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DESCRIPTION

FIELD OF INVENTION

[0001] The present invention relates to conjugates of LNA antisense oligonucleotides (oligomers) that target ApoB.

BACKGROUND

[0002] See the background sections of WO2007/031081, WO2008/113830, WO2010142805, and WO2010076248. SPC3833 and SPC4955 (which have SEQ ID NO 1 and 2) are two LNA compounds which have been previously identified as potent compounds which target human ApoB mRNA.

[0003] WO2007/146511 and WO2007/131238 reports on short bicyclic (LNA) gapmer antisense oligonucleotides which apparently are more potent and less toxic than longer compounds. The exemplified compounds appear to be 14nts in length,

[0004] According to van Poelgeest et al., (American Journal of Kidney Disease, 2013 Oct;62(4):796-800), the administration of LNA antisense oligonucleotide SPC5001 in human clinical trials may result in acute kidney injury.

[0005] According to EP 1 984 381 B1, Seth et al., Nucleic Acids Symposium Series 2008 No. 52 553-554 and Swayze et al., Nucleic Acid Research 2007, vol 35, pp 687 - 700, LNA oligonucleotides cause significant hepatotoxicity in animals. According to WO2007/146511, the toxicity of LNA oligonucleotides may be avoided by using LNA gapmers as short as 12 - 14 nucleotides in length. EP 1 984 381B1 recommends using 6' substituted bicyclic nucleotides to decrease the hepatotoxicity potential of LNA oligonucleotides. According to Hagedorn et al., Nucleic Acid Therapeutics 2013, the hepatotoxic potential of antisense oligonucleotide may be predicted from their sequence and modification pattern.

[0006] Oligonucleotide conjugates have been extensively evaluated for use in siRNAs, where they are considered essential in order to obtain sufficient *in vivo* potency. For example, see WO2004/044141 refers to modified oligomeric compounds that modulate gene expression via an RNA interference pathway. The oligomeric compounds include one or more conjugate moieties that can modify or enhance the pharmacokinetic and pharmacodynamic properties of the attached oligomeric compound.

[0007] WO2012/083046 reports on a galactose cluster-pharmacokinetic modulator targeting moiety for siRNAs.

[0008] In contrast, single stranded antisense oligonucleotides are typically administered therapeutically without conjugation or formulation. The main target tissues for antisense oligonucleotides are the liver and the kidney, although a wide range of other tissues are also accessible by the antisense modality, including lymph node, spleen, bone marrow.

[0009] WO 2005/086775 refers to targeted delivery of therapeutic agents to specific organs using a therapeutic chemical moiety, a cleavable linker and a labeling domain. The cleavable linker may be, for example, a disulfide group, a peptide or a restriction enzyme cleavable oligonucleotide domain.

[0010] WO 2011/126937 refers to targeted intracellular delivery of oligonucelotides via conjugation with small molecule ligands.

[0011] Raiur et al 1997 Bioconjugate chemistry vol 8 pp 935-940 describes oligomeric DNA complexed with asialoglycoprotein-poly(L-lysine) (ASGP-PLL) conjugates.

[0012] Zhu et al 2008 Bioconiuqate chemistry vol 19 pp 290-298 describes radiolabeled oligogonucleotides conjugated to Gal-PEG via an acid-labile ester linkage of beta-thiopropionate.

[0013] WO2009/025669 refers to polymeric (polyethylene glycol) linkers containing pyridyl disulphide moieties. See also Zhao et al., Bioconjugate Chem. 2005 16 758 - 766.

[0014] Chaltin et al., Bioconjugate Chem. 2005 16 827 - 836 reports on cholesterol modified mono- di- and tetrameric oligonucleotides used to incorporate antisense oligonucleotides into cationic liposomes, to produce a dendrimeric delivery system. Cholesterol is conjugated to the oligonucleotides via a lysine linker.

[0015] Other non-cleavable cholesterol conjugates have been used to target siRNAs and antagomirs to the liver - see for example, Soutscheck et al., Nature 2004 vol. 432 173 - 178 and Krützfeldt et al., Nature 2005 vol 438, 685 - 689. For the partially phosphorothiolated siRNAs and antagomirs, the use of cholesterol as a liver targeting entity was found to be essential for *in vivo* activity.

[0016] There is therefore a need for ApoB targeting LNA antisense compounds have enhanced efficacy and a reduced toxicity risk.

SUMMARY OF INVENTION

[0017] The invention provides for an antisense oligonucleotide conjugate (the compound of the invention) comprising a first region of an oligomer (region A - such as an LNA oligomer, a gapmer oligomer or an LNA gapmer oligomer), targeting an ApoB nucleic acid, covalently joined to a further region (region C) comprising a conjugate moiety comprising an N-acetylgalactosamine (GalNAc) moiety joined to the LNA oligomer via biocleavable linker, wherein the biocleavable linker comprises a peptide, such as a lysine linker, or a physiologically labile nucleotide linker.

[0018] The application describes a conjugate comprising an LNA antisense oligomer (the compound of the invention-) targeting to a ApoB nucleic acid (A) and at least one non-nucleotide or non-polynucleotide moiety (C) covalently attached to said oligomer (A), wherein the non-polynucleotide moiety is selected from the group consisting of an asialoglycoprotein receptor targeting conjugate and a lipophillic conjugate, wherein the lipophilic conjugate, and optionally the asialoglycoprotein receptor targeting conjugate, is covalently joined to the LNA antisense oligomer via a biocleavable linker (region B)

[0019] In some embodiments, the invention provides for an oligomeric compound (the compound of the invention), which targets an ApoB nucleic acid target, which comprises three regions:

1. i) a first region (region A), which comprises 7 - 26 contiguous nucleotides which are complementary to a ApoB nucleic acid target;
2. ii) a second region (region B) which comprises between 1 - 10 nucleotides, which is covalently linked to the 5' or 3' nucleotide of the first region, such as via a internucleoside linkage group such as a phosphodiester linkage, wherein either
    1. a. the internucleoside linkage between the first and second region is a phosphodiester linkage and the nucleoside of the second region [such as immediately] adjacent to the first region is either DNA or RNA; and/or
    2. b. at least 1 nucleoside of the second region is a phosphodiester linked DNA or RNA nucleoside;
3. iii) a third region (C), a conjugate moiety comprising an N-acetylgalactosamine (GalNAc) moiety.

[0020] In some embodiments, region A and region B form a single contiguous nucleotide sequence of 8 - 35 nucleotides in length.

[0021] In some aspects the internucleoside linkage between the first and second regions may be considered part of the second region.

[0022] In some embodiments, there is a phosphorus containing linkage group between the second and third region. The phosphorus linkage group, may, for example, be a phosphate (phosphodiester), a phosphorothioate, a phosphorodithioate or a boranophosphate group. In some embodiments, this phosphorus containing linkage group is positioned between the second region and a linker region which is attached to the third region. In some embodiments, the phosphate group is a phosphodiester.

[0023] Therefore, in some aspects the oligomeric compound comprises at least two phosphodiester groups, wherein at least one is as according to the above statement of invention, and the other is positioned between the second and third regions, optionally between a linker group and the second region.

[0024] In some embodiments region A comprises at least one, such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 internucleoside linkages other than phosphodiester, such as internucleoside linkages which are (optionally independently] selected from the group consisting of phosphorothioate, phosphorodithioate,and boranophosphate, and methylphosphonate, such as phosphorothioate. In some embodiments region A comprises at least one phosphorothioate linkage. In some embodiments at least 50%, such as at least 75%, such as at least 90% of the internucleoside linkages, such as all the internucleoside linkages within region A are other than phosphodiester, for example are phosphorothioate linkages. In some embodiments, all the internucleoside linkages in region A are other than phosphodiester.

[0025] The application describes oligomeric compounds comprising an antisense oligonucleotide, such as an antisense oligonucleotide conjugate. The antisense oligonucleotide may be or may comprise the first region, and optionally the second region. In this respect, in some embodiments, region B may form part of a contiguous nucleobase sequence which is complementary to the (nucleic acid) target. In other embodiments, region B may lack complementarity to the target.

Alternatively stated, in some embodiments, the invention provides a non-phosphodiester linked, such as a phosphorothioate linked, oligonucleotide (e.g. an antisense oligonucleotide) which has at least one terminal (5' and/or 3') DNA or RNA nucleoside linked to the adjacent nucleoside of the oligonucleotide via a phosphodiester linkage, wherein the terminal DNA or RNA nucleoside is further covalently linked to a GalNAc conjugate moiety, optionally via a linker moiety. The invention provides for pharmaceutical composition comprising the compound of the invention, and a pharmaceutically acceptable diluent, carrier, salt or adjuvant.

The invention provides for the compound or pharmaceutical composition of the invention, for use as a medicament, such as for the treatment of acute coronary syndrome, or hypercholesterolemia or related disorder, such as a disorder selected from the group consisting of atherosclerosis, hyperlipidemia, hypercholesterolemia, HDL/LDL cholesterol imbalance, dyslipidemias, e.g., familial hyperlipidemia (FCHL), acquired hyperlipidemia, statin-resistant hypercholesterolemia, coronary artery disease (CAD), and coronary heart disease (CHD).

The invention provides for the use of the compound or pharmaceutical composition of the invention, for the manufacture of a medicament for the treatment of acute coronary syndrome, or hypercholesterolemia or a related disorder, such as a disorder selected from the group consisting of atherosclerosis, hyperlipidemia, hypercholesterolemia, HDL/LDL cholesterol imbalance, dyslipidemias, e.g., familial hyperlipidemia (FCHL), acquired hyperlipidemia, statin-resistant hypercholesterolemia, coronary artery disease (CAD), and coronary heart disease (CHD).

The application describes a method of treating acute coronary syndrome, or hypercholesterolemia or a related disorder, such as a disorder selected from the group consisting atherosclerosis, hyperlipidemia, hypercholesterolemia, HDL/LDL cholesterol imbalance, dyslipidemias, e.g., familial hyperlipidemia (FCHL), acquired hyperlipidemia, statin-resistant hypercholesterolemia, coronary artery disease (CAD), and coronary heart disease (CHD), said method comprising administering an effective amount of the compound or pharmaceutical composition according to the invention, to a patient suffering from, or likely to suffer from hypercholesterolemia or a related disorder.

The invention provides for an *in vitro* method for the inhibition of ApoB in a cell which is expressing ApoB, said method comprising administering the compound of the invention to said cell so as to inhibit ApoB in said cell.

The invention provides for the compound of the invention for use in medicine, such as for use as a medicament.

[0026] The application describes an LNA oligomer, comprising a contiguous region of 12 - 24 phosphorothioate linked nucleosides which are complementary to a corresponding region of a ApoB nucleic acid target, and further comprising between 1 and 6 DNA nucleosides which are contiguous with the LNA oligomer, wherein the internucleoside linkages between the DNA, and/or adjacent to the DNA nucleoside(s), is physiologically labile, such as is / are phosphodiester linkages. Such an LNA oligomer may be in the form of a conjugate, as described herein, or may, for example be an intermediate to be used in a subsequent conjugation step. When conjugated, the conjugate may, for example be or comprise a sterol, such as cholesterol or tocopherol, or may be or comprise a (non-nucleotide) carbohydrate, such as a GalNAc conjugate, such as a GalNAc cluster, e.g. triGalNAc, or another conjugate as described herein.

[0027] The invention provides for an LNA antisense oligomer (which may be referred to as region A herein) comprising an antisense oligomer comprising a contiguous region of 12 - 24 phosphorothioate linked nucleosides which are complementary to a corresponding region of a ApoB nucleic acid target, and a GalNAc moiety, which may form part of a further region (referred to as region C). The LNA antisense oligomer may be 12 - 24, and may be in the form of a gapmer oligomer.

BRIEF DESCRIPTION OF FIGURES

[0028]

Figure 1: Non-limiting illustration of oligomers of the invention attached to an activation group (i.e. a protected reactive group - as the third region). The internucleoside linkage L may be, for example phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate or methylphosphonate, such as phosphodiester. PO is a phosphodiester linkage. Compound a) has a region B with a single DNA or RNA, the linkage between the second and the first region is PO. Compound b) has two DNA/RNA (such as DNA) nucleosides linked by a phosphodiester linkage. Compound c) has three DNA/RNA (such as DNA) nucleosides linked by a phosphodiester linkages. In some embodiments, Region B may be further extended by further phosphodiester DNA/RNA (such as DNA nucleosides). The activation group is illustrated on the left side of each compound, and may, optionally be linked to the terminal nucleoside of region B via a phosphorus nucleoside linkage group, such as phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate or methylphosphonate, or in some embodiments a triazole linkage. Compounds d), e), & f) further comprise a linker (Y) between region B and the activation group, and region Y may be linked to region B via, for example, a phosphorus nucleoside linkage group, such as phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate or methylphosphonate, or in some embodiments a triazole linkage.

Figure 2: Equivalent compounds as shown in figure 1, however a reactive group is used in place of the activation group. The reactive group may, in some embodiments be the result of activation of the activation group (e.g. deprotection). The reactive group may, in non-limiting examples, be an amine or alcohol.

Figure 3: Non-limiting Illustration of compounds of the invention. Same nomenclature as Figure 1. X may in some embodiments be a conjugate, such as a lipophilic conjugate such as cholesterol, or another conjugate such as those described herein. In addition, or alternatively X may be a targeting group or a blocking group. In some aspects X may be an activation group (see Figure 1), or a reactive group (see figure 2). X may be covalently attached to region B via a phosphorus nucleoside linkage group, such as phosphodiester, phosphorothioate, phosphorodithioate,

boranophosphate or methylphosphonate, or may be linked via via an alternative linkage, e.g. a triazol linkage (see L in compounds d), e), and f)).

Figure 4. Non-limiting Illustration of compounds of the invention, where the compounds comprise the optional linker between the third region (X) and the second region (region B). Same nomenclature as Figure 1. Suitable linkers are disclosed herein, and include, for example alkyl linkers, for example C6 linkers. In compounds A, B and C, the linker between X and region B is attached to region B via a phosphorus nucleoside linkage group, such as phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate or methylphosphonate, or may be linked via an alternative linkage eg. a triazol linkage (Li). In these compounds Lii represents the internucleoside linkage between the first (A) and second regions (B).

Figure 5a and b. 5b shows a non-limiting example of a method of synthesis of compounds of the invention. US represent a oligonucleotide synthesis support, which may be a solid support. X is the third region, such as a conjugate, a targeting group, a blocking group etc. In an optional pre-step, X is added to the oligonucleotide synthesis support. Otherwise the support with X already attached may be obtained (i). In a first step, region B is synthesized (ii), followed by region A (iii), and subsequently the cleavage of the oligomeric compound of the invention from the oligonucleotide synthesis support (iv). In an alternative method the pre-step involves the provision of a oligonucleotide synthesis support with a region X and a linker group (Y) attached (see Figure 5a).. In some embodiments, either X or Y (if present) is attached to region B via a phosphorus nucleoside linkage group, such as phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate or methylphosphonate, or an alternative linkage, such as a triazol linkage.

Figure 6. A non-limiting example of a method of synthesis of compounds of the invention which comprise a linker (Y) between the third region (X) and the second region (B). US represents a oligonucleotide synthesis support, which may be a solid support. X is the third region, such as a conjugate, a targeting group, a blocking group etc. In an optional pre-step, Y is added to the oligonucleotide synthesis support. Otherwise the support with Y already attached may be obtained (i). In a first step, region B is synthesized (ii), followed by region A (iii), and subsequently the cleavage of the oligomeric compound of the invention from the oligonucleotide synthesis support (iv). In some embodiments (as shown), region X may be added to the linker (Y) after the cleavage step (v). In some embodiments, Y is attached to region B via a phosphorus nucleoside linkage group, such as phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate or methylphosphonate, or an alternative linkage, such as a triazol linkage.

Figure 7. A non-limiting example of a method of synthesis of compounds of the invention which utilize an activation group. In an optional pre-step, the activation group is attached the oligonucleotide synthesis support (i), or the oligonucleotide synthesis support with activation group is otherwise obtained. In step ii) region B is synthesized, followed by region A (iii). The oligomer is then cleaved from the oligonucleotide synthesis support (iv). The intermediate oligomer (comprising an activation group) may then be activated (vl) or (viii) and a third region (X) added (vi), optionally via a linker (Y) (ix). In some embodiments, X (or Y when present) is attached to region B via a phosphorus nucleoside linkage group, such as phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate or methylphosphonate, or an alternative linkage, such as a triazol linkage..

Figure 8. A non-limiting example of a method of synthesis of compounds of the invention, wherein

a bifunctional oligonucleotide synthesis support is used (i). In such a method, either the oligonucleotide is synthesized in an initial series of steps (ii) - (iii), followed by the attachment of the third region (optionally via a linker group Y), the oligomeric compound of the invention may then be cleaved (v). Alternatively, as shown in steps (vi) - (ix), the third region (optionally with a linker group (Y) is attached to the oligonucleotide synthesis support (this may be an optional pre-step) - or a oligonucleotide synthesis support with the third region (optionally with Y) is otherwise provided, , the oligonucleotide is then synthesized (vii - viii). The oligomeric compound of the invention may then be cleaved (ix). In some embodiments, X (or Y when present) is attached to region B via a phosphorus nucleoside linkage group, such as phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate or methylphosphonate, or an alternative linkage, such as a triazol linkage. The US may in some embodiment, prior to the method (such as the pre-step) comprise a step of adding a bidirectional (bifunctional) group which allows the independent synthesis of the oligonucleotide and the covalent attachment of group X, Y (or X and Y) to support (as shown) - this may for example be achieved using a triazol or of nucleoside group. The bidirectional (bifunctional) group, with the oligomer attached, may then be cleaved from the support.

Figure 9. A non-limiting example of a method of synthesis of compounds of the invention: In an initial step, the first region (A) is synthesized (ii), followed by region B. In some embodiments the third region is then attached to region B (iii), optionally via a phosphate nucleoside linkage (or e.g. a trialzol linkage). The oligomeric compound of the invention may then be cleaved (iv). When a linker(Y) is used, in some embodiments the steps (v) - (viii) may be followed: after synthesis of region B, the linker group (Y) is added, and then either attached to (Y) or in a subsequent step, region X is added (vi). The oligomeric compound of the invention may then be cleaved (vii). In some embodiments, X (or Y when present) is attached to region B via a phosphorus nucleoside linkage group, such as phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate or methylphosphonate, or an alternative linkage, such as a triazol linkage.

Figure 10. A non-limiting example of a method of synthesis of compounds of the invention: In this method an activation group is used: Steps (i) - (iii) are as per Figure 9. However after the oligonucleotide synthesis (step iii), an activation group (or a reactive group) is added to region B, optionally via a phosphate nucleoside linkage. The oligonucleotide is then cleaved from the support (v). The activation group may be subsequently activated to produce a reactive group, and then the third region (X), such as the conjugate, blocking group or targeting group, is added to the reactive group (which may be the activated activation group or the reactive group), to produce the oligomer (vi). As shown in (vii) - (viii), after cleavage, a linker group (Y) is added (vii), and then either attached to (Y) or in a subsequent step, region X is added to produce the oligomer (viii). It should be recognized that in an alternative all of the steps (ii) - (viii) may be performed on the oligonucleotide synthesis support, and in such instances a final step of cleaving the oligomer from the support may be performed. In some embodiments, the reactive group or activation group is attached to region B via a phosphorus nucleoside linkage group, such as phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate or methylphosphonate, or an alternative linkage, such as a triazol linkage.

Figure 11. Silencing of ApoB mRNA with Cholesterol-conjugates *in vivo*. Mice were injected with a single dose of 1 mg/kg unconjugated LNA-antisense oligonucleotide (#3833) or equimolar amounts of LNA antisense oligonulceotides conjugated to Cholesterol with different linkers (Tab. 3) and

sacrificed at days 1, 3, 7 and 10 after dosing. RNA was isolated from liver and kidney and subjected to ApoB specific RT-qPCR A. Quantification of ApoB mRNA from liver samples normalized to GAPDH and shown as percentage of the average of equivalent saline controls B. Quantification of ApoB mRNA from kidney samples normalized to GAPDH and shown as percentage of the average of equivalent saline controls.

Figure 12. Shows the cholesterol C6 conjugate which may be used as X-Y- in compounds of the invention, as well as specific compounds used in the examples, include specific compounds of the invention.

Figure 13: Examples of tri-GalNac conjugates which may be used. Conjuagtes 1 - 4 illustrate 4 suitable GalNac conjugate moieties, and conjugates 1a - 4a refer to the same conjugates with an additional linker moiety (Y) which is used to link the conjugate to the oligomer (region A or to a biocleavable linker, such as region B). The wavy line represents the covalent link to the oligomer.

Figure 14: Examples of cholesterol and tocopherol conjugate moieties. The wavy line represents the covalent link to the oligomer.

Figure 15: *In vivo* silencing of ApoB mRNA with different conjugates (See example 4). Mice were treated with 1 mg/kg of ASO with different conjugates either without biocleavable linker, with Dithio-linker (SS) or with DNA/PO-linker (PO). RNA was isolated from liver (A)and kidney samples (B) and analysed for ApoB mRNA knock down. Data is shown compared to Saline (=1).

Figure 16: Example 8 - ApoB mRNA expression

Figure 17: Example 8 - Total cholesterol in serum

Figure 18: Example 8 - Oligonucleotide content in liver and kidney

DETAILED DESCRIPTION OF INVENTION

[0029] The application describes oligomeric compounds which targets an ApoB nucleic acid, such as LNA antisense oligonucleotides, which are covalently linked to a conjugate group, a targeting group, a reactive group, an activation group, or a blocking group, via a short region comprising (e.g. 1 - 10) of phosphodiester linked DNA or RNA nucleoside(s).

The Oligomer

[0030] The term "oligomer" in the context of the present invention, refers to a molecule formed by covalent linkage of two or more nucleotides (*i.e.* an oligonucleotide). Herein, a single nucleotide (unit) may also be referred to as a monomer or unit. In some embodiments, the terms "nucleoside", "nucleotide", "unit" and "monomer" are used interchangeably. It will be recognized that when referring to a sequence of nucleotides or monomers, what is referred to is the sequence of bases, such as A, T, G, C or U.

[0031] The oligomer of the conjugate compound of the invention is an LNA oligomer, i.e. comprises at least one LNA nucleoside unit, or a gapmer, such as an LNA gapmer.

[0032] The oligomer may comprise between 10 - 22, such as 12 - 22 nucleotides in length. The oligomer of the conjugate compound of the invention may comprise a contiguous sequence of 10 - 20 nucleotides which are complementary, such as fully complementary, to a corresponding length of the ApoB nucleic acid target (such as NM_000384 or genbank accession No: NG_011793, NM_000384.2 GI:105990531 and NG_011793.1 GI:226442987). The contiguous sequence of 10 - 20 nucleotides may belinked, for example, by phosphorothioate linkages.

[0033] For example, the oligomer of the conjugate compound of the invention may comprise the sequence of nucleobases shown in SEQ ID NO 1 or SEQ ID No 2.

[0034] The compound (e.g. oligomer conjugate) of the invention targets ApoB, and as such is capable of inhibiting ApoB, such as human ApoB, in a cell expressing said ApoB.

[0035] In some embodiments, the internucleoside linkages of the contiguous sequence may be phosphorothioate linkages, and may comprise affinity enhancing nucleotide analogues.

[0036] In some embodiments, the nucleotide analogues are sugar modified nucleotides, such as sugar modified nucleotides independently or dependently selected from the group consisting of: Locked Nucleic Acid (LNA or BNA) units; 2'-O-alkyl-RNA units, 2'-OMe-RNA units, 2'-amino-DNA units, and 2'-fluoro-DNA units.

[0037] In some embodiments, the nucleotide analogues comprise or are Locked Nucleic Acid (LNA, also known as BNA) units.

[0038] In some embodiments, the oligomer of the conjugate compound of the invention comprises or is a gapmer, such as a LNA gapmer oligonucleotide.

[0039] In some embodiments, the oligomer of the conjugate compound of the invention comprises a contiguous sequence of 13, 14, 15 or 16 nucleotides which are complementary to a corresponding length of the ApoB nucleic acid target, and may optionally comprise a further 1 -10, for example 1 - 6 nucleoteotides, which may form or comprise a biocleavable nucleotide region, such as a phosphate nucleotide linker. Suitably, the biocleavable nucleotide region is formed of a short stretch (eg. 1, 2, 3, 4, 5 or 6) of nucleotides which are physiologically labile. This may be achieved by using phosphodiester linkages with DNA/RNA nucleosides, or if physiological liability can be maintained, other nucleosides may be used.

[0040] The oligomer of the conjugate compound of the invention may therefore comprise of a contiguous nucleotide sequence of 10 - 20 nts in length which is complementary to a corresponding length of the ApoB nucleic acid target (A first region, or region A). The oligomer of the conjugate compound of the invention may comprise a further nucleotide region. In some embodiments, the further nucleotide region comprises a biocleavable nucleotide region, such as a phosphate nucleotide sequence (a second region, region B), which may covalently link region A to a non-nucleotide moiety, such as a conjugate group, (a third region, or region C). In some embodiments the contiguous nucleotide sequence of the oligomer of the conjugate compound of the invention (region A) is directly covalently linked to region C. In some embodiments region C is biocleavable.

[0041] The may oligomer consists or comprises of a contiguous nucleotide sequence of from 10 - 22, such as 13, 14, 15, 16, 17, 18, 19, 20, 21, nucleotides in length, such as 13 - 16, or 13 or 14, or 15 or 16 nucleotides in length.. The oligomer may therefore refer to the combined length of region A and region B, e.g. (Region A 10 - 16nt) and region B (1 - 6nt).

[0042] In various embodiments, the compound of the invention does not comprise RNA (units). In some embodiments, the compound according to the invention, the first region, or the first and second regions together (e.g. as a single contiguous sequence), is a linear molecule or is synthesized as a linear molecule. The oligomer may therefore be single stranded molecule. In some embodiments, the oligomer does not comprise short regions of, for example, at least 3, 4 or 5 contiguous nucleotides, which are complementary to equivalent regions within the same oligomer (*i.e.* duplexes). The oligomer, in some embodiments, may be not (essentially) double stranded. In some embodiments, the oligomer is essentially not double stranded, such as is not a siRNA.

The Target

[0043] Suitably the oligomer conjugate of the invention is capable of down-regulating expression of the APO-B gene, such as ApoB-100 or ApoB-48 (APOB). In this regards, the oligomer of the conjugate compound of the invention can affect the inhibition of APOB, typically in a mammalian such as a human cell, such as liver cells. In some embodiments, the oligomers of the conjugate compound of the invention bind to the target nucleic acid and effect inhibition of expression of at least 10% or 20% compared to the normal expression level, more preferably at least a 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% inhibition compared to the normal expression level. In some embodiments, such modulation is seen when using between 0.04 and 25nM, such as between 0.8 and 20nM concentration of the compound of the invention. In the same or a different embodiment, the inhibition of expression is less than 100%, such as less than 98% inhibition, less than 95% inhibition, less than 90% inhibition, less than 80% inhibition, such as less than 70% inhibition. Modulation of expression level may be determined by measuring protein levels, *e.g.* by the methods such as SDS-PAGE followed by western blotting using suitable antibodies raised against the target protein. Alternatively, modulation of expression levels can be determined by measuring levels of mRNA, *e.g.* by northern blotting or quantitative RT-PCR. When measuring via mRNA levels, the level of down-regulation when using an appropriate dosage, such as between 0.04 and 25nM, such as between 0.8 and 20nM concentration, is, In some embodiments, typically to a level of between 10-20% the normal levels in the absence of the compound of the invention.

[0044] The application describes a method of down-regulating or inhibiting the expression of APO-B protein and/or mRNA in a cell which is expressing APO-B protein and/or mRNA, said method comprising administering the compound of the invention to the invention to said cell to down-regulating or inhibiting the expression of APO-B protein and/or mRNA in said cell. Suitably the cell is a mammalian cell such as a human cell. The administration may occur, in some embodiments, *in* *vitro*. The administration may occur, in some embodiments, *in vivo*.

[0045] The term "target nucleic acid", as used herein refers to the DNA or RNA encoding mammalian APO-B polypeptide, such as human APO-B100, such as human APO-B100 mRNA. APO-B100 encoding nucleic acids or naturally occurring variants thereof, and RNA nucleic acids derived therefrom, preferably mRNA, such as pre-mRNA, although preferably mature mRNA. In some embodiments, for example when used in research or diagnostics the "target nucleic acid" may be a cDNA or a synthetic oligonucleotide derived from the above DNA or RNA nucleic acid targets. The oligomer according to the invention is preferably capable of hybridising to the target nucleic acid. It will be recognised that human APO-B mRNA is a cDNA sequence, and as such, corresponds to the mature mRNA target sequence, although uracil is replaced with thymidine in the cDNA sequences.

[0046] The term "naturally occurring variant thereof" refers to variants of the APO-B1 polypeptide of nucleic acid sequence which exist naturally within the defined taxonomic group, such as mammalian, such as mouse, monkey, and preferably human. Typically, when referring to "naturally occurring variants" of a polynucleotide the term also may encompass any allelic variant of the APO-B encoding genomic DNA by chromosomal translocation or duplication, and the RNA, such as mRNA derived therefrom. "Naturally occurring variants" may also include variants derived from alternative splicing of the APO-B100 mRNA. When referenced to a specific polypeptide sequence, *e.g.,* the term also includes naturally occurring forms of the protein which may therefore be processed, e.g. by co- or post-translational modifications, such as signal peptide cleavage, proteolytic cleavage, glycosylation, etc.

[0047] The oligomers (region A) may comprise or consist of a contiguous nucleotide sequence which corresponds to the reverse complement of a nucleotide sequence present in e.g. the human APO-B mRNA.

[0048] The oligomer (region A) may comprise or consist of a contiguous nucleotide sequence which is fully complementary (perfectly complementary) to the equivalent region of a nucleic acid which encodes a mammalian APO-B (e.g., human APO-B100 mRNA). Thus, the oligomer (region A) can comprise or consist of an antisense nucleotide sequence.

[0049] However, in some embodiments, the oligomer of the conjugate compound of the invention may tolerate 1 or 2 mismatches, when hybridising to the target sequence and still sufficiently bind to the target to show the desired effect, *i.e.* down-regulation of the target. Mismatches may, for example, be compensated by increased length of the oligomer nucleotide sequence and/or an increased number of nucleotide analogues, such as LNA, present within the nucleotide sequence.

[0050] It is recognised that, in some embodiments the nucleotide sequence of the oligomer may comprise additional 5' or 3' nucleotides, such as, independently, 1, 2, 3, 4, 5 or 6 additional nucleotides 5' and/or 3', which are non-complementary to the target sequence - such non-complementary oligonucleotides may form region B. In this respect the oligomer of the invention, may, in some embodiments, comprise a contiguous nucleotide sequence which is flanked 5' and or 3' by additional nucleotides. In some embodiments the additional 5' or 3' nucleotides are naturally occurring nucleotides, such as DNA or RNA. In some embodiments, the additional 5' or 3' nucleotides may represent region D as referred to in the context of gapmer oligomers herein. In some embodiments the internucleoside linkages between the additional nucleotides, and optionally between the additional nucleotides and the oligomer are phosphodiester linkages.

[0051] In some embodiments the oligomer of the conjugate compound of the invention consists or comprises of a nucleotide sequence according to SEQ ID NO:1, or a sub-sequence of at least 10 or 12 nucleobases thereof.

[0052] In some embodiments the oligomer of the conjugate compound of the invention consists or comprises of a nucleotide sequence according to SEQ ID NO:2, or a sub-sequence of at least 10 or 12 nucleobases thereof.
The following Table provides specific combinations of oligomer and conjugates:
Table 1: Oligomer/conjugate combinations.

SEQ ID	Conjugate Number (See figures)									
	Conj1	Conj2	Conj3	Conj4	Conj1a	Conj2a	Conj3a	Conj4a	Conj5	Conj6
1	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
2	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20

[0053] Please note that a- blocleavable linker (B) may or may not be present between the conjugate moiety(C) and the oligomer(A). For Conj1 - 4 and 1a - 4a the GalNac conjugate itself is biocleavable, utilizing a peptide linker in the GalNac cluster, and as such a further biocleavable linker (B) may or may not be used. However, preliminary data indicates inclusion of a biocleavable linker (B), such as the phosphate nucleotide linkers disclosed herein may enhance activity of such GalNac cluster oligomer conjugates. For use with Conj 5 and Conj 6, the use of a biocleavable linker greatly enhances compound activity inclusion of a biocleavable linker (B), such as the phosphate nucleotide linkers disclosed herein is recommended. The conjugate moiety (and region B or region Y or B and Y, may be positioned, e.g. 5' or 3' to the SEQ ID, such as 5' to region A.
The terms "corresponding to" and "corresponds to" refer to the comparison between the nucleotide sequence of the oligomer (*i.e.* the nucleobase or base sequence) or contiguous nucleotide sequence (a first region/region A) and the reverse complement of the nucleic acid target, or sub-region thereof.

[0054] Nucleotide analogues are compared directly to their equivalent or corresponding nucleotides. In a preferred embodiment, the oligomers (or first region thereof) are complementary to the target region or sub-region, such as fully complementary.

[0055] The terms "reverse complement", "reverse complementary" and "reverse complementarity" as used herein are interchangeable with the terms "complement", "complementary" and "complementarity".

[0056] The terms "corresponding nucleotide analogue" and "corresponding nucleotide" are intended to indicate that the nucleotide in the nucleotide analogue and the naturally occurring nucleotide are identical. For example, when the 2-deoxyribose unit of the nucleotide is linked to an adenine, the "corresponding nucleotide analogue" contains a pentose unit (different from 2-deoxyribose) linked to an adenine.

[0057] The term "nucleobase" refers to the base moiety of a nucleotide and covers both naturally occurring a well as non-naturally occurring variants. Thus, "nucleobase" covers not only the known purine and pyrimidine heterocycles but also heterocyclic analogues and tautomeres thereof. It will be recognised that the DNA or RNA nucleosides of region B may have a naturally occurring and/or non-naturally occurring nucleobase(s).

[0058] Examples of nucleobases include, but are not limited to adenine, guanine, cytosine, thymidine, uracil, xanthine, hypoxanthine, 5-methylcytosine, isocytosine, pseudoisocytosine, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine, and 2-chloro-6-aminopurine. In some embodiments the nucleobases may be independently selected from the group consisting of adenine, guanine, cytosine, thymidine, uracil, 5-methylcytosine. In some embodiments the nucleobases may be independently selected from the group consisting of adenine, guanine, cytosine, thymidine, and 5-methylcytosine.

[0059] In some embodiments, at least one of the nucleobases present in the oligomer is a modified nucleobase selected from the group consisting of 5-methylcytosine, isocytosine, pseudoisocytosine, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine, and 2-chloro-6-aminopurine.

Length

[0060] The oligomers may comprise or consist of a contiguous nucleotide sequence of a total of between 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22 contiguous nucleotides in length. Lengths may include region A or region A and B for example.

[0061] In some embodiments, the oligomers of the conjugate compound of the invention comprise or consist of a contiguous nucleotide sequence of a total of between 10 - 22, such as 12 - 18, such as 13 - 17 or 12 - 16, such as 13, 14, 15, 16 contiguous nucleotides in length.

[0062] In some embodiments, the oligomer of the conjugate compound of the invention consists of no more than 22 nucleotides, such as no more than 20 nucleotides, such as no more than 18 nucleotides, such as 15, 16 or 17 nucleotides. In some embodiments the oligomer of the invention comprises less than 20 nucleotides.

Nucleotide analogues

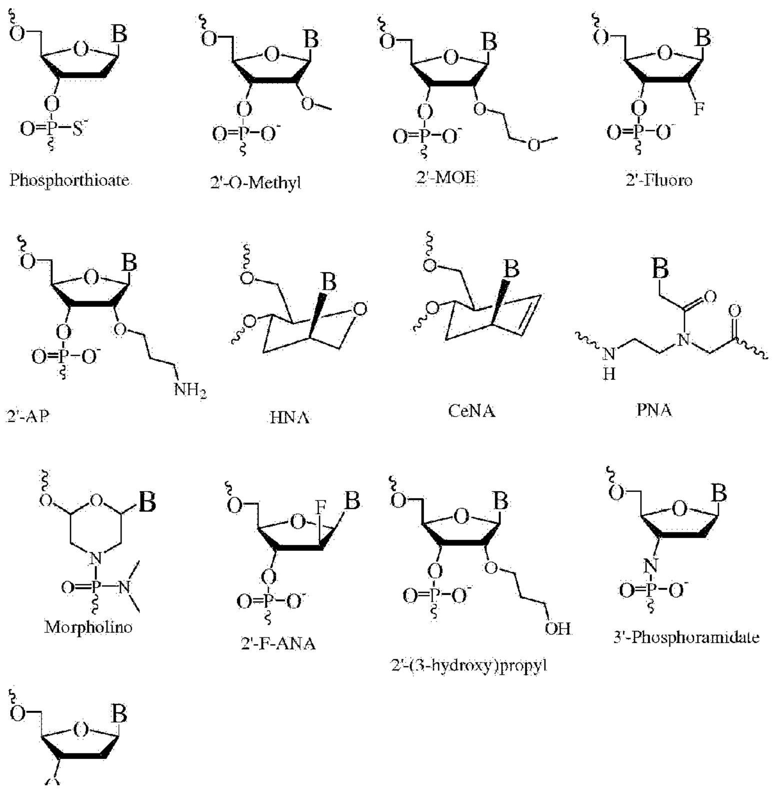
[0063] The term "nucleotide" as used herein, refers to a glycoside comprising a sugar moiety, a base moiety and a covalently linked group, such as a phosphate or phosphorothioate internucleotide linkage group, and covers both naturally occurring nucleotides, such as DNA or RNA, and non-naturally occurring nucleotides comprising modified sugar and/or base moieties, which are also referred to as "nucleotide analogues" herein. Herein, a single nucleotide (unit) may also be referred to as a monomer or nucleic acid unit.

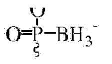
[0064] In field of biochemistry, the term "nucleoside" is commonly used to refer to a glycoside comprising a sugar moiety and a base moiety, and may therefore be used when referring to the nucleotide units, which are covalently linked by the internucleotide linkages between the nucleotides of the oligomer.

[0065] As one of ordinary skill in the art would recognise, the 5' nucleotide of an oligonucleotide does not comprise a 5' internucleotide linkage group, although may or may not comprise a 5' terminal group.

[0066] Non-naturally occurring nucleotides include nucleotides which have modified sugar moieties, such as bicyclic nucleotides or 2' modified nucleotides, such as 2' substituted nucleotides.

[0067] "Nucleotide analogues" are variants of natural nucleotides, such as DNA or RNA nucleotides, by virtue of modifications in the sugar and/or base moieties. Analogues could in principle be merely "silent" or "equivalent" to the natural nucleotides in the context of the oligonucleotide, *i.e.* have no functional effect on the way the oligonucleotide works to inhibit target gene expression. Such "equivalent" analogues may nevertheless be useful if, for example, they are easier or cheaper to manufacture, or are more stable to storage or manufacturing conditions, or represent a tag or label. Preferably, however, the analogues will have a functional effect on the way in which the oligomer works to inhibit expression; for example by producing increased binding affinity to the target and/or increased resistance to intracellular nucleases and/or increased ease of transport into the cell. Specific examples of nucleoside analogues are described by *e.g.* Freier & Altmann; Nucl. Acid Res., 1997, 25, 4429-4443 and Uhlmann; Curr. Opinion in Drug Development, 2000, 3(2), 293-213, and in Scheme 1:





Boranophosphates

Scheme 1

[0068] The oligomer may thus comprise or consist of a simple sequence of natural occurring nucleotides - preferably 2'-deoxynucleotides (referred here generally as "DNA"), but also possibly ribonucleotides (referred here generally as "RNA"), or a combination of such naturally occurring nucleotides and one or more non-naturally occurring nucleotides, *i.e.* nucleotide analogues. Such nucleotide analogues may suitably enhance the affinity of the oligomer for the target sequence.

[0069] Examples of suitable and preferred nucleotide analogues are provided by WO2007/031091 or are referenced therein. Other nucleotide analogues which may be used in the oligomer of the conjugate compound of the invention include tricyclic nucleic acids, for example please see WO2013154798 and WO2013154798.

[0070] Incorporation of affinity-enhancing nucleotide analogues in the oligomer, such as LNA or 2'-substituted sugars, can allow the size of the specifically binding oligomer to be reduced, and may also reduce the upper limit to the size of the oligomer before non-specific or aberrant binding takes place.

[0071] In some embodiments the oligomer comprises at least 2 nucleotide analogues. In some embodiments, the oligomer comprises from 3-8 nucleotide analogues, *e.g.* 6 or 7 nucleotide analogues. In the by far most preferred embodiments, at least one of said nucleotide analogues is a locked nucleic acid (LNA); for example at least 3 or at least 4, or at least 5, or at least 6, or at least 7, or 8, of the nucleotide analogues may be LNA. In some embodiments all the nucleotides analogues may be LNA.

[0072] It will be recognised that when referring to a preferred nucleotide sequence motif or nucleotide sequence, which consists of only nucleotides, the oligomers of the invention which are defined by that sequence may comprise a corresponding nucleotide analogue in place of one or more of the nucleotides present in said sequence, such as LNA units or other nucleotide analogues, which raise the duplex stability/T_m of the oligomer/target duplex (*i.e.* affinity enhancing nucleotide analogues).

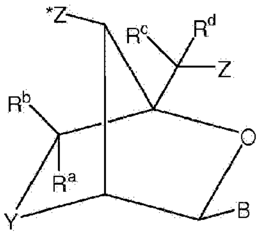
[0073] **T_m Assay:** The oligonucleotide: Oligonucleotide and RNA target (PO) duplexes are diluted to 3 mM in 500 ml RNase-free water and mixed with 500 ml 2x T_m-buffer (200mM NaCl, 0.2mM EDTA, 20mM Naphosphate, pH 7.0). The solution is heated to 95ºC for 3 min and then allowed to anneal in room temperature for 30 min. The duplex melting temperatures (T_m) is measured on a Lambda 40 UV/VIS Spectrophotometer equipped with a Peltier temperature programmer PTP6 using PE Templab software (Perkin Elmer). The temperature is ramped up from 20ºC to 95ºC and then down to 25ºC, recording absorption at 260 nm. First derivative and the local maximums of both the melting and annealing are used to assess the duplex T_m.

LNA

[0074] The term "LNA" refers to a bicyclic nucleoside analogue which comprises a C2* - C4* biradical (a bridge), and is known as "Locked Nucleic Acid". It may refer to an LNA monomer, or, when used in the context of an "LNA oligonucleotide", LNA refers to an oligonucleotide containing one or more such bicyclic nucleotide analogues. In some aspects bicyclic nucleoside analogues are LNA nucleotides, and these terms may therefore be used interchangeably, and is such embodiments, both are be characterized by the presence of a linker group (such as a bridge) between C2' and C4' of the ribose sugar ring.

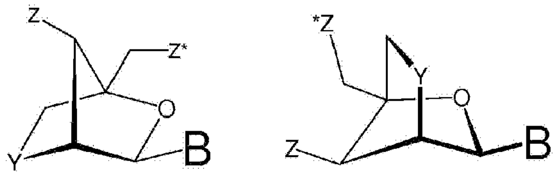
[0075] In some embodiments, at least one nucleoside analogue present in the first region (A) is a bicyclic nucleoside analogue, such as at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, (except the DNA and or RNA nucleosides of region B) are sugar modified nucleoside analogues, such as such as bicyclic nucleoside analogues, such as LNA, e.g. beta-D-X-LNA or alpha-L-X-LNA (wherein X is oxy, amino or thio), or other LNAs disclosed herein including, but not limited to,(R/S) cET, cMOE or 5'-Me-LNA.

[0076] In some embodiments the LNA used in the oligonucleotide compounds of the invention preferably has the structure of the general formula II:

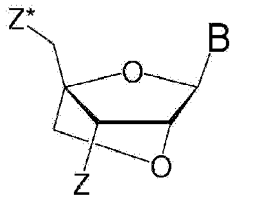


Formula II

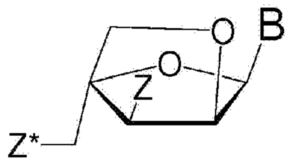
wherein Y is selected from the group consisting of -O-, -CH_2O-, -S-, -NH-, N(R^e) and/or-CH_2-; Z and Z* are independently selected among an internucleotide linkage, R^H, a terminal group or a protecting group; B constitutes a natural or non-natural nucleotide base moiety (nucleobase), and R^H is selected from hydrogen and C_{1-4}-alkyl; R^a, R^b R^c, R^d and R^e are, optionally independently, selected from the group consisting of hydrogen, optionally substituted C_{1-12}-alkyl, optionally substituted C_{2-12}-alkenyl, optionally substituted C_{2-12}-alkynyl, hydroxy, C_{1-12}-alkoxy, C_{2-12}-alkoxyalkyl, C_{2-12}-alkenyloxy, carboxy, C_{1-12}-alkoxycarbonyl, C_{1-12}-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C_{1-6}-alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)-amino-carbonyl, amino-C_{1-6}-alkyl-aminocarbonyl, mono-and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, C_{1-6}-alkanoyloxy, sulphono, C_{1-6}-alkylsulphonyloxy, nitro, azido, sulphanyl, C_{1-6}-alkylthio, halogen, DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands, where aryl and heteroaryl may be optionally substituted and where two geminal substituents R^a and R^b together may designate optionally substituted methylene (=CH_2); and R^H is selected from hydrogen and C_{1-4}-alkyl. In some embodiments R^a, R^b R^c, R^d and R^e are, optionally independently, selected from the group consisting of hydrogen and C_{1-6} alkyl, such as methyl. For all chiral centers, asymmetric groups may be found in either *R* or *S* orientation, for example, two exemplary stereochemical isomers include the beta-D and alpha-L isoforms, which may be illustrated as follows:



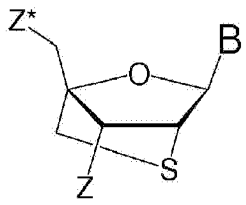
[0077] Specific exemplary LNA units are shown below:



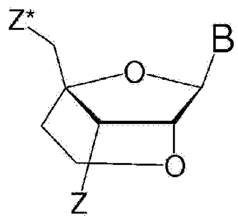
β-D-oxy-LNA



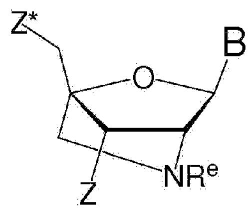
α-L-Oxy-LNA



β-D-thio-LNA



β-D-ENA



β-D-amino-LNA

[0078] The term "thio-LNA" comprises a locked nucleotide in which Y in the general formula above is selected from S or -CH_2-S-. Thio-LNA can be in both beta-D and alpha-L-configuration.

[0079] The term "amino-LNA" comprises a locked nucleotide in which Y in the general formula above is selected from -N(H)-, N(R)-, CH_2-N(H)-, and -CH_2-N(R)- where R is selected from hydrogen and C_{1-4}-alkyl. Amino-LNA can be in both beta-D and alpha-L-configuration.

[0080] The term "oxy-LNA" comprises a locked nucleotide in which Y in the general formula above represents -O-. Oxy-LNA can be in both beta-D and alpha-L-configuration.

[0081] The term "ENA" comprises a locked nucleotide in which Y in the general formula above is -CH_2-O- (where the oxygen atom of -CH_2-O- is attached to the 2'-position relative to the base B). R^e is hydrogen or methyl.

In some exemplary embodiments LNA is selected from beta-D-oxy-LNA, alpha-L-oxy-LNA, beta-D-amino-LNA and beta-D-thio-LNA, in particular beta-D-oxy-LNA.

[0082] 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 some embodiments, compounds provided herein include one or more bicyclic nucleosides wherein the bridge comprises a 4' to 2' bicyclic nucleoside. Examples of such 4' to 2' bicyclic nucleosides, include, but are not limited to, one of the formulae: 4'-(CH_2)- O -2' (LNA); 4'-(CH_2)-S-2'; 4'-$(CH_2)_2$- O -2' (ENA); 4'-$CH(CH_3)$- O -2' and 4'-$CH(CH_2OCH_3)$-O-2*, and analogs thereof (see, U.S. Patent 7,399,845, issued on July 15, 2008); 4'-$C(CH_3)(CH_3)$-O-2', and analogs thereof (see, published PCT International Application WO2009/006478, published January 8, 2009); 4'-CH_2-$N(OCH_3)$-2', and analogs thereof (see, published PCT International Application WO2008/150729, published December 11, 2008); 4'-CH_2-$ON(CH_3)$-2' (see, published U.S. Patent Application US2004/0171570, published September 2, 2004); 4'-CH_2-N(R)- O -2', wherein R is H, C_1-C_{10} alkyl, or a protecting group (see, U.S. Patent 7,427,672, issued on September 23, 2008); 4'-CH_2-C(H)(CH_3)-2' (see, Chattopadhyaya, et al, J. Org. Chem.,2009, 74, 118-134); and 4'-CH_2-C(=CH_2)-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, 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; Oram et al, Curr. Opinion Mol. Ther., 2001, 3, 239-243; U.S. Patent Nos U.S. 6,670,461, 7,053,207, 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. WO08150729, WO08154401, and WO09006478. Each of the foregoing bicyclic nucleosides can be prepared having one or more stereochemical sugar configurations including for example a-L-ribofuranose and beta -D-ribofuranose (see PCT international application PCT DK98/00393, published on March 25, 1999 as WO 99/14226).

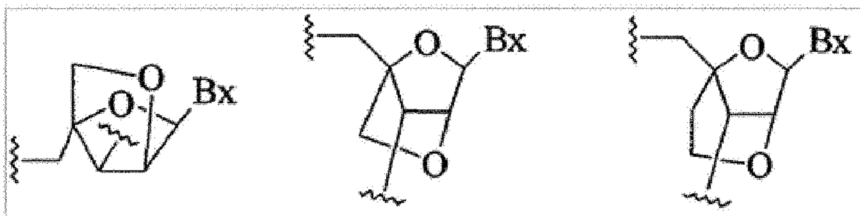
[0083] In some embodiments, bicyclic sugar moieties of BNA nucleosides include, but are not limited to, compounds having at least one bridge between the 4' and the 2' position of the pentofuranosyl sugar moiety wherein such bridges independently comprises 1 or from 2 to 4 linked groups independently selected from - $[CiR_aXR_b)]$,,-, -$C(R_a)$=$C(R_b)$-, -$C(R_a)$=N-,-C(=NR_a)-, -C(=O)-, -C(=S)-, - O -, -$Si(R_a)_2$-, -$S(=O)_x$-, and -N(Ra)-; 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 ($S(=O)_2$-J_1), or sulfoxyl (S(=O)-J_1); and each J_1 and J_2 is, independently, H, C_1-C_6 alkyl, substituted C_1-C_{12}alkyl, C_2-C_{12} alkenyl, substituted C_2-

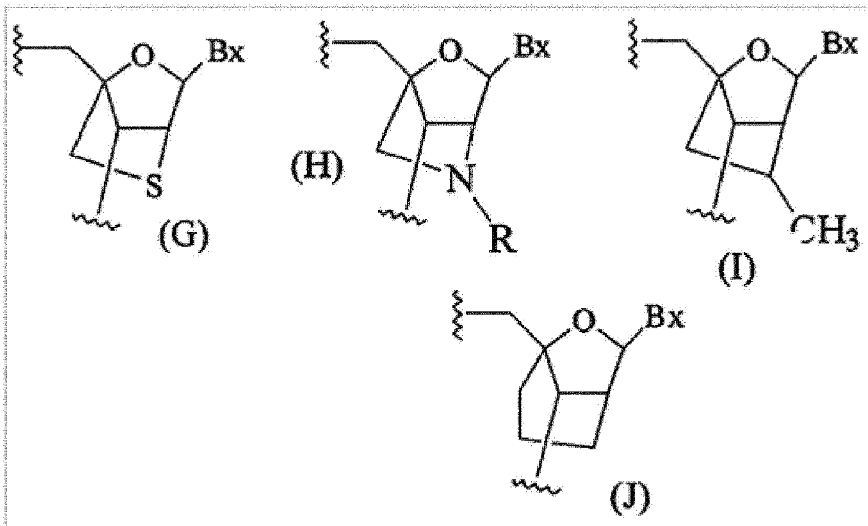
C_{12} alkenyl, C_2-C_{12} alkynyl, substituted C_2-C_{12} alkynyl, C_5-C_{20} aryl, substituted C_5-C_{20} aryl, acyl (C(=O)- H), substituted acyl, a heterocycle radical, a substituted heterocycle radical, C_1-C_{12} aminoalkyl, substituted C_1-C_{12} aminoalkyl, or a protecting group.

[0084] In some embodiments, the bridge of a bicyclic sugar moiety is, -[C(R_a)(Rb)]$_n$-,-[C(R_a)(R_b)]$_n$-O -, -C(R_aR_b)-N(R)- O - or, -C(R_aR_b)- O -N(R)-. In some 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.

[0085] In some embodiments, bicyclic nucleosides are further defined by isomeric configuration. For example, a nucleoside comprising a 4'-2' methylene-oxy bridge, may be in the a-L configuration or in the beta - D configuration. Previously, a-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).

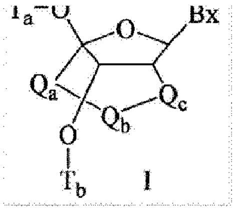
[0086] In some embodiments, bicyclic nucleosides include, but are not limited to, (A) a-L-Methyleneoxy (4'-CH_2-O-2') BNA, (B) beta -D-Methyleneoxy (4'-CH_2-O-2') BNA, (C) Ethyleneoxy (4'-$(CH_2)_2$-O-2') BNA, (D) Aminooxy (4'-CH_2-O-N(R)-2') BNA, (E) Oxyamino (4'-CH_2-N(R)-O-2') BNA, (F), Methyl(methyleneoxy) (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 C_1-C_2 alkyl. odiments, bicyclic nucleoside having Formula I:

In certain embo



wherein:

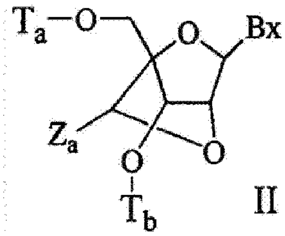
Bx is a heterocyclic base moiety;

$-Q_a-Q_b-Q_c-$ is $-CH_2-N(Rc)-CH_2-$, $-C(=O)-N(R_c)-CH_2-$, $-CH_2-O-N(Rc)-$, $-CH_2-N(Rc)-O-$, or$-N(Rc)-O-CH_2$;

R_c is C_1-C_{12} 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.

In some embodiments, bicyclic nucleoside having Formula II:



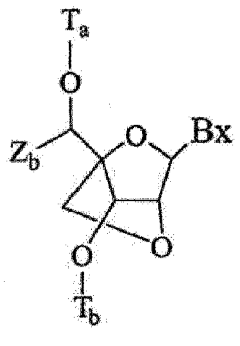
wherein:

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_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 some embodiments, each of the substituted groups is, independently, mono or poly substituted with substituent groups independently selected from halogen, oxo, hydroxyl, OJ_c, NJ_d, SJ_C, N_3, $OC(=X)J_c$, and $NJ_eC(=X)NJ_cJ_d$, 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 some embodiments, bicyclic nucleoside having Formula III:



III

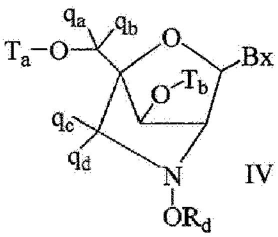
wherein:

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 some embodiments, bicyclic nucleoside having Formula IV:



IV

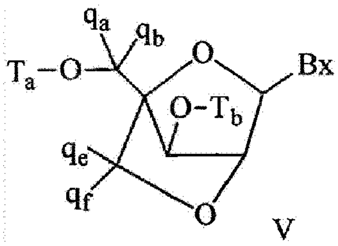
wherein:

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

[0087] In some embodiments, bicyclic nucleoside having Formula V:



V

wherein:

Bx is a heterocyclic base moiety;

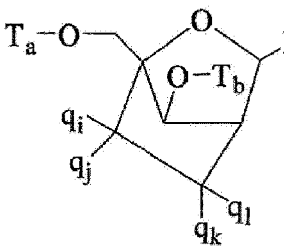
T_a and T_b are each, independently, H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety, or a covalent attachment to a support medium; q_a, q_b, q_c and q_f are each, independently, hydrogen, halogen, C_1-C_{12} alkyl, substituted C_1-C_{12} alkyl, C_2- 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_e 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.
Analogs 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 comformationally 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 some embodiments, the bicyclic nucleoside has Formula VI:

In certain embodiments, bicyc

[0088]



VI

wherein:

Bx is a heterocyclic base moiety;

T_a and T_b are each, independently, H, a hydroxyl protecting group, a conjuate group, a reactive phosphorus group, a phosphorus moiety, or a covalent attachment to a support medium; each qj, qj, q_k and ql is, independently, H, 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} alkoxyl, substituted C_2- C_{12} alkoxyl, 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 $(H)C(=S)NJ_jJ_k$; and qi and q_j or ql and q_k together are $=C(q_g)(q_h)$, wherein q_g and q_h are each, independently, H, halogen, C_1-C_{12} alkyl, or substituted C_1-C_6 alkyl.

One carbocyclic bicyclic nucleoside having a 4'-$(CH_2)_3$-2' bridge and the alkenyl analog, bridge 4'-CH=CH-CH_2-2', have been described (see, e.g., Freier et al, Nucleic Acids Research, 1997, 25(22), 4429- 4443 and Albaek et al, J. Org. Chem., 2006, 71, 7731-77 '40). 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).

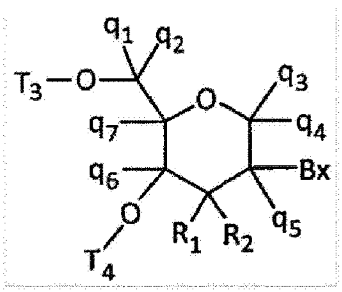
As used herein, "4'-2' bicyclic nucleoside" or "4' to 2' bicyclic nucleoside" refers to a bicyclic nucleoside comprising a furanose ring comprising a bridge connecting the 2' carbon atom and the 4' carbon atom.

As used herein, "monocylic nucleosides" refer to nucleosides comprising modified sugar moieties that are not bicyclic sugar moieties. In some 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 some 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 some embodiments, 2' modifications are selected from substituents including, but not limited to: $O[(CH_2)_nO]_mCH_3$, $O(CH_2)$,,NH_2, $O(CH_2)$,,CH_3, $O(CH_2)$,,ONH_2, $OCH_2C(=O)N(H)CH_3$, and $O(CH2)_nON[(CH_2)_nCH_3]2$, where n and m are from 1 to about 10. Other 2'- substituent groups can also be selected from: C_1-C_{12} alkyl; substituted alkyl; alkenyl; alkynyl; alkaryl; aralkyl; O-alkaryl or O-aralkyl; SH; SCH_3; OCN; Cl; Br; CN; CF_3; OCF_3; $SOCH_3$; SO_2CH_3; ONO_2; NO_2; N_3; NH_2; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an R; a 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 some embodiments, modified nucleosides comprise a 2'-MOE side chain {see, e.g., Baker et al., J. Biol. Chem., 1997, 272, 1 1944-12000). 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., He/v. 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).

[0089] 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, CJ. Bioorg. and Med. Chem. (2002) 10:841-854), fluoro HNA (F-HNA), or those compounds having Formula X:

Formula



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

Bx is a heterocyclic base moiety;

T_3 and T_4 are each, independently, an internucleoside linking group linking the tetrahydropyran nucleoside analog to the antisense compound or one of T_3 and T_4 is an internucleoside linking group linking the tetrahydropyran nucleoside analog to the antisense compound and the other of T_3 and T4 is H, a hydroxyl protecting group, a linked conjugate group, or a 5' or 3'-terminal group; q_1 q_2 q_3 q_4 q_5, q_6 and q_7 are each, independently, H, 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; and one of R_1 and R_2 is hydrogen and the other is selected from halogen, substituted or unsubstituted alkoxy, NJ,J_2, SJ,, 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 some 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 some 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 some embodiments, at least one of q_m, q_n, q_p, q_r, q_s, q_t and q_u is methyl. In some embodiments, THP nucleosides of Formula X are provided wherein one of R_1 and R_2 is F. In some 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.

[0090] 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 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$(CH_2)_2SCH_3$, 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,, is, independently, H or substituted or unsubstituted C_1-C_{10} alkyl. 2'-modifed nucleosides may further comprise other modifications, for example, at other positions of the sugar and/or at the nucleobase.

[0091] As used herein, "2'-F" refers to a sugar comprising a fluoro group at the 2' position.

[0092] As used herein, "2'-OMe" or "2'-OCH_3" or "2'-O-methyl" each refers to a nucleoside comprising a sugar comprising an -OCH_3 group at the 2' position of the sugar ring.

[0093] As used herein, "oligonucleotide" refers to a compound comprising a plurality of linked nucleosides.

[0094] In some embodiments, one or more of the plurality of nucleosides is modified. In some embodiments, an oligonucleotide comprises one or more ribonucleosides (RNA) and/or deoxyribonucleosides (DNA).

[0095] 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 and 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.

[0096] In some embodiments, antisense compounds comprise one or more nucleotides having modified sugar moieties. In some embodiments, the modified sugar moiety is 2'-MOE. In some embodiments, the 2'-MOE modified nucleotides are arranged in a gapmer motif. In some embodiments, the modified sugar moiety is a cEt. In some embodiments, the cEt modified nucleotides are arranged throughout the wings of a gapmer motif.

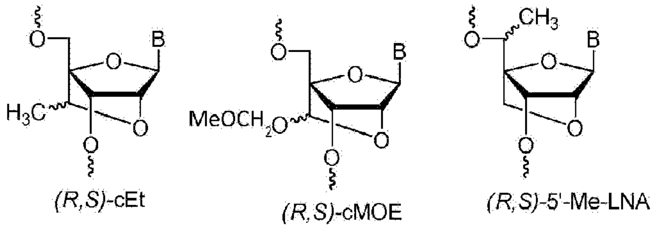
[0097] In some embodiments, in the BNA (LNA), R^{4*} and R^{2*} together designate the biradical -O-CH(CH_2OCH_3)- (2'O-methoxyethyl bicyclic nucleic acid - Seth at al., 2010, J. Org. Chem) - in either the R- or S- configuration.

[0098] In some embodiments, in the BNA (LNA), R^{4*} and R^{2*} together designate the biradical -O-CH(CH_2CH_3)- (2'O-ethyl bicyclic nucleic acid - Seth at al., 2010, J. Org. Chem). - in either the R- or S- configuration.

[0099] In some embodiments, in the BNA (LNA), R^{4*} and R^{2*} together designate the biradical -O-CH(CH_3)-. - in either the R- or S- configuration. In some embodiments, R^{4*} and R^{2*} together designate the biradical -O-CH_2-O-CH_2- - (Seth at al., 2010, J. Org. Chem).

[0100] In some embodiments, in the BNA (LNA), R^{4*} and R^{2*} together designate the biradical -O-NR-CH_3-- (Seth at al., 2010, J. Org. Chem) .

[0101] In some embodiments, the LNA units have a structure selected from the following group:



[0102] Incorporation of affinity-enhancing nucleotide analogues in the oligomer, such as BNA, (e.g.) LNA or 2'-substituted sugars, can allow the size of the specifically binding oligomer to be reduced, and may also reduce the upper limit to the size of the oligomer before non-specific or aberrant binding takes place.

[0103] In some embodiments, the oligomer of the conjugate compound of the invention comprises at least 1 nucleoside analogue. In some embodiments the oligomer comprises at least 2 nucleotide analogues. In some embodiments, the oligomer comprises from 3-8 nucleotide analogues, *e.g.* 6 or 7 nucleotide analogues. In the by far most preferred embodiments, at least one of said nucleotide analogues is a BNA, such as locked nucleic acid (LNA); for example at least 3 or at least 4, or at least 5, or at least 6, or at least 7, or 8, of the nucleotide analogues may be BNA, such as LNA. In some embodiments all the nucleotides analogues may be BNA, such as LNA.

[0104] It will be recognized that when referring to a preferred nucleotide sequence motif or nucleotide sequence, which consists of only nucleotides, the oligomers of the invention which are defined by that sequence may comprise a corresponding nucleotide analogue in place of one or more of the nucleotides present in said sequence, such as BNA units or other nucleotide analogues, which raise the duplex stability/T_m of the oligomer/target duplex (*i.e.* affinity enhancing nucleotide analogues).

[0105] A preferred nucleotide analogue is LNA, such as oxy-LNA (such as beta-D-oxy-LNA, and alpha-L-oxy-LNA), and/or amino-LNA (such as beta-D-amino-LNA and alpha-L-amino-LNA) and/or thio-LNA (such as beta-D-thio-LNA and alpha-L-thio-LNA) and/or ENA (such as beta-D-ENA and alpha-L-ENA).

[0106] In some embodiments, the oligomer of the conjugate compound of the invention, such as region A, may comprise BNA or LNA units and other nucleotide analogues. Further nucleotide analogues present within the oligomer of the invention are independently selected from, for example: 2'-O-alkyl-RNA units, 2'-amino-DNA units, 2'-fluoro-DNA units, BNA units, e.g. LNA units, arabino nucleic acid (ANA) units, 2'-fluoro-ANA units, HNA units, INA (intercalating nucleic acid - Christensen, 2002. Nucl. Acids. Res. 2002 30: 4918-4925) units and 2'MOE units. In some embodiments there is only one of the above types of nucleotide analogues present in the oligomer of the invention, such as the first region, or contiguous nucleotide sequence thereof.

[0107] In some embodiments, the oligomer of the conjugate compound of the invention (region A) may therefore comprises at least one BNA, e.g. Locked Nucleic Acid (LNA) unit, such as 1, 2, 3, 4, 5, 6, 7, or 8 BNA/LNA units, such as from 3 - 7 or 4 to 8 BNA/ LNA units, or 3, 4, 5, 6 or 7 BNA/LNA units. In some embodiments, all the nucleotide analogues are BNA, such as LNA. In some embodiments, the oligomer may comprise both beta-D-oxy-LNA, and one or more of the following LNA units: thio-LNA, amino-LNA, oxy-LNA, and/or ENA in either the beta-D or alpha-L configurations or combinations thereof. In some embodiments all BNA, such as LNA, cytosine units are 5'methyl-Cytosine. In some embodiments of the invention, the oligomer (such as the first and optionally second regions) may comprise both BNA and LNA and DNA units. In some embodiments, the combined total of LNA and DNA units is 10-25, such as 10 - 24, preferably 10-20, such as 10 - 18, such as 12-16. In some embodiments of the invention, the nucleotide sequence of the oligomer, of first region thereof, such as the contiguous nucleotide sequence consists of at least one BNA, e.g. LNA and the remaining nucleotide units are DNA units. In some embodiments the oligomer, or first region thereof, comprises only BNA, e.g. LNA, nucleotide analogues and naturally occurring nucleotides (such as RNA or DNA, most preferably DNA nucleotides), optionally with modified internucleotide linkages such as phosphorothioate.

RNAse recruitment

[0108] It is recognised that an oligomeric compound may function via non RNase mediated degradation of target mRNA, such as by steric hindrance of translation, or other methods, In some embodiments, the oligomers of the invention are capable of recruiting an endoribonuclease (RNase), such as RNase H.

[0109] It is preferable such oligomers, such as region A, or contiguous nucleotide sequence, comprises of a region of at least 6, such as at least 7 consecutive nucleotide units, such as at least 8 or at least 9 consecutive nucleotide units (residues), including 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 consecutive nucleotides, which, when formed in a duplex with the complementary target RNA is capable of recruiting RNase (such as DNA units). The contiguous sequence which is capable of recruiting RNAse may be region Y' as referred to in the context of a gapmer as described herein. In some embodiments the size of the contiguous sequence which is capable of recruiting RNAse, such as region Y', may be higher, such as 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotide units.

[0110] EP 1 222 309 provides *in vitro* methods for determining RNaseH activity, which may be used to determine the ability to recruit RNaseH. A oligomer is deemed capable of recruiting RNase H if, when provided with the complementary RNA target, it has an initial rate, as measured in pmol/l/min, of at least 1 %, such as at least 5%, such as at least 10% or ,more than 20% of the of the initial rate determined using DNA only oligonucleotide, having the same base sequence but containing only DNA monomers, with no 2' substitutions, with phosphorothioate linkage groups between all monomers in the oligonucleotide, using the methodology provided by Example 91 - 95 of EP 1 222 309.

[0111] In some embodiments, an oligomer is deemed essentially incapable of recruiting RNaseH if, when provided with the complementary RNA target, and RNaseH, the RNaseH initial rate, as measured in pmol/l/min, is less than 1%, such as less than 5%,such as less than 10% or less than 20% of the initial rate determined using the equivalent DNA only oligonucleotide, with no 2' substitutions, with phosphorothioate linkage groups between all nucleotides in the oligonucleotide, using the methodology provided by Example 91 - 95 of EP 1 222 309.

[0112] In other embodiments, an oligomer is deemed capable of recruiting RNaseH if, when provided with the complementary RNA target, and RNaseH, the RNaseH initial rate, as measured in pmol/l/min, is at least 20%, such as at least 40 %, such as at least 60 %, such as at least 80 % of the initial rate determined using the equivalent DNA only oligonucleotide, with no 2' substitutions, with phosphorothioate linkage groups between all nucleotides in the oligonucleotide, using the methodology provided by Example 91 - 95 of EP 1 222 309. Typically the region of the oligomer which forms the consecutive nucleotide units which, when formed in a duplex with the complementary target RNA is capable of recruiting RNase consists of nucleotide units which form a DNA/RNA like duplex with the RNA target. The oligomer of the invention, such as the first region, may comprise a nucleotide sequence which comprises both nucleotides and nucleotide analogues, and may be e.g. in the form of a gapmer.

Gapmer Design

[0113] In some embodiments, the oligomer of the conjugate compound of the invention, such as the first region, comprises or is a gapmer. A gapmer oligomer is an oligomer which comprises a contiguous stretch of nucleotides which is capable of recruiting an RNAse, such as RNAseH, such as a region of at least 6 or 7 DNA nucleotides, referred to herein in as region Y' (Y'), wherein region Y' is flanked both 5' and 3' by regions of affinity enhancing nucleotide analogues, such as from 1 - 6 nucleotide analogues 5' and 3' to the contiguous stretch of nucleotides which is capable of recruiting RNAse - these regions are referred to as regions X' (X') and Z' (Z') respectively. Examples of gapmers are disclosed in WO2004/046160, WO2008/113832, and WO2007/146511.

[0114] In some embodiments, the monomers which are capable of recruiting RNAse are selected from the group consisting of DNA monomers, alpha-L-LNA monomers, C4' alkylayted DNA monomers (see WO2009/090182 and Vester et al., Bioorg. Med. Chem. Lett. 18 (2008) 2296 - 2300), and UNA (unlinked nucleic acid) nucleotides (see Fluiter et al., Mol. Biosyst., 2009, 10, 1039). UNA is unlocked nucleic acid, typically where the C2 - C3 C-C bond of the ribose has been removed, forming an unlocked "sugar" residue. Preferably the gapmer comprises a (poly)nucleotide sequence of formula (5' to 3'), X'-Y'-Z', wherein; region X' (X') (5' region) consists or comprises of at least one nucleotide analogue, such as at least one BNA (e.g. LNA) unit, such as from 1-6 nucleotide analogues, such as BNA (e.g. LNA) units, and; region Y' (Y') consists or comprises of at least five consecutive nucleotides which are capable of recruiting RNAse (when formed in a duplex with a complementary RNA molecule, such as the mRNA target), such as DNA nucleotides, and; region Z' (Z') (3'region) consists or comprises of at least one nucleotide analogue, such as at least one BNA (e.g. LNA unit), such as from -1-6 nucleotide analogues, such as BNA (e.g. LNA) units.

[0115] In some embodiments, region X' consists of 1, 2, 3, 4, 5 or 6 nucleotide analogues, such as BNA (e.g. LNA) units, such as from 2-5 nucleotide analogues, such as 2-5 LNA units, such as 3 or 4 nucleotide analogues, such as 3 or 4 LNA units; and/or region Z' consists of 1, 2, 3, 4, 5 or 6 nucleotide analogues, such as BNA (e.g. LNA) units, such as from 2-5 nucleotide analogues, such as 2-5 BNA (e.g. LNA units), such as 3 or 4 nucleotide analogues, such as 3 or 4 BNA (e.g. LNA) units.

[0116] In some embodiments Y' consists or comprises of 5, 6, 7, 8, 9, 10, 11 or 12 consecutive nucleotides which are capable of recruiting RNAse, or from 6-10, or from 7-9, such as 8 consecutive nucleotides which are capable of recruiting RNAse. In some embodiments region Y' consists or comprises at least one DNA nucleotide unit, such as 1-12 DNA units, preferably from 4-12 DNA units, more preferably from 6-10 DNA units, such as from 7-10 DNA units, most preferably 8, 9 or 10 DNA units.

[0117] In some embodiments region X' consist of 3 or 4 nucleotide analogues, such as BNA (e.g. LNA), region X' consists of 7, 8, 9 or 10 DNA units, and region Z' consists of 3 or 4 nucleotide analogues, such as BNA (e.g. LNA). Such designs include (X'-Y'-Z') 3-10-3, 3-10-4, 4-10-3, 3-9-3, 3-9-4, 4-9-3, 3-8-3, 3-8-4, 4-8-3, 3-7-3, 3-7-4, 4-7-3.

[0118] Further gapmer designs are disclosed in WO2004/046160. WO2008/113832, which claims priority from US provisional application 60/977,409 refers to 'shortmer' gapmer oligomers. In some embodiments, oligomers presented here may be such shortmer gapmers.

[0119] In some embodiments the oligomer, e.g. region X', is consisting of a contiguous nucleotide sequence of a total of 10, 11, 12, 13 or 14 nucleotide units, wherein the contiguous nucleotide sequence comprises or is of formula (5' - 3'), X'-Y'-Z' wherein; X' consists of 1, 2 or 3 nucleotide analogue units, such as BNA (e.g. LNA) units; Y' consists of 7, 8 or 9 contiguous nucleotide units which are capable of recruiting RNAse when formed in a duplex with a complementary RNA molecule (such as a mRNA target); and Z' consists of 1, 2 or 3 nucleotide analogue units, such as BNA (e.g. LNA) units.

[0120] In some embodiments X' consists of 1 BNA (e.g. LNA) unit. In some embodiments X' consists of 2 BNA (e.g. LNA) units. In some embodiments X' consists of 3 BNA (e.g. LNA) units. In some embodiments Z' consists of 1 BNA (e.g. LNA) units. In some embodiments Z' consists of 2 BNA (e.g. LNA) units. In some embodiments Z' consists of 3 BNA (e.g. LNA) units. In some embodiments Y' consists of 7 nucleotide units. In some embodiments Y' consists of 8 nucleotide units. In some embodiments Y' consists of 9 nucleotide units.. In certain embodiments, region Y' consists of 10 nucleoside monomers. In certain embodiments, region Y' consists or comprises 1 - 10 DNA monomers. In some embodiments Y' comprises of from 1 - 9 DNA units, such as 2, 3, 4, 5, 6, 7 , 8 or 9 DNA units. In some embodiments Y' consists of DNA units. In some embodiments Y' comprises of at least one BNA unit which is in the alpha-L configuration, such as 2, 3, 4, 5, 6, 7, 8 or 9 LNA units in the alpha-L-configuration. In some embodiments Y' comprises of at least one alpha-L-oxy BNA/LNA unit or wherein all the LNA units in the alpha-L- configuration are alpha-L-oxy LNA units. In some embodiments the number of nucleotides present in X'-Y'-Z' are selected from the group consisting of (nucleotide analogue units - region Y' - nucleotide analogue units): 1-8-1, 1-8-2, 2-8-1, 2-8-2, 3-8-3, 2-8-3, 3-8-2, 4-8-1, 4-8-2, 1-8-4, 2-8-4, or;1 -9-1, 1-9-2, 2-9-1, 2-9-2, 2-9-3, 3-9-2, 1-9-3, 3-9-1, 4-9-1, 1-9-4, or; 1-10-1, 1-10-2, 2-10-1, 2-10-2, 1-10-3, 3-10-1. In some embodiments the number of nucleotides in X'-Y'-Z' are selected from the group consisting of: 2-7-1, 1-7-2, 2-7-2, 3-7-3, 2-7-3, 3-7-2, 3-7-4, and 4-7-3. In certain embodiments, each of regions X' and Y' consists of three BNA (e.g. LNA) monomers, and region Y' consists of 8 or 9 or 10 nucleoside monomers, preferably DNA monomers. In some embodiments both X' and Z' consists of two BNA (e.g. LNA) units each, and Y' consists of 8 or 9 nucleotide units, preferably DNA units. In various embodiments, other gapmer designs include those where regions X' and/or Z' consists of 3, 4, 5 or 6 nucleoside analogues, such as monomers containing a 2'-O-methoxyethyl-ribose sugar (2'-MOE) or monomers containing a 2'-fluoro-deoxyribose sugar, and region Y' consists of 8, 9, 10, 11 or 12 nucleosides, such as DNA monomers, where regions X'-Y'-Z' have 3-9-3, 3-10-3, 5-10-5 or 4-12-4 monomers. Further gapmer designs are disclosed in WO 2007/146511 A2.
BNA and LNA Gapmers: A BNA gapmer is a gapmer oligomer (region A) which comprises at least one BNA nucleotide. A LNA gapmer is a gapmer oligomer (region A) which comprises at least one LNA nucleotide. SEQ ID NO 2 and 3 are LNA gapmer oligomers. The oligomers with a contiguous sequence of 10 - 16 nucleotides which are complementary to a corresponding length of SEQ ID NO 33 or 34 may also be gapmer oligomers such as BNA gapmers or LNA gapmers.

Internucleotide Linkages

[0121] The nucleoside monomers of the oligomers (e.g. first and second regions) described herein are coupled together via [internucleoside] linkage groups. Suitably, each monomer is linked to the 3' adjacent monomer via a linkage group.

[0122] The person having ordinary skill in the art would understand that, in the context of the present invention, the 5' monomer at the end of an oligomer does not comprise a 5' linkage group, although it may or may not comprise a 5' terminal group.

[0123] The terms "linkage group" or "internucleotide linkage" are intended to mean a group capable of covalently coupling together two nucleotides. Specific and preferred examples include phosphate groups and phosphorothioate groups.

[0124] The nucleotides of the oligomer of the invention or contiguous nucleotides sequence thereof are coupled together via linkage groups. Suitably each nucleotide is linked to the 3' adjacent nucleotide via a linkage group.

[0125] Suitable internucleotide linkages include those listed within WO2007/031091, for example the internucleotide linkages listed on the first paragraph of page 34 of WO2007/031091.

[0126] It is, in some embodiments, other than the phosphodiester linkage(s) of region B (where present), the preferred to modify the internucleotide linkage from its normal phosphodiester to one that is more resistant to nuclease attack, such as phosphorothioate or boranophosphate - these two, being cleavable by RNase H, also allow that route of antisense inhibition in reducing the expression of the target gene.

[0127] Suitable sulphur (S) containing internucleotide linkages as provided herein may be preferred, such as phosphorothioate or phosphodithioate. Phosphorothioate internucleotide linkages are also preferred, particularly for the first region, such as in gapmers, mixmers, antimirs splice switching oligomers, and totalmers.

[0128] For gapmers, the internucleotide linkages in the oligomer may, for example be phosphorothioate or boranophosphate so as to allow RNase H cleavage of targeted RNA. Phosphorothioate is preferred, for improved nuclease resistance and other reasons, such as ease of manufacture.

[0129] In one aspect, with the exception of the phosphodiester linkage between the first and second region, and optionally within region B, the remaining internucleoside linkages of the oligomer of the invention, the nucleotides and/or nucleotide analogues are linked to each other by means of phosphorothioate groups. In some embodiments, at least 50%, such as at least 70%, such as at least 80%, such as at least 90% such as all the internucleoside linkages between nucleosides in the first region are other than phosphodiester (phosphate), such as are selected from the group consisting of phosphorothioate phosphorodithioate, or boranophosphate. In some embodiments, at least 50%, such as at least 70%, such as at least 80%, such as at least 90% such as all the internucleoside linkages between nucleosides in the first region are phosphorothioate.
WO09124238 refers to oligomeric compounds having at least one bicyclic nucleoside attached to the 3' or 5' termini by a neutral internucleoside linkage. The oligomers of the invention may therefore have at least one bicyclic nucleoside attached to the 3' or 5' termini by a neutral internucleoside linkage, such as one or more phosphotriester, methylphosphonate, MMI, amide-3,

formacetal or thioformacetal. The remaining linkages may be phosphorothioate.

Oligomer Conjugates

[0130] Representative conjugate moieties which have been used with oligonucleotides can include lipophilic molecules (aromatic and non-aromatic) including steroid molecules; proteins (e.g., antibodies, enzymes, serum proteins); peptides; vitamins (water-soluble or lipid-soluble); polymers (water-soluble or lipid-soluble); small molecules including drugs, toxins, reporter molecules, and receptor ligands; carbohydrate complexes; nucleic acid cleaving complexes; metal chelators (e.g., porphyrins, texaphyrins, crown ethers, etc.); intercalators including hybrid photonuclease/intercalators; crosslinking agents (e.g., photoactive, redox active), and combinations and derivatives thereof. Numerous suitable conjugate moieties, their preparation and linkage to oligomeric compounds are provided, for example, in WO 93/07883 and U.S. Pat. No. 6,395,492. Oligonucleotide conjugates and their syntheses are also reported in comprehensive reviews by Manoharan in Antisense Drug Technology, Principles, Strategies, and Applications, S.T. Crooke, ed., Ch. 16, Marcel Dekker, Inc., 2001 and Manoharan, Antisense and Nucleic Acid Drug Development, 2002, 12, 103.

[0131] In some embodiments the oligomer of the invention is targeted to the liver - *i.e.* after systemic administration the compound accumulates in the liver cells (such as hepatocytes). Targeting to the liver can be greatly enhanced by the addition of a conjugate moiety (C). However, in order to maximize the efficacy of the oligomer it is often desirable that the conjugate (or targeting moiety) is linked to the oligomer via a biocleavable linker (B), such as a nucleotide phosphate linker. It is therefore desirable to use a conjugate moiety which enhances uptake and activity in hepatocytes. The enhancement of activity may be due to enhanced uptake or it may be due to enhanced potency of the compound in hepatocytes.

[0132] In some embodiments, the oligomeric compound is a BNA or LNA oligomer, such as a gapmer, or for example an LNA antisense oligomer, (which may be referred to as region A herein) comprising an antisense oligomer, optionally a biocleavable linker, such as region B, and a carbohydrate conjugate (which may be referred to as region C). The LNA antisense oligomer may be 7 - 30, such as 8 - 26 nucleosides in length and it comprises at least one LNA unit (nucleoside). In some embodiments the carbohydrate moiety is not a linear carbohydrate polymer.

[0133] In some embodiments, the oligomeric compound is a LNA oligomer, for example an LNA antisense oligomer, (which may be referred to as region A herein) comprising an antisense oligomer, region B as defined herein,_and an asialoglycoprotein receptor targeting moiety conjugate moiety, such as a GalNAc moiety (which may be referred to as region C). The carbohydrate moiety may be multi-valent, such as, for example 2, 3, 4 or 4 identical or non-identical carbohydrate moieties may be covalently joined to the oligomer, optionally via a linker or linkers (such as region Y).

GalNac Conjugate Moieties

[0134] In some embodiments the carbohydrate moiety is not a linear carbohydrate polymer. The carbohydrate moiety may however be multi-valent, such as, for example 2, 3, 4 or 4 identical or non-identical carbohydrate moieties may be covalently joined to the oligomer, optionally via a linker or linkers. In some embodiments the invention provides a conjugate comprising the oligomer described herein and a carbohydrate conjugate moiety. In some embodiments the invention provides a conjugate comprising the oligomer described herein and an asialoglycoprotein receptor targeting moiety conjugate moiety, such as a GalNAc moiety, which may form part of a further region (referred to as region C).

[0135] The invention provides LNA antisense oligonucleotides which are conjugated to an asialoglycoprotein receptor targeting moiety. In some embodiments, the conjugate moiety (such as the third region or region C) comprises an asialoglycoprotein receptor targeting moiety, such as galactose, galactosamine, N-formyl-galactosamine, Nacetylgalactosamine, N-propionyl-galactosamine, N-n-butanoyl-galactosamine, and N-isobutanoylgalactos-amine. In some emboidments the conjugate comprises a galactose cluster, such as N-acetylgalactosamine trimer. In some embodiments, the conjugate moiety comprises an GalNAc (N-acetylgalactosamine), such as a mono-valent, di-valent , tri-valent of tetra-valent GalNAc. Trivalent GalNAc conjugates may be used to target the compound to the liver. GalNAc conjugates have been used with methylphosphonate and PNA antisense oligonucleotides (e.g. US 5,994517 and Hangeland et al., Bioconjug Chem. 1995 Nov-Dec;6(6):695-701) and siRNAs (e.g. WO2009/126933, WO2012/089352 & WO2012/083046). WO2012/083046 discloses siRNAs with GalNAc conjugate moieties which comprise cleavable pharmacokinetic modulators, which are suitable for use in the present invention, the preferred pharmacokinetic modulators are C16 hydrophobic groups such as palmitoyl, hexadec-8-enoyl, oleyl, (9E, 12E)-octadeca-9,12-dienoyl, dioctanoyl, and C16-C20 acyl. The '046 cleavable pharmacokinetic modulators may also be cholesterol.

[0136] The targeting moieties (conjugate moieties) may be selected from the group consisting of: galactose, galactosamine, N-formyl-galactosamine, N-acetylgalactosamine, Npropionyl-galactosamine, N-n-butanoyl-galactosamine, N-iso-butanoylgalactos-amine, galactose cluster, and N-acetylgalactosamine trimer and may have a pharmacokinetic modulator selected from the group consisting of: hydrophobic group having 16 or more carbon atoms, hydrophobic group having 16-20 carbon atoms, palmitoyl, hexadec-8-enoyl, oleyl, (9E,12E)-octadeca-9,12dienoyl, dioctanoyl, and C16-C20 acyl, and cholesterol. Certain GalNac clusters disclosed in '046 include: (E)-hexadec-8-enoyl (C16), oleyl (C18), (9,E,12E)-octadeca-9,12-dienoyl (C18), octanoyl (C8), dodececanoyl (C12), C-20 acyl, C24 acyl, dioctanoyl (2xC8). The targeting moiety-pharmacokinetic modulator targeting moiety may be linked to the polynucleotide via a physiologically labile bond or, e.g. a disulfide bond, or a PEG linker. The invention also relates to the use of phospodiester linkers between the oligomer and the conjugate group (these are referred to as region B herein, and suitably are positioned between the LNA oligomer and the carbohydrate conjugate group).

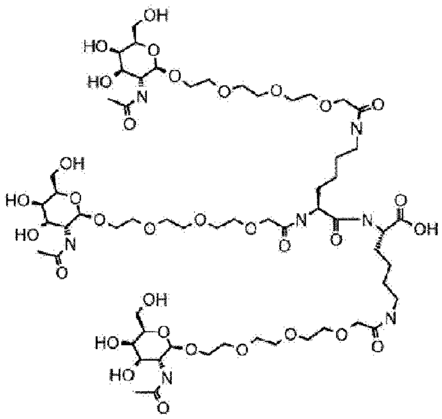
[0137] For targeting hepatocytes in liver, a preferred targeting ligand is a galactose cluster.

[0138] A galactose cluster comprises a molecule having e.g. comprising two to four terminal galactose derivatives. As used herein, the term galactose derivative includes both galactose and derivatives of galactose having affinity for the asialoglycoprotein receptor equal to or greater than that of galactose. A terminal galactose derivative is attached to a molecule through its C-I carbon.

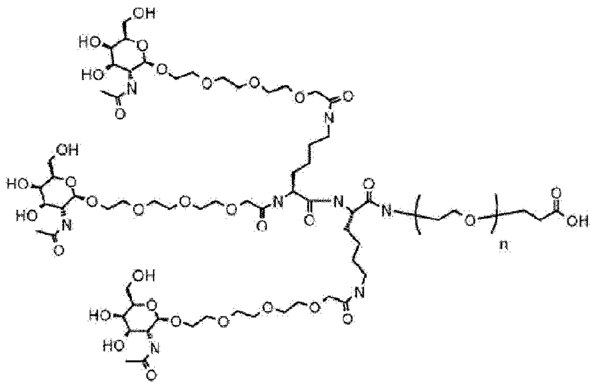
The asialoglycoprotein receptor (ASGPr) is unique to hepatocytes and binds branched galactose-terminal glycoproteins. A preferred galactose cluster has three terminal galactosamines or galactosamine derivatives each having affinity for the asialoglycoprotein receptor. A more preferred galactose cluster has three terminal N-acetyl-galactosamines. Other terms common in the art include tri-antennary galactose, tri-valent galactose and galactose trimer. It is known that tri-antennary galactose derivative clusters are bound to the ASGPr with greater affinity than bi-antennary or mono-antennary galactose derivative structures (Baenziger and Fiete, 1980, Cell, 22, 611-620; Connolly et al., 1982,1. Biol. Chern., 257,939-945). Multivalency is required to achieve nM affinity. According to WO 2012/083046 the attachment of a single galactose derivative having affinity for the asialoglycoprotein receptor does not enable functional delivery of the RNAi polynucleotide to hepatocytes in vivo when co-administered with the delivery polymer.

[0139] A galactose cluster may comprise two or preferably three galactose derivatives each linked to a central branch point. The galactose derivatives are attached to the central branch point through the C-l carbons of the saccharides. The galactose derivative is preferably linked to the branch point via linkers or spacers. A preferred spacer is a flexible hydrophilic spacer (U.S. Patent 5885968; Biessen et al. J. Med. Chern. 1995 Vol. 39 p. 1538-1546). A preferred flexible hydrophilic spacer is a PEG spacer. A preferred PEG spacer is a PEG3 spacer. The branch point can be any small molecule which permits attachment of the three galactose derivatives and further permits attachment of the branch point to the oligomer. An exemplary branch point group is a di-lysine. A di-lysine molecule contains three amine groups through which three galactose derivatives may be attached and a carboxyl reactive group through which the di-lysine may be attached to the oligomer. Attachment of the branch point to oligomer may occur through a linker or spacer. A preferred spacer is a flexible hydrophilic spacer. A preferred flexible hydrophilic spacer is a PEG spacer. A preferred PEG spacer is a PEG3 spacer (three ethylene units). The galactose cluster may be attached to the 3' or 5' end of the oligomer using methods known in the art.

[0140] A preferred galactose derivative is an N-acetyl-galactosamine (GalNAc). Other saccharides having affinity for the asialoglycoprotein receptor may be selected from the list comprising: galactosamine, N-n-butanoylgalactosamine, and N-iso-butanoylgalactosamine. The affinities of numerous galactose derivatives for the asialoglycoprotein receptor have been studied (see for example: Jobst, S.T. and Drickamer, K. JB.C. 1996,271,6686) or are readily determined using methods typical in the art.

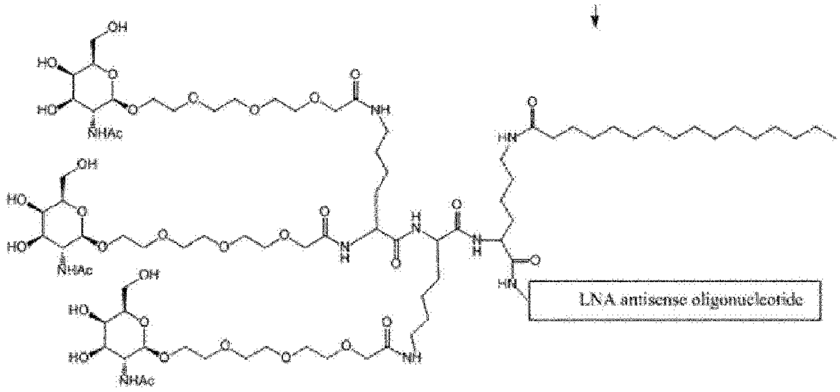


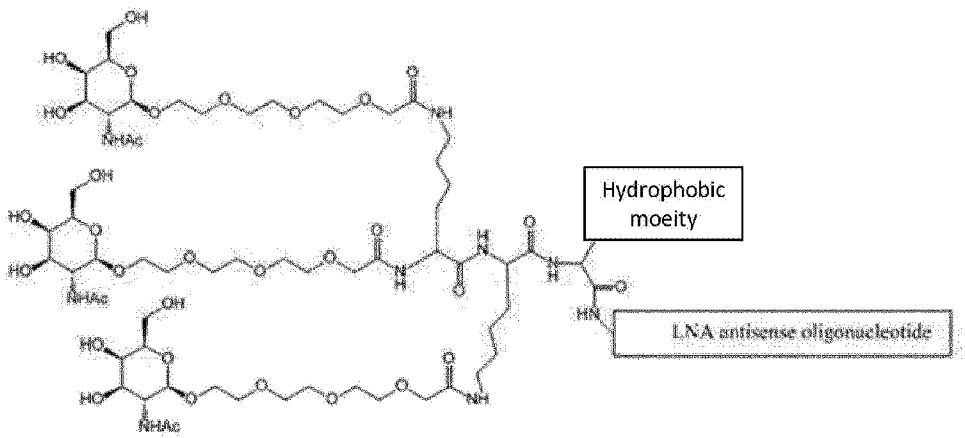
[0141] One embodiment of a Galactose cluster

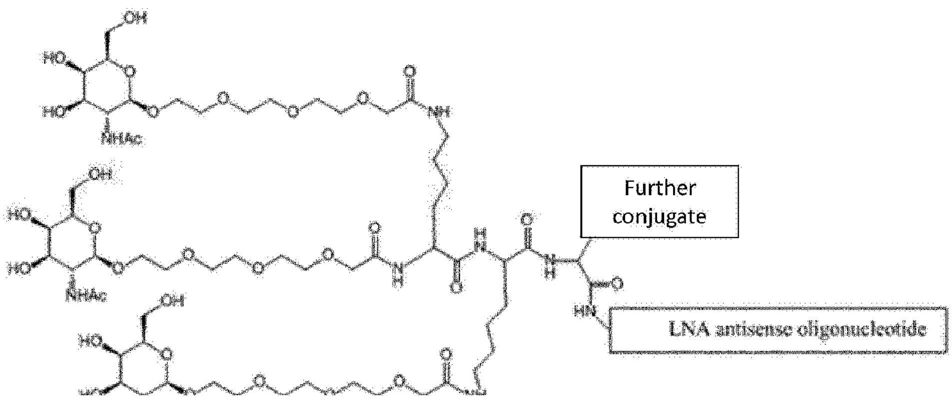


[0142] Galactose cluster with PEG spacer between branch point and nucleic acid

[0143] Further Examples of the conjugate of the invention are illustrated below:





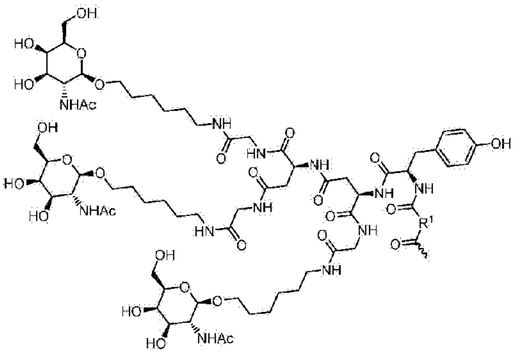


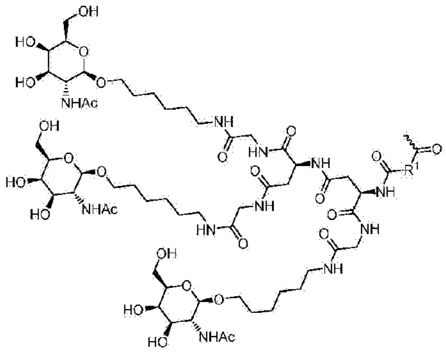
[0144] Where at the hydrophobic or lipophilic (or further conjugate) moiety (i.e. pharmacokinetic modulator) in the above GalNAc cluster conjugates is, when using BNA or LNA oligomers, such as LNA antisense oligonucleotides, optional.

[0145] See the figures for specific GalNAc clusters used in the present study, Conj 1, 2, 3, 4 and Conj1 a, 2a, 3a and 4a (which are shown with an optional C6 linker which joins the GalNAc cluster to the oligomer).

[0146] Each carbohydrate moiety of a GalNAc cluster (e.g. GalNAc) may therefore be joined to the oligomer via a spacer, such as (poly)ethylene glycol linker (PEG), such as a di, tri, tetra, penta, hexa-ethylene glycol linker. As is shown above the PEG moiety forms -a spacer between the galctose sugar moiety and a peptide (trilysine is shown) linker.

[0147] In some embodiments, the GalNAc cluster comprises a peptide linker, e.g. a Tyr-Asp(Asp) tripeptide or Asp(Asp) dipeptide, which is attached to the oligomer (or to region Y or region B) via a biradical linker, for example the GalNAc cluster may comprise the following biradical linkers:





R^1 is a biradical preferably selected from $-C_2H_4-$, $-C_3H_6-$, $-C_4H_8-$, $-C_5H_{10}-$, $-C_9H_{12}-$, 1,4-cyclohexyl ($-C_6H_{10}-$), 1,4-phenyl ($-C_6H_4-$), $-C_2H_4OC_2H_4-$, $-C_2H_4(OC_2H_4)_2-$ or $-C_2H_4(OC_2H_4)_3-$.

[0148] The carbohydrate conjugate(e.g. GalNAc), or carbohydrate-linker moiety (e.g. carbohydrate-PEG moiety) may be covalently joined (linked) to the oligomer via a branch point group such as, an amino acid, or peptide, which suitably comprises two or more amino groups (such as 3, 4, or5), such as lysine, di-lysine or tri-lysine or tetra-lysine. A tri-lysine molecule contains four amine groups through which three carbohydrate conjugate groups, such as galactose & derivatives (e.g.GalNAc) and a further conjugate such as a hydrophobic or lipophilic moiety/group may be attached and a carboxyl reactive group through which the tri-lysine may be attached to the oligomer. The further conjugate, such as lipophilic/hyrodphobic moiety may be attached to the lysine residue that is attached to the oligomer.

Pharmacokinetic Modulators

[0149] The compound of the invention may further comprise one or more additional conjugate moieties, of which lipophilic or hydrophobic moieties are particularly interesting, such as when the conjugate group is a carbohydrate moiety. Such lipophilic or hydrophobic moieties may act as pharmacokinetic modulators, and may be covalently linked to either the carbohydrate conjugate, a linker linking the carbohydrate conjugate to the oligomer or a linker linking multiple carbohydrate conjugates (multi-valent) conjugates, or to the oligomer, optionally via a linker, such as a bio cleavable linker.

[0150] The oligomer or conjugate moiety may therefore comprise a pharmacokinetic modulator, such as a lipophilic or hydrophobic moieties. Such moieties are disclosed within the context of siRNA conjugates in WO2012/082046. The hydrophobic moiety may comprise a C8 - C36 fatty acid, which may be saturated or un-saturated. In some embodiments, C10, C12, C14, C16, C18, C20, C22, C24, C26, C28, C30, C32 and C34 fatty acids may be used. The hydrophobic group may have 16 or more carbon atoms. Exemplary suitable hydrophobic groups may be selected from the group comprising:sterol, cholesterol, palmitoyl, hexadec-8-enoyl, oleyl, (9E, 12E)-octadeca-9,12-dienoyl, dioctanoyl, and C16-C20 acyl. According to WO'346, hydrophobic groups having fewer than 16 carbon atoms are less effective in enhancing polynucleotide targeting, but they may be used in multiple copies (e.g. 2x, such as 2x C8 or C10, C12 or C14) to enhance efficacy. Pharmacokinetic modulators useful as polynucleotide targeting moieties may be selected from the group consisting of: cholesterol, alkyl group, alkenyl group, alkynyl group, aryl group, aralkyl group, aralkenyl group, and aralkynyl group, each of which may be linear, branched, or cyclic. Pharmacokinetic modulators are preferably hydrocarbons, containing only carbon and hydrogen atoms. However, substitutions or heteroatoms which maintain hydrophobicity, for example fluorine, may be permitted.

[0151] The application describes a conjugate that is or may comprise a carbohydrate or comprises a carbohydrate group. In some conjugates, the carbohydrate is selected from the group consisting of galactose, lactose, n-acetylgalactosamine, mannose, and mannose-6-phosphate. In some embodiments, the conjugate group is or may comprise mannose or mannose-6-phosphate. Carbohydrate conjugates may be used to enhance delivery or activity in a range of tissues, such as liver and/or muscle. See, for example, EP1495769, WO99/65925, Yang et al., Bioconjug Chem (2009) 20(2): 213-21. Zatsepin & Oretskaya Chem Biodivers. (2004) 1(10): 1401-17.

[0152] Surprisingly, the present inventors have found that GalNAc conjugates for use with LNA oligomers do not require a pharmacokinetic modulator, and as such, in some embodiments, the GalNAc conjugate is not covalently linked to a lipophilic or hydrophobic moiety, such as those described here in, e.g. do not comprise a C8 - C36 fatty acid or a sterol. The invention therefore also provides for LNA oligomer GalNAc conjugates which do not comprise a lipophilic or hydrophobic pharmacokinetic modulator or conjugate moiety/group.

Lipophilic conjugates

[0153] The application describes a conjugate group that is or may comprise a lipophilic moiety, such as a sterol (for example, cholesterol, cholesteryl, cholestanol, stigmasterol, cholanic acid and ergosterol). In some conjugatess the conjugate group is or comprises tocopherol. In some conjugates, the conjugate group is or may comprise cholesterol.

[0154] The application describes a conjugate that is, or may comprise a lipid, a phospholipid or a lipophilic alcohol, such as a cationic lipids, a neutral lipids, sphingolipids, and fatty acids such as stearic, oleic, elaidic, linoleic, linoleaidic, linolenic, and myristic acids. In some conjugates the fatty acid comprises a C4 - C30 saturated or unsaturated alkyl chain. The alkyl chain may be linear or branched. Lipophilic conjugate moieties can be used, for example, to counter the hydrophilic nature of an oligomeric compound and enhance cellular penetration.

[0155] Lipophilic moieties include, for example, sterols stanols, and steroids and related compounds such as cholesterol (U.S. Pat. No. 4,958,013 and Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553), thiocholesterol (Oberhauser et al, Nucl Acids Res., 1992, 20, 533), lanosterol, coprostanol, stigmasterol, ergosterol, calciferol, cholic acid, deoxycholic acid, estrone, estradiol, estratriol, progesterone, stilbestrol, testosterone, androsterone, deoxycorticosterone, cortisone, 17-hydroxycorticosterone, their derivatives, and the like. In some embodiments, the conjugate may be selected from the group consisting of cholesterol , thiocholesterol, lanosterol, coprostanol, stigmasterol, ergosterol, calciferol, cholic acid, deoxycholic acid, estrone, estradiol, estratriol, progesterone, stilbestrol, testosterone, androsterone, deoxycorticosterone, cortisone, and 17-hydroxycorticosterone. Other lipophilic conjugate moieties include aliphatic groups, such as, for example, straight chain, branched, and cyclic alkyls, alkenyls, and alkynyls. The aliphatic groups can have, for example, 5 to about 50, 6 to about 50, 8 to about 50, or 10 to about 50 carbon atoms. Example aliphatic groups include undecyl, dodecyl, hexadecyl, heptadecyl, octadecyl, nonadecyl, terpenes, bornyl, adamantyl, derivatives thereof and the like. In some embodiments, one or more carbon atoms in the aliphatic group can be replaced by a heteroatom such as O, S, or N (e.g., geranyloxyhexyl). Further suitable lipophilic conjugate moieties include aliphatic derivatives of glycerols such as alkylglycerols, bis(alkyl)glycerols, tris(alkyl)glycerols, monoglycerides, diglycerides, and triglycerides. In some embodiments, the lipophilic conjugate is di-hexyldecyl-rac-glycerol or 1,2-di-O- hexyldecyl-rac-glycerol (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651; Shea, et al., Nuc. Acids Res., 1990, 18, 3777) or phosphonates thereof. Saturated and unsaturated fatty functionalities, such as, for example, fatty acids, fatty alcohols, fatty esters, and fatty amines, can also serve as lipophilic conjugate moieties. In some embodiments, the fatty functionalities can contain from about 6 carbons to about 30 or about 8 to about 22 carbons. Example fatty acids include, capric, caprylic, lauric, palmitic, myristic, stearic, oleic, linoleic, linolenic, arachidonic, eicosenoic acids and the like.

[0156] The lipophilic conjugate groups can be polycyclic aromatic groups having from 6 to about 50, 10 to about 50, or 14 to about 40 carbon atoms. Example polycyclic aromatic groups include pyrenes, purines, acridines, xanthenes, fluorenes, phenanthrenes, anthracenes, quinolines, isoquinolines, naphthalenes, derivatives thereof and the like. Other suitable lipophilic conjugate moieties include menthols, trityls (e.g., dimethoxytrityl (DMT)), phenoxazines, lipoic acid, phospholipids, ethers, thioethers (e.g., hexyl-S-tritylthiol), derivatives thereof and the like. Preparation of lipophilic conjugates of oligomeric compounds are well-described in the art, such as in, for example, Saison-Behmoaras et al, EMBO J., 1991, 10, 1111; Kabanov et al., FEBSLett., 1990, 259, 327; Svinarchuk et al, Biochimie, 1993, 75, 49; (Mishra et al., Biochim. Biophys. Acta, 1995, 1264, 229, and Manoharan et al., Tetrahedron Lett., 1995, 36, 3651.

[0157] Oligomeric compounds containing conjugate moieties with affinity for low density lipoprotein (LDL) can help provide an effective targeted delivery system. High expression levels of receptors for LDL on tumor cells makes LDL an attractive carrier for selective delivery of drugs to these cells (Rump, et al., Bioconjugate Chem., 1998, 9, 341; Firestone, Bioconjugate Chem., 1994, 5, 105; Mishra, et al., Biochim. Biophys. Acta, 1995, 1264, 229). Moieties having affinity for LDL include many lipophilic groups such as steroids (e.g., cholesterol), fatty acids, derivatives thereof and combinations thereof. In some embodiments, conjugate moieties having LDL affinity can be dioleyl esters of cholic acids such as chenodeoxycholic acid and lithocholic acid.

[0158] The lipophillic conjugates may be or may comprise biotin. In some embodiments, the lipophilic conjugate may be or may comprise a glyceride or glyceride ester.

[0159] Lipophillic conjugates, such as sterols, stanols, and stains, such as cholesterol or as disclosed herein, may be used to enhance delivery of the oligonucleotide to, for example, the liver (typically hepatocytes).

[0160] The following references also refer to the use of lipophilic conjugates: Kobylanska et al., Acta Biochim Pol. (1999); 46(3): 679 -91. Felber et al,. Biomaterials (2012) 33(25): 599-65); Grijalvo et al., J Org Chem (2010) 75(20): 6806 - 13. Koufaki et al., Curr Med Chem (2009) 16(35): 4728-42. Godeau et al J. Med. Chem. (2008) 51(15): 4374-6.

Linkers (e.g. Region Y)

[0161] A linkage or linker is a connection between two atoms that links one chemical group or segment of interest to another chemical group or segment of interest via one or more covalent bonds. Conjugate moieties (or targeting or blocking moieties) can be attached to the oligomeric compound directly or through a linking moiety (linker or tether) - a linker. Linkers are bifunctional moieties that serve to covalently connect a third region, e.g. a conjugate moiety, to an oligomeric compound (such as to region B). In some embodiments, the linker comprises a chain structure or an oligomer of repeating units such as ethylene glyol or amino acid units. The linker can have at least two functionalities, one for attaching to the oligomeric compound and the other for attaching to the conjugate moiety. Example linker functionalities can be electrophilic for reacting with nucleophilic groups on the oligomer or conjugate moiety, or nucleophilic for reacting with electrophilic groups. In some embodiments, linker functionalities include amino, hydroxyl, carboxylic acid, thiol, phosphoramidate, phosphorothioate, phosphate, phosphite, unsaturations (e.g., double or triple bonds), and the like. Some example linkers include 8-amino-3,6-dioxaoctanoic acid (ADO), succinimidyl 4-(N-maleimidomethyl)cyclohexane-l-carboxylate (SMCC), 6-aminohexanoic acid (AHEX or AHA), 6-aminohexyloxy, 4-aminobutyric acid, 4-aminocyclohexylcarboxylic acid, succinimidyl 4-(N-maleimidomethyl)cyclohexane- l-carboxy-(6-amido-caproate) (LCSMCC), succinimidyl m-maleimido-benzoylate (MBS), succinimidyl N-e-maleimido-caproylate (EMCS), succinimidyl 6-(beta - maleimido-propionamido) hexanoate (SMPH), succinimidyl N-(a-maleimido acetate) (AMAS), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), beta -alanine (beta -ALA), phenylglycine (PHG), 4-aminocyclohexanoic acid (ACHC), beta -(cyclopropyl) alanine (beta -CYPR), amino dodecanoic acid (ADC), alylene diols, polyethylene glycols, amino acids, and the like.

[0162] A wide variety of further linker groups are known in the art that can be useful in the attachment of conjugate moieties to oligomeric compounds. A review of many of the useful linker groups can be found in, for example, Antisense Research and Applications, S. T. Crooke and B. Lebleu, Eds., CRC Press, Boca Raton, Fla., 1993, p. 303-350. A disulfide linkage has been used to link the 3' terminus of an oligonucleotide to a peptide (Corey, et al., Science 1987, 238, 1401; Zuckermann, et al, J Am. Chem. Soc. 1988, 110, 1614; and Corey, et al., J Am. Chem. Soc. 1989, 111, 8524). Nelson, et al., Nuc. Acids Res. 1989, 17, 7187 describe a linking reagent for attaching biotin to the 3'-terminus of an oligonucleotide. This reagent, N-Fmoc-O- DMT-3 -amino- 1,2-propanediol is commercially available from Clontech Laboratories (Palo Alto, Calif.) under the name 3'-Amine. It is also commercially available under the name 3'-Amino-Modifier reagent from Glen Research Corporation (Sterling, Va.). This reagent was also utilized to link a peptide to an oligonucleotide as reported by Judy, et al., Tetrahedron Letters 1991, 32, 879. A similar commercial reagent for linking to the 5 '-terminus of an oligonucleotide is 5'- Amino-Modifier C6. These reagents are available from Glen Research Corporation (Sterling, Va.). These compounds or similar ones were utilized by Krieg, et al, Antisense Research and Development 1991, 1, 161 to link fluorescein to the 5'- terminus of an oligonucleotide. Other compounds such as acridine have been attached to the 3 '-terminal phosphate group of an oligonucleotide via a polymethylene linkage (Asseline, et al., Proc. Natl. Acad. Sci. USA 1984, 81, 3297). Any of the above groups can be used as a single linker or in combination with one or more further linkers.

[0163] Linkers and their use in preparation of conjugates of oligomeric compounds are provided throughout the art such as in WO 96/11205 and WO 98/52614 and U.S. Pat. Nos. 4,948,882; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,580,731; 5,486,603; 5,608,046; 4,587,044; 4,667,025; 5,254,469; 5,245,022; 5,112,963; 5,391,723; 5,510475; 5,512,667; 5,574,142; 5,684,142; 5,770,716; 6,096,875; 6,335,432; and 6,335,437,Wo2012/083046.

[0164] As used herein, a physiologically labile bond is a labile bond that is cleavable under conditions normally encountered or analogous to those encountered within a mammalian body (also referred to as a cleavable linker). Physiologically labile linkage groups are selected such that they undergo a chemical transformation (e.g., cleavage) when present in certain physiological conditions. Mammalian intracellular conditions include chemical conditions such as pH, temperature, oxidative or reductive conditions or agents, and salt concentration found in or analogous to those encountered in mammalian cells. Mammalian intracellular conditions also include the presence of enzymatic activity normally present in a mammalian cell such as from proteolytic or hydrolytic enzymes. In some embodiments, the cleavable linker is susceptible to nuclease(s) which may for example, be expressed in the target cell - and as such, as detailed herein, the linker may be a short region (e.g. 1 - 10) phosphodiester linked nucleosides, such as DNA nucleosides,

[0165] Chemical transformation (cleavage of the labile bond) may be initiated by the addition of a pharmaceutically acceptable agent to the cell or may occur spontaneously when a molecule containing the labile bond reaches an appropriate intra-and/or extra-cellular environment. For example, a pH labile bond may be cleaved when the molecule enters an acidified endosome. Thus, a pH labile bond may be considered to be an endosomal cleavable bond. Enzyme cleavable bonds may be cleaved when exposed to enzymes such as those present in an endosome or lysosome or in the cytoplasm. A disulfide bond may be cleaved when the molecule enters the more reducing environment of the cell cytoplasm. Thus, a disulfide may be considered to be a cytoplasmic cleavable bond. As used herein, a pH-labile bond is a labile bond that is selectively broken under acidic conditions (pH<7). Such bonds may also be termed endosomally labile bonds, since cell endosomes and lysosomes have a pH less than 7.

Oligomer linked biocleavable conjugates

[0166] The oligomeric compound may optionally, comprise a second region (region B) which is positioned between the oligomer (referred to as region A) and the conjugate (referred to as region C). Region B may be a linker such as a cleavable linker (also referred to as a physiologically labile linkage). (see Example 7)

[0167] Nuclease Susceptible Physiological Labile Linkages: In some embodiments, the oligomer (also referred to as oligomeric compound) of the invention (or conjugate) comprises three regions:

iv) a first region (region A), which comprises 10 - 18 contiguous nucleotides;

v) a second region (region B) which comprises a biocleavable linker

vi) a third region (C) which comprises a GalNAc conjugate moiety, wherein the third region is covalent linked to the second region.

[0168] In some embodiments, region B may be a phosphate nucleotide linker. Phosphate nucleotide linkers-may be used for GalNAc conjugates.

Peptide Linkers

[0169] In some embodiments, the biocleable linker (region B) is a peptide, such as a trilysine peptide linker which may be used in a polyGalNAc conjugate, such a a triGalNAc conjugate.

[0170] Other linkers known in the art which may be used, include disulfide linkers.

Phosphate nucleotide linkers

[0171] In some embodiments, region B comprises between 1 - 6 nucleotides, which is covalently linked to the 5' or 3' nucleotide of the first region, such as via a internucleoside linkage group such as a phosphodiester linkage, wherein either

1. a. the internucleoside linkage between the first and second region is a phosphodiester linkage and the nucleoside of the second region [such as immediately] adjacent to the first region is either DNA or RNA; and/or
2. b. at least 1 nucleoside of the second region is a phosphodiester linked DNA or RNA nucleoside;

[0172] In some embodiments, region A and region B form a single contiguous nucleotide sequence of 12 - 22 nucleotides in length.

[0173] In some aspects the internucleoside linkage between the first and second regions may be considered part of the second region.

[0174] In some embodiments, there is a phosphorus containing linkage group between the second and third region. The phosphorus linkage group, may, for example, be a phosphate (phosphodiester), a phosphorothioate, a phosphorodithioate or a boranophosphate group. In some embodiments, this phosphorus containing linkage group is positioned between the second region and a linker region which is attached to the third region. In some embodiments, the phosphate group is a phosphodiester.

[0175] Therefore, in some aspects the oligomeric compound comprises at least two phosphodiester groups, wherein at least one is as according to the above statement of invention, and the other is positioned between the second and third regions, optionally between a linker group and the second region.

[0176] In some embodiments region A comprises at least one, such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 internucleoside linkages other than phosphodiester, such as internucleoside linkages which are (optionally independently] selected from the group consisting of phosphorothioate, phosphorodithioate,and boranophosphate, and methylphosphonate, such as phosphorothioate. In some embodiments region A comprises at least one phosphorothioate linkage. In some embodiments at least 50%, such as at least 75%, such as at least 90% of the internucleoside linkages, such as all the internucleoside linkages within region A are other than phosphodiester, for example are phosphorothioate linkages. In some embodiments, all the internucleoside linkages in region A are other than phosphodiester.

[0177] In some embodiments, the oligomeric compound comprises an antisense oligonucleotide, such as an antisense oligonucleotide conjugate. The antisense oligonucleotide may be or may comprise the first region, and optionally the second region.

[0178] In this respect, in some embodiments, region B may form part of a contiguous nucleobase sequence which is complementary to the (nucleic acid) target. In other embodiments, region B may lack complementarity to the target.

[0179] Alternatively stated, in some embodiments, the invention provides a non-phosphodieser linked, such as a phosphorothioate linked, oligonucleotide (e.g. an antisense oligonucleotide) which has at least one terminal (5' and/or 3') DNA or RNA nucleoside linked to the adjacent nucleoside of the oligonucleotide via a phosphodiester linkage, wherein the terminal DNA or RNA nucleoside is further covalently linked to a conjugate moiety, a targeting moiety or a blocking moiety, optionally via a linker moiety.

[0180] In some embodiments, the oligomeric compound comprises an antisense oligonucleotide, such as an antisense oligonucleotide conjugate. The antisense oligonucleotide may be or may comprise the first region, and optionally the second region.
In this respect, in some embodiments, region B may form part of a contiguous nucleobase sequence which is complementary to the (nucleic acid) target. In other embodiments, region B may lack complementarity to the target.

[0181] In some embodiments, at least two consecutive nucleosides of the second region are DNA nucleosides (such as at least 3 or 4 or 5 consecutive DNA nucleotides).

[0182] In such an embodiment, the oligonucleotide of the invention may be described according to the following formula:

5'-A-PO-B [Y)X- 3'

or

3'-A-PO-B [Y)X- 5'

wherein A is region A, PO is a phosphodiester linkage, B is region B, Y is an optional linkage group, and X is a conjugate, a targeting, a blocking group or a reactive or activation group.

[0183] In some embodiments, region B comprises 3' - 5' or 5'-3': i) a phosphodiester linkage to the 5' nucleoside of region A, ii) a DNA or RNA nucleoside, such as a DNA nucleoside, and iii) a further phosphodiester linkage

5'-A-PO-B - PO- 3'

or

3'-A-PO-B - PO- 5'

[0184] The further phosphodiester linkage link the region B nucleoside with one or more further nucleoside, such as one or more DNA or RNA nucleosides, or may link to X (is a conjugate, a targeting or a blocking group or a reactive or activation group) optionally via a linkage group (Y).

[0185] In some embodiments, region B comprises 3' - 5' or 5'-3': i) a phosphodiester linkage to the 5' nucleoside of region A, ii) between 2 - 10 DNA or RNA phosphodiester linked nucleosides, such as a DNA nucleoside, and optionally iii) a further phosphodiester linkage:

5'-A-[PO-B]n - [Y]-X 3'

or

3'-A-[PO-B]n -[Y]-X 5'

5'-A-[PO-B]n - PO-[Y]-X 3'

or

3'-A-[PO-B]n - PO-[Y]-X 5'

[0186] Wherein A represent region A, [PO-B]n represents region B, wherein n is 1 - 10, such as 1, 2, 3,4, 5, 6, 7, 8, 9 or 10, PO is an optional phosphodiester linkage group between region B and X (or Y if present).

[0187] In some embodiments the invention provides compounds according to (or comprising) one of the following formula:

5' [Region A] - PO - [region B] 3' -Y - X

5' [Region A] - PO - [region B] -PO 3' -Y - X

5' [Region A] - PO - [region B] 3' - X

5' [Region A] - PO - [region B] -PO 3' - X

3' [Region A] - PO - [region B] 5' -Y - X

3' [Region A] - PO - [region B] -PO 5' -Y - X

3' [Region A] - PO - [region B] 5' - X

3' [Region A] - PO - [region B] -PO 5' - X

[0188] Region B, may for example comprise or consist of:

5' DNA3'

3' DNA 5'

5' DNA-PO-DNA-3'

3' DNA-PO-DNA-5'

5' DNA-PO-DNA-PO-DNA 3'

3' DNA-PO-DNA-PO-DNA 5'

5' DNA-PO-DNA-PO-DNA-PO-DNA 3'

3' DNA-PO-DNA-PO-DNA-PO-DNA 5'

5' DNA-PO-DNA-PO-DNA-PO-DNA-PO-DNA 3'

3' DNA-PO-DNA-PO-DNA-PO-DNA-PO-DNA 5'

[0189] It should be recognized that phosphate linked biocleavable linkers may employ nucleosides other than DNA and RNA. Bio cleavable nucleotide linkers may, for example, be identified using the assays in Example 7.

In some embodiments, the compound of the invention comprises a biocleavable linker (also referred to as the physiologically labile linker, Nuclease Susceptible Physiological Labile Linkages, or nuclease susceptible linker), for example the phosphate nucleotide linker (such as region B) or a peptide linker, which joins the oligomer (or contiguous nucleotide sequence or region A), to a conjugate moiety (or region C).

The susceptibility to cleavage in the assays shown in Example 7 can be used to determine whether a linker is biocleavable or physiologically labile.

Biocleavable linkers according to the present invention refers to linkers which are susceptible to cleavage in a target tissue (i.e. physiologically labile), for example liver and/or kidney. It is preferred that the cleavage rate seen in the target tissue is greater than that found in blood serum. Suitable methods for determining the level (%) of cleavage in tissue (e.g. liver or kidney) and in serum are found in example 6. In some embodiments, the biocleavable linker (also referred to as the physiologically labile linker, or nuclease susceptible linker), such as region B, in a compound of the invention, are at least about 20% cleaved, such as at least about 30% cleaved, such as at least about 40% cleaved, such as at least about 50% cleaved, such as at least about 60% cleaved, such as at least about 70% cleaved, such as at least about 75% cleaved, in the liver or kidney homogenate assay of Example 7. In some embodiments, the cleavage (%) in serum, as used in the assay in Example 7, is less than about 30%, is less than about 20%, such as less than about 10%, such as less than 5%, such as less than about 1%.

[0190] In some embodiments, which may be the same of different, the biocleavable linker (also referred to as the physiologically labile linker, or nuclease susceptible linker), such as region B, in a compound of the invention, are susceptible to S1 nuclease cleavage. Susceptibility to S1 cleavage may be evaluated using the S1 nuclease assay shown in Example 7. In some embodiments, the biocleavable linker (also referred to as the physiologically labile linker, or nuclease susceptible linker), such as region B, in a compound of the invention, are at least about 30% cleaved, such as at least about 40% cleaved, such as at least about 50% cleaved, such as at least about 60% cleaved, such as at least about 70% cleaved, such as at least about 80% cleaved, such as at least about 90% cleaved, such as at least 95% cleaved after 120min incubation with S1 nuclease according to the assay used in Example 7.

Sequence selection in the second region:

[0191] In some embodiments, region B does not form a complementary sequence when the oligonucleotide region A and B is aligned to the complementary target sequence.

[0192] In some embodiments, region B does form a complementary sequence when the oligonucleotide region A and B is aligned to the complementary target sequence. In this respect region A and B together may form a single contiguous sequence which is complementary to the target sequence.

[0193] In some embodiments, the sequence of bases in region B is selected to provide an optimal endonuclease cleavage site, based upon the predominant endonuclease cleavage enzymes present in the target tissue or cell or sub-cellular compartment. In this respect, by isolating cell extracts from target tissues and non-target tissues, endonuclease cleavage sequences for use in region B may be selected based upon a preferential cleavage activity in the desired target cell (e.g. liver/hepatocytes) as compared to a non-target cell (e.g. kidney). In this respect, the potency of the compound for target down-regulation may be optimized for the desired tissue/cell.

[0194] In some embodiments region B comprises a dinucleotide of sequence AA, AT, AC, AG, TA, TT, TC, TG, CA, CT, CC, CG, GA, GT, GC, or GG, wherein C may be 5-mthylcytosine, and/or T may be replaced with U. In some embodiments region B comprises a trinucleotide of sequence AAA, AAT, AAC, AAG, ATA, ATT, ATC, ATG, ACA, ACT, ACC, ACG, AGA, AGT, AGC, AGG, TAA, TAT, TAC, TAG, TTA, TTT, TTC, TAG, TCA, TCT, TCC, TCG, TGA, TGT, TGC, TGG, CAA, CAT, CAC, CAG, CTA, CTG, CTC, CTT, CCA, CCT, CCC, CCG, CGA, CGT, CGC, CGG, GAA, GAT, GAC, CAG, GTA, GTT, GTC, GTG, GCA, GCT, GCC, GCG, GGA, GGT, GGC, and GGG wherein C may be 5-mthylcytosine and/or T may be replaced with U. In some embodiments region B comprises a trinucleotide of sequence AAAX, AATX, AACX, AAGX, ATAX, ATTX, ATCX, ATGX, ACAX, ACTX, ACCX, ACGX, AGAX, AGTX, AGCX, AGGX, TAAX, TATX, TACX, TAGX, TTAX, TTTX, TTCX, TAGX, TCAX, TCTX, TCCX, TCGX, TGAX, TGTX, TGCX, TGGX, CAAX, CATX, CACX, CAGX, CTAX, CTGX, CTCX, CTTX, CCAX, CCTX, CCCX, CCGX, CGAX, CGTX, CGCX, CGGX, GAAX, GATX, GACX, CAGX, GTAX, GTTX, GTCX, GTGX, GCAX, GCTX, GCCX, GCGX, GGAX, GGTX, GGCX, and GGGX, wherein X may be selected from the group consisting of A, T, U, G, C and analogues thereof, wherein C may be 5-mthylcytosine and/or T may be replaced with U. It will be recognised that when referring to (naturally occurring) nucleobases A, T, U, G, C, these may be substituted with nucleobase analogues which function as the equivalent natural nucleobase (e.g. base pair with the complementary nucleoside).

Amino alkyl Intermediates

[0195] The application further describes the LNA oligomer intermediates which comprise an antisense LNA oligomer which comprises an (e.g. terminal, 5' or 3') amino alkyl, such as a C2 - C36 amino alkyl group, including, for example C6 and C12 amino alkyl groups. The amino alkyl group may be added to the LNA oligomer as part of standard oligonucleotide synthesis, for example using a (e.g. protected) amino alkyl phosphoramidite. The linkage group between the amino alkyl and the LNA oligomer may for example be a phosphorothioate or a phosphodiester, or one of the other nucleoside linkage groups referred to herein, for example. The amino alkyl group may be covalently linked to, for example, the 5' or 3' of the LNA oligomer, such as by the nucleoside linkage group, such as phosphorothioate or phosphodiester linkage.

[0196] The application also describes a method of synthesis of the LNA oligomer comprising the sequential synthesis of the LNA oligomer, such as solid phase oligonucleotide synthesis, comprising the step of adding a amino alkyl group to the oligomer, such as e.g. during the first or last round of oligonucleotide synthesis. The method of synthesis my further comprise the step of reacting the conjugate to the amino alkyl -LNA oligomer (the conjugation step). The a conjugate may comprise suitable linkers and/or branch point groups, and optionally further conjugate groups, such as hydrophobic or lipophilic groups, as described herein. The conjugation step may be performed whilst the oligomer is bound to the solid support (e.g. after oligonucleotide synthesis, but prior to elution of the oligomer from the solid support), or subsequently (i.e. after elution). The invention provides for the use of an amino alkyl linker in the synthesis of the oligomer of the invention.

Method of Manufacture/Synthesis

[0197] The application describes a -method of synthesizing (or manufacture) of an oligomeric compound, such as the oligomeric compound of the invention, said method comprising
either:

1. a) a step of providing a [solid phase] oligonucleotide synthesis support to which one of the following is attached [third region]:
   1. i) a linker group (-Y-)
   2. ii) a group selected from the group consisting of a conjugate, a targeting group, a blocking group, a reactive group [e.g. an amine or an alcohol] or an activation group(X-)
   3. iii)an -Y - X group

   and
2. b) a step of [sequential] oligonucleotide synthesis of region B followed by region A, and / or:
3. c) a step of [sequential] oligonucleotide synthesis of a first region (A) and a second region (B), wherein the synthesis step is followed by
4. d) a step of adding a third region [phosphoramidite comprising]
   1. i) a linker group (-Y-)
   2. ii) a group selected from the group consisting of a conjugate, a targeting group, a blocking group, a reactive group [e.g. an amine or an alcohol] or an activation group (X-)
   3. iii)an -Y - X group

   followed by
5. e) the cleavage of the oligomeric compound from the [solid phase] support wherein, optionally said method further comprises a further step selected from:
6. f) wherein the third group is an activation group, the step of activating the activation group to produce a reactive group, followed by adding a conjugate, a blocking, or targeting group to the reactive group, optionally via a linker group (Y);
7. g) wherein the third region is a reactive group, the step of adding a conjugate, a blocking, or targeting group to the reactive group, optionally via a linker group (Y).
8. h) wherein the third region is a linker group (Y), the step of adding a conjugate, a blocking, or targeting group to the linker group (Y)

wherein steps f), g) or h) are performed either prior to or subsequent to cleavage of the oligomeric compound from the oligonucleotide synthesis support. In some embodiments, the method may be performed using standard phosphoramidite chemistry, and as such the region X and/or region X or region X and Y may be provided, prior to incorporation into the oligomer, as a phosphoramidite. Please see figures 5 - 10 which illustrate non-limiting aspects of the method of the invention.
The invention provides for a method of synthesizing (or manufacture) of an oligomeric compound, such as the oligomeric compound of the invention, said method comprising a step of [sequential] oligonucleotide synthesis of a first region (A) and optionally a second region (B), wherein the synthesis step is followed by a step of adding a third region [phosphoramidite comprising] region X (also referred to as region C) or Y, such as a region comprising a group selected from the group consisting of a conjugate, a targeting group, a blocking group, a functional group, a reactive group [e.g. an amine or an alcohol] or an activation group (X), or an -Y - X group followed by the cleavage of the oligomeric compound from the [solid phase] support.
It is however recognized that the region X or X-Y may be added after the cleavage from the solid support. Alternatively, the method of synthesis may comprise the steps of synthesizing a first (A), and optionally second region (B), followed by the cleavage of the oligomer from the support, with a subsequent step of adding a third region , such as X or X-Y group to the oligomer. The addition of the third region may be achieved, by example, by adding an amino phosphoramidite unit in the final step of oligomer synthesis (on the support), which can, after cleavage from the support, be used to join to the X or X-Y group, optionally via an activation group on the X or Y (when present) group. In the embodiments where the cleavable linker is not a nucleotide region, region B may be a non-nucleotide cleavable linker for example a peptide linker, which may form part of region X (also referred to as region C) or be region Y (or part thereof).
Region X (such as C) or (X-Y), such as the conjugate (e.g. a GalNAc conjugate) comprises an activation group, (an activated functional group) and in the method of synthesis the activated conjugate (or region x, or X-Y) is added to the first and second regions, such as an amino linked oligomer. The amino group may be added to the oligomer by standard phosphoramidite chemistry, for example as the final step of oligomer synthesis (which typically will result in amino group at the 5' end of the oligomer). For example during the last step of the oligonucleotide synthesis a protected amino-alkyl phosphoramidite is used, for example a TFA-aminoC6 phosphoramidite (6-(Trifluoroacetylamino)-hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite).
Region X (or region C as referred to herein), such as the conjugate (e.g. a GalNac conjugate) may be activated via NHS ester method and then the aminolinked oligomer is added. For example a N-hydroxysuccinimide (NHS) may be used as activating group for region X (or region C, such as a conjugate, such as a GalNac conjugate moiety.
The invention provides an oligomer prepared by the method of the invention.

[0198] Region X and/or region X or region X and Y may be covalently joined (linked) to region B via a phosphate nucleoside linkage, such as those described herein, including phosphodiester or phosphorothioate, or via an alternative group, such as a triazol group.
The internucleoside linkage between the first and second region is a phosphodiester linked to the first (or only) DNA or RNA nucleoside of the second region, or region B comprises at least one phosphodiester linked DNA or RNA nucleoside..

[0199] The second region may comprise further DNA or RNA nucleosides which may be phosphodester linked. The second region is further covalently linked to a third region which may, for example, be a conjugate, a targeting group a reactive group, and/or a blocking group.

[0200] In some aspects, the present invention is based upon the provision of a labile region, the second region, linking the first region, e.g. an antisense oligonucleotide, and a GalNAc conjugate. The labile region comprises at least one phosphodiester linked nucleoside, such as a DNA or RNA nucleoside, such as 1, 2, 3, 4, 5, 6, 7, 8,9 or 10 phosphodiester linked nucleosides, such as DNA or RNA. In some embodiments, the oligomeric compound comprises a cleavable (labile) linker. In this respect the cleavable linker is preferably present in region B (or in some embodiments, between region A and B).

[0201] Alternatively stated, in some embodiments, the invention provides a non-phosphodiester linked, such as a phosphorothioate linked, oligonucleotide (e.g. an antisense oligonucleotide) which has at least one terminal (5' and/or 3') DNA or RNA nucleoside linked to the adjacent nucleoside of the oligonucleotide via a phosphodiester linkage, wherein the terminal DNA or RNA nucleoside is further covalently linked to a conjugate moiety, a targeting moiety or a blocking moiety, optionally via a linker moiety.

Compositions

[0202] The oligomer of the compound of the invention may be used in pharmaceutical formulations and compositions. Suitably, such compositions comprise a pharmaceutically acceptable diluent, carrier, salt or adjuvant. WO2007/031091 provides suitable and preferred pharmaceutically acceptable diluent, carrier and adjuvants. Suitable dosages, formulations, administration routes, compositions, dosage forms, combinations with other therapeutic agents, pro-drug formulations are also provided in WO2007/031091.

Applications

[0203] The oligomers of the compound of the invention may be utilized as research reagents for, for example, diagnostics, therapeutics and prophylaxis.

[0204] In research, such oligomers may be used to specifically inhibit the synthesis of APOB protein (typically by degrading or inhibiting the mRNA and thereby prevent protein formation) in cells and experimental animals thereby facilitating functional analysis of the target or an appraisal of its usefulness as a target for therapeutic intervention.

[0205] In diagnostics the oligomers may be used to detect and quantitate APOB expression in cell and tissues by northern blotting, *in-situ* hybridisation or similar techniques.

[0206] For therapeutics, an animal or a human, suspected of having a disease or disorder, which can be treated by modulating the expression of APOB is treated by administering oligomeric compounds in accordance with this invention. Further described are methods of treating a mammal, such as treating a human, suspected of having or being prone to a disease or condition, associated with expression of APOB -by administering a therapeutically or prophylactically effective amount of one or more of the oligomers or compositions of the invention. The oligomer, a conjugate or a pharmaceutical composition according to the invention is typically administered in an effective amount.

[0207] The invention also provides for the use of the compound or conjugate of the invention as described for the manufacture of a medicament for the treatment of a disorder as referred to herein, or for a method of the treatment of as a disorder as referred to herein.

[0208] The invention also provides for a method for treating a disorder as referred to herein said method comprising administering a compound according to the invention as herein described, and/or a conjugate according to the invention, and/or a pharmaceutical composition according to the invention to a patient in need thereof.

Medical Indications

[0209] The oligomer conjugates and other compositions according to the invention can be used for the treatment of conditions associated with over expression or expression of mutated version of the ApoB.

[0210] The invention further provides use of a compound of the invention in the manufacture of a medicament for the treatment of a disease, disorder or condition as referred to herein.

[0211] Generally stated, to the application describes a method of treating a mammal suffering from or susceptible to conditions associated with abnormal levels and/or activity of APOB, comprising administering to the mammal and therapeutically effective amount of an oligomer targeted to APOB that comprises one or more LNA units. The oligomer conjugate or a pharmaceutical composition according to the invention is typically administered in an effective amount.

[0212] The disease or disorder, as referred to herein, may, in some embodiments be associated with a mutation in the APOB gene or a gene whose protein product is associated with or interacts with APOB. Therefore, in some embodiments, the target mRNA is a mutated form of the APOB sequence.

[0213] An interesting aspect of the invention is directed to the use of an oligomer conjugate (compound) as defined herein for the preparation of a medicament for the treatment of a disease, disorder or condition as referred to herein.

[0214] Alternatively stated, to the application describes a method for treating abnormal levels and/or activity of APOB, said method comprising administering a oligomer of the invention, or a conjugate of the invention or a pharmaceutical composition of the invention to a patient in need thereof.

[0215] The invention also relates to an oligomer conjugate or a composition as defined herein for use as a medicament.

[0216] The invention further relates to use of composition, or a conjugate as defined herein for the manufacture of a medicament for the treatment of abnormal levels and/or activity of APOB or expression of mutant forms of APOB (such as allelic variants, such as those associated with one of the diseases referred to herein).

[0217] Moreover, the application describes a method of treating a subject suffering from a disease or condition such as those referred to herein.

[0218] A patient who is in need of treatment is a patient suffering from or likely to suffer from the disease or disorder.

[0219] In some embodiments, the term 'treatment' as used herein refers to both treatment of an existing disease (*e.g.* a disease or disorder as herein referred to), or prevention of a disease, *i.e.* prophylaxis. It will therefore be recognised that treatment as referred to herein may, in some embodiments, be prophylactic.

[0220] In one embodiment, the invention relates to compounds or compositions comprising compounds for treatment of hypercholesterolemia and related disorders, or methods of treatment using such compounds or compositions for treating hypercholesterolemia and related disorders, wherein the term "related disorders" when referring to hypercholesterolemia refers to one or more of the conditions selected from the group consisting of: atherosclerosis, hyperlipidemia, hypercholesterolemia, familiar hypercholesterolemia *e.g.* gain of function mutations in APOB, HDL/LDL cholesterol imbalance, dyslipidemias, *e.g.*, familial hyperlipidemia (FCHL), acquired hyperlipidemia, statin-resistant hypercholesterolemia, coronary artery disease (CAD), and coronary heart disease (CHD.

EXAMPLES

Oligonucloetides

ApoB Targeting Compounds

Oligonucleotide sequence motifs

[0221]

GCATTGGTATTCA (SEQ ID NO 1)
GTTGACACTGTC (SEQ ID NO 2)

#	SEQ ID NO	Seq (5'-3') (Region A)	Cleavable Linker (Region B)	Region C - Conjuqate
#1	3	GCattggtatTCA	no	no
#2	4	GCattggtatTCA	no	Cholesterol
#3	5	GCattggtatTCA	SS	Cholesterol
#4	6	GCattggtatTCA	3PO-DNA (5'tca3')	Cholesterol
#5	7	GCattggtatTCA	2PO-DNA (5'ca3')	Cholesterol
#6	8	GCattggtatTCA	1 PO-DNA (5'a3')	Cholesterol

ApoB Targeting Compounds with FAM label conjugates

[0222]

SEQ ID	Seq (5'-3')	Cleavable linker (B)	Conjugate (C)
9	GCattggtatTCA	3PO-DNA (5'tca3')	FAM
10	GCattggtatTCA	2PO-DNA (5'ca3')	FAM
11	GCattggtatTCA	1PO-DNA (5'a3')	FAM
12	GCattggtatTCA	3PO-DNA (5'gac3')	FAM
13	GCattggtatTCA	no	FAM

SEQ ID NO	Seq (5'-3')	Cleavable Linker (B)	Conjugate
14	GCattggtatTCA	no	Folic acid
15	GCattggtatTCA	SS	Folic acid
16	GCattggtatTCA	2PO-DNA (5'ca3')	Folic acid
17	GCattggtatTCA	no	monoGalNAc
18	GCattggtatTCA	SS	monoGalNAc
19	GCattggtatTCA	2PO-DNA (5'ca3')	monoGalNAc
20	GCattggtatTCA		GalNAc cluster Conj2a
21	GCattggtatTCA	*no*	FAM
22	GCattggtatTCA	SS	FAM
23	GCattggtatTCA	2PO-DNA (5'ca3')	FAM
24	GCattggtatTCA	no	Tocopherol
25	GCattggtatTCA	SS	Tocopherol
26	GCattggtatTCA	2PO-DNA (5'ca3')	Tocopherol
30	GCattggtatTCA		GalNAc cluster Conj1a

	SEQ ID NO	Seq (5'-3')	Cleavable Linker (B)
7	GCattggtatTCA	2PO-DNA (5'ca3')	Cholesterol
20	GCattggtatTCA		GalNAc cluster Conj2a

	SEQ ID NO	Seq (5'-3')	Cleavable Linker (B)
28	GTtgacactgTC	2PO-DNA (5'ca3')	Cholesterol
29	GTtgacactgTC		GalNAc cluster Conj2a
31	GTtgacactgTC		GalNAc cluster Conj1a

[0223] Mouse Experiments: Unless otherwise specified, the mouse experiments may be performed as follows:

Dose administration and sampling:

7-10 week old C57Bl6-N mice were used, animals were age and sex matched (females for study 1, 2 and 4, males in study 3). Compounds were injected i.v. into the tail vein. For intermediate serum sampling, 2-3 drops of blood were collected by puncture of the vena facialis, final bleeds were taken from the vena cava inferior. Serum was collected in gel-containing serum-separation tubes (Greiner) and kept frozen until analysis.

[0224] C57BL6 mice were dosed i.v. with a single dose of 1mg/kg ASO (or amount shown) formulated in saline or saline alone according to the information shown. Animals were sacrificed at e.g. day 4 or 7 (or time shown) after dosing and liver and kidney were sampled. RNA isolation and mRNA analysis: mRNA analysis from tissue was performed using the Qantigene mRNA quantification kit ("bDNA-assay", Panomics/Affimetrix), following the manufacturers protocol. For tissue lysates, 50-80 mg of tissue was lysed by sonication in 1 ml lysis-buffer containing Proteinase K. Lysates were used directly for bDNA-assay without RNA extraction. Probesets for the target and GAPDH were obtained custom designed from Panomics. For analysis, luminescence units obtained for target genes were normalized to the housekeeper GAPDH.

[0225] Serum analysis for ALT, AST and cholesterol was performed on the "Cobas INTEGRA 400 plus" clinical chemistry platform (Roche Diagnostics), using 10µl of serum.

[0226] For quantification of Factor VII serum levels, the BIOPHEN FVII enzyme activity kit (#221304, Hyphen BioMed) was used according to the manufacturer's protocol.

[0227] For oligonucleotide quantification, a fluorescently-labeled PNA probe is hybridized to the oligo of interest in the tissue lysate. The same lysates are used as for bDNA-assays, just with exactly weighted amounts of tissue. The heteroduplex is quantified using AEX-HPLC and fluorescent detection.

Example 1: Synthesis of compounds

[0228] Oligonucleotides were synthesized on uridine universal supports using the phosphoramidite approach on an Expedite 8900/MOSS synthesizer (Multiple Oligonucleotide Synthesis System) at 4 µmol scale. At the end of the synthesis, the oligonucleotides were cleaved from the solid support using aqueous ammonia for 1-2 hours at room temperature, and further deprotected for 16 hours at 65°C. The oligonucleotides were purified by reverse phase HPLC (RP-HPLC) and characterized by UPLC, and the molecular mass was further confirmed by ESI-MS. See below for more details.

Elongation of the oligonucleotide

[0229] The coupling of β-cyanoethyl- phosphoramidites (DNA-A(Bz), DNA- G(ibu), DNA- C(Bz), DNA-T, LNA-5-methyl-C(Bz), LNA-A(Bz), LNA- G(dmf), LNA-T or C6-S-S linker) is performed by using a solution of 0.1 M of the 5'-O-DMT-protected amidite in acetonitrile and DCI (4,5-dicyanoimidazole) in acetonitrile (0.25 M) as activator. For the final cycle a commercially available C6-linked cholesterol phosphoramidite was used at 0.1M in DCM. Thiolation for introduction of phosphorthioate linkages is carried out by using xanthane hydride (0.01 M in acetonitrile/pyridine 9:1). Phosphordiester linkages are introduced using 0.02 M iodine in THF/Pyridine/water 7:2:1. The rest of the reagents are the ones typically used for oligonucleotide synthesis. For post solid phase synthesis conjugation a commercially available C6 aminolinker phorphoramidite was used in the last cycle of the solid phase synthesis and after deprotection and cleavage from the solid support the aminolinked deprotected oligonucleotide was isolated. The conjugates was introduced via activation of the functional group using standard synthesis methods.

Purification by RP-HPLC:

[0230] The crude compounds were purified by preparative RP-HPLC on a Phenomenex Jupiter C18 10µ 150x10 mm column. 0.1 M ammonium acetate pH 8 and acetonitrile was used as buffers at a flowrate of 5 mL/min. The collected fractions were lyophilized to give the purified compound typically as a white solid.

Abbreviations:

[0231]

DCI:
: 4,5-Dicyanoimidazole

DCM:
: Dichloromethane

DMF:
: Dimethylformamide

DMT:
: 4,4'-Dimethoxytrityl

THF:
: Tetrahydrofurane

Bz:
: Benzoyl

Ibu:

Isobutyryl

RP-HPLC:

Reverse phase high performance liquid chromatography

Example 2: Design of LNA antisense oligonucleotides

[0232] Oligomers used in the examples and figures. The SEQ# is an identifier used throughout the examples and figures

Comp'ID (SEQ ID)	**Compound Sequence**	**Comment**
	GCATTGGTATTCA (SEQ ID NO 1)	Nucleobase sequence
	GTTGACACTGTC (SEQ ID NO 2)	Nucleobase sequence
#1 (3)	5'-$\mathbf{G_s}^o$ $^m\mathbf{C_s}^o$ a_s t_s t_s g_s g_s t_s a_s t_s $\mathbf{T_s}^o$ ^{m} $\mathbf{C_s}^o$ $\mathbf{A}^o$ -3'	No conjugate
#2 (4)	5'- CHOL $\mathbf{G_s}^o$ $^m\mathbf{C_s}^o$ a_s t_s t_s g_s g_s t_s a_s t_s $\mathbf{T_s}^o$ $^m\mathbf{C_s}^o$ $\mathbf{A}^o$ -3'	Chol-Compound
#3 (5)	5'- Chol_C6 C6SSC6 $\mathbf{G_s}^o$ $^m\mathbf{C_s}^o$ a_s t_s t_s g_s g_s t_s a_s t_s $\mathbf{T_s}^o$ $^m\mathbf{C_s}^o$ $\mathbf{A}^o$ -3'	Chol-SS-#1
#4 (6)	5'- Chol_C6 t c a $\mathbf{G_s}^o$ $^m\mathbf{C_s}^o$ a_s t_s t_s g_s g_s t_s a_s t_s $\mathbf{T_s}^o$ $^m\mathbf{C_s}^o$ $\mathbf{A}^o$ -3'	Chol-3PO-#1
#5 (7)	5'- Chol_C6 c a $\mathbf{G_s}^o$ $^m\mathbf{C_s}^o$ a_s t_s t_s g_s g_s t_s a_s t_s $\mathbf{T_s}^o$ $^m\mathbf{C_s}^o$ $\mathbf{A}^o$ -3'	Chol-2PO-#1

Example 3. Knock down of ApoB mRNA with Cholesterol-conjugates *in vivo.*

[0233] C57BL6/J mice were injected with a single dose saline or 1 mg/kg unconjugated LNA-antisense oligonucleotide (#3) or equimolar amounts of LNA antisense oligonulceotides conjugated to Cholesterol with different linkers and sacrificed at days 1-10 according to the table below. RNA was isolated from liver and kidney and subjected to qPCR with ApoB specific primers and probe to analyse for ApoB mRNA knockdown.

[0234] **Conclusions:** Cholesterol conjugated to an ApoB LNA antisense oligonucleotide with a linker composed of 2 or 3 DNA with Phophodiester-backbone (Seq#4 and 5) showed a preference for liver specific knock down of ApoB (Fig. 11). This means increases efficiency and duration of

ApoB mRNA knock down in liver tissue compared to the unconjugated compound (Seq #3),as well as compared to Cholesterol conjugates with stable linker (Seq#4) and with disulphide linker (Seq.#5) and concomitant less knock down activity of Seq#6 and #7 in kidney tissue.

Materials and Methods:

Experimental design:

[0235]

	Gr. no.	Animal ID no.	**No. of animals**	**Animal strain/ gender/ feed**	**Compound Dose level per day**	**Conc. at dose vol. 10 ml/kg**	**Body weight**	**Sacrifice**
A	1	1-4	4	C57BL/6J-♀-Chow	NaCl 0.9%	-	Day -1, 7 and 10	Day 10
	2	5-8	4	C57BL/6J-♀- Chow	SEQ ID 3 1 mg/kg	0.1 mg/ml	Day -1, 7 and 10	Day 10
	3	9-12	4	C57BL/6J-♀- Chow	SEQ ID 4 1,2 mg/kg	0.12mg/ml	Day -1, 7 and 10	Day 10
	4	13-16	4	C57BL/6J-♀- Chow	SEQ ID 5 1,2 mg/kg	0.12mg/ml	Day -1, 7 and 10	Day 10
	5	17-20	4	C57BL/6J-♀- Chow	SEQ ID 6 1,3 mg/kg	0.13mg/ml	Day -1, 7 and 10	Day 10
	6	21-24	4	C57BL/6J-♀- Chow	SEQ ID 7 1,3 mg/kg	0.13mg/ml	Day -1, 7 and 10	Day 10
B	7	25-28	4	C57BL/6J-♀- Chow	NaCl 0.9%	-	Day -1, 7	Day 7
	8	29-32	4	C57BL/6J-♀- Chow	SEQ ID 3 1 mg/kg	0.1 mg/ml	Day -1, 7	Day 7
	9	33-36	4	C57BL/6J-♀- Chow	SEQ ID 4 1,2 mg/kg	0.12mg/ml	Day -1, 7	Day 7
	10	37-40	4	C57BL/6J-♀- Chow	SEQ ID 5 1,2 mg/kg	0.12mg/ml	Day -1, 7	Day 7
	11	41-44	4	C57BL/6J-♀- Chow	SEQ ID 6 1,3 mg/kg	0.13mg/ml	Day -1, 7	Day 7
	12	45-48	4	C57BL/6J-♀- Chow	SEQ ID 7 1,3 mg/kg	0.13mg/ml	Day -1, 7	Day 7

	Gr. no.	Animal ID no.	No. of animals	Animal strain/ gender/ feed	Compound Dose level per day	Conc. at dose vol. 10 ml/kg	Body weight	Sacrifice
C	13	49-52	4	C57BL/6J- ♀- Chow	NaCl 0.9%	-	Day 0, 3	Day 3
	14	53-56	4	C57BL/6J- ♀- Chow	SEQ ID 3 1 mg/kg	0.1 mg/ml	Day 0, 3	Day 3
	15	57-60	4	C57BL/6J- ♀- Chow	SEQ ID 4 1,2 mg/kg	0.12mg/ml	Day 0, 3	Day 3
	16	61-64	4	C57BL/6J- ♀- Chow	SEQ ID 5 1,2 mg/kg	0.12mg/ml	Day 0, 3	Day 3
	17	65-68	4	C57BL/6J- ♀- Chow	SEQ ID 6 1,3 mg/kg	0.13mg/ml	Day 0, 3	Day 3
	18	69-72	4	C57BL/6J- ♀- Chow	SEQ ID 7 1,3 mg/kg	0.13mg/ml	Day 0, 3	Day 3
D	19	73-76	4	C57BL/6J- ♀- Chow	NaCl 0.9%	-	Day -1, 1	Day 1
	20	77-80	4	C57BL/6J- ♀- Chow	SEQ ID 3 1 mg/kg	0.1 mg/ml	Day -1, 1	Day 1
	21	81-84	4	C57BL/6J- ♀- Chow	SEQ ID 4 1,2 mg/kg	0.12mg/ml	Day -1, 1	Day 1
	22	85-88	4	C57BL/6J- ♀- Chow	SEQ ID 5 1,2 mg/kg	0.12mg/ml	Day -1, 1	Day 1
	23	89-92	4	C57BL/6J- ♀- Chow	SEQ ID 6 1,3 mg/kg	0.13mg/ml	Day -1, 1	Day 1
	24	93-96	4	C57BL/6J- ♀- Chow	SEQ ID 7 1,3 mg/kg	0.13mg/ml	Day -1, 1	Day 1

[0236] *Dose administration*. C57BL/6JBom female animals, app. 20 g at arrival, were dosed with 10 ml per kg BW (according to day 0 bodyweight) *i.v.* of the compound formulated in saline or saline alone according to the above table.

[0237] *Sampling of liver and kidney tissue*. The animals were anaesthetised with 70% CO_2-30% O_2 and sacrificed by cervical dislocation according to the table above. One half of the large liver lobe and one kidney were minced and submerged in RNAlater.

[0238] Total RNA Isolation and First strand synthesis. Total RNA was extracted from maximum 30 mg of tissue homogenized by bead-milling in the presence of RLT-Lysis buffer using the Qiagen RNeasy kit (Qiagen cat. no. 74106) according to the manufacturer's instructions. First strand synthesis was performed using Reverse Transcriptase reagents from Ambion according to the manufacturer's instructions.

[0239] For each sample 0.5 µg total RNA was adjusted to (10.8 µl) with RNase free H_2O and mixed with 2 µl random decamers (50 µM) and 4 µl dNTP mix (2.5 mM each dNTP) and heated to 70 °C for 3 min after which the samples were rapidly cooled on ice. 2 µl 10x Buffer RT, 1 µl MMLV Reverse Transcriptase (100 U/µl) and 0.25 µl RNase inhibitor (10 U/µl) were added to each sample, followed by incubation at 42 °C for 60 min, heat inactivation of the enzyme at 95°C for 10 min and then the sample was cooled to 4 °C. cDNA samples were diluted 1: 5 and subjected to RT-QPCR using Taqman Fast Universal PCR Master Mix 2x (Applied Biosystems Cat #4364103) and Taqman gene expression assay (mApoB, Mn01545150_m1 and mGAPDH #4352339E) following the manufacturers protocol and processed in an Applied Biosystems RT-qPCR instrument (7500/7900 or ViiA7) in fast mode.

Example 4. *In vivo* silencing of ApoB mRNA with different conjugates.

[0240] To explore the impact of different conjugation moieties and linkers on the activity of an ApoB compound, Seq ID #3 was conjugated to either monoGalNAc, Folic acid, FAM or Tocopherol using a non-cleavable linker or biocleavable linker (Dithio (SS) or 2 DNA nucleotides with Phosphodiester backbone (PO)). Additionally the monoGalNAc was compared to a GalNAc cluster (Conjugate 2a). C57BL6In mice were treated i.v. with saline control or with a single dose of 1 or 0,25 mg/kg of ASO conjugates. After 7 days the animals were sacrificed and RNA was isolated from liver and kidney samples and analysed for ApoB mRNA expression (Fig.15).

[0241] Conclusions: Tocopherol conjugated to the ApoB compound with a DNA/PO-linker (#26) increased ApoB knock down in the liver compared to the unconjugated ApoB compound (#3) while decreasing activity in kidney (compare Fig.15A and B).This points towards an ability of the Tocopherol to redirect the ApoB compound from kidney to liver. The non-cleavable (#24) and SS-linker (#25) were inactive in both tissues. Mono-GalNAc conjugates with a non-cleavable (#17) and with bio-cleavable DNA/PO linker (#19) show a tendency to preserve the activity of the unconjugated compound (#3) in kidney while improving activity in the Liver. Introduction of a SS-linker decreased activity in both tissues (compare Fig. 15A and B). Conjugation of different GalNAc conjugates e.g. mono GalNAcPO (#19) and a GalNAc cluster (#20) also allows fine tuning of the compound acitivity with focus on either liver or kidney (Fig.15C). Folic acid and FAM conjugates with the cleavable DNA/PO-linker (SEQ ID Nos16 and 23) behave comparable to the unconjugated compound (3). Here as well the introduction of a non-cleavable (14 and 21) or SS-linker (15 and 22) decreases compound activity in both tissues (compare Figures 15a and 15b).

Materials and Methods:

Experimental design:

[0242]

Gr. no.	Animals per group	Animal strain/ gender/ feed	Compound Seq ID #	Dose mg/kg	Adm. Route	Dosing Day	Sacrifice Day
1	5	C57BL6 ♀- Chow	3	1	i.v.	0	7
2	5	C57BL6 ♀- Chow	14	1	i.v.	0	7
3	5	C57BL6 ♀- Chow	15	1	i.v.	0	7
4	5	C57BL6 ♀- Chow	16	1	i.v..	0	7
5	5	C57BL6 ♀- Chow	17	1	i.v.	0	7
6	5	C57BL6 ♀- Chow	18	1	i.v.	0	7
7	5	C57BL6 ♀- Chow	19	1	i.v.	0	7
8	5	C57BL6 ♀- Chow	19	0,25	i.v.	0	7
9	5	C57BL6 ♀- Chow	20	0,25	i.v.	0	7
10	5	C57BL6 ♀- Chow	NaCl 0.9%		i.v.	0	7

Gr. no.	Anima ls per group.	Animal strain/ gender/ feed	Compound Seq ID #	Dose mg/kg	Adm. Route	Dosing Day	Sacrifice Day
1	5	C57BL6 ♀- Chow	1	1	i.v.	0	7
2	5	C57BL6 ♀- Chow	31	1	i.v.	0	7
3	5	C57BL6 ♀- Chow	32	1	i.v.	0	7
4	5	C57BL6 ♀- Chow	33	1	i.v..	0	7
5	5	C57BL6 ♀- Chow	34	1	i.v.	0	7
6	5	C57BL6 ♀- Chow	35	1	i.v.	0	7
7	5	C57BL6 ♀- Chow	36	1	i.v.	0	7
8	5	C57BL6 ♀- Chow	NaCl 0.9%	1	i.v.	0	7

[0243] *Dose administration and sampling*. C57BL6 mice were dosed i.v. with a single dose of 1 mg/kg or 0,25 mg/kg ASO formulated in saline or saline alone according to the above table. Animals were sacrificed at day7 after dosing and liver and kidney were sampled. RNA isolation and mRNA analysis. Total RNA was extracted from liver and kidney samples and ApoB mRNA levels were analysed using a branched DNA assay

Example 5: Non-Human Primate Study

[0244] The primary objective for this study is to investigate selected lipid markers over 7 weeks after a single slow bolus injection of anti-ApoB LNA conjugated compounds to cynomolgus monkeys and assess the potential toxicity of compounds in monkey. The compounds used in this study are SEQ ID NOs 7, 20, 28 & 29, prepared in sterile saline (0.9%) at an initial concentration of 0.625 and 2.5 mg/ml).

[0245] Female monkeys of at least 24 months old are used, and given free access to tap water and 180g of OWM(E) SQC SHORT expanded diet (Dietex France, SDS, Saint Gratien, France) will be distributed daily per animal. The total quantity of food distributed in each cage will be calculated according to the number of animals in the cage on that day. In addition, fruit or vegetables will be given daily to each animal. The animals will be acclimated to the study conditions for a period of at least 14 days before the beginning of the treatment period. During this period, pre-treatment investigations will be performed. The animals are dosed i.v. at a dose if, for example, 0.25 mg/kg or 1 mg/kg. The dose volume will be 0.4 mL/kg. 2 animals are used per group. After three weeks, the data will be analyzed and a second group of animals using a higher or lower dosing regimen may be initiated - preliminary dose setting is 0.5 mg/kg and 1 mg/kg, or lower than that based on the first data set.

[0246] The dose formulations will be administered once on Day 1. Animals will be observed for a period of 7 weeks following treatment, and will be released from the study on Day 51. Day 1 corresponds to the first day of the treatment period. Clinical observations and body weight and food intake (per group) will be recorded prior to and during the study.

[0247] Blood is sampled and analysis at the following time points:

Study Day	Parameters
-8	RCP, L, Apo-B, OA
-1	L, Apo-B, PK, OA
1	Dosing
4	LSB, L, Apo-B,OA
8	LSB, L, Apo-B, PK, OA
15	RCP, L, Apo-B, PK, OA
22	LSB, L, Apo-B, PK, OA
29	L, Apo-B, PK, OA
36	LSB, L, Apo-B, PK, OA

Study Day	Parameters
43	L, PK, Apo-B, PK, OA
50	RCP, L, Apo-B, PK, OA
RCP 0 routine clinical pathology, LSB = liver safety biochemistry, PK = pharmacokinetics, OA = other analysis, L = Lipids.	

Blood biochemistry

[0248] The following parameters will be determined for all surviving animals at the occasions indicated below:

- full biochemistry panel (complete list below) - on Days -8, 15 and 50,
- liver Safety (ASAT, ALP, ALAT, TBIL and GGT only) - on Days 4, 8, 22 and 36,
- lipid profile (Total cholesterol, HDL-C, LDL-C and Triglycerides) and Apo-B only - on Days -1, 4, 8, 22, 29, 36, and 43.

[0249] Blood (approximately 1.0 mL) is taken into lithium heparin tubes (using the ADVIA 1650 blood biochemistry analyzer): Apo-B, sodium, potassium, chloride, calcium, inorganic phosphorus, glucose, HDL-C, LDL-C, urea, creatinine, total bilirubin (TBIL), total cholesterol, triglycerides, alkaline phosphatase (ALP), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT),creatine kinase, gamma-glutamyl transferase (GGT), lactate dehydrogenase, total protein, albumin, albumin/globulin ratio.
Analysis of blood: Blood samples for ApoB analysis will be collected from Group 1-16 animals only (i.e. animals treated with anti-PCSK9 compounds) on Days -8, -1, 4, 8, 15, 22, 29, 36, 43 and 50. Venous blood (approximately 2 mL) will be collected from an appropriate vein in each animal into a Serum Separating Tube (SST) and allowed to clot for at least 60 ± 30 minutes at room temperature. Blood will be centrifuged at 1000 g for 10 minutes under refrigerated conditions (set to maintain +4°C). The serum will be transferred into 3 individual tubes and stored at -80°C until analyzed at CitoxLAB France using an ELISA method (Circulex Human PCSK9 ELISA kit, CY-8079, validated for samples from cynomolgus monkey).
Other Analysis: WO2010142805 provides the methods for the following analysis: qPCR, ApoB mRNA analysis. Other analysis includes ApoB protein ELISA, serum Lp(a) analysis with ELISA (Mercodia No. 10-1106-01), tissue and plasma oligonucleotide analysis (drug content), Extraction of samples, standard - and QC-samples, Oligonucleotide content determination by ELISA.

Example 6: Liver and Kidney toxicity Assessment in Rat.

[0250] Compounds of the invention can be evaluated for their toxicity profile in rodents, such as in mice or rats. By way of example the following protocol may be used: Wistar Han Crl:WI(Han) are used at an age of approximately 8 weeks old. At this age, the males should weigh approximately

250 g. All animals have free access to SSNIFF R/M-H pelleted maintenance diet (SSNIFF Spezialdiäten GmbH, Soest, Germany) and to tap water (filtered with a 0.22 µm filter) contained in bottles.The dose level of 10 and 40mg/kg/dose is used (sub-cutaneous administration) and dosed on days 1 and 8. The animals are euthanized on Day 15. Urine and blood samples are collected on day 7 and 14. A clinical pathology assessment is made on day 14. Body weight is determined prior to the study, on the first day of administration, and 1 week prior to necropsy. Food consumption per group will be assessed daily. Blood samples are taken via the tail vein after 6 hours of fasting. The following blood serum analysis is performed: erythrocyte count mean cell volume packed cell volume hemoglobin mean cell hemoglobin concentration mean cell hemoglobin thrombocyte count leucocyte count differential white cell count with cell morphology reticulocyte count, sodium potassium chloride calcium inorganic phosphorus glucose urea creatinine total bilirubin total cholesterol triglycerides alkaline phosphatase alanine aminotransferase aspartate aminotransferase total protein albumin albumin/globulin ratio. Urinalysis are performed α-GST, β-2 Microglobulin, Calbindin, Clusterin, Cystatin C, KIM-1,Osteopontin, TIMP-1, VEGF,and NGAL. Seven analytes (Calbindin, Clusterin, GST-α, KIM-1, Osteopontin, TIMP-1, VEGF) will be quantified under Panel 1 (MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panel 1, RKTX1 MAG-37K). Three analytes (β-2 Microglobulin, Cystatin C, Lipocalin-2/NGAL) will be quantified under Panel 2 (MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panel 2, RKTX2MAG-37K). The assay for the determination of these biomarkers' concentration in rat urines is based on the Luminex xMAP® technology. Microspheres coated with anti- α-GST / β-2 microglobulin / calbindin / clusterin / cystacin C / KIM-1 / osteopontin / TIMP-1 / VEGF / NGAL antibodies are color-coded with two different fluorescent dyes.The following parameters are determined (Urine using the ADVIA 1650): Urine protein, urine creatinine. Quantitative parameters: volume, pH (using 10-Multistix SG test strips/Clinitek 500 urine analyzer), specific gravity (using a refractometer). Semi-quantitative parameters (using 10-Multistix SG test strips/Clinitek 500 urine analyzer): proteins, glucose, ketones, bilirubin, nitrites, blood, urobilinogen, cytology of sediment (by microscopic examination).Qualitative parameters: Appearance, color. After sacrifice, the body weight and kidney, liver and spleen weight are determined and organ to body weight ratio calculated. Kidney and liver samples will be taken and either frozen or stored in formalin. Microscopic analysis is performed.

Example 7 ApoB Targeting Compounds with FAM label conjugates

[0251]

#	Seq (5'-3')	Cleavable linker (B)	Conjugate (C)
32	GCattggtatTCA	3PO-DNA (5'tca3')	FAM
33	GCattggtatTCA	2PO-DNA (5'ca3')	FAM
34	GCattggtatTCA	1 PO-DNA (5'a3')	FAM
35	GCattggtatTCA	3PO-DNA (5'gac3')	FAM
36	GCattggtatTCA	no	FAM

[0252] Capital letters are LNA nucleosides (such as beta-D-oxy LNA), lower case letters are DNA nucleosides. Subscript s represents a phosphorothioate internucleoside linkages. LNA cytosines are optionally 5-methyl cytosine.

[0253] FAM-labelled ASOs with different DNA/PO-linkers were subjected to *in vitro* cleavage either in S1 nuclease extract, Liver or kidney homogenates or Serum.

[0254] FAM-labeled ASOs 100 µM with different DNA/PO-linkers were subjected to *in vitro* cleavage by S1 nuclease in nuclease buffer (60 U pr. 100 µL) for 20 and 120 minutes (see table below). The enzymatic activity was stopped by adding EDTA to the buffer solution. The solutions were then subjected to AIE HPLC analyses on a Dionex Ultimate 3000 using an Dionex DNApac p-100 column and a gradient ranging from 10mM - 1 M sodium perchlorate at pH 7.5. The content of cleaved and non cleaved oligonucleotide were determined against a standard using both a fluoresense detector at 615 nm and a uv detector at 260 nm.

SEQ ID NO	Linker sequence	% cleaved after 20min S1	% cleaved after 120min S1
36	--	2	5
34	a	29.1	100
33	ca	40.8	100
32	tca	74.2	100
35	gac	22.9	n.d

[0255] **Conclusion:** The PO linkers (or region B as referred to herein) results in the conjugate (or group C) being cleaved off, and both the length and/or the sequence composition of the linker can be used to modulate susceptibility to nucleolytic cleavage of region B. The Sequence of DNA/PO-linkers can modulate the cleavage rate as seen after 20 min in Nuclease S1 extract Sequence selection for region B (e.g.for the DNA/PO-linker) can therefore also be used to modulate the level of cleavage in serum and in cells of target tissues.

[0256] Liver, kidney and Serum were spiked with oligonucleotide SEQ ID NO 32 to concentrations of 200 µg/g tissue (see table below). Liver and kidney samples collected from NMRI mice were homogenized in a homogenisation buffer (0,5% Igepal CA-630, 25 mM Tris pH 8.0, 100 mM NaCl, pH 8.0 (adjusted with 1 N NaOH). The homogenates were incubated for 24 hours at 37° and thereafter the homogenates were extracted with phenol - chloroform. The content of cleaved and non-cleaved oligonucleotide in the extract from liver and kidney and from the serum were determinded against a standard using the above HPLC method.

Seq ID	Linker Sequence	% cleaved after 24hrs liver homogenate	% cleaved after 24hrs kidney homogenate	% cleaved after 24hours in serum
32	tca	83	95	0

[0257] **Conclusion:** The PO linkers (or region B as referred to herein) results in cleavage of the conjugate (or group C) from the oligonucleotide in liver or kidney homogenate, but not in serum. Note: cleavage in the above assays refers to the cleavage of the cleavable linker, the oligomer or region A should remain functionally intact.

[0258] The susceptibility to cleavage in the assays shown in Example 7 may be used to determine whether a linker is biocleavable or physiologically labile.

Example 8. Knock down of ApoB mRNA, tissue content, and total cholesterol with GalNAc-conjugates *in vivo.*

Compounds

[0259]

SEQ ID NO	Seq (5'-3') (A)	Cleavable Linker (B)	Conjugate (C)
3	$G_sC_sa_st_st_sg_sg_st_sa_st_sT_sC_sA$	no	no
30	$G_sC_sa_st_st_sg_sg_st_sa_st_sT_sC_sA$		GalNAc cluster Conj1a
20	$G_sC_sa_st_st_sg_sg_st_sa_st_sT_sC_sA$		GalNAc cluster Conj2a
7	$G_sC_sa_st_st_sg_sg_st_sa_st_sT_sC_sA$	2PO-DNA (5'ca3')	cholesterol

[0260] Capital letters are LNA nucleosides (such as beta-D-oxy LNA), lower case letters are a DNA nucleoside. Subscript s represents a phosphorothioate internucleoside linkage (region A). LNA cytosines are optionally 5-methyl cytosine. The 2PO linker (region B) is 5' to the sequence region A, and comprises of two DNA nucleosides linked by phosphodiester linkage, with the internucleoside linkage between the 3' DNA nucleoside of region A and the 5' LNA nucleoside of region A also being phosphodiester. A linkage group (Y) may be used to link the conjugate group, when present, to region B, or A (SEQ ID NO 7, 20 and 30). C57BL6/J mice were injected either iv or sc with a single dose saline or 0,25 mg/kg unconjugated LNA-antisense oligonucleotide (SEQ ID NO3) or equimolar amounts of LNA antisense oligonucleotides conjugated to GalNAc1, GalNAc2, or cholesterol(2PO) and sacrificed at days 1-7 according to the table below (experimental design).

[0261] RNA was isolated from liver and kidney and subjected to qPCR with ApoB specific primers and probe to analyse for ApoB mRNA knockdown. The oligonucleotide content was measured using ELISA method and total cholesterol in serum was measured. **Conclusions:** GalNAc1 and GalNAc2 conjugated to an ApoB LNA antisense oligonucleotide (SEQ ID NO 30 and 20) showed knock down of ApoB mRNA better than the unconjugated ApoB LNA (Fig. 16). For GalNAc 1 conjugate (SEQ ID NO 30) is seems that iv dosing is better than sc dosing which is surprising since the opposite has been reported for another GalNAc clusters (Alnylam, 8th Annual Meeting of the Oligonucleotide Therapeutics Society) . The total cholesterol data show how the GalNAc cluster conjugates (SEQ ID NO 30 and 20) gives better effect than the unconjugated and the cholesterol conjugated compounds (SEQ ID NO 7) both at iv and sc administration (Fig 17, a and b). The tissue content of the oligonucleotides (Fig 18, a-f) shows how the conjugates enhances the uptake in liver while giving less uptake in kidney compared to the parent compound. This holds for both iv and sc administration. When dosing iv the GalNAc 1 (SEQ ID NO 30) gives very much uptake in liver when compared to GalNAc 2 (SEQ ID NO 20) but since activity is good for both compounds the GalNAc 2 conjugate appears to induce a higher specific activity than GalNAc 1 conjugate indicating that GalNAc conjugates without the pharmacokinetic modulator may be particularly useful with LNA antisense oligonucleotides.

Materials and Methods:

Experimental design:

[0262]

Group no.	Animal id no.	No. of Animals	Animal strain/ gender/feed	Compound Dose level per day	Conc. at dose vol. 10 ml/kg	Adm. Route	Dosing day	Sacrifice day
1	1-3	3	C57BL/6J/ ♀/Chow	Saline	-	i.v	0	1
2	4-6	3	C57BL/6J/ ♀/Chow	SEQ ID NO 3 0,25mg/kg	0,025 mg/ml	i.v	0	1
3	7-9	3	C57BL/6J/ ♀/Chow	SEQ ID NO 3 0,25mg/kg	0,025 mg/ml	s.c	0	1
4	10-12	3	C57BL/6J/ ♀/Chow	SEQ ID NO 30 0,36mg/kg	0,036 mg/ml	i.v	0	1
5	13-15	3	C57BL/6J/ ♀/Chow	SEQ ID NO 30 0,36mg/kg	0,036 mg/ml	s.c	0	1
6	16-18	3	C57BL/6J/ ♀/Chow	SEQ ID NO 7 0,32mg/kg	0,032 mg/ml	i.v	0	1
7	19-21	3	C57BL/6J/ ♀/Chow	SEQ ID NO 7 0,32mg/kg	0,032 mg/ml	s.c	0	1
8	22-24	3	C57BL/6J/ ♀/Chow	SEQ ID NO 20 0,34mg/kg	0,034 mg/ml	i.v	0	1
9	25-27	3	C57BL/6J/ ♀/Chow	SEQ ID NO 20 0,34mg/kg	0,034 mg/ml	S.C	0	1
10	28-30	3	C57BL/6J/ ♀/Chow	Saline	-	i.v	0	3

Group no.	Animal id no.	No. of Animals	Animal strain/ gender/feed	Compound Dose level per day	Conc. at dose vol. 10 ml/kg	Adm. Route	Dosing day	Sacrifice day
11	31-33	3	C57BL/6J/ ♀/Chow	SEQ ID NO 3 0,25mg/kg	0,025 mg/ml	i.v	0	3
12	34-36	3	C57BL/6J/ ♀/Chow	SEQ ID NO 3 0,25mg/kg	0,025 mg/ml	s.c	0	3
13	37-39	3	C57BL/6J/ ♀/Chow	SEQ ID NO 30 0,36mg/kg	0,036 mg/ml	i.v	0	3
14	40-42	3	C57BL/6J/ ♀/Chow	SEQ ID NO 30 0,36mg/kg	0,036 mg/ml	s.c	0	3
15	43-45	3	C57BL/6J/ ♀/Chow	SEQ ID NO 7 0,32mg/kg	0,032 mg/ml	i.v	0	3
16	46-48	3	C57BL/6J/ ♀/Chow	SEQ ID NO 7 0,32mg/kg	0,032 mg/ml	s.c	0	3
17	49-51	3	C57BL/6J/ ♀/Chow	SEQ ID NO 20 0,34mg/kg	0,034 mg/ml	i.v	0	3
18	52-54	3	C57BL/6J/ ♀/Chow	SEQ ID NO 20 0,34mg/kg	0,034 mg/ml	s.c	0	3
19	55-57	3	C57BL/6J/ ♀/Chow	Saline	-	i.v	0	7
20	58-60	3	C57BL/6J/ ♀/Chow	SEQ ID NO 3 0,25mg/kg	0,025 mg/ml	i.v	0	7
21	61-63	3	C57BL/6J/ ♀/Chow	SEQ ID NO 3 0,25mg/kg	0,025 mg/ml	s.c	0	7
22	64-66	3	C57BL/6J/ ♀/Chow	SEQ ID NO 30 0,36mg/kg	0,036 mg/ml	i.v	0	7
23	67-69	3	C57BL/6J/ ♀/Chow	SEQ ID NO 30 0,36mg/kg	0,036 mg/ml	s.c	0	7
24	70-72	3	C57BL/6J/ ♀/Chow	SEQ ID NO 7 0,32mg/kg	0,032 mg/ml	i.v	0	7

Group no.	Animal id no.	No. of Animals	Animal strain/ gender/feed	Compound Dose level per day	Conc. at dose vol. 10 ml/kg	Adm. Route	Dosing day	Sacrifice day
25	73-75	3	C57BL/6J/ ♀/Chow	SEQ ID NO 7 0,32mg/kg	0,032 mg/ml	s.c	0	7
26	76-78	3	C57BL/6J/ ♀/Chow	SEQ ID NO 10 0,34mg/kg	0,034 mg/ml	i.v	0	7
27	79-81	3	C57BL/6J/ ♀/Chow	SEQ ID NO 20 0,34mg/kg	0,034 mg/ml	s.c	0	7

[0263] *Dose administration.* C57BL/6JBom female animals, app. 20 g at arrival, were dosed with 10 ml per kg BW (according to day 0 bodyweight) *i.v.* or s.c. of the compound formulated in saline or saline alone according to the table above.

*Sampling of liver and kidney tissue.*_ The animals were anaesthetised with 70% CO_2-30% O_2 and sacrificed by cervical dislocation according to the above table. One half of the large liver lobe and one kidney were minced and submerged in RNAlater. The other half of liver and the other kidney was frozen and used for tissue analysis.

[0264] *Total RNA Isolation and First strand synthesis.* Total RNA was extracted from maximum 30 mg of tissue homogenized by bead-milling in the presence of RLT-Lysis buffer using the Qiagen RNeasy kit (Qiagen cat. no. 74106) according to the manufacturer's instructions. First strand synthesis was performed using Reverse Transcriptase reagents from Ambion according to the manufacturer's instructions.

For each sample 0.5 µg total RNA was adjusted to (10.8 µl) with RNase free H_2O and mixed with 2 µl random decamers (50 µM) and 4 µl dNTP mix (2.5 mM each dNTP) and heated to 70 °C for 3 min after which the samples were rapidly cooled on ice. 2 µl 10x Buffer RT, 1 µl MMLV Reverse Transcriptase (100 U/µl) and 0.25 µl RNase inhibitor (10 U/µl) were added to each sample, followed by incubation at 42 °C for 60 min, heat inactivation of the enzyme at 95°C for 10 min and then the sample was cooled to 4 °C. cDNA samples were diluted 1: 5 and subjected to RT-QPCR using Taqman Fast Universal PCR Master Mix 2x (Applied Biosystems Cat #4364103) and Taqman gene expression assay (mApoB, Mn01545150_m1 and mGAPDH #4352339E) following the manufacturers protocol and processed in an Applied Biosystems RT-qPCR instrument (7500/7900 or ViiA7) in fast mode. Oligonucleotide content in liver and kidney was measured by sandwich ELISA method.

Serum cholesterol analysis: Immediately before sacrifice retro-orbital sinus blood was collected using S-monovette Serum-Gel vials (Sarstedt, Nümbrecht, Germany) for serum preparation. Serum was analyzed for total cholesterol using ABX Pentra Cholesterol CP (Triolab, Brondby, Denmark) according to the manufacturer's instructions.

SEQUENCE LISTING

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PATENTKRAV

1. Antisense-oligonukleotidkonjugat omfattende et første område af en LNA-oligomer (område A, såsom en LNA-gapmer-oligomer), der er målrettet mod en ApoB-nukleinsyre, der er kovalent bundet til en konjugatdel (område C) omfattende en N-acetylgalactosamin (GalNAc)-del bundet til LNA-oligomeren via en biospaltbar linker, hvor den biospaltbare linker omfatter et peptid, såsom en lysinlinker eller en fysiologisk labil nukleotidlinker.

2. Antisense-oligonukleotidkonjugat ifølge krav 1, hvor GalNAc-konjugatdelen (C) er en trivalent GalNAc-del.

3. Antisense-oligonukleotidkonjugat ifølge et hvilket som helst af kravene 1 eller 2, hvor LNA-oligomeren er kovalent bundet til konjugatdelen via et område af et eller flere phosphatbundne nukleosider, såsom DNA- eller RNA-nukleosider (område B), såsom en phosphodiester-nukleotidlinker.

4. Antisense-oligonukleotidkonjugat ifølge et hvilket som helst af kravene 1 til 3, hvor LNA-oligonukleotidet er kovalent bundet til konjugatdelen via et område B af 1 til 6 phosphatbundne nukleosider, såsom DNA-nukleosider (område B).

5. Antisense-oligonukleotidkonjugat ifølge krav 4, hvor område B (phosphodiester-nukleotidbinding) omfatter 1, 2 eller 3 sammenhængende DNA-phosphodiester-nukleotider, såsom to sammenhængende DNA-phosphodiester-nukleotider, såsom et 5'-CA-3'-dinukleotid.

6. Antisense-oligonukleotidkonjugat ifølge et hvilket som helst af kravene 1 til 5, hvor konjugatdelen yderligere omfatter en linker (Y), der binder konjugatdelen kovalent til enten LNA-oligomeren eller området af et eller flere phosphatbundne DNA- eller RNA-nukleotider (område B).

7. Antisense-oligonukleotidkonjugat ifølge krav 6, hvor linkerområdet Y omfatter en

C6-linker.

8. Antisense-oligonukleotidkonjugat ifølge et hvilket som helst af kravene 1 til 7, hvor konjugatdelen omfatter en trivalent GalNac-del valgt fra gruppen bestående af Conj1, Conj2, Conj3, Conj4, Conj1a, Conj2a, Conj3a og Conj4a som vist i figur 13.

9. Antisense-oligonukleotidkonjugat ifølge et hvilket som helst af kravene 1 til 8, hvor LNA-oligomeren omfatter en sammenhængende nukleotidsekvens valgt fra gruppen bestående af SEQ ID NO 1 eller SEQ ID NO 2:

SEQ ID NO 1 (3833) GCattggtatTCA

SEQ ID NO 2 (4955) GTtgacactgTC

hvor store bogstaver repræsenterer LNA-nukleosider, såsom beta-D-oxy-LNA, små bogstaver repræsenterer DNA-nukleosider, LNA-cytosiner eventuelt er 5-methylcytosin og alle internukleosidbindinger er phosphorthioat.

10. Antisense-oligonukleotidkonjugat ifølge krav 9, der er valgt fra gruppen bestående af SEQ ID NO 20, 29, 30 og 31.

11. Farmaceutisk sammensætning omfattende antisense-oligonukleotidkonjugatet ifølge et hvilket som helst af kravene 1 til 10 og en farmaceutisk acceptabel fortynder, bærer eller adjuvans eller et farmaceutisk acceptabelt salt.

12. Antisense-oligonukleotidkonjugat eller farmaceutisk sammensætning ifølge et hvilket som helst af kravene 1 til 11 til anvendelse som lægemiddel, såsom til behandling af akut hjerteinfarkt, hyperkolesterolæmi eller en beslægtet sygdom, såsom en sygdom valgt fra gruppen bestående af aterosklerose, hyperlipidæmi, hyperkolesterolæmi, HDL-/LDL-kolesterolubalance, dyslipidæmi, eksempelvis familiær hyperlipidæmi (FCHL), erhvervet hyperlipidæmi, statinresistent hyperkolesterolæmi, koronararteriesygdom (CAD) og koronar hjertesygdom (CHD).

13. Anvendelse af et antisense-oligonukleotidkonjugat eller en farmaceutisk

sammensætning ifølge et hvilket som helst af kravene 1 til 11 til fremstilling af et lægemiddel til behandling af akut hjerteinfarkt, hyperkolesterolæmi eller en beslægtet sygdom, såsom en sygdom valgt fra gruppen bestående af aterosklerose, hyperlipidæmi, hyperkolesterolæmi, HDL-/LDL-kolesterolubalance, dyslipidæmi, eksempelvis familiær hyperlipidæmi (FCHL), erhvervet hyperlipidæmi, statinresistent hyperkolesterolæmi, koronararteriesygdom (CAD) og koronar hjertesygdom (CHD).

14. *In vitro*-fremgangsmåde til at hæmme ApoB i en celle, der udtrykker ApoB, hvilken fremgangsmåde omfatter indgivelse af et antisense-oligonukleotidkonjugat eller en farmaceutisk sammensætning ifølge et hvilket som helst af kravene 1 til 11 til cellen for at hæmme ApoB i cellen.

DRAWINGS

Figure 1:

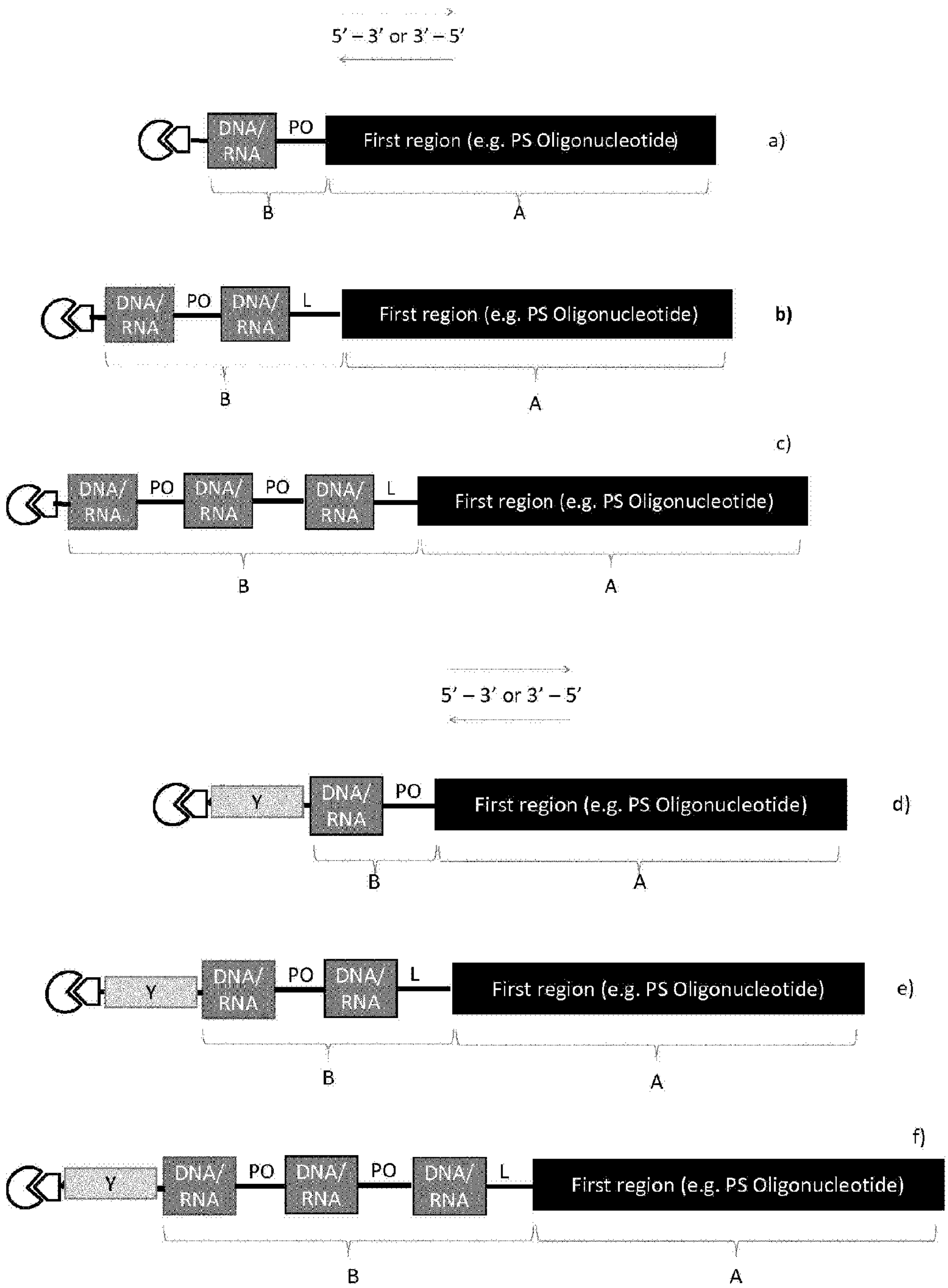


Figure 2

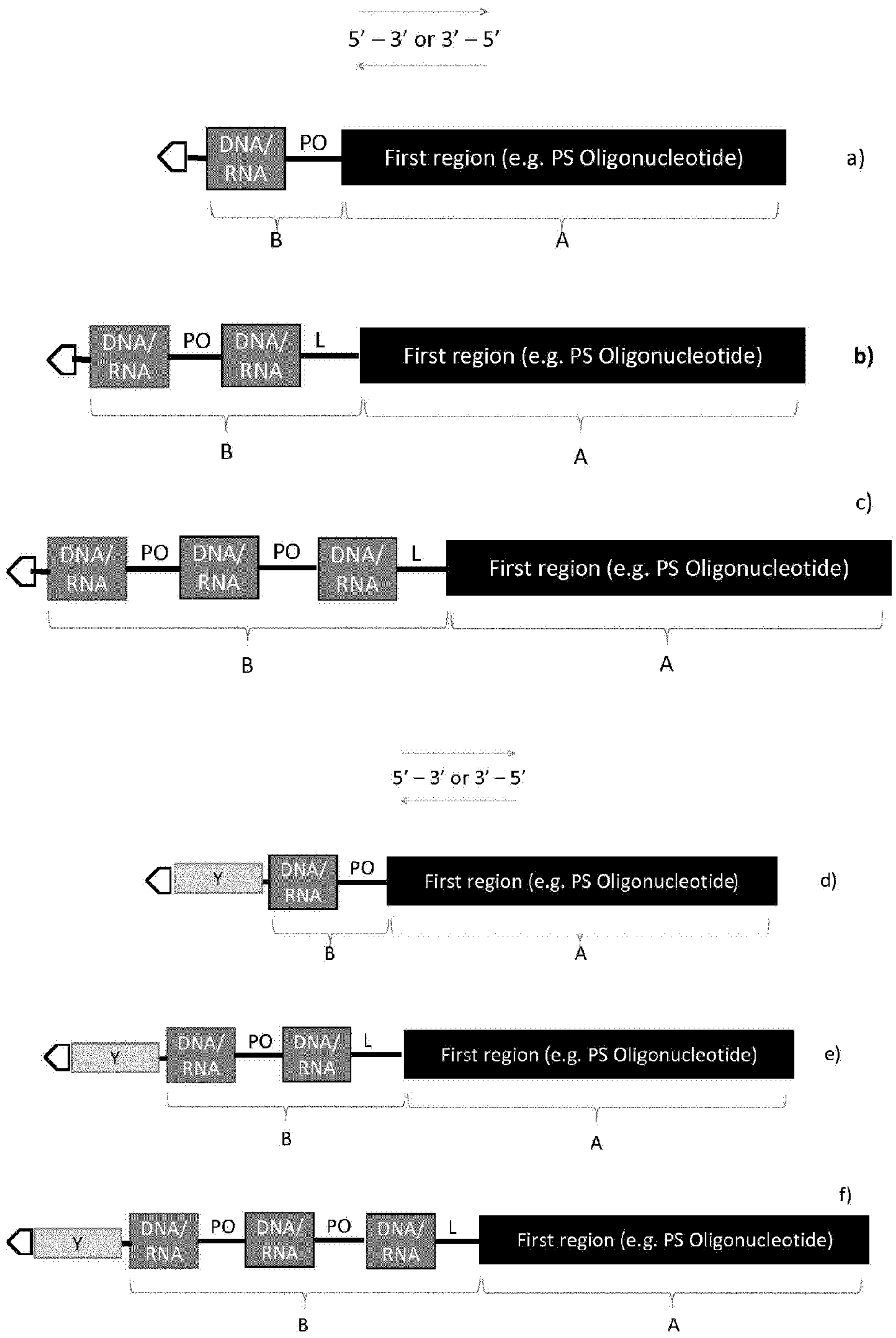

5' – 3' or 3' – 5'
DNA/ RNA
PO
First region (e.g. PS Oligonucleotide)
a)
B
A
DNA/ RNA
PO
DNA/ RNA
L
First region (e.g. PS Oligonucleotide)
b)
B
A
c)
DNA/ RNA
PO
DNA/ RNA
PO
DNA/ RNA
L
First region (e.g. PS Oligonucleotide)
B
A
5' – 3' or 3' – 5'
Y
DNA/ RNA
PO
First region (e.g. PS Oligonucleotide)
d)
B
A
Y
DNA/ RNA
PO
DNA/ RNA
L
First region (e.g. PS Oligonucleotide)
e)
B
A
f)
Y
DNA/ RNA
PO
DNA/ RNA
PO
DNA/ RNA
L
First region (e.g. PS Oligonucleotide)
B
A

Figure 3

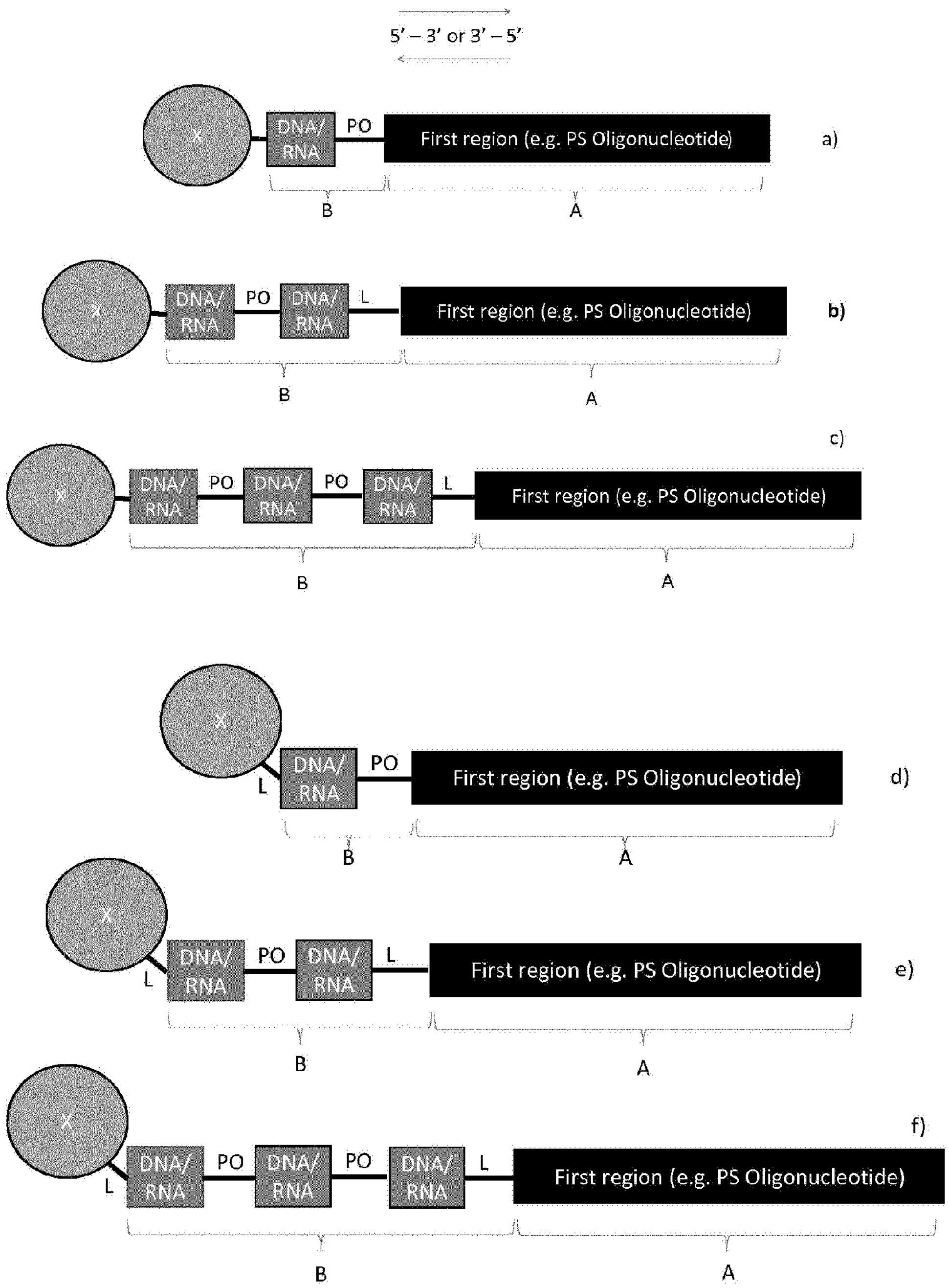

5' – 3' or 3' – 5'
X
DNA/ RNA
PO
L
First region (e.g. PS Oligonucleotide)
B
A
a)
b)
c)
d)
e)
f)

Figure 4

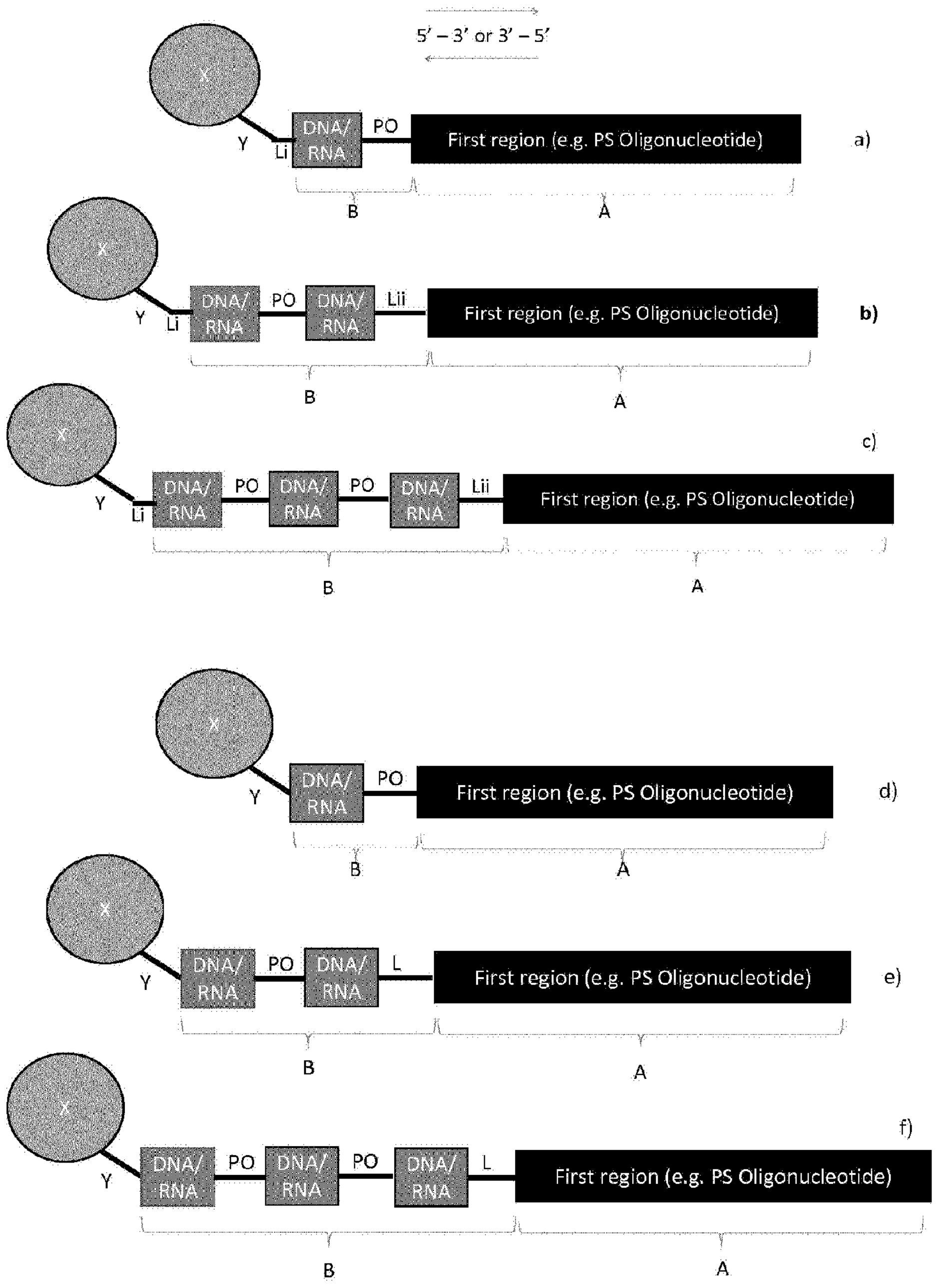

5' – 3' or 3' – 5'
X
Y
Li
DNA/ RNA
PO
First region (e.g. PS Oligonucleotide)
a)
B
A
Lii
b)
c)
d)
L
e)
f)

Figure 5a

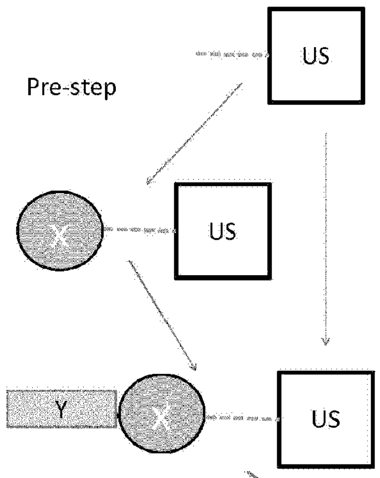


Figure 5b

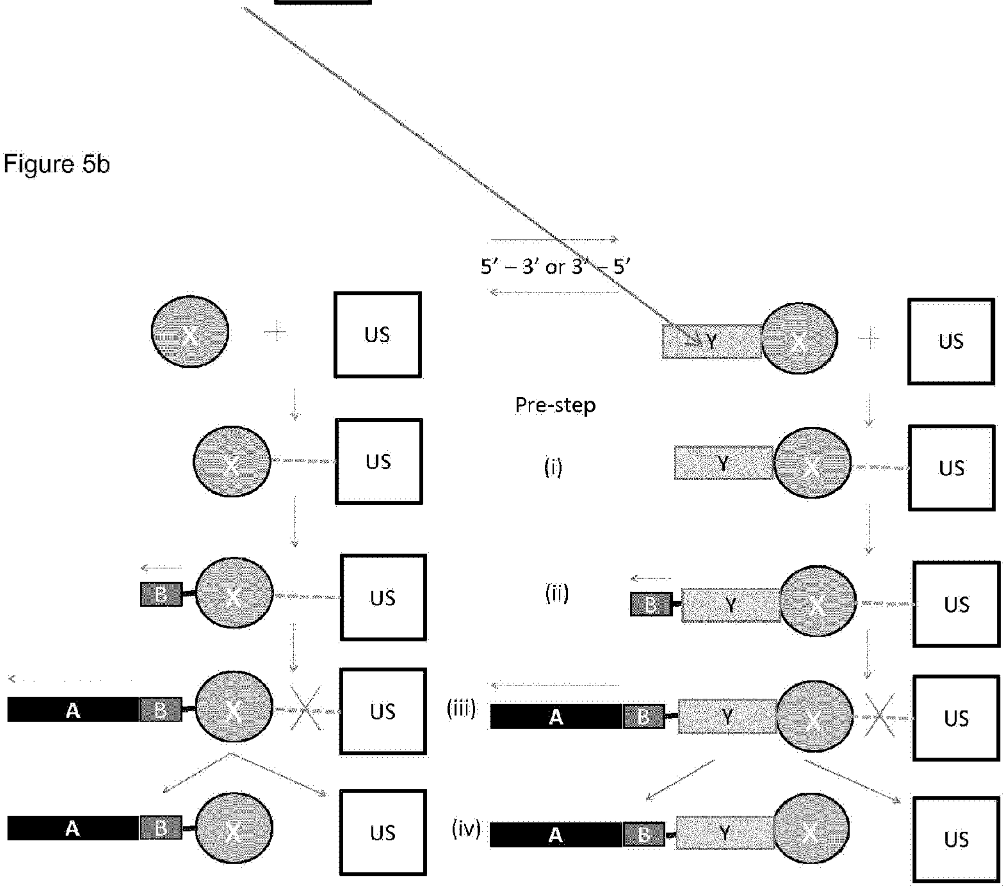


Figure 6

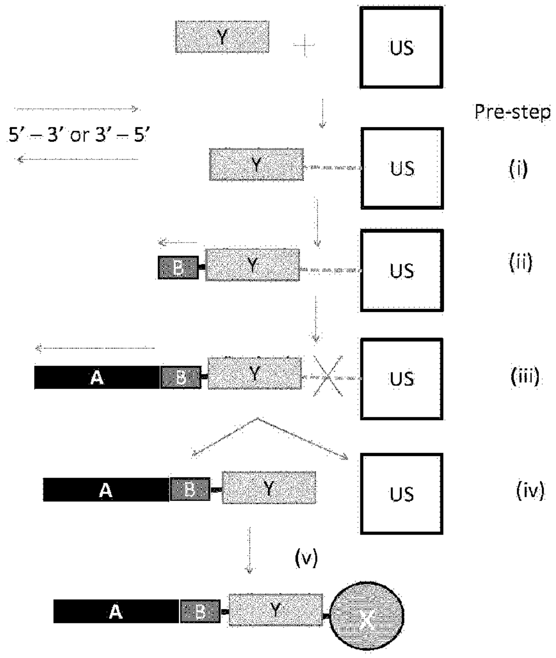


Figure 7

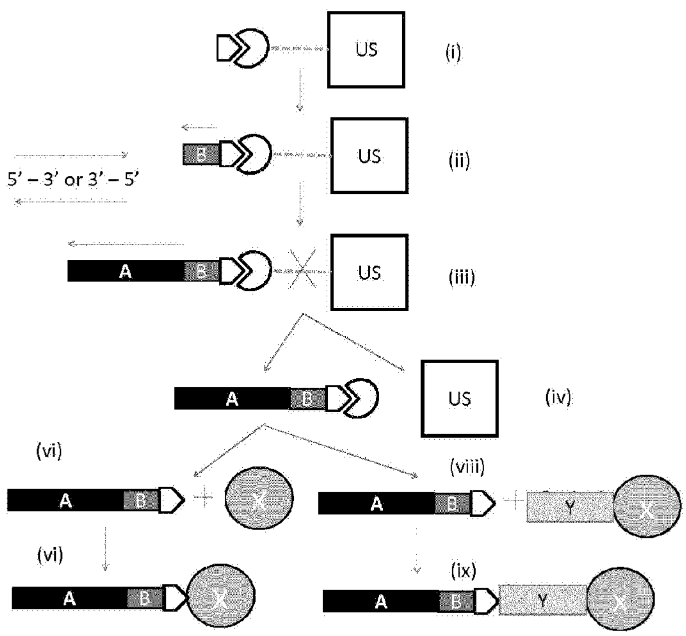
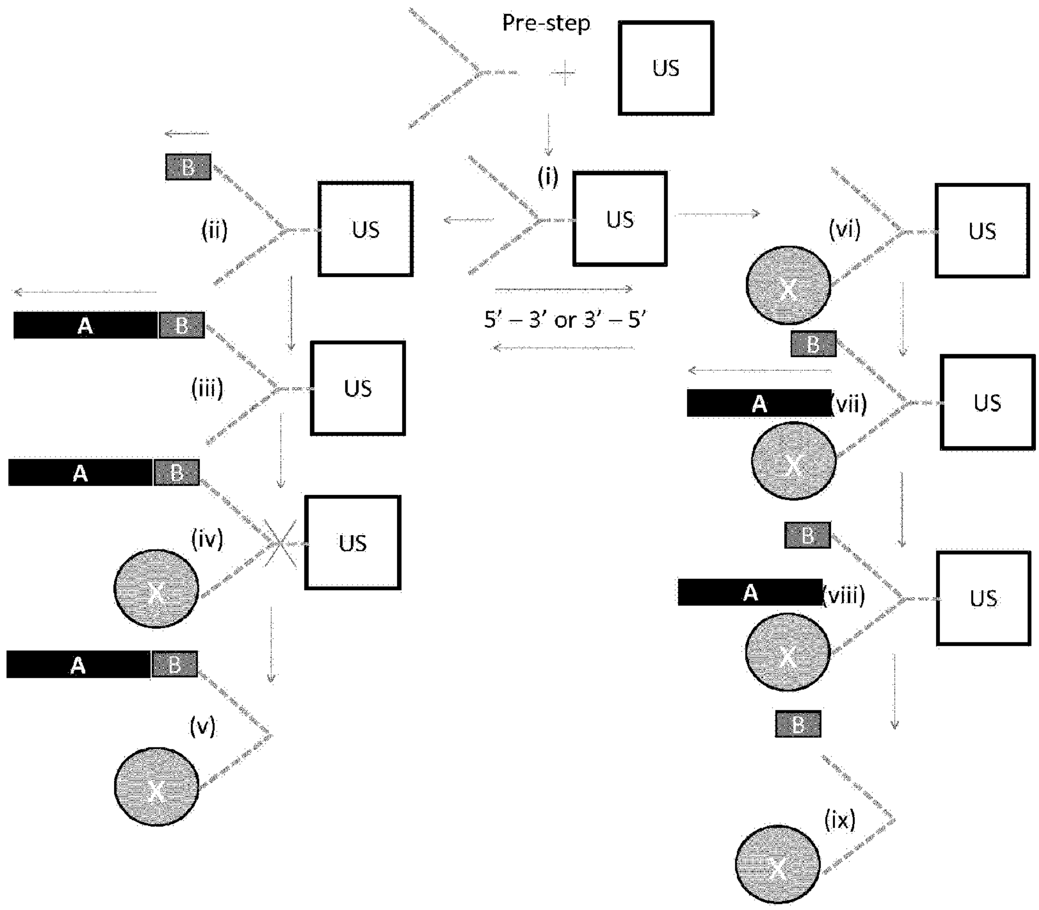


Figure 8



Pre-step
US
(i)
US
5' – 3' or 3' – 5'
B
(ii)
US
A
B
(iii)
US
A
B
(iv)
X
US
A
B
(v)
X
(vi)
X
US
B
A
(vii)
X
US
B
A
(viii)
X
US
B
(ix)
X

Figure 9

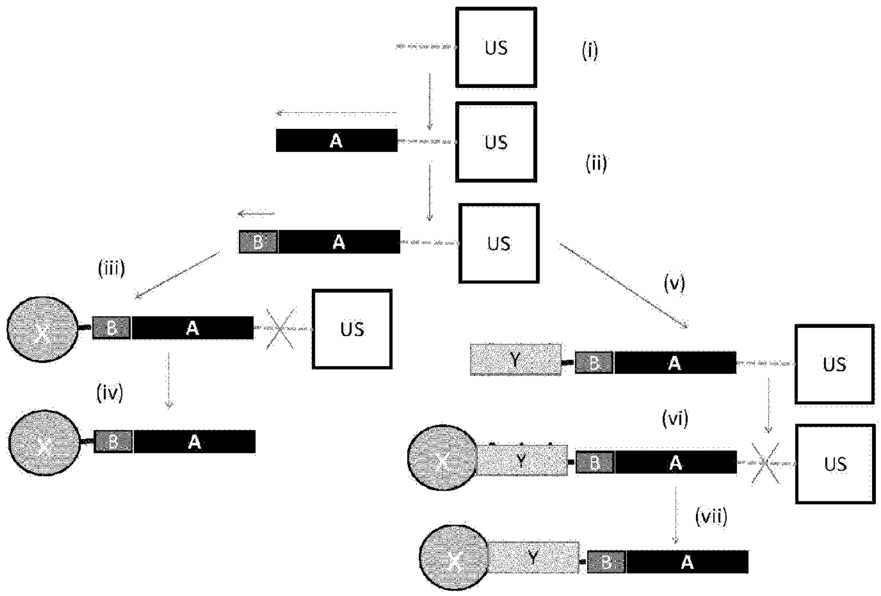


Figure 10

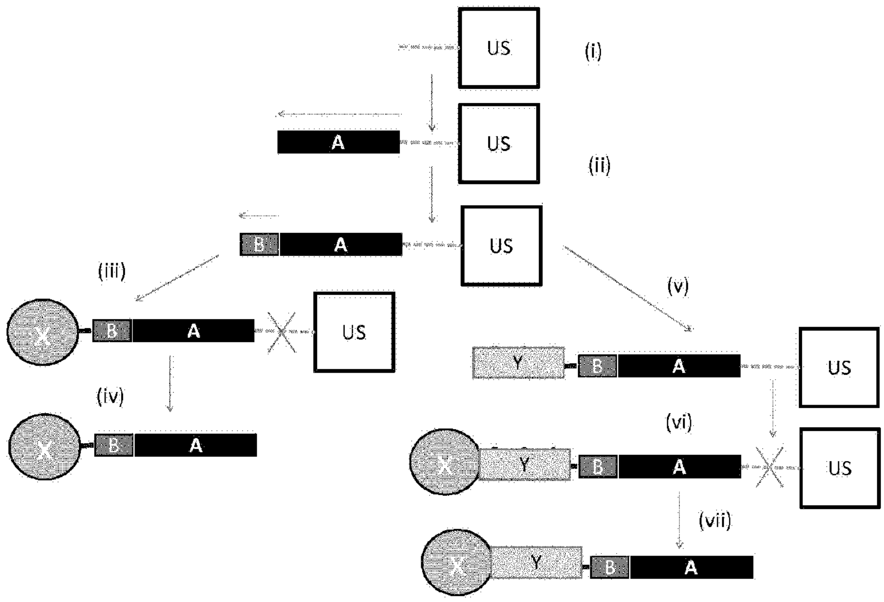
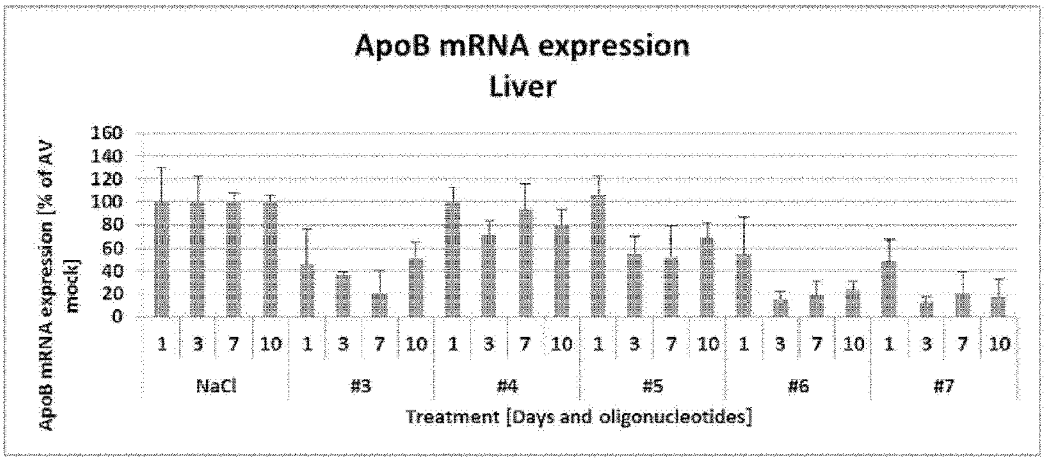


Figure 11

A


ApoB mRNA expression
Liver
ApoB mRNA expression [% of AV mock]
160
140
120
100
80
60
40
20
0
1 3 7 10 1 3 7 10 1 3 7 10 1 3 7 10 1 3 7 10 1 3 7 10
NaCl
#3
#4
#5
#6
#7
Treatment [Days and oligonucleotides]

B

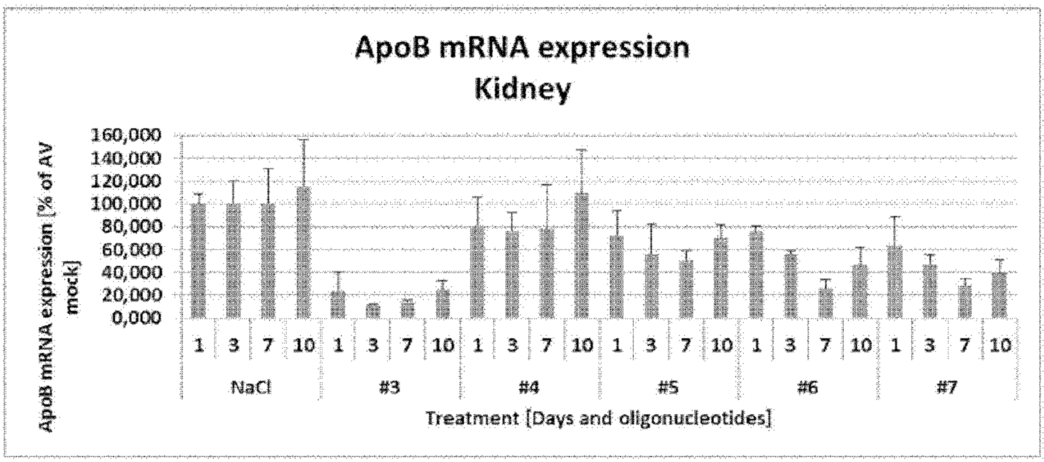
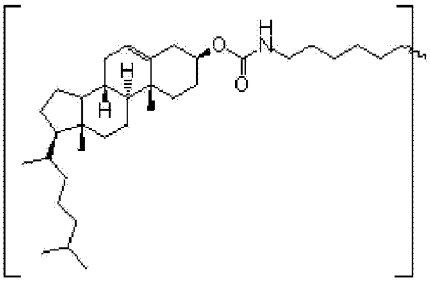

ApoB mRNA expression
Kidney
ApoB mRNA expression [% of AV mock]
160,000
140,000
120,000
100,000
80,000
60,000
40,000
20,000
0,000
1 3 7 10 1 3 7 10 1 3 7 10 1 3 7 10 1 3 7 10 1 3 7 10
NaCl
#3
#4
#5
#6
#7
Treatment [Days and oligonucleotides]

Figure 12

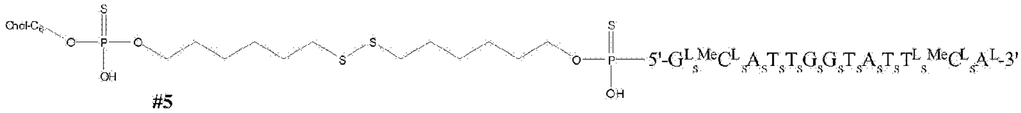
Chol-C6 =



5'-$G^{L}_{s}{}^{Me}C^{L}_{s}A_{s}T_{s}T_{s}G_{s}G_{s}T_{s}A_{s}T_{s}T^{L}_{s}{}^{Me}C^{L}_{s}A^{L}$-3'

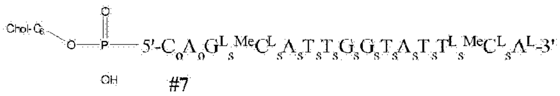
#3

#4



#5

#6



#7

#8

Figure 12 (cont)

#9

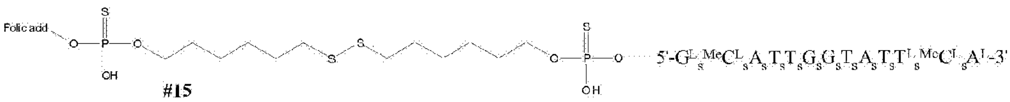
#10

#11

#12

#13

#14



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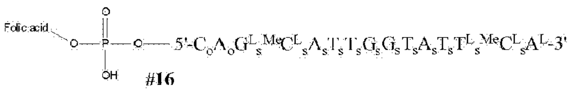

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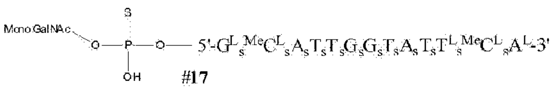

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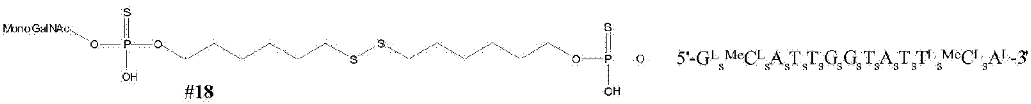
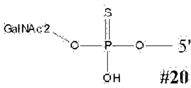


#18

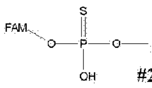
#19

Figure 12 (cont)



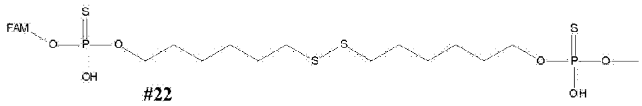
$5'\text{-}G^{L}_{s}{}^{Me}C^{L}_{s}A_{s}T_{s}T_{s}G_{s}G_{s}T_{s}A_{s}T_{s}T^{L}_{s}{}^{Me}C^{L}_{s}A^{L}\text{-}3'$

#20



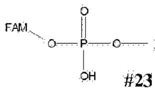
$5'\text{-}G^{L}_{s}{}^{Me}C^{L}_{s}A_{s}T_{s}T_{s}G_{s}G_{s}T_{s}A_{s}T_{s}T^{L}_{s}{}^{Me}C^{L}_{s}A^{L}\text{-}3'$

#21



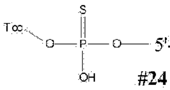
$5'\text{-}G^{L}_{s}{}^{Me}C^{L}_{s}A_{s}T_{s}T_{s}G_{s}G_{s}T_{s}A_{s}T_{s}T^{L}_{s}{}^{Me}C^{L}_{s}A^{L}\text{-}3'$

#22



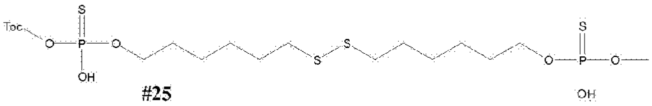
$5'\text{-}C_{o}A_{o}G^{L}_{s}{}^{Me}C^{L}_{s}A_{s}T_{s}T_{s}G_{s}G_{s}T_{s}A_{s}T_{s}T^{L}_{s}{}^{Me}C^{L}_{s}A^{L}\text{-}3'$

#23



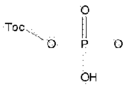
$5'\text{-}G^{L}_{s}{}^{Me}C^{L}_{s}A_{s}T_{s}T_{s}G_{s}G_{s}T_{s}A_{s}T_{s}T^{L}_{s}{}^{Me}C^{L}_{s}A^{L}\text{-}3'$

#24



$5'\text{-}G^{L}_{s}{}^{Me}C^{L}_{s}A_{s}T_{s}T_{s}G_{s}G_{s}T_{s}A_{s}T_{s}T^{L}_{s}{}^{Me}C^{L}_{s}A^{L}\text{-}3'$

#25

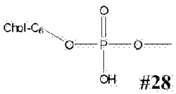


$5'\text{-}C_{o}A_{o}G^{L}_{s}{}^{Me}C^{L}_{s}A_{s}T_{s}T_{s}G_{s}G_{s}T_{s}A_{s}T_{s}T^{L}_{s}{}^{Me}C^{L}_{s}A^{L}\text{-}3'$

#26

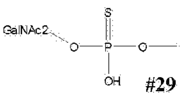
$5'\text{-}G^{L}_{s}T^{L}_{s}T_{s}G_{s}A_{s}C_{s}A_{s}C_{s}T_{s}G_{s}T^{L}_{s}{}^{Me}C^{L}\text{-}3'$

#27



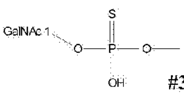
$5'\text{-}C_{o}A_{o}G^{L}_{s}T^{L}_{s}T_{s}G_{s}A_{s}C_{s}A_{s}C_{s}T_{s}G_{s}T^{L}_{s}{}^{Me}C^{L}\text{-}3'$

#28



$5'\text{-}G^{L}_{s}T^{L}_{s}T_{s}G_{s}A_{s}C_{s}A_{s}C_{s}T_{s}G_{s}T^{L}_{s}{}^{Me}C^{L}\text{-}3'$

#29

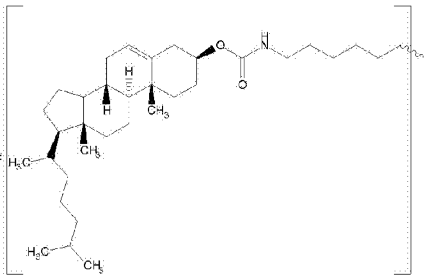


$5'\text{-}G^{L}_{s}{}^{Me}C^{L}_{s}A_{s}T_{s}T_{s}G_{s}G_{s}T_{s}A_{s}T_{s}T^{L}_{s}{}^{Me}C^{L}_{s}A^{L}\text{-}3'$

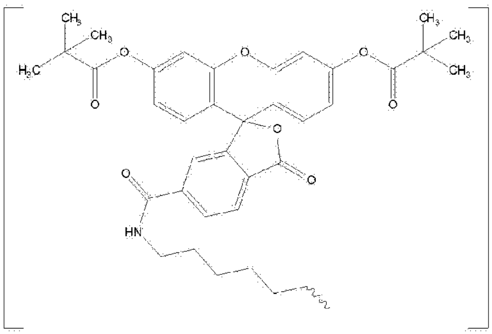
#30

Figure 12 (cont)

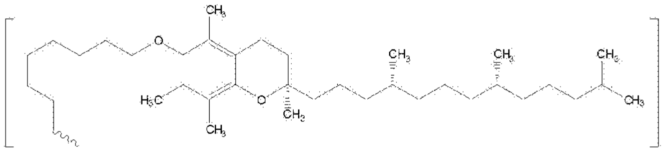
Chol-C_6 =



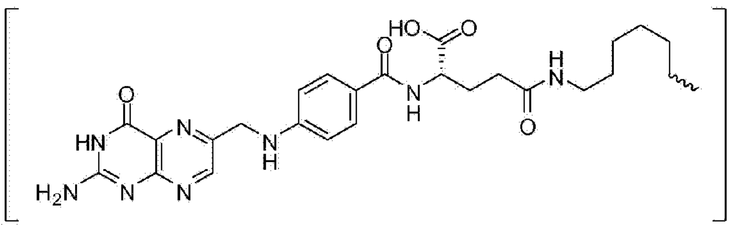
FAM=



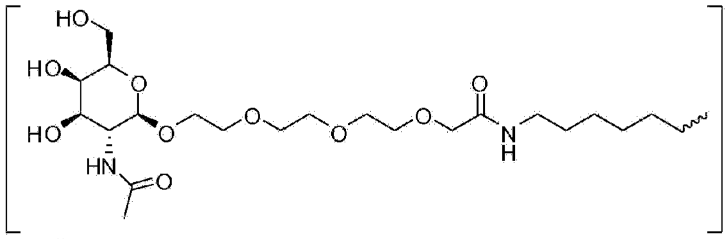
Toc=



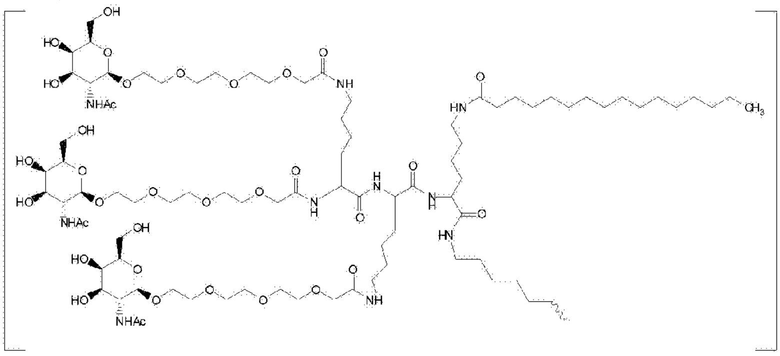
Folic acid=



Mono GalNAc=



GalNAc 1=



GalNAc2=

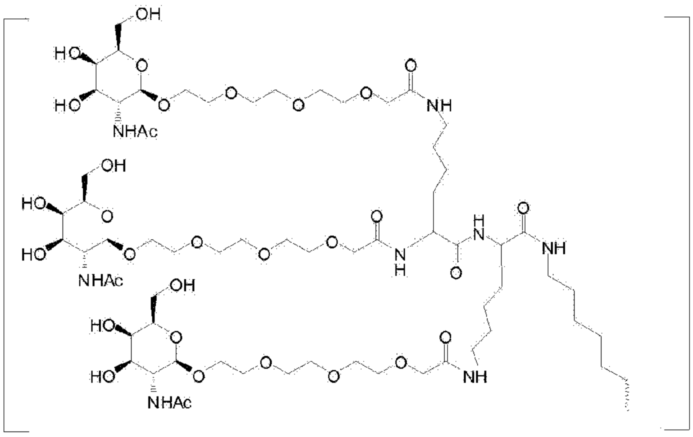
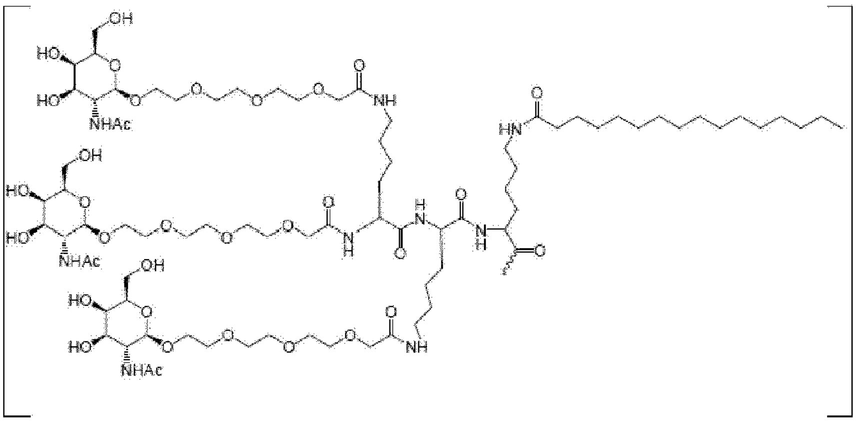


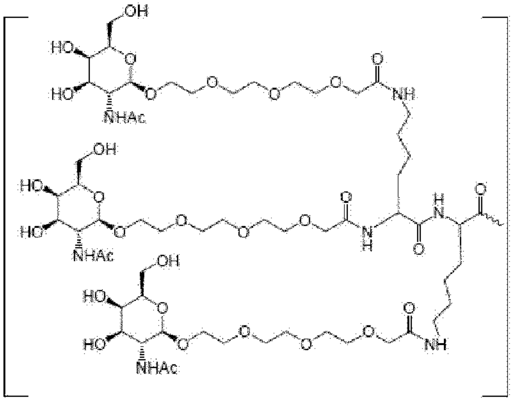
Figure 12 (cont)

Figure 13

Conj 1=



Conj 2=



Conj 3=

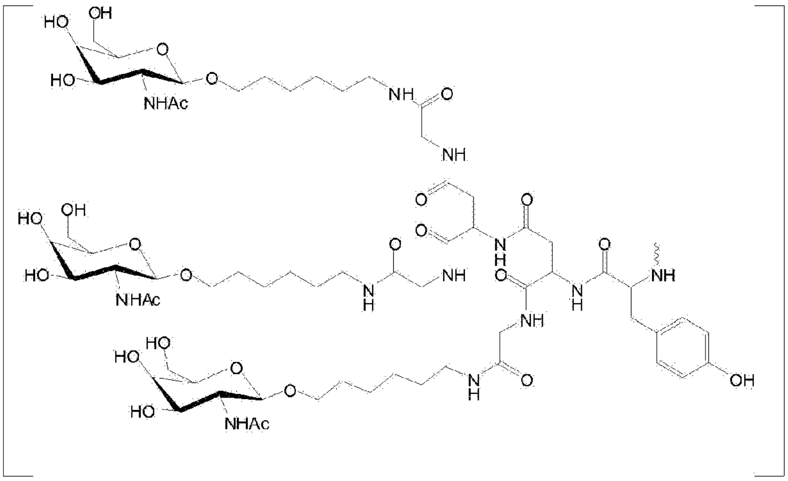


Figure 13 (cont)

Conj 4=

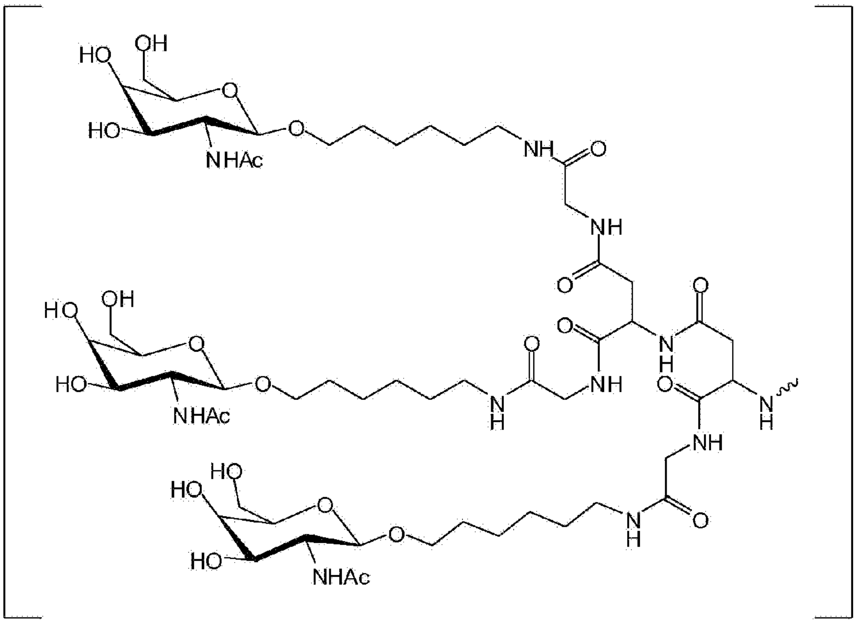
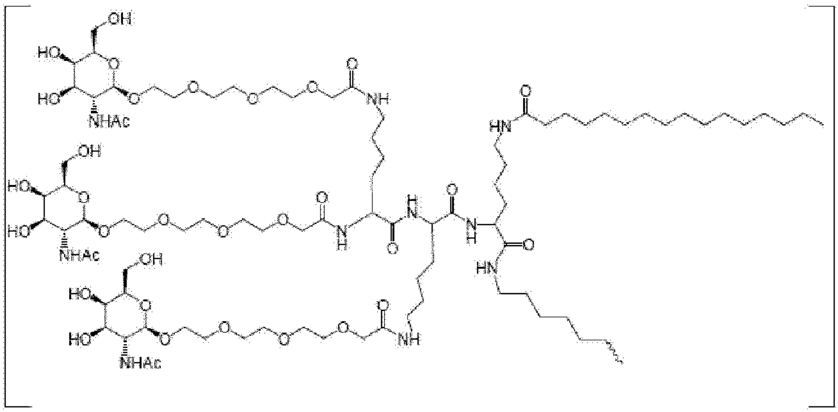
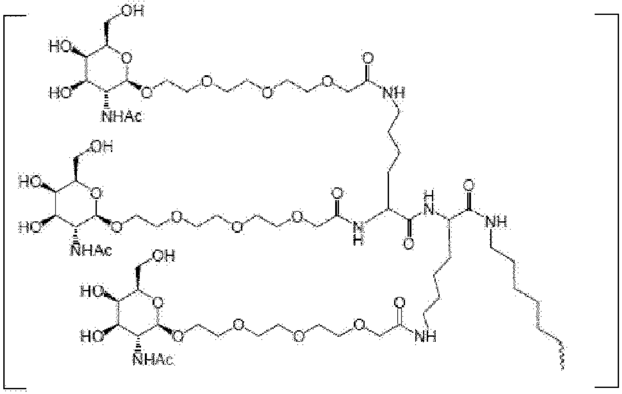


Figure 13 (cont)

Conj 1a=



Conj 2a=



Conj 3a=

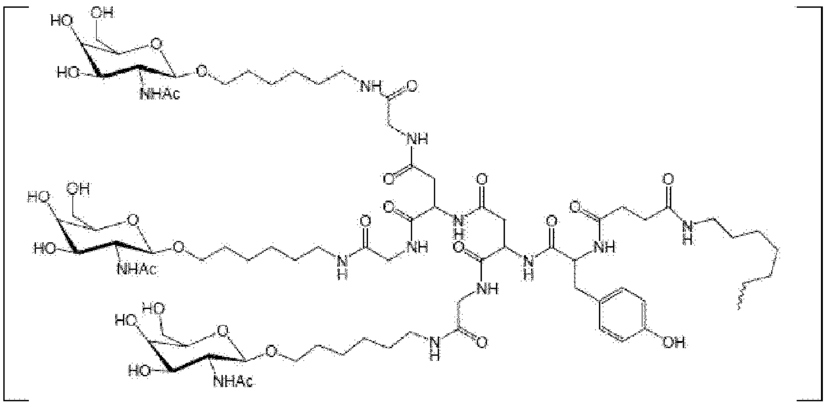


Figure 13 (cont)

Conj 4a=

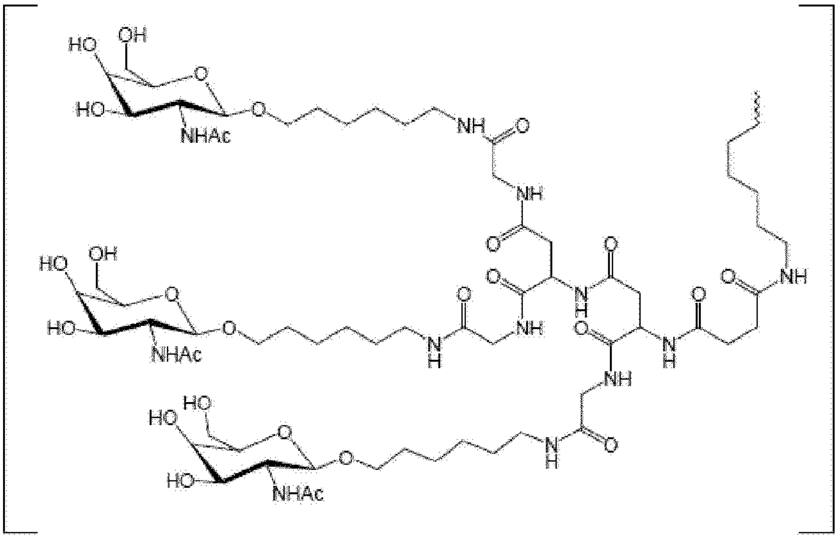
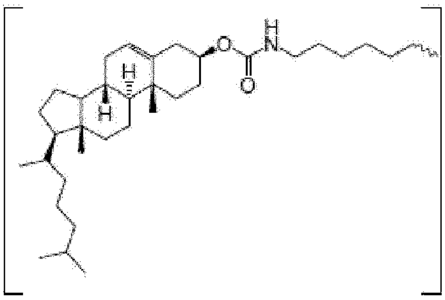


Figure 14

Conj 5a=



Conj 6a=

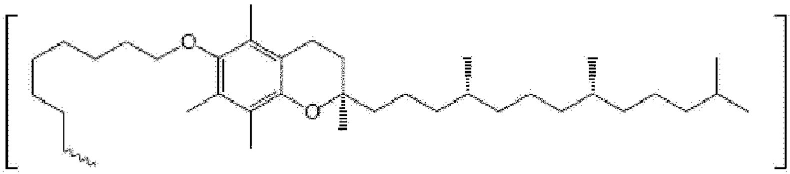
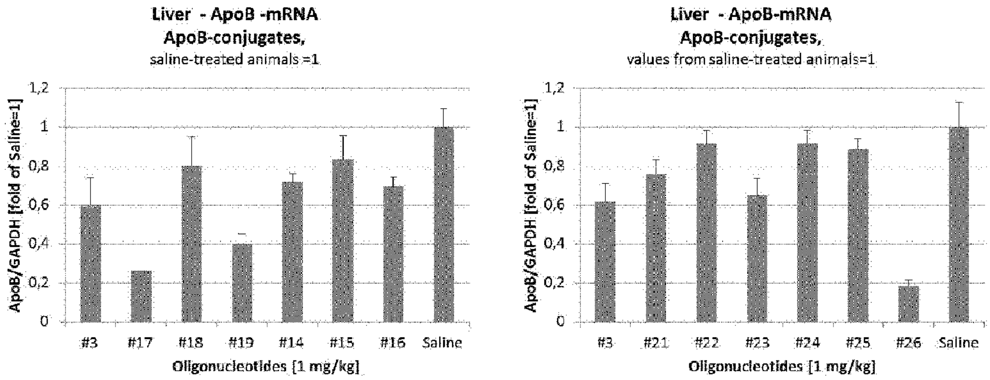
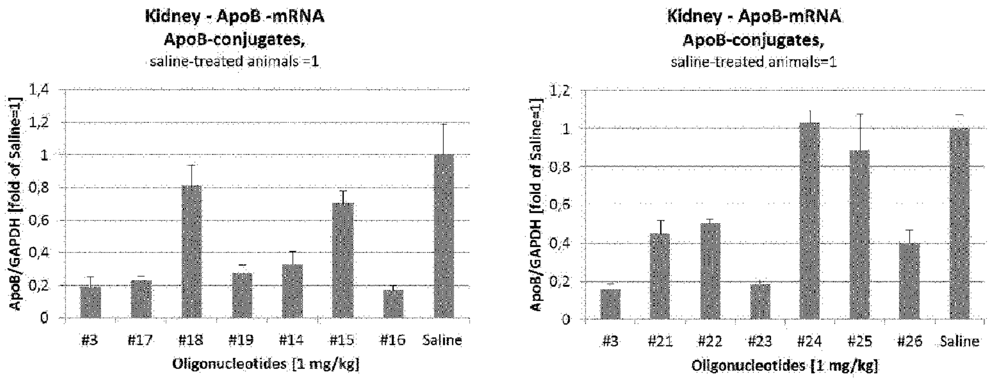


Figure 15

A



B



C

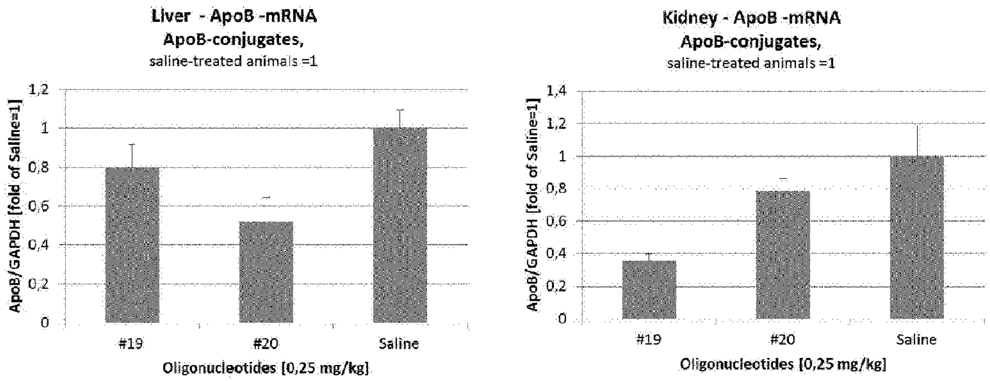


Figure 16

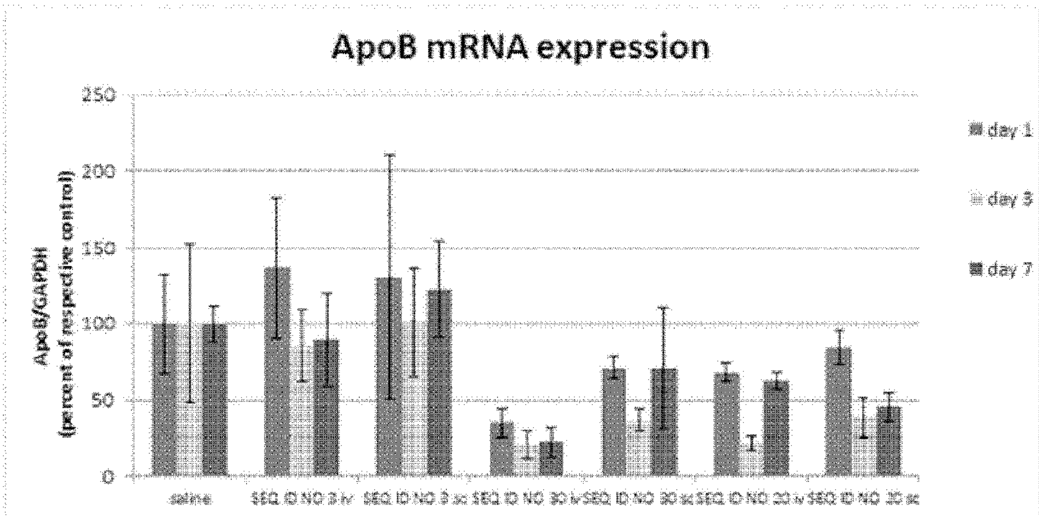


Figure 17 a)

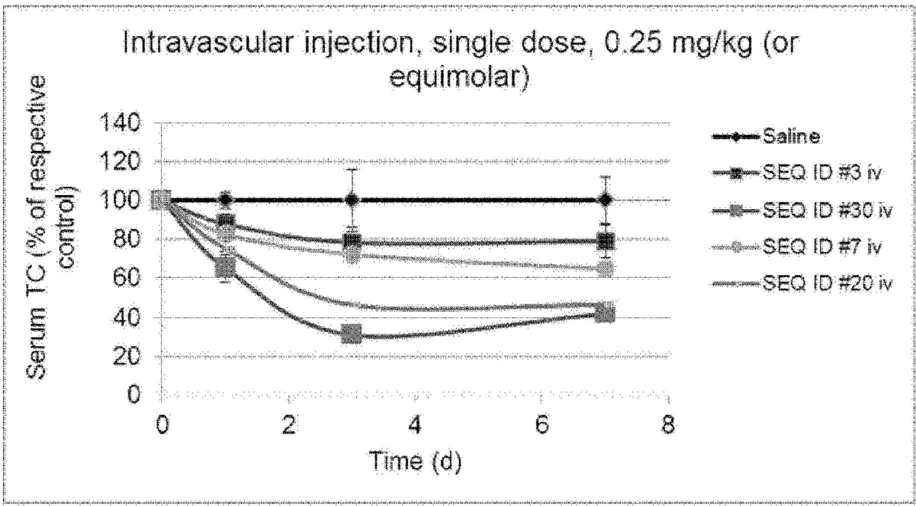


Figure 17 b)

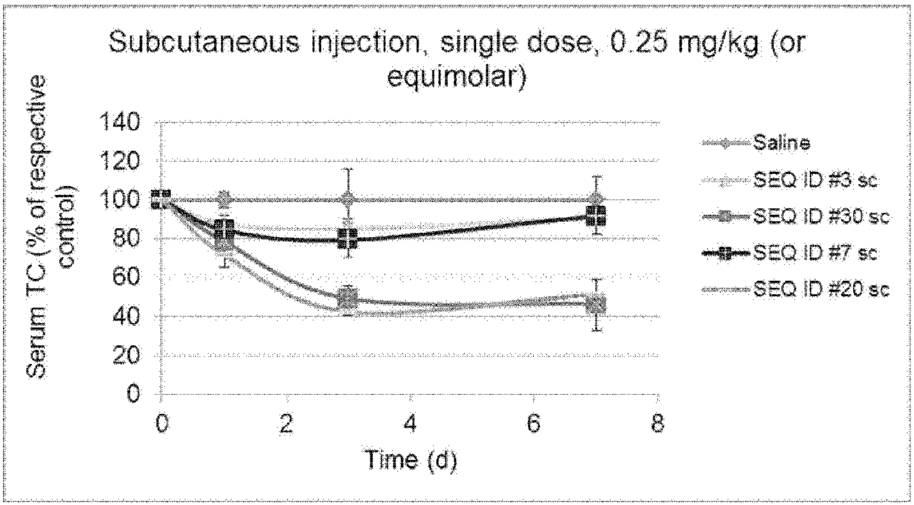


Figure 18 a)

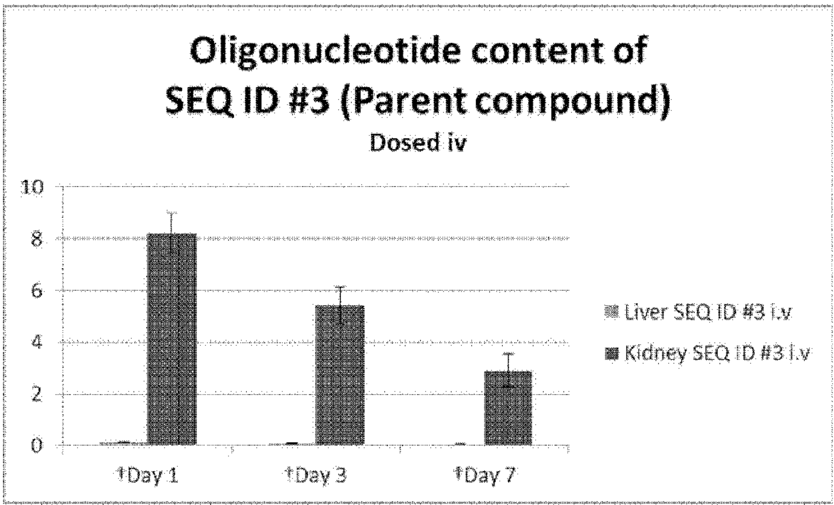


Figure 18 b)

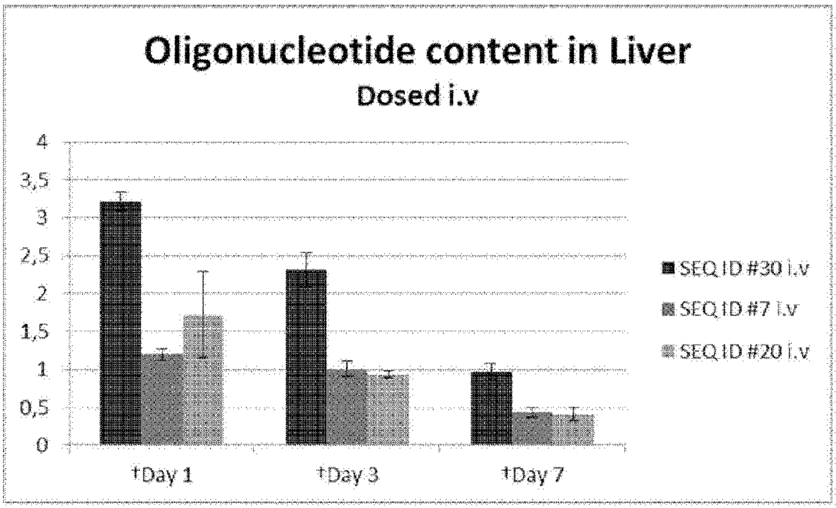


Figure 18c)

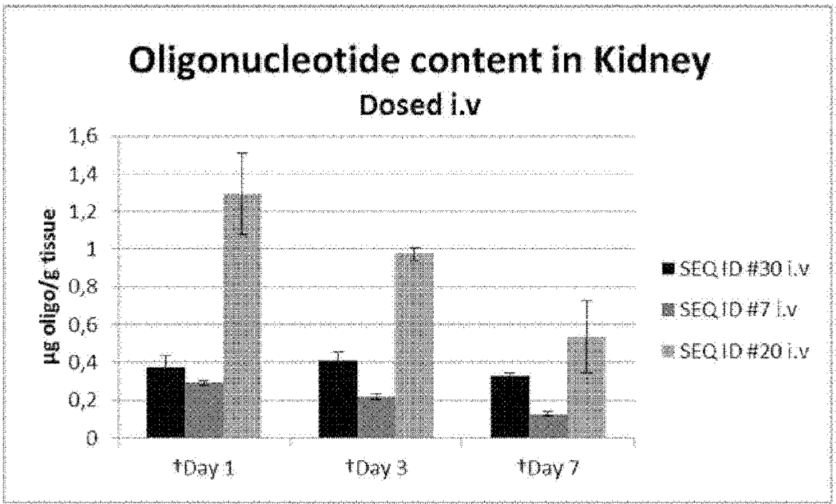


Figure 18 d)

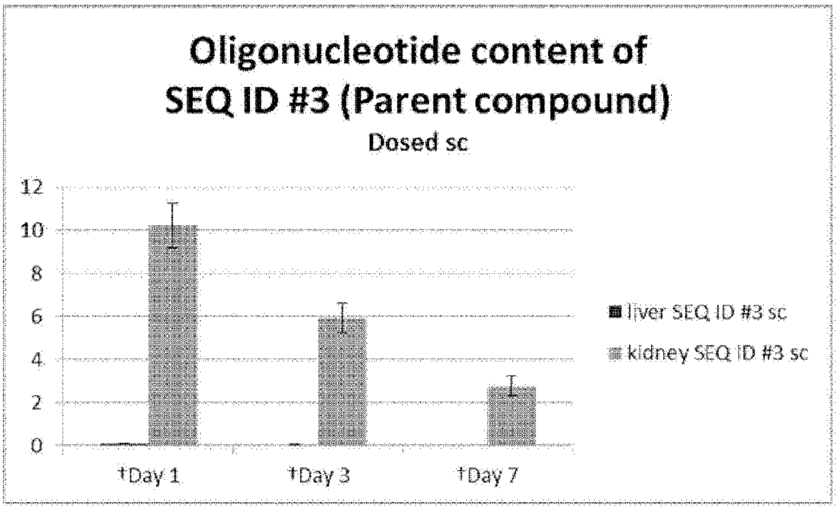


Figure 18 e)

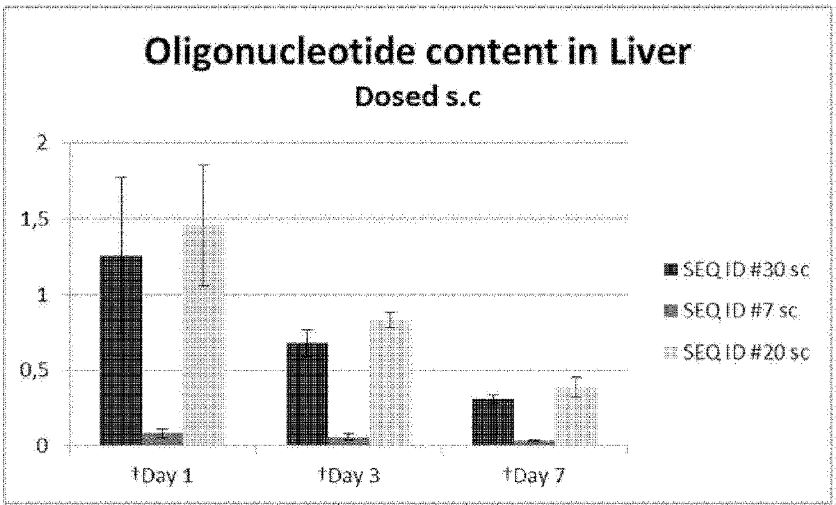
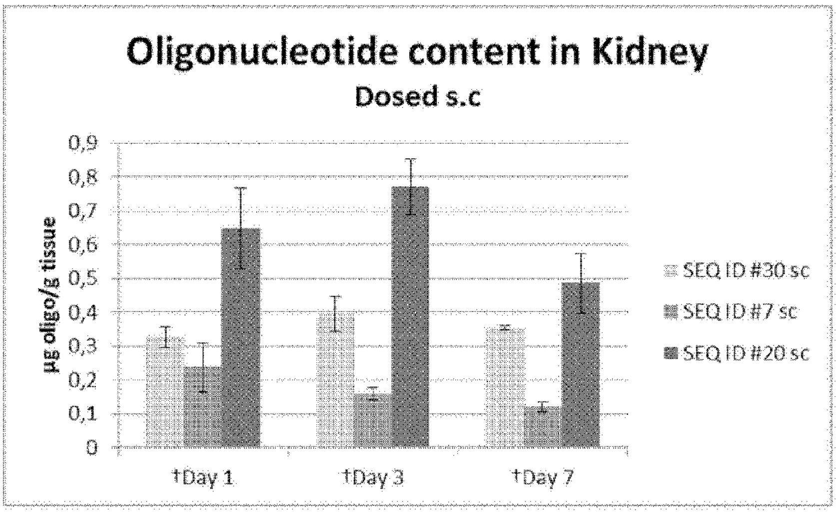


Figure 18 f)


Oligonucleotide content in Kidney
Dosed s.c
µg oligo/g tissue
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
†Day 1
†Day 3
†Day 7
SEQ ID #30 sc
SEQ ID #7 sc
SEQ ID #20 sc