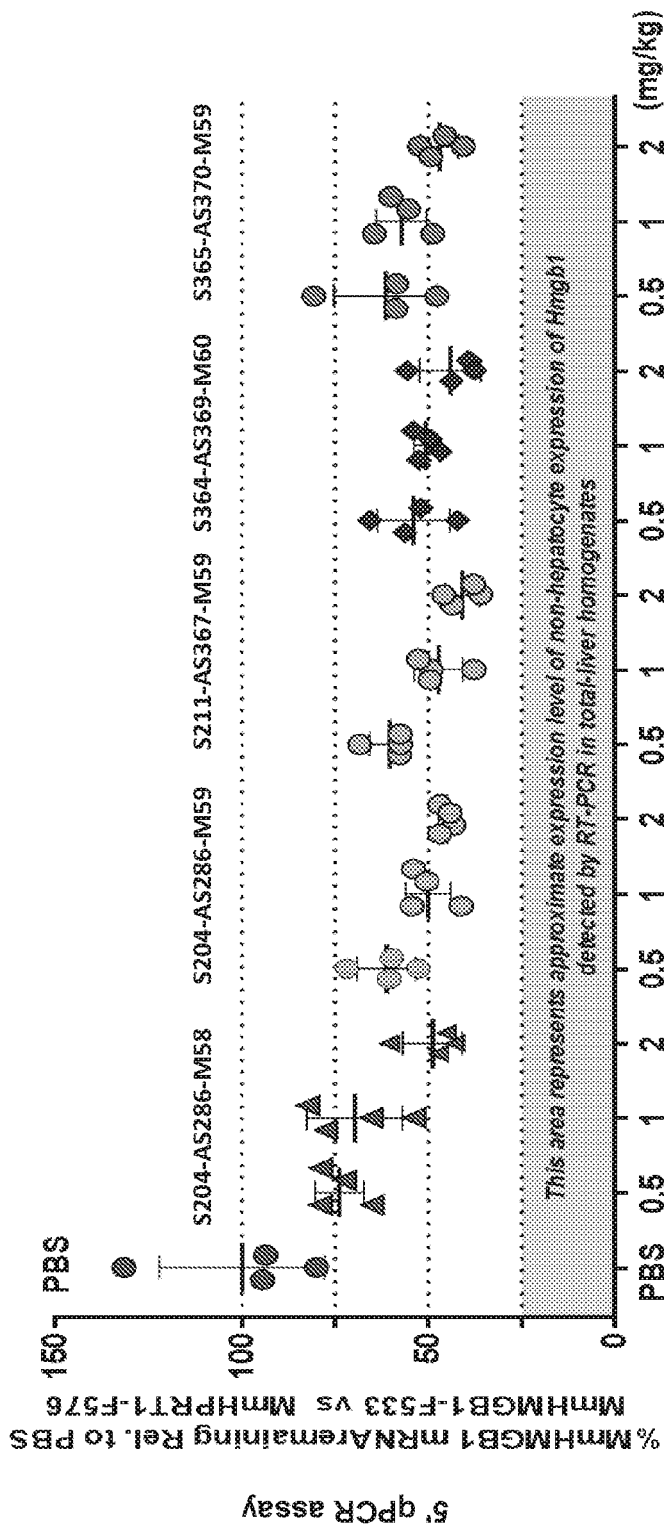

5' qPCR assay
%MmHMGB1 mRNAremaining Rel. to PBS
MmHMGB1-F533 vs MmHPRT1-F576
150
100
50
0
PBS
S204-AS286-M58
S204-AS286-M59
S211-AS367-M59
S364-AS369-M60
S365-AS370-M59
PBS 0.5 1 2 0.5 1 2 0.5 1 2 0.5 1 2 0.5 1 2 (mg/kg)
This area represents approximate expression level of non-hepatocyte expression of Hmgb1 detected by RT-PCR in total-liver homogenates

FIG. 31B

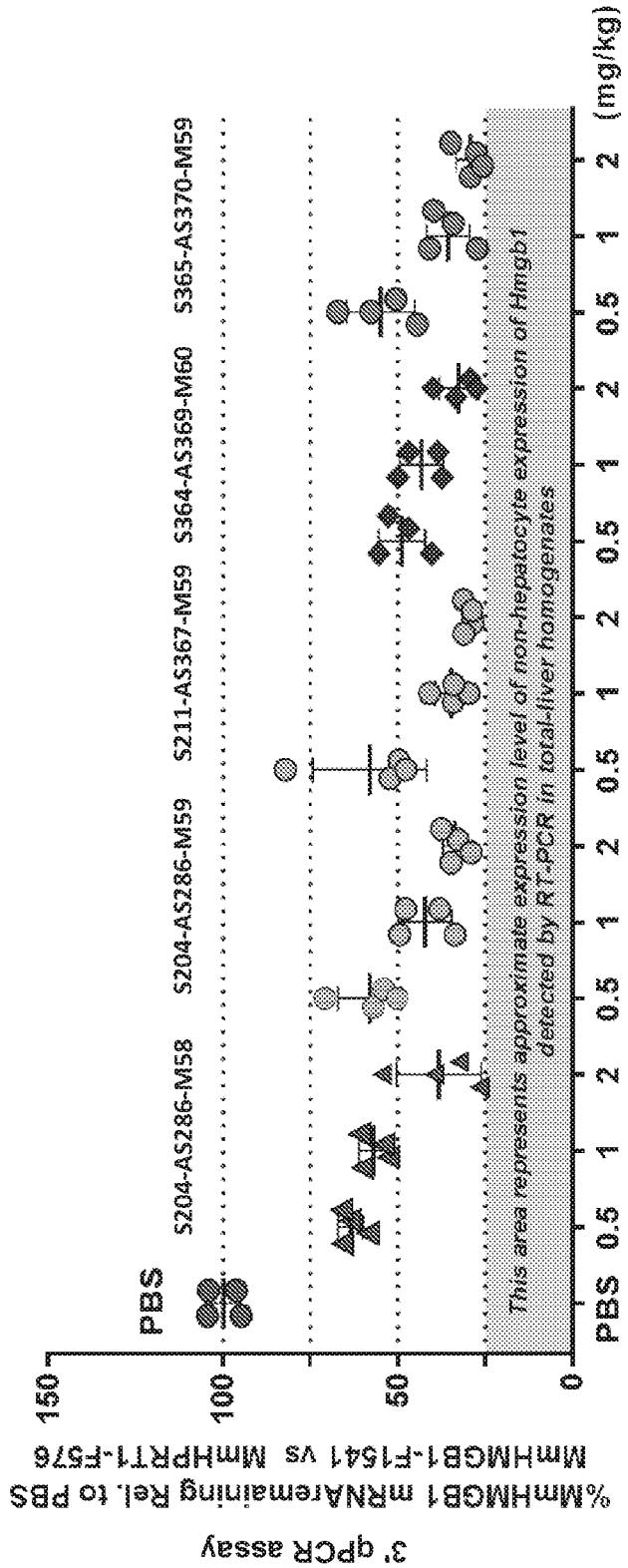

3' qPCR assay
%MmHMGB1 mRNAremaining Rel. to PBS
MmHMGB1-F1541 vs MmHPRT1-F576
150
100
50
0
PBS
S204-AS286-M58
S204-AS286-M59
S211-AS367-M59
S364-AS369-M60
S365-AS370-M59
PBS 0.5 1 2 0.5 1 2 0.5 1 2 0.5 1 2 (mg/kg)
This area represents approximate expression level of non-hepatocyte expression of Hmgb1
detected by RT-PCR in total-liver homogenates

FIG. 32A

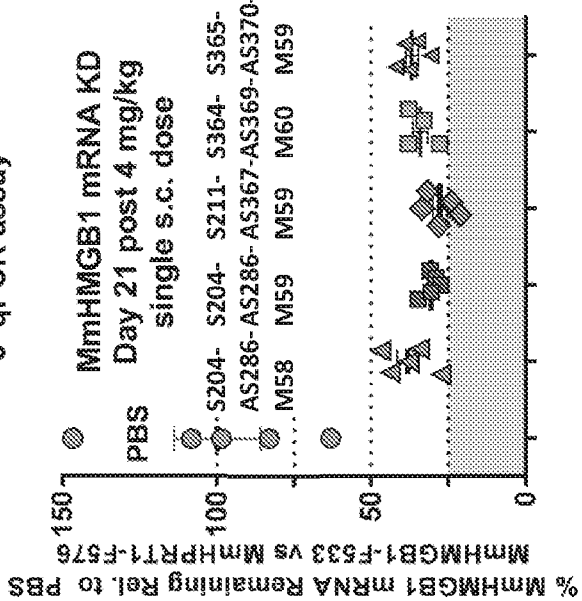


FIG. 32B

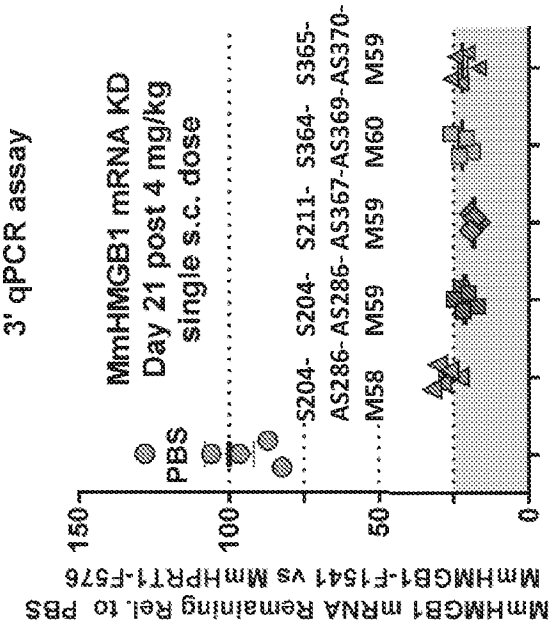


FIG. 33A

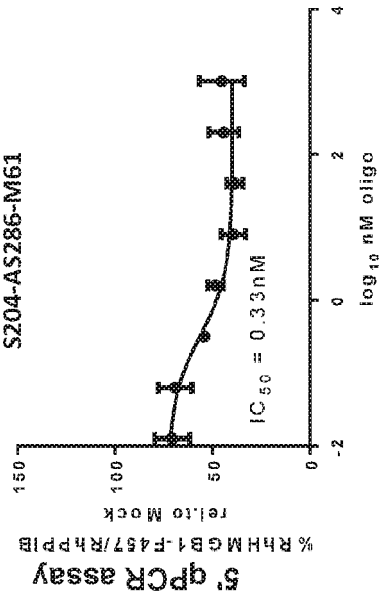


FIG. 33B

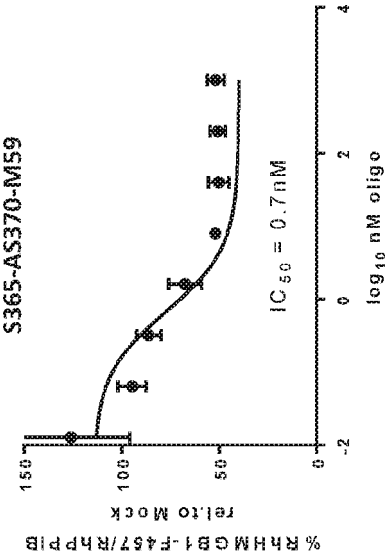


FIG. 33C

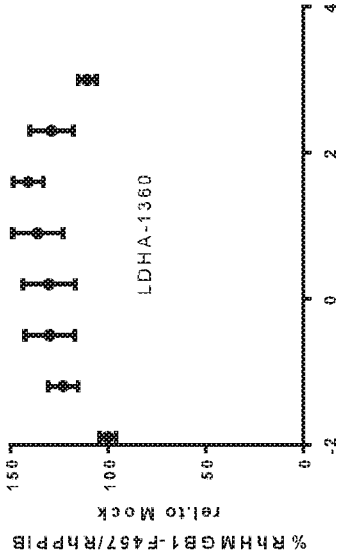


FIG. 33D

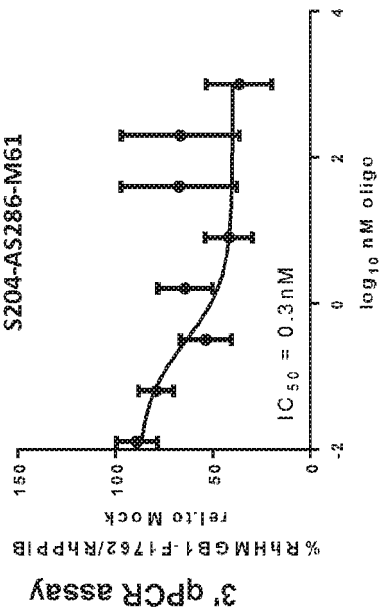


FIG. 33E

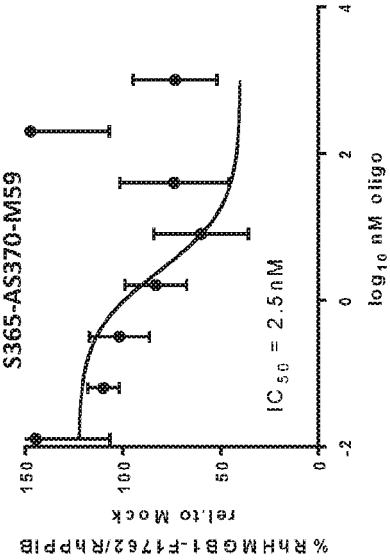


FIG. 33F

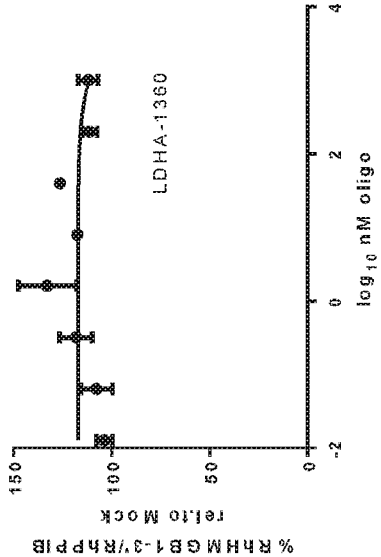


FIG. 33G

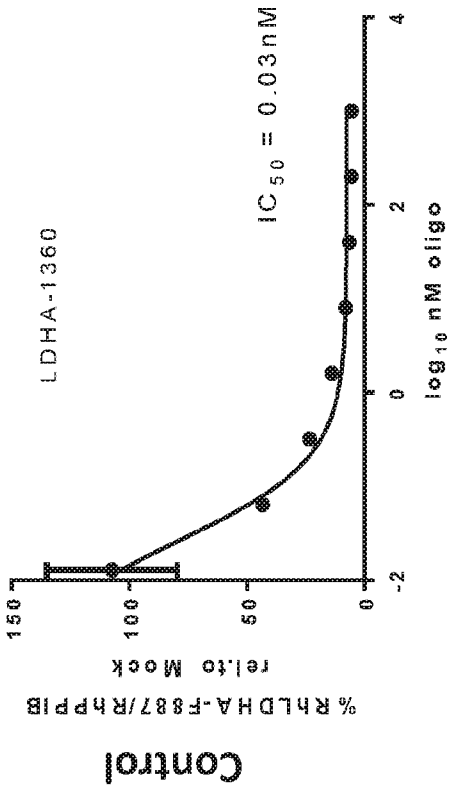

Control
%RhLDHA-F887/RhPPIB rel.to Mock
150
100
50
0
-2
0
2
4
log10 nM oligo
LDHA-1360
IC50 = 0.03nM

FIG. 34A

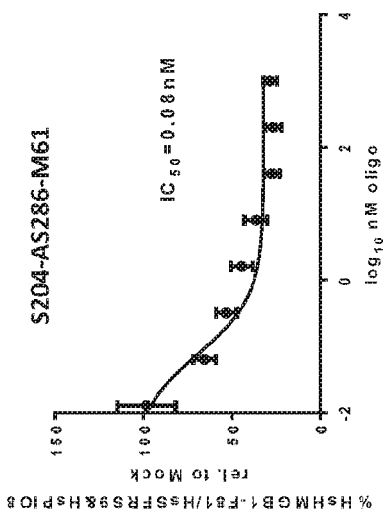


FIG. 34B

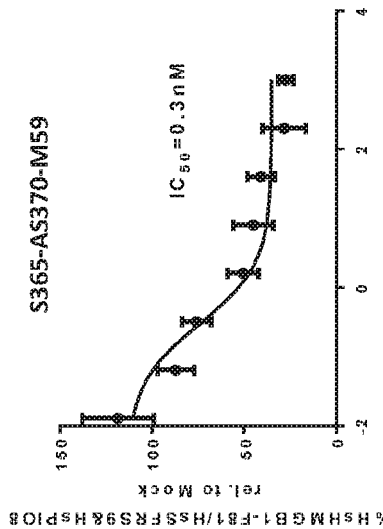


FIG. 34C

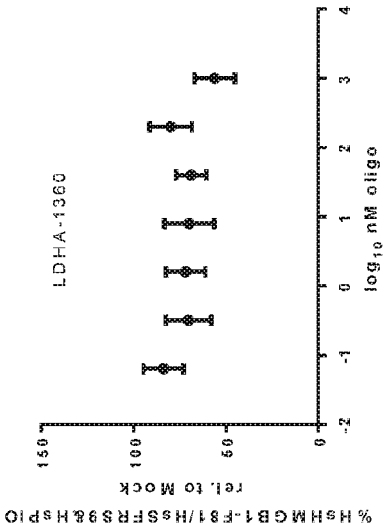


FIG. 34D

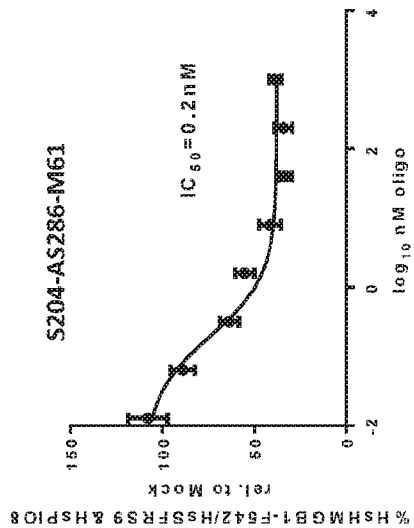


FIG. 34E

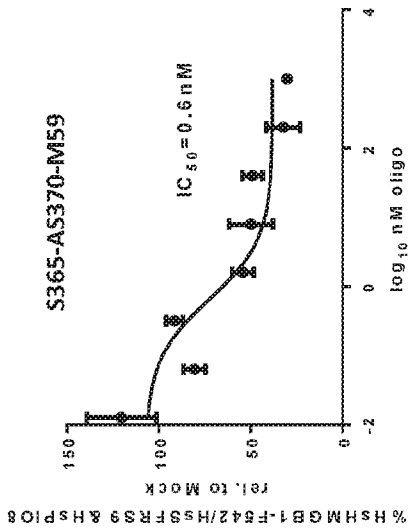


FIG. 34F

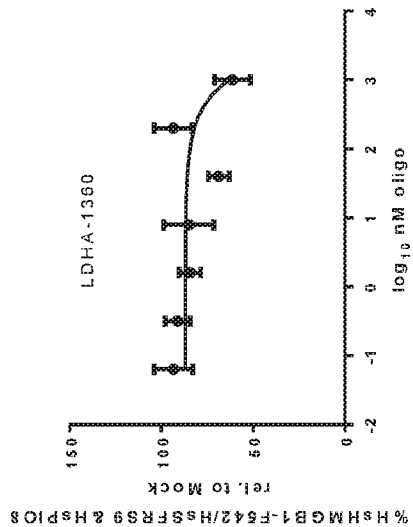
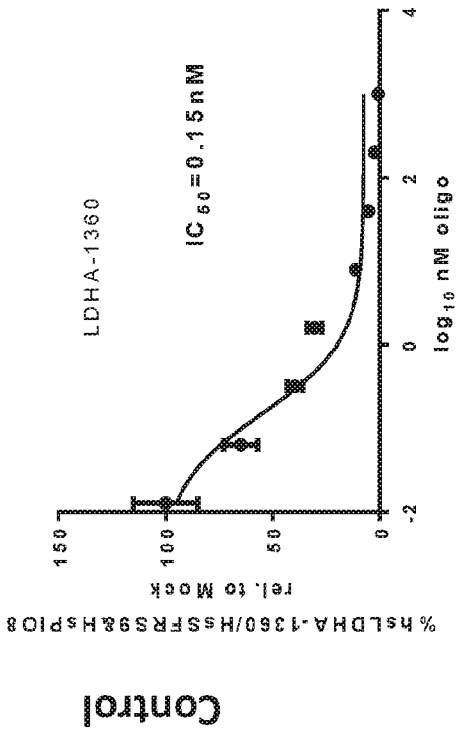


FIG. 34G


Control
%hsLDHA-1360/HssFRS98HsPIO8
rel. to Mock
150
100
50
0
-2
0
2
4
log10 nM oligo
LDHA-1360
IC60=0.15nM

SEQUENCE LISTING

<110> Dicerna Pharmaceuticals, Inc.

<120> COMPOSITIONS AND METHODS FOR INHIBITING HMGB1 EXPRESSION

<130> D0800.70002WO00

<140> Not Yet Assigned
<141> Concurrently Herewith

<150> US 62/526,971
<151> 2017-06-29

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<170> PatentIn version 3.5

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<212> DNA
<213> Artificial Sequence

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<223> Synthetic Polynucleotide

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