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(54) Title: ANTISENSE MODULATION OF PYRUVATE CARBOXYLASE EXPRESSION

(57) Abstract: Provided herein are methods, compounds, and compositions for reducing expression of pyruvate carboxylase mRNA and protein in an animal. Such methods, compounds, and compositions are useful to treat, prevent, delay, or ameliorate metabolic disease, for example, diabetes, or a symptom thereof. Antisense inhibition of pyruvate carboxylase provides a unique advantage over traditional small molecule inhibitors in that antisense inhibitors do not rely on competitive binding of the compound to the protein and inhibit activity directly by reducing the expression of pyruvate carboxylase. As such, with antisense inhibition, pyruvate carboxylase is effectively reduced without affecting islet pyruvate carboxylase activity in the pancreas. Antisense technology is emerging as an effective means for reducing the expression of certain gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of pyruvate carboxylase gene expression.



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ANTISENSE MODULATION OF PYRUVATE CARBOXYLASE EXPRESSION

SEQUENCE LISTING

5 The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled BIOL0158WO.txt created June 15, 2012, which is 196 Kb in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

10 FIELD

 Provided herein are methods, compounds, and compositions for reducing expression of pyruvate carboxylase (pyruvate carboxylase) mRNA and protein in an animal. Such methods, compounds, and compositions are useful, for example, to treat, prevent, delay or ameliorate diseases associated with metabolic disorders, particularly disorders associated with diabetes.

15 BACKGROUND

 Insulin and glucagon are two pancreatic hormones involved in regulating glucose homeostasis and metabolism. Glucagon is secreted from the α -cells of the pancreatic islets and regulates glucose homeostasis through modulation of hepatic glucose production (Quesada et al., J. Endocrinol. 2008. 199: 5-19). The main
20 function of glucagon is to counteract the actions of insulin.

 Dysregulation of glucose metabolism may be caused either by defective insulin secretion and/or action, or by impaired postprandial glucagon suppression (Shah et al., Am. J. Physiol. Endocrinol. Metab. 1999. 277: E283-E290). Inhibition of postprandial glucagon secretion in diabetic subjects has been shown to substantially reduce blood glucose, suggesting that glucagon contributes significantly to the hyperglycemia
25 seen in subjects with type 2 diabetes mellitus (Shah et al., J. Clin. Endocrinol. Metab. 2000. 85: 4053-4059).

 Type 2 diabetes is characterized by impaired insulin secretion and/or action, and many subjects also exhibit inappropriate levels of circulating glucagon in the fasting and postprandial state. An increase in the glucagon/insulin ratio is likely an important determinant of the hyperglycemia seen in type 2 diabetes patients (Baron et al., Diabetes. 1987. 36: 274-283). Lack of suppression of postprandial glucagon secretion in
30 subjects with T2DM also plays an important role in the pathogenesis of postprandial hyperglycemia (Henkel et al., Metabolism. 2005. 54: 1168-1173).

 Pyruvate carboxylase (PC), is a regulatory metabolic enzyme responsible for replenishing the intermediates of the TCA cycle and catalyzing the first committed step in gluconeogenesis, is found in a wide

variety of organisms including bacteria, fungi, plants, invertebrates and vertebrates (Wallace JC., Pyruvate Carboxylase. Boca Raton: CRC Press 1985; pp. 5-64). Pyruvate carboxylase is required to transfer carbons from pyruvate into the Krebs cycle.

Abnormalities in pyruvate carboxylase activity and regulation have been associated with the occurrence of Type II diabetes (Jitrapakdee et al., Biochem J. 2008. 413: 369-87) resulting in impaired-glucose tolerance and insulin insensitivity (Moller DE. Nature. 2001. 414(6865):821-7). In pre-diabetic patients, pancreatic islets compensate for the escalating insulin-resistance by increasing glucose stimulated insulin secretion (GSIS) (Jitrapakdee, et al., Biochem J. 2008. 413: 369-87; Jitrapakdee, et al., Cell Mol Life Sci. 2006. 63: 843-54). The use of small interfering RNA (si-RNA) to partially suppress pyruvate carboxylase activity in INS-1 83/13-derived cell lines (Hasan, et al., J Biol Chem. 2008. 283: 28048-59) demonstrated that decreases in GSIS were directly proportional to decreases in pyruvate carboxylase activity. Furthermore, Liu *et al.* (Liu, et al., J Biol Chem. 2002. 277: 39163-68) previously observed a 2-fold increase in *in vivo* pyruvate carboxylase β -cell activity and GSIS in obese, non-diabetic, insulin-resistant Zucker fatty rats as compared to non-insulin resistant Zucker lean rats. Inhibition of pyruvate carboxylase by phenylacetate resulted in not only a marked decrease in pyruvate carboxylase activity, but also a corresponding decrease in GSIS. The decreased pyruvate carboxylase activity and expression observed in the islets of diabetic rats, which are insulin resistant and show little GSIS (MacDonald, et al., Diabetes. 1996. 45: 886-90.; Macdonald, et al., J Biol Chem. 1996. 45: 1626-30) further suggests that pyruvate carboxylase plays an important role in both GSIS and β -cell adaptation to insulin resistance in fully functioning pancreatic cells. While the mechanism by which pyruvate carboxylase activity regulates and enhances GSIS is not completely understood, it has been proposed that the metabolic cycling of pyruvate through pyruvate carboxylase and the subsequent formation of anaplerotic by-products, including NADPH, aids in modulating GSIS in pancreatic islets. (MacDonald, et al., J Physiol Endocrinol Metab. 2005. 288: E1-E15; [12] Jensen, et al., Am J Physiol Endocrinol Metab. 2008. 295:E1287-E97.). It was later shown, that pyruvate carboxylase activity is preserved in the islets of obese animals but it is reduced in the islets of type 2 diabetic animals, suggesting that pyruvate carboxylase is important in beta-cell adaptation to insulin resistance and that a reduction of islet pyruvate carboxylase activity results in beta-cell failure and the development of type 2 diabetes (Han et al. J. of Endocrinology. 2010. 204: 143-152).

There is a currently a lack of acceptable options for treating diabetes. It is therefore an object herein to provide compounds and methods for the treatment of such diseases and disorder.

All documents, or portions of documents, cited in this application, including, but not limited to, patents, patent applications, articles, books, and treatises, are hereby expressly incorporated-by-reference for the portions of the document discussed herein, as well as in their entirety.

SUMMARY

Antisense inhibition of pyruvate carboxylase provides a unique advantage over traditional small molecule inhibitors in that antisense inhibitors do not rely on competitive binding of the compound to the protein and inhibit activity directly by reducing the expression of pyruvate carboxylase. As such, with
5 antisense inhibition, pyruvate carboxylase is effectively reduced without affecting islet pyruvate carboxylase activity in the pancreas. Antisense technology is emerging as an effective means for reducing the expression of certain gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of pyruvate carboxylase gene expression.

Provided herein are methods, compounds, and compositions for modulating expression of pyruvate
10 carboxylase (PC) and treating, preventing, delaying or ameliorating diseases associated with metabolic disorders, particularly disorders associated with diabetes and/or a symptom thereof.

DETAILED DESCRIPTION

It is to be understood that both the foregoing general description and the following detailed
15 description are exemplary and explanatory only and are not restrictive described herein, as claimed. Herein, the use of the singular includes the plural unless specifically stated otherwise. As used herein, the use of “or” means “and/or” unless stated otherwise. Furthermore, the use of the term “including” as well as other forms, such as “includes” and “included”, is not limiting. Also, terms such as “element” or “component” encompass both elements and components comprising one unit and elements and components that comprise more than
20 one subunit, unless specifically stated otherwise.

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including, but not limited to, patents, patent applications, articles, books, and treatises, are hereby expressly
25 incorporated-by-reference for the portions of the document discussed herein, as well as in their entirety.

Definitions

Unless specific definitions are provided, the nomenclature utilized in connection with, and the procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard
30 techniques can be used for chemical synthesis, and chemical analysis. Where permitted, all documents, or portions of documents, cited in this application, including, but not limited to, all patents, applications, published applications and other journal publications, GENBANK Accession Numbers and associated sequence information obtainable through databases such as National Center for Biotechnology Information

(NCBI) and other data referred to throughout in the disclosure herein are incorporated by reference for the portions of the document discussed herein, as well as in their entirety.

Unless specific definitions are provided, the nomenclature utilized in connection with, and the procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques can be used for chemical synthesis, and chemical analysis. Where permitted, all patents, applications, published applications and other publications, GENBANK Accession Numbers and associated sequence information obtainable through databases such as National Center for Biotechnology Information (NCBI) and other data referred to throughout in the disclosure herein are incorporated by reference for the portions of the document discussed herein, as well as in their entirety.

Unless otherwise indicated, the following terms have the following meanings:

“2’-O-methoxyethyl” (also 2’-MOE and 2’-O(CH₂)₂-OCH₃) refers to an O-methoxy-ethyl modification of the 2’ position of a furosyl ring. A 2’-O-methoxyethyl modified sugar is a modified sugar.

“2’-O-methoxyethyl nucleotide” means a nucleotide comprising a 2’-O-methoxyethyl modified sugar moiety.

“3’ target site” refers to the nucleotide of a target nucleic acid which is complementary to the 3’-most nucleotide of a particular antisense compound.

“5’ target site” refers to the nucleotide of a target nucleic acid which is complementary to the 5’-most nucleotide of a particular antisense compound.

“5-methylcytosine” means a cytosine modified with a methyl group attached to the 5’ position. A 5-methylcytosine is a modified nucleobase.

“About” means within $\pm 10\%$ of a value. For example, if it is stated, “a marker may be increased by about 50%”, it is implied that the marker may be increased between 45%-55%.

“Active pharmaceutical agent” means the substance or substances in a pharmaceutical composition that provide a therapeutic benefit when administered to an individual. For example, in certain embodiments an antisense oligonucleotide targeted to or complementary to pyruvate carboxylase is an active pharmaceutical agent.

“Active target region” or “target region” means a region to which one or more active antisense compounds is targeted. “Active antisense compounds” means antisense compounds that reduce target nucleic acid levels or protein levels.

“Adiposity” or “Obesity” refers to the state of being obese or an excessively high amount of body fat or adipose tissue in relation to lean body mass. The amount of body fat includes concern for both the distribution of fat throughout the body and the size and mass of the adipose tissue deposits. Body fat distribution can be estimated by skin-fold measures, waist-to-hip circumference ratios, or techniques such as

ultrasound, computed tomography, or magnetic resonance imaging. According to the Center for Disease Control and Prevention, individuals with a body mass index (BMI) of 30 or more are considered obese. The term "Obesity" as used herein includes conditions where there is an increase in body fat beyond the physical requirement as a result of excess accumulation of adipose tissue in the body. The term "obesity" includes, but is not limited to, the following conditions: adult-onset obesity; alimentary obesity; endogenous or inflammatory obesity; endocrine obesity; familial obesity; hyperinsular obesity; hyperplastic-hypertrophic obesity; hypogonadal obesity; hypothyroid obesity; lifelong obesity; morbid obesity and exogenous obesity.

"Administered concomitantly" refers to the co-administration of two agents in any manner in which the pharmacological effects of both are manifest in the patient at the same time. Concomitant administration does not require that both agents be administered in a single pharmaceutical composition, in the same dosage form, or by the same route of administration. The effects of both agents need not manifest themselves at the same time. The effects need only be overlapping for a period of time and need not be coextensive.

"Administering" means providing an agent to an animal, and includes, but is not limited to, administering by a medical professional and self-administering.

"Agent" means an active substance that can provide a therapeutic benefit when administered to an animal. "First Agent" means a therapeutic compound provided herein. For example, a first agent can be an antisense oligonucleotide targeting pyruvate carboxylase. "Second agent" means a second therapeutic compound described herein (e.g. a second antisense oligonucleotide targeting pyruvate carboxylase) and/or a non- pyruvate carboxylase therapeutic compound.

"Amelioration" refers to a lessening of at least one indicator, sign, or symptom of an associated disease, disorder, or condition. The severity of indicators can be determined by subjective or objective measures, which are known to those skilled in the art.

"Animal" refers to a human or non-human animal, including, but not limited to, mice, rats, rabbits, dogs, cats, pigs, and non-human primates, including, but not limited to, monkeys and chimpanzees.

"Antisense activity" means any detectable or measurable activity attributable to the hybridization of an antisense compound to its target nucleic acid. In certain embodiments, antisense activity is a decrease in the amount or expression of a target nucleic acid or protein encoded by such target nucleic acid.

"Antisense compound" means an oligomeric compound that is capable of undergoing hybridization to a target nucleic acid through hydrogen bonding.

"Antisense inhibition" means reduction of target nucleic acid levels or target protein levels in the presence of an antisense compound complementary to a target nucleic acid compared to target nucleic acid levels or target protein levels in the absence of the antisense compound.

"Antisense oligonucleotide" means a single-stranded oligonucleotide having a nucleobase sequence that permits hybridization to a corresponding region or segment of a target nucleic acid.

“Bicyclic sugar” means a furosyl ring modified by the bridging of two non-geminal ring atoms. A bicyclic sugar is a modified sugar.

“Bicyclic nucleic acid” or “BNA” refers to a nucleoside or nucleotide wherein the furanose portion of the nucleoside or nucleotide includes a bridge connecting two carbon atoms on the furanose ring, thereby forming a bicyclic ring system.

“Cap structure” or “terminal cap moiety” means chemical modifications, which have been incorporated at either terminus of an antisense compound.

“Chemically distinct region” refers to a region of an antisense compound that is in some way chemically different than another region of the same antisense compound. For example, a region having 2'-O-methoxyethyl nucleotides is chemically distinct from a region having nucleotides without 2'-O-methoxyethyl modifications.

“Chimeric antisense compound” means an antisense compound that has at least two chemically distinct regions.

“Co-administration” means administration of two or more agents to an individual. The two or more agents can be in a single pharmaceutical composition, or can be in separate pharmaceutical compositions. Each of the two or more agents can be administered through the same or different routes of administration. Co-administration encompasses parallel or sequential administration.

“Cholesterol” is a sterol molecule found in the cell membranes of all animal tissues. Cholesterol must be transported in an animal's blood plasma by lipoproteins including very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). “Plasma cholesterol” refers to the sum of all lipoproteins (VDL, IDL, LDL, HDL) esterified and/or non-esterified cholesterol present in the plasma or serum.

“Complementarity” means the capacity for pairing between nucleobases of a first nucleic acid and a second nucleic acid.

“cEt” or “constrained ethyl” means a bicyclic sugar moiety comprising a bridge connecting the 4'-carbon and the 2'-carbon, wherein the bridge has the formula: 4'-CH(CH₃)-O-2'.

“Constrained ethyl nucleoside” (also cEt nucleoside) means a nucleoside comprising a bicyclic sugar moiety comprising a 4'-CH(CH₃)-O-2' bridge.

“Contiguous nucleobases” means nucleobases immediately adjacent to each other.

“Deoxyribonucleotide” means a nucleotide having a hydrogen at the 2' position of the sugar portion of the nucleotide. Deoxyribonucleotides may be modified with any of a variety of substituents.

“Diabetes mellitus” or “diabetes” is a syndrome characterized by disordered metabolism and abnormally high blood sugar (hyperglycemia) resulting from insufficient levels of insulin or reduced insulin sensitivity. The characteristic symptoms are excessive urine production (polyuria) due to high blood glucose

levels, excessive thirst and increased fluid intake (polydipsia) attempting to compensate for increased urination, blurred vision due to high blood glucose effects on the eye's optics, unexplained weight loss, and lethargy.

“Diabetic dyslipidemia” or “type 2 diabetes with dyslipidemia” means a condition characterized by Type 2 diabetes, reduced HDL-C, elevated triglycerides, and elevated small, dense LDL particles.

“Diluent” means an ingredient in a composition that lacks pharmacological activity, but is pharmaceutically necessary or desirable. For example, the diluent in an injected composition can be a liquid, e.g. saline solution.

“Dyslipidemia” refers to a disorder of lipid and/or lipoprotein metabolism, including lipid and/or lipoprotein overproduction or deficiency. Dyslipidemias may be manifested by elevation of lipids such as cholesterol and triglycerides as well as lipoproteins such as low-density lipoprotein (LDL) cholesterol.

“Dosage unit” means a form in which a pharmaceutical agent is provided, e.g. pill, tablet, or other dosage unit known in the art. In certain embodiments, a dosage unit is a vial containing lyophilized antisense oligonucleotide. In certain embodiments, a dosage unit is a vial containing reconstituted antisense oligonucleotide.

“Dose” means a specified quantity of a pharmaceutical agent provided in a single administration, or in a specified time period. In certain embodiments, a dose can be administered in one, two, or more boluses, tablets, or injections. For example, in certain embodiments where subcutaneous administration is desired, the desired dose requires a volume not easily accommodated by a single injection, therefore, two or more injections can be used to achieve the desired dose. In certain embodiments, the pharmaceutical agent is administered by infusion over an extended period of time or continuously. Doses can be stated as the amount of pharmaceutical agent per hour, day, week, or month.

“Effective amount” or “therapeutically effective amount” means the amount of active pharmaceutical agent sufficient to effectuate a desired physiological outcome in an individual in need of the agent. The effective amount can vary among individuals depending on the health and physical condition of the individual to be treated, the taxonomic group of the individuals to be treated, the formulation of the composition, assessment of the individual’s medical condition, and other relevant factors.

“Fully complementary” or “100% complementary” means each nucleobase of a nucleobase sequence of a first nucleic acid has a complementary nucleobase in a second nucleobase sequence of a second nucleic acid. In certain embodiments, a first nucleic acid is an antisense compound and a target nucleic acid is a second nucleic acid.

“Gapmer” means a chimeric antisense compound in which an internal region having a plurality of nucleosides that support RNase H cleavage is positioned between external regions having one or more nucleosides, wherein the nucleosides comprising the internal region are chemically distinct from the

nucleoside or nucleosides comprising the external regions. The internal region can be referred to as a “gap segment” and the external regions can be referred to as “wing segments.”

“Gap-widened” means a chimeric antisense compound having a gap segment of 12 or more contiguous 2’-deoxyribonucleosides positioned between and immediately adjacent to 5’ and 3’ wing segments having from one to six nucleosides.

“Glucose” is a monosaccharide used by cells as a source of energy and inflammatory intermediate. “Plasma glucose” refers to glucose present in the plasma.

“Hybridization” means the annealing of complementary nucleic acid molecules. In certain embodiments, complementary nucleic acid molecules include an antisense compound and a target nucleic acid.

“Hyperlipidemia” or “hyperlipemia” is a condition characterized by elevated serum lipids or circulating (plasma) lipids. This condition manifests an abnormally high concentration of fats. The lipid fractions in the circulating blood are cholesterol, low density lipoproteins, very low density lipoproteins and triglycerides.

“Hypertriglyceridemia” means a condition characterized by elevated triglyceride levels.

“Identifying” or “selecting an animal with metabolic” means identifying or selecting a subject having been diagnosed with a metabolic disease, or a metabolic disorder; or, identifying or selecting a subject having any symptom of a metabolic disease, including, but not limited to, metabolic syndrome, hyperglycemia, hypertriglyceridemia, hypertension increased insulin resistance, decreased insulin sensitivity, above normal body weight, and/or above normal body fat or any combination thereof. Such identification may be accomplished by any method, including but not limited to, standard clinical tests or assessments, such as measuring serum or circulating (plasma) blood-glucose, measuring serum or circulating (plasma) triglycerides, measuring blood-pressure, measuring body fat, measuring body weight, and the like.

“Immediately adjacent” means there are no intervening elements between the immediately adjacent elements.

“Individual” or “subject” or “animal” means a human or non-human animal selected for treatment or therapy.

“Inhibiting the expression or activity” refers to a reduction or blockade of the expression or activity of a RNA or protein and does not necessarily indicate a total elimination of expression or activity.

“Insulin levels” refers to levels of insulin in the plasma.

“Insulin resistance” is defined as the condition in which normal amounts of insulin are inadequate to produce a normal insulin response from fat, muscle and liver cells. Insulin resistance in fat cells results in hydrolysis of stored triglycerides, which elevates free fatty acids in the blood plasma. Insulin resistance in muscle reduces glucose uptake whereas insulin resistance in liver reduces glucose storage, with both effects

serving to elevate blood glucose. High plasma levels of insulin and glucose due to insulin resistance often leads to metabolic syndrome and type 2 diabetes.

“Insulin sensitivity” is a measure of how effectively an individual processes glucose. An individual having high insulin sensitivity effectively processes glucose whereas an individual with low insulin sensitivity does not effectively process glucose.

“Internucleoside linkage” refers to the chemical bond between nucleosides.

“Intravenous administration” means administration into a vein.

“Linked nucleosides” means adjacent nucleosides which are bonded together.

“Lipid-lowering therapy” or “lipid lowering agent” means a therapeutic regimen provided to a subject to reduce one or more lipids in a subject. In certain embodiments, a lipid-lowering therapy is provided to reduce one or more of ApoB, total cholesterol, LDL-C, VLDL-C, IDL-C, non-HDL-C, triglycerides, small dense LDL particles, and Lp(a) in a subject. Examples of lipid-lowering therapy include statins, fibrates, and MTP inhibitors.

“Major risk factors” refers to factors that contribute to a high risk for a particular disease or condition. In certain embodiments, major risk factors for coronary heart disease include, without limitation, cigarette smoking, hypertension, low HDL-C, family history of coronary heart disease, age, and other factors disclosed herein.

“Metabolic disease” or “metabolic disorder” refers to a condition characterized by an alteration or disturbance in metabolic function. “Metabolic” and “metabolism” are terms well known in the art and generally include the whole range of biochemical processes that occur within a living organism. Metabolic diseases or disorders include, but are not limited to, obesity, diabetes, hyperglycemia, prediabetes, non-alcoholic fatty liver disease (NAFLD), metabolic syndrome, insulin resistance, diabetic dyslipidemia, or hypertriglyceridemia or a combination thereof.

“Metabolic syndrome” means a condition characterized by a clustering of lipid and non-lipid cardiovascular risk factors of metabolic origin. In certain embodiments, metabolic syndrome is identified by the presence of any 3 of the following factors: waist circumference of greater than 102 cm in men or greater than 88 cm in women; serum triglyceride of at least 150 mg/dL; HDL-C less than 40 mg/dL in men or less than 50 mg/dL in women; blood pressure of at least 130/85 mmHg; and fasting glucose of at least 110 mg/dL. These determinants can be readily measured in clinical practice (JAMA, 2001, 285: 2486-2497).

“Mismatch” or “non-complementary nucleobase” refers to the case when a nucleobase of a first nucleic acid is not capable of pairing with the corresponding nucleobase of a second or target nucleic acid.

“Mixed dyslipidemia” means a condition characterized by elevated cholesterol and elevated triglycerides.

“Modified internucleoside linkage” refers to a substitution or any change from a naturally occurring internucleoside bond (i.e. a phosphodiester internucleoside bond).

“Modified nucleobase” refers to any nucleobase other than adenine, cytosine, guanine, thymidine, or uracil. An “unmodified nucleobase” means the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C), and uracil (U).

“Modified nucleoside” means a nucleoside having, independently, a modified sugar moiety or modified nucleobase.

“Modified nucleotide” means a nucleotide having, independently, a modified sugar moiety, modified internucleoside linkage, or modified nucleobase. A “modified nucleoside” means a nucleoside having, independently, a modified sugar moiety or modified nucleobase.

“Modified oligonucleotide” means an oligonucleotide comprising at least one modified nucleotide.

“Modified sugar” refers to a substitution or change from a natural sugar.

“Motif” means the pattern of chemically distinct regions in an antisense compound.

“Naturally occurring internucleoside linkage” means a 3' to 5' phosphodiester linkage.

“Natural sugar moiety” means a sugar found in DNA (2'-H) or RNA (2'-OH).

“Non-alcoholic fatty liver disease” or “NAFLD” means a condition characterized by fatty inflammation of the liver that is not due to excessive alcohol use (for example, alcohol consumption of over 20 g/day). In certain embodiments, NAFLD is related to insulin resistance and the metabolic syndrome. NAFLD encompasses a disease spectrum ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation (steatohepatitis), fibrosis, and cirrhosis.

“Nonalcoholic steatohepatitis” (NASH) occurs from progression of NAFLD beyond deposition of triglycerides. A “second hit” capable of inducing necrosis, inflammation, and fibrosis is required for development of NASH. Candidates for the second-hit can be grouped into broad categories: factors causing an increase in oxidative stress and factors promoting expression of proinflammatory cytokines

“Nucleic acid” refers to molecules composed of monomeric nucleotides. A nucleic acid includes ribonucleic acids (RNA), deoxyribonucleic acids (DNA), single-stranded nucleic acids, double-stranded nucleic acids, small interfering ribonucleic acids (siRNA), and microRNAs (miRNA). A nucleic acid can also comprise a combination of these elements in a single molecule.

“Nucleobase” means a heterocyclic moiety capable of pairing with a base of another nucleic acid.

“Nucleobase sequence” means the order of contiguous nucleobases independent of any sugar, linkage, or nucleobase modification.

“Nucleoside” means a nucleobase linked to a sugar.

“Nucleoside mimetic” includes those structures used to replace the sugar or the sugar and the base and not necessarily the linkage at one or more positions of an oligomeric compound such as for example

nucleoside mimetics having morpholino, cyclohexenyl, cyclohexyl, tetrahydropyranyl, bicyclo or tricyclo sugar mimetics e.g. non furanose sugar units.

“Nucleotide” means a nucleoside having a phosphate group covalently linked to the sugar portion of the nucleoside.

5 “Nucleotide mimetic” includes those structures used to replace the nucleoside and the linkage at one or more positions of an oligomeric compound such as for example peptide nucleic acids or morpholinos (morpholinos linked by -N(H)-C(=O)-O- or other non-phosphodiester linkage).

10 “Nuclear ribonuclease” means a ribonuclease found in the nucleus. Nuclear ribonucleases include, but are not limited to, RNase H including RNase H1 and RNase H2, the double stranded RNase drosha and other double stranded RNases.

15 “Oligomeric compound” or “oligomer” refers to a polymeric structure comprising two or more sub-structures and capable of hybridizing to a region of a nucleic acid molecule. In certain embodiments, oligomeric compounds are oligonucleosides. In certain embodiments, oligomeric compounds are oligonucleotides. In certain embodiments, oligomeric compounds are antisense compounds. In certain
embodiments, oligomeric compounds are antisense oligonucleotides. In certain embodiments, oligomeric compounds are chimeric oligonucleotides.

“Oligonucleotide” means a polymer of linked nucleosides each of which can be modified or unmodified, independent one from another.

“Pancreatic pyruvate carboxylase” means pyruvate carboxylase expressed in the pancreas.

20 “Pancreatic islet pyruvate carboxylase” or “pancreatic islet PC” means pyruvate carboxylase expressed in the islet or β cells of the pancreas.

25 “Parenteral administration” means administration through injection or infusion. Parenteral administration includes subcutaneous administration, intravenous administration, intramuscular administration, intraarterial administration, intraperitoneal administration, or intracranial administration, e.g. intrathecal or intracerebroventricular administration. Administration can be continuous, or chronic, or short or intermittent.

“Peptide” means a molecule formed by linking at least two amino acids by amide bonds. Peptide refers to polypeptides and proteins.

30 “Pharmaceutical agent” means a substance that provides a therapeutic benefit when administered to an individual. For example, in certain embodiments, an antisense oligonucleotide targeted to or complementary to pyruvate carboxylase is pharmaceutical agent.

“Pharmaceutical composition” means a mixture of substances suitable for administering to an individual. For example, a pharmaceutical composition can comprise one or more active agents and a sterile aqueous solution.

“Pharmaceutically acceptable carrier” means a medium or diluent that does not interfere with the structure of the oligonucleotide. Certain, of such carriers enable pharmaceutical compositions to be formulated as, for example, tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspension and lozenges for the oral ingestion by a subject. For example, a pharmaceutically acceptable carrier can be a sterile aqueous solution.

“Pharmaceutically acceptable derivative” encompasses pharmaceutically acceptable salts, conjugates, prodrugs or isomers of the compounds described herein.

“Pharmaceutically acceptable salts” means physiologically and pharmaceutically acceptable salts of antisense compounds, i.e., salts that retain the desired biological activity of the parent oligonucleotide and do not impart undesired toxicological effects thereto.

“Phosphorothioate linkage” means a linkage between nucleosides where the phosphodiester bond is modified by replacing one of the non-bridging oxygen atoms with a sulfur atom. A phosphorothioate linkage is a modified internucleoside linkage.

“Portion” means a defined number of contiguous (i.e. linked) nucleobases of a nucleic acid. In certain embodiments, a portion is a defined number of contiguous nucleobases of a target nucleic acid. In certain embodiments, a portion is a defined number of contiguous nucleobases of an antisense compound.

“Preferentially reduces hepatic pyruvate carboxylase” means that pyruvate carboxylase expression is reduced in the liver affecting pyruvate carboxylase activity in the liver without affecting or substantially affecting the activity of pyruvate carboxylase in other tissues, for example, in the pancreas.

“Prevent” refers to delaying or forestalling the onset or development of a disease, disorder, or condition for a period of time from minutes to indefinitely. Prevent also means reducing risk of developing a disease, disorder, or condition.

“Prodrug” means a therapeutic agent that is prepared in an inactive form that is converted to an active form within the body or cells thereof by the action of endogenous enzymes or other chemicals or conditions.

“Pyruvate carboxylase” or “PC” means any nucleic acid or protein of pyruvate carboxylase.

“Pyruvate carboxylase expression” “PC expression” means the level of mRNA transcribed from the gene encoding pyruvate carboxylase or the level of protein translated from the mRNA. Pyruvate carboxylase expression can be determined by art known methods such as a Northern or Western blot.

“Pyruvate carboxylase nucleic acid” or “PC nucleic acid” means any nucleic acid encoding pyruvate carboxylase. For example, in certain embodiments, a pyruvate carboxylase nucleic acid includes a DNA sequence encoding pyruvate carboxylase, a RNA sequence transcribed from DNA encoding pyruvate carboxylase (including genomic DNA comprising introns and exons), and a mRNA sequence encoding pyruvate carboxylase.

“Pyruvate carboxylase mRNA” or “PC mRNA” means a mRNA encoding a pyruvate carboxylase protein.

“Side effects” means physiological responses attributable to a treatment other than the desired effects.

In certain embodiments, side effects include injection site reactions, liver function test abnormalities, renal function abnormalities, liver toxicity, renal toxicity, central nervous system abnormalities, myopathies, and malaise. For example, increased aminotransferase levels in serum can indicate liver toxicity or liver function abnormality. For example, increased bilirubin can indicate liver toxicity or liver function abnormality.

“Single-stranded oligonucleotide” means an oligonucleotide which is not hybridized to a complementary strand.

“Specifically hybridizable” refers to an antisense compound having a sufficient degree of complementarity between an antisense oligonucleotide and a target nucleic acid to induce a desired effect, while exhibiting minimal or no effects on non-target nucleic acids under conditions in which specific binding is desired, i.e. under physiological conditions in the case of *in vivo* assays and therapeutic treatments.

“Statin” means an agent that inhibits the activity of HMG-CoA reductase.

“Subcutaneous administration” means administration just below the skin.

“Targeting” or “targeted” means the process of design and selection of an antisense compound that will specifically hybridize to a target nucleic acid and induce a desired effect.

“Target nucleic acid,” “target RNA,” and “target RNA transcript” all refer to a nucleic acid capable of being targeted by antisense compounds.

“Target segment” means the sequence of nucleotides of a target nucleic acid to which an antisense compound is targeted. “5’ target site” refers to the 5’-most nucleotide of a target segment. “3’ target site” refers to the 3’-most nucleotide of a target segment.

“Therapeutically effective amount” means an amount of an agent that provides a therapeutic benefit to an individual.

“Therapeutic lifestyle change” means dietary and lifestyle changes intended to lower fat /adipose tissue mass and/or cholesterol. Such change can reduce the risk of developing heart disease, and may include recommendations for dietary intake of total daily calories, total fat, saturated fat, polyunsaturated fat, monounsaturated fat, carbohydrate, protein, cholesterol, insoluble fiber, as well as recommendations for physical activity.

“Triglyceride” or “TG” means a lipid or neutral fat consisting of glycerol combined with three fatty acid molecules.

“Type 2 diabetes,” (also known as “type 2 diabetes mellitus” or “diabetes mellitus, type 2”, and formerly called “diabetes mellitus type 2” , “non-insulin-dependent diabetes (NIDDM)”, “obesity related

diabetes”, or “adult-onset diabetes”) is a metabolic disorder that is primarily characterized by insulin resistance, relative insulin deficiency, and hyperglycemia.

“Treat” refers to administering a pharmaceutical composition to an animal to effect an alteration or improvement of a disease, disorder, or condition.

5 “Unmodified nucleotide” means a nucleotide composed of naturally occurring nucleobases, sugar moieties, and internucleoside linkages. In certain embodiments, an unmodified nucleotide is an RNA nucleotide (i.e. β -D-ribonucleosides) or a DNA nucleotide (i.e. β -D-deoxyribonucleoside).

Certain Embodiments

10 In certain embodiments, the compounds or compositions described herein comprise a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to of complementary to pyruvate carboxylase. The pyruvate carboxylase target can have a sequence selected from any one of SEQ ID NOs: 1-5.

In certain embodiments, the compounds or compositions described herein comprise a modified oligonucleotide consisting of 10 to 30 nucleosides having a nucleobase sequence complementary to any of
15 SEQ ID NOs: 1-5.

In certain embodiments, the nucleobase sequence of the modified oligonucleotide is at least 70%, 75%, 80%, 85%, 90%, 95%, 98% or 100% complementary to any one of SEQ ID NOS: 1-5 as measured over the entirety of the modified oligonucleotide.

In certain embodiments, the compounds or compositions described herein comprise a salt of the
20 modified oligonucleotide.

In certain embodiments, the compounds or compositions described herein further comprise a pharmaceutically acceptable carrier or diluent.

In certain embodiments, the compound described herein consists of a single-stranded modified oligonucleotide.

25 In certain embodiments, the modified oligonucleotide consists of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 linked nucleosides. In certain embodiments, the modified oligonucleotide consists of 20 linked nucleosides.

In certain embodiments, at least one internucleoside linkage of said modified oligonucleotide is a modified internucleoside linkage. In certain embodiments, each internucleoside linkage is a phosphorothioate
30 internucleoside linkage.

In certain embodiments, at least one nucleoside of the modified oligonucleotide comprises a modified sugar. In certain embodiments the modified oligonucleotide comprises at least one tetrahydropyran modified nucleoside wherein a tetrahydropyran ring replaces a furanose ring.

In certain embodiments, at least one nucleoside of said modified oligonucleotide comprises a modified nucleobase. In certain embodiments, the modified nucleobase is a 5-methylcytosine.

In certain embodiments, the modified oligonucleotide comprises: a) a gap segment consisting of linked deoxynucleosides; b) a 5' wing segment consisting of linked nucleosides; and c) a 3' wing segment consisting of linked nucleosides. The gap segment is positioned between the 5' wing segment and the 3' wing segment and each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, the modified oligonucleotide consists of 20 linked nucleosides, the gap segment consisting of eight to fourteen linked deoxynucleosides, the 5' wing segment consisting of two to six linked nucleosides, the 3' wing segment consisting of two to six linked nucleosides. In certain embodiments, each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, the modified sugar is a 2'-O-methoxyethyl sugar. In certain embodiments each internucleoside linkage is a phosphorothioate linkage. In certain embodiments, the modified oligonucleotide consists of 20 linked nucleosides, the gap segment consisting of ten linked deoxynucleosides, the 5' wing segment consisting of five linked nucleosides, the 3' wing segment consisting of five linked nucleosides, each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar and each internucleoside linkage is a phosphorothioate linkage.

Certain embodiments provide methods, compounds, and compositions for inhibiting pyruvate carboxylase expression.

Certain embodiments provide methods, compounds, and compositions for inhibiting hepatic pyruvate carboxylase expression.

Certain embodiments provide methods, compounds, and compositions for preferentially reducing hepatic pyruvate carboxylase expression.

Certain embodiments provide methods, compounds, and compositions for inhibiting pyruvate carboxylase expression without affecting pancreatic islet pyruvate carboxylase activity or without concomitant decrease in glucose stimulated insulin secretion (GSIS) in the pancreas or pancreatic islets. In certain embodiments, the compounds for use in the methods provided herein comprise a modified antisense oligonucleotide that activates a nuclear ribonuclease capable of cleaving pyruvate carboxylase.

Certain embodiments provide methods, compounds, and compositions for inhibiting hepatic pyruvate carboxylase expression without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to a reduction in glucose levels in the animal. In certain embodiments, the reduction in glucose levels in the animal is a reduction in fasting glucose levels. In certain embodiments, the reduction in glucose levels in the animal is a reduction in fasting plasma glucose levels. In certain

embodiments, glucose levels in the animal are reduced without affecting pancreatic or pancreatic islet pyruvate carboxylase activity or without concomitant decrease in glucose stimulated insulin secretion (GSIS) in the pancreas or pancreatic islets.

5 Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to a reduction in glucose levels in the animal. In certain embodiments, the reduction in glucose levels in the animal is a reduction in fasting glucose levels. In certain embodiments, the reduction in glucose levels in the animal is a
10 reduction in fasting plasma glucose levels. In certain embodiments, such reductions are achieved without affecting pancreatic or pancreatic islet pyruvate carboxylase activity or without concomitant decrease in glucose stimulated insulin secretion (GSIS) in the pancreas or pancreatic islets.

Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising
15 administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal leads to a reduction in glucose levels in the animal. In certain embodiments, the reduction in glucose levels in the animal is a reduction in fasting glucose levels. In certain embodiments, the reduction in glucose levels in the animal is a reduction in fasting plasma glucose levels.

20 Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing
25 segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal leads to a reduction in glucose levels in the animal. In certain embodiments, the reduction in glucose levels in the animal is a reduction in fasting glucose levels. In certain embodiments, the reduction in glucose levels in the animal is a
30 reduction in fasting plasma glucose levels.

Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to a reduction in insulin levels in the animal. In certain embodiments, the

reduction in insulin levels in the animal is a reduction in fasting insulin levels. In certain embodiments, such reductions are achieved without concomitant decrease in glucose stimulated insulin secretion (GSIS) in the pancreas or pancreatic islets.

Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to a reduction in insulin levels in the animal. In certain embodiments, the reduction in insulin levels in the animal is a reduction in fasting insulin levels.

Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal leads to a reduction in insulin levels in the animal. In certain embodiments, the reduction in insulin levels in the animal is a reduction in fasting insulin levels.

Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal leads to a reduction in insulin levels in the animal. In certain embodiments, the reduction in insulin levels in the animal is a reduction in fasting insulin levels.

Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to a reduction in body weight in the animal. In certain embodiments, a reduction in pyruvate carboxylase in an animal inhibits weight gain in the animal.

Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to a reduction in

body weight in the animal. In certain embodiments, a reduction in pyruvate carboxylase in an animal inhibits weight gain in the animal.

Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising
5 administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal leads to a reduction in body weight in the animal. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal inhibits weight gain in the animal.

Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase
10 expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing
15 segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal leads to a reduction in body weight in the animal. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal inhibits weight gain in the animal.

Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal
20 comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to a reduction in lipid levels in the animal. In certain embodiments, the reduction in lipid levels is a reduction in cholesterol, low density lipoproteins, very low density lipoproteins and triglycerides.

Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal
25 without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to a reduction in lipid levels in the animal. In certain embodiments, the reduction in lipid levels is a reduction in cholesterol,
30 low density lipoproteins, very low density lipoproteins and triglycerides.

Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic or pancreatic islet pyruvate carboxylase activity or without concomitant decrease in glucose stimulated insulin secretion (GSIS) in the pancreas or pancreatic islets comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30

linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal leads to a reduction in lipid levels in the animal. In certain embodiments, the reduction in lipid levels is a reduction in cholesterol, low density lipoproteins, very low density lipoproteins and triglycerides.

5 Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing
10 segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal leads to a reduction in lipid levels in the animal. In certain embodiments, the reduction in lipid levels is a reduction in cholesterol, low density lipoproteins, very low density lipoproteins and triglycerides.

15 Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to a reduction in triglyceride levels in the animal. In certain embodiments, the reduction in triglyceride levels in the animal is a reduction in hepatic triglyceride content.

20 Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to a reduction in triglyceride levels in the animal. In certain embodiments, the reduction in triglyceride levels in the animal is a
25 reduction in hepatic triglyceride content.

 Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in hepatic pyruvate
30 carboxylase in an animal leads to a reduction in triglyceride levels in the animal. In certain embodiments, the reduction in triglyceride levels in the animal is a reduction in hepatic triglyceride content.

 Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides

in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal leads to a reduction in triglyceride levels in the animal. In certain embodiments, the reduction in triglyceride levels in the animal is a reduction in hepatic triglyceride content.

Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to an increase in insulin sensitivity in the animal.

Certain embodiments provide a method of reducing pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in pyruvate carboxylase in an animal leads to an increase in insulin sensitivity in the animal.

Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal leads to an increase in insulin sensitivity in the animal.

Certain embodiments provide a method of preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, a reduction in hepatic pyruvate carboxylase in an animal leads to an increase in insulin sensitivity in the animal.

Certain embodiments provide a method of reducing triglyceride levels in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase, thereby reducing the level of triglyceride in the animal. In certain embodiments, the reduction in triglyceride levels is a reduction in hepatic triglyceride levels.

Certain embodiments provide a method of reducing triglyceride levels in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar, thereby reducing the level of triglyceride in the animal. In certain embodiments, the reduction in triglyceride levels is a reduction in hepatic triglyceride levels.

Certain embodiments provide a method of reducing insulin levels in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase, thereby reducing the level of insulin in the animal.

Certain embodiments provide a method of reducing insulin levels in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar, thereby reducing the level of insulin in the animal.

Certain embodiments provide a method of increasing fatty acid oxidation in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase, thereby increasing fatty acid oxidation in the animal.

Certain embodiments provide a method of increasing fatty acid oxidation in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar, thereby increasing fatty acid oxidation in the animal.

Certain embodiments provide a method of treating, preventing or ameliorating a metabolic disease in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase, thereby treating, preventing or ameliorating the a metabolic disease in the animal. In certain embodiments, the metabolic disease is diabetes. In certain embodiments, the metabolic disease is NAFLD, including but not limited to hepatic steatosis. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate

carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide a method of treating, preventing or ameliorating a metabolic disease in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar, thereby treating, preventing or ameliorating the a metabolic disease in the animal. In certain embodiments, the metabolic disease is diabetes. In certain embodiments, the metabolic disease is hepatic NAFLD, including but not limited to hepatic steatosis. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide a method for treating an animal with a pyruvate carboxylase related disease or condition comprising: a) identifying said animal with the pyruvate carboxylase related disease or condition, and b) administering to said animal a therapeutically effective amount of a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, the therapeutically effective amount of the compound administered to the animal reduces the pyruvate carboxylase related disease or condition in the animal. In certain embodiments, the modified oligonucleotide consists of 20 linked nucleosides. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, the nucleobase sequence is at least 80%, at least 85%, at least 90%, at least 95% at least 98% or 100% complementary to any of SEQ ID NOS: 1-5 as measured over the entirety of said modified oligonucleotide.

Certain embodiments provide a method for treating an animal with a pyruvate carboxylase related disease or condition comprising: a) identifying said animal with the pyruvate carboxylase related disease or condition, and b) administering to said animal a therapeutically effective amount of a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, the therapeutically effective amount

of the compound administered to the animal reduces the pyruvate carboxylase related disease or condition in the animal. In certain embodiments, the modified oligonucleotide consists of 20 linked nucleosides. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, the nucleobase sequence is at least 80%, at least 85%, at least 90%, at least 95% at least 98% or 100% complementary to any of SEQ ID NOS: 1-5 as measured over the entirety of said modified oligonucleotide.

In certain embodiments, the pyruvate carboxylase related disease or condition is a metabolic disease. In certain embodiments, the pyruvate carboxylase related disease is diabetes. In certain embodiments, the pyruvate carboxylase related disease is NAFLD, including but not limited to hepatic steatosis. In certain embodiments, reducing pyruvate carboxylase leads to a reduction in glucose levels. In certain embodiments, reducing pyruvate carboxylase leads to a reduction in fasting glucose levels. In certain embodiments, reducing pyruvate carboxylase leads to a reduction in plasma glucose levels. In certain embodiments, reducing pyruvate carboxylase leads to a reduction in insulin levels. In certain embodiments, reducing pyruvate carboxylase leads to a reduction in fasting insulin levels. In certain embodiments, reducing pyruvate carboxylase leads to a reduction in weight gain. In certain embodiments, reducing pyruvate carboxylase leads to a reduction in white adipose tissue. In certain embodiments, reducing pyruvate carboxylase leads to a reduction in triglyceride levels. In certain embodiments, reducing pyruvate carboxylase leads to a reduction in hepatic triglyceride levels. In certain embodiments, reducing pyruvate carboxylase leads to an increase in insulin sensitivity.

Certain embodiments provide a method of reducing one or more of hepatic pyruvate carboxylase levels, glucose levels, insulin levels, body weight, white adipose tissue, LDL levels, triglyceride levels, cholesterol levels, insulin resistance, metabolic disease in an animal by administering a pyruvate carboxylase inhibitor comprising a modified oligonucleotide consisting of 20 linked nucleosides and having a nucleobase sequence at least 90% complementary to SEQ ID NOS: 1-5 as measured over the entirety of said modified oligonucleotide. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, reduction in one or more of the above measures are achieved without affecting pancreatic or pancreatic islet pyruvate carboxylase activity or without concomitant decrease in glucose stimulated insulin secretion (GSIS) in the pancreas or pancreatic islets. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide a method of decreasing one or more of pyruvate carboxylase levels, glucose levels, insulin levels, body weight, white adipose tissue, LDL levels, triglyceride levels, cholesterol levels, insulin resistance, metabolic disease in an animal by administering a pyruvate carboxylase inhibitor

comprising a modified oligonucleotide consisting of 10 to 30 linked nucleosides having a nucleobase sequence at least 90% complementary to SEQ ID NOS: 1-5 as measured over the entirety of said modified oligonucleotide and wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide uses of the compounds and compositions described herein for reducing pyruvate carboxylase expression in an animal.

Certain embodiments provide uses of the compounds and compositions described herein for reducing pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide uses of the compounds and compositions described herein for preferentially reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide use of the compounds and compositions described herein for reducing one or more of pyruvate carboxylase levels, glucose levels, insulin levels, body weight, white adipose tissue, LDL levels, triglyceride levels, cholesterol levels, insulin resistance, or metabolic disease in an animal.

Certain embodiments include administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase, thereby reducing one or more of pyruvate carboxylase levels, glucose levels, insulin levels, body weight, white adipose tissue, LDL levels, triglyceride levels, cholesterol levels, insulin resistance, or metabolic disease in the animal. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide use of the compounds and compositions described herein for reducing one or more of pyruvate carboxylase levels, glucose levels, insulin levels, body weight, white adipose tissue, LDL levels, triglyceride levels, cholesterol levels, insulin resistance, or metabolic disease in an animal.

Certain embodiments include administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment

comprises a modified sugar, thereby reducing one or more of pyruvate carboxylase levels, glucose levels, insulin levels, body weight, white adipose tissue, LDL levels, triglyceride levels, cholesterol levels, insulin resistance, or metabolic disease in the animal. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide use of the compounds and compositions described herein for treating, preventing or ameliorating diabetes in an animal. Certain embodiments include administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase, thereby ameliorating diabetes in the animal. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide use of the compounds and compositions described herein for treating, preventing or ameliorating diabetes in an animal. Certain embodiments include administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar, thereby ameliorating diabetes in the animal. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide use of the compounds and compositions described herein for treating an animal with a pyruvate carboxylase related disease or condition. In certain embodiments, the pyruvate carboxylase related disease or condition is metabolic disease. Certain embodiments include: a) identifying said animal with the pyruvate carboxylase related disease or condition, and b) administering to said animal a therapeutically effective amount of a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, the therapeutically effective amount of the compound administered to the animal reduces the pyruvate carboxylase related disease or condition in the animal. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide use of the compounds and compositions described herein for treating an animal with a pyruvate carboxylase related disease or condition. In certain embodiments, the pyruvate carboxylase related disease or condition is metabolic disease. Certain embodiments include: a) identifying said animal with the pyruvate carboxylase related disease or condition, and b) administering to said animal a therapeutically effective amount of a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, the therapeutically effective amount of the compound administered to the animal reduces the pyruvate carboxylase related disease or condition in the animal. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide use of the compounds and compositions described herein for treating an animal with a pyruvate carboxylase related disease or condition. In certain embodiments, the pyruvate carboxylase related disease or condition is diabetes. Certain embodiments include: a) identifying said animal with the pyruvate carboxylase related disease or condition, and b) administering to said animal a therapeutically effective amount of a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase. In certain embodiments, the therapeutically effective amount of the compound administered to the animal reduces the pyruvate carboxylase related disease or condition in the animal. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

Certain embodiments provide use of the compounds and compositions described herein for treating an animal with a pyruvate carboxylase related disease or condition. In certain embodiments, the pyruvate carboxylase related disease or condition is diabetes. Certain embodiments include: a) identifying said animal with the pyruvate carboxylase related disease or condition, and b) administering to said animal a therapeutically effective amount of a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length targeted to pyruvate carboxylase wherein modified oligonucleotide comprises: a gap segment consisting of linked deoxynucleosides; a 5' wing segment consisting of linked nucleosides; a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, the therapeutically effective amount of the compound administered to the

animal reduces the pyruvate carboxylase related disease or condition in the animal. In certain embodiments, pyruvate carboxylase expression is reduced without affecting pancreatic islet pyruvate carboxylase activity. In certain embodiments, hepatic pyruvate carboxylase expression is preferentially reduced without affecting pancreatic islet pyruvate carboxylase activity.

5 In certain embodiments, the pyruvate carboxylase nucleic acid is any of the sequences set forth in GENBANK Accession No. NM_000920.3 (incorporated herein as SEQ ID NO: 1), the complement of GENBANK Accession No. NT_167190.1 truncated from nucleosides 11920000 to 12033000 (incorporated herein as SEQ ID NO: 2); GENBANK Accession No. NM_001040716.1 (incorporated herein as SEQ ID NO: 3); GENBANK Accession No. NM_022172.2 (incorporated herein as SEQ ID NO: 4); and GENBANK
10 Accession No. NM_001162946.1 (incorporated herein as SEQ ID NO: 5).

In certain embodiments, the animal is a human.

In certain embodiments, the compounds or compositions are designated as a first agent and the methods further comprise administering a second agent. In certain embodiments, the first agent and the second agent are co-administered. In certain embodiments the first agent and the second agent are co-
15 administered sequentially or concomitantly.

In certain embodiments, second agents include, but are not limited to, a glucose-lowering agent. The glucose lowering agent can include, but is not limited to, a therapeutic lifestyle change, PPAR agonist, a dipeptidyl peptidase (IV) inhibitor, a GLP-1 analog, insulin or an insulin analog, an insulin secretagogue, a SGLT2 inhibitor, a human amylin analog, a biguanide, an alpha-glucosidase inhibitor, or a combination
20 thereof. The glucose-lowering agent can include, but is not limited to metformin, sulfonylurea, rosiglitazone, meglitinide, thiazolidinedione, alpha-glucosidase inhibitor or a combination thereof. The sulfonylurea can be acetohexamide, chlorpropamide, tolbutamide, tolazamide, glimepiride, a glipizide, a glyburide, or a gliclazide. The meglitinide can be nateglinide or repaglinide. The thiazolidinedione can be pioglitazone or rosiglitazone. The alpha-glucosidase can be acarbose or miglitol.

25 In some embodiments, the glucose-lowering therapeutic is a GLP-1 analog. In some embodiments, the GLP-1 analog is exendin-4 or liraglutide.

In other embodiments, the glucose-lowering therapeutic is a sulfonylurea. In some embodiments, the sulfonylurea is acetohexamide, chlorpropamide, tolbutamide, tolazamide, glimepiride, a glipizide, a glyburide, or a gliclazide.

30 In some embodiments, the glucose-lowering drug is a biguanide. In some embodiments, the biguanide is metformin, and in some embodiments, blood glucose levels are decreased without increased lactic acidosis as compared to the lactic acidosis observed after treatment with metformin alone.

In some embodiments, the glucose-lowering drug is a meglitinide. In some embodiments, the meglitinide is nateglinide or repaglinide.

In some embodiments, the glucose-lowering drug is a thiazolidinedione. In some embodiments, the thiazolidinedione is pioglitazone, rosiglitazone, or troglitazone. In some embodiments, blood glucose levels are decreased without greater weight gain than observed with rosiglitazone treatment alone.

In some embodiments, the glucose-lowering drug is an alpha-glucosidase inhibitor. In some embodiments, the alpha-glucosidase inhibitor is acarbose or miglitol.

In a certain embodiment, a co-administered glucose-lowering agent is ISIS 113715.

In a certain embodiment, glucose-lowering therapy is therapeutic lifestyle change.

In certain embodiments, the second agent is a lipid-lowering therapy. In certain embodiments the lipid lowering therapy can include, but is not limited to, a therapeutic lifestyle change, HMG-CoA reductase inhibitor, triglyceride lowering agent, cholesterol absorption inhibitor, MTP inhibitor, antisense compound targeted to ApoB or any combination thereof. The HMG-CoA reductase inhibitor can be atorvastatin, rosuvastatin, fluvastatin, lovastatin, pravastatin, or simvastatin. The cholesterol absorption inhibitor can be ezetimibe. The triglyceride lowering agent can be a fibrate, niacin or fish oil.

In certain such embodiments, the lipid-lowering agent is administered prior to administration of a pharmaceutical composition described herein. In certain such embodiments, the lipid-lowering agent is administered following administration of a pharmaceutical composition described herein. In certain such embodiments the lipid-lowering agent is administered at the same time as a pharmaceutical composition described herein. In certain such embodiments the dose of a co-administered lipid-lowering agent is the same as the dose that would be administered if the lipid-lowering agent was administered alone. In certain such embodiments the dose of a co-administered lipid-lowering agent is lower than the dose that would be administered if the lipid-lowering agent was administered alone. In certain such embodiments the dose of a co-administered lipid-lowering agent is greater than the dose that would be administered if the lipid-lowering agent was administered alone.

In certain embodiments, a co-administered lipid-lowering agent is a cholesterol absorption inhibitor. In certain such embodiments, cholesterol absorption inhibitor is ezetimibe.

In certain embodiments, a co-administered lipid-lowering agent is a co-formulated HMG-CoA reductase inhibitor and cholesterol absorption inhibitor. In certain such embodiments the co-formulated lipid-lowering agent is ezetimibe/simvastatin.

In certain embodiments, a co-administered lipid-lowering agent is a microsomal triglyceride transfer protein inhibitor (MTP inhibitor).

In certain embodiments, a co-administered lipid-lowering agent is an oligonucleotide targeted to ApoB.

In certain embodiments, second agents include, but are not limited to an anti-obesity drug or agent. Such anti-obesity agents include but are not limited to Orlistat, Sibutramine, or Rimonabant, and may be administered as described above as adipose or body weight lowering agents. In certain embodiments, the antisense compound may be co-administered with appetite suppressants. Such appetite suppressants include but are not limited to diethylpropion, fenproporex, mazindol, orlistat, phendimetrazine, phentermine, and sibutramine and may be administered as described herein. In certain embodiment, the anti-obesity agents are CNS based such as, but not limited to, sibutramine or GLP-1 based such as, but not limited to, liraglutide.

In certain embodiments, administration comprises parenteral administration.

In certain embodiments, the metabolic disease includes, but is not limited to, obesity, diabetes, hyperglycemia, prediabetes, non-alcoholic fatty liver disease (NAFLD), metabolic syndrome, insulin resistance, diabetic dyslipidemia, hypertriglyceridemia or a combination thereof. The diabetic dyslipidemia can be hyperlipidemia. The NAFLD can be hepatic steatosis or steatohepatitis.

In certain embodiments, administering the compound to an animal results in a reduction of glucose levels, insulin levels, body weight, white adipose tissue, triglyceride levels, or a combination thereof. One or more of the levels can be independently reduced by at least 5%, 10%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%.

Certain embodiments provide the use of a compound as described herein in the manufacture of a medicament for treating, ameliorating, delaying or preventing a metabolic disease.

Certain embodiments provide the use of a compound as described herein in the manufacture of a medicament for treating, ameliorating, delaying or preventing diabetes.

Certain embodiments provide a kit for treating, preventing, or ameliorating a metabolic disease as described herein wherein the kit comprises: a) a compound as described herein; and optionally b) an additional agent or therapy as described herein. The kit can further include instructions or a label for using the kit to treat, prevent, or ameliorate a metabolic disease.

Certain embodiments provide a kit for treating, preventing, or ameliorating diabetes as described herein wherein the kit comprises: a) a compound as described herein; and optionally b) an additional agent or therapy as described herein. The kit can further include instructions or a label for using the kit to treat, prevent, or ameliorate diabetes.

Antisense Compounds

Oligomeric compounds include, but are not limited to, oligonucleotides, oligonucleosides, oligonucleotide analogs, oligonucleotide mimetics, antisense compounds, antisense oligonucleotides, and

siRNAs. An oligomeric compound may be “antisense” to a target nucleic acid, meaning that is capable of undergoing hybridization to a target nucleic acid through hydrogen bonding.

In certain embodiments, an antisense compound has a nucleobase sequence that, when written in the 5' to 3' direction, comprises the reverse complement of the target segment of a target nucleic acid to which it is targeted. In certain such embodiments, an antisense oligonucleotide has a nucleobase sequence that, when written in the 5' to 3' direction, comprises the reverse complement of the target segment of a target nucleic acid to which it is targeted.

In certain embodiments, an antisense compound targeted to a pyruvate carboxylase nucleic acid is 10 to 30 nucleotides in length. In other words, antisense compounds are from 10 to 30 linked nucleobases. In other embodiments, the antisense compound comprises a modified oligonucleotide consisting of 8 to 80, 10 to 50, 15 to 30, 18 to 21, 20 to 80, 20 to 35, 20 to 30, 20 to 29, 20 to 28, 20 to 27, 20 to 26, 20 to 25, 20 to 24, 20 to 23, 20 to 22, 20 to 21 or 20 linked nucleobases. In certain such embodiments, the antisense compound comprises a modified oligonucleotide consisting of 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, or 80 linked nucleobases in length, or a range defined by any two of the above values.

In certain embodiments, the antisense compound comprises a shortened or truncated modified oligonucleotide. The shortened or truncated modified oligonucleotide can have a single nucleoside deleted from the 5' end (5' truncation), or alternatively from the 3' end (3' truncation). A shortened or truncated oligonucleotide may have two nucleosides deleted from the 5' end, or alternatively may have two subunits deleted from the 3' end. Alternatively, the deleted nucleosides may be dispersed throughout the modified oligonucleotide, for example, in an antisense compound having one nucleoside deleted from the 5' end and one nucleoside deleted from the 3' end.

When a single additional nucleoside is present in a lengthened oligonucleotide, the additional nucleoside may be located at the 5' or 3' end of the oligonucleotide. When two or more additional nucleosides are present, the added nucleosides may be adjacent to each other, for example, in an oligonucleotide having two nucleosides added to the 5' end (5' addition), or alternatively to the 3' end (3' addition), of the oligonucleotide. Alternatively, the added nucleoside may be dispersed throughout the antisense compound, for example, in an oligonucleotide having one nucleoside added to the 5' end and one subunit added to the 3' end.

It is possible to increase or decrease the length of an antisense compound, such as an antisense oligonucleotide, and/or introduce mismatch bases without eliminating activity. For example, in Woolf et al. (Proc. Natl. Acad. Sci. USA 89:7305-7309, 1992), a series of antisense oligonucleotides 13-25 nucleobases in length were tested for their ability to induce cleavage of a target RNA in an oocyte injection model.

Antisense oligonucleotides 25 nucleobases in length with 8 or 11 mismatch bases near the ends of the antisense oligonucleotides were able to direct specific cleavage of the target mRNA, albeit to a lesser extent than the antisense oligonucleotides that contained no mismatches. Similarly, target specific cleavage was achieved using 13 nucleobase antisense oligonucleotides, including those with 1 or 3 mismatches.

5 Gautschi et al (J. Natl. Cancer Inst. 93:463-471, March 2001) demonstrated the ability of an oligonucleotide having 100% complementarity to the bcl-2 mRNA and having 3 mismatches to the bcl-xL mRNA to reduce the expression of both bcl-2 and bcl-xL *in vitro* and *in vivo*. Furthermore, this oligonucleotide demonstrated potent anti-tumor activity *in vivo*.

10 Maher and Dolnick (Nuc. Acid. Res. 16:3341-3358, 1988) tested a series of tandem 14 nucleobase antisense oligonucleotides, and a 28 and 42 nucleobase antisense oligonucleotides comprised of the sequence of two or three of the tandem antisense oligonucleotides, respectively, for their ability to arrest translation of human DHFR in a rabbit reticulocyte assay. Each of the three 14 nucleobase antisense oligonucleotides alone was able to inhibit translation, albeit at a more modest level than the 28 or 42 nucleobase antisense oligonucleotides.

15 *Antisense Compound Motifs*

In certain embodiments, antisense compounds targeted to a pyruvate carboxylase nucleic acid have chemically modified subunits arranged in patterns, or motifs, to confer to the antisense compounds properties such as enhanced inhibitory activity, increased binding affinity for a target nucleic acid, or resistance to degradation by *in vivo* nucleases.

20 Chimeric antisense compounds typically contain at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, increased binding affinity for the target nucleic acid, and/or increased inhibitory activity. A second region of a chimeric antisense compound may optionally serve as a substrate for the cellular endonuclease RNase H, which cleaves the RNA strand of an RNA:DNA duplex.

25 Antisense compounds having a gapmer motif are considered chimeric antisense compounds. In a gapmer an internal region having a plurality of nucleotides that supports RNaseH cleavage is positioned between external regions having a plurality of nucleotides that are chemically distinct from the nucleosides of the internal region. In the case of an antisense oligonucleotide having a gapmer motif, the gap segment generally serves as the substrate for endonuclease cleavage, while the wing segments comprise modified
30 nucleosides. In certain embodiments, the regions of a gapmer are differentiated by the types of sugar moieties comprising each distinct region. The types of sugar moieties that are used to differentiate the regions of a gapmer may in some embodiments include β -D-ribonucleosides, β -D-deoxyribonucleosides, 2'-modified nucleosides (such 2'-modified nucleosides may include 2'-MOE and 2'-O-CH₃, among others), and bicyclic sugar modified nucleosides (such bicyclic sugar modified nucleosides may include those having a

constrained ethyl). In certain embodiments, wings may include several modified sugar moieties, including, for example 2'-MOE and constrained ethyl. In certain embodiments, wings may include several modified and unmodified sugar moieties. In certain embodiments, wings may include various combinations of 2'-MOE nucleosides, constrained ethyl nucleosides, and 2'-deoxynucleosides.

5 Each distinct region may comprise uniform sugar moieties, variant, or alternating sugar moieties. The wing-gap-wing motif is frequently described as "X-Y-Z", where "X" represents the length of the 5'-wing, "Y" represents the length of the gap, and "Z" represents the length of the 3'-wing. "X" and "Z" may comprise uniform, variant, or alternating sugar moieties. In certain embodiments, "X" and "Y" may include one or more 2'-deoxynucleosides. "Y" may comprise 2'-deoxynucleosides. As used herein, a gapmer
10 described as "X-Y-Z" has a configuration such that the gap is positioned immediately adjacent to each of the 5'-wing and the 3' wing. Thus, no intervening nucleotides exist between the 5'-wing and gap, or the gap and the 3'-wing. Any of the antisense compounds described herein can have a gapmer motif. In certain embodiments, "X" and "Z" are the same, in other embodiments they are different. In certain embodiments, "Y" is between 8 and 15 nucleosides. X, Y, or Z can be any of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,
15 16, 17, 18, 19, 20, 25, 30 or more nucleosides.

In certain embodiments, antisense compounds targeted to a pyruvate carboxylase nucleic acid possess a 5-10-5 gapmer motif.

Target Nucleic Acids, Target Regions and Nucleotide Sequences

In certain embodiments, the pyruvate carboxylase nucleic acid is any of the sequences set forth in
20 GENBANK Accession No. NM_000920.3 (incorporated herein as SEQ ID NO: 1), the complement of GENBANK Accession No. NT_167190.1 truncated from nucleosides 11920000 to 12033000 (incorporated herein as SEQ ID NO: 2); GENBANK Accession No. NM_001040716.1 (incorporated herein as SEQ ID NO: 3); GENBANK Accession No. NM_022172.2 (incorporated herein as SEQ ID NO: 4); and GENBANK Accession No. NM_001162946.1 (incorporated herein as SEQ ID NO: 5).

25 It is understood that the sequence set forth in each SEQ ID NO in the Examples contained herein is independent of any modification to a sugar moiety, an internucleoside linkage, or a nucleobase. As such, antisense compounds defined by a SEQ ID NO may comprise, independently, one or more modifications to a sugar moiety, an internucleoside linkage, or a nucleobase. Antisense compounds described by Isis Number (Isis No) indicate a combination of nucleobase sequence and motif.

30 In certain embodiments, a target region is a structurally defined region of the target nucleic acid. For example, a target region may encompass a 3' UTR, a 5' UTR, an exon, an intron, an exon/intron junction, a coding region, a translation initiation region, translation termination region, or other defined nucleic acid region. The structurally defined regions for pyruvate carboxylase can be obtained by accession number from sequence databases such as NCBI and such information is incorporated herein by reference. In certain

embodiments, a target region may encompass the sequence from a 5' target site of one target segment within the target region to a 3' target site of another target segment within the same target region.

Targeting includes determination of at least one target segment to which an antisense compound hybridizes, such that a desired effect occurs. In certain embodiments, the desired effect is a reduction in mRNA target nucleic acid levels. In certain embodiments, the desired effect is reduction of levels of protein encoded by the target nucleic acid or a phenotypic change associated with the target nucleic acid.

A target region may contain one or more target segments. Multiple target segments within a target region may be overlapping. Alternatively, they may be non-overlapping. In certain embodiments, target segments within a target region are separated by no more than about 300 nucleotides. In certain embodiments, target segments within a target region are separated by a number of nucleotides that is, is about, is no more than, is no more than about, 250, 200, 150, 100, 90, 80, 70, 60, 50, 40, 30, 20, or 10 nucleotides on the target nucleic acid, or is a range defined by any two of the preceding values. In certain embodiments, target segments within a target region are separated by no more than, or no more than about, 5 nucleotides on the target nucleic acid. In certain embodiments, target segments are contiguous. Contemplated are target regions defined by a range having a starting nucleic acid that is any of the 5' target sites or 3' target sites listed herein.

Suitable target segments may be found within a 5' UTR, a coding region, a 3' UTR, an intron, an exon, or an exon/intron junction. Target segments containing a start codon or a stop codon are also suitable target segments. A suitable target segment may specifically exclude a certain structurally defined region such as the start codon or stop codon.

The determination of suitable target segments may include a comparison of the sequence of a target nucleic acid to other sequences throughout the genome. For example, the BLAST algorithm may be used to identify regions of similarity amongst different nucleic acids. This comparison can prevent the selection of antisense compound sequences that may hybridize in a non-specific manner to sequences other than a selected target nucleic acid (i.e., non-target or off-target sequences).

There may be variation in activity (e.g., as defined by percent reduction of target nucleic acid levels) of the antisense compounds within an active target region. In certain embodiments, reductions in pyruvate carboxylase mRNA levels are indicative of inhibition of pyruvate carboxylase expression. Reductions in levels of a pyruvate carboxylase protein are also indicative of inhibition of target mRNA expression. Further, phenotypic changes are indicative of inhibition of pyruvate carboxylase expression. In certain embodiments, reduced glucose levels, reduced insulin levels, reduced triglyceride levels, reduced lipid levels, reduced white adipose tissue, and reduced body weight can be indicative of inhibition of pyruvate carboxylase expression. In certain embodiments, reduced glucose levels, reduced insulin levels, reduced triglyceride levels, reduced lipid levels, reduced white adipose tissue, and reduced body weight can be indicative of inhibition of pyruvate

carboxylase expression without affecting islet pyruvate carboxylase in the pancreas. In certain embodiments, reduced glucose levels, reduced insulin levels, reduced triglyceride levels, reduced lipid levels, reduced white adipose tissue, and reduced body weight can be indicative of preferential inhibition of hepatic pyruvate carboxylase expression without affecting pancreatic islet pyruvate carboxylase activity. In certain
5 embodiments, amelioration of symptoms associated with metabolic disease can be indicative of inhibition of pyruvate carboxylase expression. In certain embodiments, amelioration of symptoms associated with diabetes can be indicative of inhibition of pyruvate carboxylase expression. In certain embodiments, amelioration of symptoms associated with diabetes can be indicative of inhibition of pyruvate carboxylase expression without affecting islet pyruvate carboxylase activity. In certain embodiments, amelioration of symptoms associated
10 with diabetes can be indicative of preferential inhibition of hepatic pyruvate carboxylase expression without affecting islet pyruvate carboxylase activity. In certain embodiments, reduction of insulin resistance is indicative of inhibition of pyruvate carboxylase expression. In certain embodiments, increase of insulin sensitivity is indicative of inhibition of pyruvate carboxylase expression. In certain embodiments, increase of insulin sensitivity is indicative of inhibition of pyruvate carboxylase expression without affecting islet
15 pyruvate carboxylase activity. In certain embodiments, increase of insulin sensitivity is indicative of preferential inhibition of hepatic pyruvate carboxylase expression without affecting islet pyruvate carboxylase activity. In certain embodiments, reduction of diabetes biomarkers can be indicative of inhibition of pyruvate carboxylase expression. In certain embodiments, reduction of diabetes biomarkers can be indicative of preferential inhibition of hepatic pyruvate carboxylase expression without affecting islet
20 pyruvate carboxylase activity.

Hybridization

In some embodiments, hybridization occurs between an antisense compound disclosed herein and a pyruvate carboxylase nucleic acid. The most common mechanism of hybridization involves hydrogen
25 bonding (e.g., Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding) between complementary nucleobases of the nucleic acid molecules.

Hybridization can occur under varying conditions. Stringent conditions are sequence-dependent and are determined by the nature and composition of the nucleic acid molecules to be hybridized.

Methods of determining whether a sequence is specifically hybridizable to a target nucleic acid are
30 well known in the art. In certain embodiments, the antisense compounds provided herein are specifically hybridizable with a pyruvate carboxylase nucleic acid.

Complementarity

An antisense compound and a target nucleic acid are complementary to each other when a sufficient number of nucleobases of the antisense compound can hydrogen bond with the corresponding nucleobases of

the target nucleic acid, such that a desired effect will occur (e.g., antisense inhibition of a target nucleic acid, such as a pyruvate carboxylase nucleic acid).

An antisense compound may hybridize over one or more segments of a pyruvate carboxylase nucleic acid such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure, mismatch or hairpin structure).

In certain embodiments, the antisense compounds provided herein, or a specified portion thereof, are, or are at least, 70%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% complementary to a pyruvate carboxylase nucleic acid, a target region, target segment, or specified portion thereof. Percent complementarity of an antisense compound with a target nucleic acid can be determined using routine methods.

For example, an antisense compound in which 18 of 20 nucleobases of the antisense compound are complementary to a target region, and would therefore specifically hybridize, would represent 90 percent complementarity. In this example, the remaining non-complementary nucleobases may be clustered or interspersed with complementary nucleobases and need not be contiguous to each other or to complementary nucleobases. As such, an antisense compound which is 18 nucleobases in length having 4 (four) non-complementary nucleobases which are flanked by two regions of complete complementarity with the target nucleic acid would have 77.8% overall complementarity with the target nucleic acid and would thus fall within the scope of the present invention. Percent complementarity of an antisense compound with a region of a target nucleic acid can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., J. Mol. Biol., 1990, 215, 403 410; Zhang and Madden, Genome Res., 1997, 7, 649 656). Percent homology, sequence identity or complementarity, can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wis.), using default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482 489).

In certain embodiments, the antisense compounds provided herein, or specified portions thereof, are fully complementary (i.e. 100% complementary) to a target nucleic acid, or specified portion thereof. For example, antisense compound may be fully complementary to a pyruvate carboxylase nucleic acid, or a target region, or a target segment or target sequence thereof. As used herein, “fully complementary” means each nucleobase of an antisense compound is capable of precise base pairing with the corresponding nucleobases of a target nucleic acid. For example, a 20 nucleobase antisense compound is fully complementary to a target sequence that is 400 nucleobases long, so long as there is a corresponding 20 nucleobase portion of the target nucleic acid that is fully complementary to the antisense compound. Fully complementary can also be used in reference to a specified portion of the first and /or the second nucleic acid. For example, a 20 nucleobase portion of a 30 nucleobase antisense compound can be “fully complementary” to a target

sequence that is 400 nucleobases long. The 20 nucleobase portion of the 30 nucleobase oligonucleotide is fully complementary to the target sequence if the target sequence has a corresponding 20 nucleobase portion wherein each nucleobase is complementary to the 20 nucleobase portion of the antisense compound. At the same time, the entire 30 nucleobase antisense compound may or may not be fully complementary to the target sequence, depending on whether the remaining 10 nucleobases of the antisense compound are also complementary to the target sequence.

The location of a non-complementary nucleobase may be at the 5' end or 3' end of the antisense compound. Alternatively, the non-complementary nucleobase or nucleobases may be at an internal position of the antisense compound. When two or more non-complementary nucleobases are present, they may be contiguous (i.e. linked) or non-contiguous. In one embodiment, a non-complementary nucleobase is located in the wing segment of a gapmer antisense oligonucleotide.

In certain embodiments, antisense compounds that are, or are up to 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleobases in length comprise no more than 4, no more than 3, no more than 2, or no more than 1 non-complementary nucleobase(s) relative to a target nucleic acid, such as a pyruvate carboxylase nucleic acid, or specified portion thereof.

In certain embodiments, antisense compounds that are, or are up to 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleobases in length comprise no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 non-complementary nucleobase(s) relative to a target nucleic acid, such as a pyruvate carboxylase nucleic acid, or specified portion thereof.

The antisense compounds provided herein also include those which are complementary to a portion of a target nucleic acid. As used herein, "portion" refers to a defined number of contiguous (i.e. linked) nucleobases within a region or segment of a target nucleic acid. A "portion" can also refer to a defined number of contiguous nucleobases of an antisense compound. In certain embodiments, the antisense compounds, are complementary to at least an 8 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 12 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 13 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 14 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 15 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 16 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 17 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 18 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 19 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are

complementary to at least a 20 nucleobase portion of a target segment. Also contemplated are antisense compounds that are complementary to at least a 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more nucleobase portion of a target segment, or a range defined by any two of these values.

Identity

5 The antisense compounds provided herein may also have a defined percent identity to a particular nucleotide sequence, SEQ ID NO, or compound represented by a specific Isis number, or portion thereof. As used herein, an antisense compound is identical to the sequence disclosed herein if it has the same nucleobase pairing ability. For example, a RNA which contains uracil in place of thymidine in a disclosed DNA
10 sequence would be considered identical to the DNA sequence since both uracil and thymidine pair with adenine. Shortened and lengthened versions of the antisense compounds described herein as well as compounds having non-identical bases relative to the antisense compounds provided herein also are contemplated. The non-identical bases may be adjacent to each other or dispersed throughout the antisense compound. Percent identity of an antisense compound is calculated according to the number of bases that have identical base pairing relative to the sequence to which it is being compared.

15 In certain embodiments, the antisense compounds, or portions thereof, are at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to one or more of the antisense compounds or SEQ ID NOs, or a portion thereof, disclosed herein.

Modifications

20 A nucleoside is a base-sugar combination. The nucleobase (also known as base) portion of the nucleoside is normally a heterocyclic base moiety. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to the 2', 3' or 5' hydroxyl moiety of the sugar. Oligonucleotides are formed through the covalent linkage of adjacent nucleosides to one another, to form a linear polymeric oligonucleotide. Within the oligonucleotide structure, the phosphate groups are commonly
25 referred to as forming the internucleoside linkages of the oligonucleotide.

 Modifications to antisense compounds encompass substitutions or changes to internucleoside linkages, sugar moieties, or nucleobases. Modified antisense compounds are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target, increased stability in the presence of nucleases, or increased inhibitory activity.

30 Chemically modified nucleosides may also be employed to increase the binding affinity of a shortened or truncated antisense oligonucleotide for its target nucleic acid. Consequently, comparable results can often be obtained with shorter antisense compounds that have such chemically modified nucleosides.

Modified Internucleoside Linkages

The naturally occurring internucleoside linkage of RNA and DNA is a 3' to 5' phosphodiester linkage. Antisense compounds having one or more modified, i.e. non-naturally occurring, internucleoside linkages are often selected over antisense compounds having naturally occurring internucleoside linkages because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for target nucleic acids, and increased stability in the presence of nucleases.

Oligonucleotides having modified internucleoside linkages include internucleoside linkages that retain a phosphorus atom as well as internucleoside linkages that do not have a phosphorus atom. Representative phosphorus containing internucleoside linkages include, but are not limited to, phosphodiesters, phosphotriesters, methylphosphonates, phosphoramidate, and phosphorothioates. Methods of preparation of phosphorous-containing and non-phosphorous-containing linkages are well known.

In certain embodiments, antisense compounds targeted to a pyruvate carboxylase nucleic acid comprise one or more modified internucleoside linkages. In certain embodiments, the modified internucleoside linkages are phosphorothioate linkages. In certain embodiments, each internucleoside linkage of an antisense compound is a phosphorothioate internucleoside linkage.

Modified Sugar Moieties

Antisense compounds provided herein can optionally contain one or more nucleosides wherein the sugar group has been modified. Such sugar modified nucleosides may impart enhanced nuclease stability, increased binding affinity, or some other beneficial biological property to the antisense compounds. In certain embodiments, nucleosides comprise a chemically modified ribofuranose ring moiety. Examples of chemically modified ribofuranose rings include, without limitation, addition of substituent groups (including 5' and 2' substituent groups); bridging of non-geminal ring atoms to form bicyclic nucleic acids (BNA); replacement of the ribosyl ring oxygen atom with S, N(R), or C(R1)(R2) (R = H, C₁-C₁₂ alkyl or a protecting group); and combinations thereof. Examples of chemically modified sugars include, 2'-F-5'-methyl substituted nucleoside (*see*, PCT International Application WO 2008/101157, published on 8/21/08 for other disclosed 5', 2'-bis substituted nucleosides), replacement of the ribosyl ring oxygen atom with S with further substitution at the 2'-position (*see*, published U.S. Patent Application US2005/0130923, published on June 16, 2005), or, alternatively, 5'-substitution of a BNA (*see*, PCT International Application WO 2007/134181, published on 11/22/07, wherein LNA is substituted with, for example, a 5'-methyl or a 5'-vinyl group).

Examples of nucleosides having modified sugar moieties include, without limitation, nucleosides comprising 5'-vinyl, 5'-methyl (R or S), 4'-S, 2'-F, 2'-OCH₃, and 2'-O(CH₂)₂OCH₃ substituent groups. The substituent at the 2' position can also be selected from allyl, amino, azido, thio, O-allyl, O-C₁-C₁₀ alkyl, OCF₃, O(CH₂)₂SCH₃, O(CH₂)₂-O-N(R_m)(R_n), and O-CH₂-C(=O)-N(R_m)(R_n), where each R_m and R_n is, independently, H or substituted or unsubstituted C₁-C₁₀ alkyl.

As used herein, "bicyclic nucleosides" refer to modified nucleosides comprising a bicyclic sugar moiety. Examples of bicyclic nucleosides include, without limitation, nucleosides comprising a bridge between the 4' and the 2' ribosyl ring atoms. In certain embodiments, antisense compounds provided herein include one or more bicyclic nucleosides wherein the bridge comprises a 4' to 2' bicyclic nucleoside.

- 5 Examples of such 4' to 2' bicyclic nucleosides, include, but are not limited to, one of the formulae: 4'-(CH₂)-O-2' (LNA); 4'-(CH₂)-S-2'; 4'-(CH₂)₂-O-2' (ENA); 4'-CH(CH₃)-O-2' and 4'-CH(CH₂OCH₃)-O-2', and analogs thereof (*see*, U.S. Patent 7,399,845, issued on July 15, 2008); 4'-C(CH₃)(CH₃)-O-2', and analogs thereof (*see*, published PCT International Application WO2009/006478, published January 8, 2009); 4'-CH₂-N(OCH₃)-2', and analogs thereof (*see*, published PCT International Application WO2008/150729, published December 11, 10 2008); 4'-CH₂-O-N(CH₃)-2' (*see*, published U.S. Patent Application US2004/0171570, published September 2, 2004); 4'-CH₂-N(R)-O-2', wherein R is H, C₁-C₁₂ alkyl, or a protecting group (*see*, U.S. Patent 7,427,672, issued on September 23, 2008); 4'-CH₂-C(H)(CH₃)-2' (*see*, Chattopadhyaya, *et al.*, *J. Org. Chem.*, 2009, 74, 118-134); and 4'-CH₂-C(=CH₂)-2', and analogs thereof (*see*, published PCT International Application WO 2008/154401, published on December 8, 2008). Also see, for example: Singh *et al.*, *Chem. Commun.*, 1998, 15 4, 455-456; Koshkin *et al.*, *Tetrahedron*, 1998, 54, 3607-3630; Wahlestedt *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, 97, 5633-5638; Kumar *et al.*, *Bioorg. Med. Chem. Lett.*, 1998, 8, 2219-2222; Singh *et al.*, *J. Org. Chem.*, 1998, 63, 10035-10039; Srivastava *et al.*, *J. Am. Chem. Soc.*, 129(26) 8362-8379 (Jul. 4, 2007); Elayadi *et al.*, *Curr. Opinion Invens. Drugs*, 2001, 2, 558-561; Braasch *et al.*, *Chem. Biol.*, 2001, 8, 1-7; Orum *et al.*, *Curr. Opinion Mol. Ther.*, 2001, 3, 239-243; U.S. Patent Nos U.S. 6,670,461, 7,053,207, 20 6,268,490, 6,770,748, 6,794,499, 7,034,133, 6,525,191, 7,399,845; published PCT International applications WO 2004/106356, WO 94/14226, WO 2005/021570, and WO 2007/134181; U.S. Patent Publication Nos. US2004/0171570, US2007/0287831, and US2008/0039618; and U.S. Patent Serial Nos. 12/129,154, 60/989,574, 61/026,995, 61/026,998, 61/056,564, 61/086,231, 61/097,787, and 61/099,844; and PCT International Application Nos. PCT/US2008/064591, PCT/US2008/066154, and PCT/US2008/068922.
- 25 Each of the foregoing bicyclic nucleosides can be prepared having one or more stereochemical sugar configurations including for example α -L-ribofuranose and β -D-ribofuranose (*see* PCT international application PCT/DK98/00393, published on March 25, 1999 as WO 99/14226).

In certain embodiments, bicyclic sugar moieties of BNA nucleosides include, but are not limited to, compounds having at least one bridge between the 4' and the 2' position of the pentofuranosyl sugar moiety wherein such bridges independently comprises 1 or from 2 to 4 linked groups independently selected from -[C(R_a)(R_b)]_n-, -C(R_a)=C(R_b)-, -C(R_a)=N-, -C(=NR_a)-, -C(=O)-, -C(=S)-, -O-, -Si(R_a)₂-, -S(=O)_x-, and -N(R_a)-, 30 wherein:

x is 0, 1, or 2;

n is 1, 2, 3, or 4;

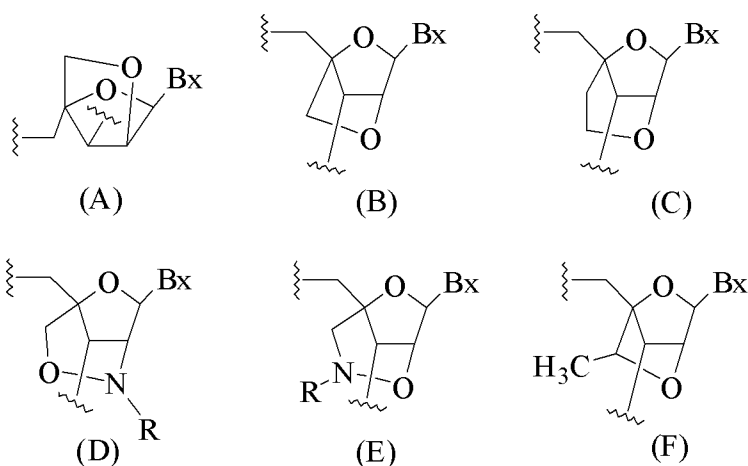
each R_a and R_b is, independently, H, a protecting group, hydroxyl, C_1 - C_{12} alkyl, substituted C_1 - C_{12} alkyl, C_2 - C_{12} alkenyl, substituted C_2 - C_{12} alkenyl, C_2 - C_{12} alkynyl, substituted C_2 - C_{12} alkynyl, C_5 - C_{20} aryl, substituted C_5 - C_{20} aryl, heterocycle radical, substituted heterocycle radical, heteroaryl, substituted heteroaryl, C_5 - C_7 alicyclic radical, substituted C_5 - C_7 alicyclic radical, halogen, OJ_1 , NJ_1J_2 , SJ_1 , N_3 , $COOJ_1$, acyl ($C(=O)$ -H), substituted acyl, CN, sulfonyl ($S(=O)_2J_1$), or sulfoxyl ($S(=O)J_1$); and

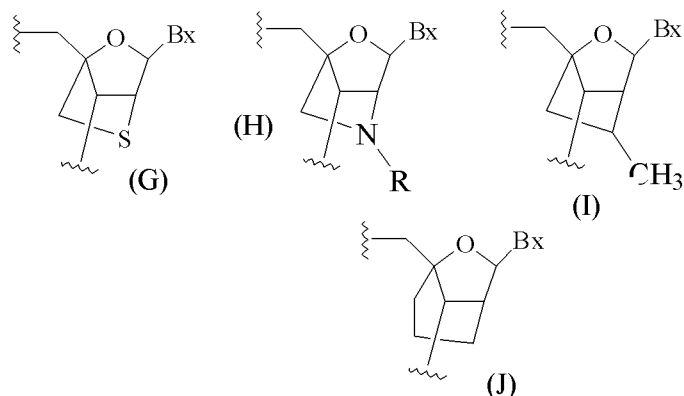
each J_1 and J_2 is, independently, H, C_1 - C_{12} alkyl, substituted C_1 - C_{12} alkyl, C_2 - C_{12} alkenyl, substituted C_2 - C_{12} alkenyl, C_2 - C_{12} alkynyl, substituted C_2 - C_{12} alkynyl, C_5 - C_{20} aryl, substituted C_5 - C_{20} aryl, acyl ($C(=O)$ -H), substituted acyl, a heterocycle radical, a substituted heterocycle radical, C_1 - C_{12} aminoalkyl, substituted C_1 - C_{12} aminoalkyl, or a protecting group.

In certain embodiments, the bridge of a bicyclic sugar moiety is, $-[C(R_a)(R_b)]_n-$, $-[C(R_a)(R_b)]_n-O-$, $-C(R_aR_b)-N(R)-O-$ or, $-C(R_aR_b)-O-N(R)-$. In certain embodiments, the bridge is 4'- CH_2 -2', 4'-(CH_2)₂-2', 4'-(CH_2)₃-2', 4'- CH_2 -O-2', 4'-(CH_2)₂-O-2', 4'- CH_2 -O-N(R)-2', and 4'- CH_2 -N(R)-O-2', wherein each R is, independently, H, a protecting group, or C_1 - C_{12} alkyl.

In certain embodiments, bicyclic nucleosides are further defined by isomeric configuration. For example, a nucleoside comprising a 4'-2' methylene-oxy bridge, may be in the α -L configuration or in the β -D configuration. Previously, α -L-methyleneoxy (4'- CH_2 -O-2') BNA's have been incorporated into antisense oligonucleotides that showed antisense activity (Frieden *et al.*, *Nucleic Acids Research*, 2003, 21, 6365-6372).

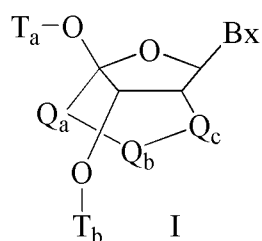
In certain embodiments, bicyclic nucleosides include, but are not limited to, (A) α -L-Methyleneoxy (4'- CH_2 -O-2') BNA, (B) β -D-Methyleneoxy (4'- CH_2 -O-2') BNA, (C) Ethyleneoxy (4'-(CH_2)₂-O-2') BNA, (D) Aminoxy (4'- CH_2 -O-N(R)-2') BNA, (E) Oxyamino (4'- CH_2 -N(R)-O-2') BNA, (F) Methyl(methyleneoxy) (4'- $CH(CH_3)$ -O-2') BNA, (G) methylene-thio (4'- CH_2 -S-2') BNA, (H) methylene-amino (4'- CH_2 -N(R)-2') BNA, (I) methyl carbocyclic (4'- CH_2 -CH(CH_3)-2') BNA, and (J) propylene carbocyclic (4'-(CH_2)₃-2') BNA as depicted below.





wherein Bx is the base moiety and R is, independently, H, a protecting group or C₁-C₁₂ alkyl.

5 In certain embodiments, bicyclic nucleoside having Formula I:



wherein:

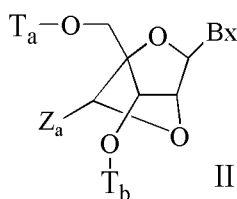
Bx is a heterocyclic base moiety;

10 -Q_a-Q_b-Q_c- is -CH₂-N(R_c)-CH₂-, -C(=O)-N(R_c)-CH₂-, -CH₂-O-N(R_c)-, -CH₂-N(R_c)-O-, or -N(R_c)-O-CH₂;

R_c is C₁-C₁₂ alkyl or an amino protecting group; and

T_a and T_b are each, independently, H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety, or a covalent attachment to a support medium.

15 In certain embodiments, bicyclic nucleoside having Formula II:



wherein:

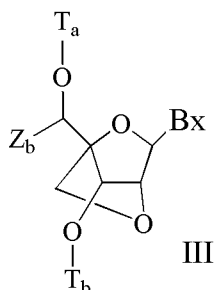
20 Bx is a heterocyclic base moiety;

T_a and T_b are each, independently, H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety, or a covalent attachment to a support medium;

Z_a is C_1 - C_6 alkyl, C_2 - C_6 alkenyl, C_2 - C_6 alkynyl, substituted C_1 - C_6 alkyl, substituted C_2 - C_6 alkenyl, substituted C_2 - C_6 alkynyl, acyl, substituted acyl, substituted amide, thiol, or substituted thio.

In one embodiment, each of the substituted groups is, independently, mono or poly substituted with substituent groups independently selected from halogen, oxo, hydroxyl, OJ_c , NJ_cJ_d , SJ_c , N_3 , $OC(=X)J_c$, and $NJ_cC(=X)NJ_cJ_d$, wherein each J_c , J_d , and J_e is, independently, H, C_1 - C_6 alkyl, or substituted C_1 - C_6 alkyl and X is O or NJ_c .

In certain embodiments, bicyclic nucleoside having Formula III:



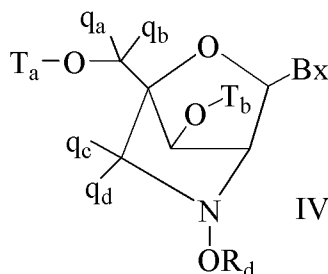
wherein:

Bx is a heterocyclic base moiety;

T_a and T_b are each, independently, H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety, or a covalent attachment to a support medium;

Z_b is C_1 - C_6 alkyl, C_2 - C_6 alkenyl, C_2 - C_6 alkynyl, substituted C_1 - C_6 alkyl, substituted C_2 - C_6 alkenyl, substituted C_2 - C_6 alkynyl, or substituted acyl ($C(=O)-$).

In certain embodiments, bicyclic nucleoside having Formula IV:



wherein:

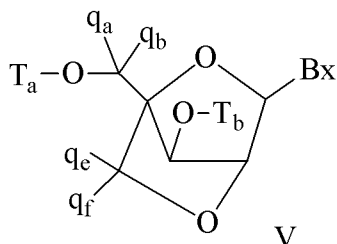
Bx is a heterocyclic base moiety;

T_a and T_b are each, independently, H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety, or a covalent attachment to a support medium;

R_d is C_1 - C_6 alkyl, substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, substituted C_2 - C_6 alkenyl, C_2 - C_6 alkynyl, or substituted C_2 - C_6 alkynyl;

each q_a , q_b , q_c and q_d is, independently, H, halogen, C_1 - C_6 alkyl, substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, substituted C_2 - C_6 alkenyl, C_2 - C_6 alkynyl, or substituted C_2 - C_6 alkynyl, C_1 - C_6 alkoxy, substituted C_1 - C_6 alkoxy, acyl, substituted acyl, C_1 - C_6 aminoalkyl, or substituted C_1 - C_6 aminoalkyl;

In certain embodiments, bicyclic nucleoside having Formula V:



wherein:

B_x is a heterocyclic base moiety;

T_a and T_b are each, independently, H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety, or a covalent attachment to a support medium;

q_a , q_b , q_c and q_f are each, independently, hydrogen, halogen, C_1 - C_{12} alkyl, substituted C_1 - C_{12} alkyl, C_2 - C_{12} alkenyl, substituted C_2 - C_{12} alkenyl, C_2 - C_{12} alkynyl, substituted C_2 - C_{12} alkynyl, C_1 - C_{12} alkoxy, substituted C_1 - C_{12} alkoxy, OJ_j , SJ_j , SOJ_j , SO_2J_j , NJ_jJ_k , N_3 , CN , $C(=O)OJ_j$, $C(=O)NJ_jJ_k$, $C(=O)J_j$, $O-C(=O)NJ_jJ_k$, $N(H)C(=NH)NJ_jJ_k$, $N(H)C(=O)NJ_jJ_k$ or $N(H)C(=S)NJ_jJ_k$;

or q_c and q_f together are $=C(q_g)(q_h)$;

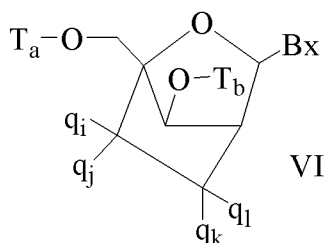
q_g and q_h are each, independently, H, halogen, C_1 - C_{12} alkyl, or substituted C_1 - C_{12} alkyl.

The synthesis and preparation of the methyleneoxy (4'- CH_2 -O-2') BNA monomers adenine, cytosine, guanine, 5-methyl-cytosine, thymine, and uracil, along with their oligomerization, and nucleic acid recognition properties have been described (*see, e.g., Koshkin et al., Tetrahedron*, 1998, 54, 3607-3630). BNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

Analogues of methyleneoxy (4'- CH_2 -O-2') BNA, methyleneoxy (4'- CH_2 -O-2') BNA, and 2'-thio-BNAs, have also been prepared (*see, e.g., Kumar et al., Bioorg. Med. Chem. Lett.*, 1998, 8, 2219-2222).

Preparation of locked nucleoside analogs comprising oligodeoxyribonucleotide duplexes as substrates for nucleic acid polymerases has also been described (*see, e.g., Wengel et al., WO 99/14226*). Furthermore, synthesis of 2'-amino-BNA, a novel conformationally restricted high-affinity oligonucleotide analog, has been described in the art (*see, e.g., Singh et al., J. Org. Chem.*, 1998, 63, 10035-10039). In addition, 2'-amino- and 2'-methylamino-BNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

In certain embodiments, bicyclic nucleoside having Formula VI:



wherein:

Bx is a heterocyclic base moiety;

Ta and Tb are each, independently, H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety, or a covalent attachment to a support medium;

each qi, qj, qk and qi is, independently, H, halogen, C₁-C₁₂ alkyl, substituted C₁-C₁₂ alkyl, C₂-C₁₂ alkenyl, substituted C₂-C₁₂ alkenyl, C₂-C₁₂ alkynyl, substituted C₂-C₁₂ alkynyl, C₁-C₁₂ alkoxy, substituted C₁-C₁₂ alkoxy, OJ_j, SJ_j, SO₂J_j, SO₂J_k, NJ_jJ_k, N₃, CN, C(=O)OJ_j, C(=O)NJ_jJ_k, C(=O)J_j, O-C(=O)NJ_jJ_k, N(H)C(=NH)NJ_jJ_k, N(H)C(=O)NJ_jJ_k, or N(H)C(=S)NJ_jJ_k; and

qi and qj or qi and qk together are =C(q_g)(q_h), wherein q_g and q_h are each, independently, H, halogen, C₁-C₁₂ alkyl, or substituted C₁-C₁₂ alkyl.

One carbocyclic bicyclic nucleoside having a 4'-(CH₂)₃-2' bridge and the alkenyl analog, bridge 4'-CH=CH-CH₂-2', have been described (*see, e.g., Freier et al., Nucleic Acids Research*, 1997, 25(22), 4429-4443 and Alback et al., *J. Org. Chem.*, 2006, 71, 7731-7740). The synthesis and preparation of carbocyclic bicyclic nucleosides along with their oligomerization and biochemical studies have also been described (*see, e.g., Srivastava et al., J. Am. Chem. Soc.* 2007, 129(26), 8362-8379).

As used herein, "4'-2' bicyclic nucleoside" or "4' to 2' bicyclic nucleoside" refers to a bicyclic nucleoside comprising a furanose ring comprising a bridge connecting the 2' carbon atom and the 4' carbon atom.

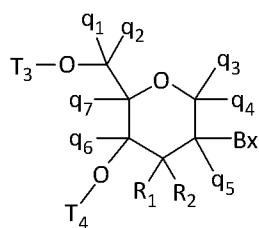
As used herein, "monocyclic nucleosides" refer to nucleosides comprising modified sugar moieties that are not bicyclic sugar moieties. In certain embodiments, the sugar moiety, or sugar moiety analogue, of a nucleoside may be modified or substituted at any position.

As used herein, "2'-modified sugar" means a furanosyl sugar modified at the 2' position. In certain embodiments, such modifications include substituents selected from: a halide, including, but not limited to substituted and unsubstituted alkoxy, substituted and unsubstituted thioalkyl, substituted and unsubstituted amino alkyl, substituted and unsubstituted alkyl, substituted and unsubstituted allyl, and substituted and unsubstituted alkynyl. In certain embodiments, 2' modifications are selected from substituents including, but not limited to: O[(CH₂)_nO]_mCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, OCH₂C(=O)N(H)CH₃, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. Other 2'-substituent groups can also be

selected from: C₁-C₁₂ alkyl; substituted alkyl; alkenyl; alkynyl; alkaryl; aralkyl; O-alkaryl or O-aralkyl; SH; SCH₃; OCN; Cl; Br; CN; CF₃; OCF₃; SOCH₃; SO₂CH₃; ONO₂; NO₂; N₃; NH₂; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving pharmacokinetic properties; and a group for improving the pharmacodynamic properties of an antisense compound, and other substituents having similar properties. In certain embodiments, modified nucleosides comprise a 2'-MOE side chain (*see, e.g.*, Baker et al., *J. Biol. Chem.*, 1997, 272, 11944-12000). Such 2'-MOE substitution have been described as having improved binding affinity compared to unmodified nucleosides and to other modified nucleosides, such as 2'-O-methyl, O-propyl, and O-aminopropyl. Oligonucleotides having the 2'-MOE substituent also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (*see, e.g.*, Martin, P., *Helv. Chim. Acta*, 1995, 78, 486-504; Altmann et al., *Chimia*, 1996, 50, 168-176; Altmann et al., *Biochem. Soc. Trans.*, 1996, 24, 630-637; and Altmann et al., *Nucleosides Nucleotides*, 1997, 16, 917-926).

As used herein, a "modified tetrahydropyran nucleoside" or "modified THP nucleoside" means a nucleoside having a six-membered tetrahydropyran "sugar" substituted in for the pentofuranosyl residue in normal nucleosides (a sugar surrogate). Modified THP nucleosides include, but are not limited to, what is referred to in the art as hexitol nucleic acid (HNA), anitol nucleic acid (ANA), manitol nucleic acid (MNA) (*see* Leumann, C.J. *Bioorg. & Med. Chem.* (2002) 10:841-854), fluoro HNA (F-HNA), or those compounds having Formula X:

Formula X:



X

wherein independently for each of said at least one tetrahydropyran nucleoside analog of Formula X:

Bx is a heterocyclic base moiety;

T₃ and T₄ are each, independently, an internucleoside linking group linking the tetrahydropyran nucleoside analog to the antisense compound or one of T₃ and T₄ is an internucleoside linking group linking the tetrahydropyran nucleoside analog to the antisense compound and the other of T₃ and T₄ is H, a hydroxyl protecting group, a linked conjugate group, or a 5' or 3'-terminal group;

q₁, q₂, q₃, q₄, q₅, q₆ and q₇ are each, independently, H, C₁-C₆ alkyl, substituted C₁-C₆ alkyl, C₂-C₆ alkenyl, substituted C₂-C₆ alkenyl, C₂-C₆ alkynyl, or substituted C₂-C₆ alkynyl; and

one of R_1 and R_2 is hydrogen and the other is selected from halogen, substituted or unsubstituted alkoxy, NJ_1J_2 , SJ_1 , N_3 , $OC(=X)J_1$, $OC(=X)NJ_1J_2$, $NJ_3C(=X)NJ_1J_2$, and CN , wherein X is O , S , or NJ_1 , and each J_1 , J_2 , and J_3 is, independently, H or C_1 - C_6 alkyl.

In certain embodiments, the modified THP nucleosides of Formula X are provided wherein q_m , q_n , q_p , q_r , q_s , q_t , and q_u are each H . In certain embodiments, at least one of q_m , q_n , q_p , q_r , q_s , q_t , and q_u is other than H . In certain embodiments, at least one of q_m , q_n , q_p , q_r , q_s , q_t , and q_u is methyl. In certain embodiments, THP nucleosides of Formula X are provided wherein one of R_1 and R_2 is F . In certain embodiments, R_1 is fluoro and R_2 is H , R_1 is methoxy and R_2 is H , and R_1 is methoxyethoxy and R_2 is H .

As used herein, "2'-modified" or "2'-substituted" refers to a nucleoside comprising a sugar comprising a substituent at the 2' position other than H or OH . 2'-modified nucleosides, include, but are not limited to, bicyclic nucleosides wherein the bridge connecting two carbon atoms of the sugar ring connects the 2' carbon and another carbon of the sugar ring and nucleosides with non-bridging 2' substituents, such as allyl, amino, azido, thio, O-allyl, O- C_1 - C_{10} alkyl, $-OCF_3$, O- $(CH_2)_2$ -O- CH_3 , 2'-O $(CH_2)_2$ S CH_3 , O- $(CH_2)_2$ -O- $N(R_m)(R_n)$, or O- CH_2 -C(=O)- $N(R_m)(R_n)$, where each R_m and R_n is, independently, H or substituted or unsubstituted C_1 - C_{10} alkyl. 2'-modified nucleosides may further comprise other modifications, for example, at other positions of the sugar and/or at the nucleobase.

As used herein, "2'-F" refers to a sugar comprising a fluoro group at the 2' position.

As used herein, "2'-OMe" or "2'-OCH₃" or "2'-O-methyl" each refers to a sugar comprising an -OCH₃ group at the 2' position of the sugar ring.

As used herein, "oligonucleotide" refers to a compound comprising a plurality of linked nucleosides. In certain embodiments, one or more of the plurality of nucleosides is modified. In certain embodiments, an oligonucleotide comprises one or more ribonucleosides (RNA) and/or deoxyribonucleosides (DNA).

Many other bicyclo and tricyclo sugar surrogate ring systems are also known in the art that can be used to modify nucleosides for incorporation into antisense compounds (*see, e.g.*, review article: Leumann, J. C, *Bioorganic & Medicinal Chemistry*, **2002**, 10, 841-854).

Such ring systems can undergo various additional substitutions to enhance activity.

Methods for the preparations of modified sugars are well known to those skilled in the art.

In nucleotides having modified sugar moieties, the nucleobase moieties (natural, modified, or a combination thereof) are maintained for hybridization with an appropriate nucleic acid target.

In certain embodiments, antisense compounds comprise one or more nucleotides having modified sugar moieties. In certain embodiments, the modified sugar moiety is 2'-MOE. In certain embodiments, the 2'-MOE modified nucleotides are arranged in a gapmer motif. In certain embodiments, the modified sugar moiety is a cEt. In certain embodiments, the cEt modified nucleotides are arranged throughout the wings of a gapmer motif.

Modified Nucleobases

Nucleobase (or base) modifications or substitutions are structurally distinguishable from, yet functionally interchangeable with, naturally occurring or synthetic unmodified nucleobases. Both natural and modified nucleobases are capable of participating in hydrogen bonding. Such nucleobase modifications may impart nuclease stability, binding affinity or some other beneficial biological property to antisense compounds. Modified nucleobases include synthetic and natural nucleobases such as, for example, 5-methylcytosine (5-me-C). Certain nucleobase substitutions, including 5-methylcytosine substitutions, are particularly useful for increasing the binding affinity of an antisense compound for a target nucleic acid. For example, 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278).

Additional unmodified nucleobases include 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ($-C\equiv C-CH_3$) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 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, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Heterocyclic base moieties may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Nucleobases that are particularly useful for increasing the binding affinity of antisense compounds include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine.

In certain embodiments, antisense compounds targeted to a pyruvate carboxylase nucleic acid comprise one or more modified nucleobases. In certain embodiments, gap-widened antisense oligonucleotides targeted to a pyruvate carboxylase nucleic acid comprise one or more modified nucleobases. In certain embodiments, the modified nucleobase is 5-methylcytosine. In certain embodiments, each cytosine is a 5-methylcytosine.

Compositions and Methods for Formulating Pharmaceutical Compositions

Antisense oligonucleotides may be admixed with pharmaceutically acceptable active or inert substance for the preparation of pharmaceutical compositions or formulations. Compositions and methods for

the formulation of pharmaceutical compositions are dependent upon a number of criteria, including, but not limited to, route of administration, extent of disease, or dose to be administered.

Antisense compound targeted to a pyruvate carboxylase nucleic acid can be utilized in pharmaceutical compositions by combining the antisense compound with a suitable pharmaceutically acceptable diluent or carrier. A pharmaceutically acceptable diluent includes phosphate-buffered saline (PBS). PBS is a diluent suitable for use in compositions to be delivered parenterally. Accordingly, in one embodiment, employed in the methods described herein is a pharmaceutical composition comprising an antisense compound targeted to a pyruvate carboxylase nucleic acid and a pharmaceutically acceptable diluent. In certain embodiments, the pharmaceutically acceptable diluent is PBS. In certain embodiments, the antisense compound is an antisense oligonucleotide.

Pharmaceutical compositions comprising antisense compounds encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other oligonucleotide which, upon administration to an animal, including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to pharmaceutically acceptable salts of antisense compounds, prodrugs, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents. Suitable pharmaceutically acceptable salts include, but are not limited to, sodium and potassium salts.

Pharmaceutically acceptable salts of the compounds described herein may be prepared by methods well-known in the art. For a review of pharmaceutically acceptable salts, see Stahl and Wermuth, Handbook of Pharmaceutical Salts: Properties, Selection and Use (Wiley-VCH, Weinheim, Germany, 2002). Sodium salts of antisense oligonucleotides are useful and are well accepted for therapeutic administration to humans. Accordingly, in one embodiment the compounds described herein are in the form of a sodium salt.

A prodrug can include the incorporation of additional nucleosides at one or both ends of an antisense compound which are cleaved by endogenous nucleases within the body, to form the active antisense compound.

Conjugated Antisense Compounds

Antisense compounds may be covalently linked to one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the resulting antisense oligonucleotides. Typical conjugate groups include cholesterol moieties and lipid moieties. Additional conjugate groups include carbohydrates, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes.

Antisense compounds can also be modified to have one or more stabilizing groups that are generally attached to one or both termini of antisense compounds to enhance properties such as, for example, nuclease stability. Included in stabilizing groups are cap structures. These terminal modifications protect the antisense

compound having terminal nucleic acid from exonuclease degradation, and can help in delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap), or at the 3'-terminus (3'-cap), or can be present on both termini. Cap structures are well known in the art and include, for example, inverted deoxy abasic caps. Further 3' and 5'-stabilizing groups that can be used to cap one or both ends of an antisense compound to impart nuclease stability include those disclosed in WO 03/004602 published on January 16, 2003.

Cell culture and antisense compounds treatment

The effects of antisense compounds on the level, activity or expression of pyruvate carboxylase nucleic acids can be tested *in vitro* in a variety of cell types. Cell types used for such analyses are available from commercial vendors (e.g. American Type Culture Collection, Manassus, VA; Zen-Bio, Inc., Research Triangle Park, NC; Clonetics Corporation, Walkersville, MD) and cells are cultured according to the vendor's instructions using commercially available reagents (e.g. Invitrogen Life Technologies, Carlsbad, CA). Illustrative cell types include, but are not limited to, HepG2 cells and primary hepatocytes.

In vitro testing of antisense oligonucleotides

Described herein are methods for treatment of cells with antisense oligonucleotides, which can be modified appropriately for treatment with other antisense compounds.

In general, cells are treated with antisense oligonucleotides when the cells reach approximately 60-80% confluence in culture.

One reagent commonly used to introduce antisense oligonucleotides into cultured cells includes the cationic lipid transfection reagent LIPOFECTIN® (Invitrogen, Carlsbad, CA). Antisense oligonucleotides are mixed with LIPOFECTIN® in OPTI-MEM® 1 (Invitrogen, Carlsbad, CA) to achieve the desired final concentration of antisense oligonucleotide and a LIPOFECTIN® concentration that typically ranges 2 to 12 ug/mL per 100 nM antisense oligonucleotide.

Another reagent used to introduce antisense oligonucleotides into cultured cells includes LIPOFECTAMINE 2000® (Invitrogen, Carlsbad, CA). Antisense oligonucleotide is mixed with LIPOFECTAMINE 2000® in OPTI-MEM® 1 reduced serum medium (Invitrogen, Carlsbad, CA) to achieve the desired concentration of antisense oligonucleotide and a LIPOFECTAMINE® concentration that typically ranges 2 to 12 ug/mL per 100 nM antisense oligonucleotide.

Another reagent used to introduce antisense oligonucleotides into cultured cells includes Cytofectin® (Invitrogen, Carlsbad, CA). Antisense oligonucleotide is mixed with Cytofectin® in OPTI-MEM® 1 reduced serum medium (Invitrogen, Carlsbad, CA) to achieve the desired concentration of antisense oligonucleotide and a Cytofectin® concentration that typically ranges 2 to 12 ug/mL per 100 nM antisense oligonucleotide.

Another technique used to introduce antisense oligonucleotides into cultured cells includes electroporation.

Cells are treated with antisense oligonucleotides by routine methods. Cells are typically harvested 16-24 hours after antisense oligonucleotide treatment, at which time RNA or protein levels of target nucleic acids are measured by methods known in the art and described herein. In general, when treatments are performed in multiple replicates, the data are presented as the average of the replicate treatments.

The concentration of antisense oligonucleotide used varies from cell line to cell line. Methods to determine the optimal antisense oligonucleotide concentration for a particular cell line are well known in the art. Antisense oligonucleotides are typically used at concentrations ranging from 1 nM to 300 nM when transfected with LIPOFECTAMINE2000®, Lipofectin or Cytofectin. Antisense oligonucleotides are used at higher concentrations ranging from 625 to 20,000 nM when transfected using electroporation.

RNA Isolation

RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are well known in the art. RNA is prepared using methods well known in the art, for example, using the TRIZOL® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocols.

Analysis of inhibition of target levels or expression

Inhibition of levels or expression of a pyruvate carboxylase nucleic acid can be assayed in a variety of ways known in the art. For example, target nucleic acid levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or quantitative real-time PCR. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are well known in the art. Northern blot analysis is also routine in the art. Quantitative real-time PCR can be conveniently accomplished using the commercially available ABI PRISM® 7600, 7700, or 7900 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Quantitative Real-Time PCR Analysis of Target RNA Levels

Quantitation of target RNA levels may be accomplished by quantitative real-time PCR using the ABI PRISM® 7600, 7700, or 7900 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Methods of quantitative real-time PCR are well known in the art.

Prior to real-time PCR, the isolated RNA is subjected to a reverse transcriptase (RT) reaction, which produces complementary DNA (cDNA) that is then used as the substrate for the real-time PCR amplification. The RT and real-time PCR reactions are performed sequentially in the same sample well. RT and real-time PCR reagents are obtained from Invitrogen (Carlsbad, CA). RT, real-time-PCR reactions are carried out by methods well known to those skilled in the art.

Gene (or RNA) target quantities obtained by real time PCR are normalized using either the expression level of a gene whose expression is constant, such as cyclophilin A, or by quantifying total RNA using RIBOGREEN® (Invitrogen, Inc. Carlsbad, CA). Cyclophilin A expression is quantified by real time PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RIBOGREEN® RNA quantification reagent (Invitrogen, Inc. Eugene, OR). Methods of RNA quantification by RIBOGREEN® are taught in Jones, L.J., et al, (Analytical Biochemistry, 1998, 265, 368-374). A CYTOFLUOR® 4000 instrument (PE Applied Biosystems) is used to measure RIBOGREEN® fluorescence.

Probes and primers are designed to hybridize to a pyruvate carboxylase nucleic acid. Methods for designing real-time PCR probes and primers are well known in the art, and may include the use of software such as PRIMER EXPRESS® Software (Applied Biosystems, Foster City, CA).

Analysis of Protein Levels

Antisense inhibition of pyruvate carboxylase nucleic acids can be assessed by measuring pyruvate carboxylase protein levels. Protein levels of pyruvate carboxylase can be evaluated or quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), enzyme-linked immunosorbent assay (ELISA), quantitative protein assays, protein activity assays (for example, caspase activity assays), immunohistochemistry, immunocytochemistry or fluorescence-activated cell sorting (FACS). Antibodies directed to a target can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional monoclonal or polyclonal antibody generation methods well known in the art. Antibodies useful for the detection of human and rat pyruvate carboxylase are commercially available.

In vivo testing of antisense compounds

Antisense compounds, for example, antisense oligonucleotides, are tested in animals to assess their ability to inhibit expression of pyruvate carboxylase and produce phenotypic changes. Testing may be performed in normal animals, or in experimental disease models. For administration to animals, antisense oligonucleotides are formulated in a pharmaceutically acceptable diluent, such as phosphate-buffered saline. Administration includes parenteral routes of administration. Following a period of treatment with antisense oligonucleotides, RNA is isolated from tissue and changes in pyruvate carboxylase nucleic acid expression are measured. Changes in pyruvate carboxylase protein levels are also measured.

Certain Indications

In certain embodiments, provided herein are methods of treating an individual comprising administering one or more pharmaceutical compositions as described herein. In certain embodiments, the individual has metabolic related disease.

As shown in the examples below, compounds targeted to pyruvate carboxylase, as described herein, have been shown to reduce the severity of physiological symptoms of metabolic related diseases, including metabolic syndrome, diabetes mellitus, insulin resistance, diabetic dyslipidemia, hypertriglyceridemia, obesity and weight gain. In certain of the experiments, the compounds reduced blood glucose levels, *e.g.*, the animals continued to experience symptoms, but the symptoms were less severe compared to untreated animals. In other of the experiments, however, the compounds appear to reduce the symptoms of diabetes; *e.g.*, animals treated for a longer period of time experienced less severe symptoms than those administered the compounds for a shorter period of time. In other of the experiments, however, the compounds appear to inhibit weight gain; *e.g.*, animals treated for a longer period of time experienced less severe symptoms than those administered the compounds for a shorter period of time. In other of the experiments, however, the compounds appear to inhibit hypertriglyceridemia; *e.g.*, animals treated for a longer period of time experienced less severe symptoms than those administered the compounds for a shorter period of time. The ability of the compounds exemplified below to restore function therefore demonstrates that symptoms of the disease may be reversed by treatment with a compound as described herein.

Diabetes mellitus is characterized by numerous physical and physiological symptoms. Any symptom known to one of skill in the art to be associated with Type 2 diabetes can be ameliorated or otherwise modulated as set forth above in the methods described above. In certain embodiments, the symptom is a physical symptom selected from the group consisting of increased glucose levels, increased weight gain, frequent urination, unusual thirst, extreme hunger, extreme fatigue, blurred vision, frequent infections, tingling or numbness at the extremities, dry and itchy skin, weight loss, slow-healing sores, and swollen gums.

In certain embodiments, the symptom is a physiological symptom selected from the group consisting of increased insulin resistance, increased glucose levels, increased fat mass, decreased metabolic rate, decreased glucose clearance, decreased glucose tolerance, decreased insulin sensitivity, decreased hepatic insulin sensitivity, increased adipose tissue size and weight, increased body fat, and increased body weight.

In certain embodiments, the physical symptom is increased weight gain. In certain embodiments, the symptom is frequent urination. In certain embodiments, the symptom is unusual thirst. In certain embodiments, the symptom is extreme hunger. In certain embodiments, the symptom is extreme fatigue. In certain embodiments, the symptom is blurred vision. In certain embodiments, the symptom is frequent infections. In certain embodiments, the symptom is tingling or numbness at the extremities. In certain embodiments, the symptom is dry and itchy skin. In certain embodiments, the symptom is weight loss. In certain embodiments, the symptom is slow-healing sores. In certain embodiments, the symptom is swollen gums. In certain embodiments, the symptom is increased insulin resistance. In certain embodiments, the symptom is increased fat mass. In certain embodiments, the symptom is decreased metabolic rate. In certain

embodiments, the symptom is decreased glucose clearance. In certain embodiments, the symptom is decreased glucose tolerance. In certain embodiments, the symptom is decreased insulin sensitivity. In certain embodiments, the symptom is decreased hepatic insulin sensitivity. In certain embodiments, the symptom is increased adipose tissue size and weight. In certain embodiments, the symptom is increased body fat. In certain embodiments, the symptom is increased body weight.

In certain embodiments, provided are methods of treating an individual comprising administering one or more pharmaceutical compositions as described herein. In certain embodiments, the individual has metabolic related disease.

In certain embodiments, administration of an antisense compound targeted to a pyruvate carboxylase nucleic acid results in reduction of pyruvate carboxylase expression by at least about 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99%, or a range defined by any two of these values.

In certain embodiments, pharmaceutical compositions comprising an antisense compound targeted to transthyretin are used for the preparation of a medicament for treating a patient suffering or susceptible to metabolic related disease.

Administration

In certain embodiments, the compounds and compositions as described herein may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical, pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal, oral or parenteral. The compounds and compositions as described herein can be administered directly to a tissue or organ.

In certain embodiments, the compounds and compositions as described herein are administered parenterally. "Parenteral administration" means administration through injection or infusion. Parenteral administration includes subcutaneous administration, intravenous administration, intramuscular administration, intraarterial administration, intraperitoneal administration, or intracranial administration, e.g. intracerebral administration, intrathecal administration, intraventricular administration, ventricular administration, intracerebroventricular administration, cerebral intraventricular administration or cerebral ventricular administration. Administration can be continuous, or chronic, or short or intermittent.

In certain embodiments, parenteral administration is by injection. The injection can be delivered with a syringe or a pump. In certain embodiments, the injection is a bolus injection. In certain embodiments, the injection is administered directly to a tissue or organ.

In certain embodiments, the compounds and compositions as described herein are administered parenterally.

In certain embodiments, parenteral administration is subcutaneous.

In further embodiments, the formulation for administration is the compounds described herein and saline.

In certain embodiments, an antisense oligonucleotide is delivered by injection or infusion once every month, every two months, every 90 days, every 3 months, every 6 months, twice a year or once a year.

5

Certain Combination Therapies

In certain embodiments, one or more pharmaceutical compositions described herein are co-administered with one or more other pharmaceutical agents. In certain embodiments, such one or more other pharmaceutical agents are designed to treat the same disease, disorder, or condition as the one or more pharmaceutical compositions described herein. In certain embodiments, such one or more other pharmaceutical agents are designed to treat a different disease, disorder, or condition as the one or more pharmaceutical compositions described herein. In certain embodiments, such one or more other pharmaceutical agents are designed to treat an undesired side effect of one or more pharmaceutical compositions as described herein. In certain embodiments, one or more pharmaceutical compositions are co-administered with another pharmaceutical agent to treat an undesired effect of that other pharmaceutical agent. In certain embodiments, one or more pharmaceutical compositions are co-administered with another pharmaceutical agent to produce a combinational effect. In certain embodiments, one or more pharmaceutical compositions are co-administered with another pharmaceutical agent to produce a synergistic effect.

In certain embodiments, a first agent and one or more second agents are administered at the same time. In certain embodiments, the first agent and one or more second agents are administered at different times. In certain embodiments, the first agent and one or more second agents are prepared together in a single pharmaceutical formulation. In certain embodiments, the first agent and one or more second agents are prepared separately.

In certain embodiments, the second compound is administered prior to administration of a pharmaceutical composition described herein. In certain embodiments, the second compound is administered following administration of a pharmaceutical composition described herein. In certain embodiments, the second compound is administered at the same time as a pharmaceutical composition described herein. In certain embodiments, the dose of a co-administered second compound is the same as the dose that would be administered if the second compound was administered alone. In certain embodiments, the dose of a co-administered second compound is lower than the dose that would be administered if the second compound was administered alone. In certain embodiments, the dose of a co-administered second compound is greater than the dose that would be administered if the second compound was administered alone.

In certain embodiments, the co-administration of a second compound enhances the effect of a first compound, such that co-administration of the compounds results in an effect that is greater than the effect of administering the first compound alone. In certain embodiments, the co-administration results in effects that are additive of the effects of the compounds when administered alone. In certain embodiments, the co-administration results in effects that are supra-additive of the effects of the compounds when administered alone. In certain embodiments, the first compound is an antisense compound. In certain embodiments, the second compound is an antisense compound.

In certain embodiments, second agents include, but are not limited to, a glucose-lowering agent. The glucose lowering agent can include, but is not limited to, a therapeutic lifestyle change, PPAR agonist, a dipeptidyl peptidase (IV) inhibitor, a GLP-1 analog, insulin or an insulin analog, an insulin secretagogue, a SGLT2 inhibitor, a human amylin analog, a biguanide, an alpha-glucosidase inhibitor, or a combination thereof. The glucose-lowering agent can include, but is not limited to metformin, sulfonylurea, rosiglitazone, meglitinide, thiazolidinedione, alpha-glucosidase inhibitor or a combination thereof. The sulfonylurea can be acetohexamide, chlorpropamide, tolbutamide, tolazamide, glimepiride, a glipizide, a glyburide, or a gliclazide. The meglitinide can be nateglinide or repaglinide. The thiazolidinedione can be pioglitazone or rosiglitazone. The alpha-glucosidase can be acarbose or miglitol.

In some embodiments, the glucose-lowering therapeutic is a GLP-1 analog. In some embodiments, the GLP-1 analog is exendin-4 or liraglutide.

In other embodiments, the glucose-lowering therapeutic is a sulfonylurea. In some embodiments, the sulfonylurea is acetohexamide, chlorpropamide, tolbutamide, tolazamide, glimepiride, a glipizide, a glyburide, or a gliclazide.

In some embodiments, the glucose-lowering drug is a biguanide. In some embodiments, the biguanide is metformin, and in some embodiments, blood glucose levels are decreased without increased lactic acidosis as compared to the lactic acidosis observed after treatment with metformin alone.

In some embodiments, the glucose-lowering drug is a meglitinide. In some embodiments, the meglitinide is nateglinide or repaglinide.

In some embodiments, the glucose-lowering drug is a thiazolidinedione. In some embodiments, the thiazolidinedione is pioglitazone, rosiglitazone, or troglitazone. In some embodiments, blood glucose levels are decreased without greater weight gain than observed with rosiglitazone treatment alone.

In some embodiments, the glucose-lowering drug is an alpha-glucosidase inhibitor. In some embodiments, the alpha-glucosidase inhibitor is acarbose or miglitol.

In a certain embodiment, a co-administered glucose-lowering agent is ISIS 113715.

In a certain embodiment, glucose-lowering therapy is therapeutic lifestyle change.

5 In certain embodiments, second agents include, but are not limited to, lipid-lowering agents. The lipid-lowering agent can include, but is not limited to atorvastatin, simvastatin, rosuvastatin, and ezetimibe. In certain such embodiments, the lipid-lowering agent is administered prior to administration of a pharmaceutical composition described herein. In certain such embodiments, the lipid-lowering agent is administered following administration of a pharmaceutical composition described herein. In certain such
10 embodiments the lipid-lowering agent is administered at the same time as a pharmaceutical composition described herein. In certain such embodiments the dose of a co-administered lipid-lowering agent is the same as the dose that would be administered if the lipid-lowering agent was administered alone. In certain such
15 embodiments the dose of a co-administered lipid-lowering agent is lower than the dose that would be administered if the lipid-lowering agent was administered alone. In certain such embodiments the dose of a co-administered lipid-lowering agent is greater than the dose that would be administered if the lipid-lowering agent was administered alone.

20 In certain embodiments, a co-administered lipid-lowering agent is a HMG-CoA reductase inhibitor. In certain such embodiments the HMG-CoA reductase inhibitor is a statin. In certain such embodiments the statin is selected from atorvastatin, simvastatin, pravastatin, fluvastatin, and rosuvastatin.

In certain embodiments, a co-administered lipid-lowering agent is a cholesterol absorption inhibitor. In certain such embodiments, cholesterol absorption inhibitor is ezetimibe.

25 In certain embodiments, a co-administered lipid-lowering agent is a co-formulated HMG-CoA reductase inhibitor and cholesterol absorption inhibitor. In certain such embodiments the co-formulated lipid-lowering agent is ezetimibe/simvastatin.

In certain embodiments, a co-administered lipid-lowering agent is a microsomal triglyceride transfer protein inhibitor (MTP inhibitor).

30 In certain embodiments, a co-administered lipid-lowering agent is an oligonucleotide targeted to ApoB.

In certain embodiments, second agents include, but are not limited to an anti-obesity drug or agent. Such anti-obesity agents include but are not limited to Orlistat, Sibutramine, or Rimonabant, and may be administered as described above as adipose or body weight lowering agents. In certain embodiments, the antisense compound may be co-administered with appetite suppressants. Such appetite suppressants include
35 but are not limited to diethylpropion, tenuate, mazindol, orlistat, phendimetrazine, phentermine, and sibutramine and may be administered as described herein. In certain embodiment, the anti-obesity agents are CNS based such as, but not limited to, sibutramine or GLP-1 based such as, but not limited to, liraglutide.

Formulations

The compounds provided herein may also be admixed, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor-targeted molecules, or other formulations, for assisting in uptake, distribution and/or absorption. Representative
5 United States patents that teach the preparation of such uptake, distribution and/or absorption-assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated
10 by reference.

The antisense compounds provided herein encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal, including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically
15 acceptable salts of the compounds provided herein: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. The term "pharmaceutically acceptable salt" includes a salt prepared from pharmaceutically acceptable non-toxic acids or bases, including inorganic or organic acids and bases. For oligonucleotides, preferred examples of pharmaceutically acceptable salts and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in
20 its entirety. Sodium salts have been shown to be suitable forms of oligonucleotide drugs.

The term "pharmaceutically acceptable derivative" encompasses, but is not limited to, pharmaceutically acceptable salts, solvates, hydrates, esters, prodrugs, polymorphs, isomers, isotopically labeled variants of the compounds described herein.

The present invention also includes pharmaceutical compositions and formulations which include the
25 antisense compounds provided herein. The pharmaceutical compositions described herein may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intracerebral administration, intrathecal administration, intraventricular administration, ventricular
30 administration, intracerebroventricular administration, cerebral intraventricular administration or cerebral ventricular administration.

Parenteral administration is preferred to target pyruvate carboxylase expression in the liver and plasma. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration. Pharmaceutical compositions and formulations for topical administration may

include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

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

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

Pharmaceutical compositions described herein include, but are not limited to, solutions, emulsions, foams and liposome-containing formulations. The pharmaceutical compositions and formulations described herein may comprise one or more penetration enhancers, carriers, excipients or other active or inactive ingredients.

Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in the aqueous phase, oily phase or itself as a separate phase. Microemulsions are included as an embodiment described herein. Emulsions and their uses are well known in the art and are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

Formulations include liposomal formulations. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers. Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior that contains the composition to be delivered. Cationic liposomes are positively charged liposomes which are believed to interact with negatively charged DNA molecules to form a stable complex. Liposomes that are pH-sensitive or negatively-charged are believed to entrap DNA rather than complex with it. Both cationic and noncationic liposomes have been used to deliver DNA to cells.

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in

enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Liposomes and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

In another embodiment, formulations include saline formulations. In certain embodiments, a formulation consists of the compounds described herein and saline. In certain embodiments, a formulation
5 consists essentially of the compounds described herein and saline. In certain embodiments, the saline is pharmaceutically acceptable grade saline. In certain embodiments, the saline is buffered saline. In certain embodiments, the saline is phosphate buffered saline (PBS).

In certain embodiments, a formulation excludes liposomes. In certain embodiments, the formulation excludes sterically stabilized liposomes. In certain embodiments, a formulation excludes phospholipids. In
10 certain embodiments, the formulation consists essentially of the compounds described herein and saline and excludes liposomes.

The pharmaceutical formulations and compositions may also include surfactants. Surfactants and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

In one embodiment, the present invention employs various penetration enhancers to affect the
15 efficient delivery of nucleic acids, particularly oligonucleotides. Penetration enhancers and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

One of skill in the art will recognize that formulations are routinely designed according to their intended use, i.e. route of administration.

Formulations for topical administration include those in which the oligonucleotides provided herein
20 are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA).

25 Compositions and formulations for parenteral administration, including intravenous, intraarterial, subcutaneous, intraperitoneal, intramuscular injection or infusion, or intracranial may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Certain embodiments provided herein provide pharmaceutical compositions containing one or more
30 oligomeric compounds and one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to cancer chemotherapeutic drugs such as daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone,

tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycytosine, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). When used with the compounds provided herein, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions provided herein. Combinations of antisense compounds and other non-antisense drugs are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions provided herein may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Alternatively, compositions provided herein may contain two or more antisense compounds targeted to different regions of the same nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

Dosing

The formulation of therapeutic compositions and their subsequent administration (dosing) is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50} s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μ g to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or at desired intervals. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μ g to 100 g per kg of body weight, once or more daily.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to

limit the same. Each of the references, GenBank accession numbers, and the like recited in the present application is incorporated herein by reference in its entirety.

EXAMPLES

Non-limiting disclosure and incorporation by reference

While certain compounds, compositions and methods described herein have been described with specificity in accordance with certain embodiments, the following examples serve only to illustrate the compounds described herein and are not intended to limit the same. Each of the references recited in the present application is incorporated herein by reference in its entirety.

Example 1: Antisense inhibition of murine pyruvate carboxylase mRNA in mouse primary hepatocytes

Antisense oligonucleotides targeted to a murine pyruvate carboxylase nucleic acid were tested for their effects on pyruvate carboxylase mRNA *in vitro*. Cultured mouse primary hepatocytes were transfected with 50 nM antisense oligonucleotide. After a treatment period of approximately 24 hours, RNA was isolated from the cells and mouse pyruvate carboxylase mRNA levels were measured by quantitative real-time PCR. Pyruvate carboxylase mRNA levels were adjusted according to total RNA content, as measured by RIBOGREEN.

ISIS 330749 (GCCAGACTTCATGGTAGCCG; SEQ ID NO: 6), which was one of the antisense oligonucleotides tested in the assay, was designed as a 5-10-5 MOE gapmer, and is 20 nucleosides in length, wherein the central gap segment is comprised of ten 2'-deoxynucleosides and is flanked on both sides (in the 5' and 3' directions) by wings comprising 5 nucleosides each. Each nucleoside in the 5' wing segment and each nucleoside in the 3' wing segment has a 2'-MOE modification. The internucleoside linkages throughout the gapmer are phosphorothioate (P=S) linkages. All cytosine residues throughout the gapmer are 5-methylcytosines. ISIS 330749 is targeted to nucleobases 2699 to 2718 of mouse pyruvate carboxylase (GENBANK Accession No. NM_001162946.1), incorporated herein as SEQ ID NO: 5. ISIS 330749 reduced murine pyruvate carboxylase mRNA expression by 85%.

Example 2: Effect of antisense inhibition of murine pyruvate carboxylase in rat primary hepatocytes

The effect of antisense inhibition of pyruvate carboxylase by ISIS 330749 on lipid oxidation in rat primary hepatocytes was evaluated.

Hepatocytes were isolated from rats and washed three times with recovery medium (4.5g/L glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 nM dexamethasone, 1 nM insulin, and 100 U/mL penicillin-streptomycin). Equal amount of cells (9×10^5) were incubated in the recovery medium in a 25 mL flask at 37°C in humidified 5% CO₂ environment for 4 hrs. The cells were then washed with PBS and incubated in 1.0 g/L glucose DMEM with 0.25% BSA and 0.25 μCi [1-¹⁴C]oleate (GE

Healthcare Biosciences, Piscataway, NJ) or 0.25 μCi [$1\text{-}^{14}\text{C}$]palmitate (Perkin Elmer, Inc., San Jose, CA) in sealed flasks containing a center well supplied with $1.5 \times 5.5 \text{ cm}^2$ of clean filter paper. After 1 hr, incubations were quenched with 300 μL of 30% perchloric acid. A quantity of 300 μL of 2M NaOH was added to the filter paper in the center well to collect $^{14}\text{CO}_2$. After 1 hr on ice to allow for quantitative trapping of [$^{14}\text{CO}_2$], the filter paper was put into 5 mL of scintillation fluid (Ultima Gold, Perkin Elmer, Inc., San Jose, CA) and the radioactivity was counted in a scintillation counter. The quenched incubation medium was transferred to centrifuge tubes and perchloric acid-soluble ^{14}C -radioactivity (representing ketone bodies, acyl-carnitine, and Kreb's cycle intermediates) was measured, as described previously (Nakae, J. et al., J. Clin. Invest. 108: 1359-1367, 2001). Protein assays were carried out from equally incubated flasks without fatty acids and counts were normalized to protein amounts. Though each incubation experiment was done in triplicate, the results were averaged to a single data point representing a single animal. The final data represents an n=5 per group. The data is presented in Table 1.

Table 1

Increase in fatty acid oxidation (%) in ISIS 330749-treated hepatocytes compared to the control

	%
Palmitate	4
Oleate	29

Example 3: *In vivo* effect of antisense inhibition of murine pyruvate carboxylase on plasma glucose and insulin levels in a Sprague Dawley rat model

Sprague-Dawley rats are a multipurpose model used for safety and efficacy evaluations. The rats were treated with ISIS antisense oligonucleotides from the study described in Example 1 and evaluated for changes in the levels of various metabolic markers.

Treatment

Sprague-Dawley rats were maintained on a 12-hour light/dark cycle and fed *ad libitum* with Purina normal rat chow, diet 5001. The rats were treated with either 75 mg/kg ISIS 330749 or with 75 mg/kg control oligonucleotide ISIS 141923 (CCTTCCCTGAAGGTTCTCC, 5-10-5 MOE gapmer with no known murine target; SEQ ID NO: 7) administered weekly for 4 weeks. After the treatment period, the rats from each group were further treated, as specified in Table 2. The rats either continued to be fed *ad libitum* or were fasted for a period of 24 hrs or 48 hrs.

Table 2

Rat groups

ISIS 141923 (N)	ISIS 330749-treated (N)
Ad lib (6)	Ad-lib (6)

Fasted 24 hrs (7)	Fasted 24 hrs (7)
Fasted 48 hrs (5)	Fasted 48 hrs (4)

Effect on fasted and fed glucose and insulin levels

After the treatment period, a single catheter was inserted into the right internal jugular vein, extending to the right atrium, and left carotid artery, extending into the aortic arch. The rats were given 1 week to recover from the surgery. Plasma glucose values were determined by using a glucose oxidase method (Beckman Glucose Analyzer II; Beckman Coulter). Plasma insulin concentrations were determined by a RIA Assay system (Linco). The results are presented in Tables 3 and 4. The data demonstrates that glucose levels were significantly reduced while plasma insulin levels remained relatively unchanged after 48 hours on treatment with ISIS 330749 in the fed and fasted states.

Table 3
Plasma glucose levels in Sprague-Dawley rats

	PBS	ISIS 330749
Ad-lib	146	132
24 hrs fasted	114	106
48 hrs fasted	107	97

Table 4
Plasma insulin levels in Sprague-Dawley rats

	PBS	ISIS 330749
Ad-lib	27	48
24 hrs fasted	13.4	8.9
48 hrs fasted	2.4	3.3

Example 4: *In vivo* effect of antisense inhibition of murine pyruvate carboxylase on glucose levels during a mixed meal tolerance test

To determine whether the alteration in plasma glucose and insulin levels were due to insulin secretion, an oral mixed meal tolerance test (MMTT) was performed. MMTT is a standardized test to assess pancreatic β -cell function (Greenbaum, C.J. et al., Diabetes Care. 31: 1966-1971, 2008).

Treatment

Sprague-Dawley rats were maintained on a 12-hour light/dark cycle and fed *ad libitum* with Purina normal rat chow, diet 5001. A group of rats was treated with 75 mg/kg ISIS 330749 administered weekly for

4 weeks. A group of rats were treated with control oligonucleotide 75 mg/kg ISIS 141923 administered weekly for 4 weeks.

Effect of mixed meal test on glucose and insulin levels

Rats were fasted overnight and then gavaged with 8 kcal/kg of liquid protein shake (SlimFast high protein shake mix). A select number of rats from each group were taken, as indicated in Tables 5 and 6, and plasma glucose and insulin levels were then measured at 0 min, 10 min, 20 min, 30 min, 60 min, 90 min, and 120 min. The results are presented in Tables 5 and 6, expressed in mg/dL.

Table 5
Plasma glucose levels (mg/dL) in MMTT

Time (min)	ISIS 141923 (N= 7)	ISIS 330749 (N= 9-10)
0	109	103
10	192	185
20	182	184
30	155	150
60	143	139
90	130	120
120	127	115

Table 6
Plasma insulin levels (mg/dL) in MMTT

Time (min)	ISIS 141923 (N = 6-8)	ISIS 330749 (N = 7-10)
0	7	6
10	103	118
20	100	147
30	56	56
60	13	9
90	11	11
120	11	10

Example 5: *In vivo* effect of antisense inhibition of murine pyruvate carboxylase in high fat diet-fed rats

The effect of antisense inhibition of pyruvate carboxylase by ISIS 330749 on Sprague-Dawley rats fed a high fat diet was evaluated.

Treatment

The rats (300-350 g) were maintained on a 12-hour light/dark cycle and fed *ad libitum* with a high-fat diet (60% fat, 20% protein, and 20% carbohydrates, Research Diets, New Brunswick, NJ). A group of rats was treated with 75 mg/kg ISIS 330749 administered weekly for 4 weeks. A group of rats were treated with control oligonucleotide 75 mg/kg ISIS 141923 administered weekly for 4 weeks.

5 *Effect on basal glucose production*

Rats were fasted overnight and then injected a primed continuous infusion of 6,6-²H glucose (0.3 mg/kg/min) for 120 min to measure basal glucose turnover. Samples were taken to evaluate basal glucose production. The results are presented in Table 7 and indicate that treatment with ISIS 330749 decreased the basal glucose production.

Table 7

Basal glucose production in high fat-fed rats

ISIS No	mg/kg-min
141923	7.9
330749	6.3

Effect on insulin sensitivity

The rats were then were fasted overnight. Hyperinsulinemic-euglycemic clamp studies were conducted for 100 min with a primed/continuous infusion of insulin (50 mU/kg primed over 10 min and 4 mU/kg per min constant infusion) (Novo Nordisk) and a variable infusion of 20% dextrose spiked with 2.5%[6,6-²H]glucose to maintain euglycemia. Once the rats maintained euglycemia for 100 min, the rats received a bolus of 30 μ Ci of 2-deoxy-D-[1-¹⁴C] glucose to measure the rate of insulin-stimulated tissue glucose uptake in skeletal muscle and adipose tissue. After the completion of the clamp, sodium pentobarbital was injected via the venous catheter administered at 150 mg/kg. After the rats were completely anesthetized, tissues were extracted for further analysis.

The HOMA-IR or homeostatic model assessment –insulin resistance was calculated based on the values obtained from the clamp test. The HOMA-IR is calculated as (glucose (mg/dL) x insulin) / 405. The results are presented in Table 8 and demonstrate that treatment with ISIS 330749 significantly increased insulin sensitivity, since the HOMA-IR for rats treated with ISIS 330749 is lower than that in the control group. Also, the rate of glucose infusion (GINF) and insulin-stimulated glucose disposal rate required to maintain euglycemia during the clamp was higher in the rat group treated with ISIS 330749 compared to that in the control group. Finally, the suppression of endogenous glucose production was higher in rats treated with ISIS 330749 compared to the control, indicating that antisense inhibition of Pyruvate Carboxylase significantly increased insulin sensitivity in the high fat-fed model.

Table 8
Hyperinsulinemic-euglycemic clamp study

	ISIS 330749	ISIS 141923
Fasted body weight (g)	355	381
White adipose tissue weight	3.4	4.8
Fasting glucose (mg/dL)	108	125
Fasting insulin (mg/dL)	8.5	12.9
HOMA-IR	2.3	4.0
Glucose infusion (GINF) (mg/kg/min)	25.0	22.8
Insulin-stimulated whole body glucose disposal	26.7	25.2
Clamp endogenous glucose production	1.3	2.4
% suppression of endogenous glucose production	80	69

5 **Example 6: *In vivo* effect of antisense inhibition of murine pyruvate carboxylase in high fat diet-fed rats**

The effect of antisense inhibition of pyruvate carboxylase by ISIS 330749 on Sprague-Dawley rats fed either normal rat diet or a high fat (HF) diet was evaluated.

Treatment

10 Male Sprague-Dawley rats (160-180 g) were obtained from Charles River Laboratories (Wilmington, MA) and given at least 3 days to acclimate. The rats were housed on a 12: 12 hr light-dark cycle and received food and water ad libitum. Chow consisted of regular rodent chow (60% carbohydrate, 10% fat, 30% protein calories), or a high fat diet (Dyets 112245: 26% carbohydrate, 59% fat, 15% protein calories). A group of rats was injected intraperitoneally with 75 mg/kg ISIS 330749 administered weekly for 4 weeks. A group of rats
15 were injected intraperitoneally with control oligonucleotide 75 mg/kg ISIS 141923 administered weekly for 4 weeks.

RNA analysis

Total RNA was extracted from ~15 mg liver, using RNeasy mini kit (Qiagen, Valencia, CA). RNA was reverse-transcribed into cDNA with the use of M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA), followed by real-time PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with a SYBR Green detection system (Stratagene, La Jolla, CA). The primer sequences used for measuring rat pyruvate carboxylase mRNA were forward sequence: AGATGCACTTCCATCCCAAG (SEQ ID NO: 8) and reverse sequence CCTTGGTCACGTGAACCTTT (SEQ ID NO: 9). The expression data
20 for each gene of interest was normalized with TATA box binding protein mRNA with forward primer

sequence GTGCCTCAGGCCAGACCCCA (SEQ ID NO: 10) and reverse primer sequence AGAGGCTGGTGTGGCAGGAGT (SEQ ID NO: 11).

Treatment with ISIS 330749 decreased hepatic and adipose pyruvate carboxylase mRNA expressions by 80-90% in both normal chow-fed and high fat diet-fed (HFF) rats. The data is presented in Table 9, expressed relative to the expression measured in the rats fed normal chow and treated with the control oligonucleotide. Data were compared using Student's unpaired t test between the two groups. '***' indicates $P < 0.01$; '****' indicates $P < 0.001$.

Table 9
Percent inhibition of Pyruvate Carboxylase mRNA expression by ISIS 330749

	Liver	Adipose tissue
Normal diet	75**	88****
HFF diet	92****	92****

Protein analysis

Tissues were homogenized in 1 mL ice-cold homogenization buffer (20 mM Tris;HCl, pH 7.4; 5 mM EDTA; 0.25 mM EGTA; 10 mM $\text{Na}_4\text{P}_2\text{O}_7$; 1% NP-40; 1 mM PMSF; 10 $\mu\text{g}/\text{mL}$ aprotinin) with a Dounce homogenizer. The homogenate was centrifuged at 12,000 rcf at 4°C for 30 min. The supernatant was removed into new tubes and protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA). Equal amounts of protein (50 μg) were resolved by SDS-PAGE using gradient gel with 15 wells, and electroblotted onto polyvinylidene difluoride membrane (DuPont, Boston, MA) using a wet-transfer cell (Bio-Rad, Hercules, CA). The membrane was then blocked for 60 min at room temperature in PBS-Tween (10 mmol/liter NaH_2PO_4 ; 80 mmol/ liter Na_2HPO_4 ; 0.145 mol/ liter NaCl; 0.1% Tween-20, pH 7.4), containing 5% (w/v) non-fat dried milk, and then incubated overnight with primary antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Antibodies were diluted 1:1000 in rinsing solution. After further washings, membranes were incubated with horseradish peroxidase-conjugated IgG fraction of goat anti-mouse IgG (Bio-Rad, Hercules, CA) diluted 1:2000 in TBS-T, containing 5% (w/v) non-fat dried milk for 90 min. Proteins were detected with enhanced chemi-luminescence.

Treatment with ISIS 330749 decreased hepatic and adipose pyruvate carboxylase protein expression by 70-90%. The data is presented in Table 10, relative to the expression measured in the rats fed normal chow and treated with the control oligonucleotide. Data were compared using Student's unpaired t test between the two groups. '***' indicates $P < 0.01$; '****' indicates $P < 0.001$.

Table 10
Percent reduction of Pyruvate Carboxylase protein expression

	Liver	Adipose tissue
Normal diet	61**	68**

HF diet	74***	94***
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Plasma chemistry markers

To evaluate the effect of ISIS oligonucleotides on liver function, plasma levels of transaminases and lactate concentrations were measured. Plasma lactate concentration was measured on Roche Cobas Mira Plus (Analytical Instruments, LLC, Golden Valley, MN), using the lactate reagent test kit (Pointe Scientific, Inc., Canton, MI). Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured on Roche Cobas Mira Plus using the AST and ALT reagent test kits (Raychem, Cliniqa Corporation, San Marcos, CA). The results are presented in Table 11. Treatment with ISIS 330749 did not have an appreciable effect on ALT, AST, or lactate concentration levels compared to control group.

Table 11

Plasma chemistry markers in Sprague-Dawley rats

	Regular chow		HF diet	
	ISIS 141923	ISIS 330749	ISIS 141923	ISIS 330749
AST (IU/L)	60	60	72	66
ALT (IU/L)	15	16	25	29
Lactate (mmol/L)	0.19	0.23	0.49	0.55

Glucose and insulin levels

Plasma glucose concentrations were measured using a YSI 2700 (YSI Life Sciences, Yellow Springs, Ohio). Plasma insulin levels and C-peptide were measured by a radioimmunoassay kit (Millipore, Billerica, MA). The results of the assays in the fasted and fed state are presented in Tables 12 and 13.

The rats also underwent a mixed meal tolerance test. In this assay, the normal chow-fed rats underwent placement of jugular venous and gastric catheters after 3 weeks of antisense oligonucleotide treatment. The rats were rested for 5-7 days, during which they regained their pre-surgical weights. The rats were then fasted overnight and mixed meal (Ensure Plus Ready-to-Drink Homemade Vanilla, Abbott Nutrition, Columbus, OH, consisting of 57% carbohydrate, 28% fat and 15% protein calories) was loaded at 10 ml/kg body weight or 15 kcal/kg body weight through the gastric catheter. Blood was taken from the venous line at the several time points. Plasma glucose, insulin, and C-peptide were subsequently measured. The results of this assay are presented in Table 14, expressed as the area under the curve (AUC) of the various time points versus glucose, insulin, or C-peptide levels at each time point.

Data were compared using Student's unpaired t test between the two groups. '*' indicates $P < 0.05$; '**' indicates $P < 0.01$; '***' indicates $P < 0.001$. Treatment with ISIS 330749 decreased both fasting and *ad-lib* fed plasma glucose concentrations in normal chow-fed rats. Plasma insulin concentration was significantly

increased in *ad-lib* fed rats. Plasma glucose excursion after a mixed-meal tolerance test was significantly reduced, but insulin secretion was not changed significantly. Hence, antisense inhibition of pyruvate carboxylase effectively lowered plasma glucose levels without suppression of insulin secretion.

Table 12

Glucose levels in normal chow-fed Sprague-Dawley rats

	ISIS 141923	ISIS 330749
Fasted	109	103**
Fed	145	132***

Table 13

Insulin levels in normal chow-fed Sprague-Dawley rats

	ISIS 141923	ISIS 330749
Fasted	7	6
Fed	27	47*

Table 14

Glucose and insulin levels in normal chow-fed Sprague-Dawley rats after mixed meal tolerance test (AUC)

	ISIS 141923	ISIS 330749
Glucose (mg/dL*hr)	292	276*
Insulin (μ U/mL*hr)	51	57
C-peptide (μ M*hr)	3	3

Body weights and adiposity

The body weights of the rats were measured weekly. Body composition in HFF diet-fed rats was assessed by ^1H magnetic resonance spectroscopy using a Bruker Minispec analyzer mq10 (Bruker Optics Inc., Billerica, MA). Hepatic triglyceride content of HFF rats after 4 weeks treatment and at the time of sacrifice was determined by using a triglyceride assay kit (Genzyme Diagnostics P.E.I. Inc., PE, Canada) and a method adapted from Storlien et al (Diabetes. 40: 280-289, 1991). The results are presented in Tables 15 and 16. Data were compared using Student's unpaired t test between the two groups. "*" indicates $P < 0.05$; "***" indicates $P < 0.01$.

Treatment with ISIS 330749 in HFF rats resulted in protection from weight gain, and was associated with decreased adiposity. The reduction in adiposity was associated with a decrease in hepatic triglyceride content.

Table 15

Body weights in Sprague-Dawley rats

	Diet	Week 0.5	Week 1	Week 1.5	Week 2
ISIS 141923	normal	261	277	290	305
ISIS 330749	normal	261	275	287	300

ISIS 141923	HF	254	291	329	359
ISIS 330749	HF	254	284	315*	340*

Table 16

Adiposity and hepatic triglyceride content in HFF Sprague-Dawley rats

	ISIS 141923	ISIS 330749
Epididymal adipose tissue weight (g/kg body weight)	9	6**
Hepatic triglyceride content (mg/g tissue)	30	20*

Hyperinsulinemic-euglycemic clamp assay

5 To determine whether reductions in hepatic steatosis altered insulin sensitivity, hyperinsulinemic-euglycemic clamp studies were performed on the HFF rats. The clamp studies were performed as previously described (Nagai, Y. et al., Cell Metab. 9: 252-264; 2009; Samuel, V.T. et al., J. Clin. Invest. 117: 739-745, 2007). After 4 weeks of treatment, rats were fasted overnight. The concentrations of glucose and insulin in the fasting condition, as well as the basal endogenous glucose production, were measured and are presented in Table 17. The following morning, the clamp study began with a primer at 1 mg/kg for 8 min of 99% labeled [6,6-²H] glucose followed by a continuous infusion at a rate of 0.1 mg/kg per min for 2 hrs to assess the basal glucose turnover. After the basal period, the hyperinsulinemic-euglycemic clamping was conducted for 140 min with a primed/ continuous infusion of human insulin (40 mU/kg over 5 min)/[4 mU/(kg-min)] (Novo Nordisk Inc., Princeton, NJ) and a variable infusion of ~20% dextrose to maintain euglycemia (approximately 100 mg/dL). The dextrose glucose was enriched with [6,6-²H] glucose to approximately 2.5% to match the enrichment in the plasma achieved after the basal period (i.e. 'hot-GINF'). A 30 μ Ci bolus of 2-deoxy-d-[1-¹⁴C] glucose (American Radiolabeled Chemicals Inc., St. Louis, MO) was injected at 140 min in the clamp to estimate the rate of insulin-stimulated tissue glucose uptake. At the end of the clamp, rats were anaesthetized with sodium pentobarbital injection (75 mg/kg) and all tissues were taken within 3 min, frozen using cooled aluminum tongs in liquid nitrogen, and stored at -80°C for subsequent analysis. The results are presented in Table 18. Data were compared using Student's unpaired t test between the two groups. '*' indicates P<0.05; '**' indicates P<0.01; '***' indicates P<0.001.

25 Treatment with ISIS 330749 reduced fasting plasma glucose concentrations and basal rates of hepatic glucose production without altering plasma insulin concentration. This suggests improved insulin sensitivity of the treatment group compared to the control. This was confirmed with the hyperinsulinemic-euglycemic clamp. Hepatic glucose production under hyperinsulinemic-euglycemic conditions was 50% lower in rats treated with ISIS 330749 compared to the control.

Table 17

Fasting plasma and hepatic glucose and plasma insulin levels

	ISIS 141923	ISIS 330749
Plasma glucose (mg/dL)	125	108**
Hepatic glucose production (mg/kg-min)	8	6**
Insulin (μ U/mL)	13	8*

Table 18

Glucose and plasma insulin levels as measured by the hyperinsulinemic-euglycemic clamp

	ISIS 141923	ISIS 330749
Insulin-stimulated peripheral glucose metabolism (mg/kg-min)	25.2	26.7
Endogenous glucose production (mg/kg-min)	2.4	1.6***
% suppression of endogenous glucose production	69.2	75.5*

Effect of the insulin signaling pathway

In order to evaluate the effect of antisense inhibition on the signaling pathway, protein levels of Akt and PKC ϵ were assessed via western blotting analysis. Akt and phosphorylated Akt (Ser⁴⁷³) were detected with whole cell lysates using antibodies purchased from Cell Signaling Technology Inc. (Danvers, MA). The changes observed in the clamp assay were associated with increased hepatic Akt Ser-473 phosphorylation. The results are presented in Table 19. Data were compared using Student's unpaired t test between the two groups. '*' indicates $P < 0.05$; '**' indicates $P < 0.01$.

Membrane translocation for PKC ϵ was performed, as previously described (Kumashiro, N. et al., Proc. Natl. Acad. Sci. USA 108: 16381-16385, 2011; Qu, X. et al., J. Endocrinol. 162: 207-214, 1999). For this assay, liver samples were separated into membrane and cytosol compartments. Tissue (100 mg) was homogenized in 500 μ l buffer A (20 mM Tris-HCl, pH 7.4; 1 mM EDTA; 0.25 mM EGTA; 250 mM sucrose; protease inhibitor (Roche Diagnostics, Indianapolis, US)) and centrifuged at 100,000 rcf at 4°C for 1 hr. The supernatants containing the cytosolic fraction were collected. Pellets were resuspended in 300 μ l buffer B (250 mM Tris-HCl, pH 7.4; 1 mM EDTA; 0.25 mM EGTA; 2% Triton-X 100; protease inhibitor cocktail) and centrifuged at 100,000 rcf at 4°C for 1 hr to obtain the plasma membrane fraction. Crude membrane and cytosol protein extracts (50 μ g) were used for western blotting. PKC ϵ translocation was expressed as the ratio of arbitrary units of membrane bands over cytosol bands. Membrane band density was corrected by sodium potassium ATPase band density (Antibody purchased from Abcam Inc., Cambridge, MA) and cytosolic band density was corrected by GAPDH band density (Antibody purchased from Cell Signaling Technology Inc., Danvers, MA). The PKC ϵ antibody was purchased from BD Transduction Laboratories (San Diego, CA). The changes observed in the clamp assay were associated with decreased activation of PKC ϵ by 67% ($P < 0.01$).

Akt is a key molecule in the insulin signaling pathway (Samuel, V.T. and Shulman, G.I. Cell. 148: 852-871, 2012). Thus, the reduction in NAFLD decreased PKC ϵ activation and improved insulin signaling.

Table 19

Hepatic Akt phosphorylation

	ISIS 141923	ISIS 330749
Basal	1.0	1.8*
Clamp	11.2	22.9**

Lipid and fatty acid levels

Plasma total cholesterol, HDL, and LDL were measured on Roche Cobas Mira Plus using the cholesterol, HDL, and LDL reagent test kit (Raychem, Clinica Corporation, San Marcos, CA), respectively. Non-esterified fatty acids (NEFA) were measured on Roche Cobas Mira Plus using the NEFA-HR (2) Color A and B reagent test kit (Wako Chemicals USA, Inc., Richmond, VA). The results are presented in Table 20. Data were compared using Student's unpaired t test between the two groups. '*' indicates $P < 0.05$; '**' indicates $P < 0.01$; '***' indicates $P < 0.001$.

Treatment with ISIS 330749 reduced plasma cholesterol concentrations in normal chow-fed and HFF rats.

Table 20
Lipid concentrations in rats

	Normal chow-fed		HF diet-fed	
	ISIS 141923	ISIS 330749	ISIS 141923	ISIS 330749
Total cholesterol (mg/dL)	83	55***	49	35***
LDL (mg/dL)	11	6***	11	8**
HDL (mg/dL)	13	9***	15	10***
NEFA (mmol/L)	0.9	0.7*	0.9	0.8*

Levels of genes regulating lipid metabolism

To evaluate the potential mechanisms underlying the lipid phenotype after antisense inhibition of Pyruvate Carboxylase, a comprehensive set of studies with the HFF rats was conducted. Total RNA was extracted from 15 mg liver using RNeasy mini kit (Qiagen, Valencia, CA). RNA was reverse-transcribed into cDNA with the use of M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA) and then assessed by real-time PCR on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with a SYBR Green detection system (Stratagene, La Jolla, CA). The results of the PCR assay are presented in Table 21. Treatment with ISIS 330749 decreased adipose expression of ATGL and PNPLA3, but did not significantly change the hepatic expression of the other key genes regulating lipid metabolism.

Table 21

% change of gene expression in ISIS 330749-treated rats compared to the control

	Liver	Adipose Tissue
Lipogenic pathway		
SREBP1c	-49	-28*
ACC	-24	n.d.
FAS	-24	+16
mGPAT	-11	-12
AGPAT1	-17	+5
AGPAT2	+18	+3
AGPAT5	+6	+22
DGAT2	-6	+14
Lipid oxidation pathway		
PPAR α	-20	+7
CPT1	-7	+9
Lipolysis pathway		
HSL	n.d.	-12
ATGL	n.d.	-21*
PNPLA3	n.d.	-21**
Cholesterol synthesis pathway		
HMGCoA reductase	-11	n.d.
SREBP2	-20	n.d.
Cholesterol uptake pathway		
LDL-R	-14	n.d.
SR-B1	+3	n.d.
Cholesterol efflux pathway		
CYP7A1	+34	n.d.
LXR α	-3	n.d.

Whole body lipolysis assay

To evaluate whole body lipolysis, HFF rats treated with ISIS oligonucleotides were fasted overnight. Then, 100 mM glycerol-1,1,2,3,3-d₅ (98 atom % D, Sigma-Aldrich, St. Louis, MO) in 0.9% PBS were infused at 75 μ L/kg-min for 2.5 hrs. Blood samples were then obtained and plasmas from the samples were used for a lipolysis assay, assessed by glycerol turnover (Weiss, R. et al., J. Clin. Endocrinol. 90: 3731-3737, 2005). For this, 100 μ L plasma was put in a tube and 50 μ L of 0.1 mM [2-¹³C]glycerol standard was added. The sample was deproteinized with 100 μ L of 0.3N ZnSO₄ and 100 μ L of 0.3N Ba(OH)₂, vortexed, and

centrifuged at 4,000 rpm at 4°C for 15 min. The supernatant was dried, derivatized with 100 µL acetic anhydride and 100 µL pyridine at 65°C for 15 min. The, [D-5]glycerol was analyzed for isotope enrichment by GC-MS (EI), by selected ion monitoring, m/z 145-148 (M_0 , M_3), as previously described (Yoon, J.C. et al., Nature. 413: 131-138, 2001). Atom percentage of enrichment of M_3 (D_5 APE) was calculated and corrected with a glycerol standard curve. Glycerol turnover rate (Glycerol Ra) was calculated with the following equation: glycerol Ra= (infusion rate)/ [(D_5 APE)/100] – (infusion rate). The data is presented in Table 22, expressed as rates of whole body lipolysis (µmol/min). The data indicates that rates of whole body lipolysis were unchanged in treatment group compared to the control.

Table 22
Rate of whole body lipolysis in HFF rats (µmol/min)

ISIS No	Rate of lipolysis
141923	16.3
330749	16.5

Glyceroneogenesis assay

To evaluate glycerol synthesis, this assay was done, as previously described (Bederman, I.R. et al., J. Biol. Chem. 284: 6101-6108, 2009). HFF rats were treated with ISIS oligonucleotides and then 20 mL/kg of 99% deuterium oxide (D_2O) (Cambridge Laboratories Inc., Andover, MA) with 0.9% NaCl was injected intraperitoneally. Drinking water with 5% D_2O was maintained *ad libitum* for three days. After an overnight fast, the rats were euthanized and tissues were taken within 3 min, frozen immediately, and stored at -80°C. Liver or epididymal tissue (100 mg) was homogenized with 1 mL of 1N KOH in 70% EtOH in a glass vial with screw cap. The homogenate was heated for 3 hrs at 75°C, then acidified with 4 mL of 6N HCl. Triglycerides from a 1 mL aliquot were extracted with 3 mL chloroform, centrifuged at 3,500 rpm for 10 min, and the upper layer was placed into a GC-MS vial and dried. Samples were derivatized with 750 µL of acetic anhydride: pyridine (1: 1) to glycerol triacetate at 75°C for 30 min, dried under N_2 gas, and reconstituted with 50 µL of 100% methanol.

Glycerol triacetate was analyzed for isotope enrichment by GC-MS using a Hewlett-Packard 6890 Gas Chromatograph interfaced to a Hewlett-Packard 5973A Mass Selective Detector (HP-1 capillary column 12m x 0.2 mm, 0.33 µM film) operating in the positive chemical ionization mode with isobutene as the reagent gas. Mass isotopomer abundances were analyzed by selected ion monitoring, m/z 219-221 (M_0 - M_2). The percentage of total newly made triglyceride-glycerol was calculated using the equation: % total newly made triglyceride-glycerol= [2H -labeling of triglyceride-glycerol/ (2H -labeling of plasma x n)] x 100, where 2H -labeling of triglyceride-glycerol is the M_1 isotopomer, the 2H -labeling of plasma is the average labeling in a given rat, and 'n' is the number of exchangeable hydrogens. Previous studies have experimentally

measured this value as 4.25 *in vivo* (Turner, S.M. et al., Am. J. Physiol. Endocrinol. Metab. 285: E790-803, 2003). The data is presented in Table 23, and indicates that treatment with ISIS 330749 did decrease glycerol synthesis in liver and adipose tissues, as measured by the incorporation of $^2\text{H}_2\text{O}$ into triglyceride-glycerol (i.e., the glycerol backbone of a triglyceride).

Table 23
% newly synthesized glycerol in tissue triglycerides

ISIS No	Liver	Adipose tissue
141923	70	9
330749	57*	7*

De novo lipogenesis assay

To evaluate hepatic *de novo* fatty acid synthesis in HFF rats, the same liver and plasma samples, as the glyceroneogenesis assay were used. Liver tissue (200 mg) was homogenized with 4 ml of chloroform: methanol (1: 2) solution. Fifty microgram of a triglyceride internal standard (Glyceryl triheptadecanoate, Sigma-Aldrich, St. Louis, MO) was added to the homogenate. The homogenate was shaken for 15 min at 4°C, followed by addition of 1.25 ml of chloroform and 1.25 ml of 1N NaCl. The mixture was centrifuged at 3,500 rpm for 10 min. The lower layer was collected, dried under a stream of nitrogen, and re-dissolved in 0.5 ml chloroform. The lipid extract was separated with a thin layer of chromatography plate (Silica Gel 60, GE Healthcare Life Sciences, Piscataway, NJ), developed with hexane: diethylether, dried and derivatized with 0.4 ml of chloroform: methanol (1:1) solution and 0.1 ml of borotrifluoride (Sigma-Aldrich, St. Louis, MO) at 70°C for 60 min. After cooling, 0.5 ml of water and 1 ml of pentane were added, vortexed, and centrifuged at 2,000 rpm for 10 min. The upper layer (fatty acid layer) was collected, dried under a stream of nitrogen, and re-dissolved in 120 µl hexane for GC-MS analysis. Then, palmitate was analyzed for isotope enrichment by GC-MS operating in the positive chemical oxidation mode (reagent gas: isobutene). Mass isotopomer abundances were analyzed by selected ion monitoring, m/z 271-273 (M_0 - M_2).

De novo lipogenesis (%), as measured by newly synthesized palmitate in the hepatic triglyceride-palmitate was calculated, as previously described (Lee, W.N. et al., Am. J. Physiol. 266: E699-708, 1994), based on incorporation of ^2H from $^2\text{H}_2\text{O}$ onto newly synthesized palmitate molecules. The data is presented in Table 24, and indicate that antisense inhibition of Pyruvate Carboxylase did not change hepatic *de novo* lipogenesis *in vivo*.

Table 24
% de novo fatty acid synthesis in HFF rats

ISIS No	%
141923	2.3

330749

3.4

Example 7: *In vivo* effect of antisense inhibition of murine pyruvate carboxylase in ZDF rats

The Zucker Diabetic Fatty (ZDF) rat model is a standard model to assess Type 2 diabetes, hyperlipidemia, glucose intolerance, obesity, and hyperinsulinemia. The effect of antisense inhibition of pyruvate carboxylase by ISIS 330749 on in the ZDF model was evaluated.

Treatment

ZDF rats were treated with either 75 mg/kg ISIS 330749 or 75 mg/kg ISIS 141923 administered weekly for 4 weeks.

Effect on basal glucose and insulin production

Rats were fasted overnight and then injected a primed continuous infusion of 6,6-²H glucose (0.3 mg/kg/min) for 120 min to measure basal glucose turnover. Blood samples were obtained to assess basal glucose production. The results are presented in Tables 25 and 26, expressed in mg/gram tissue. The results indicate that treatment with ISIS 330749 decreased the basal glucose production, while not affecting basal insulin levels.

Table 25

Basal glucose production in high fat-fed rats

ISIS No	mg/kg-min
141923	170
330749	148

Table 26

Basal insulin levels in high fat-fed rats

ISIS No	microU/mL
141923	114
330749	112

Effect on insulin sensitivity

The rats were then fasted overnight. Hyperinsulinemic-euglycemic clamp studies was conducted for 100 min with a primed/continuous infusion of insulin (50 mU/kg primed over 10 min and 4 mU/kg per min constant infusion) (Novo Nordisk) and a variable infusion of 20% dextrose spiked with 2.5% [6,6-²H]glucose to maintain euglycemia. Once rats maintained euglycemia for 100 min, the rats received a bolus of 30 μ Ci of 2-deoxy-D-[1-¹⁴C] glucose to measure the rate of insulin-stimulated tissue glucose uptake in skeletal muscles and adipose tissue. After the completion of the clamp, sodium pentobarbital was injected via the venous catheter administered at 150 mg/kg. After the rats were completely anesthetized, tissues were extracted for further analysis.

The results are presented in Table 27 and demonstrate that treatment with ISIS 330749 significantly increased insulin sensitivity, since the rate of glucose infusion (GINF) and insulin-stimulated glucose disposal rate required to maintain euglycemia during the clamp was higher in the rat group treated with ISIS 330749 compared to that in the control group. Finally, the suppression of endogenous glucose production was higher in rats treated with ISIS 330749 compared to the control, indicating that antisense inhibition of pyruvate carboxylase significantly increased insulin sensitivity in the high fat-fed model.

Table 27
Hyperinsulinemic-euglycemic clamp study

	ISIS 330749	ISIS 141923
Fasted body weight (g)	315.0	314.0
Rate of basal glucose production	6.9	9.3
Glucose infusion (GINF) (mg/kg/min)	17.1	14.3
Insulin-stimulated whole body glucose disposal	19.8	18.4
Clamp endogenous glucose production	2.7	4.0

Example 8: *In vivo* effect of antisense inhibition of murine pyruvate carboxylase in ZDF rats

The effect of antisense inhibition of pyruvate carboxylase by ISIS 330749 on ZDF rats, a widely used preclinical model for type 2 diabetes (T2D), was evaluated.

Treatment

Male ZDF rats (7 weeks of age) were obtained from Charles River Laboratories (Wilmington, MA) and given at least 3 weeks to acclimate. The rats were housed on a 12: 12 hr light-dark cycle and received food and water ad libitum. The rats were fed Purina Lab Diets 5008 (56.4% carbohydrate, 16.7% fat, 26.8% protein calories). A group of rats was injected intraperitoneally with 75 mg/kg ISIS 330749 administered weekly for 10 weeks. A group of rats were injected intraperitoneally with control oligonucleotide 75 mg/kg ISIS 141923 administered weekly for 10 weeks.

Hyperinsulinemic-euglycemic clamp assay

To determine whether reductions in hepatic steatosis altered insulin sensitivity, hyperinsulinemic-euglycemic clamp studies were performed on the rats, as described above. Fasting plasma glucose and insulin, and basal endogenous glucose production were assessed before the clamp assay. The results are presented in Table 28. The clamp assay was then performed and the plasma insulin concentration, endogenous glucose production, % suppression of endogenous glucose production, and insulin-stimulated peripheral glucose metabolism were measured. Uptake of 2-deoxy-D-glucose (2DG) at the end of the clamp in the muscle and adipose tissue was also measured. The results are presented in Table 29. All data were compared using Student's unpaired t test between two groups. '*' indicates $p < 0.05$; '**' indicates $p < 0.01$.

Treatment with ISIS 330749 reduced fasting plasma glucose concentrations and rates of hepatic glucose production during both basal and hyperinsulinemic periods, demonstrating efficacy of the antisense oligonucleotide in this model as well.

Table 28
Fasting glucose and insulin levels

	ISIS 141923	ISIS 330749
Plasma glucose (mg/dL)	169	148*
Hepatic glucose production (mg/kg-min)	10	7*
Insulin (μ U/mL)	179	142

Table 29
Levels during the hyperinsulinemic-euglycemic clamp assay

	ISIS 141923	ISIS 330749
Plasma insulin (mU/mL)	492	545
Endogenous glucose production (mg/kg-min)	4.4	2.6**
% suppression of endogenous glucose production	49	64*
Insulin-stimulated peripheral glucose metabolism (mg/kg-min)	19	19
2DG uptake in muscle (nmol/g-min)	49	55
2DG uptake in adipose tissue (nmol/g-min)	6.7	7.8

10 *Lipid and fatty acid levels*

Plasma total cholesterol, HDL, and LDL were measured on Roche Cobas Mira Plus using the cholesterol, HDL, and LDL reagent test kit (Raychem, Cliniqua Corporation, San Marcos, CA), respectively. Non-esterified fatty acids (NEFA) were measured on Roche Cobas Mira Plus using the NEFA-HR (2) Color A and B reagent test kit (Wako Chemicals USA, Inc., Richmond, VA). The results are presented in Table 30.

15 All data were compared using Student's unpaired t test between two groups. '*' indicates $p < 0.05$; '**' indicates $p < 0.01$.

Treatment with ISIS 330749 reduced plasma cholesterol concentrations in ZDF rats.

Table 30
Lipid concentrations in rats

	ISIS 141923	ISIS 330749
Total cholesterol (mg/dL)	100	69**
LDL (mg/dL)	16	10*
HDL (mg/dL)	16	12*

Triglycerides (mg/dL)	286	117
NEFA (mmol/L)	4	2

Plasma chemistry markers

To evaluate the effect of ISIS oligonucleotides on liver function, plasma levels of transaminases and lactate concentrations were measured. Plasma lactate concentration was measured on Roche Cobas Mira Plus (Analytical Instruments, LLC, Golden Valley, MN), using the lactate reagent test kit (Pointe Scientific, Inc., Canton, MI). Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured on Roche Cobas Mira Plus using the AST and ALT reagent test kits (Raychem, Clinica Corporation, San Marcos, CA). The results are presented in Table 31. Treatment with ISIS 330749 did not have any effect on the ALT, AST and lactate concentration levels compared to control group.

Table 31

Plasma chemistry markers in ZDF rats

	ISIS 141923	ISIS 330749
AST (IU/L)	65	57
ALT (IU/L)	37	28
Lactate (mmol/L)	1	2

Example 9: *In vivo* effect of antisense inhibition of murine pyruvate carboxylase in high fat diet-fed mice

The effect of antisense inhibition of pyruvate carboxylase by ISIS 330749 on C57BL/6 mice fed either normal murine diet or a high fat (HF) diet was evaluated.

Treatment

Male C57BL/6 mice (7 weeks of age) were obtained from Charles River Laboratories (Wilmington, MA) and given at least 3 weeks to acclimate. The mice were housed on a 12: 12 hr light-dark cycle and received food and water *ad libitum*. Chow consisted of regular rodent chow (60% carbohydrate, 10% fat, 30% protein calories), or a high fat diet (Dyets 112245: 26% carbohydrate, 59% fat, 15% protein calories). A group of mice was injected intraperitoneally with 75 mg/kg ISIS 330749 administered weekly for 10 weeks. A group of mice were injected intraperitoneally with control oligonucleotide 75 mg/kg ISIS 141923 administered weekly for 10 weeks.

Body composition and energy expenditure

Adipose tissue weight and hepatic triglyceride content of the mice were measured after 11 weeks and at the time of sacrifice. The data is presented in Table 32. Whole body fat weight and lean body fat weight were assessed after nine weeks of treatment by ¹H magnetic resonance spectroscopy using a Bruker Minispec

analyzer mq10 (Bruker Optics Inc., Billerica, MA). The data is presented in Table 33. Metabolic parameters, energy expenditure, food intake, and physical activity were measured using a comprehensive animal metabolic monitoring system (CLAMS; Columbus Instruments, Columbus, OH). The results are presented in Table 34. All data were compared using Student's unpaired t test between two groups. '*' indicates $p < 0.05$; '**' indicates $p < 0.01$; '***' indicates $p < 0.001$ between the control and treatment groups.

Treatment with ISIS 330749 decreased body weight gain and fat mass in the mice. There was greater reduction in liver triglyceride content compared to the effect observed in the rat model. These changes occurred without any measureable increase in energy expenditure or changes in the respiratory quotient, which was measured after 5 weeks of treatment and before significant divergence in weights. Food intake was 20% greater ($p < 0.05$) in mice treated with ISIS 330749. Taken together, these data indicate that antisense inhibition of pyruvate carboxylase in HFF mice protected against adiposity and hepatic steatosis, despite increases in food intake.

Table 332
Body composition of HFF mice at week 11

	ISIS 141923	ISIS 330749
Epididymal adipose tissue weight (g/kg body weight)	22.9	5.5***
Hepatic triglyceride content (mg/g tissue)	25.0	3.6***

Table 33
Body weights (g) of HFF mice at week 9

	ISIS 141923	ISIS 330749
Body fat weight	2.6	1.2*
Lean body weight	20.6	19.8

Table 34
Metabolic cage data of HFF mice

	Week 5		Week 9	
	ISIS 141923	ISIS 330749	ISIS 141923	ISIS 330749
Body weight (g)	27	26	28	25*
Body fat (g)	2.2	1.4*	2.6	1.2*
Body fat (%)	7.9	5.6*	9.2	4.8*
Body muscle (g)	20.2	19.8	20.6	19.8
Body muscle (%)	74.9	77.4**	73.9	77.8**
VO ₂ (ml/kg/hr)	6522	6218	6477	6072
VCO ₂ (ml/kg-hr)	5429	5211	5466	5137
Respiratory ratio	0.83	0.84	0.84	0.84
Energy expenditure (kcal/kg-hr)	31.5	30.1	31.4	29.5
Energy expenditure (kcal/day)	20.9	18.7*	21.8	18.6*

Food intake (kcal/kg-hr)	15.3	18.6*	15.7	19.5*
Food intake (kcal/day)	9.8	11.1	10.8	12.2*
Activity (counts/hr)	127	175	122	155

What is claimed is:

1. A method of preferentially reducing hepatic pyruvate carboxylase, comprising administering to an animal a compound comprising a modified antisense oligonucleotide complementary to pyruvate carboxylase and wherein the modified antisense oligonucleotide activates a nuclear ribonuclease capable of cleaving pyruvate carboxylase, wherein hepatic pyruvate carboxylase is preferentially reduced.
2. A method of reducing hepatic pyruvate carboxylase expression in an animal without affecting pyruvate carboxylase activity in the pancreas, comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length complementary to pyruvate carboxylase and wherein the modified antisense oligonucleotide activates a nuclear ribonuclease capable of cleaving pyruvate carboxylase, wherein expression of hepatic pyruvate carboxylase is reduced in the animal without affecting pyruvate carboxylase activity in the pancreas.
3. A method of reducing hepatic pyruvate carboxylase expression in an animal without affecting pancreatic islet pyruvate carboxylase activity comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length complementary to pyruvate carboxylase and wherein the modified antisense oligonucleotide activates a nuclear ribonuclease capable of cleaving pyruvate carboxylase, wherein expression of hepatic pyruvate carboxylase is reduced in the animal without affecting pancreatic islet pyruvate carboxylase activity.
4. A method of reducing glucose levels in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length complementary to pyruvate carboxylase and wherein the modified antisense oligonucleotide activates a nuclear ribonuclease capable of cleaving pyruvate carboxylase, wherein glucose levels are reduced in the animal.
5. A method of reducing insulin levels in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length complementary to pyruvate carboxylase and wherein the modified antisense oligonucleotide activates a nuclear ribonuclease capable of cleaving pyruvate carboxylase, wherein the levels of insulin is reduced in the animal.
6. A method of preventing weight gain in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length complementary to

pyruvate carboxylase and wherein the modified antisense oligonucleotide activates a nuclear ribonuclease capable of cleaving pyruvate carboxylase, wherein weight gain is prevented in the animal.

7. A method of increasing insulin sensitivity in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length complementary to pyruvate carboxylase and wherein the modified antisense oligonucleotide activates a nuclear ribonuclease capable of cleaving pyruvate carboxylase, wherein insulin sensitivity is increased in the animal.

8. A method of treating, preventing or ameliorating a metabolic disease in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length complementary to pyruvate carboxylase and wherein the modified antisense oligonucleotide activates a nuclear ribonuclease capable of cleaving pyruvate carboxylase, wherein the metabolic disease is treated, prevented or ameliorated in the animal.

9. A method of treating, preventing or ameliorating diabetes in an animal comprising administering to the animal a compound comprising a modified oligonucleotide 10 to 30 linked nucleosides in length complementary to pyruvate carboxylase and wherein the modified antisense oligonucleotide activates a nuclear ribonuclease capable of cleaving pyruvate carboxylase, wherein the diabetes is treated, prevented or ameliorated in the animal.

10. The method of any of claims 1-9, wherein the modified oligonucleotide has a nucleobase sequence at least 90% complementary to any of SEQ ID NOs: 1-5 as measured over the entirety of said modified oligonucleotide.

11. The method of any one of claims 1-9, wherein the nucleobase sequence of the modified oligonucleotide is at least 95% complementary to any of SEQ ID NOs: 1-5 as measured over the entirety of said modified oligonucleotide.

12. The method of any one of claims 1-9, wherein the nucleobase sequence of the modified oligonucleotide is 98% complementary to any of SEQ ID NOs: 1-5 as measured over the entirety of said modified oligonucleotide.

13. The method of any one of claims 1-9, wherein the nucleobase sequence of the modified oligonucleotide is 100% complementary to any of SEQ ID NOs: 1-5 as measured over the entirety of said modified oligonucleotide.
14. The method of any one of claims 1-9, wherein at least one internucleoside linkage of said modified oligonucleotide is a modified internucleoside linkage.
15. The method of claim 14, wherein each internucleoside linkage is a phosphorothioate internucleoside linkage.
16. The method of any one of claims 1-9, wherein at least one nucleoside of said modified oligonucleotide comprises a modified sugar.
17. The method of claim 16, comprising at least one tetrahydropyran modified nucleoside wherein a tetrahydropyran ring replaces a furanose ring.
18. The method of claim 16, wherein at least one modified sugar is a bicyclic sugar.
19. The method of claim 16, wherein at least one modified sugar comprises a 2'-O-methoxyethyl or a 4'-(CH₂)_n-O-2' bridge, wherein n is 1 or 2.
20. The method of any one of claims 1-9, wherein at least one nucleoside of said modified oligonucleotide comprises a modified nucleobase.
21. The method of claim 20, wherein the modified nucleobase is a 5-methylcytosine.
22. The method of any one of claims 1-9, wherein the modified oligonucleotide consists of 20 linked nucleosides.
23. The method of any one of claims 1-9, wherein the modified oligonucleotide comprises:
- a gap segment consisting of linked deoxynucleosides;
 - a 5' wing segment consisting of linked nucleosides;
 - a 3' wing segment consisting of linked nucleosides;

wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar.

24. The method of claim 1-9, wherein the modified oligonucleotide consists of 20 linked nucleosides, has a nucleobase sequence complementary to any of SEQ ID NOS: 1-5 as measured over the entirety of said modified oligonucleotide and comprises:

- a. a gap segment consisting of ten linked deoxynucleosides;
- b. a 5' wing segment consisting of five linked nucleosides;
- c. a 3' wing segment consisting of five linked nucleosides;

wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar, wherein each internucleoside linkage is a phosphorothioate linkage, and wherein each cytosine is a 5'-methylcytosine.

25. A method for treating an animal with a metabolic disease comprising

- a. identifying said animal with a metabolic disease,
- b. administering to said animal a therapeutically effective amount of a compound comprising a modified oligonucleotide consisting of 20 linked nucleosides and having a nucleobase sequence at least 90% complementary to any of SEQ ID NOS: 1-5 as measured over the entirety of said modified oligonucleotide, wherein said animal with a metabolic disease is treated.

26. The method of claim 25, wherein the therapeutically effective amount of the compound administered to the animal reduces the metabolic disease in the animal.

27. The method of claim 9 or 25, wherein the metabolic disease is diabetes.

28. The method of claims 1, 2, or 3, wherein the administration of the modified oligonucleotide results in a reduction of glucose levels, insulin levels, body weight, weight gain, white adipose tissue, triglyceride levels or a combination thereof.

29. The method of claim 28, wherein the levels are independently reduced by at least 5%, 10%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%.

30. A method of decreasing one or more of pyruvate carboxylase levels, glucose levels, insulin levels, body weight, white adipose tissue, triglyceride levels, cholesterol levels, insulin resistance, or

metabolic disease, in a human by administering a pyruvate carboxylase inhibitor comprising a modified oligonucleotide consisting of 20 linked nucleosides and having a nucleobase sequence at least 90% complementary to any of SEQ ID NOS: 1-5 as measured over the entirety of said modified oligonucleotide.

31. The method of any one of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 25 or 30, wherein the animal is a human.

32. The method of any one of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 25 or 30, wherein the compound is a first agent and further comprising administering a second agent.

33. The method of claim 32, wherein the first agent and the second agent are co-administered.

34. The method of claim 32, wherein the second agent is a glucose-lowering therapy.

35. The method of claim 34, wherein the a therapeutic lifestyle change, PPAR agonist, a dipeptidyl peptidase (IV) inhibitor, a GLP-1 analog, insulin or an insulin analog, an insulin secretagogue, a SGLT2 inhibitor, a human amylin analog, a biguanide, an alpha-glucosidase inhibitor, or a combination thereof. The glucose-lowering agent can include, but is not limited to metformin, sulfonylurea, rosiglitazone, meglitinide, thiazolidinedione, alpha-glucosidase inhibitor or a combination thereof.

36. The method of any one of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 25 or 30, wherein administration comprises parenteral administration.

37. The method of any one of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 25 or 30, wherein the compound consists of a single-stranded modified oligonucleotide.

38. A compound comprising a modified oligonucleotide consisting of 10 to 30 linked nucleosides targeting pyruvate carboxylase as shown in any of SEQ ID NOS: 1-5.

39. The compound of claim 38, wherein the nucleobase sequence of the modified oligonucleotide is at least 95% complementary to SEQ ID NOS: 1-5.

40. The compound of claim 38, wherein the nucleobase sequence of the modified oligonucleotide is 100% complementary to SEQ ID NOS: 1-5.

41. The compound of claim 38, wherein the modified oligonucleotide is a single-stranded oligonucleotide.

42. The compound of claim 38, wherein at least one internucleoside linkage is a modified internucleoside linkage.

43. The compound of claim 42, wherein each internucleoside linkage is a phosphorothioate internucleoside linkage.

44. The compound of claim 38, wherein at least one nucleoside comprises a modified sugar.

45. The compound of claim 44, wherein at least one modified sugar is a bicyclic sugar.

46. The compound of claim 44, wherein at least one modified sugar comprises a 2'-O-methoxyethyl or a 4'-(CH₂)_n-O-2' bridge, wherein n is 1 or 2.

47. The compound of claim 38, wherein at least one nucleoside comprises a modified nucleobase.

48. The compound of claim 47, wherein the modified nucleobase is a 5-methylcytosine.

49. The compound of claim 38, wherein the modified oligonucleotide comprises:
a gap segment consisting of linked deoxynucleosides;
a 5' wing segment consisting of linked nucleosides;
a 3' wing segment consisting of linked nucleosides;
wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and
wherein each nucleoside of each wing segment comprises a modified sugar.

50. The compound of claim 38, wherein the modified oligonucleotide consists of 20 linked nucleosides and comprises:

a gap segment consisting of ten linked deoxynucleosides;
a 5' wing segment consisting of five linked nucleosides;
a 3' wing segment consisting of five linked nucleosides;

wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar; and wherein each internucleoside linkage is a phosphorothioate linkage.

51. The compound of claim 38, wherein the modified oligonucleotide consists of 20 linked nucleosides.

52. Use of a compound targeting pyruvate carboxylase for treating, preventing, ameliorating or reducing at least one symptom of a metabolic disease, by decreasing hepatic pyruvate carboxylase without affecting pancreatic islet pyruvate carboxylase.

53. Use of a compound targeting pyruvate carboxylase for treating, preventing, ameliorating or reducing diabetes by decreasing hepatic pyruvate carboxylase without affecting pancreatic islet pyruvate carboxylase.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2012/042806

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A61K 31/713 (2012.01) USPC - 514/44A 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) IPC(8) - See extra sheet USPC - 514/23, 44R, 44A, 45, 46, 49; 536/1.11, 18.7, 23.1, 25.1, 25.2, 26.8, 26.9 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) Patbase, Google Patent, PubMed		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	WO 2010/019434 A1 (SI et al) 18 February 2010 (18.02.2010) entire document	1, 4-16, 19, 20, 22, 25-44, 46, 47, 51 ----- 2, 3, 17, 18, 21, 23, 24, 45, 48-50, 52, 53
Y	JENSEN et al. "Compensatory Responses to Pyruvate Carboxylase Suppression in Islet Beta-Cells", The Journal of Biological Chemistry, 04 August 2006 (04.08.2006), Vol. 281, No. 31, Pgs. 22342-22351. entire document	2, 3, 52, 53
Y	US 2010/0130595 A1 (DEAN et al) 27 May 2010 (27.05.2010) entire document	17, 18, 21, 23, 24, 45, 48-50
A	US 2009/0131348 A1 (LABOURIER et al) 21 May 2009 (21.05.2009) entire document	1-53
A	US 2010/0010068 A1 (REN et al) 14 January 2010 (14.01.2010) entire document	1-53
A	WO 2010/133970 A1 (STOFFELL et al) 25 November 2010 (25.11.2010) entire document	1-53
☐ Further documents are listed in the continuation of Box C. ☐		
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Date of the actual completion of the international search 11 September 2012		Date of mailing of the international search report 16 OCT 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201		Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2012/042806

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61K 31/70, 31/708, 31/711, 31/712, 31/713, 31/7042, 31/7052, 31/7064, 31/7072, 31/7076, 31/7088; C07H 19/00, 19/06, 19/16, 19/067, 19/073, 16/167, 19/173, 21/00, 20/02, 21/04 (2012.01)