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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 April 2023 (27.04.2023)

WIPO | PCT

(10) International Publication Number
WO 2023/069754 A2

(51) International Patent Classification:
C12N 15/113 (2010.01) *A61K 31/712* (2006.01)
C12N 9/10 (2006.01) *A61K 31/7125* (2006.01)
A61K 31/713 (2006.01) *A61P 1/16* (2006.01)
A61K 31/7115 (2006.01)

(21) International Application Number:
PCT/US2022/047491

(22) International Filing Date:
21 October 2022 (21.10.2022)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/270,813 22 October 2021 (22.10.2021) US

(71) Applicant: **AMGEN INC.** [US/US]; One Amgen Center Drive, Thousand Oaks, California 91320-1799 (US).

(72) Inventors: **RULIFSON, Ingrid**; c/o Amgen Inc., One Amgen Center Drive, Law Department-Patent Operations, Mail Stop 28-5-A, Thousand Oaks, California 91320-1799 (US). **MEADE, Bryan**; c/o Amgen Inc., One Amgen Center Drive, Law Department-Patent Operations, Mail Stop 28-5-A, Thousand Oaks, California 91320-1799 (US). **LONG, Jason C.**; c/o Amgen Inc., One Amgen Center Drive, Law Department-Patent Operations, Mail Stop 28-5-A, Thousand Oaks, California 91320-1799 (US). **MURRAY, Justin K.**; c/o Amgen Inc., One Amgen Center Drive, Law Department-Patent Operations, Mail Stop 28-5-A, Thousand Oaks, California 91320-1799 (US).

(74) Agent: **KONG, Lawrence**; 1120 Veterans Blvd., South San Francisco, California 94080 (US).

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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

Published:
— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

WO 2023/069754 A2

(54) Title: RNAI CONSTRUCTS FOR INHIBITING GPAM EXPRESSION AND METHODS OF USE THEREOF

(57) Abstract: The disclosure relates to RNAi constructs, such as siRNA, for reducing expression of the GPAM gene. Methods of using such RNAi constructs to treat or prevent liver disease, such as nonalcoholic fatty liver disease (NAFLD), are also described.

RNAI CONSTRUCTS FOR INHIBITING GPAM EXPRESSION AND METHODS OF USE THEREOF

FIELD OF THE INVENTION

[0001] The present invention relates to compositions and methods for modulating liver expression of glycerol-3-phosphate acyltransferase, mitochondrial (GPAM). In particular, the present invention relates to nucleic acid-based therapeutics for reducing GPAM expression via RNA interference and methods of using such nucleic acid-based therapeutics to treat or prevent liver disease, such as nonalcoholic fatty liver disease (NAFLD).

BACKGROUND OF THE INVENTION

[0002] Comprising a spectrum of hepatic pathologies, nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world, the prevalence of which doubled in the last 20 years and now is estimated to affect over 20% of the world's population (Sattar et al. (2014) BMJ 349: g4596; Loomba and Sanyal (2013) Nature Reviews Gastroenterology & hepatology 10(11):686-690; Kim and Kim (2017) Clin Gastroenterol Hepatol 15(4):474-485; Petta et al. (2016) Dig Liver Dis 48(3):333-342; Huang et al. (2021) Nat Rev Gastro & Hepatology (18):223-238). NAFLD begins with the accumulation of triglyceride in the liver and is defined by the presence of cytoplasmic lipid droplets in more than 5% of hepatocytes in an individual 1) without a history of significant alcohol consumption and 2) in which the diagnosis of other types of liver disease have been excluded (Zhu et al (2016) World J Gastroenterol 22(36):8226-33; Rinella (2015) JAMA 313(22):2263-73; Yki-Jarvinen (2016) Diabetologia 59(6):1104-11). In some individuals the accumulation of ectopic fat in the liver, called steatosis, triggers inflammation and hepatocellular injury leading to a more advanced stage of disease called nonalcoholic steatohepatitis (NASH) (Rinella, *supra*). As of 2015, 75-100 million Americans are predicted to have NAFLD, with NASH accounting for approximately 10-30% of NAFLD diagnoses (Rinella, *supra*; Younossi et al (2016) Hepatology 64(5):1577-1586).

[0003] Glycerol-3-phosphate acyltransferase, mitochondrial (GPAM, GPAT1), having a sequence as found in Genbank XM_005269998.1, is associated with non-alcoholic steatohepatitis (NASH). Missense mutations in GPAM associate with accumulation of excess liver fat and non-alcoholic fatty liver disease (NAFLD) related phenotypes (Jamialahmadi, O., et al., Exome-Wide Association Study on Alanine Aminotransferase Identifies Sequence Variants in the GPAM and APOE Associated With Fatty Liver Disease. Gastroenterology, 2021. 160(5): p. 1634-1646 e7).

[0004] Currently, NAFLD symptoms are managed via weight loss and treatment of any secondary conditions, as no pharmacologic treatments have been approved. Thus, there is a need for compositions and methods that treat NAFLD in affected individuals.

SUMMARY OF THE INVENTION

[0005] The present disclosure provides an RNAi construct comprising a sense strand and an antisense strand, wherein the antisense strand comprises a region having a sequence that is complementary to a GPAM mRNA sequence, such as a GPAM mRNA sequence set forth in Table 1, and wherein the RNAi construct inhibits the expression of GPAM. In certain embodiments, the RNAi construct comprises a region having at least 15 contiguous nucleotides differing by no more than 3 nucleotides from an antisense sequence listed in Table 2. In some embodiments, the antisense strand hybridizes to a GPAM mRNA sequence listed in Table 1.

[0006] In some embodiments, the sense strand of the RNAi constructs described herein comprises a sequence that is sufficiently complementary to the sequence of the antisense strand to form a duplex region of about 15 to about 30 base pairs in length. In these and other embodiments, the sense and antisense strands each are about 15 to about 30 nucleotides in length. In some embodiments, the RNAi constructs comprise at least one blunt end. In other embodiments, the RNAi constructs comprise at least one nucleotide overhang. Such nucleotide overhangs may comprise at least 1 to 6 unpaired nucleotides and can be located at the 3' end of the sense strand, the 3' end of the antisense strand, or the 3' end of both the sense and antisense strand. In certain embodiments, the RNAi constructs comprise an overhang of two unpaired nucleotides at the 3' end of the sense strand and the 3' end of the antisense strand. In other embodiments, the RNAi constructs comprise an

overhang of two unpaired nucleotides at the 3' end of the antisense strand and a blunt end of the 3' end of the sense strand/5' end of the antisense strand.

[0007] The RNAi constructs of the invention may comprise one or more modified nucleotides, including nucleotides having modifications to the ribose ring, nucleobase, or phosphodiester backbone. In some embodiments, the RNAi constructs comprise one or more 2'-modified nucleotides. Such 2'-modified nucleotides can include 2'-fluoro modified nucleotides, 2'-O-methyl modified nucleotides, 2'-O-methoxyethyl modified nucleotides, 2'-O-allyl modified nucleotides, bicyclic nucleic acids (BNA), glycol nucleic acids (GNAs), inverted bases (e.g. inverted adenosine) or combinations thereof. In one particular embodiment, the RNAi constructs comprise one or more 2'-fluoro modified nucleotides, 2'-O-methyl modified nucleotides, or combinations thereof. In some embodiments, all of the nucleotides in the sense and antisense strand of the RNAi construct are modified nucleotides.

[0008] In some embodiments, the RNAi constructs comprise at least one backbone modification, such as a modified internucleotide or internucleoside linkage. In certain embodiments, the RNAi constructs described herein comprise at least one phosphorothioate internucleotide linkage. In particular embodiments, the phosphorothioate internucleotide linkages may be positioned at the 3' or 5' ends of the sense and/or antisense strands.

[0009] In some embodiments, the antisense strand and/or the sense strand of the RNAi constructs of the invention may comprise or consist of a sequence from the antisense and sense sequences listed in Table 2. In certain embodiments, the RNAi construct may be any one of the duplex compounds listed in Table 2.

[0010] The disclosure also provides a composition comprising the aforementioned RNAi construct and a pharmaceutically acceptable carrier, excipient, or diluent, as well as methods of reducing the expression of GPAM in a patient in need thereof comprising administering to the patient the aforementioned RNAi construct or composition.

DETAILED DESCRIPTION

[0011] The present invention is based, in part, on the design and generation of RNAi constructs that target the GPAM gene and reduce expression of GPAM in liver cells. The specific inhibition of GPAM expression is useful for treating or preventing conditions

associated with GPAM expression, including liver-related diseases, such as, for example, simple fatty liver (steatosis), nonalcoholic steatohepatitis (NASH), cirrhosis (irreversible, advanced scarring of the liver), or GPAM-related obesity.

[0012] The disclosure provides compositions and methods for regulating the expression of the glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) gene. In some embodiments, the gene may be within a cell or subject, such as a mammal (e.g., a human). In some embodiments, compositions of the invention comprise RNAi constructs that target a GPAM mRNA and reduce GPAM expression in a cell or mammal. Such RNAi constructs are useful for treating or preventing various forms of liver-related diseases, such as, for example, simple fatty liver (steatosis), nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), cirrhosis (irreversible, advanced scarring of the liver), or GPAM-related obesity.

[0013] Human genetic evidence indicates that a single nucleotide polymorphism (SNP) in GPAM, rs2792751(T), is "NAFLD-promoting." This SNP is a common, missense mutation resulting in an amino acid change in GPAM: Ile43Val. Carriers of this SNP exhibit increased magnetic resonance imaging proton density fat fraction (MRI-PDFF) and increased risk (odds ratio, OR) with fatty liver and all-cause cirrhosis. Carriers also exhibit increased serum total cholesterol, LDL, HDL, triglycerides (TG), ALT and ALP, increased neutrophil and sex hormone binding globulin levels (Haas, M.E., et al., Machine learning enables new insights into clinical significance of and genetic contributions to liver fat accumulation. 2020: medRxiv 2020.09.03.20187195; Jamialahmadi, O., et al., Exome-Wide Association Study on Alanine Aminotransferase Identifies Sequence Variants in the GPAM and APOE Associated With Fatty Liver Disease. Gastroenterology, 2021. 160(5): p. 1634-1646 e7; Hammond, L.E., et al., Mitochondrial glycerol-3-phosphate acyltransferase-deficient mice have reduced weight and liver triacylglycerol content and altered glycerolipid fatty acid composition. Mol Cell Biol, 2002. 22(23): p. 8204-14). Thus, the human data evidence indicates a correct directionality for a GPAM siRNA-mediated therapy to treat patients with NASH.

[0014] The genetics of GPAM are consistent with what is known about its mechanism of action and biology. The functional enzymatic role of GPAM is well

characterized (Gimeno, R.E. and J. Cao, Thematic review series: glycerolipids. Mammalian glycerol-3-phosphate acyltransferases: new genes for an old activity. J Lipid Res, 2008. 49(10): p. 2079-88; Gonzalez-Baro, M.R., T.M. Lewin, and R.A. Coleman, Regulation of Triglyceride Metabolism. II. Function of mitochondrial GPAT1 in the regulation of triacylglycerol biosynthesis and insulin action. Am J Physiol Gastrointest Liver Physiol, 2007. 292(5): p. G1195-9). Expressed predominantly in lipogenic tissues, GPAM protein is localized to the outer mitochondrial membrane and transfers acyl-CoA from glycerol-3-phosphate to lysophosphatidic acid, serving as the rate-limiting step responsible for initiation of the TG synthesis pathway. GPAM-deficient mice are viable, fertile, and exhibit no gross abnormalities (Hammond et al. (2002)). On a high fat diet, GPAM-deficient mice are protected from fat pad increase, liver TG accumulation, increased serum lipids, and exhibit increased hepatocyte β-oxidation, plasma ketone levels, and decreased TG synthesis (Hammond et al. (2002); Hammond, L.E., et al., Mitochondrial glycerol-3-phosphate acyltransferase-1 is essential in liver for the metabolism of excess acyl-CoAs. J Biol Chem, 2005. 280(27): p. 25629-36; Kuhajda, F.P., et al., Pharmacological glycerol-3-phosphate acyltransferase inhibition decreases food intake and adiposity and increases insulin sensitivity in diet-induced obesity. Am J Physiol Regul Integr Comp Physiol, 2011. 301(1): p. R116-30; Wendel, A.A., et al., Glycerol-3-phosphate acyltransferase 1 deficiency in ob/ob mice diminishes hepatic steatosis but does not protect against insulin resistance or obesity. Diabetes, 2010. 59(6): p. 1321-9; Xu, H., et al., Hepatic knockdown of mitochondrial GPAT1 in ob/ob mice improves metabolic profile. Biochem Biophys Res Commun, 2006. 349(1): p. 439-48).

[0015] A role for GPAM in preclinical nonalcoholic steatohepatitis (NASH) models has been described (Liao, K., et al., Glycerol-3-phosphate Acyltransferase1 Is a Model-Agnostic Node in Nonalcoholic Fatty Liver Disease: Implications for Drug Development and Precision Medicine. ACS Omega, 2020. 5(29): p. 18465-18471). Using three different animal models to induce increasing degrees of NASH and fibrosis, a direct correlation between increasing GPAM mRNA and protein expression with increasing NAFLD activity score (NAS) and fibrosis was observed. GPAM-deficient mice have also been shown to be protected from hepatocellular carcinoma (HCC) (Ellis, J.M., et al., Mice deficient in

glycerol-3-phosphate acyltransferase-1 have a reduced susceptibility to liver cancer. Toxicol Pathol, 2012. 40(3): p. 513-21). Thus, in addition to regulating steatosis, data suggests silencing GPAM in the liver may improve severe liver outcomes (Li, X., et al., Genomic analysis of liver cancer unveils novel driver genes and distinct prognostic features. Theranostics, 2018. 8(6): p. 1740-1751; Ng, C.K.Y., et al., Proteogenomic characterization of hepatocellular carcinoma. 2021: bioRxiv 2021.03.05.434147).

[0016] RNA interference (RNAi) is the process of introducing exogeneous RNA into a cell leading to specific degradation of the mRNA encoding the targeted protein with a resultant decrease in protein expression. Advances in both the RNAi technology and hepatic delivery, as well as growing positive outcomes with other RNAi-based therapies, suggest RNAi as a compelling means to therapeutically treat NAFLD by directly targeting GPAM.

[0017] As used herein, the term "RNAi construct" refers to an agent comprising an RNA molecule that is capable of downregulating expression of a target gene (e.g. GPAM) via an RNA interference mechanism when introduced into a cell. "RNA interference" is the process by which a nucleic acid molecule induces the cleavage and degradation of a target RNA molecule (e.g. messenger RNA or mRNA molecule) in a sequence-specific manner, e.g. through an RNA induced silencing complex (RISC) pathway. In some embodiments, the RNAi construct comprises a double-stranded RNA (dsRNA) molecule comprising two antiparallel strands of contiguous nucleotides that are sufficiently complementary to each other to hybridize to form a duplex region. A double-stranded RNAi construct also may be referred to as an RNAi "trigger." The terms "hybridize" or "hybridization" refer to the pairing of complementary polynucleotides, typically via hydrogen bonding (e.g., Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding) between complementary bases in the two polynucleotides. The strand comprising a region having a sequence that is substantially complementary to a target sequence (e.g., target mRNA) is referred to as the "antisense strand." The "sense strand" refers to the strand that includes a region that is substantially complementary to a region of the antisense strand. In some embodiments, the sense strand may comprise a region that has a sequence that is substantially identical to the target sequence.

[0018] In certain embodiments, the sense strand and antisense strand of the double-stranded RNA may be two separate molecules that hybridize to form a duplex region but are otherwise unconnected. Such double-stranded RNA molecules formed from two separate strands are referred to as "small interfering RNAs" or "short interfering RNAs" (siRNAs). siRNAs are a class of non-coding, double-stranded RNA molecules that are typically about 20-27 base pairs and are central to RNAi. Thus, in some embodiments, the RNAi constructs of the invention comprise an siRNA. In other embodiments, the RNAi construct may be a microRNA (also known as "miRNA" or "mature miRNA"). miRNAs are small (approximately 18-24 nucleotides in length), non-coding RNA molecules present in plants, animals, and some viruses. miRNAs resemble siRNA, but miRNAs originate from hairpin mRNA structures. miRNAs regulate gene expression by base-pairing to complementary regions of target mRNAs.

[0019] In some embodiments, the invention is an RNAi construct directed to GPAM. In some embodiments, the RNAi construct is an siRNA that comprises a sense strand and an antisense strand, wherein the antisense strand comprises a region that is complementary to GPAM mRNA sequence. The region of the RNAi antisense strand may be complementary to any suitable region of a GPAM mRNA sequence.

[0020] In some embodiments, the RNAi construct binds the GPAM rs2792751(T) site. The disclosed RNAi construct, however, is not required to hybridize to a particular GPAM SNP. In some embodiments, the RNAi construct is an siRNA molecule that contains any of the sequences set forth in Table 1 or 2.

[0021] A double-stranded RNAi molecule may include chemical modifications to ribonucleotides, including modifications to the ribose sugar, base, or backbone components of the ribonucleotides, such as those described herein or known in the art. Any such modifications, as used in a double-stranded RNA molecule (e.g. siRNA, shRNA, or the like), are encompassed by the term "double-stranded RNA" for the purposes of this disclosure.

[0022] As used herein, a first sequence is "complementary" to a second sequence if a polynucleotide comprising the first sequence can hybridize to a polynucleotide comprising the second sequence to form a duplex region under certain conditions, such as physiological

conditions. Other such conditions can include moderate or stringent hybridization conditions, which are known to those of skill in the art. A first sequence is considered to be fully complementary (100% complementary) to a second sequence if a polynucleotide comprising the first sequence base pairs with a polynucleotide comprising the second sequence over the entire length of one or both nucleotide sequences without any mismatches. A sequence is "substantially complementary" to a target sequence if the sequence is at least about 80%, 85%, 90%, 95%,96%, 97%, 98%, 99% or 100% complementary to a target sequence. Percent complementarity can be calculated by dividing the number of bases in a first sequence that are complementary to bases at corresponding positions in a second or target sequence by the total length of the first sequence. A sequence may also be said to be substantially complementary to another sequence if there are no more than 5, 4, 3, 2, or 1 mismatch over a 30 base pair duplex region when the two sequences are hybridized. Generally, if any nucleotide overhangs, as defined herein, are present, the sequence of such overhangs is not considered in determining the degree of complementarity between two sequences. By way of example, a sense strand of 21 nucleotides in length and an antisense strand of 21 nucleotides in length that hybridize to form a 19 base pair duplex region with a 2 nucleotide overhang at the 3' end of each strand would be considered to be fully complementary as the term is used herein.

[0023] In some embodiments, a region of the antisense strand comprises a sequence that is fully complementary to a region of the target RNA sequence (e.g. GPAM mRNA). In such embodiments, the sense strand may comprise a sequence that is fully complementary to the sequence of the antisense strand. In other such embodiments, the sense strand may comprise a sequence that is substantially complementary to the sequence of the antisense strand, e.g., having 1, 2, 3, 4, or 5 mismatches in the duplex region formed by the sense and antisense strands. In certain embodiments, it is preferred that any mismatches occur within the terminal regions (e.g. within 6, 5, 4, 3, 2, or 1 nucleotides of the 5' and/or 3' ends of the strands). In one embodiment, any mismatches in the duplex region formed from the sense and antisense strands desirably occur within 6, 5, 4, 3, 2, or 1 nucleotides of the 5' end of the antisense strand.

[0024] Where the two substantially complementary strands of a dsRNA are comprised of separate RNA molecules, those molecules need not, but can be, covalently connected. Where the two strands are connected covalently by means other than an uninterrupted chain of nucleotides between the 3 '-end of one strand and the 5' -end of the respective other strand forming the duplex structure, the connecting structure is referred to as a "linker." The RNA strands may have the same or a different number of nucleotides. The maximum number of base pairs in the duplex is the number of nucleotides in the shortest strand of the dsRNA minus any overhangs that are present in the duplex. In addition to the duplex structure, an RNAi may comprise one or more nucleotide overhangs.

[0025] In other embodiments, the sense strand and the antisense strand that hybridize to form a duplex region may be part of a single RNA molecule, i.e., the sense and antisense strands are part of a self-complementary region of a single RNA molecule. In such cases, a single RNA molecule comprises a duplex region (also referred to as a stem region) and a loop region. The 3' end of the sense strand is connected to the 5' end of the antisense strand by a contiguous sequence of unpaired nucleotides, which will form the loop region. The loop region is typically of a sufficient length to allow the RNA molecule to fold back on itself such that the antisense strand can base pair with the sense strand to form the duplex or stem region. The loop region can comprise from about 3 to about 25, from about 5 to about 15, or from about 8 to about 12 unpaired nucleotides. As noted herein, such RNA molecules with at least partially self-complementary regions are referred to as "short hairpin RNAs" (shRNAs). In some embodiments, the loop region can comprise at least 1, 2, 3, 4, 5, 10, 20, or 25 unpaired nucleotides. In other embodiments, the loop region can have 10, 9, 8, 7, 6, 5, 4, 3, 2, or fewer unpaired nucleotides. In certain embodiments, the RNAi constructs of the invention comprise an shRNA. The length of a single, at least partially self-complementary RNA molecule can be from about 35 nucleotides to about 100 nucleotides, from about 45 nucleotides to about 85 nucleotides, or from about 50 to about 60 nucleotides and comprise a duplex region and loop region each having the lengths recited herein.

[0026] In some embodiments, the RNAi constructs of the invention comprise a sense strand and an antisense strand, wherein the antisense strand comprises a region having a sequence that is substantially or fully complementary to a GPAM messenger RNA (mRNA)

sequence. As used herein, a "GPAM mRNA sequence" refers to any messenger RNA sequence, including splice variants, encoding a GPAM protein, including GPAM protein variants or isoforms from any species (e.g. mouse, rat, non-human primate, human). GPAM protein is also known as GPAT or GPAT1.

[0027] A GPAM mRNA sequence also includes the transcript sequence expressed as its complementary DNA (cDNA) sequence. A cDNA sequence refers to the sequence of an mRNA transcript expressed as DNA bases (e.g. guanine, adenine, thymine, and cytosine) rather than RNA bases (e.g. guanine, adenine, uracil, and cytosine). Thus, the antisense strand of the RNAi constructs of the invention may comprise a region having a sequence that is substantially or fully complementary to a target GPAM mRNA sequence or GPAM cDNA sequence. A GPAM mRNA or cDNA sequence can include, but is not limited to, any GPAM mRNA or cDNA sequence such as can be derived from the NCBI Reference sequence NM_001244949.2 or NM_020918.6.

[0028] A region of the antisense strand can be substantially complementary or fully complementary to at least 15 consecutive nucleotides of the GPAM mRNA sequence. In some embodiments, the target region of the GPAM mRNA sequence to which the antisense strand comprises a region of complementarity can range from about 15 to about 30 consecutive nucleotides, from about 16 to about 28 consecutive nucleotides, from about 18 to about 26 consecutive nucleotides, from about 17 to about 24 consecutive nucleotides, from about 19 to about 25 consecutive nucleotides, from about 19 to about 23 consecutive nucleotides, or from about 19 to about 21 consecutive nucleotides. In certain embodiments, the region of the antisense strand comprising a sequence that is substantially or fully complementary to a GPAM mRNA sequence may, in some embodiments, comprise at least 15 contiguous nucleotides from an antisense sequence listed in Table 2. In other embodiments, the antisense sequence comprises at least 16, at least 17, at least 18, or at least 19 contiguous nucleotides from an antisense sequence listed in Table 2. In some embodiments, the sense and/or antisense sequence comprises at least 15 nucleotides from a sequence listed in Table 2 with no more than 1, 2, or 3 nucleotide mismatches.

[0029] The sense strand of the RNAi construct typically comprises a sequence that is sufficiently complementary to the sequence of the antisense strand such that the two strands

hybridize under physiological conditions to form a duplex region. A "duplex region" refers to the region in two complementary or substantially complementary polynucleotides that form base pairs with one another, either by Watson-Crick base pairing or other hydrogen bonding interaction, to create a duplex between the two polynucleotides. The duplex region of the RNAi construct should be of sufficient length to allow the RNAi construct to enter the RNA interference pathway, e.g. by engaging the Dicer enzyme and/or the RISC complex (described below). For instance, in some embodiments, the duplex region is about 15 to about 30 base pairs in length. Other lengths for the duplex region within this range are also suitable, such as about 15 to about 28 base pairs, about 15 to about 26 base pairs, about 15 to about 24 base pairs, about 15 to about 22 base pairs, about 17 to about 28 base pairs, about 17 to about 26 base pairs, about 17 to about 24 base pairs, about 17 to about 23 base pairs, about 17 to about 21 base pairs, about 19 to about 25 base pairs, about 19 to about 23 base pairs, or about 19 to about 21 base pairs. In one embodiment, the duplex region is about 17 to about 24 base pairs in length. In another embodiment, the duplex region is about 19 to about 21 base pairs in length.

[0030] In some embodiments, an RNAi construct of the invention contains a duplex region of about 24 to about 30 nucleotides that interacts with a target RNA sequence, e.g., an GPAM target mRNA sequence, to direct the cleavage of the target RNA. Without wishing to be bound by theory, long double-stranded RNA introduced into cells can be broken down into siRNA by a Type III endonuclease known as Dicer (Sharp et al. (2001) Genes Dev. 15:485). Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs (Bernstein, et al., (2001) Nature 409:363). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, et al., (2001) Cell107:309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleave the target to induce silencing (Elbashir, et al., (2001) Genes Dev. 15: 188).

[0031] For embodiments in which the sense strand and antisense strand are two separate molecules (e.g., an siRNA RNAi construct), the sense strand and antisense strand need not be the same length as the length of the duplex region. For instance, one or both

strands maybe longer than the duplex region and have one or more unpaired nucleotides or mismatches flanking the duplex region. Thus, in some embodiments, the RNAi construct comprises at least one nucleotide overhang. As used herein, a "nucleotide overhang" refers to the unpaired nucleotide or nucleotides that extend beyond the duplex region at the terminal ends of the strands. Nucleotide overhangs are typically created when the 3' end of one strand extends beyond the 5' end of the other strand or when the 5' end of one strand extends beyond the 3' end of the other strand. The length of a nucleotide overhang is generally between 1 and 6 nucleotides, 1 and 5 nucleotides, 1 and 4 nucleotides, 1 and 3 nucleotides, 2 and 6 nucleotides, 2 and 5 nucleotides, or 2 and 4 nucleotides. In some embodiments, the nucleotide overhang comprises 1, 2, 3, 4, 5, or 6 nucleotides. In one particular embodiment, the nucleotide overhang comprises 1 to 4 nucleotides. In certain embodiments, the nucleotide overhang comprises 2 nucleotides. The nucleotides in the overhang can be ribonucleotides, deoxyribonucleotides, or modified nucleotides as described herein. In some embodiments, the overhang comprises a 5'-uridineuridine-3' (5'-UU-3') dinucleotide. In such embodiments, the UU dinucleotide may comprise ribonucleotides or modified nucleotides, e.g., 2'-modified nucleotides. In other embodiments, the overhang comprises a 5'-deoxythymidine-deoxythymidine-3' (5'-dTdT-3') dinucleotide.

[0032] The nucleotide overhang can be at the 5' end or 3' end of one or both strands. For example, in one embodiment, the RNAi construct comprises a nucleotide overhang at the 5' end and the 3' end of the antisense strand. In another embodiment, the RNAi construct comprises a nucleotide overhang at the 5' end and the 3' end of the sense strand. In some embodiments, the RNAi construct comprises a nucleotide overhang at the 5' end of the sense strand and the 5' end of the antisense strand. In other embodiments, the RNAi construct comprises a nucleotide overhang at the 3' end of the sense strand and the 3' end of the antisense strand.

[0033] The RNAi constructs may comprise a single nucleotide overhang at one end of the double-stranded RNA molecule and a blunt end at the other. A "blunt end" means that the sense strand and antisense strand are fully base-paired at the end of the molecule and there are no unpaired nucleotides that extend beyond the duplex region. In some embodiments, the RNAi construct comprises a nucleotide overhang at the 3' end of the sense

strand and a blunt end at the 5' end of the sense strand and 3' end of the antisense strand. In other embodiments, the RNAi construct comprises a nucleotide overhang at the 3' end of the antisense strand and a blunt end at the 5' end of the antisense strand and the 3' end of the sense strand. In certain embodiments, the RNAi construct comprises a blunt end at both ends of the double-stranded RNA molecule. In such embodiments, the sense strand and antisense strand have the same length and the duplex region is the same length as the sense and antisense strands (i.e., the molecule is double-stranded over its entire length).

[0034] The sense strand and antisense strand can each independently be any suitable length, such as about 15 to about 30 nucleotides in length, about 18 to about 28 nucleotides in length, about 19 to about 27 nucleotides in length, about 19 to about 25 nucleotides in length, about 19 to about 23 nucleotides in length, about 21 to about 25 nucleotides in length, or about 21 to about 23 nucleotides in length. In certain embodiments, the sense strand and antisense strand are each about 18, about 19, about 20, about 21, about 22, about 23, about 24, or about 25 nucleotides in length. In some embodiments, the sense strand and antisense strand are of the same length but form a duplex region that is shorter than the strands such that the RNAi construct has two nucleotide overhangs. For instance, in one embodiment, the RNAi construct comprises (i) a sense strand and an antisense strand that are each 21 nucleotides in length, (ii) a duplex region that is 19 base pairs in length, and (iii) nucleotide overhangs of 2 unpaired nucleotides at both the 3' end of the sense strand and the 3' end of the antisense strand. In another embodiment, the RNAi construct comprises (i) a sense strand and an antisense strand that are each 23 nucleotides in length, (ii) a duplex region that is 21 base pairs in length, and (iii) nucleotide overhangs of 2 unpaired nucleotides at both the 3' end of the sense strand and the 3' end of the antisense strand. In other embodiments, the sense strand and antisense strand have the same length and form a duplex region over their entire length such that there are no nucleotide overhangs on either end of the double-stranded molecule. In one such embodiment, the RNAi construct is blunt ended and comprises (i) a sense strand and an antisense strand, each of which is 21 nucleotides in length, and (ii) a duplex region that is 21 base pairs in length. In another embodiment, the RNAi construct is blunt ended and comprises (i) a sense strand and an antisense strand, each of which is 23 nucleotides in length, and (ii) a duplex region that is 23 base pairs in length.

[0035] In other embodiments, the sense strand or the antisense strand is longer than the other strand and the two strands form a duplex region having a length equal to that of the shorter strand such that the RNAi construct comprises at least one nucleotide overhang. For example, in one embodiment, the RNAi construct comprises (i) a sense strand that is 19 nucleotides in length, (ii) an antisense strand that is 21 nucleotides in length, (iii) a duplex region of 19 base pairs in length, and (iv) a single nucleotide overhang of 2 unpaired nucleotides at the 3' end of the antisense strand. In another embodiment, the RNAi construct comprises (i) a sense strand that is 21 nucleotides in length, (ii) an antisense strand that is 23 nucleotides in length, (iii) a duplex region of 21 base pairs in length, and (iv) a single nucleotide overhang of 2 unpaired nucleotides at the 3' end of the antisense strand.

[0036] The antisense strand of the RNAi constructs of the invention can comprise the sequence of any one of the antisense sequences listed in Table 2.

Modified Nucleotides

[0037] The RNAi constructs of the invention may comprise one or more modified nucleotides. A "modified nucleotide" refers to a nucleotide that has one or more chemical modifications to the nucleoside, nucleobase, pentose ring, or phosphate group. As used herein, modified nucleotides do not encompass ribonucleotides containing adenosine monophosphate, guanosine monophosphate, uridine monophosphate, and cytidine monophosphate, and deoxyribonucleotides containing deoxyadenosine monophosphate, deoxyguanosine monophosphate, deoxythymidine monophosphate, and deoxycytidine monophosphate. However, the RNAi constructs may comprise combinations of modified nucleotides, ribonucleotides, and deoxyribonucleotides. Incorporation of modified nucleotides into one or both strands of double-stranded RNA molecules can improve the *in vivo* stability of the RNA molecules, e.g., by reducing the molecules' susceptibility to nucleases and other degradation processes. The potency of RNAi constructs for reducing expression of the target gene can also be enhanced by incorporation of modified nucleotides.

[0038] In certain embodiments, the modified nucleotides have a modification of the ribose sugar. These sugar modifications can include modifications at the 2' and/or 5' position of the pentose ring as well as bicyclic sugar modifications. A 2'-modified

nucleotide refers to a nucleotide having a pentose ring with a substituent at the 2' position other than H or OH. Such 2' modifications include, but are not limited to, 2'-O-alkyl (e.g. O-C1-C10 or O-C1-C10 substituted alkyl), 2'-O-allyl (O-CH2CH=CH2), 2'-C-allyl, 2'-fluoro, 2'-O-methyl (OCH3), 2'-O-methoxyethyl (O-(CH2)2OCH3), 2'-OCF3, 2'-O(CH2)2SCH3, 2'-O-aminoalkyl, 2'-amino (e.g., NH_2), 2'-O-ethylamine, and 2'-azido. Modifications at the 5' position of the pentose ring include, but are not limited to, 5'-methyl (R or S); 5'-vinyl, and 5'-methoxy.

[0039] A "bicyclic sugar modification" refers to a modification of the pentose ring where a bridge connects two atoms of the ring to form a second ring resulting in a bicyclic sugar structure. In some embodiments, the bicyclic sugar modification comprises a bridge between the 4' and 2' carbons of the pentose ring. Nucleotides comprising a sugar moiety with a bicyclic sugar modification are referred to herein as "bicyclic nucleic acids" or "BNAs." Exemplary bicyclic sugar modifications include, but are not limited to, α-L-Methyleneoxy (4'- CH_2-O-2') bicyclicnucleic acid (BNA); β-D-Methyleneoxy (4'-CH_2-O-2') BNA (also referred to as a locked nucleic acid or LNA); Ethyleneoxy (4'-(CH_2)2-O-2') BNA; Aminooxy (4'- CH_2-O-N(R)- 2')BNA; Oxyamino (4'- CH_2-N(R)-O-2') BNA; Methyl(methyleneoxy) (4'-$CH(CH_3)$-O-2') BNA (also referred to as constrained ethyl or cEt); methylene-thio (4'- CH_2-S-2') BNA; methylene-amino (4'- CH_2-N(R)-2') BNA; methyl carbocyclic (4'- CH_2-CH(CH3)-2') BNA; propylene carbocyclic (4'-(CH_2)3-2') BNA; and Methoxy(ethyleneoxy) (4'-$CH(CH_2OMe)$-O-2')BNA (also referred to as constrained MOE or cMOE). These and other sugar-modified nucleotides that can be incorporated into the RNAi constructs of the invention are described in, e.g., U.S. Patent 9,181,551, U.S. Patent Publication No. 2016/0122761, and Deleavey and Damha, *Chemistry and Biology*, *19*: 937-954 (2012.

[0040] In some embodiments, the RNAi constructs comprise one or more 2'-fluoro modified nucleotides, 2'-O-methyl modified nucleotides, 2'-O-methoxyethyl modified nucleotides, 2'-O-allyl modified nucleotides, bicyclic nucleic acids (BNAs), or combinations thereof. In certain embodiments, the RNAi constructs comprise one or more 2'-fluoro modified nucleotides, 2'-O-methyl modified nucleotides, 2'-O-methoxyethyl modified nucleotides, or combinations thereof. In one particular embodiment, the RNAi constructs

comprise one or more 2'-fluoro modified nucleotides, 2'-O-methyl modified nucleotides, or combinations thereof.

[0041] Both the sense and antisense strands of the RNAi constructs can comprise one or multiple modified nucleotides. For instance, in some embodiments, the sense strand comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more modified nucleotides. In certain embodiments, all nucleotides in the sense strand are modified nucleotides. In some embodiments, the antisense strand comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more modified nucleotides. In other embodiments, all nucleotides in the antisense strand are modified nucleotides. In certain other embodiments, all nucleotides in the sense strand and all nucleotides in the antisense strand are modified nucleotides. In these and other embodiments, the modified nucleotides can be 2'-fluoro modified nucleotides, 2'-O-methyl modified nucleotides, or combinations thereof.

[0042] In some embodiments, all pyrimidine nucleotides preceding an adenosine nucleotide in the sense strand and/or in the antisense strand are modified nucleotides. For example, where the sequence 5'-CA-3' or 5'-UA-3' appears in either strand, the cytidine and uridine nucleotides are modified nucleotides, preferably 2'-O-methyl modified nucleotides. In certain embodiments, all pyrimidine nucleotides in the sense strand are modified nucleotides (e.g. 2'-O-methyl modified nucleotides), and the 5' nucleotide in all occurrences of the sequence 5'-CA-3' or 5'-UA-3' in the antisense strand are modified nucleotides (e.g. 2'-O-methyl modified nucleotides). In other embodiments, all nucleotides in the duplex region are modified nucleotides. In such embodiments, the modified nucleotides are preferably 2'-O-methyl modified nucleotides, 2'-fluoro modified nucleotides, or combinations thereof.

[0043] In embodiments in which the RNAi construct comprises a nucleotide overhang, the nucleotides in the overhang can be ribonucleotides, deoxyribonucleotides, or modified nucleotides. In one embodiment, the nucleotides in the overhang are deoxyribonucleotides, e.g., deoxythymidine. In another embodiment, the nucleotides in the overhang are modified nucleotides. For instance, in some embodiments, the nucleotides in the overhang are 2'-O-methyl modified nucleotides, 2'-fluoro modified nucleotides, 2'-methoxyethyl modified nucleotides, or combinations thereof.

[0044] The RNAi constructs of the disclosure may also comprise one or more modified internucleotide linkages. As used herein, the term "modified internucleotide linkage" refers to an internucleotide linkage other than the natural 3' to 5' phosphodiester linkage. In some embodiments, the modified internucleotide linkage is a phosphorous-containing internucleotide linkage, such as a phosphotriester, an aminoalkyl phosphotriester, an alkylphosphonate (e.g., methylphosphonate, 3'-alkylene phosphonate), a phosphinate, a phosphoramidate (e.g., 3'-aminophosphoramidate and aminoalkylphosphoramidate), a phosphorothioate (P=S), a chiralphosphorothioate, a phosphorodithioate, a thionophosphoramidate, a thionoalkylphosphonate, athionoalkylphosphotriester, and a boranophosphate. In one embodiment, a modified internucleotide linkage is a 2' to 5' phosphodiester linkage. In other embodiments, the modified internucleotide linkage is a non-phosphorous-containing internucleotide linkage and thus can be referred to as a modified internucleoside linkage. Such non-phosphorous-containing linkages include, but are not limited to, morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane linkages (-O-Si(H)$_2$-O-); sulfide, sulfoxide and sulfone linkages; formacetyl and thioformacetyl linkages; alkene containing backbones; sulfamate backbones; methylenemethylimino (-CH_2-N(CH_3)-O-CH_2-) and methylenehydrazino linkages; sulfonate and sulfonamide linkages; amide linkages; and others having mixed N, O, S and CH_2 component parts. In one embodiment, the modified internucleoside linkage is a peptide-based linkage (e.g., aminoethylglycine) to create a peptide nucleic acid or PNA, such as those described in U.S. Patents 5,539,082; 5,714,331; and 5,719,262. Other suitable modified internucleotide and internucleoside linkages that may be employed in the disclosed RNAi constructs are described in U.S. Patents 6,693,187 and 9,181,551, U.S. Patent Publication No. 2016/0122761, and Deleavey and Damha, *supra*.

[0045] In certain embodiments, the RNAi constructs comprise one or more phosphorothioate internucleotide linkages. The phosphorothioate internucleotide linkages may be present in the sense strand, antisense strand, or both strands of the RNAi constructs. For instance, in some embodiments, the sense strand comprises 1, 2, 3, 4, 5, 6, 7, 8, or more phosphorothioate internucleotide linkages. In other embodiments, the antisense strand comprises 1, 2, 3, 4, 5, 6, 7,8, or more phosphorothioate internucleotide linkages. In still

other embodiments, both strands comprise 1, 2, 3, 4, 5, 6, 7, 8, or more phosphorothioate internucleotide linkages. The RNAi constructs can comprise one or more phosphorothioate internucleotide linkages at the 3'-end, the 5'-end, or both the 3'- and 5'- ends of the sense strand, the antisense strand, or both strands. For instance, in certain embodiments, the RNAi construct comprises about 1 to about 6 or more (e.g., about 1, 2, 3, 4, 5, 6 or more) consecutive phosphorothioate internucleotide linkages at the 3'-end of the sense strand, the antisense strand, or both strands. In other embodiments, the RNAi construct comprises about 1 to about 6 or more (e.g., about 1, 2, 3, 4, 5, 6 or more) consecutive phosphorothioate internucleotide linkages at the 5'-end of the sense strand, the antisense strand, or both strands. In one embodiment, the RNAi construct comprises a single phosphorothioate internucleotide linkage at the 3' end of the sense strand. In one embodiment, the RNAi construct comprises a single phosphorothioate internucleotide linkage at the 3' end of the sense strand and a single phosphorothioate internucleotide linkage at the 3' end of the antisense strand. In one embodiment, the RNAi construct comprises a single phosphorothioate internucleotide linkage at the 5' end of the sense strand and a single phosphorothioate internucleotide linkage at the 3' end of the sense strand. In one embodiment, the RNAi construct comprises a single phosphorothioate internucleotide linkage at the 5' end of the antisense strand and a single phosphorothioate internucleotide linkage at the 3' end of the antisense strand. In another embodiment, the RNAi construct comprises two consecutive phosphorothioate internucleotide linkages at the 3' end of the antisense strand (i.e., a phosphorothioate internucleotide linkage at the first and second internucleotide linkages at the 3' end of the antisense strand). In another embodiment, the RNAi construct comprises two consecutive phosphorothioate internucleotide linkages at both the 3' and 5' ends of the antisense strand. In yet another embodiment, the RNAi construct comprises two consecutive phosphorothioate internucleotide linkages at both the 3' and 5' ends of the antisense strand and two consecutive phosphorothioate internucleotide linkages at the 5' end of the sense strand. In still another embodiment, the RNAi construct comprises two consecutive phosphorothioate internucleotide linkages at both the 3' and 5' ends of the antisense strand and two consecutive phosphorothioate internucleotide linkages at both the 3' and 5' ends of the sense strand (i.e. a phosphorothioate internucleotide linkage at the first

and second internucleotide linkages at both the 5' and 3' ends of the antisense strand and a phosphorothioate internucleotide linkage at the first and second internucleotide linkages at both the 5' and 3' ends of the sense strand). In any of the embodiments in which one or both strands comprise one or more phosphorothioate internucleotide linkages, the remaining internucleotide linkages within the strands can be the natural 3' to 5' phosphodiester linkages. For instance, in some embodiments, each internucleotide linkage of the sense and antisense strands is selected from phosphodiester and phosphorothioate, wherein at least one internucleotide linkage is a phosphorothioate.

[0046] In embodiments in which the RNAi construct comprises a nucleotide overhang, two or more of the unpaired nucleotides in the overhang can be connected by a phosphorothioate internucleotide linkage. In certain embodiments, all the unpaired nucleotides in a nucleotide overhang at the 3' end of the antisense strand and/or the sense strand are connected by phosphorothioate internucleotide linkages. In other embodiments, all the unpaired nucleotides in a nucleotide overhang at the 5' end of the antisense strand and/or the sense strand are connected by phosphorothioate internucleotide linkages. In still other embodiments, all the unpaired nucleotides in any nucleotide overhang are connected by phosphorothioate internucleotide linkages.

[0047] In certain embodiments, the modified nucleotides incorporated into one or both of the strands of the RNAi constructs of the invention have a modification of the nucleobase (also referred to herein as "base"). A "modified nucleobase" or "modified base" refers to a base other than the naturally occurring purine bases adenine (A) and guanine (G) and pyrimidine bases thymine (T), cytosine (C), and uracil (U). Modified nucleobases can be synthetic or naturally occurring modifications and include, but are not limited to, universal bases, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine (X), hypoxanthine (I), 2-aminoadenine, 6-methyladenine, 6-methylguanine, and other alkyl derivatives of adenine and guanine, 2-propyland other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo, particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-

methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-daazaadenine and 3-deazaguanine, and 3-deazaadenine.

[0048] In some embodiments, the modified base is a universal base. A "universal base" refers to a base analog that indiscriminately forms base pairs with all of the natural bases in RNA and DNA without altering the double helical structure of the resulting duplex region. Universal bases are known to those of skill in the art and include, but are not limited to, inosine, C-phenyl, C-naphthyl and other aromatic derivatives, azole carboxamides, and nitroazole derivatives, such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole.

[0049] Other suitable modified bases that can be incorporated into the RNAi constructs of the invention include those described in, for example, Herdewijn, Antisense Nucleic Acid Drug Dev., 10: 297-310 (2000) and Peacock et al., J. Org. Chern., 76: 7295-7300 (2011). The skilled person is well aware that guanine, cytosine, adenine, thymine, and uracil may be replaced by other nucleobases, such as the modified nucleobases described above, without substantially altering the base pairing properties of a polynucleotide comprising a nucleotide bearing such replacement nucleobase.

[0050] In some embodiments, the 5' end of the sense strand, antisense strand, or both the antisense and sense strands of the disclosed RNAi constructs comprises a phosphate moiety. As used herein, the term "phosphate moiety" refers to a terminal phosphate group that includes unmodified phosphates (-O-P=O)(OH)OH) as well as modified phosphates. Modified phosphates include phosphates in which one or more of the O and OH groups are replaced with H, O, S, N(R) or alkyl where R is H, an amino protecting group or unsubstituted or substituted alkyl. Exemplary phosphate moieties include, but are not limited to, 5'-monophosphate; 5'diphosphate; 5'-triphosphate; 5'-guanosine cap (7-methylated or non-methylated); 5'-adenosinecap or any other modified or unmodified nucleotide cap structure; 5'-monothiophosphate (phosphorothioate); 5'-monodithiophosphate (phosphorodithioate); 5'-alpha-thiotriphosphate; 5'-gamma-thiotriphosphate, 5'-phosphoramidates; 5'-vinylphosphates; 5'-alkylphosphonates (wherein "alkyl" can be methyl, ethyl, isopropyl, propyl, etc.); and 5'-alkyletherphosphonates (wherein "alkylether" can be methoxymethyl, ethoxymethyl, etc.).

[0051] The modified nucleotides that can be incorporated into the RNAi constructs of the invention may have more than one chemical modification described herein. For instance, the modified nucleotide may have a modification to the ribose sugar as well as a modification to the nucleobase. By way of example, a modified nucleotide may comprise a 2' sugar modification (e.g., 2'-fluoro or 2'-methyl) and comprise a modified base (e.g., 5-methyl cytosine or pseudouracil). In other embodiments, the modified nucleotide may comprise a sugar modification in combination with a modification to the 5' phosphate that would create a modified internucleotide or internucleoside linkage when the modified nucleotide was incorporated into a polynucleotide. For instance, in some embodiments, the modified nucleotide may comprise a sugar modification, such as a 2'-fluoro modification, a 2'-O-methyl modification, or a bicyclic sugar modification, as well as a 5' phosphorothioate group. Accordingly, in some embodiments, one or both strands of the RNAi constructs of the invention comprise a combination of 2' modified nucleotides or BNAs and phosphorothioate internucleotide linkages. In certain embodiments, both the sense and antisense strands of the RNAi constructs of the invention comprise a combination of 2'-fluoro modified nucleotides, 2'-O-methyl modified nucleotides, and phosphorothioate internucleotide linkages. Exemplary RNAi constructs comprising modified nucleotides and internucleotide linkages are shown in Table 2.

Function of RNAi Constructs

[0052] The RNAi constructs of the invention desirably reduce or inhibit the expression of GPAM in cells, particularly liver cells. Accordingly, in one embodiment, the present invention provides a method of reducing GPAM expression in a cell by contacting the cell with any RNAi construct described herein. The cell may be *in vitro* or *in vivo*. GPAM expression can be assessed by measuring the amount or level of GPAM mRNA, GPAM protein, or another biomarker linked to GPAM expression. The reduction of GPAM expression in cells or animals treated with an RNAi construct of the invention can be determined relative to the GPAM expression in cells or animals not treated with the RNAi construct or treated with a control RNAi construct. For instance, in some embodiments, reduction of GPAM expression is assessed by (a) measuring the amount or level of GPAM

mRNA in liver cells treated with a RNAi construct of the invention, (b) measuring the amount or level of GPAM mRNA in liver cells treated with a control RNAi construct (e.g., RNAi construct directed to a RNA molecule not expressed in liver cells or a RNAi construct having a nonsense or scrambled sequence) or no construct, and (c) comparing the measured GPAM mRNA levels from treated cells in (a) to the measured GPAM mRNA levels from control cells in (b). The GPAM mRNA levels in the treated cells and controls cells can be normalized to RNA levels for a control gene (e.g., 18S ribosomal RNA) prior to comparison. GPAM mRNA levels can be measured by a variety of methods, including Northern blot analysis, nuclease protection assays, fluorescence *in situ* hybridization (FISH), reverse-transcriptase (RT)-PCR, real-time RT-PCR, quantitative PCR, and the like.

[0053] In other embodiments, reduction of GPAM expression is assessed by (a) measuring the amount or level of GPAM protein in liver cells treated with a RNAi construct of the invention, (b) measuring the amount or level of GPAM protein in liver cells treated with a control RNAi construct (e.g., RNAi construct directed to a RNA molecule not expressed in liver cells or a RNAi construct having a nonsense or scrambled sequence) or no construct, and (c) comparing the measured GPAM protein levels from treated cells in (a) to the measured GPAM protein levels from control cells in (b). GPAM protein levels can be measured using any suitable method known to those of skill in the art, including but not limited to, western blots, immunoassays (e.g., ELISA), and flow cytometry. Any suitable method of measuring GPAM mRNA or protein can be used to assess the efficacy of the RNAi constructs of the invention.

[0054] In some embodiments, the methods to assess GPAM expression levels are performed *in vitro* in cells that natively express GPAM (e.g., liver cells) or cells that have been engineered to express GPAM. In certain embodiments, the methods are performed *in vitro* in liver cells. Suitable liver cells include, but are not limited to, primary hepatocytes (e.g. human, non-human primate, or rodent hepatocytes), HepAD38 cells, HuH-6 cells, HuH-7 cells, HuH-5-2 cells, BNLCL2 cells, Hep3B cells, or HepG2 cells. In one embodiment, the liver cells are Hep3B cells. In another embodiment, the liver cells are HepG2 cells.

[0055] In other embodiments, the methods to assess GPAM expression levels are performed *in vivo*. For example, the RNAi constructs and any control RNAi constructs can be administered to an animal (e.g., rodent or non-human primate), and GPAM mRNA or protein levels may be assessed in liver tissue harvested from the animal following treatment. Alternatively or additionally, a biomarker or functional phenotype associated with GPAM expression can be assessed in the treated animals.

[0056] In certain embodiments, expression of GPAM is reduced in liver cells by at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, or at least 50% by an RNAi construct of the invention. In some embodiments, expression of GPAM is reduced in liver cells by at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, or at least 85% by an RNAi construct of the invention. In other embodiments, the expression of GPAM is reduced in liver cells by about 90% or more, e.g., 91%, 92%, 93%, 94%, 95%, 96%,97%, 98%, 99%, or more by an RNAi construct of the invention. The percent reduction of GPAM expression can be measured by any of the methods described herein or otherwise known in the art. For instance, in certain embodiments, the RNAi constructs of the invention inhibit at least 45% of GPAM expression at 5 nM in HepG2 cells (contains GPAM ~~having an~~ I43V mutation) *in vitro*. In related embodiments, the RNAi constructs of the invention inhibit at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, or at least 75% of GPAM expression at 5 nM in HepG2 cells *in vitro*. In other embodiments, the RNAi constructs of the invention inhibit at least 80%, at least85%, at least 90%, at least 92%, at least 94%, at least 96%, or at least 98% of GPAM expression at 5 nM in HepG2 cells *in vitro*. Reduction of GPAM can be measured using a variety of techniques including, for example, RNA FISH or droplet digital PCR (see, e.g., Kamitaki et al., Digital PCR. Methods in Molecular Biology, 1768: 401-422 (2018). doi:10.1007/978-1-4939-7778-9_23).

[0057] In some embodiments, an IC50 value is calculated to assess the potency of an RNAi construct of the invention for inhibiting GPAM expression in liver cells. An "IC50 value" is the dose/concentration required to achieve 50% inhibition of a biological or biochemical function. The IC50 value of any substance or antagonist can be determined by constructing a dose-response curve and examining the effect of different concentrations of

the substance or antagonist on expression levels or functional activity in any assay. IC50 values can be calculated for a given antagonist or substance by determining the concentration needed to inhibit half of the maximum biological response or native expression levels. Thus, the IC50 value for any RNAi construct can be calculated by determining the concentration of the RNAi construct needed to inhibit half of the native GPAM expression level in liver cells (e.g., GPAM expression level in control liver cells) in any assay, such as an immunoassay, RNA FISH assay, or a droplet digital PCR assay. The RNAi constructs of the invention may inhibit GPAM expression in liver cells (e.g. HepG2 cells) with an IC50 of less than about 20 nM (e.g., less than about 15 nM, 10 nM, 5 nM, or 1 nM). For example, the disclosed RNAi constructs may inhibit GPAM expression in liver cells with an IC50 of about 0.001 nM to about 20 nM, about 0.001 nM to about 10 nM, about 0.001 nM to about 5 nM, about 0.001 nM to about 1 nM, about 0.1 nM to about 10 nM, about 0.1 nM to about 5 nM, or about 0.1 nM to about 1 nM. In certain embodiments, the RNAi construct inhibits GPAM expression in liver cells (e.g., HepG2 cells) with an IC50 of about 1 nM to about 10 nM.

[0058] The RNAi constructs of the invention can readily be made using techniques known in the art, such as, for example, conventional nucleic acid solid phase synthesis. The polynucleotides of the RNAi constructs can be assembled on a suitable nucleic acid synthesizer utilizing standard nucleotide or nucleoside precursors (e.g., phosphoramidites). Automated nucleic acid synthesizers are sold commercially by several vendors, including DNA/RNA synthesizers from Applied Biosystems (Foster City, CA), MerMade synthesizers from BioAutomation (Irving, TX), and OligoPilot synthesizers from GE Healthcare Life Sciences (Pittsburgh, PA).

[0059] The 2' silyl protecting group can be used in conjunction with acid labile dimethoxytrityl (DMT) at the 5' position of ribonucleosides to synthesize oligonucleotides via phosphoramidite chemistry. Final deprotection conditions are known not to significantly degrade RNA products. All syntheses can be conducted in any automated or manual synthesizer on large, medium, or small scale. The syntheses may also be carried out in multiple well plates, columns, or glass slides.

[0060] The 2'-O-silyl group can be removed via exposure to fluoride ions, which can include any source of fluoride ion, e.g., those salts containing fluoride ion paired with

inorganic counterions, e.g., cesium fluoride and potassium fluoride or those salts containing fluoride ion paired with an organic counterion, e.g., a tetraalkylammonium fluoride. A crown ether catalyst can be utilized in combination with the inorganic fluoride in the deprotection reaction. Exemplary fluoride ion sources include, but are not limited to, tetrabutylammonium fluoride or aminohydrofluorides (e.g., combining aqueous HF with triethylamine in a dipolar aprotic solvent, e.g., dimethylformamide).

[0061] The choice of protecting groups for use on the phosphite triesters and phosphotriesters can alter the stability of the triesters towards fluoride. Methyl protection of the phosphotriester or phosphitetriester can stabilize the linkage against fluoride ions and improve process yields.

[0062] Since ribonucleosides have a reactive 2' hydroxyl substituent, it may be desirable to protect the reactive 2' position in RNA with a protecting group that is orthogonal to a 5'-O-dimethoxytrityl protecting group, e.g., one stable to treatment with acid. Silyl protecting groups meet this criterion and can be readily removed in a final fluoride deprotection step that can result in minimal RNA degradation.

[0063] Tetrazole catalysts can be used in the standard phosphoramidite coupling reaction. Exemplary catalysts include, e.g., tetrazole, S-ethyl-tetrazole, benzylthiotetrazole, and pnitrophenyltetrazole.

[0064] Additional methods of synthesizing the RNAi constructs described herein will be evident to those of ordinary skill in the art. Additionally, the various synthetic steps may be performed in an alternate sequence or order to give the desired compounds. Other synthetic chemistry transformations, protecting groups (e.g., for hydroxyl, amino, etc., present on the bases) and protecting group methodologies (protection and deprotection) useful in synthesizing the RNAi constructs described herein are known in the art and include, for example, those described in R. Larock, Comprehensive Organic Transformations, VCH Publishers (1989); T. W. Greene and P. G. M. Wuts, Protective Groups in Organic Synthesis, 2d. Ed., John Wiley and Sons (1991); L. Fieser and M. Fieser, Fieser and Fieser's Reagents for Organic Synthesis, John Wiley and Sons (1994); and L. Paquette, ed., Encyclopedia of Reagents for Organic Synthesis, John Wiley and Sons (1995), and subsequent editions thereof. Custom synthesis of RNAi constructs is also available from

several commercial vendors, including Dharmacon, Inc. (Lafayette, CO), AxoLabs GmbH (Kulmbach, Germany), and Ambion, Inc. (Foster City, CA).

[0065] The RNAi constructs of the invention may comprise a ligand. As used herein, a "ligand" refers to any compound or molecule that can interact with another compound or molecule, either directly or indirectly. The interaction of a ligand with another compound or molecule may elicit a biological response (e.g., initiate a signal transduction cascade, induce receptor mediated endocytosis) or may just be a physical association. The ligand can modify one or more properties of the double-stranded RNA molecule to which is attached, such as the pharmacodynamic, pharmacokinetic, binding, absorption, cellular distribution, cellular uptake, charge and/or clearance properties of the RNA molecule.

[0066] The ligand may comprise a serum protein (e.g., human serum albumin, low-density lipoprotein, globulin), a cholesterol moiety, a vitamin (e.g., biotin, vitamin E, vitamin B12), a folate moiety, a steroid, a bile acid (e.g., cholic acid), a fatty acid (e.g., palmitic acid, myristic acid), a carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid), a glycoside, a phospholipid, or an antibody or binding fragment thereof (e.g., a whole antibody or binding fragment that targets the RNAi construct to a specific cell type, such as liver cells). Other examples of ligands include dyes, intercalating agents (e.g., acridines), cross-linkers (e.g., psoralene, mitomycin C), porphyrins (e.g., TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (e.g., phenazine, dihydrophenazine), artificial endonucleases (e.g., EDTA), lipophilic molecules (e.g, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-BisO(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, 03-(oleoyl)lithocholic acid, 03-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine), peptides (e.g., antennapedia peptide, Tat peptide, RGD peptides), alkylating agents, polymers (e.g., polyethylene glycol (PEG), PEG-40K), poly amino acids, and polyamines (e.g., spermine, spermidine).

[0067] In certain embodiments, the ligands have endosomolytic properties. The endosomolytic ligands promote the lysis of the endosome and/or transport of the RNAi construct of the invention, or its components, from the endosome to the cytoplasm of the cell. The endosomolytic ligand may be a polycationic peptide or peptidomimetic which shows

pH-dependent membrane activity and fusogenicity. In one embodiment, the endosomolytic ligand assumes its active conformation at endosomal pH. The "active" conformation is that conformation in which the endosomolytic ligand promotes lysis of the endosome and/or transport of the RNAi construct of the invention, or its components, from the endosome to the cytoplasm of the cell. Exemplary endosomolytic ligands include the GALA peptide (Subbarao et al., Biochemistry, Vol. 26: 2964-2972, 1987), the EALA peptide (Vogel et al., J. Am. Chern. Soc., Vol. 118: 1581-1586, 1996), and their derivatives (Turk et al., Biochem. Biophys. Acta, Vol. 1559: 56-68, 2002). In one embodiment, the endosomolytic component may contain a chemical group (e.g., an amino acid) which will undergo a change in charge or protonation in response to a change in pH. The endosomolytic component may be linear or branched.

[0068] In some embodiments, the ligand comprises a lipid or other hydrophobic molecule. In one embodiment, the ligand comprises a cholesterol moiety or other steroid. Cholesterol conjugated oligonucleotides have been reported to be more active than their unconjugated counterparts (Manoharan, Antisense Nucleic Acid Drug Development, Vol. 12: 103-228, 2002). Ligands comprising cholesterol moieties and other lipids for conjugation to nucleic acid molecules have also been described in U.S. Patents 7,851,615; 7,745,608; and 7,833,992. In another embodiment, the ligand may comprise a folate moiety. Polynucleotides conjugated to folate moieties can be taken up by cells via a receptor-mediated endocytosis pathway. Such folate-polynucleotide conjugates are described in, e.g., U.S. Patent 8,188,247.

[0069] Given that GPAM is expressed in liver cells (e.g., hepatocytes), in certain embodiments, it is desirable to specifically deliver the RNAi construct to liver cells. In some embodiments, RNAi constructs can be specifically targeted to the liver by employing ligands that bind to or interact with proteins expressed on the surface of liver cells. For example, in certain embodiments, a ligand may comprise one or more antigen binding proteins (e.g. antibodies or binding fragments thereof (e.g. Fab, scFv)) that specifically bind to a receptor expressed on hepatocytes.

[0070] In certain embodiments, the ligand comprises a carbohydrate. A "carbohydrate" refers to a compound made up of one or more monosaccharide units having

at least 6 carbon atoms (which can be linear, branched, or cyclic) with an oxygen, nitrogen or sulfur atom bonded to each carbon atom. Carbohydrates include, but are not limited to, sugars (e.g., monosaccharides, disaccharides, trisaccharides, tetrasaccharides, and oligosaccharides containing from about 4, 5, 6, 7, 8, or 9 monosaccharide units), and polysaccharides, such as starches, glycogen, cellulose, and polysaccharide gums. In some embodiments, the carbohydrate incorporated into the ligand is a monosaccharide selected from a pentose, hexose, or heptose and di- and tri-saccharides including such monosaccharide units. In other embodiments, the carbohydrate incorporated into the ligand is an amino sugar, such as galactosamine, glucosamine, N-acetylgalactosamine, and N-acetylglucosamine.

[0071] In some embodiments, the ligand comprises a hexose or hexosamine. The hexose may be selected from glucose, galactose, mannose, fucose, or fructose. The hexosamine may be selected from fructosamine, galactosamine, glucosamine, or mannosamine. In certain embodiments, the ligand comprises glucose, galactose, galactosamine, or glucosamine. In one embodiment, the ligand comprises glucose, glucosamine, or N-acetylglucosamine. In another embodiment, the ligand comprises galactose, galactosamine, or N-acetyl-galactosamine. In particular embodiments, the ligand comprises N-acetyl-galactosamine. Ligands comprising glucose, galactose, and N-acetyl-galactosamine (GalNAc) are particularly effective in targeting compounds to liver cells (see, e.g., D'Souza and Devarajan, J. Control Release, Vol. 203: 126-139, 2015). Examples of GalNAc- or galactose-containing ligands that can be incorporated into the RNAi constructs of the invention are described in U.S. Patents 7,491,805; 8,106,022; and 8,877,917; U.S. Patent Publication No. 2003/0130186; and WIPO Publication No. WO 2013/166155.

[0072] In certain embodiments, the ligand comprises a multivalent carbohydrate moiety. As used herein, a "multivalent carbohydrate moiety" refers to a moiety comprising two or more carbohydrate units capable of independently binding or interacting with other molecules. For example, a multivalent carbohydrate moiety comprises two or more binding domains comprised of carbohydrates that can bind to two or more different molecules or two or more different sites on the same molecule. The valency of the carbohydrate moiety denotes the number of individual binding domains within the carbohydrate moiety. For

instance, the terms "monovalent," "bivalent," "trivalent," and "tetravalent" with reference to the carbohydrate moiety refer to carbohydrate moieties with one, two, three, and four binding domains, respectively. The multivalent carbohydrate moiety may comprise a multivalent lactose moiety, a multivalent galactose moiety, a multivalent glucose moiety, a multivalent N-acetyl-galactosamine moiety, a multivalent N-acetyl-glucosamine moiety, a multivalent mannose moiety, or a multivalent fucose moiety. In some embodiments, the ligand comprises a multivalent galactose moiety. In other embodiments, the ligand comprises a multivalent N-acetyl-galactosamine moiety. In these and other embodiments, the multivalent carbohydrate moiety is bivalent, trivalent, or tetravalent. In such embodiments, the multivalent carbohydrate moiety can be bi-antennary or tri-antennary. In one particular embodiment, the multivalent N-acetyl-galactosamine moiety is trivalent or tetravalent. In another particular embodiment, the multivalent galactose moiety is trivalent or tetravalent. Exemplary trivalent and tetravalent GalNAc-containing ligands for incorporation into the RNAi constructs of the invention are described in detail below.

[0073] The ligand can be attached or conjugated to the RNA molecule of the RNAi construct directly or indirectly. For instance, in some embodiments, the ligand is covalently attached directly to the sense or antisense strand of the RNAi construct. In other embodiments, the ligand is covalently attached via a linker to the sense or antisense strand of the RNAi construct. The ligand can be attached to nucleobases, sugar moieties, or internucleotide linkages of polynucleotides (e.g., sense strand or antisense strand) of the RNAi constructs of the invention. Conjugation or attachment to purine nucleobases or derivatives thereof can occur at any position including, endocyclic and exocyclic atoms. In certain embodiments, the 2-, 6-, 7-, or 8-positions of a purine nucleobase are attached to a ligand. Conjugation or attachment to pyrimidine nucleobases or derivatives thereof can also occur at any position. In some embodiments, the 2, 5-, and 6-positions of a pyrimidine nucleobase can be attached to a ligand. Conjugation or attachment to sugar moieties of nucleotides can occur at any carbon atom. Example carbon atoms of a sugar moiety that can be attached to a ligand include the 2', 3', and 5' carbon atoms. The 1' position can also be attached to a ligand, such as in a basic residue. Internucleotide linkages can also support ligand attachments. For phosphorus-containing linkages (e.g., phosphodiester,

phosphorothioate, phosphorodithiotate, phosphoroamidate, and the like), the ligand can be attached directly to the phosphorus atom or to an O, N, or S atom bound to the phosphorus atom. For amine- or amide-containing internucleoside linkages (e.g., PNA), the ligand can be attached to the nitrogen atom of the amine or amide or to an adjacent carbon atom.

[0074] In certain embodiments, the ligand may be attached to the 3' or 5' end of either the sense or antisense strand. In certain embodiments, the ligand is covalently attached to the 5' end of the sense strand. In other embodiments, the ligand is covalently attached to the 3' end of the sense strand. For example, in some embodiments, the ligand is attached to the 3'-terminal nucleotide of the sense strand. In certain such embodiments, the ligand is attached at the 3'-position of the 3'-terminal nucleotide of the sense strand. In alternative embodiments, the ligand is attached near the 3' end of the sense strand, but before one or more terminal nucleotides (i.e. before 1, 2, 3, or 4 terminal nucleotides). In some embodiments, the ligand is attached at the 2'-position of the sugar of the 3'-terminal nucleotide of the sense strand.

[0075] In certain embodiments, the ligand is attached to the sense or antisense strand via a linker. A "linker" is an atom or group of atoms that covalently joins a ligand to a polynucleotide component of the RNAi construct. The linker may be from about 1 to about 30 atoms in length, from about 2 to about 28 atoms in length, from about 3 to about 26 atoms in length, from about 4to about 24 atoms in length, from about 6 to about 20 atoms in length, from about 7 to about 20atoms in length, from about 8 to about 20 atoms in length, from about 8 to about 18 atoms in length, from about 10 to about 18 atoms in length, and from about 12 to about 18 atoms in length. In some embodiments, the linker may comprise a bifunctional linking moiety, which generally comprises an alkyl moiety with two functional groups. One of the functional groups is selected to bind to the compound of interest (e.g., sense or antisense strand of the RNAi construct) and the other is selected to bind essentially any selected group, such as a ligand as described herein. In certain embodiments, the linker comprises a chain structure or an oligomer of repeating units, such as ethylene glycol or amino acid units. Examples of functional groups that are typically employed in a bifunctional linking moiety include, but are not limited to, electrophiles for reacting with nucleophilic groups and nucleophiles for reacting with electrophilic groups. In some

embodiments, bifunctional linking moieties include amino, hydroxyl, carboxylic acid, thiol, unsaturations (e.g., double or triple bonds), and the like.

[0076] Linkers that may be used to attach a ligand to the sense or antisense strand in the RNAi constructs of the invention include, but are not limited to, pyrrolidine, 8-amino-3,6-di oxaoctanoic acid, succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, 6-aminohexanoic acid, substituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl or substituted or unsubstituted C_2-C_{10} alkynyl. Preferred substituent groups for such linkers include, but are not limited to, hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl, and alkynyl.

[0077] In certain embodiments, the linkers are cleavable. A cleavable linker is one which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding together. In some embodiments, the cleavable linker is cleaved at least 10 times, 20 times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times, or more, or at least 100 times faster in the target cell or under a first reference condition (which can, e.g., be selected to mimic or represent intracellular conditions) than in the blood of a subject, or under a second reference condition (which can, e.g., be selected to mimic or represent conditions found in the blood or serum).

[0078] Cleavable linkers are susceptible to cleavage agents, e.g., pH, redox potential, or the presence of degradative molecules. Generally, cleavage agents are more prevalent or found at higher levels or activities inside cells than in serum or blood. Examples of such degradative agents include: redox agents which are selected for particular substrates or which have no substrate specificity, including, e.g., oxidative or reductive enzymes or reductive agents such as mercaptans, present in cells, that can degrade a redox cleavable linker by reduction; esterases; endosomes or agents that can create an acidic environment, e.g., those that result in a pH of five or lower; enzymes that can hydrolyze or degrade an acid cleavable linker by acting as a general acid, peptidases (which can be substrate specific), and phosphatases.

[0079] A cleavable linker may comprise a moiety that is susceptible to pH. The pH of human serum is 7.4, while the average intracellular pH is slightly lower, ranging from about 7.1-7.3. Endosomes have a more acidic pH, in the range of 5.5-6.0, and lysosomes

have an even more acidic pH at around 5.0. Some linkers will have a cleavable group that is cleaved at a preferred pH, thereby releasing the RNA molecule from the ligand inside the cell, or into the desired compartment of the cell.

[0080] A linker can include a cleavable group that is cleavable by a particular enzyme. The type of cleavable group incorporated into a linker can depend on the cell to be targeted. For example, liver-targeting ligands can be linked to RNA molecules through a linker that includes an ester group. Liver cells are rich in esterases, and therefore the linker will be cleaved more efficiently in liver cells than in cell types that are not esterase-rich. Other types of cells rich in esterases include cells of the lung, renal cortex, and testis. Linkers that contain peptide bonds can be used when targeting cells rich in peptidases, such as liver cells and synoviocytes.

[0081] In general, the suitability of a candidate cleavable linker can be evaluated by testing the ability of a degradative agent (or condition) to cleave the candidate linker. It will also be desirable to also test the candidate cleavable linker for the ability to resist cleavage in the blood or when in contact with other non-target tissue. Thus, one can determine the relative susceptibility to cleavage between a first and a second condition, where the first is selected to be indicative of cleavage in a target cell and the second is selected to be indicative of cleavage in other tissues or biological fluids, e.g., blood or serum. The evaluations can be carried out in cell free systems, in cells, in cell culture, in organ or tissue culture, or in whole animals. It may be useful to make initial evaluations in cell-free or culture conditions and to confirm by further evaluations in whole animals. In some embodiments, useful candidate linkers are cleaved at least 2, 4, 10, 20, 50, 70, or 100 times faster in the cell (or under *in vitro* conditions selected to mimic intracellular conditions) as compared to blood or serum (or under *in vitro* conditions selected to mimic extracellular conditions).

[0082] In other embodiments, redox cleavable linkers are utilized. Redox cleavable linkers are cleaved upon reduction or oxidation. An example of reductively cleavable group is a disulfide linking group (-S-S-). To determine if a candidate cleavable linker is a suitable "reductively cleavable linker," or, for example, is suitable for use with a particular RNAi construct and particular ligand, one or more methods described herein can be used. For example, a candidate linker can be evaluated by incubation with dithiothreitol (DTT), or

other reducing agent known in the art, which mimics the rate of cleavage that would be observed in a cell, e.g., a target cell. The candidate linkers can also be evaluated under conditions which are selected to mimic blood or serum conditions. In a specific embodiment, candidate linkers are cleaved by at most 10% in the blood. In other embodiments, useful candidate linkers are degraded at least 2, 4, 10, 20, 50,70, or 100 times faster in the cell (or under *in vitro* conditions selected to mimic intracellular conditions) as compared to blood (or under *in vitro* conditions selected to mimic extracellular conditions).

[0083] In yet other embodiments, phosphate-based cleavable linkers are cleaved by agents that degrade or hydrolyze the phosphate group. An example of an agent that hydrolyzes phosphate groups in cells are enzymes, such as phosphatases in cells. Examples of phosphate-based cleavable groups are -O-P(O)(ORk)-O-, -O-P(S)(ORk)-O-, -O-P(S)(SRk)-O-, -S-P(O)(ORk)-O-, -O-P(O)(ORk)-S-, -S-P(O)(ORk)-S-, -O-P(S)(ORk)-S-, -S-P(S)(ORk)-O-, -O-P(O)(Rk)-O-, -O-P(S)(Rk)-O-, -S-P(O)(Rk)-O-, -S-P(S)(Rk)-O-, -S-P(O)(Rk)-S-, -O-P(S)(Rk)-S-. Specific embodiments include -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -SP(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O-, -S-P(S)(H)-O-, -S-P(O)(H)-S-, -O-P(S)(H)-S-. Another specific embodiment is -O-P(O)(OH)-O-. These candidate linkers can be evaluated using methods analogous to those described above.

[0084] In other embodiments, the linkers may comprise acid cleavable groups, which are groups that are cleaved under acidic conditions. In some embodiments, acid cleavable groups are cleaved in an acidic environment with a pH of about 6.5 or lower (e.g., about 6.0, 5.5, 5.0, or lower), or by agents, such as enzymes that can act as a general acid. In a cell, specific low pH organelles, such as endosomes and lysosomes, can provide a cleaving environment for acid cleavable groups. Examples of acid cleavable linking groups include, but are not limited to, hydrazones, esters, and esters of amino acids. Acid cleavable groups can have the general formula -C=NN-, C(O)O, or -OC(O). A specific embodiment is when the carbon attached to the oxygen of the ester (the alkoxy group) is an aryl group, substituted alkyl group, or tertiaryalkyl group such as dimethyl, pentyl or t-butyl. These candidates can be evaluated using methods analogous to those described above.

[0085] In other embodiments, the linkers may comprise ester-based cleavable groups, which are cleaved by enzymes, such as esterases and amidases in cells. Examples of ester-based cleavable groups include, but are not limited to, esters of alkylene, alkenylene and alkynylene groups. Ester cleavable groups have the general formula -C(O)O-, or -OC(O) -. These candidate linkers can be evaluated using methods analogous to those described above.

[0086] In further embodiments, the linkers may comprise peptide-based cleavable groups, which are cleaved by enzymes, such as peptidases and proteases in cells. Peptide-based cleavable groups are peptide bonds formed between amino acids to yield oligopeptides (e.g., dipeptides, tripeptides etc.) and polypeptides. Peptide-based cleavable groups do not include the amide group (-C(O)NH-). The amide group can be formed between any alkylene, alkenylene or alkynelene. A peptide bond is a special type of amide bond formed between amino acids to yield peptides and proteins. The peptide based cleavage group is generally limited to the peptide bond (i.e., the amide bond) formed between amino acids yielding peptides and proteins and does not include the entire amide functional group. Peptide-based cleavable linking groups have the general formula -NHCHRAC(O)NHCHRBC(O)-, where RA and RB are the R groups of the two adjacent amino acids. These candidates can be evaluated using methods analogous to those described above.

[0087] Other types of linkers suitable for attaching ligands to the sense or antisense strands in the RNAi constructs described herein are known in the art and can include the linkers described in, e.g., U.S. Patents 7,723,509; 8,017,762; 8,828,956; 8,877,917; and 9,181,551.

[0088] In certain embodiments, the ligand covalently attached to the sense or antisense strand of the RNAi constructs of the invention comprises a GalNAc moiety, e.g, a multivalent GalNAc moiety. In some embodiments, the multivalent GalNAc moiety is a trivalent GalNAc moiety and is attached to the 3' end of the sense strand. In other embodiments, the multivalent GalNAc moiety is a trivalent GalNAc moiety and is attached to the 5' end of the sense strand. In yet other embodiments, the multivalent GalNAc moiety is a tetravalent GalNAc moiety and is attached to the 3' end of the sense strand. In still other

embodiments, the multivalent GalNAc moiety is a tetravalent GalNAc moiety and is attached to the 5' end of the sense strand.

[0089] In some embodiments, the RNAi constructs of the invention may be delivered to a cell or tissue of interest by administering a vector that encodes and controls the intracellular expression of the RNAi construct. A "vector" (also referred to herein as an "expression vector") is a composition of matter which can be used to deliver a nucleic acid of interest to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated viral vectors, retroviral vectors, and the like. A vector can be replicated in a living cell, or it can be made synthetically.

[0090] Generally, a vector for expressing an RNAi construct of the invention will comprise one or more promoters operably linked to sequences encoding the RNAi construct. The phrases "operably linked," "operatively linked," or "under transcriptional control" may be used interchangeably herein to indicate when a promoter is in the correct location and orientation in relation to a polynucleotide sequence to control the initiation of transcription by RNA polymerase and expression of the polynucleotide sequence. A "promoter" refers to a sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene sequence. Suitable promoters include, but are not limited to, RNA pol I, pol II, HI or U6 RNA pol III, and viral promoters (e.g., human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, and the Rous sarcoma virus long terminal repeat). In some embodiments, an HI or U6RNA pol III promoter is employed. The promoter can be a tissue-specific or inducible promoter. Of particular interest are liver-specific promoters, such as promoter sequences from the human alpha-1 antitrypsin gene, albumin gene, hemopexin gene, and hepatic lipase gene. Inducible promoters include, for example, promoters regulated by ecdysone, estrogen, progesterone, tetracycline, and isopropyl-PD1-thiogalactopyranoside (IPTG).

[0091] When the RNAi construct comprises an siRNA, the two separate strands (sense and antisense strand) can be expressed from a single vector or two separate vectors. For example, in some embodiments, the sequence encoding the sense strand is operably linked to a promoter on a first vector and the sequence encoding the antisense strand is operably linked to a promoter on a second vector. In such an embodiment, the first and second vectors are co-introduced, e.g., by infection or transfection, into a target cell, such that the sense and antisense strands, once transcribed, will hybridize intracellularly to form the siRNA molecule. In another embodiment, the sense and antisense strands are transcribed from two separate promoters located in a single vector. In such embodiments, the sequence encoding the sense strand may be operably linked to a first promoter and the sequence encoding the antisense strand may be operably linked to a second promoter, wherein the first and second promoters are located in a single vector. In one embodiment, the vector comprises a first promoter operably linked to a sequence encoding the siRNA molecule, and a second promoter operably linked to the same sequence in the opposite direction, such that transcription of the sequence from the first promoter results in the synthesis of the sense strand of the siRNA molecule and transcription of the sequence from the second promoter results in synthesis of the antisense strand of the siRNA molecule.

[0092] When the RNAi construct comprises a shRNA, a sequence encoding the single, at least partially self-complementary RNA molecule is operably linked to a promoter to produce a single transcript. In some embodiments, the sequence encoding the shRNA comprises an inverted repeat joined by a linker polynucleotide sequence to produce the stem and loop structure of the shRNA following transcription.

[0093] In some embodiments, the vector encoding an RNAi construct of the invention is a viral vector. Various viral vector systems that are suitable to express the RNAi constructs described herein include, but are not limited to, adenoviral vectors, retroviral vectors (e.g., lentiviral vectors, maloney murine leukemia virus), adeno-associated viral vectors; herpes simplex viral vectors; SV40 vectors; polyoma viral vectors; papilloma viral vectors; picornaviral vectors; and pox viral vectors (e.g., vaccinia virus). In certain embodiments, the viral vector is a retroviral vector (e.g., lentiviral vector).

[0094] Various vectors suitable for use in the invention, methods for inserting nucleic acid sequences encoding siRNA or shRNA molecules into vectors, and methods of delivering the vectors to the cells of interest are known in the art (see, e.g., Dornburg , Gene Therap., Vol. 2: 301-310, 1995; Eglitis, Biotechniques, Vol. 6: 608-614, 1988; Miller, HumGene Therap., Vol. 1: 5-14, 1990; Anderson, Nature, Vol. 392: 25-30, 1998; Rubinson D A et al., Nat. Genet., Vol. 33: 401-406, 2003; Brummelkamp et al., Science, Vol. 296: 550-553, 2002; Brummelkamp et al., Cancer Cell, Vol. 2: 243-247, 2002; Lee et al., Nat Biotechnol, Vol. 20: 500-505, 2002; Miyagishi et al., Nat Biotechnol, Vol. 20: 497-500, 2002; Paddison et al., GenesDev, Vol. 16: 948-958, 2002; Paul et al., Nat Biotechnol, Vol. 20: 505-508, 2002; Sui et al., Proc Natl Acad Sci USA, Vol. 99: 5515-5520, 2002; and Yu et al., Proc Natl Acad Sci USA, Vol. 99: 6047-6052, 2002).

Compositions

[0095] The disclosure also provides compositions and formulations comprising the RNAi constructs described herein and pharmaceutically acceptable carriers, excipients, or diluents. Such compositions and formulations are useful for reducing expression of GPAM in a subject in need thereof. Where clinical applications are contemplated, pharmaceutical compositions and formulations will be prepared in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

[0096] The phrases "pharmaceutically acceptable" or "pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier, excipient, or diluent" includes solvents, buffers, solutions, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, etc., acceptable for use in formulating pharmaceuticals, such as pharmaceuticals suitable for administration to humans. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the RNAi constructs of the present invention, its use in therapeutic compositions is contemplated. Supplementary active

ingredients also can be incorporated into the compositions, provided they do not inactivate the vectors or RNAi constructs of the compositions.

[0097] Compositions and methods for the formulation of pharmaceutical compositions depend on several criteria, including, but not limited to, route of administration, type and extent of disease or disorder to be treated, and dose to be administered. In some embodiments, the pharmaceutical compositions are formulated based on the intended route of delivery. For instance, in certain embodiments, the pharmaceutical compositions are formulated for parenteral delivery. Parenteral forms of delivery include intravenous, intraarterial, subcutaneous, intrathecal, intraperitoneal, and intramuscular injection or infusion. In one embodiment, the pharmaceutical composition is formulated for intravenous delivery. In such an embodiment, the pharmaceutical composition may include a lipid-based delivery vehicle. In another embodiment, the pharmaceutical composition is formulated for subcutaneous delivery. In such an embodiment, the pharmaceutical composition may include a targeting ligand (e.g., GalNAc-containing ligands described herein).

[0098] In some embodiments, the pharmaceutical compositions comprise an effective amount of an RNAi construct described herein. An "effective amount" is an amount sufficient to produce a beneficial or desired clinical result. In some embodiments, an effective amount is an amount sufficient to reduce GPAM expression in hepatocytes of a subject. In some embodiments, an effective amount may be an amount sufficient to only partially reduce GPAM expression, for example, to a level comparable to expression of the wild-type GPAM allele in human heterozygotes. Human heterozygous carriers of loss of function GPAM variant alleles were reported to have lower serum levels of non- HDL cholesterol and a lower risk of coronary artery disease and myocardial infarction as compared to non-carriers (Nioi et al., New England Journal of Medicine, Vol. 374(22): 2131-2141, 2016). Thus, without being bound by theory, it is believed that partial reduction of GPAM expression may be sufficient to achieve the beneficial reduction of serum non-HDL cholesterol and reduction of risk of coronary artery disease and myocardial infarction.

[0099] An effective amount of an RNAi construct of the invention may be from about 0.01 mg/kg body weight to about 100 mg/kg body weight, about 0.05 mg/kg body weight to about 75mg/kg body weight, about 0.1 mg/kg body weight to about 50 mg/kg body

weight, about 1 mg/kg to about 30 mg/kg body weight, about 2.5 mg/kg of body weight to about 20 mg/kg bodyweight, or about 5 mg/kg body weight to about 15 mg/kg body weight. In certain embodiments, a single effective dose of an RNAi construct of the invention may be about 0.1 mg/kg, about 0.5mg/kg, about 1 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 6mg/kg, about 7 mg/kg, about 8 mg/kg, about 9 mg/kg, or about 10 mg/kg. The pharmaceutical composition comprising an effective amount of RNAi construct can be administered weekly, biweekly, monthly, quarterly, or biannually. The precise determination of what would be considered an effective amount and frequency of administration may be based on several factors, including a patient's size, age, gender, type of disorder to be treated (e.g., myocardial infarction, heart failure, coronary artery disease, hypercholesterolemia), particular RNAi construct employed, and route of administration. Estimates of effective dosages and *in vivo* half-lives for any particular RNAi construct of the invention can be ascertained using conventional methods and/or testing in appropriate animal models.

[0100] Colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems, including oil-in-water emulsions, micelles, mixed micelles, and liposomes, may be used as delivery vehicles for the RNAi constructs of the invention or vectors encoding such constructs. Commercially available fat emulsions that are suitable for delivering the nucleic acids of the invention include INTRALIPID®, LIPOSYN®, LIPOSYN®II, LIPOSYN®III, NUTRILIPID, and other similar lipid emulsions. A preferred colloidal system for use as a delivery vehicle *in vivo* is a liposome (i.e., an artificial membrane vesicle). The RNAi constructs of the invention may be encapsulated within liposomes, such as cationic liposomes. Alternatively, RNAi constructs of the invention may be complexed to lipids, such as cationic lipids. Suitable lipids and liposomes include neutral (e.g., dioleoylphosphatidyl ethanolamine (DOPE), dimyristoylphosphatidyl choline (DMPC), and dipalmitoyl phosphatidylcholine (DPPC)), distearolyphosphatidyl choline), negative (e.g., dimyristoylphosphatidyl glycerol (DMPG)), and cationic (e.g., dioleoyltetramethylaminopropyl (DOTAP) and dioleoylphosphatidyl ethanolamine (DOTMA)). The preparation and use of such colloidal dispersion systems is well known in the art. Exemplary formulations also are disclosed in, e.g., U.S. Patents

5,783,565; 5,837,533; 5,981,505; 6,127,170; 6,217,900; 6,379,965; 6,383,512; 6,747,014; 7,202,227; and WO 03/093449.

[0101] In some embodiments, the RNAi constructs of the invention are fully encapsulated in a lipid formulation, e.g., to form a SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle. As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle, including SPLP. As used herein, the term "SPLP" refers to a nucleic acid-lipid particle comprising plasmid DNA encapsulated within a lipid vesicle. SNALPs and SPLPs typically contain a cationic lipid, a noncationic lipid, and a lipid that prevents aggregation of the particle (e.g., a PEG-lipid conjugate). SNALPs and SPLPs are exceptionally useful for systemic applications, as they exhibit extended circulation lifetimes following intravenous injection and accumulate at distal sites (e.g., sites physically separated from the administration site). SPLPs include "pSPLP," which include an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683. The nucleic acid-lipid particles typically have a mean diameter of about 50 nm to about 150 nm, about 60 nm to about 130 nm, about 70 nm to about 110 nm, or about 70 nm to about 90 nm, and are substantially nontoxic. In addition, the nucleic acids present in the nucleic acid-lipid particles desirably are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, e.g., U.S. Patents 5,976,567; 5,981,501; 6,534,484; 6,586,410; and 6,815,432; and PCT Publication No. WO 96/40964.

[0102] Pharmaceutical compositions suitable for injections include, for example, sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Generally, these preparations are sterile and fluid to the extent that easy injectability exists. Preparations should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Appropriate solvents or dispersion media may contain, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by using a coating (such as lecithin), by maintaining the required particle size (in the case of dispersion), and/or by

using surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, such as, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, isotonic agents (e.g., sugars or sodium chloride) may be included in the composition. Prolonged absorption of the injectable compositions can be brought about by including absorption-delaying agents, such as, for example, aluminum monostearate and gelatin.

[0103] Sterile injectable solutions may be prepared by incorporating an appropriate amount of the RNAi construct (alone or complexed with a ligand) into a solvent along with any other ingredients (such as described above) as desired, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the desired other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions, suitable methods of preparation include vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient(s) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0104] The compositions provided herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include, for example, acid addition salts (formed with free amino groups) derived from inorganic acids (e.g., hydrochloric or phosphoric acids), or from organic acids (e.g., acetic, oxalic, tartaric, mandelic, and the like). Salts formed with free carboxyl groups can also be derived from inorganic bases (e.g., sodium, potassium, ammonium, calcium, or ferric hydroxides) or from organic bases (e.g., isopropylamine, trimethylamine, histidine, procaine, and the like).

[0105] For parenteral administration in an aqueous solution, for example, a solution generally is suitably buffered and a liquid diluent is first rendered isotonic with, e.g., sufficient saline or glucose. Such aqueous solutions may be used, for example, for intravenous, intramuscular, subcutaneous, and intraperitoneal administration. Sterile aqueous media desirably are employed as is known to those of skill in the art. By way of illustration, a single dose may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580).

For human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA standards. In certain embodiments, a pharmaceutical composition of the invention comprises or consists of a sterile saline solution and an RNAi construct described herein. In other embodiments, a pharmaceutical composition of the invention comprises or consists of an RNAi construct described herein and sterile water (e.g. water for injection, WFI). In still other embodiments, a pharmaceutical composition of the invention comprises or consists of an RNAi construct described herein and phosphate-buffered saline (PBS).

[0106] In some embodiments, the pharmaceutical compositions of the invention are packaged with or stored within a device for administration. Devices for injectable formulations include, but are not limited to, injection ports, pre-filled syringes, auto injectors, injection pumps, on-body injectors, and injection pens. Devices for aerosolized or powder formulations include, but are not limited to, inhalers, insufflators, aspirators, and the like. Thus, the present invention includes administration devices comprising a pharmaceutical composition of the invention for treating or preventing one or more of the disorders described herein.

Methods for Inhibiting GPAM Expression

[0107] The present disclosure also provides methods of inhibiting expression of a GPAM gene in a cell. The methods include contacting a cell with an RNAi construct, e.g., double-stranded RNAi construct, in an amount effective to inhibit expression of GPAM in the cell, thereby inhibiting expression of GPAM in the cell. Contacting a cell with an RNAi construct, e.g., a double-stranded RNAi construct, may be done *in vitro* or *in vivo*. Contacting a cell *in vivo* with the RNAi construct includes contacting a cell or group of cells within a subject, e.g., a human subject, with the RNAi construct. Combinations of *in vitro* and *in vivo* methods of contacting a cell also are within the scope of the present disclosure.

[0108] The present invention provides methods for reducing or inhibiting expression of GPAM in a subject in need thereof as well as methods of treating or preventing conditions, diseases, or disorders associated with GPAM expression or activity. A "condition, disease, or disorder associated with GPAM expression" refers to conditions, diseases, or disorders in

which GPAM expression levels are altered or where elevated expression levels of GPAM are associated with an increased risk of developing the condition, disease, or disorder.

[0109] Contacting a cell may be direct or indirect, as discussed above. Furthermore, contacting a cell may be accomplished via a targeting ligand, including any ligand described herein or known in the art. In preferred embodiments, the targeting ligand is a carbohydrate moiety, e.g., a GalNAc ligand, or a triantennary GalNAc structure, such as that shown in Example 1, or any other ligand that directs the RNAi construct to a site of interest.

[0110] In one embodiment, contacting a cell with an RNAi includes "introducing" or "delivering the RNAi into the cell" by facilitating or effecting uptake or absorption into the cell. Absorption or uptake of an RNAi can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. For *in vivo* introduction, for example, RNAi can be injected into a tissue site or administered systemically. *In vitro* introduction into a cell may be accomplished using methods known in the art, such as electroporation and lipofection. Additional approaches are described herein below and/or are known in the art.

[0111] The term "inhibiting," as used herein, is used interchangeably with "reducing," "silencing," "downregulating", "suppressing", and other similar terms, and includes any level of inhibition.

[0112] The phrase "inhibiting expression of a GPAM" is intended to refer to inhibition of expression of any GPAM gene (such as, e.g., a mouse GPAM gene, a rat GPAM gene, a monkey GPAM gene, or a human GPAM gene) as well as variants or mutants of a GPAM gene. Thus, the GPAM gene may be a wild-type GPAM gene, a mutant GPAM gene (such as a mutant GPAM gene giving rise to amyloid deposition), or a transgenic GPAM gene in the context of a genetically manipulated cell, group of cells, or organism.

[0113] "Inhibiting expression of a GPAM gene" includes any level of inhibition of a GPAM gene, e.g., at least partial suppression of the expression of a GPAM gene. The expression of the GPAM gene may be assessed based on the level, or the change in the level, of any variable associated with GPAM gene expression, e.g., GPAM mRNA level, GPAM protein level, or the number or extent of amyloid deposits. This level may be assessed in an individual cell or in a group of cells, including, for example, a sample derived from a subject.

[0114] Inhibition may be assessed by a decrease in an absolute or relative level of one or more variables that are associated with GPAM expression compared with a control level. The control level may be any type of control level that is utilized in the art, e.g., a pre-dose baseline level, or a level determined from a similar subject, cell, or sample that is untreated or treated with a control (such as, e.g., buffer only control or inactive agent control). In some embodiments, expression of a GPAM gene is inhibited by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%,at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%. at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%.

[0115] Inhibition of the expression of a GPAM gene may be manifested by a reduction of the amount of mRNA expressed by a first cell or group of cells (such cells may be present, for example, in a sample derived from a subject) in which a GPAM gene is transcribed and which has or have been treated (e.g., by contacting the cell or cells with an RNAi construct of the invention, or by administering an RNAi construct of the invention to a subject in which the cells are or were present), such that the expression of a GPAM gene is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has not or have not been so treated (control cell(s)). Inhibition may be assessed by expressing the level of mRNA in treated cells as a percentage of the level of mRNA in control cells, using the following formula:

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

[0116] Alternatively, inhibition of the expression of a GPAM gene may be assessed in terms of a reduction of a parameter that is functionally linked to GPAM gene expression, e.g., GPAM protein expression or Hedgehog pathway protein activities. GPAM gene

silencing may be determined in any cell expressing GPAM, either endogenously or recombinantly, by any assay known in the art.

[0117] Inhibition of the expression of a GPAM protein may be manifested by a reduction in the level of the GPAM protein that is expressed by a cell or group of cells (e.g., the level of protein expressed in a sample obtained from a subject). As explained above, for the assessment of mRNA suppression, the inhibition of protein expression levels in a treated cell or group of cells may similarly be expressed as a percentage of the level of protein in a control cell or group of cells.

[0118] A control cell or group of cells that may be used to assess the inhibition of the expression of a GPAM gene includes a cell or group of cells that has not yet been contacted with an RNAi construct of the invention. For example, the control cell or group of cells may be derived from an individual subject (e.g., a human or animal subject) prior to treatment of the subject with an RNAi construct.

[0119] The level of GPAM mRNA that is expressed by a cell or group of cells, or the level of circulating GPAM mRNA, may be determined using any method known in the art for assessing mRNA expression, such as those mentioned above. In some embodiments, the level of expression of GPAM in a sample is determined by detecting a transcribed polynucleotide, or portion thereof, e.g., mRNA of the GPAM gene. In this regard, for example, RNA may be extracted from cells using RNA extraction techniques including, for example, acid phenol/guanidine isothiocyanate extraction (RNAzol B; Biogenesis), RNeasy RNA preparation kits (Qiagen), or PAXgene (PreAnalytix, Switzerland). Typical assay formats utilizing ribonucleic acid hybridization include nuclear run-on assays, RT-PCR, RNase protection assays (Melton et al., Nuc. Acids Res., 12:7035), northern blotting, *in situ* hybridization, and microarray analysis. Circulating GPAM mRNA may be detected using methods described in WO 2012/177906.

[0120] In one embodiment, the level of expression of GPAM is determined using a nucleic acid probe. The term "probe," as used herein, refers to any molecule that is capable of selectively binding to a specific GPAM sequence. Probes can be synthesized by one of skill in the art or derived from appropriate biological preparations. Probes may be

specifically designed to be labeled. Examples of molecules that can be utilized as probes include, but are not limited to, RNA, DNA, proteins, antibodies, and organic molecules.

[0121] Isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or northern analyses, polymerase chain reaction (PCR) analyses, and probe arrays. One method for the determination of mRNA levels involves contacting isolated mRNA with a nucleic acid molecule (probe) that can hybridize to GPAM mRNA. In one embodiment, the mRNA is immobilized on a solid surface and contacted with a probe, for example by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative embodiment, the probe(s) are immobilized on a solid surface and the mRNA is contacted with the probe(s), for example, in an Affymetrix gene chip array. A skilled artisan can readily adapt known mRNA detection methods for use in determining the level of GPAM mRNA.

[0122] An alternative method for determining the level of expression of GPAM in a sample involves the process of nucleic acid amplification and/or reverse transcriptase (to prepare cDNA) of for example mRNA in the sample, e.g., by RT-PCR (see, e.g., U.S. Patent 4,683,202), ligase chain reaction (Barany (1991) Proc. Natl. Acad. Sci. USA 88: 189-193), self-sustained sequence replication (Guatelli et al. (1990) Proc. Natl. Acad. Sci. USA 87: 1874-1878), transcriptional amplification system (Kwoh et al. (1989) Proc. Natl. Acad. Sci. USA 86: 1173-1177), Q-Beta Replicase (Lizardi et al. (1988) Bio/Technology 6: 1197), rolling circle replication (Lizardi et al., *supra*; and U.S Patent 5,854,033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers. In some aspects of the invention, the level of expression of GPAM may be determined by quantitative fluorogenic RT-PCR {i.e., the TAQMAN™ System). The expression levels of GPAM mRNA may be monitored using a membrane blot (such as used in hybridization analysis such as northern, Southern, dot, and the like), or microwells, sample tubes, gels, beads or fibers (or any solid support comprising bound nucleic acids) (see, e.g., U.S. Patents 5,445,934; 5,677,195; 5,770,722; 5,744,305; and 5,874,219). The determination

of GPAM expression level may also comprise using nucleic acid probes in solution. In certain embodiments, the level of mRNA expression is assessed using branched DNA (bDNA) assays or real time PCR (qPCR).

[0123] The level of GPAM protein expression may be determined using any method known in the art for the measurement of protein levels. Such methods include, for example, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, fluid or gel precipitin reactions, absorption spectroscopy, colorimetric assays, spectrophotometric assays, flow cytometry, immunodiffusion (single or double), immunoelectrophoresis, Western blotting, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, electrochemiluminescence assays, etc.

[0124] In some embodiments, the efficacy of the methods of the invention can be monitored by detecting or monitoring a reduction in a symptom of a GPAM disease, such as reduction in edema swelling of the extremities, face, larynx, upper respiratory tract, abdomen, trunk, and genitals, prodrome; laryngeal swelling; nonpruritic rash; nausea; vomiting; or abdominal pain. These symptoms may be assessed *in vitro* or *in vivo* using any method known in the art.

[0125] In some embodiments, the RNAi construct or a composition comprising the RNAi construct is administered to a subject such that the RNAi construct is delivered to a specific site within the subject. The inhibition of expression of GPAM may be assessed using measurements of the level or change in the level of GPAM mRNA or GPAM protein in a sample derived from fluid or tissue from the specific site within the subject. In some embodiments, the RNAi construct may be delivered to a site such as the liver, choroid plexus, retina, and pancreas. The site may also be a subsection or subgroup of cells from any one of the aforementioned sites. The site may also include cells that express a particular type of receptor.

Methods of Treating or Preventing GPAM-Associated Diseases

[0126] The present invention provides therapeutic and prophylactic methods which include administering to a subject with a GPAM -associated disease, disorder, and/or

condition, or prone to developing, a GPAM- associated disease, disorder, and/or condition, an RNAi construct, compositions (e.g., pharmaceutical compositions) comprising an RNAi construct, or vectors comprising an RNAi construct as described herein. Non-limiting examples of GPAM- associated diseases include, for example, fatty liver (steatosis), nonalcoholic steatohepatitis (NASH), cirrhosis of the liver, accumulation of fat in the liver, inflammation of the liver, hepatocellular necrosis, liver fibrosis, obesity, and nonalcoholic fatty liver disease (NAFLD). In one embodiment, the GPAM-associated disease is NAFLD. In another embodiment, the GPAM-associated disease is NASH. In another embodiment, the GPAM-associated disease is fatty liver (steatosis). In another embodiment, the GPAM-associated disease is insulin resistance. In another embodiment, the GPAM-associated disease is not insulin resistance.

[0127] In certain embodiments, the present invention provides a method for reducing the expression of GPAM in a patient in need thereof comprising administering to the patient any of the RNAi constructs described herein. The term "patient," as used herein, refers to a mammal, including humans, and can be used interchangeably with the term "subject." The expression level of GPAM in hepatocytes in the patient desirably is reduced following administration of the RNAi construct as compared to the GPAM expression level in a patient not receiving the RNAi construct.

[0128] The methods of the invention are useful for treating a subject having a GPAM- associated disease, e.g., a subject that would benefit from reduction in GPAM gene expression and/or GPAM protein production. In one aspect, the present invention provides methods of reducing the level of glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) gene expression in a subject having nonalcoholic fatty liver disease (NAFLD). In another aspect, the present invention provides methods of reducing the level of GPAM protein in a subject with NAFLD. The present invention also provides methods of reducing the level of activity of the hedgehog pathway in a subject with NAFLD.

[0129] The treatment methods (and uses) of the invention include administering to the subject, e.g., a human, a therapeutically effective amount of the disclosed RNAi construct targeting a GPAM gene, a pharmaceutical composition comprising the RNAi construct, or a vector comprising the RNAi construct.

[0130] In one aspect, the invention provides methods of preventing at least one symptom in a subject having NAFLD, e.g., the presence of elevated hedgehog signaling pathways, fatigue, weakness, weight loss, loss of apetite, nausea, abdominal pain, spider-like blood vessels, yellowing of the skin and eyes (jaundice), itching, fluid buildup and swelling of the legs (edema), abdomen swelling (ascites), and mental confusion. The methods include administering to the subject a prophylactically effective amount of the RNAi construct, e.g., dsRNA, pharmaceutical compositions comprising the RNAi construct, or vectors encoding the RNAi construct, thereby preventing at least one symptom in the subject having a disorder that would benefit from reduction in GPAM gene expression. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired prophylactic result (e.g., prevention of disease onset).

[0131] In another aspect, the present invention provides uses of a therapeutically effective amount of an RNAi construct of the invention for treating a subject, e.g., a subject that would benefit from a reduction and/or inhibition of GPAM gene expression. In a further aspect, the present invention provides uses of an RNAi construct, e.g., a dsRNA, of the invention targeting an GPAM gene or pharmaceutical composition comprising an RNAi construct targeting an GPAM gene in the manufacture of a medicament for treating a subject, e.g., a subject that would benefit from a reduction and/or inhibition of GPAM gene expression and/or GPAM protein production, such as a subject having a disorder that would benefit from reduction in GPAM gene expression, e.g., a GPAM-associated disease.

[0132] The disclosure provides uses of an RNAi construct, e.g., a dsRNA, of the invention for preventing at least one symptom in a subject suffering from a disorder that would benefit from a reduction and/or inhibition of GPAM gene expression and/or GPAM protein production. For example, the disclosure provides uses of the RNAi construct described herein, compositions comprising same, and vectors comprising same, in the treatment of NAFLD.

[0133] In a further aspect, the present invention provides uses of the disclosed RNAi construct, compositions comprising same, or a vector comprising same, in the manufacture of a medicament for preventing at least one symptom in a subject suffering from a disorder that

would benefit from a reduction and/or inhibition of GPAM gene expression and/or GPAM protein production, such as a GPAM-associated disease.

[0134] In one embodiment, an RNAi construct targeting GPAM is administered to a subject having a GPAM-associated disease, e.g., nonalcoholic fatty liver disease (NAFLD), such that the expression of a GPAM gene, e.g., in a cell, tissue, blood or other tissue or fluid of the subject are reduced by at least about 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 62%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least about 99% or more when the RNAi construct is administered to the subject.

[0135] The methods and uses of the invention include administering a composition described herein such that expression of the target GPAM gene is decreased for any suitable amount of time, such as for about 1, 2, 3, 4 5, 6, 7, 8, 12, 16, 18, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, or about 80 hours. In one embodiment, expression of the target GPAM gene is decreased for an extended duration, e.g., at least about two, three, four, five, six, seven days or more, e.g., about one week, two weeks, three weeks, or about four weeks or longer.

[0136] Administration of the RNAi construct according to the methods and uses of the invention may result in a reduction of the severity, signs, symptoms, and/or markers of such diseases or disorders in a patient with a GPAM-associated disease, e.g., NAFLD. By "reduction" in this context is meant a statistically significant decrease in such level. The reduction can be, for example, at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or about 100%. Efficacy of treatment or prevention of disease can be assessed, for example by measuring disease progression, disease remission, symptom severity, reduction in pain, quality of life, dose of a medication required to sustain a treatment effect, level of a disease marker or any other measurable parameter appropriate for a given disease being treated or targeted for prevention. It is well within the ability of one skilled in the art to monitor efficacy of

treatment or prevention by measuring any one of such parameters, or any combination of parameters. For example, efficacy of treatment of NAFLD may be assessed, for example, by periodic monitoring of NAFLD symptoms, liver fat levels, or expression of downstream genes. Comparison of the later readings with the initial readings provide a physician an indication of whether the treatment is effective. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. In connection with the administration of an RNAi targeting GPAM or pharmaceutical composition thereof, "effective against" an GPAM -associated disease indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as improvement of symptoms, a cure, a reduction in disease, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating NAFLD and/or an GPAM -associated disease and the related causes.

[0137] A treatment or preventive effect is evident when there is a statistically significant improvement in one or more parameters of disease status, or by a failure to worsen or to develop symptoms where they would otherwise be anticipated. As an example, a favorable change of at least 10% in a measurable parameter of disease, and preferably at least 20%, 30%, 40%, 50% or more can be indicative of effective treatment. Efficacy for a given RNAi drug or formulation of that drug can also be judged using an experimental animal model for the given disease as known in the art. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant reduction in a marker or symptom is observed.

[0138] Subjects can be administered any therapeutically effective amount of the RNAi construct. Exemplary therapeutically effective amounts of the RNAi construct include, but are not limited to, 0.01 mg/kg, 0.02 mg/kg, 0.03 mg/kg, 0.04 mg/kg, 0.05 mg/kg, 0.1 mg/kg, 0.15 mg/kg, 0.2 mg/kg, 0.25 mg/kg, 0.3 mg/kg, 0.35 mg/kg, 0.4 mg/kg, 0.45 mg/kg, 0.5 mg/kg, 0.55 mg/kg, 0.6 mg/kg, 0.65 mg/kg, 0.7 mg/kg, 0.75 mg/kg, 0.8 mg/kg, 0.85 mg/kg, 0.9 mg/kg, 0.95 mg/kg, 1.0 mg/kg, 1.1 mg/kg, 1.2 mg/kg, 1.3 mg/kg, 1.4 mg/kg, 1.5 mg/kg, 1.6 mg/kg, 1.7 mg/kg, 1.8 mg/kg, 1.9 mg/kg, 2.0 mg/kg, 2.1 mg/kg, 2.2 mg/kg, 2.3 mg/kg, 2.4 mg/kg, 2.5 mg/kg, 2.6 mg/kg, 2.7 mg/kg, 2.8 mg/kg, 2.9 mg/kg, 3.0 mg/kg,

3.1 mg/kg, 3.2 mg/kg, 3.3 mg/kg, 3.4 mg/kg, 3.5 mg/kg, 3.6 mg/kg, 3.7 mg/kg, 3.8 mg/kg, 3.9 mg/kg, 4.0 mg/kg, 4.1 mg/kg, 4.2 mg/kg, 4.3 mg/kg, 4.4 mg/kg, 4.5 mg/kg, 4.6 mg/kg, 4.7 mg/kg, 4.8 mg/kg, 4.9 mg/kg, 5.0 mg/kg, 5.1 mg/kg, 5.2 mg/kg, 5.3 mg/kg, 5.4 mg/kg, 5.5 mg/kg, 5.6 mg/kg, 5.7 mg/kg, 5.8 mg/kg dsRNA, 5.9 mg/kg, 6.0 mg/kg, 6.1 mg/kg, 6.2 mg/kg, 6.3 mg/kg, 6.4 mg/kg, 6.5 mg/kg, 6.6 mg/kg, 6.7 mg/kg, 6.8 mg/kg, 6.9 mg/kg, 7.0 mg/kg, 7.1 mg/kg, 7.2 mg/kg, 7.3 mg/kg, 7.4 mg/kg, 7.5 mg/kg, 7.6 mg/kg, 7.7 mg/kg, 7.8 mg/kg, 7.9 mg/kg, 8.0 mg/kg, 8.1 mg/kg, 8.2 mg/kg, 8.3 mg/kg, 8.4 mg/kg, 8.5 mg/kg, 8.6 mg/kg, 8.7 mg/kg, 8.8 mg/kg, 8.9 mg/kg, 9.0 mg/kg, 9.1 mg/kg, 9.2 mg/kg, 9.3 mg/kg, 9.4 mg/kg, 9.5 mg/kg, 9.6 mg/kg, 9.7 mg/kg, 9.8 mg/kg, 9.9 mg/kg, 9.0 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30 mg/kg, 35 mg/kg, 40 mg/kg, 45 mg/kg, or about 50 mg/kg. In one embodiment, subjects can be administered 0.5 mg/kg of the RNAi construct. Values and ranges intermediate to the recited values also are encompassed by the present disclosure.

[0139] Administration of the RNAi construct, or a composition comprising same, can reduce the presence of GPAM protein levels, e.g., in a cell, tissue, blood, urine or other compartment of the patient by at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31 %, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least about 99% or more.

[0140] Before administration of a full dose of the RNAi, patients can be administered a smaller dose, such as a 5% infusion, and monitored for adverse effects, such as an allergic reaction. In another example, the patient can be monitored for unwanted immunostimulatory effects, such as increased cytokine (e.g., TNF-alpha or INF-alpha) levels.

[0141] Owing to the inhibitory effects on GPAM expression, a composition according to the invention or a pharmaceutical composition prepared therefrom can enhance the quality of life.

[0142] An RNAi of the invention may be administered in "naked" form, where the modified or unmodified RNAi construct is directly suspended in aqueous or suitable buffer

solvent, as a "free RNAi." A free RNAi is administered in the absence of a pharmaceutical composition. The free RNAi may be in a suitable buffer solution. The buffer solution may comprise acetate, citrate, prolamine, carbonate, or phosphate, or any combination thereof. In one embodiment, the buffer solution is phosphate buffered saline (PBS). The pH and osmolality of the buffer solution containing the RNAi can be adjusted such that it is suitable for administering to a subject.

[0143] Alternatively, an RNAi of the invention may be administered as a pharmaceutical composition, such as a dsRNA liposomal formulation.

[0144] Subjects that would benefit from a reduction and/or inhibition of GPAM gene expression are those having nonalcoholic fatty liver disease (NAFLD) and/or an GPAM-associated disease or disorder as described herein.

[0145] Treatment of a subject that would benefit from a reduction and/or inhibition of GPAM gene expression includes therapeutic and prophylactic treatment.

[0146] The invention further provides methods and uses of an RNAi construct or a pharmaceutical composition thereof for treating a subject that would benefit from reduction and/or inhibition of GPAM gene expression, e.g., a subject having a GPAM-associated disease, in combination with other pharmaceuticals and/or other therapeutic methods, e.g., with known pharmaceuticals and/or known therapeutic methods, such as, for example, those which are currently employed for treating these disorders.

[0147] For example, in certain embodiments, an RNAi targeting a GPAM gene is administered in combination with, e.g., an agent useful in treating an GPAM-associated disease. For example, additional therapeutics and therapeutic methods suitable for treating a subject that would benefit from reduction in GPAM expression, e.g., a subject having a GPAM-associated disease, include an RNAi construct targeting a different portion of the GPAM gene, a therapeutic agent, and/or procedures for treating a GPAM -associated disease or a combination of any of the foregoing.In certain embodiments, a first RNAi construct targeting a GPAM gene is administered in combination with a second RNAi construct targeting a different portion of the GPAM gene. For example, the first RNAi construct may comprise a first sense strand and a first antisense strand forming a double stranded region, wherein substantially all of the nucleotides of said first sense strand and substantially all of

the nucleotides of the first antisense strand are modified nucleotides, wherein said first sense strand is conjugated to a ligand attached at the 3'- terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker; and the second RNAi construct may comprise a second sense strand and a second antisense strand forming a double stranded region, wherein substantially all of the nucleotides of the second sense strand and substantially all of the nucleotides of the second antisense strand are modified nucleotides, wherein the second sense strand is conjugated to a ligand attached at the 3 '-terminus, and wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker. In one embodiment, all of the nucleotides of the first and second sense strand and/or all of the nucleotides of the first and second antisense strand comprise a modification. The modified nucleotides may be any one or combination of the modified nucleotides described herein.

[0148] In other embodiments, a first RNAi construct targeting a GPAM gene is administered in combination with a second RNAi construct targeting a gene that is different from the GPAM gene. For example, the RNAi construct targeting the GPAM gene may be administered in combination with an RNAi construct targeting the SCAP gene. SCAP (SREBP Cleavage Activating Protein) is the only known regulator of the transcription factors of the SREBP family. The SREBP (Sterol Response Element Binding Protein) family play important roles in regulating *de novo* lipogenesis and triglyceride (TG) accumulation within the liver. The first RNAi construct targeting a GPAM gene and the second RNAi construct targeting a different gene, e.g., the SCAP gene, may be administered as parts of the same pharmaceutical composition. Alternatively, the first RNAi construct targeting a GPAM gene and the second RNAi construct targeting a different gene, e.g., the SCAP gene, may be administered as parts of different pharmaceutical compositions. In addition, or alternatively, a first RNAi construct targeting a GPAM gene can be administered in combination with a second RNAi construct targeting the Patatin-Like Phospholipase Domain Containing 3 (PNPLA3) gene, or in combination with a second RNAi that targets SCAP and a third RNAi that targets PNPLA3. Patatin-like phospholipase domain-containing 3 (PNPLA3), formerly known as adiponutrin (ADPN) and calcium-independent phospholipase A2-epsilon (iPLA(2)ε), is a type II transmembrane protein (Wilson et al (2006) J Lipid Res 47(9):1940-

9; Jenkins et al (2004) J Biol Chem 279(47):48968-75). Initially identified in adipose cells as a membrane-associated, adipose-enriched protein induced during adipogenesis in mice, it is now well characterized to be expressed in other tissues, including the liver (Wilson et al, supra; Baulande et al (2001) J Biol Chem 276(36):33336-44; Moldes et al. (2006) Eur J Endocrinol 155(3):461-8; Faraj et al. (2006) J Endocrinol 191(2):427-35; Liu et al (2004) J Clin Endocrinol Metab 89(6):2684-9; Lake et al (2005) J Lipid Res 46(11) :2477-87). In cell-free biochemical systems, recombinant PNPLA3 protein can exhibit either triacylglycerol lipase or transacylation activity (Jenkins et al., supra; Kumari et al (2012) Cell Metab 15(5):691-702; He et al (2010) J Biol Chem 285(9):6706-15). In hepatocytes, PNPLA3 is expressed on the endoplasmic reticulum and lipid membranes and predominantly exhibits triacylglycerol hydrolase activity (He et al., supra; Huang et al (2010) Proc Natl Acad Sci USA 107(17):7892-7; Ruhanen et al (2014) J Lipid Res 55(4):739-46; Pingitore et al. (2014) Biochim Biophys Acta 1841(4):574-80). Although lacking a secretory signal, data indicates PNPLA3 is secreted and can be found in human plasma as disulfide-bond dependent multimers (Winberg et al. (2014) Biochem Biophys Res Commun 446(4):1114-9).

[0149] The RNAi construct and an additional therapeutic agent and/or treatment may be administered at the same time and/or in the same combination, e.g., parenterally, or the additional therapeutic agent can be administered as part of a separate composition or at separate times and/or by another method known in the art or described herein.

[0150] The present invention also provides methods of using an RNAi construct of the invention and/or a composition containing an RNAi construct of the invention to reduce and/or inhibit GPAM expression (gene or protein expression) in a cell. In yet other aspects, use of an RNAi construct of the invention and/or a composition comprising an RNAi construct of the invention for the manufacture of a medicament for reducing and/or inhibiting GPAM gene expression in a cell are provided. In still other aspects, the present invention provides an RNAi of the invention and/or a composition comprising an RNAi construct of the invention for use in reducing and/or inhibiting GPAM protein production in a cell. In yet other aspects, use of an RNAi construct of the invention and/or a composition comprising an RNAi construct of the invention for the manufacture of a medicament for reducing and/or inhibiting GPAM protein production in a cell are provided. The methods and uses include contacting the cell with an

RNAi construct, e.g., a dsRNA, of the invention and maintaining the cell for a time sufficient to obtain degradation of the mRNA transcript of a GPAM gene, thereby inhibiting expression of the GPAM gene or inhibiting GPAM protein production in the cell. Reduction in gene expression can be assessed by any methods known in the art or described herein for determining mRNA or protein levels.

[0151] In the methods and uses of the invention the cell may be contacted *in vitro* or *in vivo*, i.e., the cell may be outside (e.g., in cell culture) or within a subject. A cell suitable for treatment using the methods of the invention may be any cell that expresses an GPAM gene, e.g., a cell from a subject having NAFLD or a cell comprising an expression vector comprising a GPAM gene or portion of a GPAM gene. A suitable cell for use in the disclosed methods includes, for example, a mammalian cell, e.g., a primate cell (such as a human cell or a non-human primate cell, e.g., a monkey cell or a chimpanzee cell), a non-primate cell (such as a cow cell, a pig cell, a camel cell, a llama cell, a horse cell, a goat cell, a rabbit cell, a sheep cell, a hamster, a guinea pig cell, a cat cell, a dog cell, a rat cell, a mouse cell, a lion cell, a tiger cell, a bear cell, or a buffalo cell), a bird cell (e.g., a duck cell or a goose cell), or a whale cell. In one embodiment, the cell is a human cell.

[0152] GPAM gene expression may be inhibited in the cell by at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or about 100%.

[0153] GPAM protein production may be inhibited in the cell by at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or about 100%.

[0154] The *in vivo* methods and uses of the invention may include administering to a subject a composition containing an RNAi construct, where the RNAi construct includes a nucleotide sequence that is complementary to at least a part of an RNA transcript of the GPAM gene of the subject. When the organism to be treated is a human, the composition can be administered by any means known in the art including, but not limited to subcutaneous, intravenous, oral, intraperitoneal, or parenteral routes, including intracranial (e.g., intraventricular, intraparenchymal, and intrathecal), intramuscular, transdermal, airway (aerosol), nasal, rectal, and topical (including buccal and sublingual) administration. In certain embodiments, the compositions are administered by subcutaneous or intravenous infusion or injection. In one embodiment, the compositions are administered by subcutaneous injection.

[0155] In some embodiments, the administration is via a depot injection. A depot injection may release the RNAi construct in a consistent way over a prolonged time period. Thus, a depot injection may reduce the frequency of dosing needed to obtain a desired effect, e.g., a desired inhibition of GPAM, or a therapeutic or prophylactic effect. A depot injection may also provide more consistent serum concentrations. Depot injections may include subcutaneous injections or intramuscular injections. In some embodiments, the depot injection is a subcutaneous injection.

[0156] In some embodiments, the administration is via a pump. The pump may be an external pump or a surgically implanted pump. In certain embodiments, the pump is a subcutaneously implanted osmotic pump. In other embodiments, the pump is an infusion pump. An infusion pump may be used for intravenous, subcutaneous, arterial, or epidural infusions. In preferred embodiments, the infusion pump is a subcutaneous infusion pump. In other embodiments, the pump is a surgically implanted pump that delivers the RNAi to the subject.

[0157] The mode of administration may be chosen based upon whether local or systemic treatment is desired and based upon the area to be treated. The route and site of administration may be chosen to enhance targeting.

[0158] The methods and uses include administering to the mammal, e.g., a human, a composition comprising an RNAi construct, e.g., an siRNA, that targets an GPAM gene in a

cell of the mammal and maintaining the mammal for a time sufficient to obtain degradation of the mRNA transcript of the GPAM gene, thereby inhibiting expression of the GPAM gene in the mammal. Reduction in gene expression and/or protein expression can be assessed in a sample obtained from the RNAi construct-administered subject by any method known in the art or described herein. In one embodiment, a tissue sample serves as the tissue material for monitoring the reduction in GPAM gene and/or protein expression. In another embodiment, a blood sample serves as the tissue material for monitoring the reduction in GPAM gene and/or protein expression.

[0159] In some embodiments, verification of RISC-mediated cleavage of a target mRNA (e.g., GPAM mRNA) *in vivo* following administration of an RNAi construct may be assessed by performing 5 '-RACE or modifications of the protocol as known in the art (Lasham A et al., (2010) Nucleic Acid Res., 38 (3) p-el9; and Zimmermann et al. (2006) Nature 441: 111-4).

[0160] It is understood that all ribonucleic acid sequences disclosed herein can be converted to deoxyribonucleic acid sequences by substituting a thymine base for a uracil base in the sequence. Likewise, all deoxyribonucleic acid sequences disclosed herein can be converted to ribonucleic acid sequences by substituting a uracil base for a thymine base in the sequence. Deoxyribonucleic acid sequences, ribonucleic acid sequences, and sequences containing mixtures of deoxyribonucleotides and ribonucleotides of all sequences disclosed herein are encompassed by the present invention.

[0161] Additionally, any nucleic acid sequences disclosed herein may be modified with any combination of chemical modifications. One of skill in the art will readily appreciate that such designation as "RNA" or "DNA" to describe modified polynucleotides is, in certain instances, arbitrary. For example, a polynucleotide comprising a nucleotide having a 2'-OH substituent on the ribose sugar and a thymine base could be described as a DNA molecule having a modified sugar (2'-OH for the natural 2'-H of DNA) or as an RNA molecule having a modified base (thymine (methylated uracil) for natural uracil of RNA).

[0162] Accordingly, nucleic acid sequences provided herein, including but not limited to those set forth in the sequence listing, are intended to encompass nucleic acids containing any combination of natural or modified RNA and/or DNA, including, but not

limited to, such nucleic acids having modified nucleobases. By way of a further example and without limitation, a polynucleotide having the sequence "ATCGATCG" encompasses any polynucleotides having such a sequence, whether modified or unmodified, including, but not limited to, such compounds comprising RNA bases, such as those having sequence "AUCGAUCG" and those having some DNA bases and some RNA bases such as "AUCGATCG," and polynucleotides having other modified bases, such as "ATmeCGAUCG," wherein meC indicates a cytosine base comprising a methyl group at the 5-position.

[0163] The following examples, including the experiments conducted and the results achieved, are provided for illustrative purposes only and are not to be construed as limiting the scope of the appended claims.

EXAMPLES

[0164] All animal experiments described herein were approved by the Institutional Animal Care and Use Committee (IACUC) of Amgen and cared for in accordance to the Guide for the Care and Use of Laboratory Animals, 8th Edition (National Research Council (U.S.)). Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (U.S.), and National Academies Press (U.S.) (2011) *Guide for the care and use of laboratory animals. 8th Ed.,* National Academies Press, Washington, D.C. Mice were single-housed in an air-conditioned room at 22±2°C with a twelve-hour light; twelve-hour darkness cycle (0600-1800 hours). Animals had *ad libitum* access to a regular chow diet (Envigo, 2920X, or a diet as stated otherwise) and to water (reverse osmosis-purified) via automatic watering system, unless otherwise indicated. At termination, blood was collected by cardiac puncture under deep anesthesia, and then, following Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines, euthanized by a secondary physical method.

EXAMPLE 1: Selection, Design and Synthesis of Modified GPAM siRNA molecules

[0165] The identification and selection of optimal sequences for therapeutic siRNA molecules targeting glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) were

identified using bioinformatics analysis of a human GPAM transcript (GenBank Accession No. XM_005269998.1). Table 1 lists GPAM mRNA target sequences, having an inverted abasic nucleotide appended to the 3' end of the sense strand, identified as having therapeutic properties.

Table 1. siRNA sequences directed to GPAM

Duplex No.	Sense sequence (5'-3')	SEQ ID NO: (sense)	Antisense sequence (5'-3')	SEQ ID NO: (antisense)
1001	GGGACUCUUUCUGAGGUUAC{invAb}	1	AGUAACCUCAGAAAGAGUCCCUU	2
1002	CUUUCUGAGGUUACUGUGGA{invAb}	3	AUCCACAGUAACCUCAGAAAGUU	4
1003	CUGAGGUUACUGUGGAGCAC{invAb}	5	AGUGCUCCACAGUAACCUCAGUU	6
1004	UUUGCUAAUCGACUGAUUGG{invAb}	7	UCCAAUCAGUCGAUUAGCAAAUU	8
1005	GCUAAUCGACUGAUUGGAAA{invAb}	9	AUUUCCAAUCAGUCGAUUAGCUU	10
1006	CUAAUCGACUGAUUGGAAAU{invAb}	11	UAUUUCCAAUCAGUCGAUUAGUU	12
1007	UAAUCGACUGAUUGGAAAUA{invAb}	13	UUAUUUCCAAUCAGUCGAUUAUU	14
1008	AAUCGACUGAUUGGAAAUAA{invAb}	15	AUUAUUUCCAAUCAGUCGAUUUU	16
1009	GGAAAUAAUUCCUCAAACAC{invAb}	17	AGUGUUUGAGGAAUUAUUUCCUU	18
1010	AAUAAUUCCUCAAACACCAC{invAb}	19	AGUGGUGUUUGAGGAAUUAUUUU	20
1011	ACACCACCAAGUCAAGGAUA{invAb}	21	AUAUCCUUGACUUGGUGGUGUUU	22
1012	ACCACCAAGUCAAGGAUACA{invAb}	23	AUGUAUCCUUGACUUGGUGGUUU	24
1013	AGUCAAGGAUACAGGCAGCA{invAb}	25	AUGCUGCCUGUAUCCUUGACUUU	26
1014	AAGGAUACAGGCAGCAGCGG{invAb}	27	ACCGCUGCUGCCUGUAUCCUUUU	28
1015	GCAGCGGCUCCCCUGUUGUA{invAb}	29	AUACAACAGGGGAGCCGCUGCUU	30
1016	AGCGGCUCCCCUGUUGUAUG{invAb}	31	ACAUACAACAGGGGAGCCGCUUU	32
1017	CGGCUCCCCUGUUGUAUGGA{invAb}	33	AUCCAUACAACAGGGGAGCCGUU	34
1018	CCCCUGUUGUAUGGACAUUC{invAb}	35	AGAAUGUCCAUACAACAGGGGUU	36
1019	UGGACAUUCUGCACCCGAAA{invAb}	37	AUUUCGGGUGCAGAAUGUCCAUU	38
1020	CUGCACCCGAAACUGAUAGC{invAb}	39	AGCUAUCAGUUUCGGGUGCAGUU	40
1021	UGCACCCGAAACUGAUAGCU{invAb}	41	AAGCUAUCAGUUUCGGGUGCAUU	42
1022	CACCCGAAACUGAUAGCUGA{invAb}	43	AUCAGCUAUCAGUUUCGGGUGUU	44
1023	CCGAAACUGAUAGCUGAGUC{invAb}	45	AGACUCAGCUAUCAGUUUCGGUU	46
1024	AACUGAUAGCUGAGUCCUGA{invAb}	47	UUCAGGACUCAGCUAUCAGUUUU	48
1025	AUAGCUGAGUCCUGAAGUUU{invAb}	49	AAAACUUCAGGACUCAGCUAUUU	50
1026	CAGCACAUGAUUUGGGAAUU{invAb}	51	UAAUUCCCAAAUCAUGUGCUGUU	52
1027	ACAUGAUUUGGGAAUUACAC{invAb}	53	AGUGUAAUUCCCAAAUCAUGUUU	54
1028	AUUUGGGAAUUACACUUUGU{invAb}	55	AACAAAGUGUAAUUCCCAAAUUU	56
1029	GGGAAUUACACUUUGUGACA{invAb}	57	AUGUCACAAAGUGUAAUUCCCUU	58
1030	GAAUUACACUUUGUGACAUG{invAb}	59	ACAUGUCACAAAGUGUAAUUCUU	60

1031	ACACUUUGUGACAUGGAUGA{invAb}	61	UUCAUCCAUGUCACAAAGUGUUU	62
1032	CACUUUGUGACAUGGAUGAA{invAb}	63	AUUCAUCCAUGUCACAAAGUGUU	64
1033	AUGAAUCUGCACUGACCCUU{invAb}	65	AAAGGGUCAGUGCAGAUUCAUUU	66
1034	AUCUGCACUGACCCUUGGUA{invAb}	67	AUACCAAGGGUCAGUGCAGAUUU	68
1035	GCACUGACCCUUGGUACAAU{invAb}	69	UAUUGUACCAAGGGUCAGUGCUU	70
1036	CUGACCCUUGGUACAAUAGA{invAb}	71	AUCUAUUGUACCAAGGGUCAGUU	72
1037	AAUAGAUGUUUCUUAUCUGC{invAb}	73	AGCAGAUAAGAAACAUCUAUUUU	74
1038	AUCAGAAUACAGUGUUGGUC{invAb}	75	AGACCAACACUGUAUUCUGAUUU	76
1039	UCAGAAUACAGUGUUGGUCG{invAb}	77	UCGACCAACACUGUAUUCUGAUU	78
1040	CAGAAUACAGUGUUGGUCGA{invAb}	79	AUCGACCAACACUGUAUUCUGUU	80
1041	AGAAUACAGUGUUGGUCGAU{invAb}	81	AAUCGACCAACACUGUAUUCUUU	82
1042	GAAUACAGUGUUGGUCGAUG{invAb}	83	ACAUCGACCAACACUGUAUUCUU	84
1043	AAUACAGUGUUGGUCGAUGU{invAb}	85	UACAUCGACCAACACUGUAUUUU	86
1044	UACAGUGUUGGUCGAUGUAA{invAb}	87	AUUACAUCGACCAACACUGUAUU	88
1045	AGUGUUGGUCGAUGUAAGCA{invAb}	89	AUGCUUACAUCGACCAACACUUU	90
1046	UGUAAGCACACAAGUGAGGA{invAb}	91	UUCCUCACUUGUGUGCUUACAUU	92
1047	UAAGCACACAAGUGAGGAAU{invAb}	93	AAUUCCUCACUUGUGUGCUUAUU	94
1048	GCACACAAGUGAGGAAUGGG{invAb}	95	ACCCAUUCCUCACUUGUGUGCUU	96
1049	GGAAAGAAAGCCUAAUGAGU{invAb}	97	AACUCAUUAGGCUUUCUUUCCUU	98
1050	AAGAAAGCCUAAUGAGUCGG{invAb}	99	UCCGACUCAUUAGGCUUUCUUUU	100
1051	AGAAAGCCUAAUGAGUCGGA{invAb}	101	UUCCGACUCAUUAGGCUUUCUUU	102
1052	GAAAGCCUAAUGAGUCGGAA{invAb}	103	UUUCCGACUCAUUAGGCUUUCUU	104
1053	AAGCCUAAUGAGUCGGAAAA{invAb}	105	AUUUUCCGACUCAUUAGGCUUUU	106
1054	CCUAAUGAGUCGGAAAAGGC{invAb}	107	AGCCUUUUCCGACUCAUUAGGUU	108
1055	CUAAUGAGUCGGAAAAGGCC{invAb}	109	UGGCCUUUUCCGACUCAUUAGUU	110
1056	AAUGAGUCGGAAAAGGCCAU{invAb}	111	AAUGGCCUUUUCCGACUCAUUUU	112
1057	GUUGGAAGAUGUUGUUACUC{invAb}	113	AGAGUAACAACAUCUUCCAACUU	114
1058	AAAUUUUUCAACCCCAGUAU{invAb}	115	AAUACUGGGGUUGAAAAAUUUUU	116
1059	AAUUUUUCAACCCCAGUAUC{invAb}	117	AGAUACUGGGGUUGAAAAAUUUU	118
1060	UGGGUUUGCGGAAUGUUAUU{invAb}	119	AAAUAACAUUCCGCAAACCCAUU	120
1061	GGUUUGCGGAAUGUUAUUUA{invAb}	121	AUAAAUAACAUUCCGCAAACCUU	122
1062	UGCGGAAUGUUAUUUAUAUC{invAb}	123	UGAUAUAAAUAACAUUCCGCAUU	124
1063	GCGGAAUGUUAUUUAUAUCA{invAb}	125	UUGAUAUAAAUAACAUUCCGCUU	126
1064	AUUUAUAUCAAUGAAACUCA{invAb}	127	AUGAGUUUCAUUGAUAUAAAUUU	128
1065	UUAUAUCAAUGAAACUCACA{invAb}	129	AUGUGAGUUUCAUUGAUAUAAUU	130
1066	UCAAUGAAACUCACACAAGA{invAb}	131	AUCUUGUGUGAGUUUCAUUGAUU	132
1067	UGAAACUCACACAAGACACC{invAb}	133	AGGUGUCUUGUGUGAGUUUCAUU	134
1068	GAAACUCACACAAGACACCG{invAb}	135	ACGGUGUCUUGUGUGAGUUUCUU	136
1069	CGCGGAUGGCUUGCAAGACG{invAb}	137	ACGUCUUGCAAGCCAUCCGCGUU	138
1070	GCGGAUGGCUUGCAAGACGC{invAb}	139	AGCGUCUUGCAAGCCAUCCGCUU	140
1071	GCUUGCAAGACGCCUUUCUU{invAb}	141	UAAGAAAGGCGUCUUGCAAGCUU	142
1072	UCUUUUUAUUCAAGAGCGAG{invAb}	143	UCUCGCUCUUGAAUAAAAAGAUU	144

1073	CUUUUUAUUCAAGAGCGAGA{invAb}	145	AUCUCGCUCUUGAAUAAAAAGUU	146
1074	UUUUUAUUCAAGAGCGAGAU{invAb}	147	AAUCUCGCUCUUGAAUAAAAAUU	148
1075	UUUUAUUCAAGAGCGAGAUG{invAb}	149	ACAUCUCGCUCUUGAAUAAAAUU	150
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1508	GACUUACUAUGGGCUGUAAA{invAb}	1015	AUUUACAGCCCAUAGUAAGUCUU	1016
1509	UAUUUUAGAAACCUGAGACU{invAb}	1017	AAGUCUCAGGUUUCUAAAAUAUU	1018
1510	CAUUUGAAAGAGAUUCUUGA{invAb}	1019	AUCAAGAAUCUCUUUCAAAUGUU	1020
1511	AAAGAGAUUCUUGACCUUAU{invAb}	1021	AAUAAGGUCAAGAAUCUCUUUUU	1022
1512	AAGAGAUUCUUGACCUUAUU{invAb}	1023	AAAUAAGGUCAAGAAUCUCUUUU	1024
1513	CUUGAUGGAAGGUAUUAAAC{invAb}	1025	AGUUUAAUACCUUCCAUCAAGUU	1026
1514	UUGAUGGAAGGUAUUAAACU{invAb}	1027	UAGUUUAAUACCUUCCAUCAAUU	1028
1515	UAUUAAACUAUUUGCCUGUU{invAb}	1029	AAACAGGCAAAUAGUUUAAUAUU	1030
1516	GUGUAUUGCAAGAAACACAG{invAb}	1031	UCUGUGUUUCUUGCAAUACACUU	1032
1517	AGAAAAAUCUCAACCAAAGU{invAb}	1033	AACUUUGGUUGAGAUUUUUCUUU	1034
1518	GAUAACACUUGGGGGGACCU{invAb}	1035	AAGGUCCCCCCAAGUGUUAUCUU	1036
1519	UGAGUUUAUUAAAGAUUGAC{invAb}	1037	UGUCAAUCUUUAAUAAACUCAUU	1038
1520	AAAGAUUGACAUUUUAAGUA{invAb}	1039	AUACUUAAAAUGUCAAUCUUUUU	1040
1521	AAGAUUGACAUUUUAAGUAC{invAb}	1041	UGUACUUAAAAUGUCAAUCUUUU	1042
1522	UAAUUUGUUGGUAUAUUAUC{invAb}	1043	AGAUAAUAUACCAACAAAUUAUU	1044
1523	GUUCAUAGAUCCCAUAUUGA{invAb}	1045	AUCAAUAUGGGAUCUAUGAACUU	1046
1524	AGCAUUGCCCAAACUAUUUU{invAb}	1047	AAAAAUAGUUUGGGCAAUGCUUU	1048
1525	AAAGCCAAAGUCAGAAACCG{invAb}	1049	ACGGUUUCUGACUUUGGCUUUUU	1050
1526	GCGGAAUGUUAUUUAUAUCA{invAb}	2101	UUGAUAUAAAUAACAUUCCGCUU	2102
1527	CAGCGAGAUUGCUACCUGAA{invAb}	2103	AUUCAGGUAGCAAUCUCGCUGUU	2104
1528	ACGAAUUUGCCGCUUCUGUU{invAb}	2105	AAACAGAAGCGGCAAAUUCGUUU	2106
1529	ACCUCAGCCUCUAUUCGCAA{invAb}	2107	AUUGCGAAUAGAGGCUGAGGUUU	2108
1530	AUCAAAAGCCGUUAACAAAG{invAb}	2109	ACUUUGUUAACGGCUUUUGAUUU	2110
1531	UAGCUACAUUUUUAAUGGGA{invAb}	2111	AUCCCAUUAAAAAUGUAGCUAUU	2112
1532	UUCAGAAGAUGUAGUAAUGC{invAb}	2113	UGCAUUACUACAUCUUCUGAAUU	2114
1533	AGCGUUGUUACCAGCUAUAC{invAb}	2115	AGUAUAGCUGGUAACAACGCUUU	2116
1534	GUGUUAUUAGAAUGUUACGA{invAb}	2117	UUCGUAACAUUCUAAUAACACUU	2118

1535	UCCUAACUUGAUUAGCUUGA{invAb}	2119	AUCAAGCUAAUCAAGUUAGGAUU	2120
1536	CAUAUAGUUGAAUUACUUCG{invAb}	2121	UCGAAGUAAUUCAACUAUAUGUU	2122
1537	GGGCAUAUAGUUGAAUUACU{invAb}	2123	AAGUAAUUCAACUAUAUGCCCUU	2124
1538	UGUCAUCCCAGACAUCUUGA{invAb}	2125	AUCAAGAUGUCUGGGAUGACAUU	2126
1539	UACGAAAAAACUAUGGUUGU{invAb}	2127	AACAACCAUAGUUUUUUCGUAUU	2128
1540	CUGCCUUGCCAGACAUUUUA{invAb}	2129	AUAAAAUGUCUGGCAAGGCAGUU	2130
1541	UUUUGUCAGUUGUGGUAGAU{invAb}	2131	UAUCUACCACAACUGACAAAAUU	2132
1542	AGAAUGUUACGAAAAAACUA{invAb}	2133	AUAGUUUUUUCGUAACAUUCUUU	2134
1543	UGUAAUAUGAGUGCUCUGUG{invAb}	2135	UCACAGAGCACUCAUAUUACAUU	2136
1544	CACUUUGUGACAUGGAUGAA{invAb}	2137	AUUCAUCCAUGUCACAAAGUGUU	2138
1545	AUUUGGGAAUUACACUUUGU{invAb}	2139	AACAAAGUGUAAUUCCCAAAUUU	2140
1546	AUAGCUGAGUCCUGAAGUUU{invAb}	2141	AAAACUUCAGGACUCAGCUAUUU	2142
1547	CUGACCCUUGGUACAAUAGA{invAb}	2143	AUCUAUUGUACCAAGGGUCAGUU	2144
1548	UAAUCGACUGAUUGGAAAUA{invAb}	2145	UUAUUUCCAAUCAGUCGAUUAUU	2146
1549	AACUGAUAGCUGAGUCCUGA{invAb}	2147	UUCAGGACUCAGCUAUCAGUUUU	2148
1550	CAGCACAUGAUUUGGGAAUU{invAb}	2149	UAAUUCCCAAAUCAUGUGCUGUU	2150
1551	ACAUGAUUUGGGAAUUACAC{invAb}	2151	AGUGUAAUUCCCAAAUCAUGUUU	2152
1552	CAGAAUACAGUGUUGGUCGA{invAb}	2153	AUCGACCAACACUGUAUUCUGUU	2154
1553	CCCAGACAUCUUGAUAAUAC{invAb}	2155	AGUAUUAUCAAGAUGUCUGGGUU	2156
1554	UCUUGAUAAUACCUGUUGGA{invAb}	2157	UUCCAACAGGUAUUAUCAAGAUU	2158
1555	UGUUAUUAGAAUGUUACGAA{invAb}	2159	UUUCGUAACAUUCUAAUAACAUU	2160
1556	UGUUACGAAAAAACUAUGGU{invAb}	2161	AACCAUAGUUUUUUCGUAACAUU	2162
1557	UUACGAAAAAACUAUGGUUG{invAb}	2163	ACAACCAUAGUUUUUUCGUAAUU	2164
1558	AGGAAUAUUUAGAAAGCCAA{invAb}	2165	UUUGGCUUUCUAAAUAUUCCUUU	2166
1559	ACGAAGGAGGUUGAUUGCAA{invAb}	2167	UUUGCAAUCAACCUCCUUCGUUU	2168
1560	UGUUUUCCAACAGUGAUGGC{invAb}	2169	AGCCAUCACUGUUGGAAAACAUU	2170
1561	CAUAAGGUUAAACAAACUAG{invAb}	2171	ACUAGUUUGUUUAACCUUAUGUU	2172
1562	GGACGGAAAGAUGUUCUCUA{invAb}	2173	AUAGAGAACAUCUUUCCGUCCUU	2174
1563	GGAAAUGUGUAGAACUGUUA{invAb}	2175	UUAACAGUUCUACACAUUUCCUU	2176
1564	AUAGUUGAAUUACUUCGACA{invAb}	2177	AUGUCGAAGUAAUUCAACUAUUU	2178
1565	UCUUCGAACUCAACUUCUAC{invAb}	2179	UGUAGAAGUUGAGUUCGAAGAUU	2180
1566	CUUCGAACUCAACUUCUACA{invAb}	2181	AUGUAGAAGUUGAGUUCGAAGUU	2182
1567	UUCCUGUUUUAUGUCUGUAC{invAb}	2183	AGUACAGACAUAAAACAGGAAUU	2184
1568	CAGGUACAGCUGUUUCUUGG{invAb}	2185	UCCAAGAAACAGCUGUACCUGUU	2186
1569	CCUAACUUGAUUAGCUUGAG{invAb}	2187	ACUCAAGCUAAUCAAGUUAGGUU	2188
1570	CGUGCACAACCUGUCCAGUA{invAb}	2189	AUACUGGACAGGUUGUGCACGUU	2190
1571	CAGUAUAUGCAUGUGGUGGC{invAb}	2191	AGCCACCACAUGCAUAUACUGUU	2192
1572	ACCAUGACUUUUAACAAAUU{invAb}	2193	AAAUUUGUUAAAAGUCAUGGUUU	2194
1573	ACAUAGUGGUAAAUAAUUAU{invAb}	2195	AAUAAUUAUUUACCACUAUGUUU	2196
1574	CAUAGUGGUAAAUAAUUAUG{invAb}	2197	UCAUAAUUAUUUACCACUAUGUU	2198
1575	AUUUUUCCAGAUGAGUGUUA{invAb}	2199	AUAACACUCAUCUGGAAAAAUUU	2200
1576	GGGAUGCUGAUUGAUAUUUC{invAb}	2201	UGAAAUAUCAAUCAGCAUCCCUU	2202

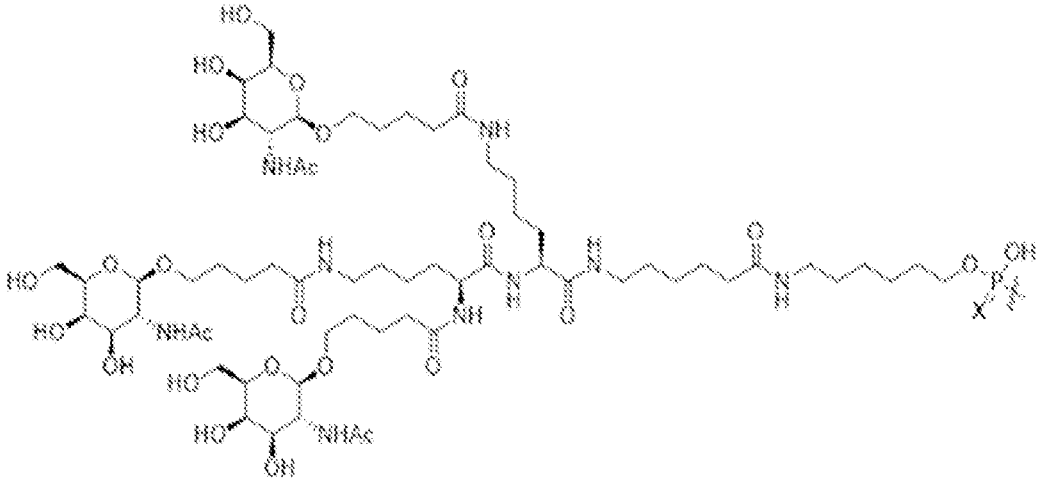
1577	UCUCAAGUUCUGCAUUUAAA{invAb}	2203	UUUUAAAUGCAGAACUUGAGAUU	2204
1578	UUCCAUUUUACUGACUAGGG{invAb}	2205	ACCCUAGUCAGUAAAAUGGAAUU	2206
1579	CAGUUUAUCACCUUCUUACA{invAb}	2207	AUGUAAGAAGGUGAUAAACUGUU	2208
1580	CGACAAAAACUUCUAGAAUA{invAb}	2209	AUAUUCUAGAAGUUUUUGUCGUU	2210
1581	AUAUAUUCUGAGUUUUGUGG{invAb}	2211	ACCACAAAACUCAGAAUAUAUUU	2212
1582	AAUGAUCCUGGCUUUUUCAC{invAb}	2213	AGUGAAAAAGCCAGGAUCAUUUU	2214
1583	UGACAGGACCUAUCGUUGAG{invAb}	2215	ACUCAACGAUAGGUCCUGUCAUU	2216
1584	UAUGGGUUAUAUGCCUAAAC{invAb}	2217	AGUUUAGGCAUAUAACCCAUAUU	2218
1585	UAAUUGGUAGGUGCCUUUUG{invAb}	2219	ACAAAAGGCACCUACCAAUUAUU	2220
1586	ACUAUGGUUGUGUCCGAGUG{invAb}	2221	ACACUCGGACACAACCAUAGUUU	2222
1587	AAGACUUACUAUGGGCUGUA{invAb}	2223	UUACAGCCCAUAGUAAGUCUUUU	2224
1588	GACUUACUAUGGGCUGUAAA{invAb}	2225	AUUUACAGCCCAUAGUAAGUCUU	2226
1589	UAAAUUAAGGAUUACUCAAG{invAb}	2227	ACUUGAGUAAUCCUUAAUUUAUU	2228
1590	CAACCAAAGUUAUGCUCAUC{invAb}	2229	AGAUGAGCAUAACUUUGGUUGUU	2230
1591	UAGACUACAAGAUGAAAUCU{invAb}	2231	AAGAUUUCAUCUUGUAGUCUAUU	2232
1592	CAAUAGUAGCUACAUUUUUA{invAb}	2233	UUAAAAAUGUAGCUACUAUUGUU	2234
1593	AAAAGCCGUUAACAAAGUGA{invAb}	2235	UUCACUUUGUUAACGGCUUUUUU	2236
1594	AAAGCCGUUAACAAAGUGAA{invAb}	2237	UUUCACUUUGUUAACGGCUUUUU	2238
1595	CAUAGAUCCCAUAUUGACUA{invAb}	2239	AUAGUCAAUAUGGGAUCUAUGUU	2240
1596	ACCUUGAUCCAUAAGCUUGG{invAb}	2241	ACCAAGCUUAUGGAUCAAGGUUU	2242
1597	UUCAUACGACGAAGGCUCGA{invAb}	2243	AUCGAGCCUUCGUCGUAUGAAUU	2244
1598	UCCAUGGGCAUAUAGUUGAA{invAb}	2245	AUUCAACUAUAUGCCCAUGGAUU	2246
1599	AUGGGCAUAUAGUUGAAUUA{invAb}	2247	AUAAUUCAACUAUAUGCCCAUUU	2248
1600	AUAUAGUUGAAUUACUUCGA{invAb}	2249	AUCGAAGUAAUUCAACUAUAUUU	2250
1601	CUUACAAUGCACUUUAGCGC{invAb}	2251	UGCGCUAAAGUGCAUUGUAAGUU	2252
1602	UUACAAUGCACUUUAGCGCA{invAb}	2253	AUGCGCUAAAGUGCAUUGUAAUU	2254
1603	ACAAUGCACUUUAGCGCAGU{invAb}	2255	UACUGCGCUAAAGUGCAUUGUUU	2256
1604	UUUAGCGCAGUAAGGGCUUG{invAb}	2257	ACAAGCCCUUACUGCGCUAAAUU	2258
1605	AUUGCCCAAACUAUUUUGAC{invAb}	2259	UGUCAAAAUAGUUUGGGCAAUUU	2260
1606	AGGUGAUCGGAGCUCUUUCC{invAb}	2261	AGGAAAGAGCUCCGAUCACCUUU	2262
1607	UAUAGUUGAAUUACUUCGAC{invAb}	2263	UGUCGAAGUAAUUCAACUAUAUU	2264
1608	UGAAUUACUUCGACAGCAGC{invAb}	2265	UGCUGCUGUCGAAGUAAUUCAUU	2266
1609	UUUGUCAGUUGUGGUAGAUA{invAb}	2267	AUAUCUACCACAACUGACAAAUU	2268
1610	CUUGAUGGAAGGUAUUAAAC{invAb}	2269	AGUUUAAUACCUUCCAUCAAGUU	2270
1611	GUGUAUUGCAAGAAACACAG{invAb}	2271	UCUGUGUUUCUUGCAAUACACUU	2272
1612	AGAAAAAUCUCAACCAAAGU{invAb}	2273	AACUUUGGUUGAGAUUUUUCUUU	2274
1613	AAGAAAGCCUAAUGAGUCGG{invAb}	2275	UCCGACUCAUUAGGCUUUCUUUU	2276
1614	UGCGGAAUGUUAUUUAUAUC{invAb}	2277	UGAUAUAAAUAACAUUCCGCAUU	2278
1615	UUAUUCAAGAGCGAGAUGUG{invAb}	2279	ACACAUCUCGCUCUUGAAUAAUU	2280
1616	AAGAGCGAGAUGUGCAUAAG{invAb}	2281	ACUUAUGCACAUCUCGCUCUUUU	2282
1617	AUGUUUGCCACCAAUGUGAC{invAb}	2283	AGUCACAUUGGUGGCAAACAUUU	2284
1618	ACAUGAUUUGGGAAUUACAC{invAb}	2285	AGUGUAAUUCCCAAAUCAUGUUU	2286

1619	AUGAUUUGGGAAUUACAC{invAb}	2287	AGUGUAAUUCCCAAAUCAUUU	2288
1620	ACAUGAUUUGGGAAUUACAC{invAb}	2289	AGUGUAAUUCCCAAAUCAUGUUU	2290
1621	AUGAUUUGGGAAUUACAC{invAb}	2291	AGUGUAAUUCCCAAAUCAUUU	2292
1622	AUUUUUCCAGAUGAGUGUUA{invAb}	2293	AUAACACUCAUCUGGAAAAAUUU	2294
1623	UUUUCCAGAUGAGUGUUA{invAb}	2295	AUAACACUCAUCUGGAAAAUU	2296
1624	UUUUCCAGAUGAGUGUUA{invAb}	2297	AUAACACUCAUCUGGAAAAUU	2298
1625	AUUUUUCCAGAUGAGUGUUA{invAb}	2299	AUAACACUCAUCUGGAAAAAUUU	2300
1626	ACCAUGACUUUUAACAAAUU{invAb}	2301	AAAUUUGUUAAAAGUCAUGGUUU	2302
1627	CAUGACUUUUAACAAAUU{invAb}	2303	AAAUUUGUUAAAAGUCAUGUU	2304
1628	CAUGACUUUUAACAAAUU{invAb}	2305	AAAUUUGUUAAAAGUCAUGUU	2306
1629	ACCAUGACUUUUAACAAAUU{invAb}	2307	AAAUUUGUUAAAAGUCAUGGUUU	2308
1630	UAGCUACAUUUUUAAUGGGA{invAb}	2309	AUCCCAUUAAAAAUGUAGCUAUU	2310
1631	GCUACAUUUUUAAUGGGA{invAb}	2311	AUCCCAUUAAAAAUGUAGCUU	2312
1632	GCUACAUUUUUAAUGGGA{invAb}	2313	AUCCCAUUAAAAAUGUAGCUU	2314
1633	UAGCUACAUUUUUAAUGGGA{invAb}	2315	AUCCCAUUAAAAAUGUAGCUAUU	2316
1634	UCUCAAGUUCUGCAUUUAAA{invAb}	2317	UUUUAAAUGCAGAACUUGAGAUU	2318
1635	UCAAGUUCUGCAUUUAAA{invAb}	2319	UUUUAAAUGCAGAACUUGAUU	2320
1636	UCAAGUUCUGCAUUUAAA{invAb}	2321	UUUUAAAUGCAGAACUUGAUU	2322
1637	UCUCAAGUUCUGCAUUUAAA{invAb}	2323	UUUUAAAUGCAGAACUUGAGAUU	2324
1638	AACUGAUAGCUGAGUCCUGA{invAb}	2325	UUCAGGACUCAGCUAUCAGUUUU	2326
1639	CUGAUAGCUGAGUCCUGA{invAb}	2327	UUCAGGACUCAGCUAUCAGUU	2328
1640	CUGAUAGCUGAGUCCUGA{invAb}	2329	UUCAGGACUCAGCUAUCAGUU	2330
1641	AACUGAUAGCUGAGUCCUGA{invAb}	2331	UUCAGGACUCAGCUAUCAGUUUU	2332
1642	UCUUGAUAAUACCUGUUGGA{invAb}	2333	UUCCAACAGGUAUUAUCAAGAUU	2334
1643	GUGUUAUUAGAAUGUUACGA{invAb}	2335	UUCGUAACAUUCUAAUAACACUU	2336
1644	UAUAGUUGAAUUACUUCGAC{invAb}	2337	UGUCGAAGUAAUUCAACUAUAUU	2338
1645	CACUUUGUGACAUGGAUGAA{invAb}	2339	AUUCAUCCAUGUCACAAAGUGUU	2340
1646	UGUCAUCCCAGACAUCUUGA{invAb}	2341	AUCAAGAUGUCUGGGAUGACAUU	2342
1647	UCUUGAUAAUACCUGUUGGA{invAb}	2343	UUCCAACAGGUAUUAUCAAGAUU	2344
1648	GUGUUAUUAGAAUGUUACGA{invAb}	2345	UUCGUAACAUUCUAAUAACACUU	2346
1649	UAUAGUUGAAUUACUUCGAC{invAb}	2347	UGUCGAAGUAAUUCAACUAUAUU	2348
1650	CACUUUGUGACAUGGAUGAA{invAb}	2349	AUUCAUCCAUGUCACAAAGUGUU	2350
1651	UGUCAUCCCAGACAUCUUGA{invAb}	2351	AUCAAGAUGUCUGGGAUGACAUU	2352
1652	UUGAUAAUACCUGUUGGA{invAb}	2353	UUCCAACAGGUAUUAUCAAUU	2354
1653	GUUAUUAGAAUGUUACGA{invAb}	2355	UUCGUAACAUUCUAAUAACUU	2356
1654	UAGUUGAAUUACUUCGAC{invAb}	2357	UGUCGAAGUAAUUCAACUAUU	2358
1655	CUUUGUGACAUGGAUGAA{invAb}	2359	AUUCAUCCAUGUCACAAAGUU	2360
1656	UCAUCCCAGACAUCUUGA{invAb}	2361	AUCAAGAUGUCUGGGAUGAUU	2362
1657	UUGAUAAUACCUGUUGGA{invAb}	2363	UUCCAACAGGUAUUAUCAAUU	2364
1658	GUUAUUAGAAUGUUACGA{invAb}	2365	UUCGUAACAUUCUAAUAACUU	2366
1659	UAGUUGAAUUACUUCGAC{invAb}	2367	UGUCGAAGUAAUUCAACUAUU	2368
1660	CUUUGUGACAUGGAUGAA{invAb}	2369	AUUCAUCCAUGUCACAAAGUU	2370

1661	UCAUCCCAGACAUCUUGA{invAb}	2371	AUCAAGAUGUCUGGGAUGAUU	2372
1662	GUUUAUCACCUUCUUACA{invAb}	2645	AUGUAAGAAGGUGAUAAACUU	2646
1663	GUUUAUCACCUUCUUACA{invAb}	2647	AUGUAAGAAGGUGAUAAACUU	2648
1664	CAGUUUAUCACCUUCUUACA{invAb}	2649	AUGUAAGAAGGUGAUAAACUGUU	2650
1665	CAGUUUAUCACCUUCUUACA{invAb}	2651	AUGUAAGAAGGUGAUAAACUGUU	2652
1666	CAGUUUAUCACCUUCUUACA{invAb}	2653	AUGUAAGAAGGUGAUAAACUGUU	2654
1667	CAGUUUAUCACCUUCUUACA{invAb}	2655	AUGUAAGAAGGUGAUAAACUGUU	2656
1668	GUUUAUCACCUUCUUACAUU{invAb}	2657	AUGUAAGAAGGUGAUAAACUU	2658
1669	CAGUUUAUCACCUUCUUACA{invAb}	2659	AUGUAAGAAGGUGAUAAACUGUU	2660
1670	CAGUUUAUCACCUUCUUACA{invAb}	2661	AUGUAAGAAGGUGAUAAACUGUU	2662
1671	GUGUUAUUAGAAUGUUACGA{invAb}	2663	UUCGUAACAUUCUAAUAACACUU	2664
1672	GUGUUAUUAGAAUGUUACGA{invAb}	2665	UUCGUAACAUUCUAAUAACACUU	2666
1673	GUUAUUAGAAUGUUACGAAU{invAb}	2667	UUCGUAACAUUCUAAUAACUU	2668
1674	GUGUUAUUAGAAUGUUACGA{invAb}	2669	UUCGUAACAUUCUAAUAACACUU	2670
1675	GUGUUAUUAGAAUGUUACGA{invAb}	2671	UUCGUAACAUUCUAAUAACACUU	2672
1676	UAUAGUUGAAUUACUUCGAC{invAb}	2673	UGUCGAAGUAAUUCAACUAUAUU	2674
1677	UAUAGUUGAAUUACUUCGAC{invAb}	2675	UGUCGAAGUAAUUCAACUAUAUU	2676
1678	UAGUUGAAUUACUUCGACAU{invAb}	2677	UGUCGAAGUAAUUCAACUAUU	2678
1679	UAUAGUUGAAUUACUUCGAC{invAb}	2679	UGUCGAAGUAAUUCAACUAUAUU	2680
1680	UAUAGUUGAAUUACUUCGAC{invAb}	2681	UGUCGAAGUAAUUCAACUAUAUU	2682
1681	UGUCAUCCCAGACAUCUUGA{invAb}	2683	AUCAAGAUGUCUGGGAUGACAUU	2684
1682	UGUCAUCCCAGACAUCUUGA{invAb}	2685	AUCAAGAUGUCUGGGAUGACAUU	2686
1683	UCAUCCCAGACAUCUUGAUU{invAb}	2687	AUCAAGAUGUCUGGGAUGAUU	2688
1684	UGUCAUCCCAGACAUCUUGA{invAb}	2689	AUCAAGAUGUCUGGGAUGACAUU	2690
1685	UGUCAUCCCAGACAUCUUGA{invAb}	2691	AUCAAGAUGUCUGGGAUGACAUU	2692
1686	AACUGAUAGCUGAGUCCUGA{invAb}	2693	UUCAGGACUCAGCUAUCAGUUUU	2694
1687	UAGCUACAUUUUUAAUGGGA{invAb}	2695	AUCCCAUUAAAAAUGUAGCUAUU	2696
1688	ACAUGAUUUGGGAAUUACAC{invAb}	2697	AGUGUAAUUCCCAAAUCAUGUUU	2698
1689	AUUUUUCCAGAUGAGUGUUA{invAb}	2699	AUAACACUCAUCUGGAAAAAUUU	2700
1690	AACUGAUAGCUGAGUCCUGA{invAb}	2701	UUCAGGACUCAGCUAUCAGUUUU	2702
1691	UAGCUACAUUUUUAAUGGGA{invAb}	2703	AUCCCAUUAAAAAUGUAGCUAUU	2704
1692	ACAUGAUUUGGGAAUUACAC{invAb}	2705	AGUGUAAUUCCCAAAUCAUGUUU	2706
1693	AUUUUUCCAGAUGAGUGUUA{invAb}	2707	AUAACACUCAUCUGGAAAAAUUU	2708
1694	CUGAUAGCUGAGUCCUGAAU{invAb}	2709	UUCAGGACUCAGCUAUCAGUU	2710
1695	GCUACAUUUUUAAUGGGAUU{invAb}	2711	AUCCCAUUAAAAAUGUAGCUU	2712
1696	AUGAUUUGGGAAUUACACUU{invAb}	2713	AGUGUAAUUCCCAAAUCAUUU	2714
1697	UUUUCCAGAUGAGUGUUAUU{invAb}	2715	AUAACACUCAUCUGGAAAAUU	2716
1698	AACUGAUAGCUGAGUCCUGA{invAb}	2717	UUCAGGACUCAGCUAUCAGUUUU	2718
1699	UAGCUACAUUUUUAAUGGGA{invAb}	2719	AUCCCAUUAAAAAUGUAGCUAUU	2720
1700	ACAUGAUUUGGGAAUUACAC{invAb}	2721	AGUGUAAUUCCCAAAUCAUGUUU	2722

[0166] To improve the potency and in vivo stability of GPAM siRNA sequences, chemical modifications were incorporated into GPAM siRNA molecules. Specifically, 2'-O-methyl and 2'-fluoro modifications of the ribose sugar were incorporated at specific positions within the GPAM siRNAs. Phosphorothioate internucleotide linkages were also incorporated at the terminal ends of the antisense and/ or sense sequences.

[0167] The antisense and sense siRNA sequences generated are shown in Table 2. The nucleotide sequences in Table 2 and other parts of the application are listed according to the following notations: A, U, G, and C =corresponding ribonucleotide; dT = deoxythymidine; dA = deoxyadenosine; dC = deoxycytidine; dG = deoxyguanosine; invDT = inverted deoxythymidine; invDA = inverted deoxyadenosine; invDC = inverted deoxycytidine; invDG = inverted deoxyguanosin; a, u, g, and c = corresponding 2'-O-methyl ribonucleotide; Af, Uf, Gf, and Cf = corresponding 2'-deoxy-2'-fluoro ("2'-fluoro") ribonucleotide; Ab = Abasic; invAb = inverted abasic; MeO-I = 2' methoxy inosine; GNA = glycol nucleic acid; sGNA = glycol nucleic acid with 3' phosphorothioate; LNA = locked nucleic acid. Insertion of an "s" in the sequence indicates that the two adjacent nucleotides are connected by a phosphorothiodiester group (e.g. a phosphorothioate internucleotide linkage). Unless indicated otherwise, all other nucleotides are connected by 3'-5' phosphodiester groups. Each of the siRNA compounds in Table 2 comprises a 19-21 base pair duplex region with either a 2 nucleotide overhang at the 3' end of both strands or bluntmer at one or both ends. Each [Phosphate] has been linked to the GalNAc structure below (sGalNAc3):



, wherein X = O or S.

Table 2. siRNA sequences directed to GPAM with modifications

Duplex No.	Sense sequence (5'-3')	SEQ ID NO: (sense)	Antisense sequence (5'-3')	SEQ ID NO: (anti-sense)
2001	gsgsgacuCfuUfUfCfUfgagguuacs{invAb}	1051	asGfsuaacCfucagaaAfgAfgucccsusu	1052
2002	csusuucuGfaGfGfUfUfacuguggas{invAb}	1053	asUfsccacAfguaaccUfcAfgaaagsusu	1054
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2627	{sGalNAc3}caugAfcUfUfUfUfaacaaauus{invAb}	2575	asAfsauuuGfuuaaaaGfuCfaugsusu	2576
2628	{sGalNAc3}caugacUfuUfUfAfAfcaaauus{invAb}	2577	asAfsauuuGfuuaaaaGfuCfaugsusu	2578
2629	{sGalNAc3}accaugacUfuUfUfAfAfcaaauus{invAb}	2579	asAfsauuuGfuuaaaaGfuCfauggususu	2580
2630	{sGalNAc3}uagcuaCfaUfUfUfUfuaaugggas{invAb}	2581	asUfscccauuaaaAfaUfgUfagcuasusu	2582
2631	{sGalNAc3}gcuaCfaUfUfUfUfuaaugggas{invAb}	2583	asUfscccaUfuaaaaaUfgUfagcsusu	2584
2632	{sGalNAc3}gcuacaUfuUfUfUfAfaugggas{invAb}	2585	asUfscccaUfuaaaaaUfgUfagcsusu	2586
2633	{sGalNAc3}uagcuacaUfuUfUfUfAfaugggas{invAb}	2587	asUfscccaUfuaaaaaUfgUfagcuasusu	2588
2634	{sGalNAc3}ucucaaGfuUfCfUfGfcauuuaaas{invAb}	2589	usUfsuuaaaugcaGfaAfcUfugagasusu	2590
2635	{sGalNAc3}ucaaGfuUfCfUfGfcauuuaaas{invAb}	2591	usUfsuuaaAfugcagaAfcUfugasusu	2592
2636	{sGalNAc3}ucaaguUfcUfGfCfAfuuuaaas{invAb}	2593	usUfsuuaaAfugcagaAfcUfugasusu	2594
2637	{sGalNAc3}ucucaaguUfcUfGfCfAfuuuaaas{invAb}	2595	usUfsuuaaAfugcagaAfcUfugagasusu	2596
2638	{sGalNAc3}aacugaUfaGfCfUfGfaguccugas{invAb}	2597	usUfscaggacucaGfcUfaUfcaguususu	2598
2639	{sGalNAc3}cugaUfaGfCfUfGfaguccugas{invAb}	2599	usUfscaggAfcucagcUfaUfcagsusu	2600
2640	{sGalNAc3}cugauaGfcUfGfAfGfuccugas{invAb}	2601	usUfscaggAfcucagcUfaUfcagsusu	2602
2641	{sGalNAc3}aacugauaGfcUfGfAfGfuccugas{invAb}	2603	usUfscaggAfcucagcUfaUfcaguususu	2604
2642	{sGalNAc3}ucuugauaAfuAfCfCfUfguuggas{invAb}	2605	usUfsccaaCfagguauUfaUfcaagasusu	2606
2643	{sGalNAc3}guguuauuAfgAfAfUfGfuuacgas{invAb}	2607	usUfscguaAfcauucuAfaUfaacacsusu	2608
2644	{sGalNAc3}uauaguugAfaUfUfAfCfuucgacs{invAb}	2609	usGfsucgaAfguaauuCfaAfcuauasusu	2610
2645	{sGalNAc3}cacuuuguGfaCfAfUfGfgaugaas{invAb}	2611	asUfsucauCfcaugucAfcAfaagugsusu	2612
2646	{sGalNAc3}ugucauccCfaGfAfCfAfucuugas{invAb}	2613	asUfscaagAfugucugGfgAfugacasusu	2614
2647	{sGalNAc3}ucuugaUfaAfUfAfCfcuguuggas{invAb}	2615	usUfsccaacagguAfuUfaUfcaagasusu	2616
2648	{sGalNAc3}guguuaUfuAfGfAfAfuguuacgas{invAb}	2617	usUfscguaacauuCfuAfaUfaacacsusu	2618
2649	{sGalNAc3}uauaguUfgAfAfUfUfacuucgacs{invAb}	2619	usGfsucgaaguaaUfuCfaAfcuauasusu	2620
2650	{sGalNAc3}cacuuuGfuGfAfCfAfuggaugaas{invAb}	2621	asUfsucauccaugUfcAfcAfaagugsusu	2622
2651	{sGalNAc3}ugucauCfcCfAfGfAfcaucuugas{invAb}	2623	asUfscaagaugucUfgGfgAfugacasusu	2624
2652	{sGalNAc3}uugauaAfuAfCfCfUfguuggas{invAb}	2625	usUfsccaaCfagguauUfaUfcaasusu	2626
2653	{sGalNAc3}guuauuAfgAfAfUfGfuuacgas{invAb}	2627	usUfscguaAfcauucuAfaUfaacsusu	2628
2654	{sGalNAc3}uaguugAfaUfUfAfCfuucgacs{invAb}	2629	usGfsucgaAfguaauuCfaAfcuasusu	2630
2655	{sGalNAc3}cuuuguGfaCfAfUfGfgaugaas{invAb}	2631	asUfsucauCfcaugucAfcAfaagsusu	2632
2656	{sGalNAc3}ucauccCfaGfAfCfAfucuugas{invAb}	2633	asUfscaagAfugucugGfgAfugasusu	2634
2657	{sGalNAc3}uugaUfaAfUfAfCfcuguuggas{invAb}	2635	usUfsccaaCfagguauUfaUfcaasusu	2636
2658	{sGalNAc3}guuaUfuAfGfAfAfuguuacgas{invAb}	2637	usUfscguaAfcauucuAfaUfaacsusu	2638
2659	{sGalNAc3}uaguUfgAfAfUfUfacuucgacs{invAb}	2639	usGfsucgaAfguaauuCfaAfcuasusu	2640
2660	{sGalNAc3}cuuuGfuGfAfCfAfuggaugaas{invAb}	2641	asUfsucauCfcaugucAfcAfaagsusu	2642
2661	{sGalNAc3}ucauCfcCfAfGfAfcaucuugas{invAb}	2643	asUfscaagAfugucugGfgAfugasusu	2644
2662	{sGalNAc3}guuuauCfaCfCfUfUfcuuacas{invAb}	2723	asUfsguaaGfaaggugAfuAfaacsusu	2724
2663	{sGalNAc3}guuuAfuCfAfCfCfuucuuacas{invAb}	2725	asUfsguaaGfaaggugAfuAfaacsusu	2726
2664	{sGalNAc3}caguuuauCfaCfCfUfUfcuuacas{invAb}	2727	asUfsguaaGfaaggugAfuAfaacugsusu	2728

2665	{sGalNAc3}caguuuAfuCfAfCfCfuucuuacas{invAb}	2729	asUfsguaagaaggUfgAfuAfaacugsusu	2730
2666	{sGalNAc3}caguuuauCfaCfCfUfUfcuuacas{invAb}	2731	asUfsguaaGfaaggUfgAfuaaacugsusu	2732
2667	{sGalNAc3}caguuuAfuCfaCfcuucuuacas{invAb}	2733	asUfsguaagaaggUfgAfuAfaacugsusu	2734
2668	{sGalNAc3}guuuAfuCfAfCfCfuucuuacausus{invAb}	2735	asUfsguaaGfaaggugAfuAfaacsusu	2736
2669	{sGalNAc3}caguuuAfuCfAfCfCfuucuuacas{invAb}	2737	asUfsguaaGfaaggUfgAfuAfaacugsusu	2738
2670	{sGalNAc3}caguuuauCfAfCfCfuucuuacas{invAb}	2739	asUfsguaaGfaaggugAfuAfaacugsusu	2740
2671	{sGalNAc3}guguuauuAfgAfAfUfGfuuacgas{invAb}	2741	usUfscguaAfcauuCfuAfauaacacsusu	2742
2672	{sGalNAc3}guguuaUfuAfgAfauguuacgas{invAb}	2743	usUfscguaacauuCfuAfaUfaacacsusu	2744
2673	{sGalNAc3}guuaUfuAfGfAfAfuguuacgaasus{invAb}	2745	usUfscguaAfcauucuAfaUfaacsusu	2746
2674	{sGalNAc3}guguuaUfuAfGfAfAfuguuacgas{invAb}	2747	usUfscguaAfcauuCfuAfaUfaacacsusu	2748
2675	{sGalNAc3}guguuauuAfGfAfAfuguuacgas{invAb}	2749	usUfscguaAfcauucuAfaUfaacacsusu	2750
2676	{sGalNAc3}uauaguugAfaUfUfAfCfuucgacs{invAb}	2751	usGfsucgaAfguaaUfuCfaacuauasusu	2752
2677	{sGalNAc3}uauaguUfgAfaUfuacuucgacs{invAb}	2753	usGfsucgaaguaaUfuCfaAfcuauasusu	2754
2678	{sGalNAc3}uaguUfgAfAfUfUfacuucgacasus{invAb}	2755	usGfsucgaAfguaauuCfaAfcuasusu	2756
2679	{sGalNAc3}uauaguUfgAfAfUfUfacuucgacs{invAb}	2757	usGfsucgaAfguaaUfuCfaAfcuauasusu	2758
2680	{sGalNAc3}uauaguugAfAfUfUfacuucgacs{invAb}	2759	usGfsucgaAfguaauuCfaAfcuauasusu	2760
2681	{sGalNAc3}ugucauccCfaGfAfCfAfucuugas{invAb}	2761	asUfscaagAfugucUfgGfgaugacasusu	2762
2682	{sGalNAc3}ugucauCfcCfaGfacaucuugas{invAb}	2763	asUfscaagaugucUfgGfgAfugacasusu	2764
2683	{sGalNAc3}ucauCfcCfAfGfAfcaucuugausus{invAb}	2765	asUfscaagAfugucugGfgAfugasusu	2766
2684	{sGalNAc3}ugucauCfcCfAfGfAfcaucuugas{invAb}	2767	asUfscaagAfugucUfgGfgAfugacasusu	2768
2685	{sGalNAc3}ugucauccCfAfGfAfcaucuugas{invAb}	2769	asUfscaagAfugucugGfgAfugacasusu	2770
2686	{sGalNAc3}aacugauaGfcUfGfAfGfuccugas{invAb}	2771	usUfscaggAfcucaGfcUfaucaguususu	2772
2687	{sGalNAc3}uagcuacaUfuUfUfUfAfaugggas{invAb}	2773	asUfscccaUfuaaaAfaUfguagcuasusu	2774
2688	{sGalNAc3}acaugauuUfgGfGfAfAfuuacacs{invAb}	2775	asGfsuguaAfuuccCfaAfaucaugususu	2776
2689	{sGalNAc3}auuuuuccAfgAfUfGfAfguguuas{invAb}	2777	asUfsaacaCfucauCfuGfgaaaaaususu	2778
2690	{sGalNAc3}aacugaUfaGfcUfgaguccugas{invAb}	2779	usUfscaggacucaGfcUfaUfcaguususu	2780
2691	{sGalNAc3}uagcuaCfaUfuUfuuaaugggas{invAb}	2781	asUfscccauuaaaAfaUfgUfagcuasusu	2782
2692	{sGalNAc3}acaugaUfuUfgGfgaauuacacs{invAb}	2783	asGfsuguaauuccCfaAfaUfcaugususu	2784
2693	{sGalNAc3}auuuuuCfcAfgAfugaguguuas{invAb}	2785	asUfsaacacucauCfuGfgAfaaaaususu	2786
2694	{sGalNAc3}cugaUfaGfCfUfGfaguccugaasus{invAb}	2787	usUfscaggAfcucagcUfaUfcagsusu	2788
2695	{sGalNAc3}gcuaCfaUfUfUfUfuaaugggausus{invAb}	2789	asUfscccaUfuaaaaaUfgUfagcsusu	2790
2696	{sGalNAc3}augaUfuUfGfGfGfaauuacacusus{invAb}	2791	asGfsuguaAfuucccaAfaUfcaususu	2792
2697	{sGalNAc3}uuuuCfcAfGfAfUfgaguguuausus{invAb}	2793	asUfsaacaCfucaucuGfgAfaaasusu	2794
2698	{sGalNAc3}aacugaUfaGfCfUfGfaguccugas{invAb}	2795	usUfscaggAfcucaGfcUfaUfcaguususu	2796
2699	{sGalNAc3}uagcuaCfaUfUfUfUfuaaugggas{invAb}	2797	asUfscccaUfuaaaAfaUfgUfagcuasusu	2798
2700	{sGalNAc3}acaugaUfuUfGfGfGfaauuacacs{invAb}	2799	asGfsuguaAfuuccCfaAfaUfcaugususu	2800

EXAMPLE 2: Efficacy of select GPAM siRNA molecules in RNA FISH assay

[0168] A panel of fully chemically modified siRNA from Example 1 were prepared and tested for potency and selectivity of mRNA knockdown in vitro. Each siRNA duplex consisted of two strands, the sense or 'passenger' strand and the antisense or 'guide' strand.

[0169] RNA FISH (fluorescence in situ hybridization) assay was carried out to measure GPAM mRNA knockdown by test siRNAs. HepG2 cells (ATCC HB-8065) were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC® 30-2003™) supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin-streptomycin (P-S, Corning). siRNAs were transfected into cells by reverse transfection using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific). 1 μL of test siRNAs (in 10 data points dosed with 1:3 dilution starting at 500 nM final concentration) or phosphate-buffered saline (PBS) vehicle and 4 μL of plain EMEM without supplements were added to PDL-coated CellCarrier-384 Ultra assay plates (PerkinElmer) by a Bravo automated liquid handling platform (Agilent). 5 μL of Lipofectamine RNAiMAX (Thermo Fisher Scientific), pre-diluted in plain EMEM without supplements (0.06 μL of RNAiMAX in 5 μL EMEM), was then dispensed into the assay plates by a Multidrop Combi reagent dispenser (Thermo Fisher Scientific). After 20-minute incubation of the siRNA/RNAiMAX mixture at room temperature (RT), 30 μL of HepG2 cells (2000 cells per well) in EMEM supplemented with 10% FBS and 1% P-S were added to the transfection complex using a Multidrop Combi reagent dispenser. The assay plates were incubated at RT for 20 mins prior to being placed in an incubator. Cells were incubated for 72 hrs at 37 °C and 5% CO2.

[0170] RNA FISH assay was performed 72 hours after siRNA transfection using the manufacturer's assay reagents and protocol (QuantiGene® ViewRNA HC Screening Assay from Thermo Fisher Scientific) on an in-house assembled automated FISH assay platform. In brief, cells were fixed in 4% formaldehyde (Thermo Fisher Scientific) for 15 mins at RT, permeabilized with detergent for 3 mins at RT and then treated with protease solution for 10 mins at RT. Target-specific probes (Thermo Fisher Scientific, VA6-3170392-VC (GPAM) and VA1-10148-VC (PPIB) or vehicle (target probe diluent without target probes as negative control) were incubated for 3 hours, whereas preamplifiers, amplifiers, and label probes were incubated for 1 hour each. All hybridization steps were carried out at 40 °C in a Cytomat 2 C-LIN automated incubator (Thermo Fisher Scientific).

[0171] After hybridization reactions, cells were stained for 30 mins with Hoechst and CellMask Blue (Thermo Fisher Scientific) and then imaged on an Opera Phenix high-content screening system (PerkinElmer). The images were analyzed using a Columbus image data storage and analysis system (PerkinElmer) to obtain the mean spot count per cell. The mean spot count per cell was normalized using the high (PBS with target probes) and low (PBS without target probes) control wells. The high and low controls have normalized values of 100 and 0, respectively. The normalized values against the test siRNA concentrations were fitted to a 4-parameter sigmoidal model using Genedata Screener data analysis software (Genedata, Basel, Switzerland) to obtain IC50 values and maximum activity.

[0172] The results of the assay are shown in Table 3. GPAM knockdown provides a percentage of knockdown compared to control samples. Negative values indicate a decrease in GPAM levels.

Table 3. RNA FISH Assay with Human/Cyno GPAM-Spanning siRNA

Duplex No.	IC50 (nM)	GPAM knockdown (%)	Duplex No.	IC50 (nM)	GPAM knockdown (%)
2001		-18.1	2021	2.55	-75.4
2002	14.70	-65.0	2022	1.43	-68.6
2003	19.30	-56.7	2023		-17.6
2004	14.00	-56.4	2024	2.45	-84.1
2005	35.60	-66.7	2025	1.84	-89.7
2006	10.94	-86.0	2026	6.65	-85.6
2007	7.32	-84.5	2027	1.70	-88.5
2008	20.30	-64.1	2028	6.58	-86.2
2009	15.40	-70.9	2029	2.71	-57.9
2010	4.87	-90.6	2030	4.26	-75.4
2011	7.84	-85.4	2031	7.42	-82.5
2012	9.94	-73.5	2032	1.33	-94.2
2013	8.85	-74.4	2033	8.79	-53.7
2014	5.80	-70.9	2034		5.9
2015	5.18	-82.0	2035	5.10	-73.6
2016	5.27	-72.3	2036	2.42	-89.1
2017	2.00	-62.2	2037	3.29	-73.7
2018	0.61	-71.5	2038	26.40	-65.5
2019	4.48	-74.8	2039	5.64	-66.4
2020	1.78	-70.6	2040	2.81	-84.4

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2041	8.27	-66.0
2042	2.62	-57.4
2043		-65.7
2044		5.9
2045	1.68	-82.0
2046	1.51	-66.2
2047	4.21	-64.4
2048	25.60	-44.6
2049	22.20	-78.8
2050	2.67	-84.5
2051	0.58	-78.2
2052		-36.3
2053		-28.8
2054	127.00	-48.6
2055	4.92	-69.5
2056	2.43	-51.1
2057	150.00	-56.3
2058	22.80	-69.1
2059	34.90	-57.7
2060	0.26	-74.5
2061	18.60	-65.7
2062	2.00	-86.3
2063	0.52	-73.3
2064	0.28	-57.0
2065		-34.5
2066	32.50	-79.6
2067	2.60	-58.2
2068	11.40	-73.6
2069	22.40	-66.8
2070	6.58	-62.6
2071	12.70	-69.8
2072	1.91	-84.8
2073	0.93	-55.8
2074	7.52	-76.9
2075		-11.0
2076		-25.1
2077	1.35	-85.1
2078	2.97	-85.0
2079	55.20	-66.0
2080		-31.4

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2081		-58.9
2082		-47.9
2083	58.10	-65.7
2084	64.90	-62.9
2085	3.55	-69.6
2086	2.43	-85.5
2087	45.20	-72.9
2088	0.54	-77.4
2089	76.90	-74.5
2090	5.37	-56.5
2091		-49.0
2092	114.00	-56.0
2093		-40.9
2094		-50.9
2095		-0.4
2096		-38.9
2097	0.99	-69.8
2098	2.33	-77.2
2099	6.31	-73.1
2100	16.00	-89.4
2101	4.08	-78.8
2102	3.08	-85.5
2103	1.46	-86.9
2104	58.30	-40.0
2105		-57.1
2106		-23.7
2107	5.10	-74.1
2108	15.90	-70.3
2109	0.92	-69.5
2110	2.26	-53.0
2111	133.00	-59.9
2112	1.77	-54.6
2113	35.00	-68.7
2114		-46.2
2115	2.75	-62.9
2116	2.34	-52.9
2117		-42.4
2118	0.23	-52.1
2119	34.30	-69.2
2120	4.32	-72.7

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2121	57.80	-64.5
2122	3.13	-75.2
2123	11.30	-77.7
2124		-14.4
2125	9.25	-45.8
2126	1.84	-83.1
2127	1.51	-92.6
2128	2.41	-84.2
2129	7.48	-78.2
2130	12.70	-60.4
2131	25.40	-77.8
2132	5.98	-77.7
2133	4.44	-70.9
2134	2.97	-59.6
2135	1.50	-84.4
2136	2.13	-65.3
2137	1.48	-76.2
2138	67.80	-67.5
2139		-59.4
2140	12.90	-55.8
2141	2.10	-89.6
2142	12.00	-68.9
2143	1.64	-86.6
2144	4.98	-81.9
2145	2.96	-79.5
2146	4.71	-63.1
2147	2.35	-86.3
2148	1.89	-80.6
2149	5.57	-78.0
2150	1.28	-82.4
2151	1.82	-70.8
2152	1.35	-87.1
2153	2.00	-84.8
2154	3.03	-64.8
2155	7.37	-61.1
2156	2.48	-73.7
2157	2.35	-86.5
2158	1.06	-90.2
2159	8.59	-86.2
2160	2.10	-81.6

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2161	3.25	-79.5
2162	7.23	-71.8
2163	2.05	-73.7
2164	4.76	-87.3
2165	9.73	-86.5
2166	2.34	-82.5
2167	18.70	-63.6
2168	1.81	-80.3
2169	1.64	-91.9
2170	3.93	-78.3
2171	3.39	-87.4
2172	4.91	-87.1
2173	1.47	-79.6
2174	1.41	-79.1
2175	2.09	-86.0
2176	2.55	-81.4
2177	17.00	-46.9
2178	1.03	-87.1
2179	1.99	-86.2
2180	3.44	-81.1
2181	2.28	-84.9
2182	1.23	-73.7
2183	3.26	-78.9
2184	1.30	-92.4
2185	1.99	-58.3
2186	2.64	-81.9
2187	3.41	-85.1
2188	2.84	-89.1
2189	2.24	-89.9
2190	2.12	-80.8
2191	2.36	-72.5
2192	7.50	-67.3
2193	1.62	-87.1
2194	1.55	-81.6
2195	1.77	-80.1
2196	2.38	-61.1
2197	1.70	-82.0
2198	11.20	-61.8
2199		2.8
2200	15.40	-73.4

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2201	1.52	-89.1
2202	75.60	-56.0
2203	13.10	-66.9
2204	1.82	-89.2
2205	33.40	-59.3
2206	16.63	-88.6
2207	13.50	-67.0
2208	1.39	-66.7
2209	0.49	-72.2
2210		-67.4
2211	2.02	-70.6
2212	19.33	-85.6
2213		-71.4
2214	21.33	-79.0
2215	17.25	-77.3
2216	3.12	-84.6
2217	13.40	-62.1
2218		-18.3
2219	1.81	-60.2
2220		-8.1
2221		-68.6
2222	6.64	-67.0
2223	1.68	-91.1
2224	43.80	-64.6
2225	18.80	-79.3
2226	71.70	-77.4
2227	2.10	-63.4
2228	99.60	-61.9
2229		-50.6
2230	16.60	-60.2
2231	7.80	-55.6
2232	3.33	-87.7
2233	4.11	-78.0
2234	2.75	-87.4
2235	2.35	-88.5
2236	7.40	-85.4
2237	2.28	-86.1
2238	12.40	-67.0
2239		5.2
2240	8.38	-53.5

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2241		-32.3
2242		-48.6
2243		6.3
2244		-19.3
2245	1.46	-89.0
2246	5.15	-67.7
2247	8.42	-82.1
2248	5.96	-79.4
2249	88.50	-50.6
2250	3.39	-86.8
2251	3.20	-93.0
2252	7.15	-79.1
2253		-52.2
2254	8.31	-84.8
2255	33.50	-59.3
2256	64.70	-53.8
2257	16.68	-84.2
2258	69.10	-66.1
2259		-38.1
2260		-44.7
2261	3.81	-79.1
2262		-16.5
2263	5.39	-89.6
2264	63.20	-60.2
2265		-38.0
2266		-23.8
2267		-23.4
2268	6.41	-58.5
2269	2.80	-81.7
2270	1.49	-84.8
2271	4.71	-79.3
2272	94.40	-61.6
2273	0.77	-57.4
2274	15.50	-55.1
2275	3.09	-56.6
2276	35.00	-57.9
2277		-0.7
2278	9.41	-59.1
2279	5.49	-78.7
2280	85.40	-50.4

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2281	2.33	-62.8
2282		-13.8
2283		-42.2
2284		-56.5
2285		-34.3
2286		-17.0
2287	97.80	-62.6
2288	7.98	-79.4
2289	4.21	-81.9
2290	3.67	-88.2
2291	5.42	-60.6
2292	4.29	-87.2
2293	3.45	-85.5
2294	1.80	-73.6
2295	6.23	-82.1
2296	3.71	-68.0
2297	14.83	-82.1
2298	7.21	-82.8
2299	15.00	-70.7
2300	10.73	-85.9
2301	14.60	-75.9
2302	12.99	-90.5
2303	5.99	-81.8
2304	15.00	-61.5
2305	5.72	-48.0
2306	3.01	-76.8
2307	4.95	-74.0
2308	7.07	-78.6
2309	5.38	-76.5
2310	5.57	-79.3
2311	41.70	-74.7
2312	23.90	-68.4
2313	13.00	-70.0
2314	168.00	-67.2
2315	18.00	-63.9
2316	29.10	-56.1
2317	54.90	-62.9
2318	13.20	-54.6
2319	10.10	-60.8
2320		-33.5

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2321	15.90	-81.3
2322	9.02	-62.9
2323	2.17	-77.9
2324	2.17	-63.3
2325	4.10	-78.3
2326		-14.3
2327	3.14	-74.2
2328	1.58	-89.1
2329		10.0
2330		11.7
2331	35.10	-68.9
2332	4.18	-69.1
2333	21.10	-79.4
2334	3.49	-60.7
2335	12.20	-46.7
2336		15.2
2337		-21.6
2338	6.03	-76.4
2339	9.95	-46.6
2340	3.74	-82.1
2341	1.95	-67.4
2342		4.8
2343	2.30	-88.0
2344		5.0
2345	3.36	-87.7
2346	3.51	-79.6
2347		6.6
2348	4.22	-79.7
2349	5.54	-87.0
2350		7.9
2351	17.10	-60.7
2352	0.55	-77.1
2353	8.18	-66.2
2354	14.10	-67.5
2355		-70.3
2356	3.93	-58.3
2357	4.83	-61.7
2358		-70.3
2359	33.70	-60.1
2360		-12.9

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2361		-6.3
2362	4.49	-53.8
2363	1.80	-64.1
2364	2.78	-65.8
2365	5.24	-74.5
2366		-55.4
2367		-27.2
2368	1.43	-68.4
2369	37.60	-67.6
2370	0.84	-67.9
2371		-46.9
2372	42.90	-71.3
2373		-31.7
2374	0.54	-47.7
2375	0.57	-59.8
2376		-39.8
2377	2.32	-77.7
2378		-52.1
2379	1.53	-86.3
2380		2.6
2381		-43.1
2382	2.88	-78.3
2383		-37.9
2384		-12.8
2385	14.00	-44.9
2386	98.50	-69.8
2387	25.80	-68.4
2388	1.27	-57.5
2389		6.0
2390		-20.9
2391	3.32	-84.8
2392	9.88	-78.2
2393	1.65	-85.2
2394	4.51	-84.9
2395	3.36	-76.7
2396	3.09	-32.7
2397	4.04	-80.0
2398	3.49	-72.5
2399	7.00	-85.2
2400	4.89	-75.7

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2401	3.33	-51.1
2402	2.76	-81.9
2403	2.11	-84.5
2404	9.06	-64.1
2405	5.22	-35.2
2406	3.07	-94.6
2407	35.40	-55.1
2408	6.99	-73.7
2409	8.04	-72.0
2410	4.60	-82.9
2411	1.69	-78.5
2412	1.97	-78.0
2413	2.20	-81.6
2414	2.41	-72.5
2415	6.32	-78.9
2416		-40.7
2417	1.22	-64.6
2418	1.32	-66.5
2419	15.00	-59.5
2420		-60.5
2421	2.33	-87.1
2422	2.09	-88.8
2423	5.29	-84.1
2424	1.52	-94.0
2425	3.50	-84.5
2426	16.90	-63.1
2427	1.58	-89.3
2428	8.65	-84.4
2429	5.23	-86.1
2430	7.23	-78.8
2431	10.20	-83.2
2432	1.49	-52.4
2433	2.77	-77.1
2434	3.36	-80.5
2435	3.57	-85.5
2436	6.18	-86.4
2437	4.40	-85.6
2438	6.92	-74.4
2439	2.49	-73.9
2440	2.91	-80.0

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2441	3.87	-91.0
2442	5.58	-86.2
2443	30.00	-86.0
2444	4.64	-68.0
2445	6.08	-61.1
2446	1.93	-89.0
2447	2.48	-89.1
2448	10.40	-63.5
2449		-42.2
2450	3.44	-85.1
2451	6.83	-71.5
2452	1.90	-68.5
2453	5.22	-78.2
2454	4.29	-89.7
2455	2.57	-91.9
2456	3.24	-91.7
2457	1.69	-93.2
2458	8.29	-83.7
2459	8.21	-77.8
2460	13.90	-86.9
2461	32.80	-57.6
2462	5.93	-82.8
2463	1.83	-83.1
2464	1.84	-87.0
2465	5.03	-90.3
2466	5.26	-86.5
2467	1.66	-91.2
2468	3.18	-79.5
2469	1.80	-87.9
2470	2.73	-73.1
2471	3.10	-87.5
2472	5.24	-73.7
2473	12.40	-79.9
2474	6.12	-76.6
2475	5.46	-91.2
2476	3.53	-89.6
2477	1.90	-85.1
2478	3.08	-85.6
2479	2.24	-88.4
2480	17.70	-78.0

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2481	0.76	-90.3
2482	3.70	-88.0
2483	1.68	-79.3
2484	4.35	-86.1
2485	23.00	-72.7
2486	5.35	-65.5
2487	7.05	-71.2
2488	4.50	-84.0
2489	5.13	-85.1
2490	1.47	-88.8
2491	2.23	-87.0
2492	4.94	-82.4
2493	4.66	-86.1
2494	4.13	-90.9
2495	2.80	-73.4
2496	14.00	-82.0
2497		-66.8
2498	22.60	-73.6
2499	6.17	-79.8
2500	3.62	-93.4
2501	3.28	-92.2
2502	1.54	-87.9
2503	3.00	-85.9
2504	0.81	-75.8
2505	1.15	-87.9
2506	0.56	-89.7
2507	0.97	-81.3
2508	1.33	-87.4
2509	0.70	-85.1
2510	5.94	-92.8
2511	5.52	-82.4
2512	5.34	-82.1
2513	6.76	-92.6
2514		-73.8
2515		-28.8
2516	3.24	-92.8
2517	3.18	-87.3
2518	9.68	-72.9
2519	1.36	-79.9
2520	1.24	-82.5

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2521	1.39	-85.4
2522	2.50	-83.3
2523	3.37	-82.2

Duplex No.	IC50 (nM)	GPAM knockdown (%)
2524	4.14	-72.9
2525	3.34	-84.6

Example 3: Efficacy screening of select PNPLA3 siRNA molecules in AAV-based mouse models containing human PNPLA3 sequences

[0173] Adeno-associated adenovirus (AAV; serotype AAVDJ8; endotoxin-free, prepared internally by Amgen) diluted in phosphate buffered saline (Thermo Fisher Scientific,14190-136) was administered at $1x10^{12}$ viral particles per animal into the tail vein of C57BL/6NCrl male or female mice (Charles River Laboratories Inc.) to drive expression of human GPAM sequences in the liver. Five AAV constructs were designed from the GPAM_XM_005269998.1 transcript for *in vivo* screening; one containing the full-length coding sequence for $GPAM^{I43V}$, and four enhanced green fluorescence protein (eGFP) reporter constructs containing stretches of the 5' untranslated region, coding region, and 3' untranslated region (nucleotides (nt) 1-1700, nt 1600-3300, nt 3200-4900, and nt 4800-6527 (AAV-A, AAV-B, AAV-C, and AAV-D). The eGFP-containing constructs also contained a benchmark siRNA target sequence to compare siRNA-mediated knockdown efficacy across AAVs and studies.

[0174] GalNAc-conjugated siRNAs shown in Table 4 were tested against $GPAM^{I43V}$, AAV-A, AAV-B, AAV-C, or AAV-D. Two weeks after AAV injection, mice (generally 10-12 weeks of age and an n=3-4 animals per group) were treated with a single dose of siRNA via subcutaneous injection, at 0.5, 1.0, or 3.0 milligrams per kilogram of animal, diluted in phosphate buffered saline (Thermo Fisher Scientific,14190-136). After 28 days post-siRNA injection, animals were euthanized, and livers collected from the animals and snap-frozen in liquid nitrogen. A portion of the liver was processed for purified RNA using a QIACube HT instrument (Qiagen, 9001793) and RNeasy 96 QIACube HT kits (Qiagen, 74171) according to manufacturer's instructions. Samples were analyzed using a QIAxpert system (Qiagen, 9002340). RNA was treated with RQ1 RNase-Free DNase (Promega, M6101) and prepared for Real-Time qPCR using the TAQMAN™ RNA-to-CT™ 1-Step kit (Applied Biosystems,

4392653). Real-Time qPCR was run on a QuantStudio Real-Time PCR machine. Results were based on gene expression of human *GPAM* (Invitrogen, Hs00326039), *GFP2* (IDT custom assay: Forward primer: TCATCTGCACCACTGGAAAG (Sense; SEQ ID NO: 2801), Reverse primer: CTGCTTCATATGGTCTGGGTATC (AntiSense; SEQ ID NO: 2802), Probe: 5'-6FAM CCAACACTGGTCACTACCCTCACC TAMRA-3' (Sense; SEQ ID NO: 2803), and/or bovine growth hormone polyadenylation (*BghpA*, which is included in each AAV construct; IDT custom assay: Forward: 5'-GCCAGCCATCTGTTGT-3' (SEQ ID NO: 2804), Reverse: 5'-GGAGTGGCACCTTCCA-3' (SEQ ID NO: 2805), Probe: 5'-6FAM-TCCCCCGTGCCTTCCTTGACC TAMRA-3' (SEQ ID NO: 2806), as normalized to mouse TATA-binding protein (Tbp) (IDT, Hex Mm.PT.39a.22214839), and presented as the relative percent knockdown of human *GPAM*, *GFP*, and/or *BghpA* mRNA expression, normalized to mouse *Tbp*, compared to vehicle-treated control animals. Negative results indicate knockdown. (nd=not determined).

Table 4. Day 28 GPAM knockdown assay

Duplex number	Dose administered (mg/kg)	GFP2 Avg KD	BghpA Avg KD	GPAM Avg KD
D-2548	1	-32.8	-38.3	
D-2549	1	-61.0	-56.5	
D-2546	1	-54.8	-56.7	
D-2550	1	-47.0	-43.1	
D-2551	1	-73.3	-67.0	
D-2545	1	-55.4	-50.4	
D-2544	1	-65.2	-57.2	
D-2614	1	-61.6	-50.7	
D-2615	1	-58.3	-53.9	
D-2529	1	53.6	42.2	
D-2591	1	31.3	38.5	
D-2592	1	-54.2	-51.5	
D-2531	1	-66.3	-62.2	
D-2567	1	-53.6	-48.8	
D-2568	1	-3.4	8.1	
D-2535	1	-52.9	-51.7	
D-2569	1	32.3	27.9	

D-2570	1	0.2	-2.5	
D-2571	1	-30.5	-23.3	
D-2572	1	-67.6	-64.2	
D-2584	1	-41.0	-33.9	
D-2573	1	-0.4	10.7	
D-2574	1	nd	-45.3	
D-2585	1	6.2	1.4	
D-2575	1	-72.7	-72.7	
D-2576	1	-36.9	-40.4	
D-2577	1	-65.7	-65.8	
D-2578	1	-49.4	-45.8	
D-2589	1	-71.4	-69.7	
D-2612	1	2.0	11.8	
D-2590	1	-20.2	-14.8	
D-2560	1	-2.6	-6.0	
D-2561	1	-12.3	1.6	
D-2563	1	-14.1	-10.2	
D-2543	1	-39.9	-31.8	
D-2601	1	-20.5	-30.3	
D-2602	1	-26.4	-11.4	
D-2603	1	-26.9	-19.9	
D-2604	1	-27.5	-25.0	
D-2605	1	-31.3	-8.0	
D-2606	1	18.3	46.1	
D-2582	1	-4.2	0.1	
D-2583	1	-13.9	-4.0	
D-2587	1	-43.0	-34.3	
D-2588	1	38.7	48.4	
D-2610	1	20.3	23.2	
D-2611	1	15.1	20.7	
D-2566	1	-56.9	-57.4	
D-2540	1	-40.4	-39.8	
D-2527	1	-58.1	-55.5	
D-2579	1	-54.3	-59.9	
D-2580	1	-37.3	-49.0	
D-2581	1	-54.5	-55.5	
D-2529	1	-46.5	-48.4	
D-2591	1	-62.7	-60.8	
D-2544	1			0.6
D-2547	1			-24.2

D-2552	1			-16.1
D-2613	1			-39.5
D-2614	1			-36.7
D-2615	1			-49.1
D-2616	1			-39.0
D-2617	1			-44.8
D-2530	1			-29.3
D-2593	1			-22.4
D-2594	1			-2.2
D-2528	1			-30.0
D-2595	1			-42.1
D-2596	1			-38.6
D-2597	1			-12.1
D-2562	1			-0.3
D-2598	1			-43.2
D-2599	1			3.7
D-2537	1			-16.2
D-2536	1			-8.8
D-2600	1			-21.6
D-2607	1			-62.3
D-2564	1			-48.6
D-2608	1			-20.9
D-2541	1			-41.7
D-2609	1			-36.4
D-2538	1			-60.5
D-2553	1			-56.5
D-2554	1			-52.8
D-2534	1			-61.8
D-2555	1			-37.6
D-2542	1			-47.9
D-2556	1			-42.9
D-2586	1			-36.4
D-2558	1			-42.3
D-2559	1			-20.0
D-2532	1			-22.9
D-2565	1			-24.8
D-2600	1			-21.6
D-2598	1			-43.2
D-2607	0.5	-6.6	-2.9	
D-2607	1	-36.7	-36.5	

D-2607	3	-30.7	-46.7	
D-2549	0.5	-38.5	-49.5	
D-2549	1	-38.8	-51.9	
D-2549	3	-34.6	-49.6	
D-2551	0.5	-52.8	-56.4	
D-2551	1	-40.1	-48.3	
D-2551	3	-49.9	-64.9	
D-2607	0.5		4.5	-1.2
D-2607	1		-37.9	-35.6
D-2607	3		-45.6	-46.7
D-2544	0.5		-35.7	-32.5
D-2544	1		5.9	7.6
D-2544	3		-29.1	-30.5
D-2554	0.5		-22.7	-22.2
D-2554	1		-40.3	-40.3
D-2554	3		-18.7	-16.3
D-2534	0.5		-41.4	-41.4
D-2534	1		13.8	2.1
D-2534	3		-63.3	-56.7
D-2538	0.5		-40.2	-27.0
D-2538	1		-31.2	-31.3
D-2538	3		-53.7	-54.1
D-2551	0.5	-33.0	-42.5	
D-2618	0.5	-5.0	-13.1	
D-2619	0.5	-47.4	-49.4	
D-2620	0.5	-47.5	-49.7	
D-2621	0.5	4.3	-8.3	
D-2549	0.5	-40.5	-43.1	
D-2638	0.5	-37.9	-32.4	
D-2639	0.5	-29.0	-35.2	
D-2640	0.5	-50.9	-56.0	
D-2641	0.5	-25.8	-28.7	
D-2531	0.5	-27.6	-26.4	
D-2630	0.5	-10.1	-8.1	
D-2631	0.5	-15.1	-14.2	
D-2632	0.5	-23.2	-26.2	
D-2633	0.5	-51.8	-51.7	
D-2575	0.5	-42.4	-48.8	
D-2622	0.5	-59.3	-59.7	
D-2623	0.5	-42.9	-44.7	

D-2624	0.5	-48.2	-52.1	
D-2625	0.5	-15.8	-24.2	
D-2572	0.5	-38.5	-36.5	
D-2626	0.5	-43.7	-41.4	
D-2627	0.5	-33.8	-33.3	
D-2628	0.5	-9.1	-12.2	
D-2629	0.5	-2.2	-2.5	
D-2577	0.5	-5.4	-12.6	
D-2634	0.5	2.1	13.2	
D-2635	0.5	2.8	5.2	
D-2636	0.5	-3.4	-13.0	
D-2637	0.5	1.3	0.1	
D-2557	0.5		-36.2	-40.0
D-2530	0.5		-27.0	-26.6
D-2533	0.5		-15.3	-11.4
D-2566	0.5		19.0	17.8
D-2540	0.5		-48.6	-48.5
D-2527	0.5		-30.0	-30.9
D-2579	0.5		-60.2	-62.0
D-2580	0.5		-14.9	-8.9
D-2581	0.5		-31.0	-35.7
D-2544	0.5		0.2	2.8
D-2645	0.5		-28.0	-32.4
D-2650	0.5		-21.8	-28.6
D-2655	0.5		-34.1	-36.8
D-2660	0.5		-16.8	-15.3
D-2607	0.5		-47.6	-43.5
D-2644	0.5		-55.7	-51.2
D-2649	0.5		-40.8	-45.0
D-2654	0.5		0.8	-6.1
D-2659	0.5		-28.8	-35.2
D-2538	0.5		-21.4	-16.7
D-2646	0.5		2.1	0.6
D-2651	0.5		-3.5	-6.7
D-2656	0.5		-37.7	-40.9
D-2661	0.5		-53.3	-53.3
D-2554	0.5		-16.9	-19.1
D-2642	0.5		-33.9	-28.4
D-2647	0.5		-32.8	-34.8
D-2652	0.5		-24.0	-22.6

D-2657	0.5		-42.9	-46.1
D-2534	0.5		-55.0	-50.6
D-2643	0.5		-51.6	-57.0
D-2648	0.5		-49.1	-46.5
D-2653	0.5		-41.2	-44.9
D-2658	0.5		-24.0	-21.6
D-2579	0.5			-21.2
D-2579	1			24.1
D-2662	0.5			-13.8
D-2663	0.5			-0.1
D-2664	0.5			-10.9
D-2665	0.5			8.6
D-2666	0.5			-8.0
D-2667	0.5			130.6
D-2668	0.5			47.5
D-2669	0.5			23.0
D-2670	0.5			-3.8
D-2534	0.5			-28.1
D-2534	1			-61.9
D-2671	0.5			10.8
D-2672	0.5			-42.1
D-2673	0.5			-2.6
D-2674	0.5			14.6
D-2675	0.5			1.9
D-2643	0.5			-13.4
D-2643	1			-20.0
D-2676	0.5			-24.0
D-2677	0.5			-47.1
D-2678	0.5			-37.8
D-2679	0.5			45.9
D-2680	0.5			2.8
D-2661	0.5			35.7
D-2661	1			31.7
D-2681	0.5			65.2
D-2682	0.5			-2.2
D-2683	0.5			-5.2
D-2684	0.5			4.1
D-2685	0.5			-45.6
D-2619	0.5	-28.7	-34.4	
D-2619	1	-18.9	-29.0	

D-2688	0.5	-32.7	-40.7	
D-2692	0.5	13.0	2.5	
D-2696	0.5	13.8	8.1	
D-2700	0.5	-34.8	-38.8	
D-2640	0.5	-44.6	-47.6	
D-2640	1	-59.7	-69.0	
D-2686	0.5	-19.1	-22.4	
D-2690	0.5	-11.6	-15.3	
D-2694	0.5	-37.1	-45.5	
D-2698	0.5	nd	nd	
D-2661	1	-3.4	-12.3	
D-2622	0.5	-43.4	-37.4	
D-2622	1	-64.7	-69.0	
D-2689	0.5	-53.8	-54.3	
D-2693	0.5	-53.2	-56.4	
D-2697	0.5	-64.9	-64.7	
D-2624	0.5	-62.8	-66.8	
D-2633	0.5	-57.2	-62.4	
D-2633	1	-69.8	-77.1	
D-2687	0.5	-53.9	-61.7	
D-2691	0.5	-16.9	-21.8	
D-2695	0.5	-33.6	-37.5	
D-2699	0.5	-44.2	-47.6	
D-2640	0.5	-58.9	-61.4	
D-2640	1	-65.9	-74.7	
D-2640	3	-86.0	-89.4	
D-2551	0.5	-42.3	-45.5	
D-2551	1	-60.6	-66.7	
D-2551	3	-79.9	-85.1	
D-2531	0.5	-29.0	-41.9	
D-2531	1	-52.1	-64.0	
D-2531	3	-76.8	-89.8	
D-2633	0.5	-5.9	-50.6	
D-2633	1	-55.5	-71.3	
D-2633	3	-82.9	-90.0	
D-2575	0.5	-17.6	-45.3	
D-2575	1	-55.5	-69.9	
D-2575	3	-79.8	-89.7	
D-2622	0.5	-17.7	-41.8	
D-2622	1	-39.3	-54.9	

D-2622	3	-73.8	-84.0	
D-2589	0.5	-33.1	-59.3	
D-2589	1	-65.0	-77.3	
D-2589	3	-81.4	-91.9	

[0175] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. However, the citation of a reference herein should not be construed as an acknowledgement that such reference is prior art to the present invention. To the extent that any of the definitions or terms provided in the references incorporated by reference differ from the terms and discussion provided herein, the present terms and definitions control.

[0176] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The foregoing description and examples detail certain preferred embodiments of the invention and describe the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the invention may be practiced in many ways and the invention should be construed in accordance with the appended claims and any equivalents thereof.

What is Claimed

1. An RNAi construct comprising a sense strand and an antisense strand, wherein the antisense strand comprises a region that is complementary to a glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) mRNA sequence listed in Table 1, and wherein the RNAi construct inhibits the expression of GPAM.

2. The RNAi construct of claim 1, which comprises a region having at least 15 contiguous nucleotides differing by no more than 3 nucleotides from an antisense sequence listed in Table 2.

3. The RNAi construct of claim 1 or claim 2, wherein the antisense strand hybridizes to a GPAM mRNA sequence listed in Table 1.

4. The RNAi construct of any one of claims 1-3, wherein the sense strand comprises a sequence that is sufficiently complementary to the sequence of the antisense strand to form a duplex region of about 15 to about 30 base pairs in length.

5. The RNAi construct of claim 4, wherein the duplex region is about 17 to about 24 base pairs in length.

6. The RNAi construct of claim 4, wherein the duplex region is about 19 to about 21 base pairs in length.

7. The RNAi construct of claim 6, wherein the duplex region is 19 base pairs in length.

8. The RNAi construct of claim 6, wherein the duplex region is 20 base pairs in length.

9. The RNAi construct of claim 6, wherein the duplex region is 21 base pairs in length.

10. The RNAi construct of any one of claims 4-10, wherein the sense strand and the antisense strand are each about 15 to about 30 nucleotides in length.

11. The RNAi construct of claim 10, wherein the sense strand and the antisense strand are each about 19 to about 27 nucleotides in length.

12. The RNAi construct of claim 10, wherein the sense strand and the antisense strand are each about 21 to about 25 nucleotides in length.

13. The RNAi construct of claim 12, wherein the sense strand and the antisense strand are each about 21 to about 23 nucleotides in length.

14. The RNAi construct of any one of claims 1 to 13, which comprises at least one blunt end.

15. The RNAi construct of any one of claims 1 to 13, which comprises at least one nucleotide overhang of 1 to 4 unpaired nucleotides.

16. The RNAi construct of claim 15, wherein the nucleotide overhang has two unpaired nucleotides.

17. The RNAi construct of claim 15 or 16, wherein the RNAi construct comprises a nucleotide overhang at the 3' end of the sense strand, the 3' end of the antisense strand, or the 3' end of both the sense strand and the antisense strand.

18. The RNAi construct of any one of claims 15-17, wherein the nucleotide overhang comprises a 5'-UU-3' dinucleotide or a 5'-dTdT-3' dinucleotide.

19. The RNAi construct of any one of claims 1 to 18, wherein the RNAi construct comprises at least one modified nucleotide.

20. The RNAi construct of claim 19, wherein the modified nucleotide is a 2'-modified nucleotide.

21. The RNAi construct of claim 19, wherein the modified nucleotide is a 2'-fluoro modified nucleotide, a 2'-O-methyl modified nucleotide, a 2'-O-methoxyethyl modified nucleotide, a 2'-O-allyl modified nucleotide, a bicyclic nucleic acid (BNA), a glycol nucleic acid, an inverted base, or combinations thereof.

22. The RNAi construct of claim 21, wherein the modified nucleotide is a 2'-O-methyl modified nucleotide, a 2'-O-methoxyethyl modified nucleotide, a 2'-fluoro modified nucleotide, or combinations thereof.

23. The RNAi construct of claim 19, wherein all of the nucleotides in the sense and antisense strands are modified nucleotides.

24. The RNAi construct of claim 23, wherein the modified nucleotides are 2'-O-methylmodified nucleotides, 2'-fluoro modified nucleotides, or combinations thereof.

25. The RNAi construct of any one of claims 1 to 24, which comprises at least one phosphorothioate internucleotide linkage.

26. The RNAi construct of claim 25, wherein the RNAi construct comprises at least one phosphorothioate internucleotide linkages at the 3' end of the sense strand.

27. The RNAi construct of claim 25, wherein the RNAi construct comprises at least one phosphorothioate internucleotide linkages at both the 3' and 5' ends of the sense strand.

28. The RNAi construct of any one of claims 1-27, wherein the antisense strand comprises a sequence selected from the antisense sequences listed in Table 2.

29. The RNAi construct of claim 28, wherein the sense strand comprises a sequence selected from the sense sequences listed in Table 2.

30. The RNAi construct of any one of claims 1-29, wherein the RNAi construct is any one of the duplex compounds listed in Table 2.

31. The RNAi construct of any one of claims 1-30, wherein the RNAi construct reduces the expression level of GPAM in liver cells following incubation with the RNAi construct as compared to the GPAM expression level in liver cells that have been incubated with a control RNAi construct.

32. The RNAi construct of claim 31, wherein the liver cells are HepG2cells.

33. The RNAi construct of any one of claims 1-32, wherein the RNAi construct inhibits at least 10% of GPAM expression at 5 nM in HepG2 cells *in vitro*.

34. The RNAi construct of any one of claims 1-33, wherein the RNAi construct inhibits GPAM expression in HepG2 cells with an IC50 of less than about 1 nM.

35. The RNAi construct of any one of claims 1-34, further comprising a ligand that binds to one or more proteins expressed on the surface of liver cells.

36. A composition comprising the RNAi construct of any one of claims 1-35 and a pharmaceutically acceptable carrier, excipient, or diluent.

37. A method for reducing the expression of GPAM in a patient in need thereof comprising administering to the patient the RNAi construct of any one of claims 1-36.

38. A method for reducing the expression of GPAM in a patient in need thereof comprising administering to the patient the composition of claim 36.

39. The method of claim 37 or claim 38, wherein the expression level of GPAM in hepatocytes is reduced in the patient following administration of the RNAi construct as compared to the GPAM expression level in a patient not receiving the RNAi construct.

40. The method of any one of claims 37-39, wherein the patient suffers from nonalcoholic fatty liver disease (NAFLD).

41. The method of claim 40, wherein the patient suffers from non-alcoholic steatohepatitis (NASH).

42. An RNAi construct of any one of claims 1-35 or a composition of claim 36 for use in the treatment of NAFLD.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 April 2023 (27.04.2023)

WIPO | PCT

(10) International Publication Number
WO 2023/069754 A3

(51) International Patent Classification:
C12N 15/113 (2010.01) *A61K 31/712* (2006.01)
C12N 9/10 (2006.01) *A61K 31/7125* (2006.01)
A61K 31/713 (2006.01) *A61P 1/16* (2006.01)
A61K 31/7115 (2006.01)

(21) International Application Number:
PCT/US2022/047491

(22) International Filing Date:
21 October 2022 (21.10.2022)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/270,813 22 October 2021 (22.10.2021) US

(71) Applicant: **AMGEN INC.** [US/US]; One Amgen Center Drive, Thousand Oaks, California 91320-1799 (US).

(72) Inventors: **RULIFSON, Ingrid**; c/o Amgen Inc., One Amgen Center Drive, Law Department-Patent Operations, Mail Stop 28-5-A, Thousand Oaks, California 91320-1799 (US). **MEADE, Bryan**; c/o Amgen Inc., One Amgen Center Drive, Law Department-Patent Operations, Mail Stop 28-5-A, Thousand Oaks, California 91320-1799 (US). **LONG, Jason C.**; c/o Amgen Inc., One Amgen Center Drive, Law Department-Patent Operations, Mail Stop 28-5-A, Thousand Oaks, California 91320-1799 (US). **MURRAY, Justin K.**; c/o Amgen Inc., One Amgen Center Drive, Law Department-Patent Operations, Mail Stop 28-5-A, Thousand Oaks, California 91320-1799 (US).

(74) Agent: **KONG, Lawrence**; 1120 Veterans Blvd., South San Francisco, California 94080 (US).

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(88) Date of publication of the international search report:
25 May 2023 (25.05.2023)

WO 2023/069754 A3

(54) Title: RNAI CONSTRUCTS FOR INHIBITING GPAM EXPRESSION AND METHODS OF USE THEREOF

(57) Abstract: The disclosure relates to RNAi constructs, such as siRNA, for reducing expression of the GPAM gene. Methods of using such RNAi constructs to treat or prevent liver disease, such as nonalcoholic fatty liver disease (NAFLD), are also described.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/047491

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113 C12N9/10 A61K31/713 A61K31/7115 A61K31/712 A61K31/7125 A61P1/16
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12N A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KIM CHAI-WAN ET AL: "Acetyl CoA Carboxylase Inhibition Reduces Hepatic Steatosis but Elevates Plasma Triglycerides in Mice and Humans: A Bedside to Bench Investigation", CELL METABOLISM, CELL PRESS, UNITED STATES, vol. 26, no. 2, 1 August 2017 (2017-08-01) , page 394, XP085151036, ISSN: 1550-4131, DOI: 10.1016/J.CMET.2017.07.009 page e2 page e3, paragraph 4 figure 7 page 402, left-hand column, paragraph 1 - page 403, right-hand column, paragraph 3 ----- -/--	1-42

[X] Further documents are listed in the continuation of Box C.
[X] See patent family annex.

* Special categories of cited documents :
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 February 2023	18/04/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Solyga-Zurek, A

2

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/047491

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:

1-42 (partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-42(partially)

an RNAi construct comprising a sense strand and an antisense strand, wherein the antisense strand comprises a region that is complementary to a glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) mRNA sequence, of SEQ ID NO: 1, as listed in Table 1, and wherein the RNAi construct inhibits the expression of GPAM

2-700. claims: 1-42(partially)

an RNAi construct comprising a sense strand and an antisense strand, wherein the antisense strand comprises a region that is complementary to a glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) mRNA sequence, having a sequence of SEQ ID NO: 3 - 2721 of Table 1, respectively, and wherein the RNAi construct inhibits the expression of GPAM

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/047491

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

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

   a. ☐ forming part of the international application as filed.

   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☒ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/047491

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2016/271137 A1 (KOYUNCU EMRE [US] ET AL) 22 September 2016 (2016-09-22) table 2 sequences 91-96	1-42
A	XU ET AL: "Hepatic knockdown of mitochondrial GPAT1 in ob/ob mice improves metabolic profile", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ELSEVIER, AMSTERDAM NL, vol. 349, no. 1, 13 October 2006 (2006-10-13), pages 439-448, XP005669579, ISSN: 0006-291X, DOI: 10.1016/J.BBRC.2006.08.071 page 440, right-hand column, paragraph 2 figures 1, 3-5 table 1 page 439, right-hand column, paragraph 2 - page 440, left-hand column, paragraph 2	1-42
A	US 2012/004276 A1 (LINDHOLM MARIE [SE] ET AL) 5 January 2012 (2012-01-05) table 1 claim 34 paragraph [0012] - paragraph [0014]	1-42

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2022/047491

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2016271137 A1	22-09-2016	EP 2536411 A2	26-12-2012
		US 2013190381 A1	25-07-2013
		US 2016271137 A1	22-09-2016
		WO 2011103516 A2	25-08-2011
US 2012004276 A1	05-01-2012	AU 2009265836 A1	07-01-2010
		BR PI0915837 A2	03-11-2015
		CA 2729897 A1	07-01-2010
		CN 102076854 A	25-05-2011
		EA 201170131 A1	30-08-2011
		EP 2310504 A1	20-04-2011
		JP 2011526482 A	13-10-2011
		KR 20110031368 A	25-03-2011
		US 2012004276 A1	05-01-2012
		WO 2010000656 A1	07-01-2010

摘要

本披露涉及用于降低 GPAM 基因的表达的 RNAi 构建体，如 siRNA。还描述了使用这样的 RNAi 构建体治疗或预防肝病如非酒精性脂肪性肝病（NAFLD）的方法。