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(54) **Title:** POLYNUCLEOTIDE AGENTS TARGETING COMPLEMENT COMPONENT C5 AND METHODS OF USE THEREOF

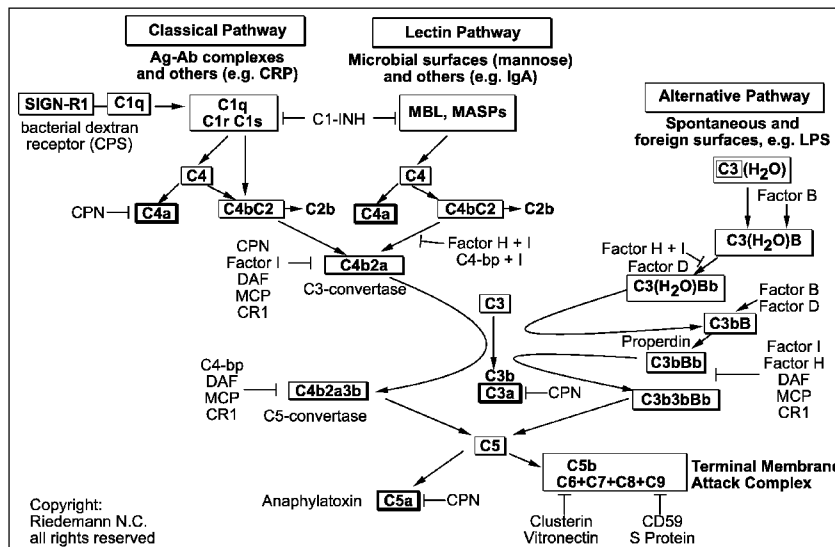


Figure 1

(57) **Abstract:** The invention relates to polynucleotide agents targeting the complement component C5 gene, and methods of using such polynucleotide agents to inhibit expression of C5 and to treat subjects having a complement component C5-associated disease, e.g., paroxysmal nocturnal hemoglobinuria.

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POLYNUCLEOTIDE AGENTS TARGETING COMPLEMENT COMPONENT C5 AND METHODS OF USE THEREOF

RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Patent Application No. 62/049,775, filed September 12, 2014. The entire contents of the aforementioned priority application are incorporated by reference herein.

SEQUENCE LISTING

This application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on September 9, 2015, is named 121301-02220_SL.txt and is 240,575 bytes in size.

BACKGROUND OF THE INVENTION

Complement was first discovered in the 1890s when it was found to aid or “complement” the killing of bacteria by heat-stable antibodies present in normal serum (Walport, M.J. (2001) *N Engl J Med.* 344:1058). The complement system consists of more than 30 proteins that are either present as soluble proteins in the blood or are present as membrane-associated proteins. Activation of complement leads to a sequential cascade of enzymatic reactions, known as complement activation pathways, resulting in the formation of the potent anaphylatoxins C3a and C5a that elicit a plethora of physiological responses that range from chemoattraction to apoptosis. Initially, complement was thought to play a major role in innate immunity where a robust and rapid response is mounted against invading pathogens. However, recently it is becoming increasingly evident that complement also plays an important role in adaptive immunity involving T and B cells that help in elimination of pathogens (Dunkelberger JR and Song WC. (2010) *Cell Res.* 20:34; Molina H, *et al.* (1996) *Proc Natl Acad Sci U S A.* 93:3357), in maintaining immunologic memory preventing pathogenic re-invasion, and is involved in numerous human pathological states (Qu, H, *et al.* (2009) *Mol Immunol.* 47:185; Wagner, E. and Frank MM. (2010) *Nat Rev Drug Discov.* 9:43).

Complement activation is known to occur through three different pathways: alternate, classical, and lectin (Figure 1), involving proteins that mostly exist as inactive zymogens that are then sequentially cleaved and activated. All pathways of complement activation lead to

cleavage of the C5 molecule generating the anaphylatoxin C5a and, C5b that subsequently forms the terminal complement complex (C5b-9). C5a exerts a predominant pro-inflammatory activity through interactions with the classical G-protein coupled receptor C5aR (CD88) as well as with the non-G protein coupled receptor C5L2 (GPR77), expressed on various immune and non-immune cells. C5b-9 causes cytolysis through the formation of the membrane attack complex (MAC), and sub-lytic MAC and soluble C5b-9 also possess a multitude of non-cytolytic immune functions. These two complement effectors, C5a and C5b-9, generated from C5 cleavage, are key components of the complement system responsible for propagating and/or initiating pathology in different diseases, including paroxysmal nocturnal hemoglobinuria, rheumatoid arthritis, ischemia-reperfusion injuries and neurodegenerative diseases.

To date, only one therapeutic that targets the C5-C5a axis is available for the treatment of complement component C5-associated diseases, the anti-C5 antibody, eculizumab (Soliris®). Although eculizumab has been shown to be effective for the treatment of paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS) and is currently being evaluated in clinical trials for additional complement component C5-associated diseases, eculizumab therapy requires weekly high dose infusions followed by biweekly maintenance infusions at a yearly cost of about \$400,000. Accordingly, there is a need in the art for alternative therapies for subjects having a complement component C5-associated disease.

SUMMARY OF THE INVENTION

The present invention provides antisense polynucleotide agents and compositions comprising such agents which target nucleic acids encoding complement component C5 and interfere with the normal function of the targeted nucleic acid. The C5 nucleic acid may be within a cell, *e.g.*, a cell within a subject, such as a human. The present invention also provides methods and combination therapies for treating a subject having a disorder that would benefit from inhibiting or reducing the expression of a C5 mRNA, *e.g.*, a complement component C5-associated disease, such as paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS) using the antisense polynucleotide agents and compositions of the invention.

Accordingly, in one aspect, the present invention provides antisense polynucleotide agents for inhibiting expression of complement component C5. The agents comprise about 4 to about 50 contiguous nucleotides, wherein at least one of the contiguous nucleotides is a modified nucleotide, and wherein the nucleotide sequence of the agent is about 80%

complementary over its entire length to the equivalent region of the nucleotide sequence of any one of SEQ ID NOs:1-4.

In one embodiment, the equivalent region is one of the target regions of SEQ ID NO:1 provided in Table 3, *e.g.*, residues 1-20, 12-31, 23-42, 34-53, 188-207, 287-306, 430-449, 716-735, 969-988, 1244-1263, 1695-1714, 2025-2044, 2289-2308, 2531-2550, 2817-2836, 3092-3111, or 3884-3903 of SEQ ID NO:1.

In another aspect, the present invention provides antisense polynucleotide agents for inhibiting expression of complement component C5, wherein the agent comprises at least 8 contiguous nucleotides differing by no more than 3 nucleotides from any one of the nucleotide sequences listed in Table 3.

In some embodiments, substantially all of the nucleotides of the antisense polynucleotide agents of the invention are modified nucleotides. In other embodiment, all of the nucleotides of the antisense polynucleotide agent are modified nucleotides.

The antisense polynucleotide agent may be 10 to 40 nucleotides in length; 10 to 30 nucleotides in length; 18 to 30 nucleotides in length; 10 to 24 nucleotides in length; 18 to 24 nucleotides in length; or 20 nucleotides in length.

In one embodiment, the modified nucleotide comprises a modified sugar moiety selected from the group consisting of: a 2'-O-methoxyethyl modified sugar moiety, a 2'-methoxy modified sugar moiety, a 2'-O-alkyl modified sugar moiety, and a bicyclic sugar moiety.

In one embodiment, the bicyclic sugar moiety has a $(\text{---CH}_2\text{---})_n$ group forming a bridge between the 2' oxygen and the 4' carbon atoms of the sugar ring, wherein n is 1 or 2.

In another embodiment, the modified nucleotide is a 5-methylcytosine.

In one embodiment, the modified nucleotide comprises a modified internucleoside linkage, such as a phosphorothioate internucleoside linkage.

In one embodiment, an agent of the invention comprises a plurality of 2'-deoxynucleotides flanked on each side by at least one nucleotide having a modified sugar moiety.

In one embodiment, the agent is a gapmer comprising a gap segment comprised of linked 2'-deoxynucleotides positioned between a 5' and a 3' wing segment.

In one embodiment, the modified sugar moiety is selected from the group consisting of a 2'-O-methoxyethyl modified sugar moiety, a 2'-methoxy modified sugar moiety, a 2'-O-alkyl modified sugar moiety, and a bicyclic sugar moiety.

In one embodiment, the 5'-wing segment is 1 to 6 nucleotides in length, *e.g.*, 2, 3, 4, or 5 nucleotides in length.

In one embodiment, the 3'-wing segment is 1 to 6 nucleotides in length, *e.g.*, 2, 3, 4, or 5 nucleotides in length.

In one embodiment, the gap segment is 5 to 14 nucleotides in length, *e.g.*, 10 nucleotides in length.

In one aspect, the present invention provides antisense polynucleotide agent for inhibiting complement component C5 expression, comprising a gap segment consisting of linked deoxynucleotides; a 5'-wing segment consisting of linked nucleotides; a 3'-wing segment consisting of linked nucleotides; wherein the gap segment is positioned between the 5'-wing segment and the 3'-wing segment and wherein each nucleotide of each wing segment comprises a modified sugar.

In one embodiment, the gap segment is ten 2'-deoxynucleotides in length and each of the wing segments is five nucleotides in length.

In another embodiment, the gap segment is ten 2'-deoxynucleotides in length and each of the wing segments is four nucleotides in length.

In yet another embodiment, the gap segment is ten 2'-deoxynucleotides in length and each of the wing segments is three nucleotides in length.

In another embodiment, the gap segment is ten 2'-deoxynucleotides in length and each of the wing segments is two nucleotides in length.

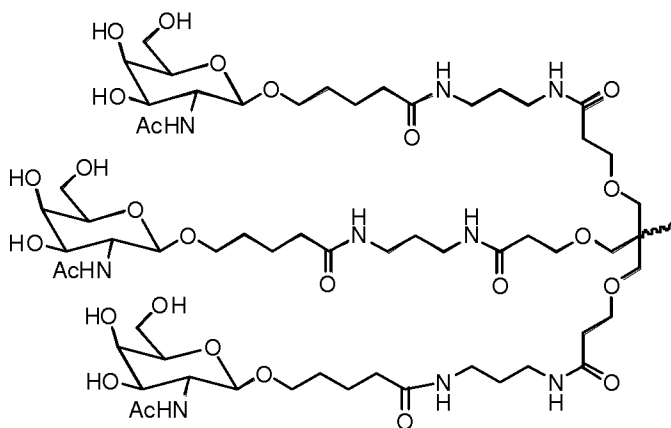
In one embodiment, the modified sugar moiety is selected from the group consisting of a 2'-O-methoxyethyl modified sugar moiety, a 2'-methoxy modified sugar moiety, a 2'-O-alkyl modified sugar moiety, and a bicyclic sugar moiety.

In some embodiments, the agents of the invention further comprise a ligand.

In one embodiment, the agent is conjugated to the ligand at the 3'-terminus.

In one embodiment, the ligand is an N-acetylgalactosamine (GalNAc) derivative.

In one embodiment, the ligand is



In one aspect, the present invention provides pharmaceutical compositions for inhibiting expression of a complement component C5 gene comprising the agents of the invention.

In one embodiment, the agent is present in an unbuffered solution, such as saline or water.

In another embodiment, the agent is present in a buffer solution, such as a buffer comprising acetate, citrate, prolamine, carbonate, or phosphate or any combination thereof.

In one embodiment, the buffer solution is phosphate buffered saline (PBS).

In another aspect, the present invention provides pharmaceutical composition comprising an agent of the invention and a lipid formulation, such as a lipid formulation comprising an LNP or a MC3.

In one aspect, the present invention provides methods of inhibiting complement component C5 expression in a cell. The methods include contacting the cell with the agent of the invention or a pharmaceutical composition of the invention; and maintaining the cell for a time sufficient to obtain antisense inhibition of a complement component C5 gene, thereby inhibiting expression of the complement component C5 gene in the cell.

In one embodiment, the cell is within a subject.

In one embodiment, the subject is a human.

In one embodiment, the complement component C5 expression is inhibited by at least about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%.

In another aspect, the present invention provides methods of treating a subject having a disease or disorder that would benefit from reduction in complement component C5 expression. The methods include administering to the subject a therapeutically effective amount of an agent of the invention or a pharmaceutical composition of the invention, thereby treating the subject.

In yet another aspect, the present invention provides methods of preventing at least one symptom in a subject having a disease or disorder that would benefit from reduction in complement component C5 expression. The methods include administering to the subject a prophylactically effective amount of the agent of the invention or a pharmaceutical composition of the invention, thereby preventing at least one symptom in the subject having a disorder that would benefit from reduction in C5 expression.

In one embodiment, the administration of the antisense polynucleotide agent to the subject causes a decrease in intravascular hemolysis, a stabilization of hemoglobin levels and/or a decrease in C5 protein levels.

In one embodiment, the disorder is a complement component C5-associated disease.

In another embodiment, the complement component C5-associated disease is selected from the group consisting of paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), asthma, rheumatoid arthritis (RA); antiphospholipid antibody syndrome; lupus nephritis; ischemia-reperfusion injury; typical or infectious hemolytic uremic syndrome (tHUS); dense deposit disease (DDD); neuromyelitis optica

(NMO); multifocal motor neuropathy (MMN); multiple sclerosis (MS); macular degeneration (e.g., age-related macular degeneration (AMD)); hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome; thrombotic thrombocytopenic purpura (TTP); spontaneous fetal loss; Pauci-immune vasculitis; epidermolysis bullosa; recurrent fetal loss; pre-eclampsia, traumatic brain injury, myasthenia gravis, cold agglutinin disease, dermatomyositis bullous pemphigoid, Shiga toxin *E. coli*-related hemolytic uremic syndrome, C3 nephropathy, anti-neutrophil cytoplasmic antibody-associated vasculitis, humoral and vascular transplant rejection, graft dysfunction, myocardial infarction, an allogenic transplant, sepsis, Coronary artery disease, dermatomyositis, Graves' disease, atherosclerosis, Alzheimer's disease, systemic inflammatory response sepsis, septic shock, spinal cord injury, glomerulonephritis, Hashimoto's thyroiditis, type I diabetes, psoriasis, pemphigus, autoimmune hemolytic anemia (AIHA), ITP, Goodpasture syndrome, Degos disease, antiphospholipid syndrome (APS), catastrophic APS (CAPS), a cardiovascular disorder, myocarditis, a cerebrovascular disorder, a peripheral vascular disorder, a renovascular disorder, a mesenteric/enteric vascular disorder, vasculitis, Henoch-Schönlein purpura nephritis, systemic lupus erythematosus-associated vasculitis, vasculitis associated with rheumatoid arthritis, immune complex vasculitis, Takayasu's disease, dilated cardiomyopathy, diabetic angiopathy, Kawasaki's disease (arteritis), venous gas embolus (VGE), and restenosis following stent placement, rotational atherectomy, membranous nephropathy, Guillain-Barre syndrome, and percutaneous transluminal coronary angioplasty (PTCA).

In one embodiment, the complement component C5-associated disease is paroxysmal nocturnal hemoglobinuria (PNH).

In another embodiment, the complement component C5-associated disease is atypical hemolytic uremic syndrome (aHUS).

In one embodiment the subject is human.

In one embodiment, the methods of the invention further include administering an anti-complement component C5 antibody, or antigen-binding fragment thereof, to the subject.

In one embodiment, the agent is administered at a dose of about 0.01 mg/kg to about 10 mg/kg or about 0.5 mg/kg to about 50 mg/kg.

In one embodiment, the agent is administered at a dose of about 10 mg/kg to about 30 mg/kg.

In one embodiment, the agent is administered to the subject once a week.

In another embodiment, the agent is administered to the subject twice a week.

In yet another embodiment, the agent is administered to the subject twice a month.

In one embodiment, the agent is administered to the subject subcutaneously.

In one embodiment, the methods of the invention further include measuring hemoglobin and/or LDH levels in the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of the three complement pathways: alternative, classical and lectin.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides antisense polynucleotide agents and compositions comprising such agents which target nucleic acids encoding complement component C5 (*e.g.*, mRNA encoding C5 as provided in, for example, any one of SEQ ID NOs:1-4). The antisense polynucleotide agents bind to nucleic acids encoding C5 *via, e.g.*, Watson-Crick base pairing, and interfere with the normal function of the targeted nucleic acid.

The antisense polynucleotide agents of the invention include a nucleotide sequence which is about 4 to about 50 nucleotides or less in length and which is about 80% complementary to at least part of an mRNA transcript of a C5 gene. The use of these antisense polynucleotide agents enables the targeted inhibition of RNA expression and/or activity of a C5 gene in mammals.

The present inventors have demonstrated that antisense polynucleotide agents targeting C5 can mediate antisense inhibition *in vitro* resulting in significant inhibition of expression of a C5 gene. Thus, methods and compositions including these antisense polynucleotide agents are useful for treating a subject who would benefit by a reduction in the levels and/or activity of a C5 protein, such as a subject having a complement component C5-associated disease, such as paroxysmal nocturnal hemoglobinuria (PNH).

The present invention also provides methods and combination therapies for treating a subject having a disorder that would benefit from inhibiting or reducing the expression of a C5 gene, *e.g.*, a complement component C5-associated disease, such as paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS) using the antisense polynucleotide agents and compositions of the invention.

The present invention also provides methods for preventing at least one symptom, *e.g.*, hemolysis, in a subject having a disorder that would benefit from inhibiting or reducing the expression of a C5 gene, *e.g.*, a complement component C5-associated disease, such as paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS). The present invention further provides compositions comprising antisense polynucleotide agents which effect antisense inhibition of a complement component C5 gene. The C5 gene may be within a cell, *e.g.*, a cell within a subject, such as a human.

The combination therapies of the present invention include administering to a subject having a complement component C5-associated disease, an antisense polynucleotide agent of the invention and an additional therapeutic, such as anti-complement component C5 antibody, or antigen-binding fragment thereof, *e.g.*, eculizumab. The combination therapies of the invention reduce C5 levels in the subject (*e.g.*, by about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or about 99%) by targeting C5 mRNA with an antisense polynucleotide agent of the invention and, accordingly, allow the therapeutically (or prophylactically) effective amount of eculizumab required to treat the subject to be reduced, thereby decreasing the costs of treatment and permitting easier and more convenient ways of administering eculizumab, such as subcutaneous administration.

The following detailed description discloses how to make and use antisense polynucleotide agents to inhibit the mRNA and/or protein expression of a C5 gene, as well as compositions, uses, and methods for treating subjects having diseases and disorders that would benefit from inhibition and/or reduction of the expression of this gene.

I. Definitions

In order that the present invention may be more readily understood, certain terms are first defined. In addition, it should be noted that whenever a value or range of values of a parameter are recited, it is intended that values and ranges intermediate to the recited values are also intended to be part of this invention.

The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element, *e.g.*, a plurality of elements.

The term “including” is used herein to mean, and is used interchangeably with, the phrase “including but not limited to”.

The term “or” is used herein to mean, and is used interchangeably with, the term “and/or,” unless context clearly indicates otherwise.

As used herein, “complement component C5,” used interchangeably with the term “C5” refers to the well-known gene and polypeptide, also known in the art as CPAMD4, C3 and PZP-like alpha-2-macroglobulin domain-containing protein, anaphylatoxin C5a analog, hemolytic complement (Hc), and complement C5. The sequence of a human C5 mRNA transcript can be found at, for example, GenBank Accession No. GI:38016946 (NM_001735.2; SEQ ID NO:1). The sequence of rhesus C5 mRNA can be found at, for example, GenBank Accession No. GI:297270262 (XM_001095750.2; SEQ ID NO:2). The sequence of mouse C5 mRNA can be found at, for example, GenBank Accession No. GI:291575171 (NM_010406.2; SEQ ID NO:3). The sequence of rat C5 mRNA can be found at, for example, GenBank Accession No. GI:392346248 (XM_345342.4; SEQ ID

NO:4). Additional examples of C5 mRNA sequences are readily available using publicly available databases, *e.g.*, GenBank.

The term “C5,” as used herein, also refers to naturally occurring DNA sequence variations of the C5 gene, such as a single nucleotide polymorphism in the C5 gene. Numerous SNPs within the C5 gene have been identified and may be found at, for example, NCBI dbSNP (see, *e.g.*, ncbi.nlm.nih.gov/snp). Non-limiting examples of SNPs within the C5 gene may be found at, NCBI dbSNP Accession Nos. rs121909588 and rs121909587.

The terms “antisense polynucleotide agent” “antisense compound”, and “agent” as used interchangeably herein, refer to an agent comprising a single-stranded oligonucleotide that contains RNA as that term is defined herein, and which targets nucleic acid molecules encoding complement component C5 (*e.g.*, mRNA encoding C5 as provided in, for example, any one of SEQ ID NOs:1-4). The antisense polynucleotide agents specifically bind to the target nucleic acid molecules *via* hydrogen bonding (*e.g.*, Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding) and interfere with the normal function of the targeted nucleic acid (*e.g.*, by an antisense mechanism of action). This interference with or modulation of the function of a target nucleic acid by the polynucleotide agents of the present invention is referred to as “antisense inhibition.”

The functions of the target nucleic acid molecule to be interfered with may include functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA.

In some embodiments, antisense inhibition refers to “inhibiting the expression” of target nucleic acid levels and/or target protein levels in a cell, *e.g.*, a cell within a subject, such as a mammalian subject, in the presence of the antisense polynucleotide agent complementary to a target nucleic acid as compared to target nucleic acid levels and/or target protein levels in the absence of the antisense polynucleotide agent. For example, the antisense polynucleotide agents of the invention can inhibit translation in a stoichiometric manner by base pairing to the mRNA and physically obstructing the translation machinery, see Dias, N. *et al.*, (2002) *Mol Cancer Ther* 1:347-355.

As used herein, “target sequence” refers to a contiguous portion of the nucleotide sequence of an mRNA molecule formed during the transcription of a C5 gene, including mRNA that is a product of RNA processing of a primary transcription product.

As used herein, “target nucleic acid” refers to a nucleic acid molecule to which an antisense polynucleotide agent specifically hybridizes.

As used herein, the term “specifically hybridizes” refers to an antisense polynucleotide agent having a sufficient degree of complementarity between the antisense polynucleotide agent and a target nucleic acid to induce a desired effect, while exhibiting minimal or no effects on non-target nucleic acids under conditions in which specific binding

is desired, *e.g.*, under physiological conditions in the case of *in vivo* assays and therapeutic treatments.

A target sequence may be from about 4-50 nucleotides in length, *e.g.*, 8-45, 10-45, 10-40, 10-35, 10-30, 10-20, 11-45, 11-40, 11-35, 11-30, 11-20, 12-45, 12-40, 12-35, 12-30, 12-25, 12-20, 13-45, 13-40, 13-35, 13-30, 13-25, 13-20, 14-45, 14-40, 14-35, 14-30, 14-25, 14-20, 15-45, 15-40, 15-35, 15-30, 15-25, 15-20, 16-45, 16-40, 16-35, 16-30, 16-25, 16-20, 17-45, 17-40, 17-35, 17-30, 17-25, 17-20, 18-45, 18-40, 18-35, 18-30, 18-25, 18-20, 19-45, 19-40, 19-35, 19-30, 19-25, 19-20, *e.g.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 contiguous nucleotides of the nucleotide sequence of an mRNA molecule formed during the transcription of a C5 gene. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the invention.

The terms “complementary,” “fully complementary” and “substantially complementary” are used herein with respect to the base matching between an antisense polynucleotide agent and a target sequence. The term “complementarity” refers to the capacity for pairing between nucleobases of a first nucleic acid and a second nucleic acid.

As used herein, an antisense polynucleotide agent that is “substantially complementary to at least part of” a messenger RNA (mRNA) refers to an antisense polynucleotide agent that is substantially complementary to a contiguous portion of the mRNA of interest (*e.g.*, an mRNA encoding C5). For example, a polynucleotide is complementary to at least a part of a C5 mRNA if the sequence is substantially complementary to a non-interrupted portion of an mRNA encoding C5.

As used herein, the term “region of complementarity” refers to the region of the antisense polynucleotide agent that is substantially complementary to a sequence, for example a target sequence, *e.g.*, a C5 nucleotide sequence, as defined herein. Where the region of complementarity is not fully complementary to the target sequence, the mismatches can be in the internal or terminal regions of the molecule. Generally, the most tolerated mismatches are in the terminal regions, *e.g.*, within 5, 4, 3, or 2 nucleotides of the 5'- and/or 3'-terminus of the antisense polynucleotide.

As used herein, and unless otherwise indicated, the term “complementary,” when used to describe a first nucleotide sequence in relation to a second nucleotide sequence, refers to the ability of a polynucleotide comprising the first nucleotide sequence to hybridize and form a duplex structure under certain conditions with the second nucleotide sequence, as will be understood by the skilled person. Such conditions can, for example, be stringent conditions, where stringent conditions can include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C for 12-16 hours followed by washing (see, *e.g.*, “Molecular Cloning: A Laboratory Manual, Sambrook, *et al.* (1989) Cold Spring Harbor Laboratory Press). Other conditions, such as physiologically relevant conditions as can be encountered inside an

organism, can apply. The skilled person will be able to determine the set of conditions most appropriate for a test of complementarity of two sequences in accordance with the ultimate application of the nucleotides.

Complementary sequences include those nucleotide sequences of an antisense polynucleotide agent of the invention that base-pair to a second nucleotide sequence over the entire length of one or both nucleotide sequences. Such sequences can be referred to as “fully complementary” with respect to each other herein. However, where a first sequence is referred to as “substantially complementary” with respect to a second sequence herein, the two sequences can be fully complementary, or they can form one or more, but generally not more than 5, 4, 3 or 2 mismatched base pairs upon hybridization for a duplex up to 30 base pairs, while retaining the ability to hybridize under the conditions most relevant to their ultimate application, *e.g.*, antisense inhibition of target gene expression.

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

As used herein, the term “strand comprising a sequence” refers to an oligonucleotide comprising a chain of nucleotides that is described by the sequence referred to using the standard nucleotide nomenclature.

“G,” “C,” “A,” “T” and “U” each generally stand for a nucleotide that contains guanine, cytosine, adenine, thymidine and uracil as a base, respectively. However, it will be understood that the terms “deoxyribonucleotide”, “ribonucleotide” and “nucleotide” can also refer to a modified nucleotide, as further detailed below, or a surrogate replacement moiety (see, *e.g.*, Table 2). The skilled person is well aware that guanine, cytosine, adenine, and uracil can be replaced by other moieties without substantially altering the base pairing properties of an oligonucleotide comprising a nucleotide bearing such replacement moiety. For example, without limitation, a nucleotide comprising inosine as its base can base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine can be replaced in the nucleotide sequences of the agents featured in the invention by a nucleotide containing, for example, inosine. In another example, adenine and cytosine anywhere in the oligonucleotide can be replaced with guanine and uracil, respectively to form G-U Wobble base pairing with the target mRNA. Sequences containing such replacement moieties are suitable for the compositions and methods featured in the invention.

A “nucleoside” is a base-sugar combination. The “nucleobase” (also known as “base”) portion of the nucleoside is normally a heterocyclic base moiety. “Nucleotides” are nucleosides that further include a phosphate group covalently linked to the sugar portion of

the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to the 2', 3' or 5' hydroxyl moiety of the sugar. "Polynucleotides," also referred to as "oligonucleotides," are formed through the covalent linkage of adjacent nucleosides to one another, to form a linear polymeric oligonucleotide. Within the polynucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside linkages of the polynucleotide.

In general, the majority of nucleotides of the antisense polynucleotide agents are ribonucleotides, but as described in detail herein, the agents may also include one or more non-ribonucleotides, *e.g.*, a deoxyribonucleotide. In addition, as used in this specification, an "antisense polynucleotide agent" may include nucleotides (*e.g.*, ribonucleotides or deoxyribonucleotides) with chemical modifications; an antisense polynucleotide agent may include substantial modifications at multiple nucleotides.

As used herein, the term "modified nucleotide" refers to a nucleotide having, independently, a modified sugar moiety, a modified internucleotide linkage, and/or modified nucleobase. Thus, the term modified nucleotide encompasses substitutions, additions or removal of, *e.g.*, a functional group or atom, to internucleoside linkages, sugar moieties, or nucleobases. The modifications suitable for use in the antisense polynucleotide agents of the invention include all types of modifications disclosed herein or known in the art. Any such modifications, as used in nucleotides, are encompassed by "antisense polynucleotide agent" for the purposes of this specification and claims.

As used herein, a "subject" is an animal, such as a mammal, including a primate (such as a human, a non-human primate, *e.g.*, a monkey, and a chimpanzee), a non-primate (such as a cow, a pig, a camel, a llama, a horse, a goat, a rabbit, a sheep, a hamster, a guinea pig, a cat, a dog, a rat, a mouse, a horse, and a whale), or a bird (*e.g.*, a duck or a goose). In an embodiment, the subject is a human, such as a human being treated or assessed for a disease, disorder or condition that would benefit from reduction in C5 expression; a human at risk for a disease, disorder or condition that would benefit from reduction in C5 expression; a human having a disease, disorder or condition that would benefit from reduction in C5 expression; and/or human being treated for a disease, disorder or condition that would benefit from reduction in C5 expression as described herein.

As used herein, the terms "treating" or "treatment" refer to a beneficial or desired result including, but not limited to, alleviation or amelioration of one or more symptoms associated with unwanted complement pathway activation (*e.g.*, hemolysis and/or chronic inflammation); diminishing the extent of unwanted complement pathway activation; stabilization (*i.e.*, not worsening) of the state of chronic inflammation and/or hemolysis; amelioration or palliation of unwanted complement pathway activation (*e.g.*, chronic inflammation and/or hemolysis) whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival in the absence of treatment.

The term “lower” in the context of the level of a complement component C5 in a subject or a disease marker or symptom refers to a statistically significant decrease in such level. The decrease can be, for example, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or more and is preferably down to a level accepted as within the range of normal for an individual without such disorder.

As used herein, “prevention” or “preventing,” when used in reference to a disease, disorder or condition thereof, that would benefit from a reduction in expression of a C5 gene, refers to a reduction in the likelihood that a subject will develop a symptom associated with such a disease, disorder, or condition, *e.g.*, a symptom of unwanted complement activation, such as a chronic inflammation, hemolysis and/or thrombosis. The likelihood of developing a thrombosis is reduced, for example, when an individual having one or more risk factors for a thrombosis either fails to develop a thrombosis or develops a thrombosis with less severity relative to a population having the same risk factors and not receiving treatment as described herein. The failure to develop a disease, disorder or condition, or the reduction in the development of a symptom associated with such a disease, disorder or condition (*e.g.*, by at least about 10% on a clinically accepted scale for that disease or disorder), or the exhibition of delayed symptoms delayed (*e.g.*, by days, weeks, months or years) is considered effective prevention.

As used herein, the term “complement component C5-associated disease” is a disease or disorder that is caused by, or associated with complement activation. Such diseases are typically associated with inflammation and/or immune system activation, *e.g.*, membrane attack complex-mediated lysis, anaphylaxis, and/or hemolysis. Non-limiting examples of complement component C5-associated diseases include paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), asthma, rheumatoid arthritis (RA); antiphospholipid antibody syndrome; lupus nephritis; ischemia-reperfusion injury; typical or infectious hemolytic uremic syndrome (tHUS); dense deposit disease (DDD); neuromyelitis optica (NMO); multifocal motor neuropathy (MMN); multiple sclerosis (MS); macular degeneration (*e.g.*, age-related macular degeneration (AMD)); hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome; thrombotic thrombocytopenic purpura (TTP); spontaneous fetal loss; Pauci-immune vasculitis; epidermolysis bullosa; recurrent fetal loss; pre-eclampsia, traumatic brain injury, myasthenia gravis, cold agglutinin disease, dermatomyositis bullous pemphigoid, Shiga toxin E. coli-related hemolytic uremic syndrome, C3 nephropathy, anti-neutrophil cytoplasmic antibody-associated vasculitis (*e.g.*, granulomatosis with polyangiitis (previously known as Wegener granulomatosis), Churg-Strauss syndrome, and microscopic polyangiitis), humoral and vascular transplant rejection, graft dysfunction, myocardial infarction (*e.g.*, tissue damage

and ischemia in myocardial infarction), an allogenic transplant, sepsis (*e.g.*, poor outcome in sepsis), Coronary artery disease, dermatomyositis, Graves' disease, atherosclerosis, Alzheimer's disease, systemic inflammatory response sepsis, septic shock, spinal cord injury, glomerulonephritis, Hashimoto's thyroiditis, type I diabetes, psoriasis, pemphigus, autoimmune hemolytic anemia (AIHA), ITP, Goodpasture syndrome, Degos disease, antiphospholipid syndrome (APS), catastrophic APS (CAPS), a cardiovascular disorder, myocarditis, a cerebrovascular disorder, a peripheral (*e.g.*, musculoskeletal) vascular disorder, a renovascular disorder, a mesenteric/enteric vascular disorder, vasculitis, Henoch-Schönlein purpura nephritis, systemic lupus erythematosus-associated vasculitis, vasculitis associated with rheumatoid arthritis, immune complex vasculitis, Takayasu's disease, dilated cardiomyopathy, diabetic angiopathy, Kawasaki's disease (arteritis), venous gas embolus (VGE), and restenosis following stent placement, rotational atherectomy, membranous nephropathy, Guillain-Barre syndrome, and percutaneous transluminal coronary angioplasty (PTCA) (see, *e.g.*, Holers (2008) *Immunological Reviews* 223:300-316; Holers and Thurman (2004) *Molecular Immunology* 41:147-152; U.S. Patent Publication No. 20070172483).

In one embodiment, a complement component C5-associated disease is paroxysmal nocturnal hemoglobinuria (PNH). The PNH may be classical PNH or PNH in the setting of another bone marrow failure syndrome and/or myelodysplastic syndromes (MDS), *e.g.*, cytopenias. In another embodiment, a complement component C5-associated disease is atypical hemolytic uremic syndrome (aHUS).

II. Antisense Polynucleotide Agents of the Invention

The present invention provides antisense polynucleotide agents, and compositions comprising such agents, which target a complement component C5 gene and inhibit the expression of the C5 gene. In one embodiment, the antisense polynucleotide agents inhibit the expression of a C5 gene in a cell, such as a cell within a subject, *e.g.*, a mammal, such as a human having a complement component C5-associated disease, *e.g.*, PNH.

The antisense polynucleotide agents of the invention include a region of complementarity which is complementary to at least a part of an mRNA formed in the expression of a C5 gene. The region of complementarity may be about 50 nucleotides or less in length (*e.g.*, about 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 nucleotides or less in length). Upon contact with a cell expressing the C5 gene, the antisense polynucleotide agent inhibits the expression of the C5 gene (*e.g.*, a human, a primate, a non-primate, or a bird C5 gene) by at least about 10% as assayed by, for example, a PCR or branched DNA (bDNA)-based method, or by a protein-based method, such as by immunofluorescence analysis, using, for example, Western Blotting or flow cytometric techniques.

The region of complementarity between an antisense polynucleotide agent and a target sequence may be substantially complementary (*e.g.*, there is a sufficient degree of complementarity between the antisense polynucleotide agent and a target nucleic acid to so that they specifically hybridize and induce a desired effect), but is generally fully complementary to the target sequence. The target sequence can be derived from the sequence of an mRNA formed during the expression of a C5 gene.

Accordingly, in one aspect, an antisense polynucleotide agent of the invention specifically hybridizes to a target nucleic acid molecule, such as the mRNA encoding complement component C5, and comprises a contiguous nucleotide sequence which corresponds to the reverse complement of a nucleotide sequence of any one of SEQ ID NOs:1-4, or a fragment of any one of SEQ ID NOs:1-4.

In some embodiments, the antisense polynucleotide agents of the invention may be substantially complementary to the target sequence. For example, an antisense polynucleotide agent that is substantially complementary to the target sequence may include a contiguous nucleotide sequence comprising no more than 5 mismatches (*e.g.*, no more than 1, no more than 2, no more than 3, no more than 4, or no more than 5 mismatches) when hybridizing to a target sequence, such as to the corresponding region of a nucleic acid which encodes a mammalian C5 mRNA. In some embodiments, the contiguous nucleotide sequence comprises no more than a single mismatch when hybridizing to the target sequence, such as the corresponding region of a nucleic acid which encodes a mammalian C5 mRNA.

In some embodiments, the antisense polynucleotide agents of the invention that are substantially complementary to the target sequence comprise a contiguous nucleotide sequence which is at least about 80% complementary over its entire length to the equivalent region of the nucleotide sequence of any one of SEQ ID NOs:1-4, or a fragment of any one of SEQ ID NOs:1-4, such as about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In some embodiments, an antisense polynucleotide agent comprises a contiguous nucleotide sequence which is fully complementary over its entire length to the equivalent region of the nucleotide sequence of any one of SEQ ID NOs:1-4 (or a fragment of any one of SEQ ID NOs:1-4). For example, the nucleotide sequence of Sequence ID A-128563.1 is fully complementary over its entire length to the equivalent region of nucleotides 1-20 of NM_001735.2 (SEQ ID NO:1) (*see, e.g.*, Table 3). Similarly, the nucleotide sequence of Sequence ID A-128637.1 is fully complementary over its entire length to the equivalent region of nucleotides 815-834 of NM_001735.2 (SEQ ID NO:1) (*see, e.g.*, Table 3) and the nucleotide sequence of Sequence ID A-128915.1 is fully complementary over its entire length to the equivalent region of nucleotides 3873-3892 of NM_001735.2 (SEQ ID NO:1) (*see, e.g.*, Table 3).

An antisense polynucleotide agent may comprise a contiguous nucleotide sequence of about 4 to about 50 nucleotides in length, *e.g.*, 8-49, 8-48, 8-47, 8-46, 8-45, 8-44, 8-43, 8-42, 8-41, 8-40, 8-39, 8-38, 8-37, 8-36, 8-35, 8-34, 8-33, 8-32, 8-31, 8-30, 8-29, 8-28, 8-27, 8-26, 8-25, 8-24, 8-23, 8-22, 8-21, 8-20, 8-19, 8-18, 8-17, 8-16, 8-15, 8-14, 8-13, 8-12, 8-11, 8-10, 8-9, 10-49, 10-48, 10-47, 10-46, 10-45, 10-44, 10-43, 10-42, 10-41, 10-40, 10-39, 10-38, 10-37, 10-36, 10-35, 10-34, 10-33, 10-32, 10-31, 10-30, 10-29, 10-28, 10-27, 10-26, 10-25, 10-24, 10-23, 10-22, 10-21, 10-20, 10-19, 10-18, 10-17, 10-16, 10-15, 10-14, 10-13, 10-12, 10-11, 11-49, 11-48, 11-47, 11-46, 11-45, 11-44, 11-43, 11-42, 11-41, 11-40, 11-39, 11-38, 11-37, 11-36, 11-35, 11-34, 11-33, 11-32, 11-31, 11-30, 11-29, 11-28, 11-27, 11-26, 11-25, 11-24, 11-23, 11-22, 11-21, 11-20, 11-19, 11-18, 11-17, 11-16, 11-15, 11-14, 11-13, 11-12, 12-49, 12-48, 12-47, 12-46, 12-45, 12-44, 12-43, 12-42, 12-41, 12-40, 12-39, 12-38, 12-37, 12-36, 12-35, 12-34, 12-33, 12-32, 12-31, 12-30, 12-29, 12-28, 12-27, 12-26, 12-25, 12-24, 12-23, 12-22, 12-21, 12-20, 12-19, 12-18, 12-17, 12-16, 12-15, 12-14, 12-13, 13-49, 13-48, 13-47, 13-46, 13-45, 13-44, 13-43, 13-42, 13-41, 13-40, 13-39, 13-38, 13-37, 13-36, 13-35, 13-34, 13-33, 13-32, 13-31, 13-30, 13-29, 13-28, 13-27, 13-26, 13-25, 13-24, 13-23, 13-22, 13-21, 13-20, 13-19, 13-18, 13-17, 13-16, 13-15, 13-14, 14-49, 14-48, 14-47, 14-46, 14-45, 14-44, 14-43, 14-42, 14-41, 14-40, 14-39, 14-38, 14-37, 14-36, 14-35, 14-34, 14-33, 14-32, 14-31, 14-30, 14-29, 14-28, 14-27, 14-26, 14-25, 14-24, 14-23, 14-22, 14-21, 14-20, 14-19, 14-18, 14-17, 14-16, 14-15, 15-49, 15-48, 15-47, 15-46, 15-45, 15-44, 15-43, 15-42, 15-41, 15-40, 15-39, 15-38, 15-37, 15-36, 15-35, 15-34, 15-33, 15-32, 15-31, 15-30, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 15-16, 16-49, 16-48, 16-47, 16-46, 16-45, 16-44, 16-43, 16-42, 16-41, 16-40, 16-39, 16-38, 16-37, 16-36, 16-35, 16-34, 16-33, 16-32, 16-31, 16-30, 16-29, 16-28, 16-27, 16-26, 16-25, 16-24, 16-23, 16-22, 16-21, 16-20, 16-19, 16-18, 16-17, 17-49, 17-48, 17-47, 17-46, 17-45, 17-44, 17-43, 17-42, 17-41, 17-40, 17-39, 17-38, 17-37, 17-36, 17-35, 17-34, 17-33, 17-32, 17-31, 17-30, 17-29, 17-28, 17-27, 17-26, 17-25, 17-24, 17-23, 17-22, 17-21, 17-20, 17-19, 17-18, 18-49, 18-48, 18-47, 18-46, 18-45, 18-44, 18-43, 18-42, 18-41, 18-40, 18-39, 18-38, 18-37, 18-36, 18-35, 18-34, 18-33, 18-32, 18-31, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-49, 19-48, 19-47, 19-46, 19-45, 19-44, 19-43, 19-42, 19-41, 19-40, 19-39, 19-38, 19-37, 19-36, 19-35, 19-34, 19-33, 19-32, 19-31, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-49, 20-48, 20-47, 20-46, 20-45, 20-44, 20-43, 20-42, 20-41, 20-40, 20-39, 20-38, 20-37, 20-36, 20-35, 20-34, 20-33, 20-32, 20-31, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24, 20-23, 20-22, 20-21, 21-49, 21-48, 21-47, 21-46, 21-45, 21-44, 21-43, 21-42, 21-41, 21-40, 21-39, 21-38, 21-37, 21-36, 21-35, 21-34, 21-33, 21-32, 21-31, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, 21-22, 22-49, 22-48, 22-47, 22-46, 22-45, 22-44, 22-43, 22-42, 22-41, 22-40, 22-39, 22-38, 22-37, 22-36, 22-35, 22-34, 22-33, 22-32, 22-31, 22-30, 22-29, 22-28, 22-27, 22-26, 22-25, 22-24, 22-23, 23-49, 23-48, 23-47, 23-46, 23-45, 23-44, 23-43, 23-42, 23-41, 23-40, 23-39, 23-38, 23-37, 23-

36, 23-35, 23-34, 23-33, 23-32, 23-31, 23-30, 23-29, 23-28, 23-27, 23-26, 23-25, 23-24, 24-49, 24-48, 24-47, 24-46, 24-45, 24-44, 24-43, 24-42, 24-41, 24-40, 24-39, 24-38, 24-37, 24-36, 24-35, 24-34, 24-33, 24-32, 24-31, 24-30, 24-29, 24-28, 24-27, 24-26, 24-25, 25-49, 25-48, 25-47, 25-46, 25-45, 25-44, 25-43, 25-42, 25-41, 25-40, 25-39, 25-38, 25-37, 25-36, 25-35, 25-34, 25-33, 25-32, 25-31, 25-30, 25-29, 25-28, 25-27, 25-26, 26-49, 26-48, 26-47, 26-46, 26-45, 26-44, 26-43, 26-42, 26-41, 26-40, 26-39, 26-38, 26-37, 26-36, 26-35, 26-34, 26-33, 26-32, 26-31, 26-30, 26-29, 26-28, 26-27, 27-49, 27-48, 27-47, 27-46, 27-45, 27-44, 27-43, 27-42, 27-41, 27-40, 27-39, 27-38, 27-37, 27-36, 27-35, 27-34, 27-33, 27-32, 27-31, 27-30, 27-29, 27-28, 28-49, 28-48, 28-47, 28-46, 28-45, 28-44, 28-43, 28-42, 28-41, 28-40, 28-39, 28-38, 28-37, 28-36, 28-35, 28-34, 28-33, 28-32, 28-31, 28-30, 28-29, 29-49, 29-48, 29-47, 29-46, 29-45, 29-44, 29-43, 29-42, 29-41, 29-40, 29-39, 29-38, 29-37, 29-36, 29-35, 29-34, 29-33, 29-32, 29-31, 29-30, 30-49, 30-48, 30-47, 30-46, 30-45, 30-44, 30-43, 30-42, 30-41, 30-40, 30-39, 30-38, 30-37, 30-36, 30-35, 30-34, 30-33, 30-32, or 30-31 nucleotides in length, *e.g.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides in length.

In some embodiments, an antisense polynucleotide agent may comprise a contiguous nucleotide sequence of no more than 22 nucleotides, such as no more than 21 nucleotides, 20 nucleotides, 19 nucleotides, or no more than 18 nucleotides. In some embodiments the antisense polynucleotide agent of the invention comprises less than 20 nucleotides. In other embodiments, the antisense polynucleotide agents of the invention comprise 20 nucleotides.

In one aspect, an antisense polynucleotide agent of the invention includes a sequence selected from the group of sequences provided in Table 3. It will be understood that, although some of the sequences in Table 3 are described as modified and/or conjugated sequences, an antisense polynucleotide agent of the invention, may also comprise any one of the sequences set forth in Table 3 that is un-modified, un-conjugated, and/or modified and/or conjugated differently than described therein.

By virtue of the nature of the nucleotide sequences provided in Table 3, antisense polynucleotide agents of the invention may include one of the sequences of Table 3 minus only a few nucleotides on one or both ends and yet remain similarly effective as compared to the antisense polynucleotide agents described above. Hence, antisense polynucleotide agents having a sequence of at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more contiguous nucleotides derived from one of the sequences of Table 3 and differing in their ability to inhibit the expression of a C5 gene by not more than about 5, 10, 15, 20, 25, or 30% inhibition from an antisense polynucleotide agent comprising the full sequence, are contemplated to be within the scope of the present invention.

In addition, the antisense polynucleotide agents provided in Table 3 identify a region(s) in a C5 transcript that is susceptible to antisense inhibition (*e.g.*, the regions encompassed by the start and end positions relative to NM_001735.2 in Table 3). As such, the present invention further features antisense polynucleotide agents that target within one of these sites. As used herein, an antisense polynucleotide agent is said to target within a particular site of an RNA transcript if the antisense polynucleotide agent promotes antisense inhibition of the target at that site. Such an antisense polynucleotide agent will generally include at least about 15 contiguous nucleotides from one of the sequences provided in Table 3 coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in a C5 gene.

While a target sequence is generally about 4-50 nucleotides in length, there is wide variation in the suitability of particular sequences in this range for directing antisense inhibition of any given target RNA. Various software packages and the guidelines set out herein provide guidance for the identification of optimal target sequences for any given gene target, but an empirical approach can also be taken in which a “window” or “mask” of a given size (as a non-limiting example, 20 nucleotides) is literally or figuratively (including, *e.g.*, *in silico*) placed on the target RNA sequence to identify sequences in the size range that can serve as target sequences. By moving the sequence “window” progressively one nucleotide upstream or downstream of an initial target sequence location, the next potential target sequence can be identified, until the complete set of possible sequences is identified for any given target size selected. This process, coupled with systematic synthesis and testing of the identified sequences (using assays as described herein or as known in the art) to identify those sequences that perform optimally can identify those RNA sequences that, when targeted with an antisense polynucleotide agent, mediate the best inhibition of target gene expression. Thus, while the sequences identified, for example, in Table 3 represent effective target sequences, it is contemplated that further optimization of antisense inhibition efficiency can be achieved by progressively “walking the window” one nucleotide upstream or downstream of the given sequences to identify sequences with equal or better inhibition characteristics.

Further, it is contemplated that for any sequence identified, *e.g.*, in Table 3, further optimization could be achieved by systematically either adding or removing nucleotides to generate longer or shorter sequences and testing those sequences generated by walking a window of the longer or shorter size up or down the target RNA from that point. Again, coupling this approach to generating new candidate targets with testing for effectiveness of antisense polynucleotide agents based on those target sequences in an inhibition assay as known in the art and/or as described herein can lead to further improvements in the efficiency of inhibition. Further still, such optimized sequences can be adjusted by, *e.g.*, the introduction of modified nucleotides as described herein or as known in the art, addition or

changes in length, or other modifications as known in the art and/or discussed herein to further optimize the molecule (*e.g.*, increasing serum stability or circulating half-life, increasing thermal stability, enhancing transmembrane delivery, targeting to a particular location or cell type, increasing interaction with silencing pathway enzymes, increasing release from endosomes) as an expression inhibitor.

III. Modified Antisense Polynucleotide Agents of the Invention

In one embodiment, the nucleotides of an antisense polynucleotide agent of the invention are un-modified, and do not comprise, *e.g.*, chemical modifications and/or conjugations known in the art and described herein. In another embodiment, at least one of the nucleotides of an antisense polynucleotide agent of the invention is chemically modified to enhance stability or other beneficial characteristics. In certain embodiments of the invention, substantially all of the nucleotides of an antisense polynucleotide agent of the invention are modified. In other embodiments of the invention, all of the nucleotides of an antisense polynucleotide agent of the invention are modified. Antisense polynucleotide agents of the invention in which “substantially all of the nucleotides are modified” are largely but not wholly modified and can include not more than 5, 4, 3, 2, or 1 unmodified nucleotides.

The nucleic acids featured in the invention can be synthesized and/or modified by standard methods known in the art as further discussed below, *e.g.*, solution-phase or solid-phase organic synthesis or both, *e.g.*, by use of an automated DNA synthesizer, such as are commercially available from, for example, Biosearch, Applied Biosystems, Inc. Well-established methods for the synthesis and/or modification of the nucleic acids featured in the invention are described in, for example, “Current protocols in nucleic acid chemistry,” Beaucage, S.L. *et al.* (Eds.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby incorporated herein by reference. Modifications include, for example, end modifications, *e.g.*, 5'-end modifications (phosphorylation, conjugation, inverted linkages) or 3'-end modifications (conjugation, DNA nucleotides, inverted linkages, *etc.*); base modifications, *e.g.*, replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners, removal of bases (abasic nucleotides), or conjugated bases; sugar modifications (*e.g.*, at the 2'-position or 4'-position) or replacement of the sugar; and/or backbone modifications, including modification or replacement of the phosphodiester linkages.

Specific examples of modified nucleotides useful in the embodiments described herein include, but are not limited to nucleotides containing modified backbones or no natural internucleoside linkages. Nucleotides having modified backbones include, among others, those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified nucleotides that do not have a

phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. In some embodiments, a modified antisense polynucleotide agent will have a phosphorus atom in its internucleoside backbone.

Modified nucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5'-linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative U.S. patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Patent Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,195; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,316; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; 6,028,188; 6,124,445; 6,160,109; 6,169,170; 6,172,209; 6,239,265; 6,277,603; 6,326,199; 6,346,614; 6,444,423; 6,531,590; 6,534,639; 6,608,035; 6,683,167; 6,858,715; 6,867,294; 6,878,805; 7,015,315; 7,041,816; 7,273,933; 7,321,029; and US Pat RE39464, the entire contents of each of which are hereby incorporated herein by reference.

Modified nucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatoms and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative U.S. patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patent Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,64,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and, 5,677,439, the entire contents of each of which are hereby incorporated herein by reference.

In other embodiments, suitable nucleotide mimetics are contemplated for use in antisense polynucleotide agents, in which both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an RNA mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar backbone of an RNA is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patent Nos. 5,539,082; 5,714,331; and 5,719,262, the entire contents of each of which are hereby incorporated herein by reference. Additional PNA compounds suitable for use in the antisense polynucleotide agents of the invention are described in, for example, in Nielsen *et al.*, *Science*, 1991, 254, 1497-1500.

Some embodiments featured in the invention include polynucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH₂--NH--CH₂--, --CH₂--N(CH₃)--O--CH₂--[known as a methylene (methylimino) or MMI backbone], --CH₂--O--N(CH₃)--CH₂--, --CH₂--N(CH₃)--N(CH₃)--CH₂-- and --N(CH₃)--CH₂--CH₂--[wherein the native phosphodiester backbone is represented as --O--P--O--CH₂--] of the above-referenced U.S. Patent No. 5,489,677, and the amide backbones of the above-referenced U.S. Patent No. 5,602,240. In some embodiments, the antisense polynucleotide agents featured herein have morpholino backbone structures of the above-referenced U.S. Patent No. 5,034,506.

Modified nucleotides can also contain one or more modified or substituted sugar moieties. The antisense polynucleotide agents featured herein can include one of the following at the 2'-position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl can be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Exemplary suitable modifications include O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10.

In other embodiments, antisense polynucleotide agents include one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an antisense polynucleotide, or a group for improving the pharmacodynamic properties of an antisense polynucleotide agent, and other substituents having similar properties. In some embodiments, the modification includes a

2'-methoxyethoxy (2'-O--CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, 1995, 78:486-504) *i.e.*, an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminoethoxy, *i.e.*, a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-O--CH₂--O--CH₂--N(CH₂)₂.

Other modifications include 2'-methoxy (2'-OCH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications can also be made at other positions on a nucleotide of an antisense polynucleotide agent, particularly the 3' position of the sugar on the 3' terminal nucleotide. Antisense polynucleotide agents can also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Patent Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application. The entire contents of each of the foregoing are hereby incorporated herein by reference.

Additional nucleotides having modified or substituted sugar moieties for use in the polynucleotide agents of the invention include nucleotides comprising a bicyclic sugar. A "bicyclic sugar" is a furanosyl ring modified by the bridging of two atoms. A "bicyclic nucleoside" ("BNA") is a nucleoside having a sugar moiety comprising a bridge connecting two carbon atoms of the sugar ring, thereby forming a bicyclic ring system. In certain embodiments, the bridge connects the 4'-carbon and the 2'-carbon of the sugar ring. Thus, in some embodiments an antisense polynucleotide agent may include one or more locked nucleic acids. A "locked nucleic acid" ("LNA") is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting the 2' and 4' carbons. In other words, an LNA is a nucleotide comprising a bicyclic sugar moiety comprising a 4'-CH₂-O-2' bridge. This structure effectively "locks" the ribose in the 3'-endo structural conformation. The addition of locked nucleic acids to antisense polynucleotide agents has been shown to increase antisense polynucleotide agent stability in serum, and to reduce off-target effects (Elmen, J. *et al.*, (2005) *Nucleic Acids Research* 33(1):439-447; Mook, OR. *et al.*, (2007) *Mol Canc Ther* 6(3):833-843; Grunweller, A. *et al.*, (2003) *Nucleic Acids Research* 31(12):3185-3193).

Examples of bicyclic nucleosides for use in the polynucleotides of the invention include without limitation nucleosides comprising a bridge between the 4' and the 2' ribosyl ring atoms. In certain embodiments, the antisense polynucleotide agents of the invention include one or more bicyclic nucleosides comprising a 4' to 2' bridge. Examples of such 4' to 2' bridged bicyclic nucleosides, include but are not limited to 4'-(CH₂)—O-2' (LNA); 4'-

(CH₂)—S-2'; 4'-(CH₂)₂—O-2' (ENA); 4'-CH(CH₃)—O-2' (also referred to as "constrained ethyl" or "cEt") and 4'-CH(CH₂OCH₃)—O-2' (and analogs thereof; see, *e.g.*, U.S. Pat. No. 7,399,845); 4'-C(CH₃)(CH₃)—O-2' (and analogs thereof; see *e.g.*, US Patent No. 8,278,283); 4'-CH₂—N(OCH₃)-2' (and analogs thereof; see *e.g.*, US Patent No. 8,278,425); 4'-CH₂—O—N(CH₃)-2' (see, *e.g.*, U.S. Patent Publication No. 2004/0171570); 4'-CH₂—N(R)—O-2', wherein R is H, C1-C12 alkyl, or a protecting group (see, *e.g.*, U.S. Pat. No. 7,427,672); 4'-CH₂—C(H)(CH₃)-2' (see, *e.g.*, Chattopadhyaya *et al.*, *J. Org. Chem.*, 2009, 74, 118-134); and 4'-CH₂—C(=CH₂)-2' (and analogs thereof; see, *e.g.*, US Patent No. 8,278,426). The entire contents of each of the foregoing are hereby incorporated herein by reference.

Additional representative U.S. Patents and US Patent Publications that teach the preparation of locked nucleic acid nucleotides include, but are not limited to, the following: U.S. Patent Nos. 6,268,490; 6,525,191; 6,670,461; 6,770,748; 6,794,499; 6,998,484; 7,053,207; 7,034,133; 7,084,125; 7,399,845; 7,427,672; 7,569,686; 7,741,457; 8,022,193; 8,030,467; 8,278,425; 8,278,426; 8,278,283; US 2008/0039618; and US 2009/0012281, the entire contents of each of which are hereby incorporated herein by reference.

Any of the foregoing bicyclic nucleosides can be prepared having one or more stereochemical sugar configurations including for example α -L-ribofuranose and β -D-ribofuranose (see WO 99/14226).

In one particular embodiment of the invention, an antisense polynucleotide agent can include one or more constrained ethyl nucleotides. As used herein, a "constrained ethyl nucleotide" or "cEt" is a locked nucleic acid comprising a bicyclic sugar moiety comprising a 4'-CH(CH₃)-O-2' bridge. In one embodiment, a constrained ethyl nucleotide is in an S conformation and is referred to as an "S-constrained ethyl nucleotide" or "S-cEt."

Modified nucleotides included in the antisense polynucleotide agents of the invention can also contain one or more sugar mimetics. For example, the antisense polynucleotide agent may include a "modified tetrahydropyran nucleotide" or "modified THP nucleotide." A "modified tetrahydropyran nucleotide" has a six-membered tetrahydropyran "sugar" substituted in for the pentofuranosyl residue in normal nucleotides (a sugar surrogate). Modified THP nucleotides 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, *e.g.*, Leumann, *Bioorg. Med. Chem.*, 2002, 10, 841-854), or fluoro HNA (F-HNA).

In some embodiments of the invention, sugar surrogates comprise rings having more than 5 atoms and more than one heteroatom. For example nucleotides comprising morpholino sugar moieties and their use in oligomeric compounds has been reported (see for example: Braasch *et al.*, *Biochemistry*, 2002, 41, 4503-4510; and U.S. Patent Nos. 5,698,685; 5,166,315; 5,185,444; and 5,034,506). Morpholinos may be modified, for example by adding or altering various substituent groups from the above morpholino structure. Such sugar surrogates are referred to herein as "modified morpholinos."

Combinations of modifications are also provided without limitation, such as 2'-F-5'-methyl substituted nucleosides (see PCT International Application WO 2008/101157 published on Aug. 21, 2008 for other disclosed 5', 2'-bis substituted nucleosides) and replacement of the ribosyl ring oxygen atom with S and further substitution at the 2'-position (see published U.S. Patent Application US2005-0130923, published on Jun. 16, 2005) or alternatively 5'-substitution of a bicyclic nucleic acid (see PCT International Application WO 2007/134181, published on 11/22/07 wherein a 4'-CH₂-O-2' bicyclic nucleoside is further substituted at the 5' position with a 5'-methyl or a 5'-vinyl group). 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).

In certain embodiments, antisense compounds comprise one or more modified cyclohexenyl nucleosides, which is a nucleoside having a six-membered cyclohexenyl in place of the pentofuranosyl residue in naturally occurring nucleosides. Modified cyclohexenyl nucleosides include, but are not limited to those described in the art (see for example commonly owned, published PCT Application WO 2010/036696, published on Apr. 10, 2010, Robeyns et al., *J. Am. Chem. Soc.*, 2008, 130(6), 1979-1984; Horvath et al., *Tetrahedron Letters*, 2007, 48, 3621-3623; Nauwelaerts et al., *J. Am. Chem. Soc.*, 2007, 129(30), 9340-9348; Gu et al., *Nucleosides, Nucleotides & Nucleic Acids*, 2005, 24(5-7), 993-998; Nauwelaerts et al., *Nucleic Acids Research*, 2005, 33(8), 2452-2463; Robeyns et al., *Acta Crystallographica, Section F: Structural Biology and Crystallization Communications*, 2005, F61(6), 585-586; Gu et al., *Tetrahedron*, 2004, 60(9), 2111-2123; Gu et al., *Oligonucleotides*, 2003, 13(6), 479-489; Wang et al., *J. Org. Chem.*, 2003, 68, 4499-4505; Verbeure et al., *Nucleic Acids Research*, 2001, 29(24), 4941-4947; Wang et al., *J. Org. Chem.*, 2001, 66, 8478-82; Wang et al., *Nucleosides, Nucleotides & Nucleic Acids*, 2001, 20(4-7), 785-788; Wang et al., *J. Am. Chem.*, 2000, 122, 8595-8602; Published PCT application, WO 06/047842; and Published PCT Application WO 01/049687; the text of each is incorporated by reference herein, in their entirety).

An antisense polynucleotide agent can also include nucleobase modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as deoxythymine (dT), 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo, particularly 5-

bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-daazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Patent No. 3,687,808, those disclosed in "Modified Nucleosides in Biochemistry," *Biotechnology and Medicine*, Herdewijn, P. ed. Wiley-VCH, 2008; those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J. L, ed. John Wiley & Sons, 1990, these disclosed by Englisch *et al.*, *Angewandte Chemie, International Edition*, 1991, 30, 613, and those disclosed by Sanghvi, Y S., Chapter 15, *antisense polynucleotide agent Research and Applications*, pages 289-302, Crooke, S. T. and Lebleu, B., Ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the agents featured in the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., Eds., *antisense polynucleotide agent Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are exemplary base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Patent Nos. 3,687,808, 4,845,205; 5,130,30; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,681,941; 5,750,692; 6,015,886; 6,147,200; 6,166,197; 6,222,025; 6,235,887; 6,380,368; 6,528,640; 6,639,062; 6,617,438; 7,045,610; 7,427,672; and 7,495,088, the entire contents of each of which are hereby incorporated herein by reference.

One or more of the nucleotides of an iRNA of the invention may also include a hydroxymethyl substituted nucleotide. A "hydroxymethyl substituted nucleotide" is an acyclic 2'-3'-seco-nucleotide, also referred to as an "unlocked nucleic acid" ("UNA") modification. Representative U.S. publications that teach the preparation of UNA include, but are not limited to, US Patent No. 8,314,227; and US Patent Publication Nos. 2013/0096289; 2013/0011922; and 2011/0313020, the entire contents of each of which are hereby incorporated herein by reference.

Additional modification which may potentially stabilize the ends of antisense polynucleotide agents can include N-(acetylaminocaproyl)-4-hydroxyprolinol (Hyp-C6-NHAc), N-(caproyl-4-hydroxyprolinol (Hyp-C6), N-(acetyl-4-hydroxyprolinol (Hyp-NHAc), thymidine-2'-O-deoxythymidine (ether), N-(aminocaproyl)-4-hydroxyprolinol (Hyp-C6-

amino), 2-docosanoyl-uridine-3"- phosphate, inverted base dT(idT) and others. Disclosure of this modification can be found in US Patent Publication No. 2012/0142101.

Any of the antisense polynucleotide agents of the invention may be optionally conjugated with a GalNAc derivative ligand, as described in Section IV, below.

As described in more detail below, an agent that contains conjugations of one or more carbohydrate moieties to an antisense polynucleotide agent can optimize one or more properties of the agent. In many cases, the carbohydrate moiety will be attached to a modified subunit of the antisense polynucleotide agent. For example, the ribose sugar of one or more ribonucleotide subunits of an agent can be replaced with another moiety, *e.g.*, a non-carbohydrate (preferably cyclic) carrier to which is attached a carbohydrate ligand. A ribonucleotide subunit in which the ribose sugar of the subunit has been so replaced is referred to herein as a ribose replacement modification subunit (RRMS). A cyclic carrier may be a carbocyclic ring system, *i.e.*, all ring atoms are carbon atoms, or a heterocyclic ring system, *i.e.*, one or more ring atoms may be a heteroatom, *e.g.*, nitrogen, oxygen, sulfur. The cyclic carrier may be a monocyclic ring system, or may contain two or more rings, *e.g.* fused rings. The cyclic carrier may be a fully saturated ring system, or it may contain one or more double bonds.

The ligand may be attached to the polynucleotide via a carrier. The carriers include (i) at least one "backbone attachment point," preferably two "backbone attachment points" and (ii) at least one "tethering attachment point." A "backbone attachment point" as used herein refers to a functional group, *e.g.* a hydroxyl group, or generally, a bond available for, and that is suitable for incorporation of the carrier into the backbone, *e.g.*, the phosphate, or modified phosphate, *e.g.*, sulfur containing, backbone, of a ribonucleic acid. A "tethering attachment point" (TAP) in some embodiments refers to a constituent ring atom of the cyclic carrier, *e.g.*, a carbon atom or a heteroatom (distinct from an atom which provides a backbone attachment point), that connects a selected moiety. The moiety can be, *e.g.*, a carbohydrate, *e.g.* monosaccharide, disaccharide, trisaccharide, tetrasaccharide, oligosaccharide and polysaccharide. Optionally, the selected moiety is connected by an intervening tether to the cyclic carrier. Thus, the cyclic carrier will often include a functional group, *e.g.*, an amino group, or generally, provide a bond, that is suitable for incorporation or tethering of another chemical entity, *e.g.*, a ligand to the constituent ring.

The antisense polynucleotide agents may be conjugated to a ligand *via* a carrier, wherein the carrier can be cyclic group or acyclic group; preferably, the cyclic group is selected from pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolane, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuryl and decalin; preferably, the acyclic group is selected from serinol backbone or diethanolamine backbone.

In certain specific embodiments, the antisense polynucleotide agent for use in the methods of the invention is an agent selected from the group of agents listed in Table 3. These agents may further comprise a ligand, as described in Section IV, below.

A. Antisense Polynucleotide Agents Comprising Motifs

In certain embodiments of the invention, at least one of the contiguous nucleotides of the antisense polynucleotide agents of the invention may be a modified nucleotide. In one embodiment, the modified nucleotide comprises one or more modified sugars. In other embodiments, the modified nucleotide comprises one or more modified nucleobases. In yet other embodiments, the modified nucleotide comprises one or more modified internucleoside linkages. In some embodiments, the modifications (sugar modifications, nucleobase modifications, and/or linkage modifications) define a pattern or motif. In one embodiment, the patterns of modifications of sugar moieties, internucleoside linkages, and nucleobases are each independent of one another.

Antisense polynucleotide agents having modified oligonucleotides arranged in patterns, or motifs may, for example, confer to the agents properties such as enhanced inhibitory activity, increased binding affinity for a target nucleic acid, or resistance to degradation by *in vivo* nucleases. For example, such agents may contain at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, increased binding affinity for the target nucleic acid, and/or increased inhibitory activity. A second region of such agents may optionally serve as a substrate for the cellular endonuclease RNase H, which cleaves the RNA strand of an RNA:DNA duplex.

An exemplary antisense polynucleotide agent having modified oligonucleotides arranged in patterns, or motifs is a gapmer. In a "gapmer", an internal region or "gap" having a plurality of linked nucleotides that supports RNaseH cleavage is positioned between two external flanking regions or "wings" having a plurality of linked nucleotides that are chemically distinct from the linked nucleotides of the internal region. The gap segment generally serves as the substrate for endonuclease cleavage, while the wing segments comprise modified nucleotides.

The three regions of a gapmer motif (the 5'-wing, the gap, and the 3'-wing) form a contiguous sequence of nucleotides and may be described as "X-Y-Z", wherein "X" represents the length of the 5-wing, "Y" represents the length of the gap, and "Z" represents the length of the 3'-wing. In one embodiment, a gapmer described as "X-Y-Z" has a configuration such that the gap segment is positioned immediately adjacent to each of the 5' wing segment and the 3' wing segment. Thus, no intervening nucleotides exist between the 5' wing segment and gap segment, or the gap segment and the 3' wing segment. Any of the antisense compounds described herein can have a gapmer motif. In some embodiments, X and Z are the same, in other embodiments they are different.

In certain embodiments, the regions of a gapmer are differentiated by the types of modified nucleotides in the region. The types of modified nucleotides that may be used to differentiate the regions of a gapmer, in some embodiments, include β -D-ribonucleotides, β -D-deoxyribonucleotides, 2'-modified nucleotides, *e.g.*, 2'-modified nucleotides (*e.g.*, 2'-MOE, and 2'-O—CH₃), and bicyclic sugar modified nucleotides (*e.g.*, those having a 4'-(CH₂)_n-O-2' bridge, where n=1 or n=2).

In one embodiment, at least some of the modified nucleotides of each of the wings may differ from at least some of the modified nucleotides of the gap. For example, at least some of the modified nucleotides of each wing that are closest to the gap (the 3'-most nucleotide of the 5'-wing and the 5'-most nucleotide of the 3'-wing) differ from the modified nucleotides of the neighboring gap nucleotides, thus defining the boundary between the wings and the gap. In certain embodiments, the modified nucleotides within the gap are the same as one another. In certain embodiments, the gap includes one or more modified nucleotides that differ from the modified nucleotides of one or more other nucleotides of the gap.

The length of the 5'- wing (X) of a gapmer may be 1 to 6 nucleotides in length, *e.g.*, 2 to 6, 2 to 5, 3 to 6, 3 to 5, 1 to 5, 1 to 4, 1 to 3, 2 to 4 nucleotides in length, *e.g.*, 1, 2, 3, 4, 5, or 6 nucleotides in length.

The length of the 3'- wing (Z) of a gapmer may be 1 to 6 nucleotides in length, *e.g.*, 2 to 6, 2-5, 3 to 6, 3 to 5, 1 to 5, 1 to 4, 1 to 3, 2 to 4 nucleotides in length, *e.g.*, 1, 2, 3, 4, 5, or 6 nucleotides in length.

The length of the gap (Y) of a gapmer may be 5 to 14 nucleotides in length, *e.g.*, 5 to 13, 5 to 12, 5 to 11, 5 to 10, 5 to 9, 5 to 8, 5 to 7, 5 to 6, 6 to 14, 6 to 13, 6 to 12, 6 to 11, 6 to 10, 6 to 9, 6 to 8, 6 to 7, 7 to 14, 7 to 13, 7 to 12, 7 to 11, 7 to 10, 7 to 9, 7 to 8, 8 to 14, 8 to 13, 8 to 12, 8 to 11, 8 to 10, 8 to 9, 9 to 14, 9 to 13, 9 to 12, 9 to 11, 9 to 10, 10 to 14, 10 to 13, 10 to 12, 10 to 11, 11 to 14, 11 to 13, 11 to 12, 12 to 14, 12 to 13, or 13 to 14 nucleotides in length, *e.g.*, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 nucleotides in length.

In some embodiments of the invention X consists of 2, 3, 4, 5 or 6 nucleotides, Y consists of 7, 8, 9, 10, 11, or 12 nucleotides, and Z consists of 2, 3, 4, 5 or 6 nucleotides. Such gapmers include (X-Y-Z) 2-7-2, 2-7-3, 2-7-4, 2-7-5, 2-7-6, 3-7-2, 3-7-3, 3-7-4, 3-7-5, 3-7-6, 4-7-3, 4-7-4, 4-7-5, 4-7-6, 5-7-3, 5-7-4, 5-7-5, 5-7-6, 6-7-3, 6-7-4, 6-7-5, 6-7-6, 3-7-3, 3-7-4, 3-7-5, 3-7-6, 4-7-3, 4-7-4, 4-7-5, 4-7-6, 5-7-3, 5-7-4, 5-7-5, 5-7-6, 6-7-3, 6-7-4, 6-7-5, 6-7-6, 2-8-2, 2-8-3, 2-8-4, 2-8-5, 2-8-6, 3-8-2, 3-8-3, 3-8-4, 3-8-5, 3-8-6, 4-8-3, 4-8-4, 4-8-5, 4-8-6, 5-8-3, 5-8-4, 5-8-5, 5-8-6, 6-8-3, 6-8-4, 6-8-5, 6-8-6, 2-9-2, 2-9-3, 2-9-4, 2-9-5, 2-9-6, 3-9-2, 3-9-3, 3-9-4, 3-9-5, 3-9-6, 4-9-3, 4-9-4, 4-9-5, 4-9-6, 5-9-3, 5-9-4, 5-9-5, 5-9-6, 6-9-3, 6-9-4, 6-9-5, 6-9-6, 2-10-2, 2-10-3, 2-10-4, 2-10-5, 2-10-6, 3-10-2, 3-10-3, 3-10-4, 3-10-5, 3-10-6, 4-10-3, 4-10-4, 4-10-5, 4-10-6, 5-10-3, 5-10-4, 5-10-5, 5-10-6, 6-10-3, 6-10-4, 6-10-5, 6-10-6, 2-11-2, 2-11-3, 2-11-4, 2-11-5, 2-11-6, 3-11-2, 3-11-3, 3-11-4, 3-11-5, 3-11-6, 4-11-

3, 4-11-4, 4-11-5, 4-11-6, 5-11-3, 5-11-4, 5-11-5, 5-11-6, 6-11-3, 6-11-4, 6-11-5, 6-11-6, 2-12-2, 2-12-3, 2-12-4, 2-12-5, 2-12-6, 3-12-2, 3-12-3, 3-12-4, 3-12-5, 3-12-6, 4-12-3, 4-12-4, 4-12-5, 4-12-6, 5-12-3, 5-12-4, 5-12-5, 5-12-6, 6-12-3, 6-12-4, 6-12-5, or 6-12-6.

In some embodiments of the invention, antisense polynucleotide agents targeting C5 include a 5-10-5 gapmer motif. In other embodiments of the invention, antisense polynucleotide agents targeting C5 include a 4-10-4 gapmer motif. In another embodiment of the invention, antisense polynucleotide agents targeting C5 include a 3-10-3 gapmer motif. In yet other embodiments of the invention, antisense polynucleotide agents targeting C5 include a 2-10-2 gapmer motif.

The 5'-wing and/or 3'-wing of a gapmer may independently include 1-6 modified nucleotides, *e.g.*, 1, 2, 3, 4, 5, or 6 modified nucleotides.

In some embodiment, the 5'-wing of a gapmer includes at least one modified nucleotide. In one embodiment, the 5'-wing of a gapmer comprises at least two modified nucleotides. In another embodiment, the 5'-wing of a gapmer comprises at least three modified nucleotides. In yet another embodiment, the 5'-wing of a gapmer comprises at least four modified nucleotides. In another embodiment, the 5'-wing of a gapmer comprises at least five modified nucleotides. In certain embodiments, each nucleotide of the 5'-wing of a gapmer is a modified nucleotide.

In some embodiments, the 3'-wing of a gapmer includes at least one modified nucleotide. In one embodiment, the 3'-wing of a gapmer comprises at least two modified nucleotides. In another embodiment, the 3'-wing of a gapmer comprises at least three modified nucleotides. In yet another embodiment, the 3'-wing of a gapmer comprises at least four modified nucleotides. In another embodiment, the 3'-wing of a gapmer comprises at least five modified nucleotides. In certain embodiments, each nucleotide of the 3'-wing of a gapmer is a modified nucleotide.

In certain embodiments, the regions of a gapmer are differentiated by the types of sugar moieties of the nucleotides. In one embodiment, the nucleotides of each distinct region comprise uniform sugar moieties. In other embodiments, the nucleotides of each distinct region comprise different sugar moieties. In certain embodiments, the sugar nucleotide modification motifs of the two wings are the same as one another. In certain embodiments, the sugar nucleotide modification motifs of the 5'-wing differs from the sugar nucleotide modification motif of the 3'-wing.

The 5'-wing of a gapmer may include 1-6 modified nucleotides, *e.g.*, 1, 2, 3, 4, 5, or 6 modified nucleotides.

In one embodiment, at least one modified nucleotide of the 5'-wing of a gapmer is a bicyclic nucleotide, such as a constrained ethyl nucleotide, or an LNA. In another embodiment, the 5'-wing of a gapmer includes 2, 3, 4, or 5 bicyclic nucleotides. In some embodiments, each nucleotide of the 5'-wing of a gapmer is a bicyclic nucleotide.

In one embodiment, the 5'-wing of a gapmer includes at least 1, 2, 3, 4, or 5 constrained ethyl nucleotides. In some embodiments, each nucleotide of the 5'- wing of a gapmer is a constrained ethyl nucleotide.

In one embodiment, the 5'-wing of a gapmer comprises at least one LNA nucleotide. In another embodiment, the 5'-wing of a gapmer includes 2, 3, 4, or 5 LNA nucleotides. In other embodiments, each nucleotide of the 5'- wing of a gapmer is an LNA nucleotide.

In certain embodiments, at least one modified nucleotide of the 5'- wing of a gapmer is a non-bicyclic modified nucleotide, *e.g.*, a 2'-substituted nucleotide. A "2'-substituted nucleotide" is a nucleotide comprising a modification at the 2'-position which is other than H or OH, such as a 2'-OMe nucleotide, or a 2'-MOE nucleotide. In one embodiment, the 5'- wing of a gapmer comprises 2, 3, 4, or 5 2'-substituted nucleotides. In one embodiment, each nucleotide of the 5'-wing of a gapmer is a 2'-substituted nucleotide.

In one embodiment, the 5'- wing of a gapmer comprises at least one 2'-OMe nucleotide. In one embodiment, the 5'- wing of a gapmer comprises at least 2, 3, 4, or 5 2'-OMe nucleotides. In one embodiment, each of the nucleotides of the 5'- wing of a gapmer comprises a 2'-OMe nucleotide.

In one embodiment, the 5'- wing of a gapmer comprises at least one 2'- MOE nucleotide. In one embodiment, the 5'- wing of a gapmer comprises at least 2, 3, 4, or 5 2'-MOE nucleotides. In one embodiment, each of the nucleotides of the 5'- wing of a gapmer comprises a 2'- MOE nucleotide.

In certain embodiments, the 5'- wing of a gapmer comprises at least one 2'-deoxynucleotide. In certain embodiments, each nucleotide of the 5'- wing of a gapmer is a 2'-deoxynucleotide. In a certain embodiments, the 5'- wing of a gapmer comprises at least one ribonucleotide. In certain embodiments, each nucleotide of the 5'- wing of a gapmer is a ribonucleotide.

The 3'-wing of a gapmer may include 1-6 modified nucleotides, *e.g.*, 1, 2, 3, 4, 5, or 6 modified nucleotides.

In one embodiment, at least one modified nucleotide of the 3'-wing of a gapmer is a bicyclic nucleotide, such as a constrained ethyl nucleotide, or an LNA. In another embodiment, the 3'-wing of a gapmer includes 2, 3, 4, or 5 bicyclic nucleotides. In some embodiments, each nucleotide of the 3'-wing of a gapmer is a bicyclic nucleotide.

In one embodiment, the 3'-wing of a gapmer includes at least one constrained ethyl nucleotide. In another embodiment, the 3'-wing of a gapmer includes 2, 3, 4, or 5 constrained ethyl nucleotides. In some embodiments, each nucleotide of the 3'-wing of a gapmer is a constrained ethyl nucleotide.

In one embodiment, the 3'-wing of a gapmer comprises at least one LNA nucleotide. In another embodiment, the 3'-wing of a gapmer includes 2, 3, 4, or 5 LNA nucleotides. In other embodiments, each nucleotide of the 3'-wing of a gapmer is an LNA nucleotide.

In certain embodiments, at least one modified nucleotide of the 3'-wing of a gapmer is a non-bicyclic modified nucleotide, *e.g.*, a 2'-substituted nucleotide. In one embodiment, the 3'-wing of a gapmer comprises 2, 3, 4, or 5 2'-substituted nucleotides. In one embodiment, each nucleotide of the 3'-wing of a gapmer is a 2'-substituted nucleotide.

In one embodiment, the 3'-wing of a gapmer comprises at least one 2'-OMe nucleotide. In one embodiment, the 3'-wing of a gapmer comprises at least 2, 3, 4, or 5 2'-OMe nucleotides. In one embodiment, each of the nucleotides of the 3'-wing of a gapmer comprises a 2'-OMe nucleotide.

In one embodiment, the 3'-wing of a gapmer comprises at least one 2'-MOE nucleotide. In one embodiment, the 3'-wing of a gapmer comprises at least 2, 3, 4, or 5 2'-MOE nucleotides. In one embodiment, each of the nucleotides of the 3'-wing of a gapmer comprises a 2'-MOE nucleotide.

In certain embodiments, the 3'-wing of a gapmer comprises at least one 2'-deoxynucleotide. In certain embodiments, each nucleotide of the 3'-wing of a gapmer is a 2'-deoxynucleotide. In certain embodiments, the 3'-wing of a gapmer comprises at least one ribonucleotide. In certain embodiments, each nucleotide of the 3'-wing of a gapmer is a ribonucleotide.

The gap of a gapmer may include 5-14 modified nucleotides, *e.g.*, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 modified nucleotides.

In one embodiment, the gap of a gapmer comprises at least one 5-methylcytosine. In one embodiment, the gap of a gapmer comprises at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 5-methylcytosines. In one embodiment, all of the nucleotides of the the gap of a gapmer are 5-methylcytosines.

In one embodiment, the gap of a gapmer comprises at least one 2'-deoxynucleotide. In one embodiment, the gap of a gapmer comprises at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 2'-deoxynucleotides. In one embodiment, all of the nucleotides of the the gap of a gapmer are 2'-deoxynucleotides.

A gapmer may include one or more modified internucleotide linkages. In some embodiments, a gapmer includes one or more phosphodiester internucleotide linkages. In other embodiments, a gapmer includes one or more phosphorothioate internucleotide linkages.

In one embodiment, each nucleotide of a 5'-wing of a gapmer are linked *via* a phosphorothioate internucleotide linkage. In another embodiment, each nucleotide of a 3'-wing of a gapmer are linked *via* a phosphorothioate internucleotide linkage. In yet another embodiment, each nucleotide of a gap segment of a gapmer is linked *via* a phosphorothioate internucleotide linkage. In one embodiment, all of the nucleotides in a gapmer are linked *via* phosphorothioate internucleotide linkages.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising five nucleotides and a 3'-wing segment comprising 5 nucleotides.

In another embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising four nucleotides and a 3'-wing segment comprising four nucleotides.

In another embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising three nucleotides and a 3'-wing segment comprising three nucleotides.

In another embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising two nucleotides and a 3'-wing segment comprising two nucleotides.

In one embodiment, each nucleotide of a 5-wing flanking a gap segment of 10 2'-deoxyribonucleotides comprises a modified nucleotide. In another embodiment, each nucleotide of a 3-wing flanking a gap segment of 10 2'-deoxyribonucleotides comprises a modified nucleotide. In one embodiment, each of the modified 5'-wing nucleotides and each of the modified 3'-wing nucleotides comprise a 2'-sugar modification. In one embodiment, the 2'-sugar modification is a 2'-OMe modification. In another embodiment, the 2'-sugar modification is a 2'-MOE modification. In one embodiment, each of the modified 5'-wing nucleotides and each of the modified 3'-wing nucleotides comprise a bicyclic nucleotide. In one embodiment, the bicyclic nucleotide is a constrained ethyl nucleotide. In another embodiment, the bicyclic nucleotide is an LNA nucleotide. In one embodiment, each cytosine in an antisense polynucleotide agent targeting a C5 gene is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising five nucleotides comprising a 2'OMe modification and a 3'-wing segment comprising five nucleotides comprising a 2'OMe modification, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine. In one embodiment, the agent further comprises a ligand.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising five nucleotides comprising a 2'MOE modification and a 3'-wing segment comprising five nucleotides comprising a 2'MOE modification,

wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine. In one embodiment, the agent further comprises a ligand.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising five constrained ethyl nucleotides and a 3'-wing segment comprising five constrained ethyl nucleotides, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising five LNA nucleotides and a 3'-wing segment comprising five LNA nucleotides, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising four nucleotides comprising a 2'OMe modification and a 3'-wing segment comprising four nucleotides comprising a 2'OMe modification, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising four nucleotides comprising a 2'MOE modification and a 3'-wing segment comprising four nucleotides comprising a 2'MOE modification, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising four constrained ethyl nucleotides and a 3'-wing segment comprising four constrained ethyl nucleotides, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising four LNA nucleotides and a 3'-wing segment comprising four LNA nucleotides, wherein each internucleotide linkage of the agent is a

phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising three nucleotides comprising a 2'OMe modification and a 3'-wing segment comprising three nucleotides comprising a 2'OMe modification, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising three nucleotides comprising a 2'MOE modification and a 3'-wing segment comprising three nucleotides comprising a 2'MOE modification, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising three constrained ethyl nucleotides and a 3'-wing segment comprising three constrained ethyl nucleotides, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising three LNA nucleotides and a 3'-wing segment comprising three LNA nucleotides, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising two nucleotides comprising a 2'OMe modification and a 3'-wing segment comprising two nucleotides comprising a 2'OMe modification, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising two nucleotides comprising a 2'MOE modification and a 3'-wing segment comprising two nucleotides comprising a 2'MOE modification, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising two constrained ethyl nucleotides and a 3'-wing segment comprising two constrained ethyl nucleotides, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

In one embodiment, an antisense polynucleotide agent targeting a C5 gene comprises a gap segment of ten 2'-deoxyribonucleotides positioned immediately adjacent to and between a 5'-wing segment comprising two LNA nucleotides and a 3'-wing segment comprising two LNA nucleotides, wherein each internucleotide linkage of the agent is a phosphorothioate linkage. In one embodiment, each cytosine of the agent is a 5-methylcytosine.

Further gapmer designs suitable for use in the agents, compositions, and methods of the invention are disclosed in, for example, U.S. Patent Nos. 7,687,617 and 8,580,756; U.S. Patent Publication Nos. 20060128646, 20090209748, 20140128586, 20140128591, 20100210712, and 20080015162A1; and International Publication No. WO 2013/159108, the entire content of each of which are incorporated herein by reference.

IV. Antisense Polynucleotide Agents Conjugated to Ligands

Another modification of the polynucleotide agents of the invention involves chemically linking to the agent one or more ligands, moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the antisense polynucleotide agent. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 1989, 86: 6553-6556), cholic acid (Manoharan *et al.*, *Biorg. Med. Chem. Lett.*, 1994, 4:1053-1060), a thioether, *e.g.*, beryl-S-tritylthiol (Manoharan *et al.*, *Ann. N.Y. Acad. Sci.*, 1992, 660:306-309; Manoharan *et al.*, *Biorg. Med. Chem. Lett.*, 1993, 3:2765-2770), a thiocholesterol (Oberhauser *et al.*, *Nucl. Acids Res.*, 1992, 20:533-538), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, *EMBO J*, 1991, 10:1111-1118; Kabanov *et al.*, *FEBS Lett.*, 1990, 259:327-330; Svinarchuk *et al.*, *Biochimie*, 1993, 75:49-54), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-phosphonate (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36:3651-3654; Shea *et al.*, *Nucl. Acids Res.*, 1990, 18:3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, *Nucleosides & Nucleotides*, 1995, 14:969-973), or adamantane acetic acid (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36:3651-3654), a palmityl moiety (Mishra *et al.*, *Biochim. Biophys. Acta*, 1995, 1264:229-237), or an octadecylamine or hexylamino-carbonyloxycholesterol moiety (Crooke *et al.*, *J. Pharmacol. Exp. Ther.*, 1996, 277:923-937).

In one embodiment, a ligand alters the distribution, targeting or lifetime of an antisense polynucleotide agent into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, *e.g.*, molecule, cell or cell type, compartment, *e.g.*, a cellular or organ compartment, tissue, organ or region of the body, as, *e.g.*, compared to a species absent such a ligand. Preferred ligands will not take part in hybridization of an antisense polynucleotide agent to the targeted mRNA.

Ligands can include a naturally occurring substance, such as a protein (*e.g.*, human serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (*e.g.*, a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin, N-acetylgalactosamine, or hyaluronic acid); or a lipid. The ligand can also be a recombinant or synthetic molecule, such as a synthetic polymer, *e.g.*, a synthetic polyamino acid. Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolid) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

Ligands can also include targeting groups, *e.g.*, a cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-galucoseamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, vitamin A, biotin, or an RGD peptide or RGD peptide mimetic.

Other examples of ligands include dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), lipophilic molecules, *e.g.*, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine) and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, [MPEG]₂, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin),

transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu³⁺ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

Ligands can be proteins, *e.g.*, glycoproteins, or peptides, *e.g.*, molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a hepatic cell. Ligands can also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF- κ B.

The ligand can be a substance, *e.g.*, a drug, which can increase the uptake of the antisense polynucleotide agent into the cell, for example, by disrupting the cell's cytoskeleton, *e.g.*, by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

In some embodiments, a ligand attached to an antisense polynucleotide agent as described herein acts as a pharmacokinetic modulator (PK modulator). PK modulators include lipophiles, bile acids, steroids, phospholipid analogues, peptides, protein binding agents, PEG, vitamins *etc.* Exemplary PK modulators include, but are not limited to, cholesterol, fatty acids, cholic acid, lithocholic acid, dialkylglycerides, diacylglyceride, phospholipids, sphingolipids, naproxen, ibuprofen, vitamin E, biotin *etc.* Oligonucleotides that comprise a number of phosphorothioate linkages are also known to bind to serum protein, thus short oligonucleotides, *e.g.*, oligonucleotides of about 5 bases, 10 bases, 15 bases or 20 bases, comprising multiple of phosphorothioate linkages in the backbone are also amenable to the present invention as ligands (*e.g.* as PK modulating ligands). In addition, aptamers that bind serum components (*e.g.* serum proteins) are also suitable for use as PK modulating ligands in the embodiments described herein.

Ligand-conjugated polynucleotides of the invention may be synthesized by the use of a polynucleotide that bears a pendant reactive functionality, such as that derived from the attachment of a linking molecule onto the oligonucleotide (described below). This reactive polynucleotide may be reacted directly with commercially-available ligands, ligands that are synthesized bearing any of a variety of protecting groups, or ligands that have a linking moiety attached thereto.

The polynucleotides used in the conjugates of the present invention may be conveniently and routinely made through the well-known technique of solid-phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may

additionally or alternatively be employed. It is also known to use similar techniques to prepare other polynucleotides, such as the phosphorothioates and alkylated derivatives.

In the ligand-conjugated polynucleotides and ligand-molecule bearing sequence-specific linked nucleosides of the present invention, the polynucleotides and polynucleosides may be assembled on a suitable DNA synthesizer utilizing standard nucleotide or nucleoside precursors, or nucleotide or nucleoside conjugate precursors that already bear the linking moiety, ligand-nucleotide or nucleoside-conjugate precursors that already bear the ligand molecule, or non-nucleoside ligand-bearing building blocks.

When using nucleotide-conjugate precursors that already bear a linking moiety, the synthesis of the sequence-specific linked nucleosides is typically completed, and the ligand molecule is then reacted with the linking moiety to form the ligand-conjugated oligonucleotide. In some embodiments, the polynucleotides or linked nucleosides of the present invention are synthesized by an automated synthesizer using phosphoramidites derived from ligand-nucleoside conjugates in addition to the standard phosphoramidites and non-standard phosphoramidites that are commercially available and routinely used in oligonucleotide synthesis.

A. Lipid Conjugates

In one embodiment, the ligand or conjugate is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, *e.g.*, human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, *e.g.*, a non-kidney target tissue of the body. For example, the target tissue can be the liver, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, naproxen or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, *e.g.*, HSA.

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

In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

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

In another aspect, the ligand is a moiety, *e.g.*, a vitamin, which is taken up by a target cell, *e.g.*, a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, *e.g.*, of the malignant or non-malignant type, *e.g.*, cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include are B vitamin, *e.g.*, folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by target cells such as liver cells. Also included are HSA and low density lipoprotein (LDL).

B. Cell Permeation Agents

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

The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to antisense polynucleotide agents can affect pharmacokinetic distribution of the agent, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, *e.g.*, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (*e.g.*, consisting primarily of Tyr, Trp or Phe). The peptide moiety can be a dendrimer peptide, constrained peptide or crosslinked peptide. In another alternative, the peptide moiety can include a hydrophobic membrane translocation sequence (MTS). An exemplary hydrophobic MTS-containing peptide is RFGF having the amino acid sequence AAVALLPAVLLALLAP (SEQ ID NO: 9). An RFGF analogue (*e.g.*, amino acid sequence AALLPVLLAAP (SEQ ID NO: 10) containing a hydrophobic MTS can also be a targeting moiety. The peptide moiety can be a "delivery" peptide, which can carry large polar molecules including peptides, oligonucleotides, and protein across cell membranes. For example, sequences from the HIV Tat protein (GRKKRRQRRRPPQ (SEQ ID NO: 11) and the *Drosophila* Antennapedia protein (RQIKIWFQNRRMKWKK (SEQ ID NO: 12) have been found to be capable of functioning as delivery peptides. A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam *et al.*, *Nature*, 354:82-84, 1991). Examples of a peptide or peptidomimetic tethered to an antisense polynucleotide agent *via* an incorporated monomer unit for cell targeting purposes is an arginine-glycine-aspartic acid (RGD)-peptide,

or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 40 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.

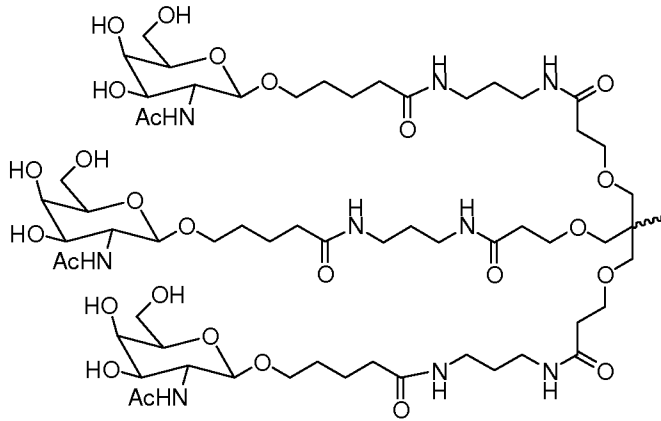
An RGD peptide for use in the compositions and methods of the invention may be linear or cyclic, and may be modified, *e.g.*, glycosylated or methylated, to facilitate targeting to a specific tissue(s). RGD-containing peptides and peptidomimetics may include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the integrin ligand. Preferred conjugates of this ligand target PECAM-1 or VEGF.

A “cell permeation peptide” is capable of permeating a cell, *e.g.*, a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an α -helical linear peptide (*e.g.*, LL-37 or Ceropin P1), a disulfide bond-containing peptide (*e.g.*, α -defensin, β -defensin or bactenecin), or a peptide containing only one or two dominating amino acids (*e.g.*, PR-39 or indolicidin). A cell permeation peptide can also include a nuclear localization signal (NLS). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni *et al.*, Nucl. Acids Res. 31:2717-2724, 2003).

C. Carbohydrate Conjugates

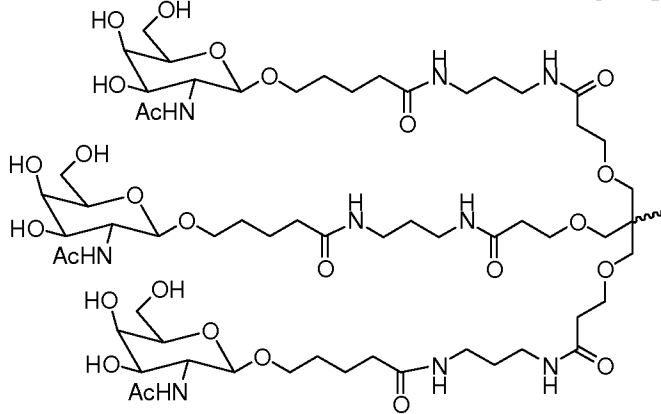
In some embodiments of the compositions and methods of the invention, an antisense polynucleotide agent further comprises a carbohydrate. The carbohydrate conjugated agents are advantageous for the *in vivo* delivery of nucleic acids, as well as compositions suitable for *in vivo* therapeutic use, as described herein (see, *e.g.*, Prakash, *et al.* (2014) *Nuc Acid Res* doi 10.1093/nar/gku531). As used herein, “carbohydrate” refers to a compound which is either a carbohydrate *per se* made up of one or more monosaccharide units having at least 6 carbon atoms (which can be linear, branched or cyclic) with an oxygen, nitrogen or sulfur atom bonded to each carbon atom; or a compound having as a part thereof a carbohydrate moiety made up of one or more monosaccharide units each having at least six carbon atoms (which can be linear, branched or cyclic), with an oxygen, nitrogen or sulfur atom bonded to each carbon atom. Representative carbohydrates include the sugars (mono-, di-, tri- and oligosaccharides containing from about 4, 5, 6, 7, 8, or 9 monosaccharide units), and polysaccharides such as starches, glycogen, cellulose and polysaccharide gums. Specific monosaccharides include C5 and above (*e.g.*, C5, C6, C7, or C8) sugars; di- and trisaccharides include sugars having two or three monosaccharide units (*e.g.*, C5, C6, C7, or C8).

In one embodiment, a carbohydrate conjugate for use in the compositions and methods of the invention is a monosaccharide. In one embodiment, the monosaccharide is an N-acetylgalactosamine, such as

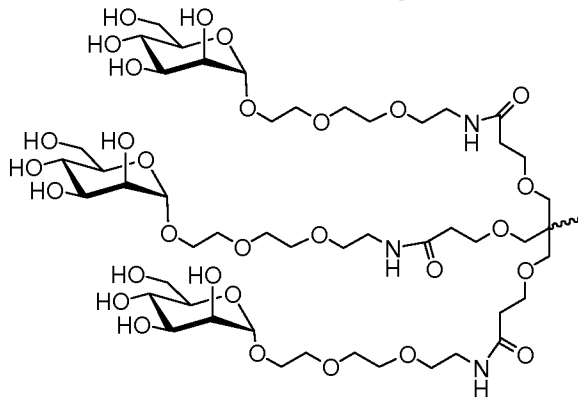


Formula II.

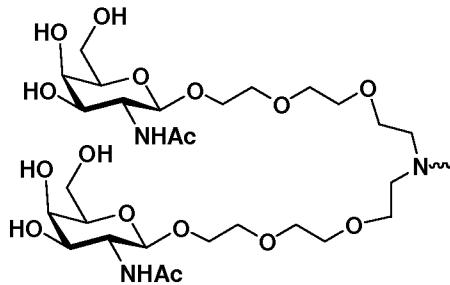
In another embodiment, a carbohydrate conjugate for use in the compositions and methods of the invention is selected from the group consisting of:



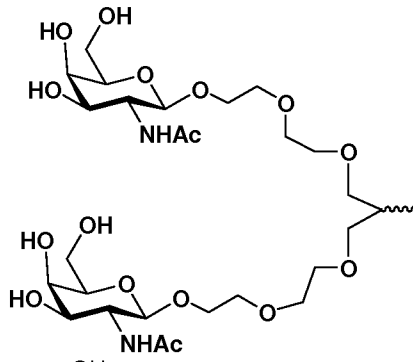
Formula II,



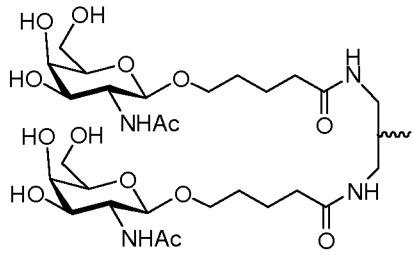
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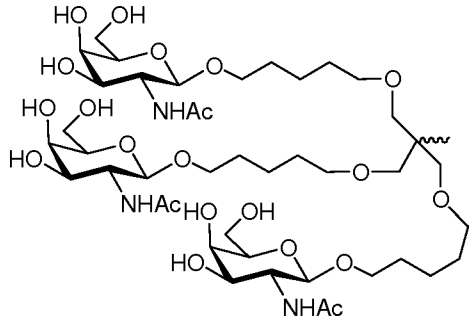
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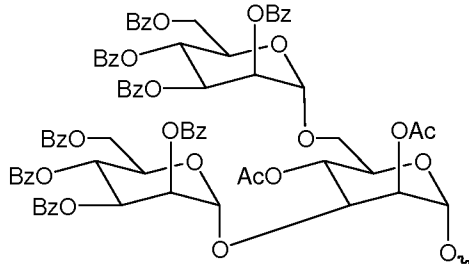
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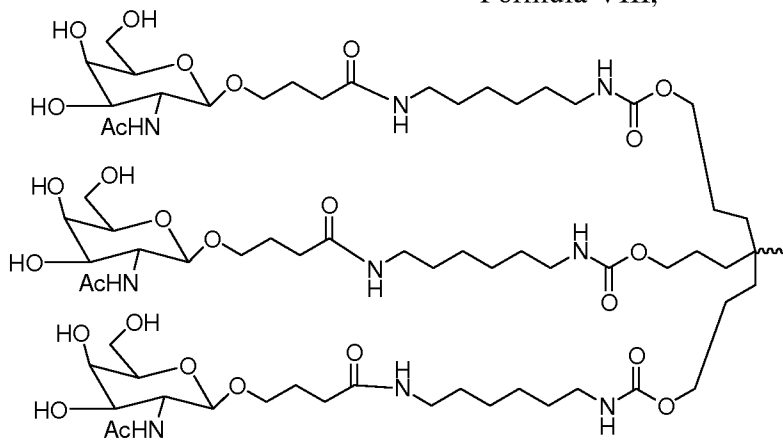
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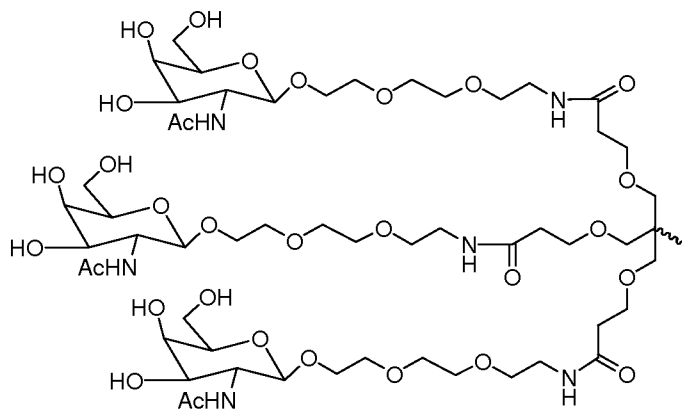
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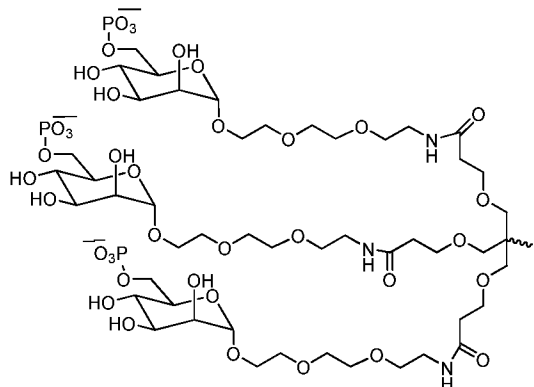
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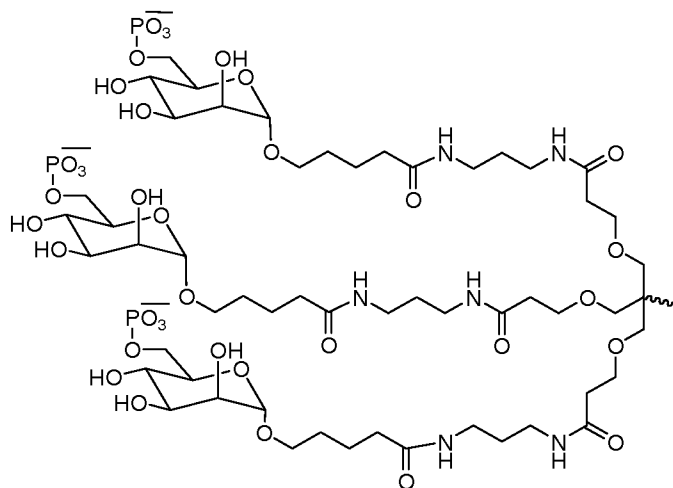
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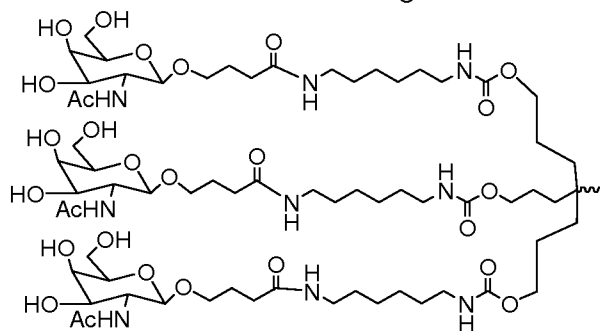
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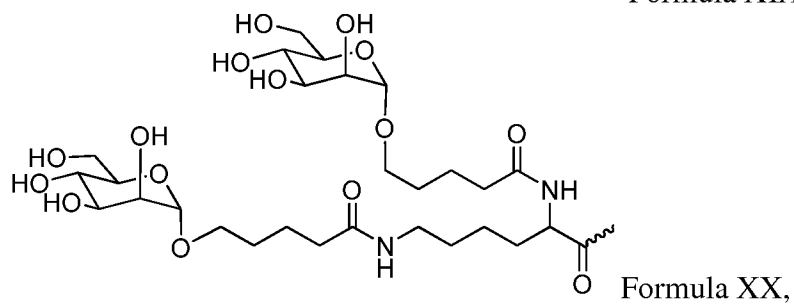
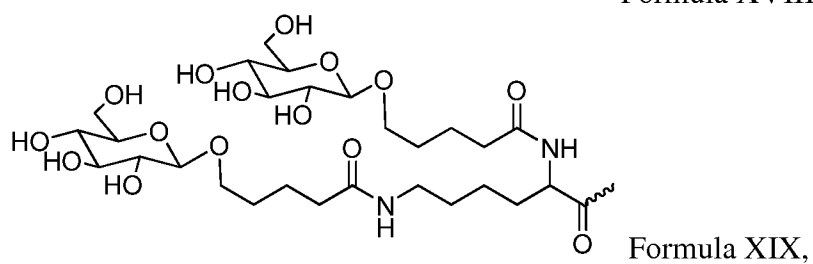
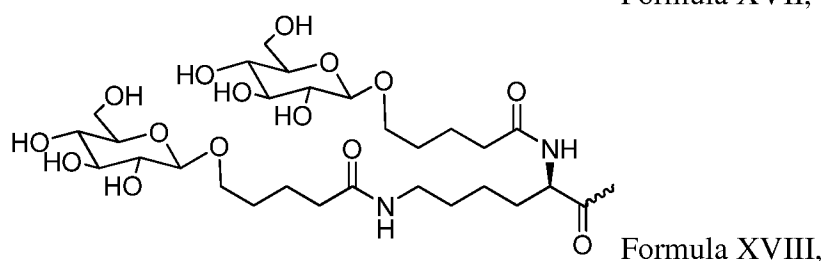
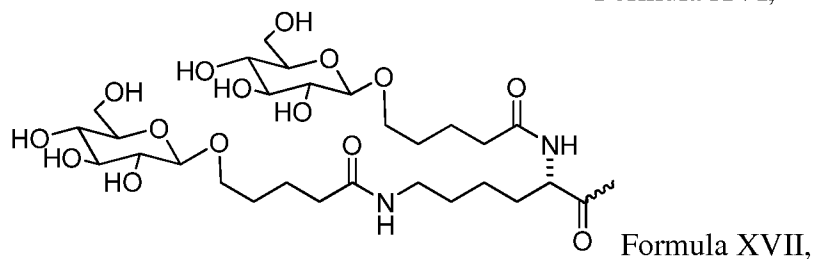
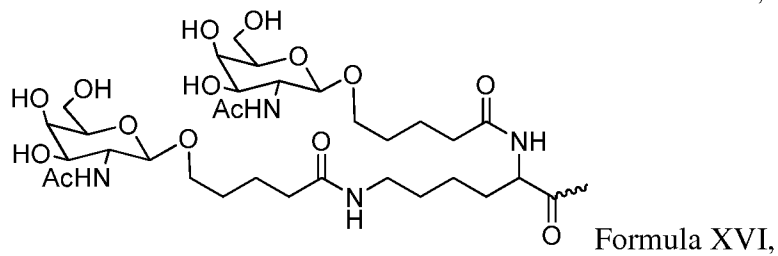
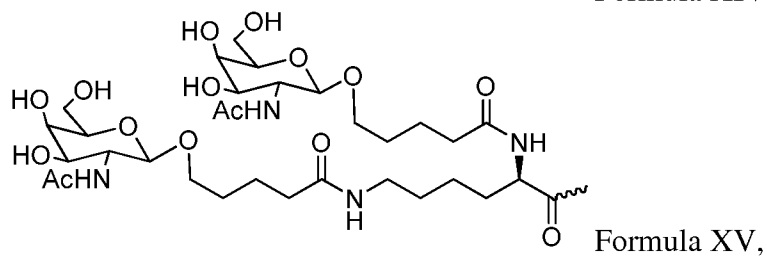
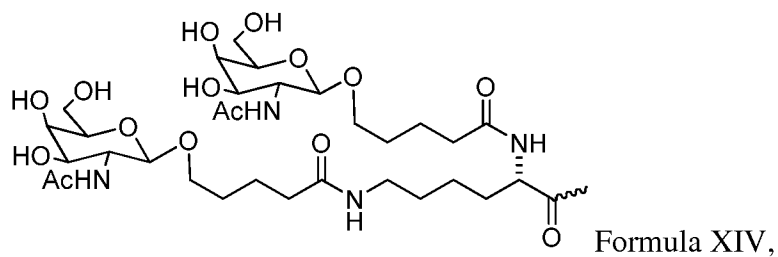
Formula XI,

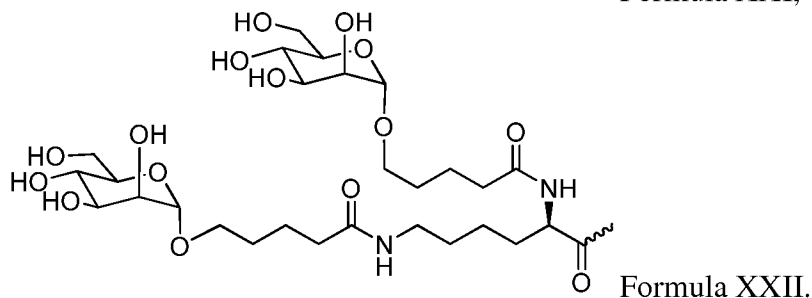
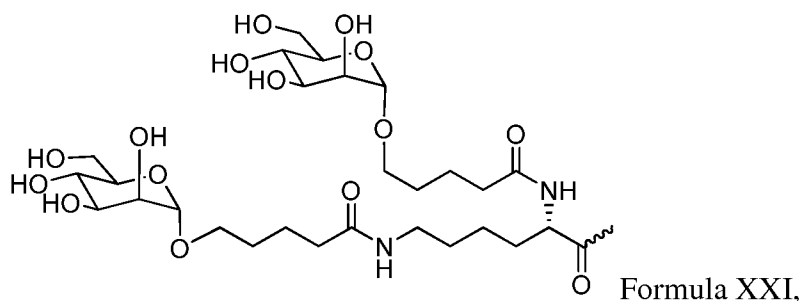


Formula XII,

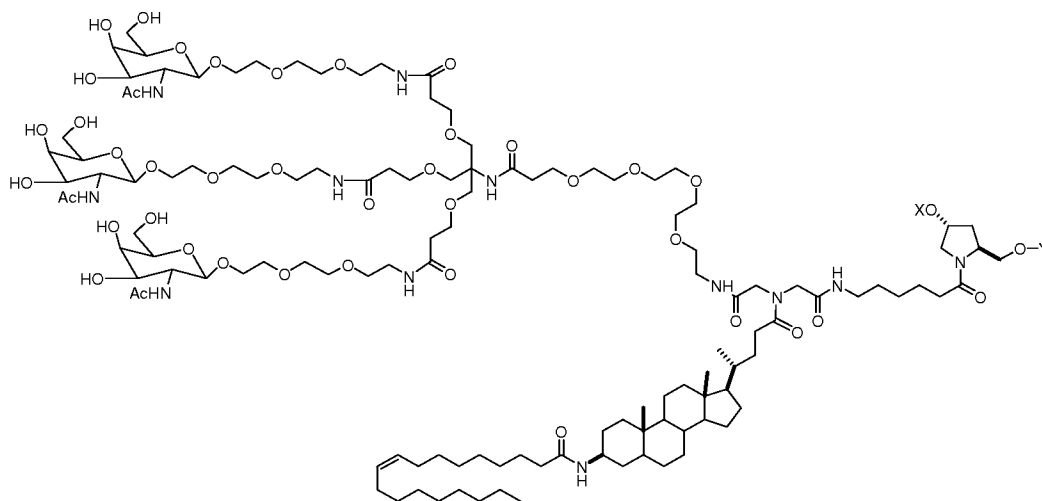


Formula XIII,





Another representative carbohydrate conjugate for use in the embodiments described herein includes, but is not limited to



(Formula XXIII),

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

In some embodiments, the carbohydrate conjugate further comprises one or more additional ligands as described above, such as, but not limited to, a PK modulator and/or a cell permeation peptide.

D. Linkers

In some embodiments, the conjugate or ligand described herein can be attached to an antisense polynucleotide agent with various linkers that can be cleavable or non-cleavable.

The term "linker" or "linking group" means an organic moiety that connects two parts of a compound, *e.g.*, covalently attaches two parts of a compound. Linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NR₈, C(O), C(O)NH, SO, SO₂, SO₂NH or a chain of atoms, such as, but not limited to, substituted or unsubstituted

alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, arylalkyl, arylalkenyl, arylalkynyl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, heterocyclalkyl, heterocyclalkenyl, heterocyclalkynyl, aryl, heteroaryl, heterocycl, cycloalkyl, cycloalkenyl, alkylarylalkyl, alkylarylalkenyl, alkylarylalkynyl, alkenylarylalkyl, alkenylarylalkenyl, alkenylarylalkynyl, alkynylarylalkyl, alkynylarylalkenyl, alkynylarylalkynyl, alkylheteroarylalkyl, alkylheteroarylalkenyl, alkylheteroarylalkynyl, alkenylheteroarylalkyl, alkenylheteroarylalkenyl, alkenylheteroarylalkynyl, alkynylheteroarylalkyl, alkynylheteroarylalkenyl, alkynylheteroarylalkynyl, alkylheterocyclalkyl, alkylheterocyclalkenyl, alkylheterocyclalkynyl, alkenylheterocyclalkyl, alkenylheterocyclalkenyl, alkenylheterocyclalkynyl, alkynylheterocyclalkyl, alkynylheterocyclalkenyl, alkynylheterocyclalkynyl, alkylaryl, alkenylaryl, alkynylaryl, alkylheteroaryl, alkenylheteroaryl, alkynylheteroaryl, which one or more methylenes can be interrupted or terminated by O, S, S(O), SO₂, N(R₈), C(O), substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocyclic; where R₈ is hydrogen, acyl, aliphatic or substituted aliphatic. In one embodiment, the linker is between about 1-24 atoms, 2-24, 3-24, 4-24, 5-24, 6-24, 6-18, 7-18, 8-18 atoms, 7-17, 8-17, 6-16, 7-16, or 8-16 atoms.

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

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

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

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

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

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

In general, the suitability of a candidate cleavable linking group can be evaluated by testing the ability of a degradative agent (or condition) to cleave the candidate linking group. It will also be desirable to also test the candidate cleavable linking group for the ability to resist cleavage in the blood or when in contact with other non-target tissue. Thus, one can determine the relative susceptibility to cleavage between a first and a second condition, where the first is selected to be indicative of cleavage in a target cell and the second is selected to be indicative of cleavage in other tissues or biological fluids, *e.g.*, blood or serum. The evaluations can be carried out in cell free systems, in cells, in cell culture, in organ or tissue culture, or in whole animals. It can be useful to make initial evaluations in cell-free or culture conditions and to confirm by further evaluations in whole animals. In preferred embodiments, useful candidate compounds are cleaved at least about 2, 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, or about 100 times faster in the cell (or under *in vitro* conditions selected to mimic intracellular conditions) as compared to blood or serum (or under *in vitro* conditions selected to mimic extracellular conditions).

i. Redox cleavable linking groups

In one embodiment, a cleavable linking group is a redox cleavable linking group that is cleaved upon reduction or oxidation. An example of reductively cleavable linking group is a disulphide linking group (-S-S-). To determine if a candidate cleavable linking group is a suitable "reductively cleavable linking group," or for example is suitable for use with a particular antisense polynucleotide agent moiety and particular targeting agent one can look to methods described herein. For example, a candidate can be evaluated by incubation with dithiothreitol (DTT), or other reducing agent using reagents known in the art, which mimic the rate of cleavage which would be observed in a cell, *e.g.*, a target cell. The candidates can also be evaluated under conditions which are selected to mimic blood or serum conditions. In one, candidate compounds are cleaved by at most about 10% in the blood. In other embodiments, useful candidate compounds are degraded at least about 2, 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, or about 100 times faster in the cell (or under *in vitro* conditions selected

to mimic intracellular conditions) as compared to blood (or under *in vitro* conditions selected to mimic extracellular conditions). The rate of cleavage of candidate compounds can be determined using standard enzyme kinetics assays under conditions chosen to mimic intracellular media and compared to conditions chosen to mimic extracellular media.

ii. Phosphate-based cleavable linking groups

In another embodiment, a cleavable linker comprises a phosphate-based cleavable linking group. A phosphate-based cleavable linking group is cleaved by agents that degrade or hydrolyze the phosphate group. An example of an agent that cleaves phosphate groups in cells are enzymes such as phosphatases in cells. Examples of phosphate-based linking groups are -O-P(O)(ORk)-O-, -O-P(S)(ORk)-O-, -O-P(S)(SRk)-O-, -S-P(O)(ORk)-O-, -O-P(O)(ORk)-S-, -S-P(O)(ORk)-S-, -O-P(S)(ORk)-S-, -S-P(S)(ORk)-O-, -O-P(O)(Rk)-O-, -O-P(S)(Rk)-O-, -S-P(O)(Rk)-O-, -S-P(S)(Rk)-O-, -S-P(O)(Rk)-S-, -O-P(S)(Rk)-S-. Preferred embodiments are -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O-, -S-P(S)(H)-O-, -S-P(O)(H)-S-, -O-P(S)(H)-S-. A preferred embodiment is -O-P(O)(OH)-O-. These candidates can be evaluated using methods analogous to those described above.

iii. Acid cleavable linking groups

In another embodiment, a cleavable linker comprises an acid cleavable linking group. An acid cleavable linking group is a linking group that is cleaved under acidic conditions. In preferred embodiments acid cleavable linking groups are cleaved in an acidic environment with a pH of about 6.5 or lower (*e.g.*, about 6.0, 5.75, 5.5, 5.25, 5.0, or lower), or by agents such as enzymes that can act as a general acid. In a cell, specific low pH organelles, such as endosomes and lysosomes can provide a cleaving environment for acid cleavable linking groups. Examples of acid cleavable linking groups include but are not limited to hydrazones, esters, and esters of amino acids. Acid cleavable groups can have the general formula -C=NN-, C(O)O, or -OC(O). A preferred embodiment is when the carbon attached to the oxygen of the ester (the alkoxy group) is an aryl group, substituted alkyl group, or tertiary alkyl group such as dimethyl pentyl or t-butyl. These candidates can be evaluated using methods analogous to those described above.

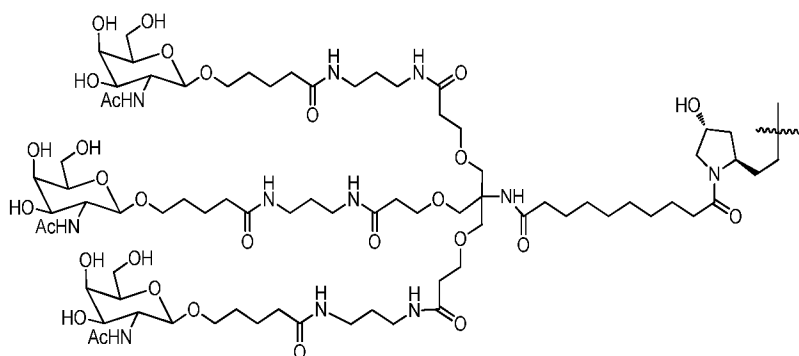
iv. Ester-based linking groups

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

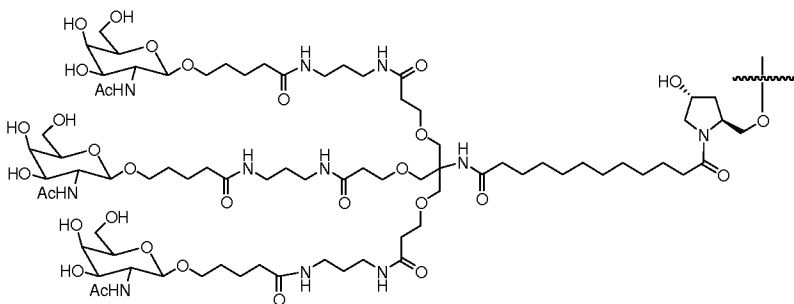
v. *Peptide-based cleaving groups*

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

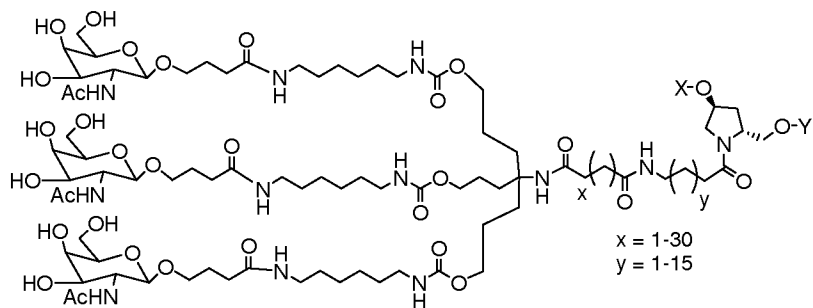
In one embodiment, an antisense polynucleotide agent of the invention is conjugated to a carbohydrate through a linker. Non-limiting examples of antisense polynucleotide agent carbohydrate conjugates with linkers of the compositions and methods of the invention include, but are not limited to,



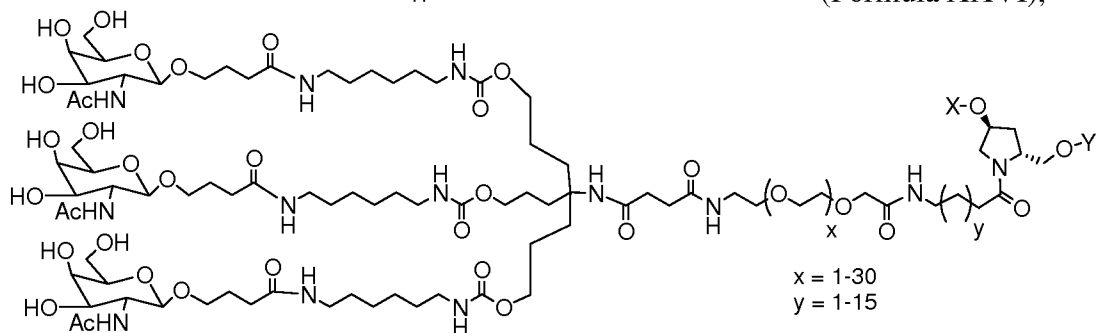
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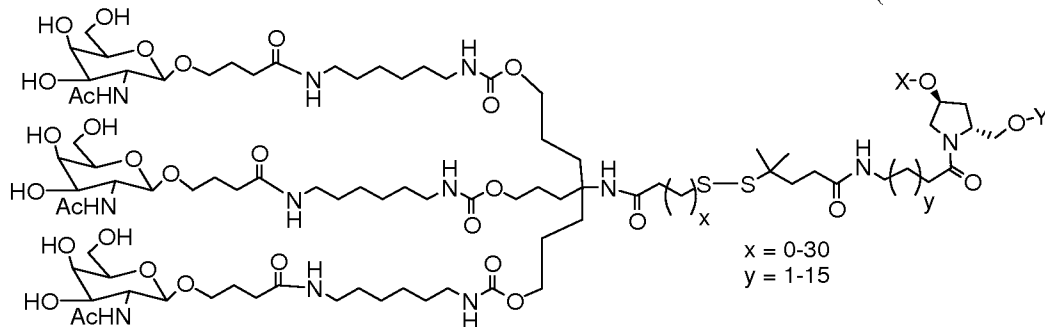
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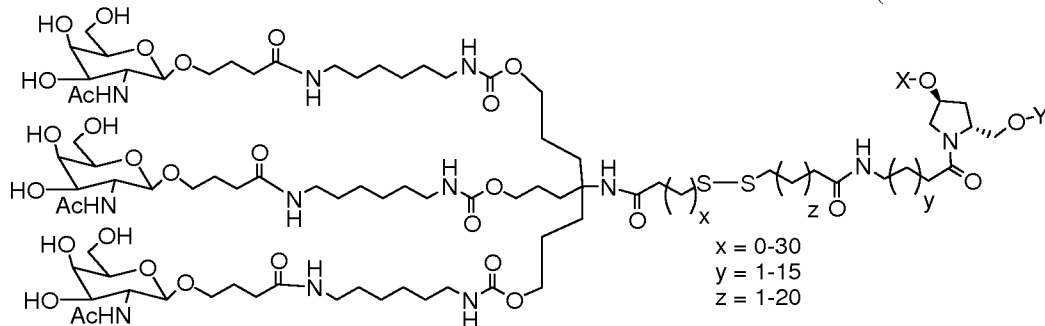
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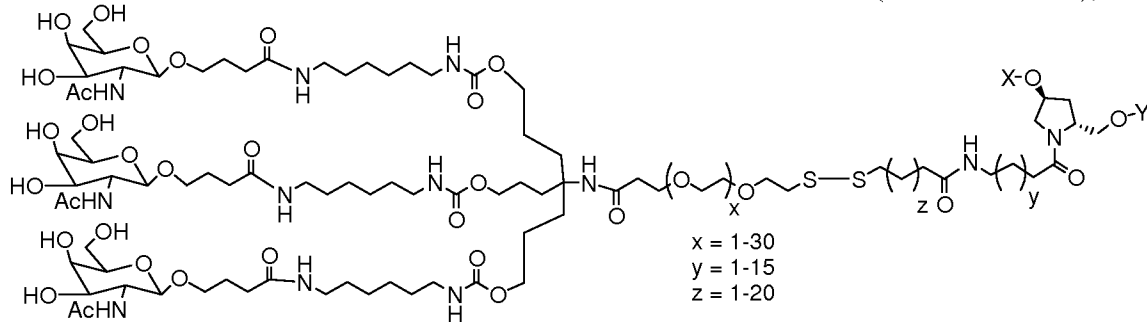
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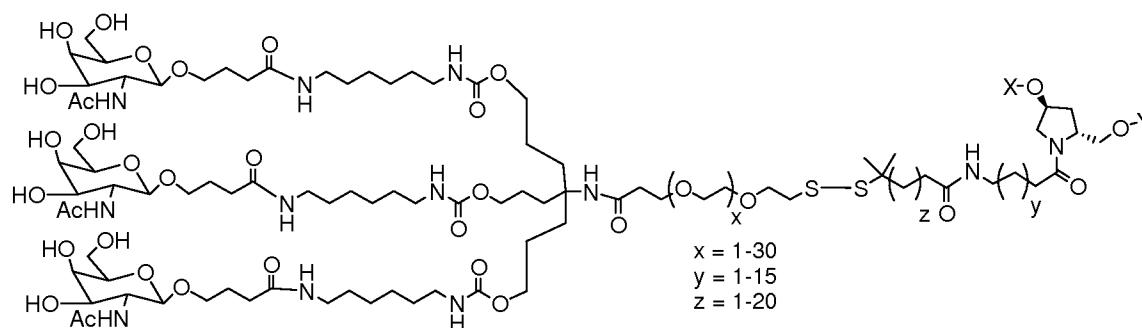
(Formula XXVIII),



(Formula XXIX),



(Formula XXX), and

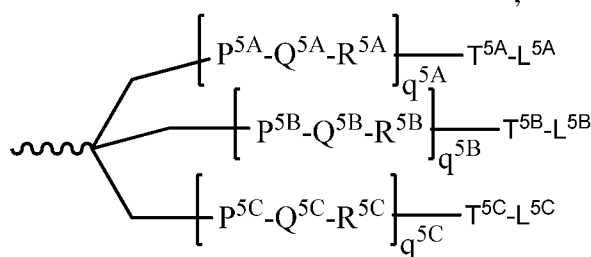
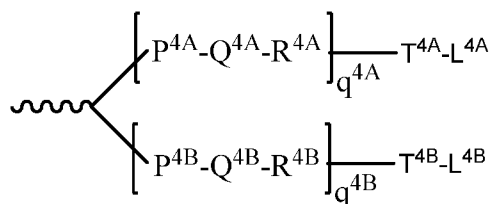
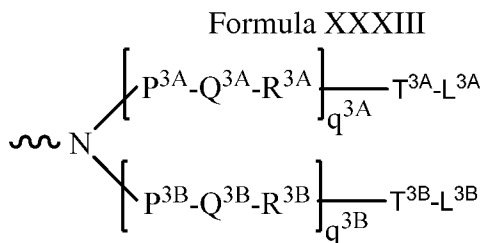
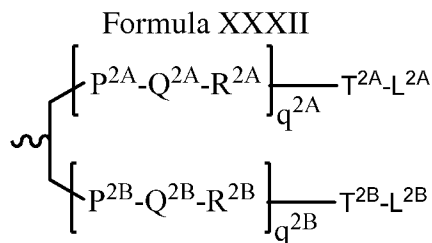


(Formula XXXI),

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

In certain embodiments of the compositions and methods of the invention, a ligand is one or more “GalNAc” (N-acetylgalactosamine) derivatives attached through a bivalent or trivalent branched linker.

In one embodiment, an antisense polynucleotide agent of the invention is conjugated to a bivalent or trivalent branched linker selected from the group of structures shown in any of formula (XXXII) – (XXXV):



, or

;

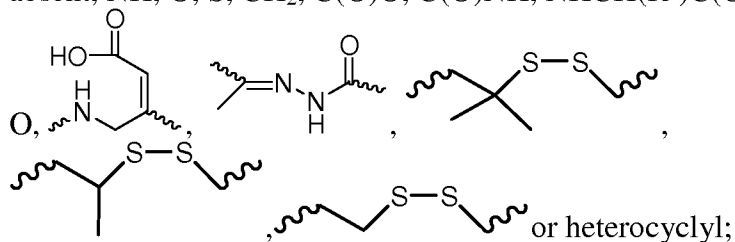
wherein:

q^{2A}, q^{2B}, q^{3A}, q^{3B}, q^{4A}, q^{4B}, q^{5A}, q^{5B} and q^{5C} represent independently for each occurrence 0-20 and wherein the repeating unit can be the same or different;

P^{2A}, P^{2B}, P^{3A}, P^{3B}, P^{4A}, P^{4B}, P^{5A}, P^{5B}, P^{5C}, T^{2A}, T^{2B}, T^{3A}, T^{3B}, T^{4A}, T^{4B}, T^{4A}, T^{5B}, T^{5C} are each independently for each occurrence absent, CO, NH, O, S, OC(O), NHC(O), CH₂, CH₂NH or CH₂O;

Q^{2A}, Q^{2B}, Q^{3A}, Q^{3B}, Q^{4A}, Q^{4B}, Q^{5A}, Q^{5B}, Q^{5C} are independently for each occurrence absent, alkylene, substituted alkylene wherein one or more methylenes can be interrupted or terminated by one or more of O, S, S(O), SO₂, N(R^N), C(R')=C(R''), C≡C or C(O);

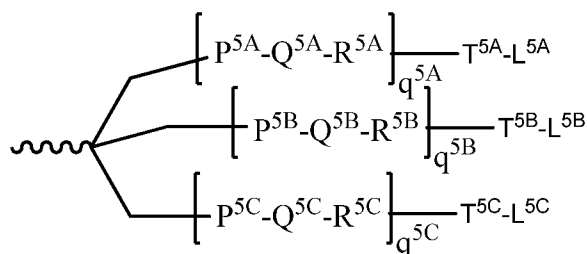
R^{2A}, R^{2B}, R^{3A}, R^{3B}, R^{4A}, R^{4B}, R^{5A}, R^{5B}, R^{5C} are each independently for each occurrence absent, NH, O, S, CH₂, C(O)O, C(O)NH, NHCH(R^a)C(O), -C(O)-CH(R^a)-NH-, CO, CH=N-



L^{2A}, L^{2B}, L^{3A}, L^{3B}, L^{4A}, L^{4B}, L^{5A}, L^{5B} and L^{5C} represent the ligand; *i.e.* each

independently for each occurrence a monosaccharide (such as GalNAc), disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, or polysaccharide; and R^a is H or amino acid side chain. Trivalent conjugating GalNAc derivatives are particularly useful for use with antisense polynucleotide agents for inhibiting the expression of a target gene, such as those of formula (XXXVI):

Formula XXXVI



wherein $\text{L}^{5\text{A}}$, $\text{L}^{5\text{B}}$ and $\text{L}^{5\text{C}}$ represent a monosaccharide, such as GalNAc derivative.

Examples of suitable bivalent and trivalent branched linker groups conjugating GalNAc derivatives include, but are not limited to, the structures recited above as formulas II, VII, XI, X, and XIII.

Representative U.S. patents that teach the preparation of RNA conjugates include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941; 6,294,664; 6,320,017; 6,576,752; 6,783,931; 6,900,297; 7,037,646; 8,106,022, the entire contents of each of which are hereby incorporated herein by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications can be incorporated in a single compound or even at a single nucleoside within an antisense polynucleotide agent. The present invention also includes antisense polynucleotide agents that are chimeric compounds.

“Chimeric” antisense polynucleotide agents or “chimeras,” in the context of this invention, are antisense polynucleotide agent compounds, which contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of an antisense polynucleotide agent. These antisense polynucleotide agents typically contain at least one region wherein the RNA is modified so as to confer upon the antisense polynucleotide agent increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the antisense polynucleotide agent can serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense polynucleotide agent inhibition of gene expression. Consequently, comparable results can

often be obtained with shorter antisense polynucleotide agents when chimeric antisense polynucleotide agents are used, compared to phosphorothioate deoxy antisense polynucleotide agents hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

In certain instances, the nucleotide of an antisense polynucleotide agent can be modified by a non-ligand group. A number of non-ligand molecules have been conjugated to antisense polynucleotide agents in order to enhance the activity, cellular distribution or cellular uptake of the antisense polynucleotide agent, and procedures for performing such conjugations are available in the scientific literature. Such non-ligand moieties have included lipid moieties, such as cholesterol (Kubo, T. *et al.*, *Biochem. Biophys. Res. Comm.*, 2007, 365(1):54-61; Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 1989, 86:6553), cholic acid (Manoharan *et al.*, *Bioorg. Med. Chem. Lett.*, 1994, 4:1053), a thioether, *e.g.*, hexyl-S-tritylthiol (Manoharan *et al.*, *Ann. N.Y. Acad. Sci.*, 1992, 660:306; Manoharan *et al.*, *Bioorg. Med. Chem. Lett.*, 1993, 3:2765), a thiocholesterol (Oberhauser *et al.*, *Nucl. Acids Res.*, 1992, 20:533), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, *EMBO J.*, 1991, 10:111; Kabanov *et al.*, *FEBS Lett.*, 1990, 259:327; Svinarchuk *et al.*, *Biochimie*, 1993, 75:49), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36:3651; Shea *et al.*, *Nucl. Acids Res.*, 1990, 18:3777), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, *Nucleosides & Nucleotides*, 1995, 14:969), or adamantane acetic acid (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36:3651), a palmityl moiety (Mishra *et al.*, *Biochim. Biophys. Acta*, 1995, 1264:229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.*, *J. Pharmacol. Exp. Ther.*, 1996, 277:923). Representative United States patents that teach the preparation of such RNA conjugates have been listed above. Typical conjugation protocols involve the synthesis of an RNAs bearing an aminolinker at one or more positions of the sequence. The amino group is then reacted with the molecule being conjugated using appropriate coupling or activating reagents. The conjugation reaction can be performed either with the RNA still bound to the solid support or following cleavage of the RNA, in solution phase. Purification of the RNA conjugate by HPLC typically affords the pure conjugate.

V. Delivery of Antisense Polynucleotide Agents of the Invention

The delivery of an antisense polynucleotide agent of the invention to a cell *e.g.*, a cell within a subject, such as a human subject (*e.g.*, a subject in need thereof, such as a subject having a complement component C5-associated disease) can be achieved in a number of different ways. For example, delivery may be performed by contacting a cell with an antisense polynucleotide agent of the invention either *in vitro* or *in vivo*. *In vivo* delivery

may also be performed directly by administering a composition comprising an antisense polynucleotide agent to a subject.

In general, any method of delivering a nucleic acid molecule (*in vitro* or *in vivo*) can be adapted for use with an antisense polynucleotide agent of the invention (see *e.g.*, Akhtar S. and Julian RL. (1992) *Trends Cell. Biol.* 2(5):139-144 and WO94/02595, which are incorporated herein by reference in their entireties). For *in vivo* delivery, factors to consider in order to deliver an antisense polynucleotide agent include, for example, biological stability of the delivered molecule, prevention of non-specific effects, and accumulation of the delivered molecule in the target tissue. The non-specific effects of an antisense polynucleotide agent can be minimized by local administration, for example, by direct injection or implantation into a tissue or topically administering the preparation. Local administration to a treatment site maximizes local concentration of the agent, limits the exposure of the agent to systemic tissues that can otherwise be harmed by the agent or that can degrade the agent, and permits a lower total dose of the antisense polynucleotide agent to be administered. Several studies have shown successful knockdown of gene products when an antisense polynucleotide agent is administered locally. For example, intraocular delivery of a VEGF antisense polynucleotide agent by intravitreal injection in cynomolgus monkeys (Tolentino, MJ., *et al* (2004) *Retina* 24:132-138) and subretinal injections in mice (Reich, SJ., *et al* (2003) *Mol. Vis.* 9:210-216) were both shown to prevent neovascularization in an experimental model of age-related macular degeneration. In addition, direct intratumoral injection of a antisense polynucleotide agent in mice reduces tumor volume (Pille, J., *et al* (2005) *Mol. Ther.* 11:267-274) and can prolong survival of tumor-bearing mice (Kim, WJ., *et al* (2006) *Mol. Ther.* 14:343-350; Li, S., *et al* (2007) *Mol. Ther.* 15:515-523). RNA interference has also shown success with local delivery to the CNS by direct injection (Dorn, G., *et al.* (2004) *Nucleic Acids* 32:e49; Tan, PH., *et al* (2005) *Gene Ther.* 12:59-66; Makimura, H., *et al* (2002) *BMC Neurosci.* 3:18; Shishkina, GT., *et al* (2004) *Neuroscience* 129:521-528; Thakker, ER., *et al* (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101:17270-17275; Akaneya, Y., *et al* (2005) *J. Neurophysiol.* 93:594-602) and to the lungs by intranasal administration (Howard, KA., *et al* (2006) *Mol. Ther.* 14:476-484; Zhang, X., *et al* (2004) *J. Biol. Chem.* 279:10677-10684; Bitko, V., *et al* (2005) *Nat. Med.* 11:50-55). For administering an antisense polynucleotide agent systemically for the treatment of a disease, the agent can be modified or alternatively delivered using a drug delivery system; both methods act to prevent the rapid degradation of the antisense polynucleotide agent by endo- and exo-nucleases *in vivo*. Modification of the agent or the pharmaceutical carrier can also permit targeting of the antisense polynucleotide agent composition to the target tissue and avoid undesirable off-target effects. Antisense polynucleotide agent can be modified by chemical conjugation to lipophilic groups such as cholesterol to enhance cellular uptake and prevent degradation. In an alternative embodiment, the antisense polynucleotide agent can be delivered using drug

delivery systems such as a nanoparticle, a dendrimer, a polymer, liposomes, or a cationic delivery system. Positively charged cationic delivery systems facilitate binding of an antisense polynucleotide agent molecule (negatively charged) and also enhance interactions at the negatively charged cell membrane to permit efficient uptake of an antisense polynucleotide agent by the cell. Cationic lipids, dendrimers, or polymers can either be bound to an antisense polynucleotide agent, or induced to form a vesicle or micelle (see *e.g.*, Kim SH., *et al* (2008) *Journal of Controlled Release* 129(2):107-116) that encases an antisense polynucleotide agent. The formation of vesicles or micelles further prevents degradation of the antisense polynucleotide agent when administered systemically. Methods for making and administering cationic- antisense polynucleotide agent complexes are well within the abilities of one skilled in the art (see *e.g.*, Sorensen, DR., *et al* (2003) *J. Mol. Biol* 327:761-766; Verma, UN, *et al* (2003) *Clin. Cancer Res.* 9:1291-1300; Arnold, AS *et al* (2007) *J. Hypertens.* 25:197-205, which are incorporated herein by reference in their entirety). Some non-limiting examples of drug delivery systems useful for systemic delivery of antisense polynucleotide agents include DOTAP (Sorensen, DR., *et al* (2003), *supra*; Verma, UN., *et al* (2003), *supra*), Oligofectamine, "solid nucleic acid lipid particles" (Zimmermann, TS., *et al* (2006) *Nature* 441:111-114), cardiolipin (Chien, PY., *et al* (2005) *Cancer Gene Ther.* 12:321-328; Pal, A., *et al* (2005) *Int J. Oncol.* 26:1087-1091), polyethyleneimine (Bonnet ME., *et al* (2008) *Pharm. Res.* Aug 16 Epub ahead of print; Aigner, A. (2006) *J. Biomed. Biotechnol.* 71659), Arg-Gly-Asp (RGD) peptides (Liu, S. (2006) *Mol. Pharm.* 3:472-487), and polyamidoamines (Tomalia, DA., *et al* (2007) *Biochem. Soc. Trans.* 35:61-67; Yoo, H., *et al* (1999) *Pharm. Res.* 16:1799-1804). In some embodiments, an antisense polynucleotide agent forms a complex with cyclodextrin for systemic administration. Methods for administration and pharmaceutical compositions of antisense polynucleotide agents and cyclodextrins can be found in U.S. Patent No. 7,427,605, which is herein incorporated by reference in its entirety.

VI. Pharmaceutical Compositions of the Invention

The present invention also includes pharmaceutical compositions and formulations which include the antisense polynucleotide agents of the invention. In one embodiment, provided herein are pharmaceutical compositions containing an antisense polynucleotide agent, as described herein, and a pharmaceutically acceptable carrier.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human subjects and animal subjects without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, manufacturing aid (*e.g.*, lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject being treated. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum components, such as serum albumin, HDL and LDL; and (22) other non-toxic compatible substances employed in pharmaceutical formulations.

The pharmaceutical compositions containing the antisense polynucleotide agents are useful for treating a disease or disorder associated with the expression or activity of a C5 gene, *e.g.* a complement component C5-associated disease. Such pharmaceutical compositions are formulated based on the mode of delivery. One example is compositions that are formulated for systemic administration *via* parenteral delivery, *e.g.*, by subcutaneous (SC) or intravenous (IV) delivery. Another example is compositions that are formulated for direct delivery into the brain parenchyma, *e.g.*, by infusion into the brain, such as by continuous pump infusion. The pharmaceutical compositions of the invention may be administered in dosages sufficient to inhibit expression of a C5 gene. In general, a suitable dose of an antisense polynucleotide agent of the invention will be in the range of about 0.001 to about 200.0 milligrams per kilogram body weight of the recipient per day, generally in the range of about 1 to 50 mg per kilogram body weight per day. For example, the antisense polynucleotide agent can be administered at about 0.01 mg/kg, about 0.05 mg/kg, about 0.5 mg/kg, about 1 mg/kg, about 1.5 mg/kg, about 2 mg/kg, about 3 mg/kg, about 10 mg/kg, about 20 mg/kg, about 30 mg/kg, about 40 mg/kg, or about 50 mg/kg per single dose.

For example, the antisense polynucleotide agent may be administered at a dose of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 2, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or about 50 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In another embodiment, the antisense polynucleotide agent is administered at a dose of about 0.1 to about 50 mg/kg, about 0.25 to about 50 mg/kg, about 0.5 to about 50 mg/kg, about 0.75 to about 50 mg/kg, about 1 to about 50 mg/mg, about 1.5 to about 50 mg/kg, about 2 to about 50 mg/kg, about 2.5 to about 50 mg/kg, about 3 to about 50 mg/kg, about 3.5 to about 50 mg/kg, about 4 to about 50 mg/kg, about 4.5 to about 50 mg/kg, about 5 to about 50 mg/kg, about 7.5 to about 50 mg/kg, about 10 to about 50 mg/kg, about 15 to about 50 mg/kg, about 20 to about 50 mg/kg, about 20 to about 50 mg/kg, about 25 to about 50 mg/kg, about 25 to about 50 mg/kg, about 30 to about 50 mg/kg, about 35 to about 50 mg/kg, about 40 to about 50 mg/kg, about 45 to about 50 mg/kg, about 0.1 to about 45 mg/kg, about 0.25 to about 45 mg/kg, about 0.5 to about 45 mg/kg, about 0.75 to about 45 mg/kg, about 1 to about 45 mg/mg, about 1.5 to about 45 mg/kg, about 2 to about 45 mg/kg, about 2.5 to about 45 mg/kg, about 3 to about 45 mg/kg, about 3.5 to about 45 mg/kg, about 4 to about 45 mg/kg, about 4.5 to about 45 mg/kg, about 5 to about 45 mg/kg, about 7.5 to about 45 mg/kg, about 10 to about 45 mg/kg, about 15 to about 45 mg/kg, about 20 to about 45 mg/kg, about 20 to about 45 mg/kg, about 25 to about 45 mg/kg, about 25 to about 45 mg/kg, about 30 to about 45 mg/kg, about 35 to about 45 mg/kg, about 40 to about 45 mg/kg, about 0.1 to about 40 mg/kg, about 0.25 to about 40 mg/kg, about 0.5 to about 40 mg/kg, about 0.75 to about 40 mg/kg, about 1 to about 40 mg/mg, about 1.5 to about 40 mg/kg, about 2 to about 40 mg/kg, about 2.5 to about 40 mg/kg, about 3 to about 40 mg/kg, about 3.5 to about 40 mg/kg, about 4 to about 40 mg/kg, about 4.5 to about 40 mg/kg, about 5 to about 40 mg/kg, about 7.5 to about 40 mg/kg, about 10 to about 40 mg/kg, about 15 to about 40 mg/kg, about 20 to about 40 mg/kg, about 20 to about 40 mg/kg, about 25 to about 40 mg/kg, about 25 to about 40 mg/kg, about 30 to about 40 mg/kg, about 35 to about 40 mg/kg, about 0.1 to about 30 mg/kg, about 0.25 to about 30 mg/kg, about 0.5 to about 30 mg/kg, about 0.75 to about 30 mg/kg, about 1 to about 30 mg/mg, about 1.5 to about 30 mg/kg, about 2 to about 30 mg/kg, about 2.5 to about 30 mg/kg, about 3 to about 30 mg/kg, about 3.5 to about 30 mg/kg, about 4 to about 30 mg/kg, about 4.5 to about 30 mg/kg, about 5 to about 30 mg/kg, about 7.5 to about 30 mg/kg, about 10 to about 30 mg/kg, about 15 to about 30 mg/kg, about 20 to about 30 mg/kg, about 20 to about 30 mg/kg, about 25 to about 30 mg/kg, about 0.1 to about 20

mg/kg, about 0.25 to about 20 mg/kg, about 0.5 to about 20 mg/kg, about 0.75 to about 20 mg/kg, about 1 to about 20 mg/mg, about 1.5 to about 20 mg/kg, about 2 to about 20 mg/kg, about 2.5 to about 20 mg/kg, about 3 to about 20 mg/kg, about 3.5 to about 20 mg/kg, about 4 to about 20 mg/kg, about 4.5 to about 20 mg/kg, about 5 to about 20 mg/kg, about 7.5 to about 20 mg/kg, about 10 to about 20 mg/kg, or about 15 to about 20 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

For example, the antisense polynucleotide agent may be administered at a dose of about 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 2, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or about 50 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In another embodiment, the antisense polynucleotide agent is administered at a dose of about 0.5 to about 50 mg/kg, about 0.75 to about 50 mg/kg, about 1 to about 50 mg/mg, about 1.5 to about 50 mg/kg, about 2 to about 50 mg/kg, about 2.5 to about 50 mg/kg, about 3 to about 50 mg/kg, about 3.5 to about 50 mg/kg, about 4 to about 50 mg/kg, about 4.5 to about 50 mg/kg, about 5 to about 50 mg/kg, about 7.5 to about 50 mg/kg, about 10 to about 50 mg/kg, about 15 to about 50 mg/kg, about 20 to about 50 mg/kg, about 20 to about 50 mg/kg, about 25 to about 50 mg/kg, about 25 to about 50 mg/kg, about 30 to about 50 mg/kg, about 35 to about 50 mg/kg, about 40 to about 50 mg/kg, about 45 to about 50 mg/kg, about 0.5 to about 45 mg/kg, about 0.75 to about 45 mg/kg, about 1 to about 45 mg/mg, about 1.5 to about 45 mg/kg, about 1.5 to about 45 mg/kg, about 2 to about 45 mg/kg, about 2.5 to about 45 mg/kg, about 3 to about 45 mg/kg, about 3.5 to about 45 mg/kg, about 4 to about 45 mg/kg, about 4.5 to about 45 mg/kg, about 5 to about 45 mg/kg, about 7.5 to about 45 mg/kg, about 10 to about 45 mg/kg, about 15 to about 45 mg/kg, about 20 to about 45 mg/kg, about 20 to about 45 mg/kg, about 25 to about 45 mg/kg, about 25 to about 45 mg/kg, about 30 to about 45 mg/kg, about 35 to about 45 mg/kg, about 40 to about 45 mg/kg, about 0.5 to about 40 mg/kg, about 0.75 to about 40 mg/kg, about 1 to about 40 mg/mg, about 1.5 to about 40 mg/kg, about 2 to about 40 mg/kg, about 2.5 to about 40 mg/kg, about 3 to about 40 mg/kg, about 3.5 to about 40 mg/kg, about 4 to about 40 mg/kg, about 4.5 to about 40 mg/kg, about 5 to about 40 mg/kg, about 7.5 to about 40 mg/kg, about 10 to about 40 mg/kg, about 15 to about 40 mg/kg, about 20 to about 40 mg/kg, about 20 to about 40 mg/kg, about 25 to about 40 mg/kg, about 25 to about 40 mg/kg, about 30 to about 40 mg/kg, about 35 to about 40 mg/kg, about 0.5 to about 30 mg/kg, about 0.75 to about 30 mg/kg, about 1 to about 30 mg/mg, about 1.5 to about 30

mg/kg, about 2 to about 30 mg/kg, about 2.5 to about 30 mg/kg, about 3 to about 30 mg/kg, about 3.5 to about 30 mg/kg, about 4 to about 30 mg/kg, about 4.5 to about 30 mg/kg, about 5 to about 30 mg/kg, about 7.5 to about 30 mg/kg, about 10 to about 30 mg/kg, about 15 to about 30 mg/kg, about 20 to about 30 mg/kg, about 20 to about 30 mg/kg, about 25 to about 30 mg/kg, about 0.5 to about 20 mg/kg, about 0.75 to about 20 mg/kg, about 1 to about 20 mg/kg, about 1.5 to about 20 mg/kg, about 2 to about 20 mg/kg, about 2.5 to about 20 mg/kg, about 3 to about 20 mg/kg, about 3.5 to about 20 mg/kg, about 4 to about 20 mg/kg, about 4.5 to about 20 mg/kg, about 5 to about 20 mg/kg, about 7.5 to about 20 mg/kg, about 10 to about 20 mg/kg, or about 15 to about 20 mg/kg. In one embodiment, the antisense polynucleotide agent is administered at a dose of about 10mg/kg to about 30 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

For example, subjects can be administered, *e.g.*, subcutaneously or intravenously, a single therapeutic amount of antisense polynucleotide agent, such as about 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, 0.275, 0.3, 0.325, 0.35, 0.375, 0.4, 0.425, 0.45, 0.475, 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75, 0.775, 0.8, 0.825, 0.85, 0.875, 0.9, 0.925, 0.95, 0.975, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, 30, 31, 32, 33, 34, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or about 50 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In some embodiments, subjects are administered, *e.g.*, subcutaneously or intravenously, multiple doses of a therapeutic amount of antisense polynucleotide agent, such as a dose about 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, 0.275, 0.3, 0.325, 0.35, 0.375, 0.4, 0.425, 0.45, 0.475, 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75, 0.775, 0.8, 0.825, 0.85, 0.875, 0.9, 0.925, 0.95, 0.975, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, 30, 31, 32, 33, 34, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or about 50 mg/kg. A multi-dose regimen may include administration of a therapeutic amount of antisense polynucleotide agent daily, such as for two days, three days, four days, five days, six days, seven days, or longer.

In other embodiments, subjects are administered, *e.g.*, subcutaneously or intravenously, a repeat dose of a therapeutic amount of antisense polynucleotide agent, such as a dose about 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, 0.275, 0.3, 0.325, 0.35, 0.375, 0.4, 0.425, 0.45, 0.475, 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75, 0.775, 0.8, 0.825, 0.85, 0.875, 0.9, 0.925, 0.95, 0.975, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, 30, 31, 32, 33, 34, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or about 50 mg/kg. A repeat-dose regimen may include administration of a therapeutic amount of antisense polynucleotide agent on a regular basis, such as every other day, every third day, every fourth day, twice a week, once a week, every other week, or once a month.

The pharmaceutical composition can be administered by intravenous infusion over a period of time, such as over a 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, and 21, 22, 23, 24, or about a 25 minute period. The administration may be repeated, for example, on a regular basis, such as weekly, biweekly (*i.e.*, every two weeks) for one month, two months, three months, four months or longer. After an initial treatment regimen, the treatments can be administered on a less frequent basis. For example, after administration weekly or biweekly for three months, administration can be repeated once per month, for six months or a year or longer.

The pharmaceutical composition can be administered once daily, or the antisense polynucleotide agent can be administered as two, three, or more sub-doses at appropriate intervals throughout the day or even using continuous infusion or delivery through a controlled release formulation. In that case, the antisense polynucleotide agent contained in each sub-dose must be correspondingly smaller in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, *e.g.*, using a conventional sustained release formulation which provides sustained release of the antisense polynucleotide agent over a several day period. Sustained release formulations are well known in the art and are particularly useful for delivery of agents at a particular site, such as could be used with the agents of the present invention. In this embodiment, the dosage unit contains a corresponding multiple of the daily dose.

In other embodiments, a single dose of the pharmaceutical compositions can be long lasting, such that subsequent doses are administered at not more than 3, 4, or 5 day intervals, or at not more than 1, 2, 3, or 4 week intervals. In some embodiments of the invention, a single dose of the pharmaceutical compositions of the invention is administered once per

week. In other embodiments of the invention, a single dose of the pharmaceutical compositions of the invention is administered bi-monthly.

The skilled artisan will appreciate that certain factors can influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment or a series of treatments. Estimates of effective dosages and *in vivo* half-lives for the individual antisense polynucleotide agents encompassed by the invention can be made using conventional methodologies or on the basis of *in vivo* testing using an appropriate animal model, as described elsewhere herein.

Advances in mouse genetics have generated a number of mouse models for the study of various human diseases, such as a disorder that would benefit from reduction in the expression of C5. Such models can be used for *in vivo* testing of an antisense polynucleotide agent, as well as for determining a therapeutically effective dose. Suitable mouse models are known in the art and include, for example, collagen-induced arthritis mouse model (Courtenay, J.S., *et al.* (1980) *Nature* 283, 666–668), myocardial ischemia (Homeister JW and Lucchesi BR (1994) *Annu Rev Pharmacol Toxicol* 34:17–40), ovalbumin induced asthma mouse models (*e.g.*, Tomkinson A., *et al.* (2001). *J. Immunol.* 166, 5792–5800), (NZB×NZW)F1, MRL/Fas^{lpr} (MRL/lpr) and BXSB mouse models (Theofilopoulos, A. N. and Kono, D. H. 1999. Murine lupus models: gene-specific and genome-wide studies. In Lahita R. G., ed., *Systemic Lupus Erythematosus*, 3rd edn, p. 145. Academic Press, San Diego, CA), mouse aHUS model (Goicoechea de Jorge *et al.* (2011) *The development of atypical hemolytic uremic syndrome depends on complement C5*, *J Am Soc Nephrol* 22:137-145).

The pharmaceutical compositions of the present invention can be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration can be topical (*e.g.*, by a transdermal patch), pulmonary, *e.g.*, by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal, oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; subdermal, *e.g.*, via an implanted device; or intracranial, *e.g.*, by intraparenchymal, intrathecal or intraventricular, administration.

The antisense polynucleotide agent can be delivered in a manner to target a particular tissue, such as the liver (*e.g.*, the hepatocytes of the liver).

Pharmaceutical compositions and formulations for topical administration can include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like can be necessary or desirable. Coated condoms, gloves and the like can also be

useful. Suitable topical formulations include those in which the antisense polynucleotide agents featured in the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Suitable lipids and liposomes include neutral (*e.g.*, dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (*e.g.*, dimyristoylphosphatidyl glycerol DMPG) and cationic (*e.g.*, dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Antisense polynucleotide agents featured in the invention can be encapsulated within liposomes or can form complexes thereto, in particular to cationic liposomes. Alternatively, antisense polynucleotide agents can be complexed to lipids, in particular to cationic lipids. Suitable fatty acids and esters include but are not limited to arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C₁₋₂₀ alkyl ester (*e.g.*, isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof). Topical formulations are described in detail in U.S. Patent No. 6,747,014, which is incorporated herein by reference.

A. Antisense Polynucleotide Agent Formulations Comprising Membranous Molecular Assemblies

An antisense polynucleotide agent for use in the compositions and methods of the invention can be formulated for delivery in a membranous molecular assembly, *e.g.*, a liposome or a micelle. As used herein, the term “liposome” refers to a vesicle composed of amphiphilic lipids arranged in at least one bilayer, *e.g.*, one bilayer or a plurality of bilayers. Liposomes include unilamellar and multilamellar vesicles that have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the antisense polynucleotide agent composition. The lipophilic material isolates the aqueous interior from an aqueous exterior, which typically does not include the antisense polynucleotide agent composition, although in some examples, it may. Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomal bilayer fuses with bilayer of the cellular membranes. As the merging of the liposome and cell progresses, the internal aqueous contents that include the antisense polynucleotide agent are delivered into the cell where the antisense polynucleotide agent can specifically bind to a target RNA and can mediate antisense inhibition. In some cases the liposomes are also specifically targeted, *e.g.*, to direct the antisense polynucleotide agent to particular cell types.

A liposome containing an antisense polynucleotide agent can be prepared by a variety of methods. In one example, the lipid component of a liposome is dissolved in a detergent so that micelles are formed with the lipid component. For example, the lipid component can be an amphipathic cationic lipid or lipid conjugate. The detergent can have a high critical micelle concentration and may be nonionic. Exemplary detergents include cholate, CHAPS, octylglucoside, deoxycholate, and lauroyl sarcosine. The antisense polynucleotide agent preparation is then added to the micelles that include the lipid component. The cationic groups on the lipid interact with the antisense polynucleotide agent and condense around the antisense polynucleotide agent to form a liposome. After condensation, the detergent is removed, *e.g.*, by dialysis, to yield a liposomal preparation of antisense polynucleotide agent.

If necessary a carrier compound that assists in condensation can be added during the condensation reaction, *e.g.*, by controlled addition. For example, the carrier compound can be a polymer other than a nucleic acid (*e.g.*, spermine or spermidine). pH can also be adjusted to favor condensation.

Methods for producing stable polynucleotide delivery vehicles, which incorporate a polynucleotide/cationic lipid complex as structural components of the delivery vehicle, are further described in, *e.g.*, WO 96/37194, the entire contents of which are incorporated herein by reference. Liposome formation can also include one or more aspects of exemplary methods described in Felgner, P. L. *et al.*, *Proc. Natl. Acad. Sci., USA* 8:7413-7417, 1987; U.S. Pat. No. 4,897,355; U.S. Pat. No. 5,171,678; Bangham, *et al. M. Mol. Biol.* 23:238, 1965; Olson, *et al. Biochim. Biophys. Acta* 557:9, 1979; Szoka, *et al. Proc. Natl. Acad. Sci.* 75: 4194, 1978; Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984; Kim, *et al. Biochim. Biophys. Acta* 728:339, 1983; and Fukunaga, *et al. Endocrinol.* 115:757, 1984. Commonly used techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion (see, *e.g.*, Mayer, *et al. Biochim. Biophys. Acta* 858:161, 1986). Microfluidization can be used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984). These methods are readily adapted to packaging antisense polynucleotide agent preparations into liposomes.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged nucleic acid molecules to form a stable complex. The positively charged nucleic acid/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, *Biochem. Biophys. Res. Commun.*, 1987, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap nucleic acids rather than complex with it. Since both the nucleic acid and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some nucleic acid is entrapped

within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver nucleic acids encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled Release*, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Examples of other methods to introduce liposomes into cells *in vitro* and *in vivo* include U.S. Pat. No. 5,283,185; U.S. Pat. No. 5,171,678; WO 94/00569; WO 93/24640; WO 91/16024; Felgner, *J. Biol. Chem.* 269:2550, 1994; Nabel, *Proc. Natl. Acad. Sci.* 90:11307, 1993; Nabel, *Human Gene Ther.* 3:649, 1992; Gershon, *Biochem.* 32:7143, 1993; and Strauss *EMBO J.* 11:417, 1992.

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporine A into different layers of the skin (Hu *et al. S.T.P. Pharma. Sci.*, 1994, 4(6) 466).

Liposomes also include “sterically stabilized” liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, *FEBS Letters*, 1987, 223, 42; Wu *et al.*, *Cancer Research*, 1993, 53, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, 1987, 507, 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 6949). U.S. Pat. No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Pat. No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim *et al.*).

In one embodiment, cationic liposomes are used. Cationic liposomes possess the advantage of being able to fuse to the cell membrane. Non-cationic liposomes, although not able to fuse as efficiently with the plasma membrane, are taken up by macrophages *in vivo* and can be used to deliver antisense polynucleotide agents to macrophages.

Further advantages of liposomes include: liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated antisense polynucleotide agents in their internal compartments from metabolism and degradation (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

A positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) can be used to form small liposomes that interact spontaneously with nucleic acid to form lipid-nucleic acid complexes which are capable of fusing with the negatively charged lipids of the cell membranes of tissue culture cells, resulting in delivery of Antisense polynucleotide agent (see, *e.g.*, Felgner, P. L. *et al.*, *Proc. Natl. Acad. Sci., USA* 8:7413-7417, 1987 and U.S. Pat. No. 4,897,355 for a description of DOTMA and its use with DNA).

A DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonia)propane (DOTAP) can be used in combination with a phospholipid to form DNA-complexing vesicles. Lipofectin™ (Bethesda Research Laboratories, Gaithersburg, Md.) is an effective agent for the delivery of highly anionic nucleic acids into living tissue culture cells that comprise positively charged DOTMA liposomes which interact spontaneously with negatively charged polynucleotides to form complexes. When enough positively charged liposomes are used, the net charge on the resulting complexes is also positive. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces, fuse with the plasma membrane, and efficiently deliver functional nucleic acids into, for example, tissue culture cells. Another commercially available cationic lipid, 1,2-bis(oleoyloxy)-3,3-(trimethylammonia)propane ("DOTAP") (Boehringer Mannheim, Indianapolis, Indiana)

differs from DOTMA in that the oleoyl moieties are linked by ester, rather than ether linkages.

Other reported cationic lipid compounds include those that have been conjugated to a variety of moieties including, for example, carboxyspermine which has been conjugated to one of two types of lipids and includes compounds such as 5-carboxyspermylglycine dioctaoleoylamide (“DOGS”) (Transfectam™, Promega, Madison, Wisconsin) and dipalmitoylphosphatidylethanolamine 5-carboxyspermyl-amide (“DPPE”) (see, e.g., U.S. Pat. No. 5,171,678).

Another cationic lipid conjugate includes derivatization of the lipid with cholesterol (“DC-Chol”) which has been formulated into liposomes in combination with DOPE (See, Gao, X. and Huang, L., *Biochim. Biophys. Res. Commun.* 179:280, 1991). Lipopolylysine, made by conjugating polylysine to DOPE, has been reported to be effective for transfection in the presence of serum (Zhou, X. et al., *Biochim. Biophys. Acta* 1065:8, 1991). For certain cell lines, these liposomes containing conjugated cationic lipids, are said to exhibit lower toxicity and provide more efficient transfection than the DOTMA-containing compositions. Other commercially available cationic lipid products include DMRIE and DMRIE-HP (Vical, La Jolla, California) and Lipofectamine (DOSPA) (Life Technology, Inc., Gaithersburg, Maryland). Other cationic lipids suitable for the delivery of oligonucleotides are described in WO 98/39359 and WO 96/37194.

Liposomal formulations are particularly suited for topical administration; liposomes present several advantages over other formulations. Such advantages include reduced side effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer an antisense polynucleotide agent into the skin. In some implementations, liposomes are used for delivering antisense polynucleotide agent to epidermal cells and also to enhance the penetration of antisense polynucleotide agent into dermal tissues, e.g., into skin. For example, the liposomes can be applied topically. Topical delivery of drugs formulated as liposomes to the skin has been documented (see, e.g., Weiner et al., *Journal of Drug Targeting*, 1992, vol. 2,405-410 and du Plessis et al., *Antiviral Research*, 18, 1992, 259-265; Mannino, R. J. and Fould-Fogerite, S., *Biotechniques* 6:682-690, 1988; Itani, T. et al. *Gene* 56:267-276, 1987; Nicolau, C. et al. *Meth. Enz.* 149:157-176, 1987; Straubinger, R. M. and Papahadjopoulos, D. *Meth. Enz.* 101:512-527, 1983; Wang, C. Y. and Huang, L., *Proc. Natl. Acad. Sci. USA* 84:7851-7855, 1987).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver a drug into the dermis of

mouse skin. Such formulations with antisense polynucleotide agents are useful for treating a dermatological disorder.

Liposomes that include antisense polynucleotide agent can be made highly deformable. Such deformability can enable the liposomes to penetrate through pore that are smaller than the average radius of the liposome. For example, transfersomes are a type of deformable liposomes. Transfersomes can be made by adding surface edge activators, usually surfactants, to a standard liposomal composition. Transfersomes that include antisense polynucleotide agents can be delivered, for example, subcutaneously by infection in order to deliver antisense polynucleotide agents to keratinocytes in the skin. In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. In addition, due to the lipid properties, these transfersomes can be self-optimizing (adaptive to the shape of pores, *e.g.*, in the skin), self-repairing, and can frequently reach their targets without fragmenting, and often self-loading.

Other formulations amenable to the present invention are described in United States provisional application serial Nos. 61/018,616, filed January 2, 2008; 61/018,611, filed January 2, 2008; 61/039,748, filed March 26, 2008; 61/047,087, filed April 22, 2008 and 61/051,528, filed May 8, 2008. PCT application no PCT/US2007/080331, filed October 3, 2007 also describes formulations that are amenable to the present invention.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes can be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.*, they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in "Pharmaceutical Dosage Forms", Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in "Pharmaceutical Dosage Forms", Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

The antisense polynucleotide agent for use in the compositions and methods of the invention can also be provided as micellar formulations. "Micelles" are defined herein as a particular type of molecular assembly in which amphipathic molecules are arranged in a spherical structure such that all the hydrophobic portions of the molecules are directed inward, leaving the hydrophilic portions in contact with the surrounding aqueous phase. The converse arrangement exists if the environment is hydrophobic.

A mixed micellar formulation suitable for delivery through transdermal membranes may be prepared by mixing an aqueous solution of the antisense polynucleotide agent composition, an alkali metal C₈ to C₂₂ alkyl sulphate, and a micelle forming compounds. Exemplary micelle forming compounds include lecithin, hyaluronic acid, pharmaceutically acceptable salts of hyaluronic acid, glycolic acid, lactic acid, chamomile extract, cucumber extract, oleic acid, linoleic acid, linolenic acid, monoolein, monooleates, monolaurates, borage oil, evening of primrose oil, menthol, trihydroxy oxo cholanyl glycine and

pharmaceutically acceptable salts thereof, glycerin, polyglycerin, lysine, polylysine, triolein, polyoxyethylene ethers and analogues thereof, polidocanol alkyl ethers and analogues thereof, chenodeoxycholate, deoxycholate, and mixtures thereof. The micelle forming compounds may be added at the same time or after addition of the alkali metal alkyl sulphate. Mixed micelles will form with substantially any kind of mixing of the ingredients but vigorous mixing in order to provide smaller size micelles.

In one method a first micellar composition is prepared which contains the antisense polynucleotide agent composition and at least the alkali metal alkyl sulphate. The first micellar composition is then mixed with at least three micelle forming compounds to form a mixed micellar composition. In another method, the micellar composition is prepared by mixing the antisense polynucleotide agent composition, the alkali metal alkyl sulphate and at least one of the micelle forming compounds, followed by addition of the remaining micelle forming compounds, with vigorous mixing.

Phenol and/or m-cresol may be added to the mixed micellar composition to stabilize the formulation and protect against bacterial growth. Alternatively, phenol and/or m-cresol may be added with the micelle forming ingredients. An isotonic agent such as glycerin may also be added after formation of the mixed micellar composition.

For delivery of the micellar formulation as a spray, the formulation can be put into an aerosol dispenser and the dispenser is charged with a propellant. The propellant, which is under pressure, is in liquid form in the dispenser. The ratios of the ingredients are adjusted so that the aqueous and propellant phases become one, *i.e.*, there is one phase. If there are two phases, it is necessary to shake the dispenser prior to dispensing a portion of the contents, *e.g.*, through a metered valve. The dispensed dose of pharmaceutical agent is propelled from the metered valve in a fine spray.

Propellants may include hydrogen-containing chlorofluorocarbons, hydrogen-containing fluorocarbons, dimethyl ether and diethyl ether. In certain embodiments, HFA 134a (1,1,1,2 tetrafluoroethane) may be used.

The specific concentrations of the essential ingredients can be determined by relatively straightforward experimentation. For absorption through the oral cavities, it is often desirable to increase, *e.g.*, at least double or triple, the dosage for through injection or administration through the gastrointestinal tract.

B. *Lipid particles*

Antisense polynucleotide agents of in the invention may be fully encapsulated in a lipid formulation, *e.g.*, a LNP, or other nucleic acid-lipid particle.

As used herein, the term "LNP" refers to a stable nucleic acid-lipid particle comprising a lipid layer encapsulating a pharmaceutically active molecule. LNPs typically contain a cationic lipid, a non-cationic lipid, and a lipid that prevents aggregation of the particle (*e.g.*, a PEG-lipid conjugate). LNPs are extremely useful for systemic applications, as

they exhibit extended circulation lifetimes following intravenous (i.v.) injection and accumulate at distal sites (*e.g.*, sites physically separated from the administration site). LNPs include "pSPLP," which include an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683. The particles of the present invention typically have a mean diameter of about 50 nm to about 150 nm, more typically about 60 nm to about 130 nm, more typically about 70 nm to about 110 nm, most typically about 70 nm to about 90 nm, and are substantially nontoxic. In addition, the nucleic acids when present in the nucleic acid-lipid particles of the present invention are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Nos. 5,976,567; 5,981,501; 6,534,484; 6,586,410; 6,815,432; 6,858,225; 8,158,601; and 8,058,069; U.S. Publication No. 2010/0324120 and PCT Publication No. WO 96/40964.

In one embodiment, the lipid to drug ratio (mass/mass ratio) (*e.g.*, lipid to antisense polynucleotide agent ratio) will be in the range of from about 1:1 to about 50:1, from about 1:1 to about 25:1, from about 3:1 to about 15:1, from about 4:1 to about 10:1, from about 5:1 to about 9:1, or about 6:1 to about 9:1. Ranges intermediate to the above recited ranges are also contemplated to be part of the invention.

The cationic lipid can be, for example, N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleoyloxypropylamine (DODMA), 1,2-DiLinoleoyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-Dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-Dilinoleoxy-3-(dimethylamino)acetoxyp propane (DLin-DAC), 1,2-Dilinoleoxy-3-morpholinopropane (DLin-MA), 1,2-Dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-Dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-Linoleoyl-2-linoleoyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-Dilinoleoyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-Dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-Dilinoleoyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), or 3-(N,N-Dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-Dioleylamino)-1,2-propanedio (DOAP), 1,2-Dilinoleoxy-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA), 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA) or analogs thereof, (3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALN100), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3), 1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediyl)didodecan-2-ol (Tech G1), or a mixture thereof. The cationic lipid can

comprise from about 20 mol % to about 50 mol % or about 40 mol % of the total lipid present in the particle.

In another embodiment, the compound 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane can be used to prepare lipid-santisense polynucleotide agent nanoparticles. Synthesis of 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane is described in United States provisional patent application number 61/107,998 filed on October 23, 2008, which is herein incorporated by reference.

In one embodiment, the lipid-antisense polynucleotide agent particle includes 40% 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane: 10% DSPC: 40% Cholesterol: 10% PEG-C-DOMG (mole percent) with a particle size of 63.0 ± 20 nm and a 0.027 antisense polynucleotide agent/Lipid Ratio.

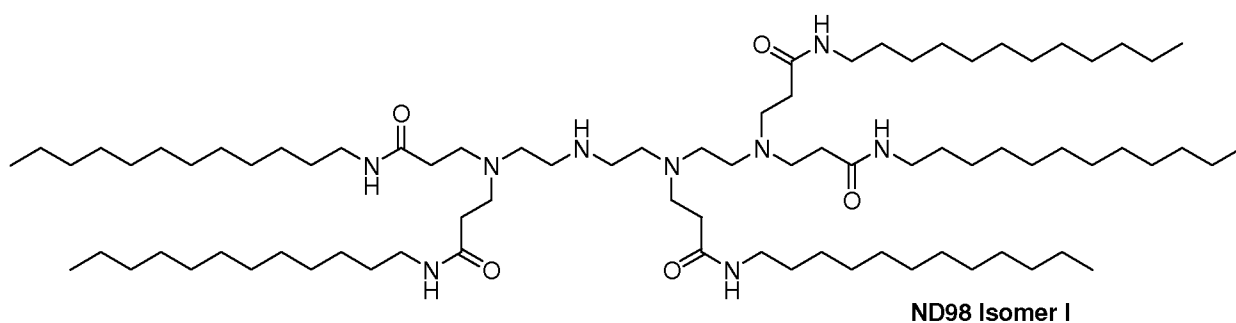
The ionizable/non-cationic lipid can be an anionic lipid or a neutral lipid including, but not limited to, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1 -trans PE, 1 -stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE), cholesterol, or a mixture thereof. The non-cationic lipid can be from about 5 mol % to about 90 mol %, about 10 mol %, or about 58 mol % if cholesterol is included, of the total lipid present in the particle.

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

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

In one embodiment, the lipidoid ND98-4HCl (MW 1487) (see U.S. Patent Application No. 12/056,230, filed 3/26/2008, which is incorporated herein by reference), Cholesterol (Sigma-Aldrich), and PEG-Ceramide C16 (Avanti Polar Lipids) can be used to prepare lipid-antisense polynucleotide agent nanoparticles (*i.e.*, LNP01 particles). Stock solutions of each in ethanol can be prepared as follows: ND98, 133 mg/ml; Cholesterol, 25 mg/ml, PEG-

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



Formula 1

LNP01 formulations are described, *e.g.*, in International Application Publication No. WO 2008/042973, which is hereby incorporated by reference.

Additional exemplary lipid-antisense polynucleotide agent formulations are described in Table 1.

Table 1

	Ionizable/Cationic Lipid	cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate Lipid:santisense polynucleotide agent ratio
SNALP-1	1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)	DLinDMA/DPPC/Cholesterol/PEG-cDMA (57.1/7.1/34.4/1.4) lipid:santisense polynucleotide agent ~ 7:1
2-XTC	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DPPC/Cholesterol/PEG-cDMA 57.1/7.1/34.4/1.4 lipid:santisense polynucleotide agent ~ 7:1
LNP05	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:santisense polynucleotide agent ~ 6:1

LNP06	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:santisense polynucleotide agent ~ 11:1
LNP07	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:santisense polynucleotide agent ~ 6:1
LNP08	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:santisense polynucleotide agent ~ 11:1
LNP09	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:santisense polynucleotide agent 10:1
LNP10	(3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALN100)	ALN100/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:santisense polynucleotide agent 10:1
LNP11	(6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3)	MC-3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:santisense polynucleotide agent 10:1
LNP12	1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediy)didodecan-2-ol (Tech G1)	Tech G1/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:santisense polynucleotide agent 10:1
LNP13	XTC	XTC/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:santisense polynucleotide agent: 33:1
LNP14	MC3	MC3/DSPC/Chol/PEG-DMG 40/15/40/5 Lipid:santisense polynucleotide agent: 11:1
LNP15	MC3	MC3/DSPC/Chol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5 Lipid:santisense polynucleotide agent: 11:1
LNP16	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5

		Lipid:santisense polynucleotide agent: 7:1
LNP17	MC3	MC3/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:santisense polynucleotide agent: 10:1
LNP18	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:santisense polynucleotide agent: 12:1
LNP19	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/35/5 Lipid:santisense polynucleotide agent: 8:1
LNP20	MC3	MC3/DSPC/Chol/PEG-DPG 50/10/38.5/1.5 Lipid:santisense polynucleotide agent: 10:1
LNP21	C12-200	C12-200/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:santisense polynucleotide agent: 7:1
LNP22	XTC	XTC/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:santisense polynucleotide agent: 10:1

DSPC: distearoylphosphatidylcholine

DPPC: dipalmitoylphosphatidylcholine

PEG-DMG: PEG-didimyrystoyl glycerol (C14-PEG, or PEG-C14) (PEG with avg mol wt of 2000)

PEG-DSG: PEG-distyryl glycerol (C18-PEG, or PEG-C18) (PEG with avg mol wt of 2000)

PEG-cDMA: PEG-carbamoyl-1,2-dimyristyloxypropylamine (PEG with avg mol wt of 2000)

SNALP (1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)) comprising formulations are described in International Publication No. WO2009/127060, filed April 15, 2009, which is hereby incorporated by reference.

XTC comprising formulations are described, *e.g.*, in U.S. Provisional Serial No. 61/148,366, filed January 29, 2009; U.S. Provisional Serial No. 61/156,851, filed March 2, 2009; U.S. Provisional Serial No. filed June 10, 2009; U.S. Provisional Serial No. 61/228,373, filed July 24, 2009; U.S. Provisional Serial No. 61/239,686, filed September 3, 2009, and International Application No. PCT/US2010/022614, filed January 29, 2010, which are hereby incorporated by reference.

MC3 comprising formulations are described, *e.g.*, in U.S. Publication No. 2010/0324120, filed June 10, 2010, the entire contents of which are hereby incorporated by reference.

ALNY-100 comprising formulations are described, *e.g.*, International patent application number PCT/US09/63933, filed on November 10, 2009, which is hereby incorporated by reference.

C12-200 comprising formulations are described in U.S. Provisional Serial No. 61/175,770, filed May 5, 2009 and International Application No. PCT/US10/33777, filed May 5, 2010, which are hereby incorporated by reference.

Synthesis of ionizable/cationic lipids

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

“Alkyl” means a straight chain or branched, noncyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms. Representative saturated straight chain alkyls include methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, and the like; while saturated branched alkyls include isopropyl, sec-butyl, isobutyl, tert-butyl, isopentyl, and the like. Representative saturated cyclic alkyls include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and the like; while unsaturated cyclic alkyls include cyclopentenyl and cyclohexenyl, and the like.

“Alkenyl” means an alkyl, as defined above, containing at least one double bond between adjacent carbon atoms. Alkenyls include both cis and trans isomers. Representative straight chain and branched alkenyls include ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, 2,3-dimethyl-2-butenyl, and the like.

“Alkynyl” means any alkyl or alkenyl, as defined above, which additionally contains at least one triple bond between adjacent carbons. Representative straight chain and branched alkynyls include acetylenyl, propynyl, 1-butylnyl, 2-butylnyl, 1-pentylnyl, 2-pentylnyl, 3-methyl-1 butynyl, and the like.

“Acyl” means any alkyl, alkenyl, or alkynyl wherein the carbon at the point of attachment is substituted with an oxo group, as defined below. For example, -C(=O)alkyl, -C(=O)alkenyl, and -C(=O)alkynyl are acyl groups.

“Heterocycle” means a 5- to 7-membered monocyclic, or 7- to 10-membered bicyclic, heterocyclic ring which is either saturated, unsaturated, or aromatic, and which contains from 1 or 2 heteroatoms independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and sulfur heteroatoms can be optionally oxidized, and the nitrogen heteroatom can be optionally quaternized, including bicyclic rings in which any of the above heterocycles are

fused to a benzene ring. The heterocycle can be attached *via* any heteroatom or carbon atom. Heterocycles include heteroaryls as defined below. Heterocycles include morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, and the like.

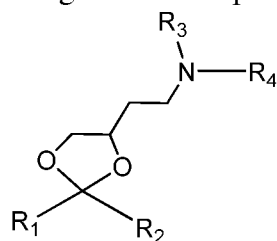
The terms “optionally substituted alkyl”, “optionally substituted alkenyl”, “optionally substituted alkynyl”, “optionally substituted acyl”, and “optionally substituted heterocycle” means that, when substituted, at least one hydrogen atom is replaced with a substituent. In the case of an oxo substituent (=O) two hydrogen atoms are replaced. In this regard, substituents include oxo, halogen, heterocycle, -CN, -OR_x, -NR_xR_y, -NR_xC(=O)R_y, -NR_xSO₂R_y, -C(=O)R_x, -C(=O)OR_x, -C(=O)NR_xR_y, -SOnR_x and -SOnNR_xR_y, wherein n is 0, 1 or 2, R_x and R_y are the same or different and independently hydrogen, alkyl or heterocycle, and each of said alkyl and heterocycle substituents can be further substituted with one or more of oxo, halogen, -OH, -CN, alkyl, -OR_x, heterocycle, -NR_xR_y, -NR_xC(=O)R_y, -NR_xSO₂R_y, -C(=O)R_x, -C(=O)OR_x, -C(=O)NR_xR_y, -SOnR_x and -SOnNR_xR_y.

“Halogen” means fluoro, chloro, bromo and iodo.

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

Synthesis of Formula A

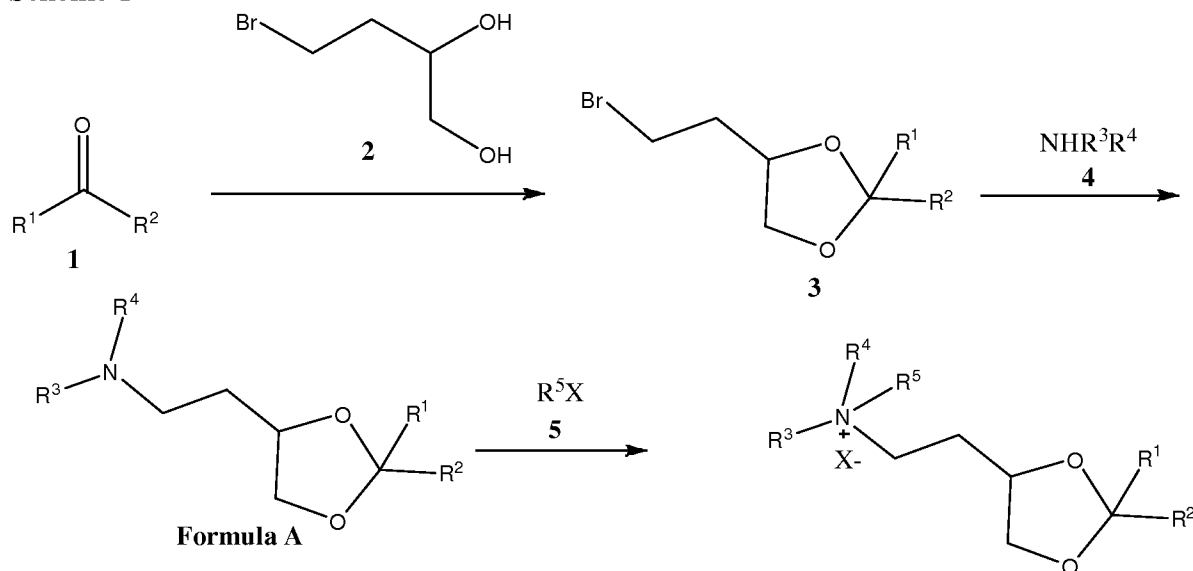
In some embodiments, nucleic acid-lipid particles of the invention are formulated using a cationic lipid of formula A:



where R₁ and R₂ are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R₃ and R₄ are independently lower alkyl or R₃ and R₄ can be taken together to form an optionally substituted heterocyclic ring. In some embodiments, the

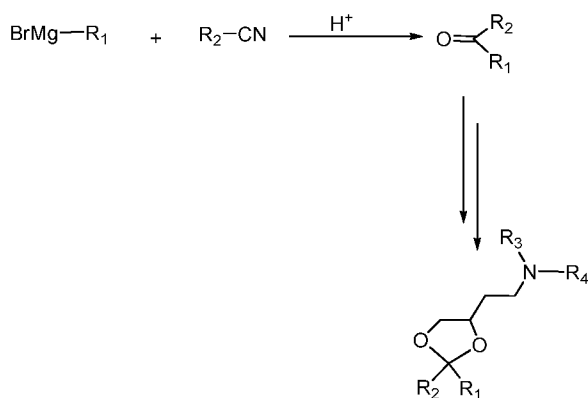
cationic lipid is XTC (2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane). In general, the lipid of formula A above can be made by the following Reaction Schemes 1 or 2, wherein all substituents are as defined above unless indicated otherwise.

Scheme 1



Lipid A, where R¹ and R² are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R³ and R⁴ are independently lower alkyl or R³ and R⁴ can be taken together to form an optionally substituted heterocyclic ring, can be prepared according to Scheme 1. Ketone **1** and bromide **2** can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of **1** and **2** yields ketal **3**. Treatment of ketal **3** with amine **4** yields lipids of formula A. The lipids of formula A can be converted to the corresponding ammonium salt with an organic salt of formula **5**, where X is anion counter ion selected from halogen, hydroxide, phosphate, sulfate, or the like.

Scheme 2

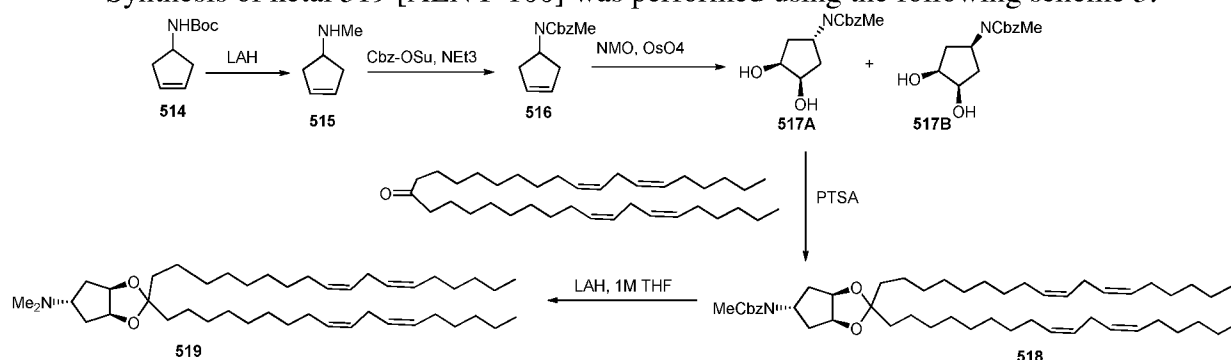


Alternatively, the ketone **1** starting material can be prepared according to Scheme 2. Grignard reagent **6** and cyanide **7** can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of **6** and **7** yields ketone **1**. Conversion of ketone **1** to the corresponding lipids of formula A is as described in Scheme 1.

Synthesis of MC3

Preparation of DLin-M-C3-DMA (*i.e.*, (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate) was as follows. A solution of (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-ol (0.53 g), 4-N,N-dimethylaminobutyric acid hydrochloride (0.51 g), 4-N,N-dimethylaminopyridine (0.61g) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.53 g) in dichloromethane (5 mL) was stirred at room temperature overnight. The solution was washed with dilute hydrochloric acid followed by dilute aqueous sodium bicarbonate. The organic fractions were dried over anhydrous magnesium sulphate, filtered and the solvent removed on a rotovap. The residue was passed down a silica gel column (20 g) using a 1-5% methanol/dichloromethane elution gradient. Fractions containing the purified product were combined and the solvent removed, yielding a colorless oil (0.54 g). *Synthesis of ALNY-100*

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



Synthesis of 515

To a stirred suspension of LiAlH₄ (3.74 g, 0.09852 mol) in 200 ml anhydrous THF in a two neck RBF (1L), was added a solution of 514 (10g, 0.04926mol) in 70 mL of THF slowly at 0 °C under nitrogen atmosphere. After complete addition, reaction mixture was warmed to room temperature and then heated to reflux for 4 h. Progress of the reaction was monitored by TLC. After completion of reaction (by TLC) the mixture was cooled to 0 °C and quenched with careful addition of saturated Na₂SO₄ solution. Reaction mixture was stirred for 4 h at room temperature and filtered off. Residue was washed well with THF. The filtrate and washings were mixed and diluted with 400 mL dioxane and 26 mL conc. HCl and stirred for 20 minutes at room temperature. The volatilities were stripped off under vacuum to furnish the hydrochloride salt of 515 as a white solid. Yield: 7.12 g ¹H-NMR (DMSO, 400MHz): δ= 9.34 (broad, 2H), 5.68 (s, 2H), 3.74 (m, 1H), 2.66-2.60 (m, 2H), 2.50-2.45 (m, 5H).

Synthesis of 516

To a stirred solution of compound 515 in 100 mL dry DCM in a 250 mL two neck RBF, was added NEt₃ (37.2 mL, 0.2669 mol) and cooled to 0 °C under nitrogen atmosphere. After a slow addition of N-(benzyloxy-carbonyloxy)-succinimide (20 g, 0.08007 mol) in 50

mL dry DCM, reaction mixture was allowed to warm to room temperature. After completion of the reaction (2-3 h by TLC) mixture was washed successively with 1N HCl solution (1 x 100 mL) and saturated NaHCO₃ solution (1 x 50 mL). The organic layer was then dried over anhyd. Na₂SO₄ and the solvent was evaporated to give crude material which was purified by silica gel column chromatography to get 516 as sticky mass. Yield: 11g (89%). ¹H-NMR (CDCl₃, 400MHz): δ = 7.36-7.27(m, 5H), 5.69 (s, 2H), 5.12 (s, 2H), 4.96 (br., 1H) 2.74 (s, 3H), 2.60(m, 2H), 2.30-2.25(m, 2H). LC-MS [M+H] -232.3 (96.94%).

Synthesis of 517A and 517B

The cyclopentene 516 (5 g, 0.02164 mol) was dissolved in a solution of 220 mL acetone and water (10:1) in a single neck 500 mL RBF and to it was added N-methyl morpholine-N-oxide (7.6 g, 0.06492 mol) followed by 4.2 mL of 7.6% solution of OsO₄ (0.275 g, 0.00108 mol) in tert-butanol at room temperature. After completion of the reaction (~ 3 h), the mixture was quenched with addition of solid Na₂SO₃ and resulting mixture was stirred for 1.5 h at room temperature. Reaction mixture was diluted with DCM (300 mL) and washed with water (2 x 100 mL) followed by saturated NaHCO₃ (1 x 50 mL) solution, water (1 x 30 mL) and finally with brine (1x 50 mL). Organic phase was dried over an.Na₂SO₄ and solvent was removed in vacuum. Silica gel column chromatographic purification of the crude material was afforded a mixture of diastereomers, which were separated by prep HPLC.

Yield: - 6 g crude

517A - Peak-1 (white solid), 5.13 g (96%). ¹H-NMR (DMSO, 400MHz): δ = 7.39-7.31(m, 5H), 5.04(s, 2H), 4.78-4.73 (m, 1H), 4.48-4.47(d, 2H), 3.94-3.93(m, 2H), 2.71(s, 3H), 1.72- 1.67(m, 4H). LC-MS - [M+H]-266.3, [M+NH₄ +]-283.5 present, HPLC-97.86%. Stereochemistry confirmed by X-ray.

Synthesis of 518

Using a procedure analogous to that described for the synthesis of compound 505, compound 518 (1.2 g, 41%) was obtained as a colorless oil. ¹H-NMR (CDCl₃, 400MHz): δ = 7.35-7.33(m, 4H), 7.30-7.27(m, 1H), 5.37-5.27(m, 8H), 5.12(s, 2H), 4.75(m,1H), 4.58-4.57(m,2H), 2.78-2.74(m,7H), 2.06-2.00(m,8H), 1.96-1.91(m, 2H), 1.62(m, 4H), 1.48(m, 2H), 1.37-1.25(br m, 36H), 0.87(m, 6H). HPLC-98.65%.

General Procedure for the Synthesis of Compound 519

A solution of compound 518 (1 eq) in hexane (15 mL) was added in a drop-wise fashion to an ice-cold solution of LAH in THF (1 M, 2 eq). After complete addition, the mixture was heated at 40°C over 0.5 h then cooled again on an ice bath. The mixture was carefully hydrolyzed with saturated aqueous Na₂SO₄ then filtered through celite and reduced to an oil. Column chromatography provided the pure 519 (1.3 g, 68%) which was obtained as a colorless oil. ¹³C NMR δ = 130.2, 130.1 (x2), 127.9 (x3), 112.3, 79.3, 64.4, 44.7, 38.3, 35.4, 31.5, 29.9 (x2), 29.7, 29.6 (x2), 29.5 (x3), 29.3 (x2), 27.2 (x3), 25.6, 24.5, 23.3, 22.6,

14.1; Electrospray MS (+ve): Molecular weight for C₄₄H₈₀NO₂ (M + H)⁺ Calc. 654.6, Found 654.6.

Formulations prepared by either the standard or extrusion-free method can be characterized in similar manners. For example, formulations are typically characterized by visual inspection. They should be whitish translucent solutions free from aggregates or sediment. Particle size and particle size distribution of lipid-nanoparticles can be measured by light scattering using, for example, a Malvern Zetasizer Nano ZS (Malvern, USA). Particles should be about 20-300 nm, such as 40-100 nm in size. The particle size distribution should be unimodal. The total antisense polynucleotide agent concentration in the formulation, as well as the entrapped fraction, is estimated using a dye exclusion assay. A sample of the formulated antisense polynucleotide agent can be incubated with an RNA-binding dye, such as Ribogreen (Molecular Probes) in the presence or absence of a formulation disrupting surfactant, *e.g.*, 0.5% Triton-X100. The total antisense polynucleotide agent in the formulation can be determined by the signal from the sample containing the surfactant, relative to a standard curve. The entrapped fraction is determined by subtracting the “free” antisense polynucleotide agent content (as measured by the signal in the absence of surfactant) from the total antisense polynucleotide agent content. Percent entrapped antisense polynucleotide agent is typically >85%. For SNALP formulation, the particle size is at least 30 nm, at least 40 nm, at least 50 nm, at least 60 nm, at least 70 nm, at least 80 nm, at least 90 nm, at least 100 nm, at least 110 nm, and at least 120 nm. The suitable range is typically about at least 50 nm to about at least 110 nm, about at least 60 nm to about at least 100 nm, or about at least 80 nm to about at least 90 nm.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitables. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders can be desirable. In some embodiments, oral formulations are those in which the antisense polynucleotide agents featured in the invention are administered in conjunction with one or more penetration enhancer surfactants and chelators. Suitable surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Suitable bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Suitable fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (*e.g.*, sodium). In some

embodiments, combinations of penetration enhancers are used, for example, fatty acids/salts in combination with bile acids/salts. One exemplary combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Antisense polynucleotide agents featured in the invention can be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Antisense polynucleotide agent complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pullulans, celluloses and starches. Suitable complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylaminomethylethylene P(TDAE), polyaminostyrene (*e.g.*, p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for antisense polynucleotide agents and their preparation are described in detail in U.S. Patent 6,887,906, US Publ. No. 20030027780, and U.S. Patent No. 6,747,014, each of which is incorporated herein by reference.

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

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

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

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

C. Additional Formulations

i. Emulsions

The compositions of the present invention can be prepared and formulated as emulsions. Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μ m in diameter (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 301). Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions can be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions can contain additional components in addition to the dispersed phases, and the active drug which can be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants can also be present in emulsions as needed. Pharmaceutical emulsions can also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or

continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion can be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that can be incorporated into either phase of the emulsion. Emulsifiers can broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants can be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and

antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that can readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used can be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (see *e.g.*, Ansel's *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (see *e.g.*, Ansel's *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

ii. Microemulsions

In one embodiment of the present invention, the compositions of antisense polynucleotide agents are formulated as microemulsions. A microemulsion can be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: Controlled Release of Drugs: Polymers and Aggregate Systems, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared *via* a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol,

serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions can, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase can typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase can include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (see *e.g.*, U.S. Patent Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides *et al.*, *Pharmaceutical Research*, 1994, 11, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (see *e.g.*, U.S. Patent Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides *et al.*, *Pharmaceutical Research*, 1994, 11, 1385; Ho *et al.*, *J. Pharm. Sci.*, 1996, 85, 138-143). Often microemulsions can form spontaneously when their components are brought together at ambient temperature. This can be particularly advantageous when formulating thermolabile drugs, peptides or antisense polynucleotide agents. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of antisense polynucleotide agents from the gastrointestinal tract, as well as improve the local cellular uptake of antisense polynucleotide agents and nucleic acids.

Microemulsions of the present invention can also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the antisense polynucleotide agents of the present invention. Penetration enhancers used in the microemulsions of the present invention can be classified as belonging to one of five broad categories--surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed above.

iii. Microparticles

An antisense polynucleotide agent of the invention may be incorporated into a particle, *e.g.*, a microparticle. Microparticles can be produced by spray-drying, but may also be produced by other methods including lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination of these techniques.

iv. Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly antisense polynucleotide agents, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs can cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers can be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (see *e.g.*, Malmsten, M. *Surfactants and polymers in drug delivery*, Informa Health Care, New York, NY, 2002; Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of antisense polynucleotide agents through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (see *e.g.*, Malmsten, M. *Surfactants and polymers in drug delivery*, Informa Health Care, New York, NY, 2002; Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, *J. Pharm. Pharmacol.*, 1988, 40, 252).

Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprinate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C₁₋₂₀ alkyl esters thereof (*e.g.*, methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (see *e.g.*, Touitou, E., *et al.* *Enhancement in Drug Delivery*, CRC Press, Danvers, MA, 2006; Lee *et al.*, *Critical Reviews*

in Therapeutic Drug Carrier Systems, 1991, p.92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; El Hariri *et al.*, *J. Pharm. Pharmacol.*, 1992, 44, 651-654).

The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (see *e.g.*, Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. Suitable bile salts include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (see *e.g.*, Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Yamamoto *et al.*, *J. Pharm. Exp. Ther.*, 1992, 263, 25; Yamashita *et al.*, *J. Pharm. Sci.*, 1990, 79, 579-583).

Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of antisense polynucleotide agents through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, 1993, 618, 315-339). Suitable chelating agents include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines)(see *e.g.*, Katdare, A. *et al.*, Excipient development for pharmaceutical, biotechnology, and drug delivery, CRC Press, Danvers, MA, 2006; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur *et al.*, *J. Control Rel.*, 1990, 14, 43-51).

As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of antisense polynucleotide agents through the alimentary mucosa (see *e.g.*, Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33). This class of penetration enhancers includes, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, 1987, 39, 621-626).

Agents that enhance uptake of antisense polynucleotide agents at the cellular level can also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al.*, U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of antisense polynucleotide agents. Examples of commercially available transfection reagents include, for example Lipofectamine™ (Invitrogen; Carlsbad, CA), Lipofectamine 2000™ (Invitrogen; Carlsbad, CA), 293fectin™ (Invitrogen; Carlsbad, CA), Cellfectin™ (Invitrogen; Carlsbad, CA), DMRIE-C™ (Invitrogen; Carlsbad, CA), FreeStyle™ MAX (Invitrogen; Carlsbad, CA), Lipofectamine™ 2000 CD (Invitrogen; Carlsbad, CA), Lipofectamine™ (Invitrogen; Carlsbad, CA), RNAiMAX (Invitrogen; Carlsbad, CA), Oligofectamine™ (Invitrogen; Carlsbad, CA), Optifect™ (Invitrogen; Carlsbad, CA), X-tremeGENE Q2 Transfection Reagent (Roche; Grenzacherstrasse, Switzerland), DOTAP Liposomal Transfection Reagent (Grenzacherstrasse, Switzerland), DOSPER Liposomal Transfection Reagent (Grenzacherstrasse, Switzerland), or Fugene (Grenzacherstrasse, Switzerland), Transfectam® Reagent (Promega; Madison, WI), TransFast™ Transfection Reagent (Promega; Madison, WI), Tfx™-20 Reagent (Promega; Madison, WI), Tfx™-50 Reagent (Promega; Madison, WI), DreamFect™ (OZ Biosciences; Marseille, France), EcoTransfect (OZ Biosciences; Marseille, France), TransPass^a D1 Transfection Reagent (New England Biolabs; Ipswich, MA, USA), LyoVec™/LipoGen™ (Invitrogen; San Diego, CA, USA), PerFectin Transfection Reagent (Genlantis; San Diego, CA, USA), NeuroPORTER Transfection Reagent (Genlantis; San Diego, CA, USA), GenePORTER Transfection reagent (Genlantis; San Diego, CA, USA), GenePORTER 2 Transfection reagent (Genlantis; San Diego, CA, USA), Cytofectin Transfection Reagent (Genlantis; San Diego, CA, USA), BaculoPORTER Transfection Reagent (Genlantis; San Diego, CA, USA), TroganPORTER™ transfection Reagent (Genlantis; San Diego, CA, USA), RiboFect (Bioline; Taunton, MA, USA), PlasFect (Bioline; Taunton, MA, USA), UniFECTOR (B-Bridge International; Mountain View, CA, USA), SureFECTOR (B-Bridge International; Mountain View, CA, USA), or HiFect™ (B-Bridge International, Mountain View, CA, USA), among others.

Other agents can be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

v. Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, “carrier compound” or “carrier” can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioated antisense polynucleotide agent in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, Antisense polynucleotide agent Res. Dev., 1995, 5, 115-121; Takakura *et al.*, Antisense polynucleotide agent & Nucl. Acid Drug Dev., 1996, 6, 177-183).

vi. Excipients

In contrast to a carrier compound, a “pharmaceutical carrier” or “excipient” is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient can be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*).

Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols,

gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids can include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions can also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

vii. Other Components

The compositions of the present invention can additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions can contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or can contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

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

In some embodiments, pharmaceutical compositions featured in the invention include (a) one or more antisense polynucleotide agents and (b) one or more agents which function by a non-antisense inhibition mechanism and which are useful in treating a hemolytic disorder. Examples of such agents include, but are not limited to an anti-inflammatory agent, anti-steatosis agent, anti-viral, and/or anti-fibrosis agent. In addition, other substances commonly used to protect the liver, such as silymarin, can also be used in conjunction with the antisense polynucleotide agents described herein. Other agents useful for treating liver diseases include telbivudine, entecavir, and protease inhibitors such as telaprevir and other disclosed, for example, in Tung *et al.*, U.S. Application Publication Nos. 2005/0148548,

2004/0167116, and 2003/0144217; and in Hale *et al.*, U.S. Application Publication No. 2004/0127488.

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

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

In addition to their administration, as discussed above, the antisense polynucleotide agents featured in the invention can be administered in combination with other known agents effective in treatment of pathological processes mediated by C5 expression. In any event, the administering physician can adjust the amount and timing of antisense polynucleotide agent administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

VII. Methods For Inhibiting C5 Expression

The present invention provides methods of inhibiting expression of C5 in a cell. The methods include contacting a cell with an antisense polynucleotide agent of the invention in an amount effective to inhibit expression of the C5 in the cell, thereby inhibiting expression of the C5 in the cell.

Contacting of a cell with an antisense polynucleotide agent may be done *in vitro* or *in vivo*. Contacting a cell *in vivo* with the antisense polynucleotide agent includes contacting a cell or group of cells within a subject, *e.g.*, a human subject, with the antisense polynucleotide agent. Combinations of *in vitro* and *in vivo* methods of contacting are also possible. Contacting may be direct or indirect, as discussed above. Furthermore, contacting

a cell may be accomplished *via* a targeting ligand, including any ligand described herein or known in the art. In preferred embodiments, the targeting ligand is a carbohydrate moiety, *e.g.*, a GalNAc₃ ligand, or any other ligand that directs the antisense polynucleotide agent to a site of interest, *e.g.*, the liver of a subject.

The term “inhibiting,” as used herein, is used interchangeably with “reducing,” “silencing,” “downregulating” and other similar terms, and includes any level of inhibition.

The phrase “inhibiting expression of a C5” is intended to refer to inhibition of expression of any C5 gene (such as, *e.g.*, a mouse C5 gene, a rat C5 gene, a monkey C5 gene, or a human C5 gene) as well as variants or mutants of a C5 gene. Thus, the C5 gene may be a wild-type C5 gene, a mutant C5 gene, or a transgenic C5 gene in the context of a genetically manipulated cell, group of cells, or organism.

“Inhibiting expression of a C5 gene” includes any level of inhibition of a C5 gene, *e.g.*, at least partial suppression of the expression of a C5 gene. The expression of the C5 gene may be assessed based on the level, or the change in the level, of any variable associated with C5 gene expression, *e.g.*, C5 mRNA level, C5 protein level, or for example, CH₅₀ activity as a measure of total hemolytic complement, AH₅₀ to measure the hemolytic activity of the alternate pathway of complement, and/or lactate dehydrogenase (LDH) levels as a measure of intravascular hemolysis, and/or hemoglobin levels. Levels of C5a, C5b, and soluble C5b-9 complex may also be measured to assess C5 expression. This level may be assessed in an individual cell or in a group of cells, including, for example, a sample derived from a subject.

Inhibition may be assessed by a decrease in an absolute or relative level of one or more variables that are associated with C5 expression compared with a control level. The control level may be any type of control level that is utilized in the art, *e.g.*, a pre-dose baseline level, or a level determined from a similar subject, cell, or sample that is untreated or treated with a control (such as, *e.g.*, buffer only control or inactive agent control).

In some embodiments of the methods of the invention, expression of a C5 gene is inhibited by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%.

Inhibition of the expression of a C5 gene may be manifested by a reduction of the amount of mRNA expressed by a first cell or group of cells (such cells may be present, for example, in a sample derived from a subject) in which a C5 gene is transcribed and which has or have been treated (*e.g.*, by contacting the cell or cells with an antisense polynucleotide agent of the invention, or by administering an antisense polynucleotide agent of the invention

to a subject in which the cells are or were present) such that the expression of a C5 gene is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has not or have not been so treated (control cell(s)). In preferred embodiments, the inhibition is assessed by expressing the level of mRNA in treated cells as a percentage of the level of mRNA in control cells, using the following formula:

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

Alternatively, inhibition of the expression of a C5 gene may be assessed in terms of a reduction of a parameter that is functionally linked to C5 gene expression, *e.g.*, C5 protein expression, CH₅₀ activity, AH₅₀, lactate dehydrogenase (LDH) levels, hemoglobin levels, mRNA or protein levels of C5a, C5b, and soluble C5b-9 complex in tissues or serum. C5 gene silencing may be determined in any cell expressing C5, either constitutively or by genomic engineering, and by any assay known in the art. The liver is the major site of C5 expression. Other significant sites of expression include the kidneys and the uterus.

Inhibition of the expression of a C5 protein may be manifested by a reduction in the level of the C5 protein that is expressed by a cell or group of cells (*e.g.*, the level of protein expressed in a sample derived from a subject). As explained above for the assessment of mRNA suppression, the inhibition of protein expression levels in a treated cell or group of cells may similarly be expressed as a percentage of the level of protein in a control cell or group of cells.

A control cell or group of cells that may be used to assess the inhibition of the expression of a C5 gene includes a cell or group of cells that has not yet been contacted with an antisense polynucleotide agent of the invention. For example, the control cell or group of cells may be derived from an individual subject (*e.g.*, a human or animal subject) prior to treatment of the subject with an antisense polynucleotide agent.

The level of C5 mRNA that is expressed by a cell or group of cells may be determined using any method known in the art for assessing mRNA expression. In one embodiment, the level of expression of C5 in a sample is determined by detecting a transcribed polynucleotide, or portion thereof, *e.g.*, mRNA of the C5 gene. RNA may be extracted from cells using RNA extraction techniques including, for example, using acid phenol/guanidine isothiocyanate extraction (RNAzol B; Biogenesis), RNeasy RNA preparation kits (Qiagen) or PAXgene (PreAnalytix, Switzerland). Typical assay formats utilizing ribonucleic acid hybridization include nuclear run-on assays, RT-PCR, RNase protection assays (Melton *et al.*, *Nuc. Acids Res.* 12:7035), Northern blotting, *in situ* hybridization, and microarray analysis.

In one embodiment, the level of expression of C5 is determined using a nucleic acid probe. The term "probe", as used herein, refers to any molecule that is capable of selectively binding to a specific C5. Probes can be synthesized by one of skill in the art, or derived from

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

Isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, polymerase chain reaction (PCR) analyses and probe arrays. One method for the determination of mRNA levels involves contacting the isolated mRNA with a nucleic acid molecule (probe) that can hybridize to C5 mRNA. In one embodiment, the mRNA is immobilized on a solid surface and contacted with a probe, for example by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative embodiment, the probe(s) are immobilized on a solid surface and the mRNA is contacted with the probe(s), for example, in an Affymetrix gene chip array. A skilled artisan can readily adapt known mRNA detection methods for use in determining the level of C5 mRNA.

An alternative method for determining the level of expression of C5 in a sample involves the process of nucleic acid amplification and/or reverse transcriptase (to prepare cDNA) of for example mRNA in the sample, *e.g.*, by RT-PCR (the experimental embodiment set forth in Mullis, 1987, U.S. Pat. No. 4,683,202), ligase chain reaction (Barany (1991) *Proc. Natl. Acad. Sci. USA* 88:189-193), self sustained sequence replication (Guatelli et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi *et al.* (1988) *Bio/Technology* 6:1197), rolling circle replication (Lizardi et al., U.S. Pat. No. 5,854,033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers. In particular aspects of the invention, the level of expression of C5 is determined by quantitative fluorogenic RT-PCR (*i.e.*, the TaqMan™ System).

The expression levels of C5 mRNA may be monitored using a membrane blot (such as used in hybridization analysis such as Northern, Southern, dot, and the like), or microwells, sample tubes, gels, beads or fibers (or any solid support comprising bound nucleic acids). See U.S. Pat. Nos. 5,770,722, 5,874,219, 5,744,305, 5,677,195 and 5,445,934, which are incorporated herein by reference. The determination of C5 expression level may also comprise using nucleic acid probes in solution.

In preferred embodiments, the level of mRNA expression is assessed using branched DNA (bDNA) assays or real time PCR (qPCR). The use of these methods is described and exemplified in the Examples presented herein.

The level of C5 protein expression may be determined using any method known in the art for the measurement of protein levels. Such methods include, for example, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC),

thin layer chromatography (TLC), hyperdiffusion chromatography, fluid or gel precipitin reactions, absorption spectroscopy, a colorimetric assays, spectrophotometric assays, flow cytometry, immunodiffusion (single or double), immunoelectrophoresis, Western blotting, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, electrochemiluminescence assays, and the like.

The term “sample” as used herein refers to a collection of similar fluids, cells, or tissues isolated from a subject, as well as fluids, cells, or tissues present within a subject. Examples of biological fluids include blood, serum and serosal fluids, plasma, lymph, urine, cerebrospinal fluid, saliva, ocular fluids, and the like. Tissue samples may include samples from tissues, organs or localized regions. For example, samples may be derived from particular organs, parts of organs, or fluids or cells within those organs. In certain embodiments, samples may be derived from the liver (*e.g.*, whole liver or certain segments of liver or certain types of cells in the liver, such as, *e.g.*, hepatocytes). In preferred embodiments, a “sample derived from a subject” refers to blood or plasma drawn from the subject. In further embodiments, a “sample derived from a subject” refers to liver tissue derived from the subject.

In some embodiments of the methods of the invention, the antisense polynucleotide agent is administered to a subject such that the antisense polynucleotide agent is delivered to a specific site within the subject. The inhibition of expression of C5 may be assessed using measurements of the level or change in the level of C5 mRNA or C5 protein in a sample derived from fluid or tissue from the specific site within the subject. In preferred embodiments, the site is the liver. The site may also be a subsection or subgroup of cells from any one of the aforementioned sites. The site may also include cells that express a particular type of receptor.

The phrase “contacting a cell with an antisense polynucleotide agent,” as used herein, includes contacting a cell by any possible means. Contacting a cell with an antisense polynucleotide agent includes contacting a cell *in vitro* with the antisense polynucleotide agent or contacting a cell *in vivo* with the antisense polynucleotide agent. The contacting may be done directly or indirectly. Thus, for example, the antisense polynucleotide agent may be put into physical contact with the cell by the individual performing the method, or alternatively, the antisense polynucleotide agent may be put into a situation that will permit or cause it to subsequently come into contact with the cell.

Contacting a cell *in vitro* may be done, for example, by incubating the cell with the antisense polynucleotide agent. Contacting a cell *in vivo* may be done, for example, by injecting the antisense polynucleotide agent into or near the tissue where the cell is located, or by injecting the antisense polynucleotide agent into another area, *e.g.*, the bloodstream or the subcutaneous space, such that the agent will subsequently reach the tissue where the cell to be contacted is located. For example, the antisense polynucleotide agent may contain and/or be

coupled to a ligand, *e.g.*, GalNAc3, that directs the antisense polynucleotide agent to a site of interest, *e.g.*, the liver. Combinations of *in vitro* and *in vivo* methods of contacting are also possible. For example, a cell may also be contacted *in vitro* with an antisense polynucleotide agent and subsequently transplanted into a subject.

In one embodiment, contacting a cell with an antisense polynucleotide agent includes “introducing” or “delivering the antisense polynucleotide agent into the cell” by facilitating or effecting uptake or absorption into the cell. Absorption or uptake of an antisense polynucleotide agent can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. Introducing an antisense polynucleotide agent into a cell may be *in vitro* and/or *in vivo*. For example, for *in vivo* introduction, antisense polynucleotide agent can be injected into a tissue site or administered systemically. *In vivo* delivery can also be done by a beta-glucan delivery system, such as those described in U.S. Patent Nos. 5,032,401 and 5,607,677, and U.S. Publication No. 2005/0281781, the entire contents of which are hereby incorporated herein by reference. *In vitro* introduction into a cell includes methods known in the art such as electroporation and lipofection. Further approaches are described herein below and/or are known in the art.

VIII. Methods for Treating or Preventing a Complement Component C5-Associated Disorder

The present invention also provides therapeutic and prophylactic methods which include administering to a subject having a complement component C5-associated disease, *e.g.*, PNH or aHUS, an antisense polynucleotide agent or pharmaceutical compositions comprising an antisense polynucleotide agent of the invention. In some aspects of the invention, the methods further include administering to the subject an additional therapeutic agent, such as an anti-complement component C5 antibody, or antigen-binding fragment thereof (*e.g.*, eculizumab).

In one aspect, the present invention provides methods of treating a subject having a disorder that would benefit from reduction in C5 expression, *e.g.*, a complement component C5-associated disease, *e.g.*, PNH or aHUS. The treatment methods (and uses) of the invention include administering to the subject, *e.g.*, a human, a therapeutically effective amount of an antisense polynucleotide agent targeting a C5 gene or a pharmaceutical composition comprising an antisense polynucleotide agent targeting a C5 gene, thereby treating the subject having a disorder that would benefit from reduction in C5 expression.

In another aspect, the present invention provides methods of treating a subject having a disorder that would benefit from reduction in C5 expression, *e.g.*, a complement component C5-associated disease, *e.g.*, PNH or aHUS, which include administering to the subject, *e.g.*, a human, a therapeutically effective amount of an antisense polynucleotide agent targeting a C5 gene or a pharmaceutical composition comprising an antisense polynucleotide agent targeting

a C5 gene, and an additional therapeutic agent, such as an anti-complement component C5 antibody, or antigen-binding fragment thereof (*e.g.*, eculizumab), thereby treating the subject having a disorder that would benefit from reduction in C5 expression.

In one aspect, the invention provides methods of preventing at least one symptom in a subject having a disorder that would benefit from reduction in C5 expression, *e.g.*, a complement component C5-associated disease, *e.g.*, PNH or aHUS. The methods include administering to the subject a prophylactically effective amount of an antisense polynucleotide agent targeting a C5 gene or a pharmaceutical composition comprising an antisense polynucleotide agent targeting a C5 gene, thereby preventing at least one symptom in the subject having a disorder that would benefit from reduction in C5 expression. For example, the invention provides methods for preventing hemolysis in a subject suffering from a disorder that would benefit from reduction in C5 expression, *e.g.*, a complement component C5-associated disease, *e.g.*, PNH or aHUS.

In another aspect, the invention provides methods of preventing at least one symptom in a subject having a disorder that would benefit from reduction in C5 expression, *e.g.*, a complement component C5-associated disease, *e.g.*, PNH or aHUS. The methods include administering to the subject a prophylactically effective amount of an antisense polynucleotide agent targeting a C5 gene or a pharmaceutical composition comprising an antisense polynucleotide agent targeting a C5 gene, and an additional therapeutic agent, such as an anti-complement component C5 antibody, or antigen-binding fragment thereof (*e.g.*, eculizumab), thereby preventing at least one symptom in the subject having a disorder that would benefit from reduction in C5 expression.

"Therapeutically effective amount," as used herein, is intended to include the amount of an antisense polynucleotide agent or anti-complement component C5 antibody, or antigen-binding fragment thereof (*e.g.*, eculizumab), that, when administered to a subject having a complement component C5-associated disease, is sufficient to effect treatment of the disease (*e.g.*, by diminishing, ameliorating or maintaining the existing disease or one or more symptoms of disease). The "therapeutically effective amount" may vary depending on the antisense polynucleotide agent or antibody, or antigen-binding fragment thereof, how the agent is administered, the disease and its severity and the history, age, weight, family history, genetic makeup, the types of preceding or concomitant treatments, if any, and other individual characteristics of the subject to be treated.

"Prophylactically effective amount," as used herein, is intended to include the amount of an antisense polynucleotide agent or anti-complement component C5 antibody, or antigen-binding fragment thereof (*e.g.*, eculizumab), that, when administered to a subject having a complement component C5-associated disease but not yet (or currently) experiencing or displaying symptoms of the disease, and/or a subject at risk of developing a complement component C5-associated disease, *e.g.*, a subject having a graft and/or transplant, *e.g.*, a

sensitized or allogenic recipient, a subject having sepsis, and/or a subject having a myocardial infarction, is sufficient to prevent or ameliorate the disease or one or more symptoms of the disease. Ameliorating the disease includes slowing the course of the disease or reducing the severity of later-developing disease. The "prophylactically effective amount" may vary depending on the antisense polynucleotide agent or anti-complement component C5 antibody, or antigen-binding fragment thereof, how the agent or anti-complement component C5 antibody, or antigen-binding fragment thereof, is administered, the degree of risk of disease, and the history, age, weight, family history, genetic makeup, the types of preceding or concomitant treatments, if any, and other individual characteristics of the patient to be treated.

A "therapeutically effective amount" or "prophylactically effective amount" also includes an amount of an antisense polynucleotide agent or anti-complement component C5 antibody, or antigen-binding fragment thereof (*e.g.*, eculizumab), that produces some desired local or systemic effect at a reasonable benefit/risk ratio applicable to any treatment. antisense polynucleotide agents employed in the methods of the present invention may be administered in a sufficient amount to produce a reasonable benefit/risk ratio applicable to such treatment.

In another aspect, the present invention provides uses of a therapeutically effective amount of an antisense polynucleotide agent of the invention for treating a subject, *e.g.*, a subject that would benefit from a reduction and/or inhibition of C5 expression.

In another aspect, the present invention provides uses of a therapeutically effective amount of an antisense polynucleotide agent of the invention and an additional therapeutic agent, such as an anti-complement component C5 antibody, or antigen-binding fragment thereof (*e.g.*, eculizumab), for treating a subject, *e.g.*, a subject that would benefit from a reduction and/or inhibition of C5 expression.

In yet another aspect, the present invention provides use of an antisense polynucleotide agent of the invention targeting a C5 gene or a pharmaceutical composition comprising an antisense polynucleotide agent targeting a C5 gene in the manufacture of a medicament for treating a subject, *e.g.*, a subject that would benefit from a reduction and/or inhibition of C5 expression, such as a subject having a disorder that would benefit from reduction in C5 expression, *e.g.*, a complement component C5-associated disease, *e.g.*, PNH or aHUS.

In another aspect, the present invention provides uses of an antisense polynucleotide agent of the invention targeting a C5 gene or a pharmaceutical composition comprising an antisense polynucleotide agent targeting a C5 gene in the manufacture of a medicament for use in combination with an additional therapeutic agent, such as an anti-complement component C5 antibody, or antigen-binding fragment thereof (*e.g.*, eculizumab), for treating a

subject, *e.g.*, a subject that would benefit from a reduction and/or inhibition of C5 expression, *e.g.*, a complement component C5-associated disease, *e.g.*, PNH or aHUS.

In another aspect, the invention provides uses of an antisense polynucleotide agent of the invention for preventing at least one symptom in a subject suffering from a disorder that would benefit from a reduction and/or inhibition of C5 expression, such as a complement component C5-associated disease, *e.g.*, PNH or aHUS.

In yet another aspect, the invention provides uses of an antisense polynucleotide agent of the invention, and an additional therapeutic agent, such as an anti-complement component C5 antibody, or antigen-binding fragment thereof (*e.g.*, eculizumab), for preventing at least one symptom in a subject suffering from a disorder that would benefit from a reduction and/or inhibition of C5 expression, such as a complement component C5-associated disease, *e.g.*, PNH or aHUS.

In a further aspect, the present invention provides uses of an antisense polynucleotide agent of the invention in the manufacture of a medicament for preventing at least one symptom in a subject suffering from a disorder that would benefit from a reduction and/or inhibition of C5 expression, such as a a complement component C5-associated disease, *e.g.*, PNH or aHUS.

In a further aspect, the present invention provides uses of an antisense polynucleotide agent of the invention in the manufacture of a medicament for use in combination with an additional therapeutic agent, such as an anti-complement component C5 antibody, or antigen-binding fragment thereof (*e.g.*, eculizumab), for preventing at least one symptom in a subject suffering from a disorder that would benefit from a reduction and/or inhibition of C5 expression, such as a a complement component C5-associated disease, *e.g.*, PNH or aHUS.

In one embodiment, an antisense polynucleotide agent targeting C5 is administered to a subject having a complement component C5-associated disease such that C5 levels, *e.g.*, in a cell, tissue, blood, urine or other tissue or fluid of the subject are reduced by at least about 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 62%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least about 99% or more and, subsequently, an additional therapeutic (as described below) is administered to the subject.

The additional therapeutic may be an anti-complement component C5 antibody, or antigen-binding fragment or derivative thereof. In one embodiment, the anti-complement component C5 antibody is eculizumab (SOLIRIS[®]), or antigen-binding fragment or derivative thereof. Eculizumab is a humanized monoclonal IgG2/4, kappa light chain antibody that specifically binds complement component C5 with high affinity and inhibits

cleavage of C5 to C5a and C5b, thereby inhibiting the generation of the terminal complement complex C5b-9. Eculizumab is described in U.S. Patent No. 6,355,245, the entire contents of which are incorporated herein by reference.

The methods of the invention comprising administration of an antisense polynucleotide agent of the invention and eculizumab to a subject may further comprise administration of a meningococcal vaccine to the subject.

The additional therapeutic, *e.g.*, eculizumab and/or a meningococcal vaccine, may be administered to the subject at the same time as the antisense polynucleotide agent targeting C5 or at a different time.

Moreover, the additional therapeutic, *e.g.*, eculizumab, may be administered to the subject in the same formulation as the antisense polynucleotide agent targeting C5 or in a different formulation as the antisense polynucleotide agent targeting C5.

Eculizumab dosage regimens are described in, for example, the product insert for eculizumab (SOLIRIS[®]) and in U.S. Patent Application No. 2012/0225056, the entire contents of each of which are incorporated herein by reference. In exemplary methods of the invention for treating a complement component C5-associated disease, *e.g.*, PNH or aHUS, an antisense polynucleotide agent targeting C5 is administered (*e.g.*, subcutaneously) to the subject first, such that the C5 levels in the subject are reduced (*e.g.*, by at least about 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 62%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least about 99% or more) and subsequently eculizumab is administered at doses lower than the ones described in the product insert for SOLIRIS[®]. For example, eculizumab may be administered to the subject weekly at a dose less than about 600 mg for 4 weeks followed by a fifth dose at about one week later of less than about 900 mg, followed by a dose less than about 900 mg about every two weeks thereafter. Eculizumab may also be administered to the subject weekly at a dose less than about 900 mg for 4 weeks followed by a fifth dose at about one week later of less than about 1200 mg, followed by a dose less than about 1200 mg about every two weeks thereafter. If the subject is less than 18 years of age, eculizumab may be administered to the subject weekly at a dose less than about 900 mg for 4 weeks followed by a fifth dose at about one week later of less than about 1200 mg, followed by a dose less than about 1200 mg about every two weeks thereafter; or if the subject is less than 18 years of age, eculizumab may be administered to the subject weekly at a dose less than about 600 mg for 2 weeks followed by a third dose at about one week later of less than about 900 mg, followed by a dose less than about 900 mg about every two weeks thereafter; or if the subject is less than 18 years of age, eculizumab may be administered to the subject weekly at a dose less

than about 600 mg for 2 weeks followed by a third dose at about one week later of less than about 600 mg, followed by a dose less than about 600 mg about every two weeks thereafter; or if the subject is less than 18 years of age, eculizumab may be administered to the subject weekly at a dose less than about 600 mg for 1 week followed by a second dose at about one week later of less than about 300 mg, followed by a dose less than about 300 mg about every two weeks thereafter; or if the subject is less than 18 years of age, eculizumab may be administered to the subject weekly at a dose less than about 300 mg for 1 week followed by a second dose at about one week later of less than about 300 mg, followed by a dose less than about 300 mg about every two weeks thereafter. If the subject is receiving plasmapheresis or plasma exchange, eculizumab may be administered to the subject at a dose less than about 300 mg (*e.g.*, if the most recent dose of eculizumab was about 300 mg) or less than about 600 mg (*e.g.*, if the most recent dose of eculizumab was about 600 mg or more). If the subject is receiving plasma infusion, eculizumab may be administered to the subject at a dose less than about 300 mg (*e.g.*, if the most recent dose of eculizumab was about 300 mg or more). The lower doses of eculizumab allow for either subcutaneous or intravenous administration of eculizumab.

In the combination therapies of the present invention comprising eculizumab, eculizumab may be administered to the subject, *e.g.*, subcutaneously, at a dose of about 0.01 mg/kg to about 10 mg/kg, or about 5 mg/kg to about 10 mg/kg, or about 0.5 mg/kg to about 15 mg/kg. For example, eculizumab may be administered to the subject, *e.g.*, subcutaneously, at a dose of 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 3.5 mg/kg, 4 mg/kg, 4.5 mg/kg, 5 mg/kg, 5.5 mg/kg, 6 mg/kg, 6.5 mg/kg, 7 mg/kg, 7.5 mg/kg, 8 mg/kg, 8.5 mg/kg, 9 mg/kg, 9.5 mg/kg, 10 mg/kg, 10.5 mg/kg, 11 mg/kg, 11.5 mg/kg, 12 mg/kg, 12.5 mg/kg, 13 mg/kg, 13.5 mg/kg, 14 mg/kg, 14.5 mg/kg, or 15 mg/kg.

The methods and uses of the invention include administering a composition described herein such that expression of the target C5 gene is decreased, such as for about 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 18, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68, 72, 76, or about 80 hours. In one embodiment, expression of the target C5 gene is decreased for an extended duration, *e.g.*, at least about two, three, four, five, six, seven days or more, *e.g.*, about one week, two weeks, three weeks, or about four weeks or longer.

Administration of the antisense polynucleotide agent according to the methods and uses of the invention may result in a reduction of the severity, signs, symptoms, and/or markers of such diseases or disorders in a patient with a complement component C5-associated disease. By “reduction” in this context is meant a statistically significant decrease in such level. The reduction can be, for example, at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or about 100%.

Efficacy of treatment or prevention of disease can be assessed, for example by measuring disease progression, disease remission, symptom severity, reduction in pain, quality of life, dose of a medication required to sustain a treatment effect, level of a disease marker or any other measurable parameter appropriate for a given disease being treated or targeted for prevention. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. For example, efficacy of treatment of a hemolytic disorder may be assessed, for example, by periodic monitoring of LDH and CH₅₀ levels. Comparisons of the later readings with the initial readings provide a physician an indication of whether the treatment is effective. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. In connection with the administration of an antisense polynucleotide agent targeting C5 or pharmaceutical composition thereof, "effective against" a complement component C5-associated disease indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as improvement of symptoms, a cure, a reduction in disease, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating a complement component C5-associated disease and the related causes.

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

Alternatively, the efficacy can be measured by a reduction in the severity of disease as determined by one skilled in the art of diagnosis based on a clinically accepted disease severity grading scale, as but one example the Rheumatoid Arthritis Severity Scale (RASS). Any positive change resulting in *e.g.*, lessening of severity of disease measured using the appropriate scale, represents adequate treatment using an antisense polynucleotide agent or antisense polynucleotide agent formulation as described herein.

Subjects can be administered a therapeutic amount of antisense polynucleotide agent, such as about 0.01 mg/kg, 0.02 mg/kg, 0.03 mg/kg, 0.04 mg/kg, 0.05 mg/kg, 0.1 mg/kg, 0.15 mg/kg, 0.2 mg/kg, 0.25 mg/kg, 0.3 mg/kg, 0.35 mg/kg, 0.4 mg/kg, 0.45 mg/kg, 0.5 mg/kg, 0.55 mg/kg, 0.6 mg/kg, 0.65 mg/kg, 0.7 mg/kg, 0.75 mg/kg, 0.8 mg/kg, 0.85 mg/kg, 0.9

mg/kg, 0.95 mg/kg, 1.0 mg/kg, 1.1 mg/kg, 1.2 mg/kg, 1.3 mg/kg, 1.4mg/kg, 1.5 mg/kg, 1.6 mg/kg, 1.7 mg/kg, 1.8 mg/kg, 1.9 mg/kg, 2.0 mg/kg, 2.1mg/kg, 2.2mg/kg, 2.3 mg/kg, 2.4 mg/kg, 2.5 mg/kg, 2.6 mg/kg, 2.7 mg/kg, 2.8 mg/kg, 2.9 mg/kg, 3.0 mg/kg, 3.1 mg/kg, 3.2 mg/kg, 3.3 mg/kg, 3.4 mg/kg, 3.5 mg/kg, 3.6 mg/kg, 3.7 mg/kg, 3.8 mg/kg, 3.9 mg/kg, 4.0 mg/kg, 4.1 mg/kg, 4.2 mg/kg, 4.3 mg/kg, 4.4 mg/kg, 4.5 mg/kg, 4.6 mg/kg, 4.7 mg/kg, 4.8 mg/kg, 4.9 mg/kg, 5.0 mg/kg, 5.1 mg/kg, 5.2 mg/kg, 5.3 mg/kg, 5.4 mg/kg, 5.5 mg/kg, 5.6 mg/kg, 5.7 mg/kg, 5.8 mg/kg, 5.9 mg/kg, 6.0 mg/kg, 6.1 mg/kg, 6.2 mg/kg, 6.3 mg/kg, 6.4 mg/kg, 6.5 mg/kg, 6.6 mg/kg, 6.7 mg/kg, 6.8 mg/kg, 6.9 mg/kg, 7.0 mg/kg, 7.1 mg/kg, 7.2 mg/kg, 7.3 mg/kg, 7.4 mg/kg, 7.5 mg/kg, 7.6 mg/kg, 7.7 mg/kg, 7.8 mg/kg, 7.9 mg/kg, 8.0 mg/kg, 8.1 mg/kg, 8.2 mg/kg, 8.3 mg/kg, 8.4 mg/kg, 8.5 mg/kg, 8.6 mg/kg, 8.7 mg/kg, 8.8 mg/kg, 8.9 mg/kg, 9.0 mg/kg, 9.1 mg/kg, 9.2 mg/kg, 9.3 mg/kg, 9.4 mg/kg, 9.5 mg/kg, 9.6 mg/kg, 9.7 mg/kg, 9.8 mg/kg, 9.9 mg/kg, 9.0 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30 mg/kg, 35 mg/kg, 40 mg/kg, 45 mg/kg, or about 50 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In certain embodiments, for example, when a composition of the invention comprises a antisense polynucleotide agent as described herein and a lipid, subjects can be administered a therapeutic amount of antisense polynucleotide agent, such as about 0.01 mg/kg to about 5 mg/kg, about 0.01 mg/kg to about 10 mg/kg, about 0.05 mg/kg to about 5 mg/kg, about 0.05 mg/kg to about 10 mg/kg, about 0.1 mg/kg to about 5 mg/kg, about 0.1 mg/kg to about 10 mg/kg, about 0.2 mg/kg to about 5 mg/kg, about 0.2 mg/kg to about 10 mg/kg, about 0.3 mg/kg to about 5 mg/kg, about 0.3 mg/kg to about 10 mg/kg, about 0.4 mg/kg to about 5 mg/kg, about 0.4 mg/kg to about 10 mg/kg, about 0.5 mg/kg to about 5 mg/kg, about 0.5 mg/kg to about 10 mg/kg, about 1 mg/kg to about 5 mg/kg, about 1 mg/kg to about 10 mg/kg, about 1.5 mg/kg to about 5 mg/kg, about 1.5 mg/kg to about 10 mg/kg, about 2 mg/kg to about about 2.5 mg/kg, about 2 mg/kg to about 10 mg/kg, about 3 mg/kg to about 5 mg/kg, about 3 mg/kg to about 10 mg/kg, about 3.5 mg/kg to about 5 mg/kg, about 4 mg/kg to about 5 mg/kg, about 4.5 mg/kg to about 5 mg/kg, about 4 mg/kg to about 10 mg/kg, about 4.5 mg/kg to about 10 mg/kg, about 5 mg/kg to about 10 mg/kg, about 5.5 mg/kg to about 10 mg/kg, about 6 mg/kg to about 10 mg/kg, about 6.5 mg/kg to about 10 mg/kg, about 7 mg/kg to about 10 mg/kg, about 7.5 mg/kg to about 10 mg/kg, about 8 mg/kg to about 10 mg/kg, about 8.5 mg/kg to about 10 mg/kg, about 9 mg/kg to about 10 mg/kg, or about 9.5 mg/kg to about 10 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

For example, the antisense polynucleotide agent may be administered at a dose of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7,

8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, or about 10 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

In other embodiments, for example, when a composition of the invention comprises a antisense polynucleotide agent as described herein and an N-acetylgalactosamine, subjects can be administered a therapeutic amount of antisense polynucleotide agent, such as a dose of about 0.1 to about 50 mg/kg, about 0.25 to about 50 mg/kg, about 0.5 to about 50 mg/kg, about 0.75 to about 50 mg/kg, about 1 to about 50 mg/mg, about 1.5 to about 50 mg/kb, about 2 to about 50 mg/kg, about 2.5 to about 50 mg/kg, about 3 to about 50 mg/kg, about 3.5 to about 50 mg/kg, about 4 to about 50 mg/kg, about 4.5 to about 50 mg/kg, about 5 to about 50 mg/kg, about 7.5 to about 50 mg/kg, about 10 to about 50 mg/kg, about 15 to about 50 mg/kg, about 20 to about 50 mg/kg, about 20 to about 50 mg/kg, about 25 to about 50 mg/kg, about 25 to about 50 mg/kg, about 30 to about 50 mg/kg, about 35 to about 50 mg/kg, about 40 to about 50 mg/kg, about 45 to about 50 mg/kg, about 0.1 to about 45 mg/kg, about 0.25 to about 45 mg/kg, about 0.5 to about 45 mg/kg, about 0.75 to about 45 mg/kg, about 1 to about 45 mg/mg, about 1.5 to about 45 mg/kb, about 2 to about 45 mg/kg, about 2.5 to about 45 mg/kg, about 3 to about 45 mg/kg, about 3.5 to about 45 mg/kg, about 4 to about 45 mg/kg, about 4.5 to about 45 mg/kg, about 5 to about 45 mg/kg, about 7.5 to about 45 mg/kg, about 10 to about 45 mg/kg, about 15 to about 45 mg/kg, about 20 to about 45 mg/kg, about 20 to about 45 mg/kg, about 25 to about 45 mg/kg, about 25 to about 45 mg/kg, about 30 to about 45 mg/kg, about 35 to about 45 mg/kg, about 40 to about 45 mg/kg, about 0.1 to about 40 mg/kg, about 0.25 to about 40 mg/kg, about 0.5 to about 40 mg/kg, about 0.75 to about 40 mg/kg, about 1 to about 40 mg/mg, about 1.5 to about 40 mg/kb, about 2 to about 40 mg/kg, about 2.5 to about 40 mg/kg, about 3 to about 40 mg/kg, about 3.5 to about 40 mg/kg, about 4 to about 40 mg/kg, about 4.5 to about 40 mg/kg, about 5 to about 40 mg/kg, about 7.5 to about 40 mg/kg, about 10 to about 40 mg/kg, about 15 to about 40 mg/kg, about 20 to about 40 mg/kg, about 20 to about 40 mg/kg, about 25 to about 40 mg/kg, about 25 to about 40 mg/kg, about 30 to about 40 mg/kg, about 35 to about 40 mg/kg, about 0.1 to about 30 mg/kg, about 0.25 to about 30 mg/kg, about 0.5 to about 30 mg/kg, about 0.75 to about 30 mg/kg, about 1 to about 30 mg/mg, about 1.5 to about 30 mg/kb, about 2 to about 30 mg/kg, about 2.5 to about 30 mg/kg, about 3 to about 30 mg/kg, about 3.5 to about 30 mg/kg, about 4 to about 30 mg/kg, about 4.5 to about 30 mg/kg, about 5 to about 30 mg/kg, about 7.5 to about 30 mg/kg, about 10 to about 30 mg/kg, about 15 to about 30 mg/kg, about 20 to about 30 mg/kg, about 20 to about 30 mg/kg, about 25 to about 30 mg/kg, about 0.1 to about 20 mg/kg, about 0.25 to about 20 mg/kg, about 0.5 to about 20 mg/kg, about 0.75 to about 20 mg/kg, about 1 to about 20 mg/mg, about 1.5 to about 20 mg/kb, about 2 to about 20 mg/kg, about 2.5 to about 20 mg/kg, about 3 to about 20 mg/kg, about 3.5 to about 20 mg/kg, about 4 to about 20 mg/kg, about 4.5 to about 20 mg/kg, about 5 to about 20 mg/kg, about 7.5 to about 20 mg/kg, about 10 to about 20 mg/kg, or about 15 to about 20 mg/kg. In one

embodiment, when a composition of the invention comprises a antisense polynucleotide agent as described herein and an N-acetylgalactosamine, subjects can be administered a therapeutic amount of about 10 to about 30 mg/kg of antisense polynucleotide agent. Values and ranges intermediate to the recited values are also intended to be part of this invention.

For example, subjects can be administered a therapeutic amount of antisense polynucleotide agent, such as about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16, 16.5, 17, 17.5, 18, 18.5, 19, 19.5, 20, 20.5, 21, 21.5, 22, 22.5, 23, 23.5, 24, 24.5, 25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, 30, 31, 32, 33, 34, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or about 50 mg/kg. Values and ranges intermediate to the recited values are also intended to be part of this invention.

The antisense polynucleotide agent can be administered by intravenous infusion over a period of time, such as over a 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or about a 25 minute period. The administration may be repeated, for example, on a regular basis, such as weekly, biweekly (*i.e.*, every two weeks) for one month, two months, three months, four months or longer. After an initial treatment regimen, the treatments can be administered on a less frequent basis. For example, after administration weekly or biweekly for three months, administration can be repeated once per month, for six months or a year or longer.

Administration of the antisense polynucleotide agent can reduce C5 levels, *e.g.*, in a cell, tissue, blood, urine or other compartment of the patient by at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least about 99% or more.

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

Owing to the inhibitory effects on C5 expression, a composition according to the invention or a pharmaceutical composition prepared therefrom can enhance the quality of life.

An antisense polynucleotide agent of the invention may be administered in “naked” form, or as a “free antisense polynucleotide agent.” A naked antisense polynucleotide agent is administered in the absence of a pharmaceutical composition. The naked antisense polynucleotide agent may be in a suitable buffer solution. The buffer solution may comprise acetate, citrate, prolamine, carbonate, or phosphate, or any combination thereof. In one embodiment, the buffer solution is phosphate buffered saline (PBS). The pH and osmolarity of the buffer solution containing the antisense polynucleotide agent can be adjusted such that it is suitable for administering to a subject.

Alternatively, an antisense polynucleotide agent of the invention may be administered as a pharmaceutical composition, such as an antisense polynucleotide agent liposomal formulation.

Subjects that would benefit from a reduction and/or inhibition of C5 gene expression are those having a complement component C5-associated disease or disorder as described herein. In one embodiment, a subject having a complement component C5-associated disease has paroxysmal nocturnal hemoglobinuria (PNH). In another embodiment, a subject having a complement component C5-associated disease has asthma. In another embodiment, a subject having a complement component C5-associated disease has rheumatoid arthritis. In yet another embodiment, a subject having a complement component C5-associated disease has systemic lupus erythematosus. In one embodiment, a subject having a complement component C5-associated disease has glomerulonephritis. In another embodiment, a subject having a complement component C5-associated disease has psoriasis. In yet another embodiment, a subject having a complement component C5-associated disease has dermatomyositis bullous pemphigoid. In one embodiment, a subject having a complement component C5-associated disease has atypical hemolytic uremic syndrome. In another embodiment, a subject having a complement component C5-associated disease has Shiga toxin *E. coli*-related hemolytic uremic syndrome. In another embodiment, a subject having a complement component C5-associated disease has myasthenia gravis. In yet another embodiment, a subject having a complement component C5-associated disease has neuromyelitis optica. In one embodiment, a subject having a complement component C5-associated disease has dense deposit disease. In one embodiment, a subject having a complement component C5-associated disease has C3 neuropathy. In another embodiment, a subject having a complement component C5-associated disease has age-related macular degeneration. In another embodiment, a subject having a complement component C5-associated disease has cold agglutinin disease. In one embodiment, a subject having a complement component C5-associated disease has anti-neutrophil cytoplasmic antibody-

associated vasculitis. In another embodiment, a subject having a complement component C5-associated disease has humoral and vascular transplant rejection. In one embodiment, a subject having a complement component C5-associated disease has graft dysfunction. In one embodiment, a subject having a complement component C5-associated disease has had a myocardial infarction. In another embodiment, a subject having a complement component C5-associated disease is a sensitized recipient of a transplant. In yet another embodiment, a subject having a complement component C5-associated disease has sepsis.

Treatment of a subject that would benefit from a reduction and/or inhibition of C5 gene expression includes therapeutic and prophylactic (*e.g.*, the subject is to undergo sensitized (or allogenic) transplant surgery) treatment.

The invention further provides methods and uses of an antisense polynucleotide agent or a pharmaceutical composition thereof (including methods and uses of an antisense polynucleotide agent or a pharmaceutical composition comprising an antisense polynucleotide agent and an anti-complement component C5 antibody, or antigen-binding fragment thereof) for treating a subject that would benefit from reduction and/or inhibition of C5 expression, *e.g.*, a subject having a complement component C5-associated disease, in combination with other pharmaceuticals and/or other therapeutic methods, *e.g.*, with known pharmaceuticals and/or known therapeutic methods, such as, for example, those which are currently employed for treating these disorders. For example, in certain embodiments, an antisense polynucleotide agent targeting C5 is administered in combination with, *e.g.*, an agent useful in treating a complement component C5-associated disease as described elsewhere herein.

For example, additional therapeutics and therapeutic methods suitable for treating a subject that would benefit from reduction in C5 expression, *e.g.*, a subject having a complement component C5-associated disease, include plasmaphoresis, thrombolytic therapy (*e.g.*, streptokinase), antiplatelet agents, folic acid, corticosteroids; immunosuppressive agents; estrogens, methotrexate, 6-MP, azathioprine, sulphasalazine, mesalazine, olsalazine, chloroquine/hydroxychloroquine, pencillamine, aurothiomalate (intramuscular and oral), azathioprine, cochlincine, corticosteroids (oral, inhaled and local injection), beta-2 adrenoreceptor agonists (salbutamol, terbutaline, salmeterol), xanthines (theophylline, aminophylline), cromoglycate, nedocromil, ketotifen, ipratropium and oxitropium, cyclosporin, FK506, rapamycin, mycophenolate mofetil, leflunomide, NSAIDs, for example, ibuprofen, corticosteroids such as prednisolone, phosphodiesterase inhibitors, adenosine agonists, antithrombotic agents, complement inhibitors, adrenergic agents, agents which interfere with signalling by proinflammatory cytokines, such as TNF- α or IL-1 (*e.g.*, IRAK, NIK, IKK, p38 or MAP kinase inhibitors), IL-1 β converting enzyme inhibitors, TNF α converting enzyme (TACE) inhibitors, T-cell signalling inhibitors, such as kinase inhibitors, metalloproteinase inhibitors, sulfasalazine, azathioprine, 6-mercaptopurines,

angiotensin converting enzyme inhibitors, soluble cytokine receptors and derivatives thereof (e.g., soluble p55 or p75 TNF receptors and the derivatives p75TNFR1gG (Enbrel™ and p55TNFR1gG (Lenercept)), sIL-1RI, sIL-1RII, and sIL-6R), antiinflammatory cytokines (e.g., IL-4, IL-10, IL-11, IL-13 and TGFβ), celecoxib, folic acid, hydroxychloroquine sulfate, rofecoxib, etanercept, infliximono-clonal antibody, naproxen, valdecoxib, sulfasalazine, methylprednisolone, meloxicam, methylprednisolone acetate, gold sodium thiomalate, aspirin, triamcinolone acetonide, propoxyphene napsylate/apap, folate, nabumetone, diclofenac, piroxicam, etodolac, diclofenac sodium, oxaprozin, oxycodone hcl, hydrocodone bitartrate/apap, diclofenac sodium/misoprostol, fentanyl, anakinra, human recombinant, tramadol hcl, salsalate, sulindac, cyanocobalamin/fa/pyridoxine, acetaminophen, alendronate sodium, prednisolone, morphine sulfate, lidocaine hydrochloride, indomethacin, glucosamine sulf/chondroitin, amitriptyline hcl, sulfadiazine, oxycodone hcl/acetaminophen, olopatadine hcl, misoprostol, naproxen sodium, omeprazole, cyclophosphamide, rituximono-clonal antibody, IL-1 TRAP, MRA, CTLA4-IG, IL-18 BP, anti-IL-18, Anti-IL15, BIRB-796, SCIO-469, VX-702, AMG-548, VX-740, Roflumilast, IC-485, CDC-801, Mesopram, cyclosporine, cytokine suppressive anti-inflammatory drug(s) (CSAIDs); CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2/infliximono-clonal antibody (chimeric anti-TNFα antibody; Centocor); 75 kDTNFR-IgG/etanercept (75 kD TNF receptor-IgG fusion protein; Immunex; see e.g., (1994) *Arthr. Rheum.* 37: S295; (1996) *J. Invest. Med.* 44: 235A); 55 kDTNF-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IDEC-CE9.1/SB 210396 (non-depleting primatized anti-CD4 antibody; IDEC/SmithKline; see e.g., (1995) *Arthr. Rheum.* 38: S185); DAB 486-IL-2 and/or DAB 389-IL-2 (IL-2 fusion proteins; Seragen; see e.g., (1993) *Arthrit. Rheum.* 36: 1223); Anti-Tac (humanized anti-IL-2Rα; Protein Design Labs/Roche); IL-4 (anti-inflammatory cytokine; DNAX/Schering); IL-10 (SCH 52000; recombinant IL-10, anti-inflammatory cytokine; DNAX/Schering); IL-4; IL-10 and/or IL-4 agonists (e.g., agonist antibodies); IL-1RA (IL-1 receptor antagonist; Synergen/Amgen); anakinra (Kineret®/Amgen); TNF-bp/s-TNF (soluble TNF binding protein; see e.g., (1996) *Arthr. Rheum.* 39(9 (supplement)): S284; (1995) *Amer. J. Physiol. - Heart and Circ. Physiol.* 268: 37-42); R973401 (phosphodiesterase Type IV inhibitor; see e.g., (1996) *Arthr. Rheum.* 39(9 (supplement)): S282); MK-966 (COX-2 Inhibitor; see e.g., (1996) *Arthr. Rheum.* 39(9 (supplement)): S81); Iloprost (see e.g., (1996) *Arthr. Rheum.* 39(9 (supplement)): S82); methotrexate; thalidomide (see e.g., (1996) *Arthr. Rheum.* 39(9 (supplement)): S282) and thalidomide-related drugs (e.g., Celgen); leflunomide (anti-inflammatory and cytokine inhibitor; see e.g., (1996) *Arthr. Rheum.* 39(9 (supplement)): S131; (1996) *Inflamm. Res.* 45: 103-107); tranexamic acid (inhibitor of plasminogen activation; see e.g., (1996) *Arthr. Rheum.* 39(9 (supplement)): S284); T-614 (cytokine inhibitor; see e.g., (1996) *Arthr. Rheum.* 39(9 (supplement)): S282); prostaglandin E1 (see e.g., (1996) *Arthr. Rheum.* 39(9 (supplement)): S282); Tenidap (non-steroidal anti-

inflammatory drug; see *e.g.*, (1996) *Arthr. Rheum.* 39(9 (supplement): S280); Naproxen (non-steroidal anti-inflammatory drug; see *e.g.*, (1996) *Neuro. Report* 7: 1209-1213); Meloxicam (non-steroidal anti-inflammatory drug); Ibuprofen (non-steroidal anti-inflammatory drug); Piroxicam (non-steroidal anti-inflammatory drug); Diclofenac (non-steroidal anti-inflammatory drug); Indomethacin (non-steroidal anti-inflammatory drug); Sulfasalazine (see *e.g.*, (1996) *Arthr. Rheum.* 39(9 (supplement): S281); Azathioprine (see *e.g.*, (1996) *Arthr. Rheum.* 39(9 (supplement): S281); ICE inhibitor (inhibitor of the enzyme interleukin-1 β converting enzyme); zap-70 and/or lck inhibitor (inhibitor of the tyrosine kinase zap-70 or lck); VEGF inhibitor and/or VEGF-R inhibitor (inhibitors of vascular endothelial cell growth factor or vascular endothelial cell growth factor receptor; inhibitors of angiogenesis); corticosteroid anti-inflammatory drugs (*e.g.*, SB203580); TNF-convertase inhibitors; anti-IL-12 antibodies; anti-IL-18 antibodies; interleukin-11 (see *e.g.*, (1996) *Arthr. Rheum.* 39(9 (supplement): S296); interleukin-13 (see *e.g.*, (1996) *Arthr. Rheum.* 39(9 (supplement): S308); interleukin -17 inhibitors (see *e.g.*, (1996) *Arthr. Rheum.* 39(9 (supplement): S120); gold; penicillamine; chloroquine; chlorambucil; hydroxychloroquine; cyclosporine; cyclophosphamide; total lymphoid irradiation; anti-thymocyte globulin; anti-CD4 antibodies; CD5-toxins; orally-administered peptides and collagen; lobenzarit disodium; Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten Pharmaceuticals, Inc.); ICAM-1 antisense phosphorothioate oligo-deoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); prednisone; orgotein; glycosaminoglycan polysulphate; minocycline; anti-IL2R antibodies; marine and botanical lipids (fish and plant seed fatty acids; see *e.g.*, DeLuca *et al.* (1995) *Rheum. Dis. Clin. North Am.* 21: 759-777); auranofin; phenylbutazone; meclofenamic acid; flufenamic acid; intravenous immune globulin; zileuton; azaribine; mycophenolic acid (RS-61443); tacrolimus (FK-506); sirolimus (rapamycin); amiprilose (therafectin); cladribine (2-chlorodeoxyadenosine); methotrexate; bcl-2 inhibitors (see Bruncko, M. *et al.* (2007) *J. Med. Chem.* 50(4): 641-662); antivirals and immune-modulating agents, small molecule inhibitor of KDR, small molecule inhibitor of Tie-2; methotrexate; prednisone; celecoxib; folic acid; hydroxychloroquine sulfate; rofecoxib; etanercept; infliximono-clonal antibody; leflunomide; naproxen; valdecoxib; sulfasalazine; methylprednisolone; ibuprofen; meloxicam; methylprednisolone acetate; gold sodium thiomalate; aspirin; azathioprine; triamcinolone acetonide; propoxyphene napsylate/apap; folate; nabumetone; diclofenac; piroxicam; etodolac; diclofenac sodium; oxaprozin; oxycodone hcl; hydrocodone bitartrate/apap; diclofenac sodium/misoprostol; fentanyl; anakinra, human recombinant; tramadol hcl; salsalate; sulindac; cyanocobalamin/fa/pyridoxine; acetaminophen; alendronate sodium; prednisolone; morphine sulfate; lidocaine hydrochloride; indomethacin; glucosamine sulfate/chondroitin; cyclosporine; amitriptyline hcl; sulfadiazine; oxycodone hcl/acetaminophen; olopatadine hcl; misoprostol; naproxen sodium; omeprazole; mycophenolate mofetil; cyclophosphamide;

rituximono-clonal antibody; IL-1 TRAP; MRA; CTLA4-IG; IL-18 BP; IL-12/23; anti-IL 18; anti-IL 15; BIRB-796; SCIO-469; VX-702; AMG-548; VX-740; Roflumilast; IC-485; CDC-801; mesopram, albuterol, salmeterol/fluticasone, montelukast sodium, fluticasone propionate, budesonide, prednisone, salmeterol xinafoate, levalbuterol hcl, albuterol sulfate/ipratropium, prednisolone sodium phosphate, triamcinolone acetonide, beclomethasone dipropionate, ipratropium bromide, azithromycin, pirbuterol acetate, prednisolone, theophylline anhydrous, methylprednisolone sodium succinate, clarithromycin, zafirlukast, formoterol fumarate, influenza virus vaccine, methylprednisolone, amoxicillin trihydrate, flunisolide, allergy injection, cromolyn sodium, fexofenadine hydrochloride, flunisolide/menthol, amoxicillin/clavulanate, levofloxacin, inhaler assist device, guaifenesin, dexamethasone sodium phosphate, moxifloxacin hcl, doxycycline hyclate, guaifenesin/d-methorphan, p-ephedrine/cod/chlorphenir, gatifloxacin, cetirizine hydrochloride, mometasone furoate, salmeterol xinafoate, benzonatate, cephalexin, pe/hydrocodone/chlorphenir, cetirizine hcl/pseudoephed, phenylephrine/cod/promethazine, codeine/promethazine, cefprozil, dexamethasone, guaifenesin/pseudoephedrine, chlorpheniramine/hydrocodone, nedocromil sodium, terbutaline sulfate, epinephrine, methylprednisolone, metaproterenol sulfate, aspirin, nitroglycerin, metoprolol tartrate, enoxaparin sodium, heparin sodium, clopidogrel bisulfate, carvedilol, atenolol, morphine sulfate, metoprolol succinate, warfarin sodium, lisinopril, isosorbide mononitrate, digoxin, furosemide, simvastatin, ramipril, tenecteplase, enalapril maleate, torsemide, retavase, losartan potassium, quinapril hcl/mag carb, bumetanide, alteplase, enalaprilat, amiodarone hydrochloride, tirofiban hcl m-hydrate, diltiazem hydrochloride, captopril, irbesartan, valsartan, propranolol hydrochloride, fosinopril sodium, lidocaine hydrochloride, eptifibatide, cefazolin sodium, atropine sulfate, aminocaproic acid, spironolactone, interferon, sotalol hydrochloride, potassium chloride, docusate sodium, dobutamine hcl, alprazolam, pravastatin sodium, atorvastatin calcium, midazolam hydrochloride, meperidine hydrochloride, isosorbide dinitrate, epinephrine, dopamine hydrochloride, bivalirudin, rosuvastatin, ezetimibe/simvastatin, avasimibe, and cariporide.

The antisense polynucleotide agent (and/or an anti-complement component C5 antibody) and an additional therapeutic agent and/or treatment may be administered at the same time and/or in the same combination, *e.g.*, parenterally, or the additional therapeutic agent can be administered as part of a separate composition or at separate times and/or by another method known in the art or described herein.

The present invention also provides methods of using an antisense polynucleotide agent of the invention and/or a composition containing an antisense polynucleotide agent of the invention to reduce and/or inhibit complement component C5 expression in a cell. In other aspects, the present invention provides an antisense polynucleotide agent of the invention and/or a composition comprising an antisense polynucleotide agent of the invention

for use in reducing and/or inhibiting C5 expression in a cell. In yet other aspects, use of an antisense polynucleotide agent of the invention and/or a composition comprising an antisense polynucleotide agent of the invention for the manufacture of a medicament for reducing and/or inhibiting C5 expression in a cell are provided.

The methods and uses include contacting the cell with an antisense polynucleotide agent, *e.g.*, a antisense polynucleotide agent, of the invention and maintaining the cell for a time sufficient to obtain antisense inhibition of a C5 gene, thereby inhibiting expression of the C5 gene in the cell.

Reduction in gene expression can be assessed by any methods known in the art. For example, a reduction in the expression of C5 may be determined by determining the mRNA expression level of C5 using methods routine to one of ordinary skill in the art, *e.g.*, Northern blotting, qRT-PCR, by determining the protein level of C5 using methods routine to one of ordinary skill in the art, such as Western blotting, immunological techniques, flow cytometry methods, ELISA, and/or by determining a biological activity of C5, such as CH₅₀ or AH₅₀ hemolysis assay, and/or by determining the biological activity of one or more molecules associated with the complement system, *e.g.*, C5 products, such as C5a and C5b (or, in an *in vivo* setting, *e.g.*, hemolysis).

In the methods and uses of the invention the cell may be contacted *in vitro* or *in vivo*, *i.e.*, the cell may be within a subject. In embodiments of the invention in which the cell is within a subject, the methods may include further contacting the cell with an anti-complement component C5 antibody, *e.g.*, eculizumab.

A cell suitable for treatment using the methods of the invention may be any cell that expresses a C5 gene. A cell suitable for use in the methods and uses of the invention may be a mammalian cell, *e.g.*, a primate cell (such as a human cell or a non-human primate cell, *e.g.*, a monkey cell or a chimpanzee cell), a non-primate cell (such as a cow cell, a pig cell, a camel cell, a llama cell, a horse cell, a goat cell, a rabbit cell, a sheep cell, a hamster, a guinea pig cell, a cat cell, a dog cell, a rat cell, a mouse cell, a lion cell, a tiger cell, a bear cell, or a buffalo cell), a bird cell (*e.g.*, a duck cell or a goose cell), or a whale cell. In one embodiment, the cell is a human cell, *e.g.*, a human liver cell.

C5 expression may be inhibited in the cell by at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or about 100%.

The *in vivo* methods and uses of the invention may include administering to a subject a composition containing an antisense polynucleotide agent, where the antisense polynucleotide agent includes a nucleotide sequence that is complementary to at least a part of an RNA transcript of the C5 gene of the mammal to be treated. When the organism to be treated is a mammal such as a human, the composition can be administered by any means known in the art including, but not limited to subcutaneous, intravenous, oral, intraperitoneal, or parenteral routes, including intracranial (*e.g.*, intraventricular, intraparenchymal and intrathecal), intramuscular, transdermal, airway (aerosol), nasal, rectal, and topical (including buccal and sublingual) administration. In certain embodiments, the compositions are administered by subcutaneous or intravenous infusion or injection.

In some embodiments, the administration is *via* a depot injection. A depot injection may release the antisense polynucleotide agent in a consistent way over a prolonged time period. Thus, a depot injection may reduce the frequency of dosing needed to obtain a desired effect, *e.g.*, a desired inhibition of C5, or a therapeutic or prophylactic effect. A depot injection may also provide more consistent serum concentrations. Depot injections may include subcutaneous injections or intramuscular injections. In preferred embodiments, the depot injection is a subcutaneous injection.

In some embodiments, the administration is *via* a pump. The pump may be an external pump or a surgically implanted pump. In certain embodiments, the pump is a subcutaneously implanted osmotic pump. In other embodiments, the pump is an infusion pump. An infusion pump may be used for intravenous, subcutaneous, arterial, or epidural infusions. In preferred embodiments, the infusion pump is a subcutaneous infusion pump. In other embodiments, the pump is a surgically implanted pump that delivers the antisense polynucleotide agent to the liver.

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

In one aspect, the present invention also provides methods for inhibiting the expression of a C5 gene in a mammal, *e.g.*, a human. The present invention also provides a composition comprising an antisense polynucleotide agent that targets a C5 gene in a cell of a mammal for use in inhibiting expression of the C5 gene in the mammal. In another aspect, the present invention provides use of an antisense polynucleotide agent that targets a C5 gene in a cell of a mammal in the manufacture of a medicament for inhibiting expression of the C5 gene in the mammal.

The methods and uses include administering to the mammal, *e.g.*, a human, a composition comprising an antisense polynucleotide agent that targets a C5 gene in a cell of the mammal and maintaining the mammal for a time sufficient to obtain antisense inhibition of the mRNA transcript of the C5 gene, thereby inhibiting expression of the C5 gene in the

mammal. In some embodiment, the methods further comprise administering an anti-complement component C5 antibody, *e.g.*, eculizumab, to the subject.

Reduction in gene expression can be assessed by any methods known in the art and by methods, *e.g.* qRT-PCR, described herein. Reduction in protein production can be assessed by any methods known in the art and by methods, *e.g.*, ELISA or Western blotting, described herein. In one embodiment, a puncture liver biopsy sample serves as the tissue material for monitoring the reduction in C5 gene and/or protein expression. In another embodiment, a blood sample serves as the tissue material for monitoring the reduction in C5 gene and/or protein expression. In other embodiments, inhibition of the expression of a C5 gene is monitored indirectly by, for example, determining the expression and/or activity of a gene in a C5 pathway, including, for example, C5a, C5b, and soluble C5b-9 (see, *e.g.*, Figure 1). For example, the activity of CD59 may be monitored to determine the inhibition of expression of a C5 gene. CH₅₀, AH₅₀, clot formation and/or serum lactate dehydrogenase (LDH), in a sample, *e.g.*, a blood or liver sample, may also be measured. Suitable assays are further described in the Examples section below.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the antisense polynucleotide agents and methods featured in the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

EXAMPLES

Example 1. Antisense Synthesis

The antisense polynucleotides targeting C5 were synthesized using standard synthesis methods well known in the art.

A detailed list of antisense molecules targeting complement component C5 is shown in Table 3.

Example 2. *In vitro* screening

In vitro screening of the antisense polynucleotides was performed by transfecting Huh7 cells with a single 5nM dose of an antisense polynucleotide using methods well known in the art. Table 4 shows the results of single dose transfection screen in cells transfected with the indicated antisense polynucleotide.

Table 2: Abbreviations of nucleotide monomers used in nucleic acid sequence representation. It will be understood that these monomers, when present in an oligonucleotide, are mutually linked by 5'-3'-phosphodiester bonds.

Abbreviation	Nucleotide(s)
A	Adenosine-3'-phosphate
Af	2'-fluoroadenosine-3'-phosphate
Afs	2'-fluoroadenosine-3'-phosphorothioate
As	adenosine-3'-phosphorothioate
a	2'-O-methyladenosine-3'-phosphate
as	2'-O-methyladenosine-3'- phosphorothioate
C	cytidine-3'-phosphate
dA	2'-deoxyadenosine-3'-phosphate
dAs	2'-deoxyadenosine-3'-phosphorothioate
Cf	2'-fluorocytidine-3'-phosphate
Cfs	2'-fluorocytidine-3'-phosphorothioate
Cs	cytidine-3'-phosphorothioate
c	2'-O-methylcytidine-3'-phosphate
cs	2'-O-methylcytidine-3'- phosphorothioate
dC	2'-deoxycytidine-3'-phosphate
dCs	2'-deoxycytidine-3'-phosphorothioate
G	guanosine-3'-phosphate
Gf	2'-fluoroguanosine-3'-phosphate
Gfs	2'-fluoroguanosine-3'-phosphorothioate

Abbreviation	Nucleotide(s)
Gs	guanosine-3'-phosphorothioate
g	2'-O-methylguanosine-3'-phosphate
gs	2'-O-methylguanosine-3'-phosphorothioate
dG	2'-deoxyguanosine-3'-phosphate
dGs	2'-deoxyguanosine-3'-phosphorothioate
T	5'-methyluridine-3'-phosphate
Tf	2'-fluoro-5-methyluridine-3'-phosphate
Tfs	2'-fluoro-5-methyluridine-3'-phosphorothioate
Ts	5-methyluridine-3'-phosphorothioate
t	2'-O-methyl-5-methyluridine-3'-phosphate
ts	2'-O-methyl-5-methyluridine-3'-phosphorothioate
dT	2'-deoxythymidine-3'-phosphate
dTs	2'-deoxythymidine-3'-phosphorothioate
U	Uridine-3'-phosphate
Uf	2'-fluorouridine-3'-phosphate
Ufs	2'-fluorouridine-3'-phosphorothioate
Us	uridine-3'-phosphorothioate
u	2'-O-methyluridine-3'-phosphate
us	2'-O-methyluridine-3'-phosphorothioate
dU	2'-deoxyuridine-3'-phosphate
dUs	2'-deoxyuridine-3'-phosphorothioate
s	phosphorothioate linkage
N	any nucleotide (G, A, C, T or U)
L96	N-[tris(GalNAc-alkyl)-amidodecanoyl]-4-hydroxyprolinol Hyp-(GalNAc-alkyl) ₃
(dt)	deoxy-thymine
(5MdC)	5'-methyl-deoxycytidine-3'-phosphate
(5MdC) _s	5'-methyl-deoxycytidine-3'-phosphorothioate

Table 3. Antisense polynucleotides targeting complement component C5.

Sequence ID	SEQ ID NO:	Modified Sequence (5' to 3')	Start position relative to NM_001735.2 (SEQ ID NO:1)	End position relative to NM_001735.2 (SEQ ID NO:1)
A-128563.1	13	asgscsasgsdGsdAsdAsdAs(5MdC)s(5MdC)sdAs(5MdC)sdGsdGsasusasusa	1	20
A-128564.1	14	usggsususdGsdGsdAsdGsdGsdTsdAsdGs(5MdC)sdAsggsasasa	12	31
A-128565.1	15	csasasasasdGsdGs(5MdC)s(5MdC)s(5MdC)sdAsdGsdGsdTsdusggsasag	23	42
A-128566.1	16	csasasasgsdTsdAsdTsdTs(5MdC)s(5MdC)s(5MdC)sdAsdAsdAsasgsgscsc	34	53
A-128567.1	17	asgsasususdAsdAsdAsdAsdAs(5MdC)sdAsdAsdAsggsusasusu	45	64
A-128568.1	18	usususcscs(5MdC)s(5MdC)sdAsdGsdGsdAsdAsdGsdAsdTsdusasasasa	56	75
A-128569.1	19	usggsuscscs(5MdC)s(5MdC)sdAsdGsdGsdTsdTsdTs(5MdC)scscscsasag	67	86
A-128570.1	20	asusgsususdTsdGs(5MdC)sdTs(5MdC)s(5MdC)sdTsdGsdTs(5MdC)scscscsasag	78	97
A-128571.1	21	usgsasasasdTsdGsdAs(5MdC)sdAsdTsdAsdTsdGsdTsdusggsusu	89	108
A-128572.1	22	asususususdTsdGsdGsdTsdGs(5MdC)sdTsdGsdAsdAsdasusgsasac	100	119
A-128573.1	23	csasascsas(5MdC)sdGsdGsdAsdAsdTsdAsdTsdTsdTsdusggsusu	111	130
A-128574.1	24	ususcasgsdAsdTsdGs(5MdC)sdTs(5MdC)s(5MdC)sdAsdAs(5MdC)sascsggsa	122	141
A-128575.1	25	asuscascsdAsdAsdTsdAsdTsdTsdTsdTs(5MdC)sdAsggsasusc	133	152
A-128576.1	26	csasusasasdAs(5MdC)sdTsdGsdAsdAsdTsdTs(5MdC)sdAscscsasasa	144	163
A-128577.1	27	ususcsasgsdTsdGsdTsdAsdTsdTs(5MdC)s(5MdC)sdAsdTsdAsasascusu	155	174
A-128578.1	28	gscsasuscscdAsdAsdAsdTsdGs(5MdC)sdTsdTs(5MdC)sdAsggsusggsusa	166	185
A-128579.1	29	usasgsasgsdAsdTsdGsdTsdTs(5MdC)sdAsdTsdscscsasasa	177	196

Table 4.

	meanval% (w/o correction)	sd %	Corrected Transfection Efficiency (tfe)
A-128636.1	16	4	6
A-128659.1	23	5	19
A-128693.1	23	7	19
A-128637.1	30	5	20
A-128671.1	28	6	23
A-128655.1	28	7	24
A-128594.1	29	10	24
A-128689.1	29	4	25
A-128720.1	29	6	26
A-128591.1	31	8	27
A-128686.1	31	4	27
A-128597.1	31	9	27
A-128606.1	32	6	27
A-128574.1	30	3	27
A-128773.1	38	4	28
A-128570.1	31	9	29
A-128713.1	32	5	29
A-128669.1	34	8	29
A-128619.1	34	6	29
A-128780.1	40	7	30
A-128688.1	34	4	30
A-128678.1	36	5	31
A-128645.1	35	6	31
A-128658.1	35	5	31
A-128603.1	36	4	31
A-128711.1	34	7	31
A-128623.1	42	3	32
A-128630.1	42	5	32
A-128598.1	36	6	32
A-128583.1	36	9	32
A-128714.1	35	7	32
A-128756.1	42	7	33

A-128577.1	36	8	33
A-128709.1	36	8	33
A-128710.1	36	5	33
A-128729.1	41	8	33
A-128648.1	38	7	34
A-128696.1	38	8	34
A-128566.1	37	8	34
A-128596.1	39	9	34
A-129058.1	35	7	34
A-128643.1	39	8	35
A-128703.1	38	6	35
A-128674.1	40	7	35
A-128687.1	39	4	35
A-128668.1	40	6	35
A-128639.1	47	6	36
A-128677.1	41	6	36
A-128787.1	47	5	37
A-128622.1	42	2	38
A-128734.1	46	8	38
A-128584.1	42	8	38
A-128759.1	47	7	38
A-128706.1	41	10	38
A-128676.1	43	5	39
A-129044.1	39	4	39
A-128763.1	49	7	39
A-128642.1	49	1	39
A-128679.1	44	8	39
A-128765.1	50	8	39
A-128667.1	44	7	39
A-128649.1	43	8	39
A-128694.1	43	12	39
A-128695.1	43	17	39
A-128672.1	44	5	40
A-128705.1	42	8	40
A-128646.1	44	10	40
A-128776.1	51	11	40
A-128749.1	49	12	40

A-128592.1	44	6	40
A-128579.1	43	11	40
A-128565.1	43	5	40
A-128975.1	41	4	41
A-128593.1	45	9	41
A-128738.1	49	9	41
A-128793.1	51	6	41
A-128717.1	44	9	41
A-128607.1	46	6	41
A-128867.1	52	5	41
A-128602.1	46	8	42
A-128600.1	46	8	42
A-128760.1	51	7	42
A-128660.1	46	6	42
A-128973.1	43	10	43
A-128682.1	48	2	43
A-128933.1	45	9	43
A-128675.1	48	11	43
A-128628.1	54	8	44
A-128684.1	48	7	44
A-128692.1	48	2	44
A-128611.1	49	4	44
A-128725.1	52	7	44
A-128563.1	47	6	44
A-128727.1	53	8	44
A-128620.1	49	5	44
A-128712.1	48	4	45
A-128770.1	56	7	46
A-128762.1	55	2	46
A-128728.1	54	8	46
A-128716.1	49	9	46
A-128626.1	57	8	46
A-128617.1	51	11	46
A-128721.1	49	8	47
A-128575.1	49	3	47
A-128690.1	51	2	47
A-128967.1	47	2	47

A-128769.1	58	7	47
A-128666.1	52	5	47
A-128612.1	53	5	48
A-128707.1	51	7	48
A-128698.1	52	5	48
A-128578.1	51	7	48
A-128761.1	57	4	48
A-128766.1	59	6	49
A-128588.1	53	7	49
A-128652.1	53	9	49
A-128618.1	54	5	49
A-129056.1	49	16	49
A-128590.1	53	7	49
A-128571.1	52	9	49
A-128625.1	60	7	49
A-128925.1	51	2	49
A-128654.1	53	8	49
A-128640.1	60	4	49
A-128656.1	54	6	50
A-128875.1	60	14	50
A-128893.1	51	16	50
A-128673.1	55	6	50
A-128573.1	53	7	50
A-128779.1	61	6	50
A-128853.1	61	3	50
A-128624.1	61	8	50
A-128856.1	61	4	50
A-128662.1	54	6	50
A-128670.1	55	8	51
A-128585.1	55	9	51
A-128976.1	51	6	51
A-128715.1	53	11	51
A-128968.1	51	7	51
A-128748.1	60	12	51
A-128841.1	60	3	51
A-128730.1	59	8	51
A-129025.1	51	8	51

A-128680.1	56	5	51
A-128732.1	59	7	51
A-129015.1	51	10	51
A-128691.1	55	3	51
A-129046.1	52	7	51
A-128788.1	62	10	51
A-129014.1	52	7	52
A-128934.1	53	1	52
A-128845.1	62	8	52
A-128792.1	62	6	52
A-128638.1	62	8	52
A-128897.1	53	23	52
A-128699.1	56	12	52
A-128737.1	60	9	52
A-129055.1	53	22	52
A-128764.1	63	8	53
A-128569.1	55	8	53
A-128633.1	63	7	53
A-128826.1	62	6	53
A-128742.1	61	3	53
A-128878.1	64	6	53
A-129031.1	53	8	53
A-129022.1	53	4	53
A-128685.1	58	3	54
A-128775.1	64	7	54
A-128783.1	64	7	54
A-128774.1	65	9	54
A-129024.1	54	8	54
A-128888.1	55	9	54
A-128977.1	54	9	54
A-128993.1	54	15	54
A-128966.1	54	6	54
A-128927.1	56	16	55
A-128863.1	65	7	55
A-128789.1	65	10	55
A-128825.1	64	4	55
A-128757.1	64	14	55

A-128908.1	56	12	55
A-128861.1	66	4	55
A-128874.1	66	9	56
A-128681.1	60	3	56
A-128700.1	60	7	56
A-128854.1	66	3	56
A-128842.1	66	2	56
A-128797.1	66	8	56
A-128650.1	60	9	56
A-128859.1	67	3	56
A-128733.1	64	6	56
A-129043.1	57	10	56
A-128922.1	57	11	56
A-128731.1	65	5	57
A-128882.1	67	2	57
A-128855.1	67	5	57
A-128610.1	62	5	57
A-128857.1	68	9	57
A-129045.1	58	5	57
A-128852.1	68	6	57
A-128726.1	65	7	57
A-128723.1	66	12	58
A-128884.1	59	10	58
A-128784.1	68	5	58
A-129013.1	58	7	58
A-128974.1	58	17	58
A-128647.1	62	6	58
A-128866.1	68	7	58
A-129035.1	58	18	58
A-128837.1	67	5	58
A-128772.1	69	5	58
A-128635.1	69	9	58
A-128586.1	63	9	58
A-128777.1	69	14	58
A-128794.1	69	25	58
A-128755.1	68	13	58
A-128807.1	68	10	59

A-128657.1	63	12	59
A-128785.1	69	5	59
A-128663.1	64	6	59
A-128804.1	68	13	59
A-129034.1	59	7	59
A-129004.1	59	12	59
A-128970.1	59	16	59
A-128786.1	69	4	59
A-128704.1	62	8	59
A-128876.1	70	7	59
A-128605.1	64	2	59
A-128939.1	61	8	59
A-128815.1	68	6	59
A-128819.1	68	4	59
A-128864.1	70	9	59
A-128858.1	70	3	59
A-128781.1	70	4	59
A-128814.1	69	5	60
A-128747.1	69	10	60
A-128896.1	61	7	60
A-128894.1	61	8	60
A-128964.1	60	8	60
A-128634.1	71	4	60
A-128833.1	70	5	60
A-128840.1	70	3	60
A-128641.1	71	1	60
A-128771.1	71	8	60
A-128744.1	70	11	61
A-128587.1	65	6	61
A-128938.1	63	7	61
A-128915.1	62	16	61
A-128829.1	70	7	61
A-128741.1	69	6	61
A-128778.1	72	9	61
A-128801.1	71	6	61
A-128665.1	66	7	61
A-128979.1	61	15	61

A-128883.1	63	13	61
A-128899.1	63	20	62
A-128719.1	64	12	62
A-128621.1	67	5	62
A-128805.1	71	10	62
A-129005.1	62	13	62
A-128629.1	73	7	62
A-128836.1	72	5	62
A-128782.1	73	5	62
A-128564.1	65	3	62
A-128918.1	63	20	62
A-128963.1	63	10	62
A-128661.1	66	5	62
A-128601.1	67	6	63
A-128768.1	73	4	63
A-128608.1	67	3	63
A-128935.1	64	14	63
A-128616.1	67	10	63
A-128999.1	63	7	63
A-128898.1	64	13	63
A-128887.1	64	18	63
A-128572.1	66	8	63
A-128902.1	65	7	63
A-129054.1	64	18	63
A-128834.1	73	9	63
A-128724.1	72	8	64
A-128767.1	74	7	64
A-128847.1	74	4	64
A-128937.1	66	13	64
A-128984.1	64	23	64
A-128627.1	74	5	64
A-128980.1	64	14	64
A-128822.1	73	7	64
A-128905.1	65	22	64
A-129047.1	65	16	64
A-128949.1	66	19	65
A-128895.1	66	7	65

A-128802.1	75	6	65
A-128953.1	66	11	65
A-129008.1	65	16	65
A-128972.1	65	12	65
A-128800.1	76	7	66
A-129040.1	66	7	66
A-128820.1	75	2	66
A-128983.1	66	18	66
A-128992.1	66	21	66
A-128885.1	67	31	66
A-128886.1	67	13	66
A-128809.1	75	9	66
A-128921.1	67	6	66
A-128930.1	68	18	66
A-128936.1	68	4	67
A-128982.1	67	17	67
A-128806.1	76	9	67
A-128892.1	68	19	67
A-128739.1	75	11	67
A-128917.1	68	8	67
A-128881.1	77	2	67
A-128873.1	78	10	67
A-128743.1	76	4	67
A-128736.1	75	17	67
A-128848.1	78	6	67
A-128745.1	77	10	68
A-128683.1	72	5	68
A-129006.1	68	18	68
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A-129009.1	95	10	95
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A-129029.1	102	19	101
A-128961.1	107	32	105
A-129028.1	108	30	108
A-129030.1	117	11	117
A-129002.1	129	27	129

1. An antisense polynucleotide agent for inhibiting expression of complement component C5, wherein the agent comprises about 4 to about 50 contiguous nucleotides, wherein at least one of the contiguous nucleotides is a modified nucleotide, and wherein the nucleotide sequence of the agent is about 80% complementary over its entire length to the equivalent region of the nucleotide sequence of any one of SEQ ID NOs:1-4.
2. The agent of claim 1, wherein the equivalent region is one of the target regions of SEQ ID NO:1 provided in Table 3.
3. An antisense polynucleotide agent for inhibiting expression of complement component C5, wherein the agent comprises at least 8 contiguous nucleotides differing by no more than 3 nucleotides from any one of the nucleotide sequences listed in Table 3.
4. The agent of claim 1, wherein substantially all of the nucleotides of the antisense polynucleotide agent are modified nucleotides.
5. The agent of claim 1, wherein all of the nucleotides of the antisense polynucleotide agent are modified nucleotides.
6. The agent of claim 1, which is 10 to 40 nucleotides in length.
7. The agent of claim 1, which is 10 to 30 nucleotides in length.
8. The agent of claim 1, which is 18 to 30 nucleotides in length.
9. The agent of claim 1, which is 10 to 24 nucleotides in length.
10. The agent of claim 1, which is 18 to 24 nucleotides in length.
11. The agent of claim 1, which is 20 nucleotides in length.
12. The agent of claim 1, wherein the modified nucleotide comprises a modified sugar moiety selected from the group consisting of: a 2'-O-methoxyethyl modified sugar moiety, a 2'-methoxy modified sugar moiety, a 2'-O-alkyl modified sugar moiety, and a bicyclic sugar moiety.

group forming a bridge between the 2' oxygen and the 4' carbon atoms of the sugar ring, wherein n is 1 or 2.

14. The agent of claim 1, wherein the modified nucleotide is a 5-methylcytosine.
15. The agent of claim 1, wherein the modified nucleotide comprises a modified internucleoside linkage.
16. The agent of claim 15, wherein the modified internucleoside linkage is a phosphorothioate internucleoside linkage.
17. The agent of claim 1, comprising a plurality of 2'-deoxynucleotides flanked on each side by at least one nucleotide having a modified sugar moiety.
18. The agent of claim 17, wherein the agent is a gapmer comprising a gap segment comprised of linked 2'-deoxynucleotides positioned between a 5' and a 3' wing segment.
19. The agent of claim 17, wherein the modified sugar moiety is selected from the group consisting of a 2'-O-methoxyethyl modified sugar moiety, a 2'-methoxy modified sugar moiety, a 2'-O-alkyl modified sugar moiety, and a bicyclic sugar moiety.
20. The agent of claim 18, wherein the 5'-wing segment is 1 to 6 nucleotides in length.
21. The agent of claim 18, wherein the 3'-wing segment is 1 to 6 nucleotides in length.
22. The agent of claim 18, wherein the gap segment is 5 to 14 nucleotides in length.
23. The agent of claim 18, wherein the 5'-wing segment is 2 nucleotides in length.
24. The agent of claim 18, wherein the 3'-wing segment is 2 nucleotides in length.
25. The agent of claim 18, wherein the 5'-wing segment is 3 nucleotides in length.
26. The agent of claim 18, wherein the 3'-wing segment is 3 nucleotides in length.

28. The agent of claim 18, wherein the 3'-wing segment is 4 nucleotides in length.
29. The agent of claim 18, wherein the 5'-wing segment is 5 nucleotides in length.
30. The agent of claim 18, wherein the 3'-wing segment is 5 nucleotides in length.
31. The agent of claim 18, wherein gap segment is 10 nucleotides in length.
32. An antisense polynucleotide agent for inhibiting complement component C5 expression, comprising
 - a gap segment consisting of linked deoxynucleotides;
 - a 5'-wing segment consisting of linked nucleotides;
 - a 3'-wing segment consisting of linked nucleotides;wherein the gap segment is positioned between the 5'-wing segment and the 3'-wing segment and wherein each nucleotide of each wing segment comprises a modified sugar.
33. The agent of claim 32, wherein the gap segment is ten 2'-deoxynucleotides in length and each of the wing segments is five nucleotides in length.
34. The agent of claim 32, wherein the gap segment is ten 2'-deoxynucleotides in length and each of the wing segments is four nucleotides in length.
35. The agent of claim 32, wherein the gap segment is ten 2'-deoxynucleotides in length and each of the wing segments is three nucleotides in length.
36. The antisense compound of claim 32, wherein the gap segment is ten 2'-deoxynucleotides in length and each of the wing segments is two nucleotides in length.
37. The agent of claim 32, wherein the modified sugar moiety is selected from the group consisting of a 2'-O-methoxyethyl modified sugar moiety, a 2'-methoxy modified sugar moiety, a 2'-O-alkyl modified sugar moiety, and a bicyclic sugar moiety.
38. The agent of any one of claims 1 or 32, wherein the agent further comprises a ligand.
39. The agent of claim 38, wherein the antisense polynucleotide agent is conjugated to the ligand at the 3'-terminus.

comprises a MC3.

51. A method of inhibiting complement component C5 expression in a cell, the method comprising:

- (a) contacting the cell with the agent of claim 1 or 32 or a pharmaceutical composition of claim 42 or 48; and
- (b) maintaining the cell produced in step (a) for a time sufficient to obtain antisense inhibition of a complement component C5 gene, thereby inhibiting expression of the complement component C5 gene in the cell.

52. The method of claim 51, wherein the cell is within a subject.

53. The method of claim 52, wherein the subject is a human.

54. The method of claim 51, wherein the complement component C5 expression is inhibited by at least about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%.

55. A method of treating a subject having a disease or disorder that would benefit from reduction in complement component C5 expression, the method comprising administering to the subject a therapeutically effective amount of the agent of claim 1 or 32 or a pharmaceutical composition of claim 42 or 48, thereby treating the subject.

56. A method of preventing at least one symptom in a subject having a disease or disorder that would benefit from reduction in complement component C5 expression, the method comprising administering to the subject a prophylactically effective amount of the agent of claim 1 or 32 or a pharmaceutical composition of claim 42 or 48, thereby preventing at least one symptom in the subject having a disorder that would benefit from reduction in C5 expression.

57. The method of claim 55 or 56, wherein the administration of the antisense polynucleotide agent to the subject causes a decrease in intravascular hemolysis, a stabilization of hemoglobin levels and/or a decrease in C5 protein levels.

58. The method of claim 55 or 56, wherein the disorder is a complement component C5-associated disease.

disease is selected from the group consisting of paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), asthma, rheumatoid arthritis (RA); antiphospholipid antibody syndrome; lupus nephritis; ischemia-reperfusion injury; typical or infectious hemolytic uremic syndrome (tHUS); dense deposit disease (DDD); neuromyelitis optica (NMO); multifocal motor neuropathy (MMN); multiple sclerosis (MS); macular degeneration (e.g., age-related macular degeneration (AMD)); hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome; thrombotic thrombocytopenic purpura (TTP); spontaneous fetal loss; Pauci-immune vasculitis; epidermolysis bullosa; recurrent fetal loss; pre-eclampsia, traumatic brain injury, myasthenia gravis, cold agglutinin disease, dermatomyositis bullous pemphigoid, Shiga toxin *E. coli*-related hemolytic uremic syndrome, C3 nephropathy, anti-neutrophil cytoplasmic antibody-associated vasculitis, humoral and vascular transplant rejection, graft dysfunction, myocardial infarction, an allogenic transplant, sepsis, Coronary artery disease, dermatomyositis, Graves' disease, atherosclerosis, Alzheimer's disease, systemic inflammatory response sepsis, septic shock, spinal cord injury, glomerulonephritis, Hashimoto's thyroiditis, type I diabetes, psoriasis, pemphigus, autoimmune hemolytic anemia (AIHA), ITP, Goodpasture syndrome, Degos disease, antiphospholipid syndrome (APS), catastrophic APS (CAPS), a cardiovascular disorder, myocarditis, a cerebrovascular disorder, a peripheral vascular disorder, a renovascular disorder, a mesenteric/enteric vascular disorder, vasculitis, Henoch-Schönlein purpura nephritis, systemic lupus erythematosus-associated vasculitis, vasculitis associated with rheumatoid arthritis, immune complex vasculitis, Takayasu's disease, dilated cardiomyopathy, diabetic angiopathy, Kawasaki's disease (arteritis), venous gas embolus (VGE), and restenosis following stent placement, rotational atherectomy, membranous nephropathy, Guillain-Barre syndrome, and percutaneous transluminal coronary angioplasty (PTCA).

60. The method of claim 59, wherein the complement component C5-associated disease is paroxysmal nocturnal hemoglobinuria (PNH).

61. The method of claim 59, wherein the complement component C5-associated disease is atypical hemolytic uremic syndrome (aHUS).

62. The method of claim 55 or 56, wherein the subject is human.

63. The method of claim 55 or 56, further comprising administering an anti-complement component C5 antibody, or antigen-binding fragment thereof, to the subject.

64. The method of claim 55 or 56, wherein the agent is administered at a dose of about 0.01 mg/kg to about 10 mg/kg or about 0.5 mg/kg to about 50 mg/kg.

65. The method of claim 64, wherein the agent is administered at a dose of about 10 mg/kg to about 30 mg/kg.
66. The method of claim 64, wherein the agent is administered to the subject once a week.
67. The method of claim 64, wherein the agent is administered to the subject twice a week.
68. The method of claim 64, wherein the agent is administered to the subject twice a month.
69. The method of claim 55 or 56, wherein the agent is administered to the subject subcutaneously.
70. The method of claim 55 or 56, further comprising measuring hemoglobin and/or LDH levels in the subject.

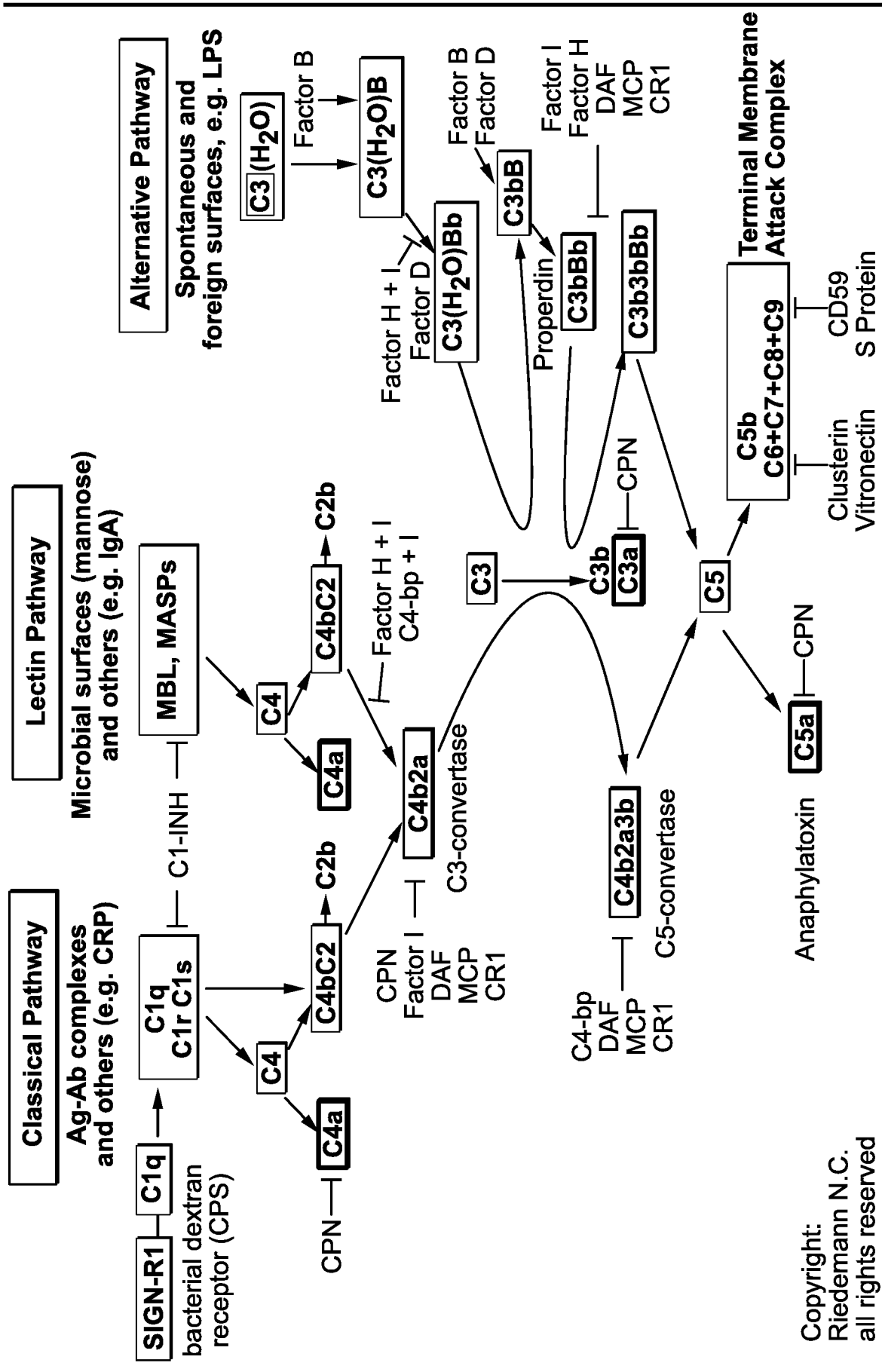


Figure 1

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INV. C12N15/113 A61K31/7088
 ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 C12N A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, Sequence Search, FSTA, COMPENDEX, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/054403 A1 (ALEXION PHARMA INC [US]; ROTHER RUSSELL P [US]; BEDROSIAN CAMILLE [US]) 14 May 2010 (2010-05-14) page 36 - page 38	1-70
X	WO 2008/113834 A2 (NOVARTIS AG [CH]; GUILD BRAYDON CHARLES [US]; KEATING MARK TAYLOR [US]) 25 September 2008 (2008-09-25) page 10 - page 14	1-70
X	WO 2007/103549 A2 (ARCHEMIX CORP) 13 September 2007 (2007-09-13) paragraph [0336] - paragraph [0344]	1-70
	-/--	

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 November 2015

Date of mailing of the international search report

05/02/2016

Name and mailing address of the ISA/

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Authorized officer

Piret, Bernard

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/049368

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. forming part of the international application as filed:
 - in the form of an Annex C/ST.25 text file.
 - on paper or in the form of an image file.
 - b. furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. furnished subsequent to the international filing date for the purposes of international search only:
 - in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/049368

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

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

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

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

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

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

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

see additional sheet

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

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

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

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

1-70(partially)

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/049368

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TANG KAI ET AL: "Protective effect of C5 shRNA on myocardial ischemia-reperfusion injury in", CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, NRC RESEARCH PRESS, CANADA, vol. 90, no. 10, 1 October 2012 (2012-10-01), pages 1394-1402, XP008170044, ISSN: 0008-4212, DOI: 10.1139/Y2012-114 [retrieved on 2012-10-15] -----	1-70
X,P	WO 2014/160129 A2 (ALNYLAM PHARMACEUTICALS INC [US]) 2 October 2014 (2014-10-02) the whole document -----	1-70

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2015/049368

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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		WO 2014160129 A2	02-10-2014

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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

1. claims: 1-70(partially)

Antisense polynucleotide targeting the region of SEQ ID NO:1 corresponding to SEQ ID NO:13 (positions 1-20).

2-496. claims: 1-70(partially)

Antisense polynucleotide targeting the region of SEQ ID NO:1 corresponding to SEQ ID NO:14 (positions 12-31), SEQ ID NO:15 (positions 23-42), SEQ ID NO:16 (positions 34-53), and so on until SEQ ID NO:508 (positions 5446-5465).
