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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 April 2005 (07.04.2005)

PCT

(10) International Publication Number
WO 2005/030259 A2

(51) International Patent Classification[7]: A61K 47/48

(21) International Application Number:
PCT/US2004/031748

(22) International Filing Date:
27 September 2004 (27.09.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/505,977 25 September 2003 (25.09.2003) US

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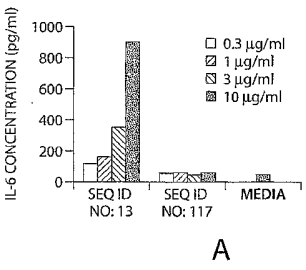
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

[Continued on next page]

(54) Title: NUCLEIC ACID-LIPOPHILIC CONJUGATES

(57) Abstract: The invention relates to a nucleic acid-lipophilic conjugates and methods for modulating an immune response using the conjugates. The lipophilic moiety associated with an immunostimulatory nucleic acid.



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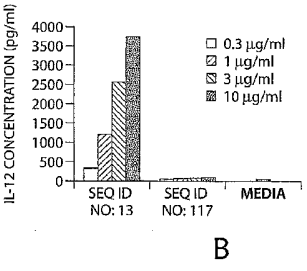

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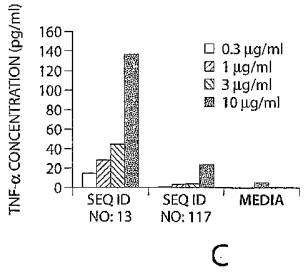

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WO 2005/030259 A2

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

NUCLEIC ACID-LIPOPHILIC CONJUGATES

FIELD OF THE INVENTION

The present invention relates generally to nucleic acids-lipophilic conjugates, compositions thereof and methods of using the conjugates.

BACKGROUND OF THE INVENTION

Bacterial DNA has immune stimulatory effects to activate B cells and natural killer cells, but vertebrate DNA does not (Tokunaga, T., et al., 1988. *Jpn. J. Cancer Res.* 79:682-686; Tokunaga, T., et al., 1984, *JNCI* 72:955-962; Messina, J.P., et al., 1991, *J. Immunol.* 147:1759-1764; and reviewed in Krieg, 1998, In: Applied Oligonucleotide Technology, C.A. Stein and A.M. Krieg, (Eds.), John Wiley and Sons, Inc., New York, NY, pp. 431-448) and Krieg. A. M. CpG motifs in bacterial DNA and their immune effects (2002) Annu. Rev. Immunol. 20: 709-760. It is now understood that these immune stimulatory effects of bacterial DNA are a result of the presence of unmethylated CpG dinucleotides in particular base contexts (CpG motifs), which are common in bacterial DNA, but methylated and underrepresented in vertebrate DNA (Krieg et al, 1995 Nature 374:546-549; Krieg, 1999 Biochim. Biophys. Acta 93321:1-10). The immune stimulatory effects of bacterial DNA can be mimicked with synthetic oligodeoxynucleotides (ODN) containing these CpG motifs. Such CpG ODN have highly stimulatory effects on human and murine leukocytes, inducing B cell proliferation; cytokine and immunoglobulin secretion; natural killer (NK) cell lytic activity and IFN-γ secretion; and activation of dendritic cells (DCs) and other antigen presenting cells to express costimulatory molecules and secrete cytokines, especially the Th1-like cytokines that are important in promoting the development of Th1-like T cell responses. These immune stimulatory effects of native phosphodiester backbone CpG ODN are highly CpG specific in that the effects are dramatically reduced if the CpG motif is methylated, changed to a GpC, or otherwise eliminated or altered (Krieg et al, 1995 Nature 374:546-549; Hartmann et al, 1999 Proc. Natl. Acad. Sci USA 96:9305-10).

In early studies, it was thought that the immune stimulatory CpG motif followed the formula purine-purine-CpG-pyrimidine-pyrimidine (Krieg et al, 1995 Nature 374:546-549; Pisetsky, 1996 J. Immunol. 156:421-423; Hacker et al., 1998 EMBO J. 17:6230-6240; Lipford et al, 1998 Trends in Microbiol. 6:496-500). However, it is now clear that mouse lymphocytes respond quite well to phosphodiester CpG motifs that do

not follow this "formula" (Yi et al., 1998 J. Immunol. 160:5898-5906) and the same is true of human B cells and dendritic cells (Hartmann et al, 1999 Proc. Natl. Acad. Sci USA 96:9305-10; Liang, 1996 J. Clin. Invest. 98:1119-1129). Nevertheless, the term "CpG motif" is generally used to refer to a hexamer motif in which the CpG dinucleotide is located at the center.

SUMMARY OF THE INVENTION

The present invention relates in part to immunostimulatory nucleic acids linked to a lipophilic group. It has been discovered that specific immunostimulatory nucleic acids linked to lipophilic groups have enhanced activity, whereas the linkage of lipophilic groups to other immunostimulatory nucleic acids has minimal effect on the immunostimulatory capability of the molecule.

The invention, in one aspect, relates to a composition of (N_1PN_2) L, wherein N_1 and N_2 are independently nucleic acids of 0-100 nucleotides in length, P is a palindrome containing nucleic acid and comprising at least one YR dinucleotide, wherein Y is a cytosine or a modified cytosine and R is a guanine or a modified guanine, and wherein L is a lipophilic group. In one embodiment N_1PN_2 is 3-14 nucleotides in length. In another embodiment L is linked to the nucleotide at the 3' end of N_1PN_2. Optionally the nucleotide is selected from the group consisting of a nucleotide at the 3' end of N_1PN_2 and an internal nucleotide. In one embodiment P is 2-100 nucleotides in length. In another embodiment P is 4-14 nucleotides in length.

In other embodiments L is linked by a linker to a 2'-position of a nucleotide in N_1PN_2, to a heterocyclic base of a nucleotide in N_1PN_2, or a phosphodiester linkage in N_1PN_2.

L is a lipophilic group which is a cholesteryl, a modified cholesteryl, a cholesterol derivative, a reduced cholesterol, a substituted cholesterol, cholestan, C16 alkyl chain, bile acids, cholic acid, taurocholic acid, deoxycholate, oleyl litocholic acid, oleoyl cholenic acid, glycolipids, phospholipids, sphingolipids, isoprenoids, such as steroids, vitamins, such as vitamin E, saturated fatty acids, unsaturated fatty acids, fatty acid esters, such as triglycerides, pyrenes, porphyrines, Texaphyrine, adamantane, acridines, biotin, coumarin, fluorescein, rhodamine, Texas-Red, digoxygenin, dimethoxytrityl, t-butyldimethylsilyl, t-butyldiphenylsilyl, cyanine dyes (e.g. Cy3 or

Cy5), Hoechst 33258 dye, psoralen, or ibuprofen. The composition may include at least 2 L.

In some embodiments the formula comprises N_1PN_2-L- N_3PN_4, wherein N_3 and N_4 are independently nucleic acids of 0-100 nucleotides in length. L may be linked to N_1PN_2 and N_3PN_4 through a linkage selected from the group consisting of -3'-L-3'-, -2'-L-2'-, -3'-L-2'- and -2'-L-3'-. In some embodiments N_1PN_2 and N_3PN_4 are identical. In other embodiments N_1PN_2 and N_3PN_4 are different.

The composition in other aspects of the invention is the following formula $([N_1PN_2]_n\text{-}(X_3)_m) \cdot (L)_p$. X_3 is a linker, m is an integer from 0 to 20 (preferably from 1-10), n is an integer from 0 to 20 (preferably from 1-10), and p is an integer from 1 to 10 (preferably 1). The oligonucleotide N_1PN_2 has a length of 4 to 40 nucleotides. n may be greater than 1 and the multiple $[N_1PN_2]$ are linked through 3'-ends.

In some embodiments X_3 is a non-nucleotidic linker selected from the group consisting of abasic residues (dSpacer), oligoethyleneglycol, such as triethyleneglycol (spacer 9) or hexaethylenegylcol (spacer 18), and alkane-diol, such as butanediol.

In other embodiments the linker is attached to the oligonucleotide through a linkage selected from the group consisting of phosphodiester, phosphorothioate, methylphosphonate, and amide linkages.

Optionally N_1PN_2 is a branched ODN and wherein N_1 includes at least one CG dinucleotide.

L may be attached to the 3' end of the oligonucleotide $[N_1PN_2]$. The linkage between L and N_1PN_2- may be metabolically stable or metabolically labile.

P may have the formula X_1-Y-R-X_2, wherein X_1 and X_2 are independently from 0 to 4 nucleotides. In some embodiments X_1 is 1 to 2 nucleotides. In other embodiments X_1 is a pyrimidine. Optionally the pyrimidine is selected from the group consisting of a thymidine, deoxyuridine and a 5-substituted deoxyuridine. In other embodiments X_2 is a palindrome or an inverted repeat (partial palindrome). The palindrome or inverted repeat (partial palindrome) may contain at least one unmethylated CpG motif. P may be selected from the group consisting of C_G_A_C, C_G_T_C, T_C_G_A_C, C_G_A_C_G_T_C, C_G_G_C_G_G_and G_A_C_G_A.

In some embodiments the oligonucleotide N_1PN_2 has a length of 4 to 20 nucleotides or 6 to 14 nucleotides.

Optionally the oligonucleotide includes at least one linear or branched non-nucleoside linkage.

An immune stimulatory molecule may be associated with the composition. An example of an immune stimulatory molecule is a TLR9 ligand.

The oligonucleotide may include at least one stabilized internucleotide linkage. Preferably the stabilized internucleotide linkage is the linkage between Y and R and is a phosphorothioate linkage in an Rp configuration. Preferably the internucleotide linkages of the oligonucleotide are all phosphodiester linkages.

At least one nucleotide in the oligonucleotide may be a substituted or modified purine or pyrimidine. In one embodiment the substituted pyrimidine is a C5 substitution or the substituted purine is a C8 or C7 substitution. In another embodiment the substituted or modified purine or pyrimidine is selected from the group consisting of 5-substituted cytosines (e.g. 5-methyl-cytosine, 5-fluoro-cytosine, 5-chloro-cytosine, 5-bromo-cytosine, 5-iodo-cytosine, 5-hydroxy-cytosine, 5-hydroxymethyl-cytosine, 5-difluoromethyl-cytosine, and unsubstituted or substituted 5-alkynyl-cytosine), 6-substituted cytosines, N4-substituted cytosines (e.g. N4-ethyl-cytosine), 5-aza-cytosine, 2-mercapto-cytosine, isocytosine, pseudo-isocytosine, cytosine analogs with condensed ring systems (e.g. N,N'-propylene cytosine or phenoxazine), and uracil and its derivatives (e.g. 5-fluoro-uracil, 5-bromo-uracil, 5-bromovinyl-uracil, 4-thio-uracil, 5-hydroxy-uracil, 5-propynyl-uracil), thymine derivatives (e.g. 2-thiothymine, 4-thiothymine, 6-substituted thymines), 7-deazaguanine, 7-deaza-7-substituted guanine (such as 7-deaza-7-(C2-C6)alkynylguanine), 7-deaza-8-substituted guanine, 7-deaza-8-aza guanine, hypoxanthine, N2-substituted guanines (e.g. N2-methyl-guanine), 5-amino-3-methyl-3H,6H-thiazolo[4,5-d]pyrimidine-2,7-dione, 2,6-diaminopurine, 2-aminopurine, purine, indole, adenine, substituted adenines (e.g. N6-methyl-adenine, 8-oxo-adenine) 8-substituted guanine (e.g. 8-hydroxyguanine and 8-bromoguanine), and 6-thioguanine. In another embodiment of the invention, the base is substituted by a universal base (e.g. 4-methyl-indole, 5-nitro-indole, 3-nitropyrrole, P-base, and K-base), an aromatic ring system (e.g. benzimidazole or dichloro-

benzimidazole, 1-methyl-1H-[1,2,4]triazole-3-carboxylic acid amide) an aromatic ring system (e.g. fluorobenzene or difluorobenzene) and a hydrogen atom (dSpacer).

Multiple oligonucleotides may be linked by multiple doubler or trebler moieties and form a dendrimer.

The composition may include at least one amino acid residue linked by an amide linkage.

Optionally the oligonucleotide includes at least one internucleotide linkage selected from the group consisting of a 3'5'-, a 2'5'-, a 3'3'-and a 5'5'-linkage.

In one embodiment L is associated with a carrier. Optionally the carrier is selected from the group consisting of a liposome, ISCOM, a hydrophobic bead, a hydrophobic formulation, a polymer, a peptide, a protein, and a nucleic acid. The composition may also include a therapeutic agent.

The invention in other aspects is a composition that further comprises a nucleic acid having at least one exposed 5' end comprising, at least one YR dinucleotide, wherein Y is a cytosine or a modified cytosine and R is a guanine or a modified guanine, at least one single stranded region, at least one double stranded region and wherein the nucleic acid is linked to at least one lipophilic group.

The nucleic acid may be a single chain nucleic acid or have a double stranded region involving base pairing of at least two nucleic acids on each side of the double stranded region. In one embodiment the nucleic acid forms a double stranded region involving base pairing of at least three nucleic acids on each side of the double stranded region.

In some embodiments the nucleic acid is a branched nucleic acid.

In other embodiments the nucleic acid is two single chain nucleic acids at least partially hybridized to one another.

The nucleic acid may be linked to at least two lipophilic groups. Optionally the lipophilic group is linked to the nucleotide at the 3' end of the nucleic acid.

In another aspect the invention is a lipophilic composition of $(N_1YRN_2) \cdot L$ wherein N_1 and N_2 are independently nucleic acids of 0-100 nucleotides in length, wherein Y is a cytosine or a modified cytosine and R is a guanine or a modified guanine, and N_1YRN_2 is

at least 10 nucleotides in length. L is a lipophilic group linked to a 2'-position of a nucleotide in N_1YRN_2, or to a heterocyclic base of a nucleotide in N_1YRN_2. In one embodiment N_1YRN_2 is 5'TCCG3', 5'TTCG3' or 5'TCGTCG3'.

In yet another aspect, the invention is a composition of $(N_1PN_2) \cdot L$ wherein N_1 and N_2 are independently nucleic acids of 0-100 nucleotides in length, P is a palindromic containing nucleic acid and comprising at least one YR dinucleotide, wherein Y is a cytosine or a modified cytosine and R is a guanine or a modified guanine, and wherein L is cholesterol. In certain embodiments L is linked to the nucleotide at the 3' end of N_1PN_2. In certain embodiments N_1PN_2 is selected from the group consisting of 5'TCGACGTCGT3' (SEQ ID NO: 111) and 5'TCGACGTCGA3' SEQ ID NO: 112).

In another aspect, the invention relates to a method for treating allergy or asthma. The method is performed by administering to a subject having or at risk of having allergy or asthma an immunostimulatory CpG oligonucleotide described herein in an effective amount to treat allergy or asthma. In one embodiment the oligonucleotide is administered to a mucosal surface, such as a respiratory tissue. In other embodiments the oligonucleotide is administered in an aerosol formulation. Optionally the oligonucleotide is administered intranasally. In other embodiments the subject has or is at risk of developing allergic asthma.

A method for inducing cytokine production is provided according to another aspect of the invention. The method is performed by administering to a subject an immunostimulatory CpG oligonucleotide described herein in an effective amount to induce a cytokine selected from the group consisting of IP10, IL6, IL 8, IL12, IL18, TNF, IFN-α, chemokines, and IFN-γ.

In another aspect the invention is a composition of the Lipophilic conjugates described herein in combination with an antigen or other therapeutic compound, such as an anti-microbial agent or an anti-cancer agent. The anti-microbial agent may be, for instance, an anti-viral agent, an anti-parasitic agent, an anti-bacterial agent or an anti-fungal agent.

The composition may optionally include a pharmaceutical carrier and/or be formulated in a delivery device. In some embodiments the delivery device is selected from the group consisting of cationic lipids, cell permeating proteins, and sustained

release devices. In one embodiment the sustained release device is a biodegradable polymer or a microparticle.

According to another aspect of the invention a method of stimulating an immune response is provided. The method involves administering a Lipophilic conjugate to a subject in an amount effective to induce an immune response in the subject. Preferably the Lipophilic conjugate is administered orally, locally, in a sustained release device, mucosally, systemically, parenterally, or intramuscularly. When the Lipophilic conjugate is administered to the mucosal surface it may be delivered in an amount effective for inducing a mucosal immune response or a systemic immune response. In preferred embodiments the mucosal surface is an oral, nasal, rectal, vaginal, or ocular surface.

In some embodiments the method includes exposing the subject to an antigen wherein the immune response is an antigen-specific immune response. In some embodiments the antigen is selected from the group consisting of a tumor antigen, a viral antigen, a bacterial antigen, a parasitic antigen and a peptide antigen.

CpG immunostimulatory oligonucleotides are capable of provoking a broad spectrum of immune response. For instance these Lipophilic conjugates can be used to redirect a Th2 to a Th1 immune response. Lipophilic conjugates may also be used to activate an immune cell, such as a lymphocyte (e.g., B and T cells), a dendritic cell, and an NK cell. The activation can be performed *in vivo, in vitro,* or *ex vivo*, i.e., by isolating an immune cell from the subject, contacting the immune cell with an effective amount to activate the immune cell of the Lipophilic conjugate and re-administering the activated immune cell to the subject. In some embodiments the dendritic cell presents a cancer antigen. The dendritic cell can be exposed to the cancer antigen *ex vivo*.

The immune response produced by Lipophilic conjugates may also result in induction of cytokine production, e.g., production of IP10, IL6, IL 8, IL12, IL18, TNF, IFN-α, chemokines, and IFN-γ.

In still another embodiment, the Lipophilic conjugates are useful for treating cancer in a subject having or at risk of developing a cancer. The cancer may be selected from the group consisting of biliary tract cancer, breast cancer, cervical cancer, choriocarcinoma, colon cancer, endometrial cancer, gastric cancer, intraepithelial neoplasms, lymphomas, liver cancer, lung cancer (e.g. small cell and non-small cell),

melanoma, neuroblastomas, oral cancer, ovarian cancer, pancreatic cancer, prostate cancer, rectal cancer, sarcomas, thyroid cancer, and renal cancer, as well as other carcinomas and sarcomas. In some important embodiments, the cancer is selected from the group consisting of bone cancer, brain and CNS cancer, connective tissue cancer, esophageal cancer, eye cancer, Hodgkin's lymphoma, larynx cancer, oral cavity cancer, skin cancer, and testicular cancer.

Lipophilic conjugates may also be used for increasing the responsiveness of a cancer cell to a cancer therapy (i.e., an anti-cancer therapy), optionally when the Lipophilic conjugate is administered in conjunction with an anti-cancer therapy. The anti-cancer therapy may be, for instance, a chemotherapy, a vaccine (e.g., an in vitro primed dendritic cell vaccine or a cancer antigen vaccine) or an immunotherapeutic agent such as an antibody based therapy. This latter therapy may also involve administering an antibody specific for a cell surface antigen of, for example, a cancer cell, wherein the immune response results in antibody dependent cellular cytotoxicity (ADCC). In one embodiment, the antibody may be selected from the group consisting of Ributaxin, Herceptin, Quadramet, Panorex, IDEC-Y2B8, BEC2, C225, Oncolym, SMART M195, ATRAGEN, Ovarex, Bexxar, LDP-03, ior t6, MDX-210, MDX-11, MDX-22, OV103, 3622W94, anti-VEGF, Zenapax, MDX-220, MDX-447, MELIMMUNE-2, MELIMMUNE-1, CEACIDE, Pretarget, NovoMAb-G2, TNT, Gliomab-H, GNI-250, EMD-72000, LymphoCide, CMA 676, Monopharm-C, 4B5, ior egf.r3, ior c5, BABS, anti-FLK-2, MDX-260, ANA Ab, SMART 1D10 Ab, SMART ABL 364 Ab and ImmuRAIT-CEA.

Thus, according to some aspects of the invention, a subject having cancer or at risk of having a cancer is administered a Lipophilic conjugate and an anti-cancer therapy. In some embodiments, the anti-cancer therapy is selected from the group consisting of a chemotherapeutic agent, an immunotherapeutic agent and a cancer vaccine.

In still another embodiment of the methods directed to treating cancer, the subject may be further administered interferon-α.

In other aspects, the invention is a method for inducing an innate immune response by administering to the subject a Lipophilic conjugate in an amount effective for activating an innate immune response. Thus the ODN are useful for treating pathogens such as Leishmania, Listeria, and Anthrax.

According to another aspect of the invention a method for treating a viral or retroviral infection is provided. The method involves administering to a subject having or at risk of having a viral or retroviral infection, an effective amount for treating the viral or retroviral infection of any of the compositions of the invention. In some embodiments the virus is caused by hepatitis virus e.g., hepatitis B or hepatitis C, HIV, herpes virus, or papillomavirus.

A method for treating a bacterial infection is provided according to another aspect of the invention. The method involves administering to a subject having or at risk of having a bacterial infection, an effective amount for treating the bacterial infection of any of the compositions of the invention. In one embodiment the bacterial infection is due to an intracellular bacteria.

In another aspect the invention is a method for treating a parasite infection by administering to a subject having or at risk of having a parasite infection, an effective amount for treating the parasite infection of any of the compositions of the invention. In one embodiment the parasite infection is due to an intracellular parasite. In another embodiment the parasite infection is due to a non-helminthic parasite.

In some embodiments the subject is a human and in other embodiments the subject is a non-human vertebrate such as a dog, cat, horse, cow, pig, turkey, goat, fish, monkey, chicken, rat, mouse, or sheep.

In another aspect the invention relates to a method for inducing a TH1 immune response by administering to a subject any of the compositions of the invention in an effective amount to produce a TH1 immune response.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention may be more easily and completely understood when taken in conjunction with the accompanying figures.

Figure 1 is a graph depicting the effect of linkage of a lipophilic group to an immunostimulatory nucleic acid through induction of IFN-α.

Figure 2 is a bar graph depicting effect of linkage of a lipophilic group to an immunostimulatory nucleic acid through induction of IL-6.

Figure 3 is a bar graph depicting effect of linkage of a lipophilic group to an immunostimulatory nucleic acid through induction of IL-10.

Figure 4 is a bar graph demonstrating that a lipophilic group conjugated to an immunostimulatory nucleic acid enhances induction of TLR9-dependent NF□B signaling.

Figure 5 is a bar graph depicting the *in vitro* mouse splenocyte stimulation effect of linkage of a lipophilic group to an immunostimulatory nucleic acid through induction of IL-6, IL-12, and TNF-α.

Figure 6 is a bar graph depicting the *in vitro* $TLR9^{+/+}$ and $TLR9^{-/-}$ mouse splenocyte stimulation effect of linkage of a lipophilic group to an immunostimulatory nucleic acid through induction of IL-12.

Figure 7 is a graph depicting the *in vivo* time-dependent effect of linkage of a lipophilic group to an immunostimulatory nucleic acid through induction of IP-10.

Figure 8 is a bar graph depicting the *in vivo* stimulation effect of linkage of a lipophilic group to an immunostimulatory nucleic acid through induction of IP-10, IL-12 and IL-6.

DETAILED DESCRIPTION

The invention in one aspect involves the finding that specific sub-classes of immunostimulatory oligonucleotides linked to a lipophilic group are highly effective in mediating immune stimulatory effects. These conjugates are useful therapeutically and prophylactically for stimulating the immune system to treat cancer, infectious diseases, allergy, asthma and other disorders.

A-Class immunostimulatory CpG oligonucleotides, such as oligonucleotide SEQ ID NO: 40, are characterized by their very efficient induction of IFN-α secretion, but low B cell stimulation. SEQ ID NO: 40 is composed of a palindromic phosphodiester CpG sequence (SEQ ID NO: 110) clamped by phosphorothioate (G)n stretches. ODN sequences are presented in Table 1 below. A-Class immunostimulatory CpG oligonucleotides, in which the 3'- and 5'- ends are phosphorothioate-modified and the center portion is phosphodiester, have runs of at least four G residues at both ends of the

oligonucleotide. As a result of intermolecular tetrad formation which results in high molecular weight aggregates, the development of G-rich oligonucleotides has been difficult. Issues related to the biophysical properties of this class of compounds include tendency to aggregation, poor solubility, difficulty in quality control and solid phase extraction (SPE) used in PK studies.

It is known that (G)n stretches in oligonucleotides, where $n \geq 4$, lead to intermolecular tetrad formation resulting in non homogeneous high molecular weight aggregates. The uptake of oligonucleotides with (G)n stretches is about 20 to 40-times higher than of non-aggregated oligonucleotides and the intracellular localization appears also to be different. It is not understood how these observations correlate with biological activity.

In an attempt to discover new immunostimulatory oligonucleotides having similar potency to A-class oligonucleotides such as SEQ ID NO: 40 but more favorable biophysical properties than G-rich oligonucleotides, a series of oligonucleotides without (G)n stretches but having lipophilic residues covalently attached have been developed according to the invention. Surprisingly, high Interferon-alpha (IFN-α) induction was detected, when an oligonucleotide having a palindromic center region, preferably with phosphodiester linkages, and at least one lipophilic group attached, even without the G_n sequences believed to be critical for A-class activity. For highest IFN-α induction, it is preferable that the number of phosphorothioate residues is kept to a minimum. An unexpectedly high induction of IFN-α secretion was observed with compositions composed of an L (lipophilic group) attached to the 3'-end of an oligonucleotide with a 5'-TCG and having only few or no phosphorothioate linkages.

It is also of interest that B-Class CpG oligonucleotides, when modified at the 3 '-end with Cholesterol (SEQ ID NO: 38), are less immunostimulatory than the corresponding 3'-unmodified (SEQ ID NO: 36) both in IFN-α induction and in a TLR9 assay. Similarly, the activity of a 5'-Cholesterol modified ODN (SEQ ID NO: 37) is much lower than that of the 5'-unmodified SEQ ID NO: 36. B-class ODN consist of non-palindromic sequences and are usually fully phosphorothioate modified. The decreased activity of B-class CpG ODN resulting from cholesterol modification is in contrast to the palindromic phosphodiester CpG ODN described herein. Cholesterol

modification of the latter at the 3'-end results in increased immunostimulatory activity (SEQ ID NO: 4) while 5'-cholesterol modification of the same sequence completely abolishes activity (SEQ ID NO: 6).

In some instances non-palindromic YR containing oligonucleotides having phosphodiester backbones also have increased immune stimulatory activity when a lipophilic group is conjugated at the 3' end of the oligonucleotide. Chimeric oligonucleotides having at least one YR motif that is phosphodiester but having at least one phosphorothioate or other modified linkage at the 5' and 3' end of the oligonucleotide also have increased immune stimulatory activity if a lipophilic group is conjugated at the 3'end of the molecule. The YR motifs in such chimeric oligonucleotides may be palindromic or nonpalindromic.

Thus, the invention involves, in one aspect, the discovery that a subset of immunostimulatory oligonucleotides linked to lipophilic groups have improved immune stimulatory properties. In some aspects the invention is a conjugate having the following formula $(N_1PN_2) \cdot L$. L is a lipophilic group.

The lipophilic group L is preferably a cholesterol, a cholesteryl or modified cholesteryl residue. The cholesterol moiety may be reduced (e.g. as in cholestan) or may be substituted (e.g. by halogen). A combination of different lipophilic groups in one molecule is also possible. Other lipophilic groups include but are not limited to bile acids, cholic acid or taurocholic acid, deoxycholate, oleyl litocholic acid, oleoyl cholenic acid, glycolipids, phospholipids, sphingolipids, isoprenoids, such as steroids, vitamins, such as vitamin E, fatty acids either saturated or unsaturated, fatty acid esters, such as triglycerides, pyrenes, porphyrines, Texaphyrine, adamantane, acridines, biotin, coumarin, fluorescein, rhodamine, Texas-Red, digoxygenin, dimethoxytrityl, t-butyldimethylsilyl, t-butyldiphenylsilyl, cyanine dyes (e.g. Cy3 or Cy5), Hoechst 33258 dye, psoralen, or ibuprofen. In some embodiements L is not a cholesterol.

The highest immunostimulatory activity was brought about by cholesterol modification as compared to other end-modifications, such as hexedecyl, vitamine E or triethylenglycole. It is expected, however, that these agents will produce more activity when more than one agent is attached to an oligonucleotide. Thus, in some embodiments the compositions of the invention have multiple L groups.

L is preferably at or near the 3' end of the oligonucleotide, unless it is in a branched oligonucleotide where there is at least one unobstructed 5' CpG motif. Cholesterol substitution at the only available 5'-end of the oligonucleotides tested was detrimental to the immunostimulatory effect (SEQ ID NO: 5 and SEQ ID NO: 6).

L may be connected to the oligonucleotide by a linker moiety. Optionally the linker moiety is a non-nucleotidic linker moiety. Non-nucleotidic linkers are e.g. abasic residues (dSpacer), oligoethyleneglycol, such as triethyleneglycol (spacer 9) or hexaethylenegylcol (spacer 18), or alkane-diol, such as butanediol. The spacer units are preferably linked by phosphodiester or phosphorothioate bonds. The linker units may appear just once in the molecule or may be incorporated several times, e.g. via phosphodiester, phosphorothioate, methylphosphonate, or amide linkages.

The lipophilic group L may be attached at various positions of the oligonucleotide. As described above, the lipophilic group L is linked to the 3'-end of the oligonucleotide, where it also serves the purpose to enhance the stability of the oligomer against 3'-exonucleoases. Alternatively, it may be linked to an internal nucleotide or a nucleotide on a branch. The lipophilic group L may be attached to a 2'-position of the nucleotide. The lipophilic group L may also be linked to the heterocyclic base of the nucleotide.

The oligonucleotides may have one or more than one accessible 5' ends. This may be achieved, for instance by attaching two oligonucleotides through a 3'-3' or other linkage or to connect two 3' ends through an L group to generate an oligonucleotide having one or two accessible 5' ends. Such a structure might have a formula such as 5'TCGN_1-L-N_1GCT5'. The 3'3'-linkage may be, for instance, a phosphodiester, phosphorothioate or any other modified internucleoside bridge. Methods for accomplishing such linkages are known in the art. For instance, such linkages have been described in Seliger, H.; et al., Oligonucleotide analogs with terminal 3'-3'- and 5'-5'-internucleotidic linkages as antisense inhibitors of viral gene expression, Nucleosides & Nucleotides (1991), 10(1-3), 469-77 and Jiang, et al., Pseudo-cyclic oligonucleotides: in vitro and in vivo properties, Bioorganic & Medicinal Chemistry (1999), 7(12), 2727-2735.

Additionally, 3'3'-linked ODNs where the linkage between the 3'-terminal nucleosides is not a phosphodiester, phosphorothioate or other modified bridge, can be

prepared using an additional spacer, such as tri- or tetra-ethylenglycol phosphate moiety (Durand, M. et al, Triple-helix formation by an oligonucleotide containing one (dA)12 and two (dT)12 sequences bridged by two hexaethylene glycol chains, Biochemistry (1992), 31(38), 9197-204, US Patent No. 5658738, and US Patent No. 5668265). Alternatively, the non-nucleotidic linker may be derived from ethanediol, propanediol, or from an abasic deoxyribose (dSpacer) unit (Fontanel, Marie Laurence et al., Sterical recognition by T4 polynucleotide kinase of non-nucleosidic moieties 5'-attached to oligonucleotides; Nucleic Acids Research (1994), 22(11), 2022-7) using standard phosphoramidite chemistry. The non-nucleotidic linkers can be incorporated once or multiple times, or combined with each other allowing for any desirable distance between the 3'-ends of the two ODNs to be linked.

Further preferred are oligonucleotides of the formula in which the lipophilic modification is part of the inter-nucleotide linkage which connects two adjacent nucleosides. If the lipophilic residue is within the sequence, thus linking different sequence parts together, then the sequence parts are preferentially not connected via their 5'-ends. In this case, two or more 3'3'-linked sequences are preferred. Also preferred are 2'2'-, 3'2'- or 2'3'-linked sequences, respectively. Optionally the linkage could be a 5'3' linkage. If two or more sequences are linked, these can be identical or different. Preferred linkages are phosphodiester, phosphorothioate, amide, ether, thioether, urea, thiourea, sulfonamide, Schiff' Base and disulfide linkages. Another possibility is the use of the Solulink BioConjugation System.

The lipophilic group may be linked to the oligonucleotide without additional spacers (m=0) or can be linked via one or more linker units (m>1). The linkage between the oligonucleotide and the lipophilic residue may be a metabolically stable or metabolically labile one.

Thus, in some embodiments the conjugate may have the following formula:

$([N_1PN_2]_n\text{-}(X_3)_m) \cdot (L)_p$

N_1 and N_2 are independently nucleic acids of 0-100 nucleotides in length, P is a palindromic containing nucleic acid and comprising at least one YR dinucleotide, wherein Y is a cytosine or a modified cytosine and R is a guanine or a modified guanine.

N may optionally have interspersed linear or branched non-nucleoside linkages or other immune stimulatory conjugates such as ligands for TLR molecules. It has been discovered that oligonucleotides having a 5'TCG or 5' UCG have particularly strong immunostimulatory capability.

The oligonucleotide of the formula (separate from the linkers connecting nucleotides to L) may also contain non-nucleotidic linkers, in particular abasic linkers (dSpacers), trietyhlene glycol units or hexaethylene glycol units. Further preferred linkers are alkylamino linkers, such as C3, C6, C12 aminolinkers, and also alkylthiol linkers, such as C3 or C6 thiol linkers. Oligonucleotides with a 3'3'-linkeage may also contain a Doubler or Trebler unit. Branching of the oligonucleotides by multiple doubler or trebler moieties leads to dendrimers which are a further embodiment of this invention. The oligonucleotide of formula I may also contain linker units resulting from peptide modifying reagents or oligonucleotide modifying reagents. Furthermore, it may contain one or more natural or unnatural amino acid residues which are connected by peptide (amide) linkages. The nucleotides in the formula may be linked through 3'5'- and/or 2'5'-linkages. It may further contain independently from each other one or more 3'3'-linkages and/or 5'5'-linkages.

P is a palindrome or inverted repeat, i.e. a partial palindrome. Preferably, the palindrome or inverted repeat (partial palindrome) contains at least one unmethylated CpG motif. In some embodiments it includes at least 2 or 3 CpG motifs. In SEQ ID NO: 4, the sequence (TCGACGTCGT, SEQ ID NO: 111) is only partially palindromic (CGACGTCG), i.e. inverted repeat, whereas in SEQ ID NO: 13, the sequence forms a complete palindrome. Preferably, at least one of the CpG motifs in the palindrome or inverted repeat (partial palindrome) is TCGA, ACGT, or CGGCCG. Some preferred palindromes include:

C_G_A_C_G_T_C_G	
C_G_T_C_G_A_C_G	
T_C_G_A_C_G_T_C_G_A	SEQ ID NO: 112
C_G_A_C_G_T_C_G_A_C_G_T_C_G	SEQ ID NO: 113
C_G_G_C_G_G_C_C_G_C_C_G	SEQ ID NO: 114
G_A_C_G_A_T_C_G_T_C	SEQ ID NO: 115

The immunostimulatory oligonucleotides generally have a length in the range of between 4 and 100 nucleotides. In some embodiments the length is in the range of 4-40, 13-100, 13-40, 13-30, 15-50, or 15- 30 nucleotides or any integer range therebetween.

The terms "nucleic acid" and "oligonucleotide" are used interchangeably to mean multiple nucleotides (i.e., molecules comprising a sugar (e.g., ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g., cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g., adenine (A) or guanine (G)). As used herein, the terms "nucleic acid" and "oligonucleotide" refer to oligoribonucleotides as well as oligodeoxyribonucleotides. The terms "nucleic acid" and "oligonucleotide" shall also include polynucleosides (i.e., a polynucleotide minus the phosphate) and any other organic base containing polymer. Nucleic acid molecules can be obtained from existing nucleic acid sources (e.g., genomic or cDNA), but are preferably synthetic (e.g., produced by nucleic acid synthesis). The term oligonucleotide generally refers to a shorter molecule, i.e. 100 nucleotides or less in length.

The terms "nucleic acid" and "oligonucleotide" also encompass nucleic acids or oligonucleotides with substitutions or modifications, such as in the bases and/or sugars. For example, they include nucleic acids having backbone sugars that are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 2' position and other than a phosphate group or hydroxy group at the 5' position. Thus modified nucleic acids may include a 2'-O-alkylated ribose group. In addition, modified nucleic acids may include sugars such as arabinose or 2'-fluoroarabinose instead of ribose. Thus the nucleic acids may be heterogeneous in backbone composition thereby containing any possible combination of polymer units linked together such as peptide-nucleic acids (which have an amino acid backbone with nucleic acid bases). Other examples are described in more detail below.

The immunostimulatory oligonucleotides of the instant invention can encompass various chemical modifications and substitutions, in comparison to natural RNA and DNA, involving a phosphodiester internucleoside bridge, a β-D-ribose unit and/or a natural nucleoside base (adenine, guanine, cytosine, thymine, uracil). Examples of chemical modifications are known to the skilled person and are described, for example, in Uhlmann E et al. (1990) *Chem Rev* 90:543; "Protocols for Oligonucleotides and

Analogs" Synthesis and Properties & Synthesis and Analytical Techniques, S. Agrawal, Ed, Humana Press, Totowa, USA 1993; Crooke ST et al. (1996) *Annu Rev Pharmacol Toxicol* 36:107-129; and Hunziker J et al. (1995) *Mod Synth Methods* 7:331-417. An oligonucleotide according to the invention may have one or more modifications, wherein each modification is located at a particular phosphodiester internucleoside bridge and/or at a particular β-D-ribose unit and/or at a particular natural nucleoside base position in comparison to an oligonucleotide of the same sequence which is composed of natural DNA or RNA.

For example, the oligonucleotides may comprise one or more modifications and wherein each modification is independently selected from:

a) the replacement of a phosphodiester internucleoside bridge located at the 3' and/or the 5' end of a nucleoside by a modified internucleoside bridge,

b) the replacement of phosphodiester bridge located at the 3' and/or the 5' end of a nucleoside by a dephospho bridge,

c) the replacement of a sugar phosphate unit from the sugar phosphate backbone by another unit,

d) the replacement of a β-D-ribose unit by a modified sugar unit, and

e) the replacement of a natural nucleoside base by a modified nucleoside base.

More detailed examples for the chemical modification of an oligonucleotide are as follows.

The oligonucleotides may include modified internucleotide linkages, such as those described in a or b above. These modified linkages may be partially resistant to degradation (*e.g.*, are stabilized). A "stabilized oligonucleotide molecule" shall mean an oligonucleotide that is relatively resistant to *in vivo* degradation (e.g. via an exo- or endo-nuclease) resulting form such modifications. Oligonucleotides having phosphorothioate linkages, in some embodiments, may provide maximal activity and protect the oligonucleotide from degradation by intracellular exo- and endo-nucleases.

A phosphodiester internucleoside bridge located at the 3' and/or the 5' end of a nucleoside can be replaced by a modified internucleoside bridge, wherein the modified internucleoside bridge is for example selected from phosphorothioate, phosphorodithioate, NR^1R^2-phosphoramidate, boranophosphate, α-hydroxybenzyl phosphonate, phosphate-(C_1-C_{21})-O-alkyl ester, phosphate-[(C_6-C_{12})aryl-(C_1-C_{21})-O-

alkyl]ester, (C_1-C_8)alkylphosphonate and/or (C_6-C_{12})arylphosphonate bridges, (C_7-C_{12})-□-hydroxymethyl-aryl (e.g., disclosed in WO 95/01363), wherein (C_6-C_{12})aryl, (C_6-C_{20})aryl and (C_6-C_{14})aryl are optionally substituted by halogen, alkyl, alkoxy, nitro, cyano, and where R^1 and R^2 are, independently of each other, hydrogen, (C_1-C_{18})-alkyl, (C_6-C_{20})-aryl, (C_6-C_{14})-aryl-(C_1-C_8)-alkyl, preferably hydrogen, (C_1-C_8)-alkyl, preferably (C_1-C_4)-alkyl and/or methoxyethyl, or R^1 and R^2 form, together with the nitrogen atom carrying them, a 5-6-membered heterocyclic ring which can additionally contain a further heteroatom from the group O, S and N.

The replacement of a phosphodiester bridge located at the 3' and/or the 5' end of a nucleoside by a dephospho bridge (dephospho bridges are described, for example, in Uhlmann E and Peyman A in "Methods in Molecular Biology", Vol. 20, "Protocols for Oligonucleotides and Analogs", S. Agrawal, Ed., Humana Press, Totowa 1993, Chapter 16, pp. 355 ff), wherein a dephospho bridge is for example selected from the dephospho bridges formacetal, 3'-thioformacetal, methylhydroxylamine, oxime, methylenedimethyl-hydrazo, dimethylenesulfone and/or silyl groups.

A sugar phosphate unit (i.e., a β-D-ribose and phosphodiester internucleoside bridge together forming a sugar phosphate unit) from the sugar phosphate backbone (i.e., a sugar phosphate backbone is composed of sugar phosphate units) can be replaced by another unit, wherein the other unit is for example suitable to build up a "morpholino-derivative" oligomer (as described, for example, in Stirchak EP et al. (1989) *Nucleic Acids Res* 17:6129-41), that is, e.g., the replacement by a morpholino-derivative unit; or to build up a polyamide nucleic acid ("PNA"; as described for example, in Nielsen PE et al. (1994) *Bioconjug Chem* 5:3-7), that is, e.g., the replacement by a PNA backbone unit, e.g., by 2-aminoethylglycine. The oligonucleotide may have other carbohydrate backbone modifications and replacements, such as peptide nucleic acids with phosphate groups (PHONA), locked nucleic acids (LNA), and oligonucleotides having backbone sections with alkyl linkers or amino linkers. The alkyl linker may be branched or unbranched, substituted or unsubstituted, and chirally pure or a racemic mixture.

A β-ribose unit or a β-D-2'-deoxyribose unit can be replaced by a modified sugar unit, wherein the modified sugar unit is for example selected from β-D-ribose, α-D-2'-deoxyribose, L-2'-deoxyribose, 2'-F-2'-deoxyribose, 2'-F-arabinose, 2'-O-(C_1-C_6)alkyl-ribose, preferably 2'-O-(C_1-C_6)alkyl-ribose is 2'-O-methylribose, 2'-O-(C_2-C_6)alkenyl-

ribose, 2'-[O-(C_1-C_6)alkyl-O-(C_1-C_6)alkyl]-ribose, 2'-NH_2-2'-deoxyribose, β-D-xylo-furanose, α-arabinofuranose, 2,4-dideoxy-β-D-erythro-hexo-pyranose, and carbocyclic (described, for example, in Froehler J (1992) *Am Chem Soc* 114:8320) and/or open-chain sugar analogs (described, for example, in Vandendriessche et al. (1993) *Tetrahedron* 49:7223) and/or bicyclosugar analogs (described, for example, in Tarkov M et al. (1993) *Helv Chim Acta* 76:481).

In some embodiments the sugar is 2'-O-methylribose, particularly for one or both nucleotides linked by a phosphodiester or phosphodiester-like internucleoside linkage.

Nucleic acids also include substituted purines and pyrimidines such as C-5 propyne pyrimidine and 7-deaza-7-substituted purine modified bases. Wagner RW et al. (1996) *Nat Biotechnol* 14:840-4. Purines and pyrimidines include but are not limited to adenine, cytosine, guanine, and thymine, and other naturally and non-naturally occurring nucleobases, substituted and unsubstituted aromatic moieties.

A modified base is any base which is chemically distinct from the naturally occurring bases typically found in DNA and RNA such as T, C, G, A, and U, but which share basic chemical structures with these naturally occurring bases. The modified nucleoside base may be, for example, selected from hypoxanthine, uracil, dihydrouracil, pseudouracil, 2-thiouracil, 4-thiouracil, 5-aminouracil, 5-(C_1-C_6)-alkyluracil, 5-(C_2-C_6)-alkenyluracil, 5-(C_2-C_6)-alkynyluracil, 5-(hydroxymethyl)uracil, 5-chlorouracil, 5-fluorouracil, 5-bromouracil, 5-hydroxycytosine, 5-(C_1-C_6)-alkylcytosine, 5-(C_2-C_6)-alkenylcytosine, 5-(C_2-C_6)-alkynylcytosine, 5-chlorocytosine, 5-fluorocytosine, 5-bromocytosine, N^2-dimethylguanine, 2,4-diamino-purine, 8-azapurine, a substituted 7-deazapurine, preferably 7-deaza-7-substituted and/or 7-deaza-8-substituted purine, 5-hydroxymethylcytosine, N4-alkylcytosine, e.g., N4-ethylcytosine, 5-hydroxydeoxycytidine, 5-hydroxymethyldeoxycytidine, N4-alkyldeoxycytidine, e.g., N4-ethyldeoxycytidine, 6-thiodeoxyguanosine, and deoxyribonucleosides of nitropyrrole, C5-propynylpyrimidine, and diaminopurine e.g., 2,6-diaminopurine, inosine, 5-methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, hypoxanthine or other modifications of a natural nucleoside bases. This list is meant to be exemplary and is not to be interpreted to be limiting.

In particular formulas described herein a set of modified bases is defined. For instance the letter Y is used to refer to a nucleotide containing a cytosine or a modified

cytosine. A modified cytosine as used herein is a naturally occurring or non-naturally occurring pyrimidine base analog of cytosine which can replace this base without impairing the immunostimulatory activity of the oligonucleotide. Modified cytosines include but are not limited to 5-substituted cytosines (e.g. 5-methyl-cytosine, 5-fluoro-cytosine, 5-chloro-cytosine, 5-bromo-cytosine, 5-iodo-cytosine, 5-hydroxy-cytosine, 5-hydroxymethyl-cytosine, 5-difluoromethyl-cytosine, and unsubstituted or substituted 5-alkynyl-cytosine), 6-substituted cytosines, N4-substituted cytosines (e.g. N4-ethyl-cytosine), 5-aza-cytosine, 2-mercapto-cytosine, isocytosine, pseudo-isocytosine, cytosine analogs with condensed ring systems (e.g. N,N'-propylene cytosine or phenoxazine), and uracil and its derivatives (e.g. 5-fluoro-uracil, 5-bromo-uracil, 5-bromovinyl-uracil, 4-thio-uracil, 5-hydroxy-uracil, 5-propynyl-uracil). Some of the preferred cytosines include 5-methyl-cytosine, 5-fluoro-cytosine, 5-hydroxy-cytosine, 5-hydroxymethyl-cytosine, and N4-ethyl-cytosine. In another embodiment of the invention, the cytosine base is substituted by a universal base (e.g. 3-nitropyrrole, P-base), an aromatic ring system (e.g. fluorobenzene or difluorobenzene) or a hydrogen atom (dSpacer).

The letter R is used to refer to guanine or a modified guanine base. A modified guanine as used herein is a naturally occurring or non-naturally occurring purine base analog of guanine which can replace this base without impairing the immunostimulatory activity of the oligonucleotide. Modified guanines include but are not limited to 7-deazaguanine, 7-deaza-7-substituted guanine (such as 7-deaza-7-(C2-C6)alkynylguanine), 7-deaza-8-substituted guanine, hypoxanthine, N2-substituted guanines (e.g. N2-methyl-guanine), 5-amino-3-methyl-3H,6H-thiazolo[4,5-d]pyrimidine-2,7-dione, 2,6-diaminopurine, 2-aminopurine, purine, indole, adenine, substituted adenines (e.g. N6-methyl-adenine, 8-oxo-adenine) 8-substituted guanine (e.g. 8-hydroxyguanine and 8-bromoguanine), and 6-thioguanine. In another embodiment of the invention, the guanine base is substituted by a universal base (e.g. 4-methyl-indole, 5-nitro-indole, and K-base), an aromatic ring system (e.g. benzimidazole or dichloro-benzimidazole, 1-methyl-1H-[1,2,4]triazole-3-carboxylic acid amide) or a hydrogen atom (dSpacer).

Certain base modifications were also allowed. SEQ ID NO: 29, in which the terminal T residues at either end were replaced by deoxyuridine (U), turned out to be a potent inducer of IFN-α. In contrast, replacing G by deoxyinosine (I) in all CpG motifs

(as in SEQ ID NO: 30) completely abolished IFN-α induction. Surprisingly, modification of G residues as 7-deaza deoxyguanosine (SEQ ID NO: 31) resulted in high IFN-α induction. Therefore, the need for tetrad formation via Hoogsteen base-pairing, a prerequisite for high activity of the previously described G-rich A-Class oligonucleotides, can be excluded for the new cholesterol modified A-Class immunostimulatory oligonucleotides.

For use in the instant invention, the oligonucleotides of the invention can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the β-cyanoethyl phosphoramidite method (Beaucage, S.L., and Caruthers, M.H., *Tet. Let.* 22:1859, 1981); nucleoside H-phosphonate method (Garegg *et al.*, *Tet. Let.* 27:4051-4054, 1986; Froehler *et al.*, *Nucl. Acid. Res.* 14:5399-5407, 1986, ; Garegg *et al.*, *Tet. Let.* 27:4055-4058, 1986, Gaffney *et al.*, *Tet. Let.* 29:2619-2622, 1988). These chemistries can be performed by a variety of automated nucleic acid synthesizers available in the market. These oligonucleotides are referred to as synthetic oligonucleotides. An isolated oligonucleotide generally refers to an oligonucleotide which is separated from components which it is normally associated with in nature. As an example, an isolated oligonucleotide may be one which is separated from a cell, from a nucleus, from mitochondria or from chromatin.

The internucleotide linkages in the oligonucleotide, may be a non-stabilized or stabilized linkage (against nucleases), preferably a phosphodiester (non stabilized), a phosphorothioate (stabilized) or another charged backbone, most preferably a phosphodiester linkage. If the internucleotide linkage at Y-R is a phosphorothioate, the chirality of this linkage may be random, or is preferably a phosphorothioate linkage of Rp configuration. Increasing numbers of phosphorothioate linkages (SEQ ID NO: 3, SEQ ID NO: 15, SEQ ID NO: 25), in particular at the 5'-end, resulted in diminished or no IFN-α induction.

Modified backbones such as phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl-and alkyl-phosphonates can be made, *e.g.*, as described in U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents.

Methods for making other DNA backbone modifications and substitutions have been described (e.g., Uhlmann, E. and Peyman, A., *Chem. Rev.* 90:544, 1990; Goodchild, J., *Bioconjugate Chem.* 1:165, 1990).

Table 1

ODN #	Sequence
SEQ ID NO: 1	T*C_G_A_C_G_T_C_G_T_teg
SEQ ID NO: 2	T*C_G_A_C_G_T_C_G_T_L
SEQ ID NO: 3	T*C_G_A_C_G_T_C_G*T-Chol
SEQ ID NO: 4	T_C_G_A_C_G_T_C_G_T_Chol
SEQ ID NO: 5	Chol-T_C_G_A_C_G_T_C_G_T-Chol
SEQ ID NO: 6	Chol_T_C_G_A_C_G_T_C_G_T_teg
SEQ ID NO: 7	T_C_G_T_C_G_A_C_G_T_G_Chol
SEQ ID NO: 8	T_C_G_A_C_G_T_C_G_T_T_Chol
SEQ ID NO: 9	G_T_C_G_A_C_G_T_C_G_T_Chol
SEQ ID NO: 10	G_T_C_G_A_C_G_T_C_G_T_T_Chol
SEQ ID NO: 11	T_C_G_T_C_G_A_C_G_T_T_Chol
SEQ ID NO: 12	A_C_G_A_C_G_T_C_G_T_Chol
SEQ ID NO: 13	T_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 14	G_A_C_G_A_C_G_T_C_G_T_T_Chol
SEQ ID NO: 15	T*C*G*A*C*G*T*C*G*T_Chol
SEQ ID NO: 16	T*C_G_A_C_G_T_C_G_T_Chol
SEQ ID NO: 17	T_C_G_A_C_G_T_C_G*T_Chol
SEQ ID NO: 18	T_C_G_A_C_G_T_C_G_T_teg
SEQ ID NO: 19	T_C_G_A_C_G_T_C_G_A_C_G_T_C_G_T_Chol
SEQ ID NO: 20	T_C_G_T_C_G_T_C_G_T_Chol
SEQ ID NO: 21	T_G_C_A_G_C_T_G_C_T-Chol
SEQ ID NO: 22	..C_G_A_C_G_T_C_G.. Chol
SEQ ID NO: 23	T_A_A_C_G_T_T_T_Chol
SEQ ID NO: 24	T_G_A_C_G_T_T_T_Chol
SEQ ID NO: 25	T*C*G*T_C_G_A_C_G_T_C_G_T_Chol

SEQ ID NO: 26	T*C*G*T*C*G*T*T*T*T_C_G_A_C_G_T_C_G_T_Chol
SEQ ID NO: 27	T_C_G_G_C_G_G_C_C_G_C_C_G_Chol
SEQ ID NO: 28	T*C*G*T_C_G_G_C_G_G_C_C_G_C_C_G_T_Chol
SEQ ID NO: 29	U_C_G_A_C_G_T_C_G_U-Chol
SEQ ID NO: 30	T_C_I_A_C_I_T_C_I_T-Chol
SEQ ID NO: 31	T_C_7_A_C_7_T_C_7_T-Chol
SEQ ID NO: 32	T_C_A_T_C_G_A_T_G_A_Chol
SEQ ID NO: 33	....G_A_C_G_A_T_C_G_T_C_Chol
SEQ ID NO: 34	T_C_A_C_C_G_G_T_G_A_Chol
SEQ ID NO: 35	G_A_C_G_T_T_A_A_C_G_T_C_Chol
SEQ ID NO: 36	T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T (B-Class ODN)
SEQ ID NO: 37	Chol_T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T
SEQ ID NO: 38	T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T_Chol
SEQ ID NO: 39	T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G (C-Class ODN)
SEQ ID NO: 40	G*G*G_G_A_C_G_A_C_G_T_C_G_T_G_G*G*G*G*G*G (A-Class ODN)
SEQ ID NO: 41	T_C_G_A_Chol
SEQ ID NO: 42	T_C_G_C_G_A_Chol
SEQ ID NO: 43	T_C_G_C_G_C_G_A_Chol
SEQ ID NO: 44	T_C_G_C_C_G_G_C_G_A_Chol
SEQ ID NO: 45	T_C_G_G_C_G_C_C_G_A_Chol
SEQ ID NO: 46	T_C_G_C_G_C_G_C_G_A_Chol
SEQ ID NO: 47	T_C_G_T_C_G_A_C_G_A_Chol
SEQ ID NO: 48	T_C_G_T_A_C_G_A_Chol
SEQ ID NO: 49	T_C_G_A_A_T_T_C_G_A_Chol
SEQ ID NO: 50	T_C_G_T_T_A_A_C_G_A_Chol
SEQ ID NO: 51	T_C_G_A_A_C_G_T_T_C_G_A_Chol

SEQ ID NO: 52	T_C_G_T_T_C_G_A_A_C_G_A_Chol
SEQ ID NO: 53	T_C_G_A_C_G_A_T_C_G_T_C_G_A_Chol
SEQ ID NO: 54	T_C_G_G_A_C_G_A_T_C_G_T_C_C_G_A_Chol
SEQ ID NO: 55	T_C_G_A_C_G_A_G_C_T_C_G_T_C_G_A_Chol
SEQ ID NO: 56	T_C_G_G_C_G_G_C_C_G_C_C_G_A_Chol
SEQ ID NO: 57	T_C_G_A_C_G_T_C_G_A*Chol
SEQ ID NO: 58	T_C_G_A_C_G_T_C_G*A_Chol
SEQ ID NO: 59	T_C_G_A_C_G_T_C*G*A_Chol
SEQ ID NO: 60	T_C_G_A_C_G_T*C*G*A_Chol
SEQ ID NO: 61	G_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 62	C_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 63	I_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 64	U_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 65	Z_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 66	T_T_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 67	T_T_T_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 68	T_C_G_T_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 69	T_C_G_A_A_T_A_T_A_T_A_T_T_A_C_G_A_chol
SEQ ID NO: 70	T_C_G_A_A_T_A_T_A_T_A_T_T_A_chol
SEQ ID NO: 71	T_C_A_T_C_G_A_T_G_A_Chol
SEQ ID NO: 72	T_C_G_A_C_G_T_T_G_A_Chol
SEQ ID NO: 73	F_C_G_A_C_G_F_C_G_A_Chol
SEQ ID NO: 74	T_H_G_A_H_G_T_H_G_A_Chol
SEQ ID NO: 75	T_Z_G_A_Z_G_T_Z_G_A_Chol
SEQ ID NO: 76	T_C_G_V_C_G_T_C_G_V_Chol

SEQ ID NO: 77	T_C_V_A_C_V_T_C_V_A_Chol
SEQ ID NO: 78	T_C_R_A_C_R_T_C_R_A_Chol
SEQ ID NO: 79	T_C_O_A_C_O_T_C_O_A_Chol
SEQ ID NO: 80	T_C_S_A_C_S_T_C_S_A_Chol
SEQ ID NO: 81	T_C_G_S_C_G_T_C_G_S_Chol
SEQ ID NO: 82	T_S_G_A_S_G_T_S_G_A_Chol
SEQ ID NO: 83	T_C_G_A_C_G_S_C_G_A_Chol
SEQ ID NO: 84	T_C_6G_A_C_6G_T_C_6G_A_Chol
SEQ ID NO: 85	ff_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 86	4T_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 87	yU_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 88	5U_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 89	T_C_D_A_C_G_T_C_G_A_Chol
SEQ ID NO: 90	T_C_G_A_D_G_T_C_G_A_Chol
SEQ ID NO: 91	T_C_G_A_C_G_T_C_D_A_Chol
SEQ ID NO: 92	T_C_G_A_D_D_T_C_G_A_Chol
SEQ ID NO: 93	5T_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 94	3T_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 95	T_aC_G_A_aC_G_T_aC_G_A_Chol
SEQ ID NO: 96	T_fC_G_A_fC_G_T_fC_G_A_Chol
SEQ ID NO: 97	fU_C_G_A_C_G_fU_C_G_A_Chol
SEQ ID NO: 98	mU_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 99	mU_mC_mG_mA_mC_mG_mU_mC_mG_mA_Chol
SEQ ID NO: 100	rU_C_G_A_C_G_T_C_G_A_Chol
SEQ ID NO: 101	rU_rC_rG_rA_rC_rG_rU_rC_rG_rA_Chol

SEQ ID NO: 102	mU&mC&mG&mA&mC&mG&mU&mC&mG&mA_Chol
SEQ ID NO: 103	T_C_G_A_C_G_T_C_G_A_D_D_D_D_T_C_G_A_C_G_T_C_G_A_chol
SEQ ID NO: 104	3'-teg_A_G_C_T_G_C_A_G_C_T_(5'5'link)D_D_D_D_T_C_G_A_C_G_T_C_G_A_chol-3'

The symbol * refers to the presence of a stabilized internucleotide linkage and _: refers to the presence of a phosphodiester linkage. The following are definitions of symbols and letters in table 1:

&	2'5'-linkage as phosphodiester
*	phosphorothioate
*p	5′-Thiophosphate
_	phosphodiester (PO-bonds)
A, C, G, T	2'-Deoxynucleotide (dA, dC, dG, T)
chol	Cholesterol
D	dSpacer (abasic residue)
7	7-Deaza-dG
F	5-Fluoro-dU
H	5-Hydroxy-dC
I	Inosine (deoxy)
J	Spacer C3 (propanediol phosphate)
L	Spacer 18 (hexaethylenglycol phosphate)
mA, mC, mG	2'-oder 3'-O-Methyl Ribonucleotide (A, C, G)
mA, mC, mG	3'-O-Methyl-A (C, G)
mT	3'-O-Methyl-T
mU	2'-O-Methyl Uridine
O	8-Oxo-dG
p*	3′-Thiophosphate
Q	8-Oxo-dA
R	2-Aminopurine (deoxyribofuranoside)
rA, rC, rG, rU	RNA
S	5NI = 5-Nitroindol
teg	Spacer 9 (triethylenglycol phosphate)
U	2'-Deoxyuridine
V	2.6-Diaminopurine (deoxyribofuranoside)
vitE	Vitamin E
W	Nebularine (deoxyribofuranoside)
Z	5-Methyl-deoxycytidine
5T	5-Methoxy-deoxythymidine
doub	Doubler (Glenresearch)
doub2	Doubler2 (Chemgenes)
but	1,4-Butandiole
6G	6-Thiodeoxyguanosine
ff	Difluorotoluyldeoxyribonucleotide

4T	4- Thiothymidine
yU	Pseudodeoxyuridine
5U	5- Hydroxymethyldeoxyuridine
5T	5- Methoxythymidine
3T	2′3′- Dideoxythymidine
aC	Ara-cytidine (5′5′- linked)
fC	2′- Fluoro-cytidine
fU	2′- Fluoro-uridine
rU	Ribo-uridine
bC	5′- Bromo-cytidine
eC	N-4- Ethyl-cytidine
dP	P-Base
cC	Amino-Modifier-C6-cytidine

The invention also relates to compositions that are a set of oligonucleotides forming a duplex. As shown in the examples below, the oligonucleotides have minimal or no activity when used alone. However when they are prepared as a duplex the activity of the duplex is greatly enhanced.

The duplex that forms between the two oligonucleotides has partial complementarity. Partial complementarity refers to at least a portion of the duplex that includes nucleotides that base-pair with one another. Thus one region of the first oligonucleotide may include at least some nucleotides that form a base pair with complementary nucleotides in a region of the second oligonucleotide. The partial complementarity is that amount that is sufficient to stabilize the duplex in the presence or absence of an exogenous stabilizer. In general the region of partial complementarity should include at least 2 nucleotides on each oligonucleotide that are capable of base pairing with the other oligonucleotide, depending on the length of the oligonucleotide pair. In some embodiments it is preferred that the region of partial complementarity is greater than 2 nucleotides. For instance it may include at least 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides on each oligonucleotide. Thus, the region of the nucleotides that has partial complementarity may include one or more nucleotide mis-matches.

Alternatively the entire region of the nucleotide participating in the duplex may be perfectly complementary. A region that is perfectly complementary is one that includes only nucleotides that base-pair with a complementary nucleotide on the other oligonucleotide.

The duplex can be stabilized by the interaction between the base-pairing nucleotides. In some instances, the duplex may be stabilized or further stabilized with

the use of an exogenous stabilizer. An exogenous stabilizer is any molecule, such as a linker that reduces the level of disassociation of the duplex, or in other words increases the stability of the duplex.

At least one of the oligonucleotides includes a YR motif and preferably a CG motif. One or both oligonucleotides may include a palindrome, but it is not necessary. In some embodiments neither oligonucleotide includes a palindrome.

An example of a functionally active duplex of oligonucleotides is SEQ ID NO: 108 and SEQ ID NO: 109.

SEQ ID NO: 108	5'-T_C_G_T_C_G_T_C_G_A_Chol
SEQ ID NO: 109	Chol-A_G_C_A_G_C_A_G_C_T-5'

It has been discovered according to the invention that the subsets of lipophilic conjugates have dramatic immune stimulatory effects on human cells, suggesting that these conjugates are effective therapeutic agents for human vaccination, cancer immunotherapy, asthma immunotherapy, general enhancement of immune function, enhancement of hematopoietic recovery following radiation or chemotherapy, and other immune modulatory applications.

As used herein, the terms treat, treated, or treating when used with respect to a disorder such as an infectious disease, cancer, allergy, or asthma refers to a prophylactic treatment which increases the resistance of a subject to development of the disease (e.g., to infection with a pathogen) or, in other words, decreases the likelihood that the subject will develop the disease (e.g., become infected with the pathogen) as well as a treatment after the subject has developed the disease in order to fight the disease (e.g., reduce or eliminate the infection) or prevent the disease from becoming worse.

Thus the Lipophilic conjugates are useful in some aspects of the invention as a vaccine for the treatment of a subject having or at risk of developing allergy or asthma, an infection with an infectious organism or a cancer in which a specific cancer antigen has been identified. The Lipophilic conjugates can also be given alone without the antigen or allergen for protection against infection, allergy or cancer or may be administered with other therapeutic agents. Repeated doses may allow longer term protection. A subject at risk as used herein is a subject who has any risk of exposure to an infection causing pathogen or a cancer or an allergen or a risk of developing cancer. For instance, a subject at risk may be a subject who is planning to travel to an area where

a particular type of infectious agent is found or it may be a subject who through lifestyle or medical procedures is exposed to bodily fluids which may contain infectious organisms or directly to the organism or even any subject living in an area where an infectious organism or an allergen has been identified. Subjects at risk of developing infection also include general populations to which a medical agency recommends vaccination with a particular infectious organism antigen. If the antigen is an allergen and the subject develops allergic responses to that particular antigen and the subject may be exposed to the antigen, i.e., during pollen season, then that subject is at risk of exposure to the antigen. A subject at risk of developing an allergy to asthma includes those subjects that have been identified as having an allergy or asthma but that don't have the active disease during the Lipophilic conjugate treatment as well as subjects that are considered to be at risk of developing these diseases because of genetic or environmental factors.

A subject at risk of developing a cancer is one who has a high probability of developing cancer. These subjects include, for instance, subjects having a genetic abnormality, the presence of which has been demonstrated to have a correlative relation to a higher likelihood of developing a cancer and subjects exposed to cancer causing agents such as tobacco, asbestos, or other chemical toxins, or a subject who has previously been treated for cancer and is in apparent remission. When a subject at risk of developing a cancer is treated with a Lipophilic conjugate and optionally an antigen specific for the type of cancer to which the subject is at risk of developing, the subject may be able to kill the cancer cells as they develop. If a tumor begins to form in the subject, the subject will develop an innate immune response or a specific immune response against the tumor antigen.

In addition to the use of the Lipophilic conjugates for prophylactic treatment, the invention also encompasses the use of the Lipophilic conjugates for the treatment of a subject having an infection, an allergy, asthma, or a cancer.

A subject having an infection is a subject that has been exposed to an infectious pathogen and has acute or chronic detectable levels of the pathogen in the body. The Lipophilic conjugates can be used with or without an antigen or other therapeutic to mount an innate or an antigen specific systemic or mucosal immune response that is capable of reducing the level of or eradicating the infectious pathogen. An infectious

disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body. It is particularly important to develop effective vaccine strategies and treatments to protect the body's mucosal surfaces, which are the primary site of pathogenic entry.

A subject having an allergy is a subject that is capable of developing an allergic reaction in response to an allergen. An allergy refers to acquired hypersensitivity to a substance (allergen). Allergic conditions include but are not limited to eczema, allergic rhinitis or coryza, hay fever, conjunctivitis, bronchial asthma, allergic asthma, urticaria (hives) and food allergies, and other atopic conditions.

Allergies are generally caused by IgE antibody generation against harmless allergens. The cytokines that are induced by systemic or mucosal administration of Lipophilic conjugates are predominantly of a class called Th1 (examples are IL-12, IP-10, IFN-α and IFN-γ) and these induce both humoral and cellular immune responses. The other major type of immune response, which is associated with the production of IL-4 and IL-5 cytokines, is termed a Th2 immune response. In general, it appears that allergic diseases are mediated by Th2 type immune responses. Based on the ability of the Lipophilic conjugates described herein to shift the immune response in a subject from a predominant Th2 (which is associated with production of IgE antibodies and allergy) to a balanced Th2/Th1 response (which is protective against allergic reactions), an effective dose for inducing an immune response of a Lipophilic conjugate can be administered to a subject to treat asthma and allergy.

Thus, the Lipophilic conjugates have significant therapeutic utility in the treatment of allergic conditions and asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotope switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN-γ and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines. Asthma refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms. Thus, asthma includes allergic asthma and non-allergic asthma.

A subject having a cancer is a subject that has detectable cancerous cells. The cancer may be a malignant or non-malignant cancer. Cancers or tumors include but are not limited to biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; intraepithelial neoplasms; lymphomas; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid cancer; and renal cancer, as well as other carcinomas and sarcomas. In one embodiment the cancer is hairy cell leukemia, chronic myelogenous leukemia, cutaneous T-cell leukemia, multiple myeloma, follicular lymphoma, malignant melanoma, squamous cell carcinoma, renal cell carcinoma, prostate carcinoma, bladder cell carcinoma, or colon carcinoma.

A subject shall mean a human or vertebrate animal or mammal including but not limited to a dog, cat, horse, cow, pig, sheep, goat, turkey, chicken, primate, e.g., monkey, and fish (aquaculture species), e.g. salmon. Thus, the compounds may be used to treat cancer and tumors, infections, and allergy/asthma in human and non human subjects. Cancer is one of the leading causes of death in companion animals (i.e., cats and dogs).

In the instances when the CpG oligonucleotide is administered with an antigen, the subject may be exposed to the antigen. As used herein, the term exposed to refers to either the active step of contacting the subject with an antigen or the passive exposure of the subject to the antigen *in vivo*. Methods for the active exposure of a subject to an antigen are well-known in the art. In general, an antigen is administered directly to the subject by any means such as intravenous, intramuscular, oral, transdermal, mucosal, intranasal, intratracheal, or subcutaneous administration. The antigen can be administered systemically or locally. Methods for administering the antigen and the Lipophilic conjugate are described in more detail below. A subject is passively exposed to an antigen if an antigen becomes available for exposure to the immune cells in the body. A subject may be passively exposed to an antigen, for instance, by entry of a foreign pathogen into the body or by the development of a tumor cell expressing a foreign antigen on its surface.

The methods in which a subject is passively exposed to an antigen can be particularly dependent on timing of administration of the Lipophilic conjugate. For

instance, in a subject at risk of developing a cancer or an infectious disease or an allergic or asthmatic response, the subject may be administered the Lipophilic conjugate on a regular basis when that risk is greatest, i.e., during allergy season or after exposure to a cancer causing agent. Additionally the Lipophilic conjugate may be administered to travelers before they travel to foreign lands where they are at risk of exposure to infectious agents. Likewise the Lipophilic conjugate may be administered to soldiers or civilians at risk of exposure to biowarfare to induce a systemic or mucosal immune response to the antigen when and if the subject is exposed to it.

An antigen as used herein is a molecule capable of provoking an immune response. Antigens include but are not limited to cells, cell extracts, proteins, polypeptides, peptides, polysaccharides, polysaccharide conjugates, peptide and non-peptide mimics of polysaccharides and other molecules, small molecules, lipids, glycolipids, carbohydrates, viruses and viral extracts and muticellular organisms such as parasites and allergens. The term antigen broadly includes any type of molecule which is recognized by a host immune system as being foreign. Antigens include but are not limited to cancer antigens, microbial antigens, and allergens.

A cancer antigen as used herein is a compound, such as a peptide or protein, associated with a tumor or cancer cell surface and which is capable of provoking an immune response when expressed on the surface of an antigen presenting cell in the context of an MHC molecule. Cancer antigens can be prepared from cancer cells either by preparing crude extracts of cancer cells, for example, as described in Cohen, et al., 1994, *Cancer Research*, 54:1055, by partially purifying the antigens, by recombinant technology, or by de novo synthesis of known antigens. Cancer antigens include but are not limited to antigens that are recombinantly expressed, an immunogenic portion thereof, or a whole tumor or cancer cell. Such antigens can be isolated or prepared recombinantly or by any other means known in the art.

As used herein, the terms "cancer antigen" and "tumor antigen" are used interchangeably to refer to antigens which are differentially expressed by cancer cells and can thereby be exploited in order to target cancer cells. Cancer antigens are antigens which can potentially stimulate apparently tumor-specific immune responses. Some of these antigens are encoded, although not necessarily expressed, by normal cells. These antigens can be characterized as those which are normally silent (i.e., not expressed) in

normal cells, those that are expressed only at certain stages of differentiation and those that are temporally expressed such as embryonic and fetal antigens. Other cancer antigens are encoded by mutant cellular genes, such as oncogenes (e.g., activated ras oncogene), suppressor genes (e.g., mutant p53), fusion proteins resulting from internal deletions or chromosomal translocations. Still other cancer antigens can be encoded by viral genes such as those carried on RNA and DNA tumor viruses.

A microbial antigen as used herein is an antigen of a microorganism and includes but is not limited to virus, bacteria, parasites, and fungi. Such antigens include the intact microorganism as well as natural isolates and fragments or derivatives thereof and also synthetic compounds which are identical to or similar to natural microorganism antigens and induce an immune response specific for that microorganism. A compound is similar to a natural microorganism antigen if it induces an immune response (humoral and/or cellular) to a natural microorganism antigen. Such antigens are used routinely in the art and are well known to those of ordinary skill in the art.

Examples of viruses that have been found in humans include but are not limited to: *Retroviridae* (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; *Picornaviridae* (e.g. polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (e.g. strains that cause gastroenteritis); *Togaviridae* (e.g. equine encephalitis viruses, rubella viruses); *Flaviridae* (e.g. dengue viruses, encephalitis viruses, yellow fever viruses); *Coronoviridae* (e.g. coronaviruses); *Rhabdoviradae* (e.g. vesicular stomatitis viruses, rabies viruses); *Coronaviridae* (e.g. coronaviruses); *Rhabdoviridae* (e.g. vesicular stomatitis viruses, rabies viruses); *Filoviridae* (e.g. ebola viruses); *Paramyxoviridae* (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (e.g. influenza viruses); *Bungaviridae* (e.g. Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); *Reoviridae* (e.g. reoviruses, orbiviurses and rotaviruses); *Birnaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvovirida* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (e.g. African swine fever virus); and

unclassified viruses (e.g. the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), Hepatitis C; Norwalk and related viruses, and astroviruses).

Both gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to, *Pasteurella* species, *Staphylococci* species, and *Streptococcus* species. Gram negative bacteria include, but are not limited to, *Escherichia coli*, *Pseudomonas* species, and *Salmonella* species. Specific examples of infectious bacteria include but are not limited to, *Helicobacter pyloris*, *Borelia burgdorferi*, *Legionella pneumophilia*, *Mycobacteria sps* (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansaii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter sp.*, *Enterococcus sp.*, *Haemophilus influenzae*, *Bacillus antracis*, *corynebacterium diphtheriae*, *corynebacterium sp.*, *Erysipelothrix rhusiopathiae*, *Clostridium perfringers*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides sp.*, *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidium*, *Treponema pertenue*, *Leptospira*, *Rickettsia*, and *Actinomyces israelli*.

Examples of fungi include *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Chlamydia trachomatis*, *Candida albicans*.

Other infectious organisms (i.e., protists) include *Plasmodium spp.* such as *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium* vivax and *Toxoplasma gondii*. Blood-borne and/or tissues parasites include *Plasmodium* spp., *Babesia microti*, *Babesia divergens*, *Leishmania tropica*, *Leishmania* spp., *Leishmania braziliensis*, *Leishmania donovani*, *Trypanosoma gambiense* and *Trypanosoma rhodesiense* (African sleeping sickness), *Trypanosoma cruzi* (Chagas' disease), and *Toxoplasma gondii*.

Other medically relevant microorganisms have been described extensively in the literature, e.g., see C.G.A Thomas, *Medical Microbiology*, Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference.

An allergen refers to a substance (antigen) that can induce an allergic or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, animal dander dust, fungal spores and drugs (e.g. penicillin). Examples of natural, animal and plant allergens include but are not limited to proteins specific to the following genuses: *Canine (Canis familiaris)*; *Dermatophagoides* (e.g. *Dermatophagoides farinae*); *Felis* (*Felis domesticus*); *Ambrosia* (*Ambrosia artemiisfolia*; *Lolium* (e.g. *Lolium perenne* or *Lolium multiflorum*); *Cryptomeria* (*Cryptomeria japonica*); *Alternaria* (*Alternaria alternata*); *Alder*; *Alnus* (*Alnus gultinoasa*); *Betula* (*Betula verrucosa*); *Quercus* (*Quercus alba*); *Olea* (*Olea europa*); *Artemisia* (*Artemisia vulgaris*); *Plantago* (e.g. *Plantago lanceolata*); *Parietaria* (e.g. *Parietaria officinalis* or *Parietaria judaica*); *Blattella* (e.g. *Blattella germanica*); *Apis* (e.g. *Apis multiflorum*); *Cupressus* (e.g. *Cupressus sempervirens*, *Cupressus arizonica* and *Cupressus macrocarpa*); *Juniperus* (e.g. *Juniperus sabinoides*, *Juniperus virginiana*, *Juniperus communis* and *Juniperus ashei*); *Thuya* (e.g. *Thuya orientalis*); *Chamaecyparis* (e.g. *Chamaecyparis obtusa*); *Periplaneta* (e.g. *Periplaneta americana*); *Agropyron* (e.g. *Agropyron repens*); *Secale* (e.g. *Secale cereale*); *Triticum* (e.g. *Triticum aestivum*); *Dactylis* (e.g. *Dactylis glomerata*); *Festuca* (e.g. *Festuca elatior*); *Poa* (e.g. *Poa pratensis* or *Poa compressa*); *Avena* (e.g. *Avena sativa*); *Holcus* (e.g. *Holcus lanatus*); *Anthoxanthum* (e.g. *Anthoxanthum odoratum*); *Arrhenatherum* (e.g. *Arrhenatherum elatius*); *Agrostis* (e.g. *Agrostis alba*); *Phleum* (e.g. *Phleum pratense*); *Phalaris* (e.g. *Phalaris arundinacea*); *Paspalum* (e.g. *Paspalum notatum*); *Sorghum* (e.g. *Sorghum halepensis*); and *Bromus* (e.g. *Bromus inermis*).

The antigen may be substantially purified. The term substantially purified as used herein refers to an antigen, i.e., a polypeptide which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify polypeptide antigens using standard techniques for protein purification. The substantially pure polypeptide will often yield a single major band on a non-reducing polyacrylamide gel. In the case of partially glycosylated polypeptides or those that have several start codons, there may be several bands on a non-reducing polyacrylamide gel, but these will form a distinctive pattern for that polypeptide. The purity of the polypeptide antigen may also be determined by amino-terminal amino acid sequence analysis. Other types of antigens such as

polysaccharides, small molecule, mimics etc are included within the invention and may optionally be substantially pure.

The conjugates of the invention may be administered to a subject with an anti-microbial agent. An anti-microbial agent, as used herein, refers to a naturally-occurring or synthetic compound which is capable of killing or inhibiting infectious microorganisms. The type of anti-microbial agent useful according to the invention will depend upon the type of microorganism with which the subject is infected or at risk of becoming infected. Anti-microbial agents include but are not limited to anti-bacterial agents, anti-viral agents, anti-fungal agents and anti-parasitic agents. Phrases such as "anti-infective agent", "anti-bacterial agent", "anti-viral agent", "anti-fungal agent", "anti-parasitic agent" and "parasiticide" have well-established meanings to those of ordinary skill in the art and are defined in standard medical texts. Briefly, anti-bacterial agents kill or inhibit bacteria, and include antibiotics as well as other synthetic or natural compounds having similar functions. Antibiotics are low molecular weight molecules which are produced as secondary metabolites by cells, such as microorganisms. In general, antibiotics interfere with one or more bacterial functions or structures which are specific for the microorganism and which are not present in host cells. Anti-viral agents can be isolated from natural sources or synthesized and are useful for killing or inhibiting viruses. Anti-fungal agents are used to treat superficial fungal infections as well as opportunistic and primary systemic fungal infections. Anti-parasitic agents kill or inhibit parasites.

Examples of anti-parasitic agents, also referred to as parasiticides useful for human administration include but are not limited to albendazole, amphotericin B, benznidazole, bithionol, chloroquine HCl, chloroquine phosphate, clindamycin, dehydroemetine, diethylcarbamazine, diloxanide furoate, eflornithine, furazolidaone, glucocorticoids, halofantrine, iodoquinol, ivermectin, mebendazole, mefloquine, meglumine antimoniate, melarsoprol, metrifonate, metronidazole, niclosamide, nifurtimox, oxamniquine, paromomycin, pentamidine isethionate, piperazine, praziquantel, primaquine phosphate, proguanil, pyrantel pamoate, pyrimethanmine-sulfonamides, pyrimethanmine-sulfadoxine, quinacrine HCl, quinine sulfate, quinidine gluconate, spiramycin, stibogluconate sodium (sodium antimony gluconate), suramin,

tetracycline, doxycycline, thiabendazole, tinidazole, trimethroprim-sulfamethoxazole, and tryparsamide some of which are used alone or in combination with others.

Antibacterial agents kill or inhibit the growth or function of bacteria. A large class of antibacterial agents is antibiotics. Antibiotics, which are effective for killing or inhibiting a wide range of bacteria, are referred to as broad spectrum antibiotics. Other types of antibiotics are predominantly effective against the bacteria of the class gram-positive or gram-negative. These types of antibiotics are referred to as narrow spectrum antibiotics. Other antibiotics which are effective against a single organism or disease and not against other types of bacteria, are referred to as limited spectrum antibiotics. Antibacterial agents are sometimes classified based on their primary mode of action. In general, antibacterial agents are cell wall synthesis inhibitors, cell membrane inhibitors, protein synthesis inhibitors, nucleic acid synthesis or functional inhibitors, and competitive inhibitors.

Antiviral agents are compounds which prevent infection of cells by viruses or replication of the virus within the cell. There are many fewer antiviral drugs than antibacterial drugs because the process of viral replication is so closely related to DNA replication within the host cell, that non-specific antiviral agents would often be toxic to the host. There are several stages within the process of viral infection which can be blocked or inhibited by antiviral agents. These stages include, attachment of the virus to the host cell (immunoglobulin or binding peptides), uncoating of the virus (e.g. amantadine), synthesis or translation of viral mRNA (e.g. interferon), replication of viral RNA or DNA (e.g. nucleoside analogues), maturation of new virus proteins (e.g. protease inhibitors), and budding and release of the virus.

Nucleotide analogues are synthetic compounds which are similar to nucleotides, but which have an incomplete or abnormal deoxyribose or ribose group. Once the nucleotide analogues are in the cell, they are phosphorylated, producing the triphosphate form which competes with normal nucleotides for incorporation into the viral DNA or RNA. Once the triphosphate form of the nucleotide analogue is incorporated into the growing nucleic acid chain, it causes irreversible association with the viral polymerase and thus chain termination. Nucleotide analogues include, but are not limited to, acyclovir (used for the treatment of herpes simplex virus and varicella-zoster virus), gancyclovir (useful for the treatment of cytomegalovirus), idoxuridine, ribavirin (useful

for the treatment of respiratory syncitial virus), dideoxyinosine, dideoxycytidine, zidovudine (azidothymidine), imiquimod, and resimiquimod.

The interferons are cytokines which are secreted by virus-infected cells as well as immune cells. The interferons function by binding to specific receptors on cells adjacent to the infected cells, causing the change in the cell which protects it from infection by the virus. α and β-interferon also induce the expression of Class I and Class II MHC molecules on the surface of infected cells, resulting in increased antigen presentation for host immune cell recognition. α and β-interferons are available as recombinant forms and have been used for the treatment of chronic hepatitis B and C infection. At the dosages which are effective for anti-viral therapy, interferons have severe side effects such as fever, malaise and weight loss.

Anti-viral agents useful in the invention include but are not limited to immunoglobulins, amantadine, interferons, nucleoside analogues, and protease inhibitors. Specific examples of anti-virals include but are not limited to Acemannan; Acyclovir; Acyclovir Sodium; Adefovir; Alovudine; Alvircept Sudotox; Amantadine Hydrochloride; Aranotin; Arildone; Atevirdine Mesylate; Avridine; Cidofovir; Cipamfylline; Cytarabine Hydrochloride; Delavirdine Mesylate; Desciclovir; Didanosine; Disoxaril; Edoxudine; Enviradene; Enviroxime; Famciclovir; Famotine Hydrochloride; Fiacitabine; Fialuridine; Fosarilate; Foscarnet Sodium; Fosfonet Sodium; Ganciclovir; Ganciclovir Sodium; Idoxuridine; Kethoxal; Lamivudine; Lobucavir; Memotine Hydrochloride; Methisazone; Nevirapine; Penciclovir; Pirodavir; Ribavirin; Rimantadine Hydrochloride; Saquinavir Mesylate; Somantadine Hydrochloride; Sorivudine; Statolon; Stavudine; Tilorone Hydrochloride; Trifluridine; Valacyclovir Hydrochloride; Vidarabine; Vidarabine Phosphate; Vidarabine Sodium Phosphate; Viroxime; Zalcitabine; Zidovudine; and Zinviroxime.

Anti-fungal agents are useful for the treatment and prevention of infective fungi. Anti-fungal agents are sometimes classified by their mechanism of action. Some anti-fungal agents function as cell wall inhibitors by inhibiting glucose synthase. These include, but are not limited to, basiungin/ECB. Other anti-fungal agents function by destabilizing membrane integrity. These include, but are not limited to, immidazoles, such as clotrimazole, sertaconzole, fluconazole, itraconazole, ketoconazole, miconazole, and voriconacole, as well as FK 463, amphotericin B, BAY 38-9502, MK 991,

pradimicin, UK 292, butenafine, and terbinafine. Other anti-fungal agents function by breaking down chitin (e.g. chitinase) or immunosuppression (501 cream).

Lipophilic conjugates can be combined with other therapeutic agents such as adjuvants to enhance immune responses. The Lipophilic conjugate and other therapeutic agent may be administered simultaneously or sequentially. When the other therapeutic agents are administered simultaneously they can be administered in the same or separate formulations, but are administered at the same time. The other therapeutic agents are administered sequentially with one another and with Lipophilic conjugate, when the administration of the other therapeutic agents and the Lipophilic conjugate is temporally separated. The separation in time between the administration of these compounds may be a matter of minutes or it may be longer. Other therapeutic agents include but are not limited to adjuvants, cytokines, antibodies, antigens, etc.

The compositions of the invention may also be administered with non-nucleic acid adjuvants. A non-nucleic acid adjuvant is any molecule or compound except for the Lipophilic conjugates described herein which can stimulate the humoral and/or cellular immune response. Non-nucleic acid adjuvants include, for instance, adjuvants that create a depo effect, immune stimulating adjuvants, and adjuvants that create a depo effect and stimulate the immune system.

The Lipophilic conjugates are also useful as mucosal adjuvants. It has previously been discovered that both systemic and mucosal immunity are induced by mucosal delivery of CpG nucleic acids. Thus, the oligonucleotides may be administered in combination with other mucosal adjuvants.

Immune responses can also be induced or augmented by the co-administration or co-linear expression of cytokines (Bueler & Mulligan, 1996; Chow *et al.,* 1997; Geissler *et al.,*1997; Iwasaki *et al.,*1997; Kim *et al.,*1997) or co-stimulatory molecules such as B7 (Iwasaki *et al.,*1997; Tsuji *et al.,*1997) with the Lipophilic conjugates. The term cytokine is used as a generic name for a diverse group of soluble proteins and peptides which act as humoral regulators at nano- to picomolar concentrations and which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment. Examples of cytokines include, but are not limited to IP-10, IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-

15, IL-18, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interferon-γ (IFN-γ), IFN-α, tumor necrosis factor (TNF), TGF-β, FLT-3 ligand, and CD40 ligand. In addition to cytokines the CpG oligonucleotides may be used in combination with antibodies against certain cytokines, such as anti-IL-10 and anti-TGF-β, as well as Cox inhibitors, i.e. COX-1 and COX-2 inhibitors.

The oligonucleotides are also useful in mediating immune responses through cellular toll-like receptors (TLRs). TLRs are a series of signaling pattern-recognition receptors known as play a major role in the inflammatory responses and the induction of immunity. Different TLRs directly or indirectly bind different microbial molecules. For example, TLR-2 recognizes peptidoglycan and lipoproteins; TLR-4 recognizes lipopolysaccharide and lipoteichoic acid; TLR-5 recognizes bacterial flagellin; and TLR-9 recognizes bacterial DNA. The stimulation of TLR transmits a signal to the cell's nucleus inducting the expression of genes coding for the synthesis of intracellular regulatory molecules such as cytokines. The cytokines, in turn, bind to cytokine receptors on other defense cells. These cytokines trigger innate immune defenses such as inflammation, fever, and phagocytosis and provide an immediate response against the invading microorganism. TLRs also participate in adoptive immunity by triggering various secondary signals needed for humoral immunity (the production of antibodies) and cell-mediated immunity (the production of cytotoxic T-lymphocytes and additional cytokines). The oligonucleotides of the invention are useful in mediating TLR immune responses, and can the oligonucleotides of the invention can stimulate the production of certain cytokines in a TLR dependent manner.

The oligonucleotides are also useful for redirecting an immune response from a Th2 immune response to a Th1 immune response. This results in the production of a relatively balanced Th1/Th2 environment. Redirection of an immune response from a Th2 to a Th1 immune response can be assessed by measuring the levels of cytokines produced in response to the nucleic acid (*e.g.*, by inducing monocytic cells and other cells to produce Th1 cytokines, including IL-12, IFN-γ and GM-CSF). The redirection or rebalance of the immune response from a Th2 to a Th1 response is particularly useful for the treatment of asthma. For instance, an effective amount for treating asthma can be that amount; useful for redirecting a Th2 type of immune response that is associated with

asthma to a Th1 type of response or a balanced Th1/Th2 environment. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. The Lipophilic conjugates described herein cause an increase in Th1 cytokines which helps to rebalance the immune system, preventing or reducing the adverse effects associated with a predominately Th2 immune response.

The Lipophilic conjugates have the unique capability to promote cell survival, differentiation, activation and maturation of dendritic cells, and are useful for *in vitro, in vivo,* and *ex vivo* methods involving dendritic cells.

Lipophilic conjugates also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). ADCC can be performed using a Lipophilic conjugate in combination with an antibody specific for a cellular target, such as a cancer cell. When the Lipophilic conjugate is administered to a subject in conjunction with the antibody the subject's immune system is induced to kill the tumor cell. The antibodies useful in the ADCC procedure include antibodies which interact with a cell in the body. Many such antibodies specific for cellular targets have been described in the art and many are commercially available.

The Lipophilic conjugates may also be administered in conjunction with an anti-cancer therapy. Anti-cancer therapies include cancer medicaments, radiation and surgical procedures. As used herein, a "cancer medicament" refers to an agent which is administered to a subject for the purpose of treating a cancer. As used herein, "treating cancer" includes preventing the development of a cancer, reducing the symptoms of cancer, and/or inhibiting the growth of an established cancer. In other aspects, the cancer medicament is administered to a subject at risk of developing a cancer for the purpose of reducing the risk of developing the cancer. Various types of medicaments for the treatment of cancer are described herein. For the purpose of this specification, cancer medicaments are classified as chemotherapeutic agents, immunotherapeutic agents, cancer vaccines, hormone therapy, and biological response modifiers.

Additionally, the methods of the invention are intended to embrace the use of more than one cancer medicament along with the Lipophilic conjugates. As an example, where appropriate, the Lipophilic conjugates may be administered with both a chemotherapeutic agent and an immunotherapeutic agent. Alternatively, the cancer medicament may embrace an immunotherapeutic agent and a cancer vaccine, or a

chemotherapeutic agent and a cancer vaccine, or a chemotherapeutic agent, an immunotherapeutic agent and a cancer vaccine all administered to one subject for the purpose of treating a subject having a cancer or at risk of developing a cancer.

The chemotherapeutic agent may be, for instance, methotrexate, vincristine, adriamycin, cisplatin, non-sugar containing chloroethylnitrosoureas, 5-fluorouracil, mitomycin C, bleomycin, doxorubicin, dacarbazine, taxol, fragyline, Meglamine GLA, valrubicin, carmustaine and poliferposan, MMI270, BAY 12-9566, RAS famesyl transferase inhibitor, famesyl transferase inhibitor, MMP, MTA/LY231514, LY264618/Lometexol, Glamolec, CI-994, TNP-470, Hycamtin/Topotecan, PKC412, Valspodar/PSC833, Novantrone/Mitroxantrone, Metaret/Suramin, Batimastat, E7070, BCH-4556, CS-682, 9-AC, AG3340, AG3433, Incel/VX-710, VX-853, ZD0101, ISI641, ODN 698, TA 2516/Marmistat, BB2516/Marmistat, CDP 845, D2163, PD183805, DX8951f, Lemonal DP 2202, FK 317, Picibanil/OK-432, AD 32/Valrubicin, Metastron/strontium derivative, Temodal/Temozolomide, Evacet/liposomal doxorubicin, Yewtaxan/Paclitaxel, Taxol/Paclitaxel, Xeload/Capecitabine, Furtulon/Doxifluridine, Cyclopax/oral paclitaxel, Oral Taxoid, SPU-077/Cisplatin, HMR 1275/Flavopiridol, CP-358 (774)/EGFR, CP-609 (754)/RAS oncogene inhibitor, BMS-182751/oral platinum, UFT(Tegafur/Uracil), Ergamisol/Levamisole, Eniluracil/776C85/5FU enhancer, Campto/Levamisole, Camptosar/Irinotecan, Tumodex/Ralitrexed, Leustatin/Cladribine, Paxex/Paclitaxel, Doxil/liposomal doxorubicin, Caelyx/liposomal doxorubicin, Fludara/Fludarabine, Pharmarubicin/Epirubicin, DepoCyt, ZD1839, LU 79553/Bis-Naphtalimide, LU 103793/Dolastain, Caetyx/liposomal doxorubicin, Gemzar/Gemcitabine, ZD 0473/Anormed, YM 116, Iodine seeds, CDK4 and CDK2 inhibitors, PARP inhibitors, D4809/Dexifosamide, Ifes/Mesnex/Ifosamide, Vumon/Teniposide, Paraplatin/Carboplatin, Plantinol/cisplatin, Vepeside/Etoposide, ZD 9331, Taxotere/Docetaxel, prodrug of guanine arabinoside, Taxane Analog, nitrosoureas, alkylating agents such as melphelan and cyclophosphamide, Aminoglutethimide, Asparaginase, Busulfan, Carboplatin, Chlorombucil, Cytarabine HCI, Dactinomycin, Daunorubicin HCl, Estramustine phosphate sodium, Etoposide (VP16-213), Floxuridine, Fluorouracil (5-FU), Flutamide, Hydroxyurea (hydroxycarbamide), Ifosfamide, Interferon Alfa-2a, Alfa-2b, Leuprolide acetate (LHRH-releasing factor analogue), Lomustine (CCNU), Mechlorethamine HCl (nitrogen mustard), Mercaptopurine, Mesna,

Mitotane (o.p'-DDD), Mitoxantrone HCl, Octreotide, Plicamycin, Procarbazine HCl, Streptozocin, Tamoxifen citrate, Thioguanine, Thiotepa, Vinblastine sulfate, Amsacrine (m-AMSA), Azacitidine, Erthropoietin, Hexamethylmelamine (HMM), Interleukin 2, Mitoguazone (methyl-GAG; methyl glyoxal bis-guanylhydrazone; MGBG), Pentostatin (2'deoxycoformycin), Semustine (methyl-CCNU), Teniposide (VM-26) or Vindesine sulfate, but it is not so limited.

The immunotherapeutic agent may be, for instance, Ributaxin, Herceptin, Quadramet, Panorex, IDEC-Y2B8, BEC2, C225, Oncolym, SMART M195, ATRAGEN, Ovarex, Bexxar, LDP-03, ior t6, MDX-210, MDX-11, MDX-22, OV103, 3622W94, anti-VEGF, Zenapax, MDX-220, MDX-447, MELIMMUNE-2, MELIMMUNE-1, CEACIDE, Pretarget, NovoMAb-G2, TNT, Gliomab-H, GNI-250, EMD-72000, LymphoCide, CMA 676, Monopharm-C, 4B5, ior egf.r3, ior c5, BABS, anti-FLK-2, MDX-260, ANA Ab, SMART 1D10 Ab, SMART ABL 364 Ab or ImmuRAIT-CEA, but it is not so limited.

The cancer vaccine may be, for instance, EGF, Anti-idiotypic cancer vaccines, Gp75 antigen, GMK melanoma vaccine, MGV ganglioside conjugate vaccine, Her2/neu, Ovarex, M-Vax, O-Vax, L-Vax, STn-KHL theratope, BLP25 (MUC-1), liposomal idiotypic vaccine, Melacine, peptide antigen vaccines, toxin/antigen vaccines, MVA-based vaccine, PACIS, BCG vacine, TA-HPV, TA-CIN, DISC-virus or ImmuCyst/TheraCys, but it is not so limited.

The use of Lipophilic conjugates in conjunction with immunotherapeutic agents such as monoclonal antibodies is able to increase long-term survival through a number of mechanisms including significant enhancement of ADCC (as discussed above), activation of natural killer (NK) cells and an increase in IFN-α levels. The nucleic acids when used in combination with monoclonal antibodies serve to reduce the dose of the antibody required to achieve a biological result.

The invention also includes methods for inducing antigen non-specific innate immune activation and broad spectrum resistance to infectious challenge using the Lipophilic conjugates. The term innate immune activation as used herein refers to the activation of immune cells other than memory B cells and for instance can include the activation of NK cells, T cells and/or other immune cells that can respond in an antigen independent fashion. A broad spectrum resistance to infectious challenge is induced

because the immune cells are in active form and are primed to respond to any invading compound or microorganism. The cells do not have to be specifically primed against a particular antigen. This is particularly useful in biowarfare, and the other circumstances described above such as travelers.

The conjugates of the invention may be formulated as other oligonucleotides, or with variations due to the lipophilic group, e.g., the formation of multimers by the binding or embedding of the L group in a surface, such as a liposome, ISCOM, or other suitable hydrophobic bead or formulation. The conjugates may be formulated in a complex with a desired carrier structure, such as a polymer, a peptide, a protein, or a nucleic acid of interest. The conjugates may be formulated in vesicles comprising mainly or almost exclusively a lipophilic compound as described herein. The present invention also provides a method for increasing the lipophilicity of an immunostimulatory oligonucleotide in order to increase its affinity to a formulation reagent. Therefore, the oligonucleotides described herein posses favorable properties when encapsulated in a lipid composition. In conventional liposomes, it is often difficult to entrap a high concentration of a drug. By lipophilic derivatisation of the immunostimulatory oligonucleotide and incorporation into liposomes, the oligonucleotide may be more appropriate for long-term storage, since there will be less leakage of drug from the liposome. The lipophilic ligand may also lead to improved bioavailability and favorable biodistribution to certain organs, such as liver, and may also reduce toxic side effects. Without being bound to any particular mechanism of action. The free 5' ends of the ODN protruding from such multimeric macromolecules will be available to interact with the TLR9 receptor in such a way that leads to the crosslinking of the receptor, which may induce even further increased production of IFN-α.

The Lipophilic conjugate and/or the antigen and/or other therapeutics may be administered alone (e.g., in saline or buffer) or using any delivery vehicles known in the art. For instance the following delivery vehicles have been described: Cochleates (Gould-Fogerite et al., 1994, 1996); Emulsomes (Vancott et al., 1998, Lowell et al., 1997); ISCOMs (Mowat et al., 1993, Carlsson et al., 1991, Hu et., 1998, Morein et al., 1999); Liposomes (Childers et al., 1999, Michalek et al., 1989, 1992, de Haan 1995a,

1995b); Live bacterial vectors (e.g., *Salmonella, Escherichia coli, Bacillus calmatte-guerin, Shigella, Lactobacillus*) (Hone et al., 1996, Pouwels et al., 1998, Chatfield et al., 1993, Stover et al., 1991, Nugent et al., 1998); Live viral vectors (e.g., Vaccinia, adenovirus, Herpes Simplex) (Gallichan et al., 1993, 1995, Moss et al., 1996, Nugent et al., 1998, Flexner et al., 1988, Morrow et al., 1999); Microspheres (Gupta et al., 1998, Jones et al., 1996, Maloy et al., 1994, Moore et al., 1995, O'Hagan et al., 1994, Eldridge et al., 1989); Nucleic acid vaccines (Fynan et al., 1993, Kuklin et al., 1997, Sasaki et al., 1998, Okada et al., 1997, Ishii et al., 1997); Polymers (e.g. carboxymethylcellulose, chitosan) (Hamajima et al., 1998, Jabbal-Gill et al., 1998); Polymer rings (Wyatt et al., 1998); Proteosomes (Vancott et al., 1998, Lowell et al., 1988, 1996, 1997); Sodium Fluoride (Hashi et al., 1998); Transgenic plants (Tacket et al., 1998, Mason et al., 1998, Haq et al., 1995); Virosomes (Gluck et al., 1992, Mengiardi et al., 1995, Cryz et al., 1998); Virus-like particles (Jiang et al., 1999, Leibl et al., 1998). Other delivery vehicles are known in the art.

The term effective amount of a Lipophilic conjugate refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of a Lipophilic conjugate administered with an antigen for inducing mucosal immunity is that amount necessary to cause the development of IgA in response to an antigen upon exposure to the antigen, whereas that amount required for inducing systemic immunity is that amount necessary to cause the development of IgG in response to an antigen upon exposure to the antigen. Combined with the teachings provided herein, by choosing among the various active compounds and weighing factors such as potency, relative bioavailability, patient body weight, severity of adverse side-effects and preferred mode of administration, an effective prophylactic or therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the particular subject. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular Lipophilic conjugate being administered the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular Lipophilic conjugate and/or antigen and/or other therapeutic agent without necessitating undue experimentation.

Subject doses of the compounds described herein for mucosal or local delivery typically range from about 10 μg to 1000 mg per administration, which depending on the application could be given daily, weekly, or monthly and any other amount of time therebetween or as otherwise required. More typically mucosal or local doses range from about 100 μg to 50 mg per administration, and most typically from about 500 μg to 5 mg, with 2 - 4 administrations being spaced days or weeks apart. More typically, immune stimulant doses range from 100 μg to 1000 mg per administration, and most typically 500 μg to 50 mg, with daily or weekly administrations. Doses of the compounds described herein for parenteral delivery for the purpose of inducing an innate immune response or for increasing ADCC or for inducing an antigen specific immune response when the Lipophilic conjugates are administered in combination with other therapeutic agents or in specialized delivery vehicles typically range from about 10 μg to 1000 mg per administration, which depending on the application could be given daily, weekly, or monthly and any other amount of time therebetween or as otherwise required. More typically parenteral doses for these purposes range from about 100 μg to 50 mg per administration, and most typically from about 1000 μg to 10 mg, with 2 - 4 administrations being spaced days or weeks apart. In some embodiments, however, parenteral doses for these purposes may be used in a range of 5 to 10,000 times higher than the typical doses described above.

For any compound described herein the therapeutically effective amount can be initially determined from animal models. A therapeutically effective dose can also be determined from human data for other CpG oligonucleotides which have been tested in humans (human clinical trials are ongoing) and for compounds which are known to exhibit similar pharmacological activities, such as other adjuvants, e.g., LT and other antigens for vaccination purposes. Higher doses may be required for parenteral administration. The applied dose can be adjusted based on the relative bioavailability and potency of the administered compound. Adjusting the dose to achieve maximal efficacy based on the methods described above and other methods as are well-known in the art is well within the capabilities of the ordinarily skilled artisan.

The formulations of the invention are administered in pharmaceutically acceptable solutions, which may routinely contain pharmaceutically acceptable

concentrations of salt, buffering agents, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients.

For use in therapy, an effective amount of the Lipophilic conjugate an/or other therapeutics can be administered to a subject by any mode that delivers the compound to the desired surface, e.g., local, mucosal, systemic. Administering the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. Preferred routes of administration include but are not limited to oral, parenteral, intramuscular, intranasal, sublingual, intratracheal, inhalation, ocular, vaginal, and rectal.

For oral administration, the compounds (i.e., Lipophilic conjugates, antigens and/or other therapeutic agents) can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. Pharmaceutical preparations for oral use can be obtained as solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Optionally the oral formulations may also be formulated in saline or buffers for neutralizing internal acid conditions or may be administered without any carriers.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. Microspheres formulated for oral administration may also be used. Such microspheres have been well defined in the art. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

The compounds may be administered by inhalation to pulmonary tract, especially the bronchi and more particularly into the alveoli of the deep lung, using standard inhalation devices. The compounds may be delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. An inhalation apparatus may be used to deliver the compounds to a subject. An inhalation apparatus, as used herein, is any device for administering an aerosol, such as dry powdered form of the compounds. This type of equipment is well known in the art and has been described in detail, such as that description found in Remington: The Science and Practice of Pharmacy, 19th Edition, 1995, Mac Publishing Company, Easton, Pennsylvania, pages 1676-1692. Many U.S. patents also describe inhalation devices, such as U.S. Patent No. 6,116,237.

"Powder" as used herein refers to a composition that consists of finely dispersed solid particles. Preferably the compounds are relatively free flowing and capable of being dispersed in an inhalation device and subsequently inhaled by a subject so that the compounds reach the lungs to permit penetration into the alveoli. A "dry powder" refers to a powder composition that has a moisture content such that the particles are readily dispersible in an inhalation device to form an aerosol. The moisture content is generally

below about 10% by weight (% w) water, and in some embodiments is below about 5% w and preferably less than about 3% w. The powder may be formulated with polymers or optionally may be formulated with other materials such as liposomes, albumin and/or other carriers.

Aerosol dosage and delivery systems may be selected for a particular therapeutic application by one of skill in the art, such as described, for example in Gonda, I. "Aerosols for delivery of therapeutic and diagnostic agents to the respiratory tract," in Critical Reviews in Therapeutic Drug Carrier Systems, 6:273-313 (1990), and in Moren, "Aerosol dosage forms and formulations," in Aerosols in Medicine. Principles, Diagnosis and Therapy, Moren, et al., Eds., Esevier, Amsterdam, 1985.

The compounds, when it is desirable to deliver them systemically, may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active compounds may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Suitable liquid or solid pharmaceutical preparation forms are, for example, aqueous or saline solutions for inhalation, microencapsulated, encochleated, coated onto microscopic gold particles, contained in liposomes, nebulized, aerosols, pellets for implantation into the skin, or dried onto a sharp object to be scratched into the skin. The pharmaceutical compositions also include granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, drops or preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of methods for drug delivery, see Langer, *Science* 249:1527-1533, 1990, which is incorporated herein by reference.

The Lipophilic conjugates and optionally other therapeutics and/or antigens may be administered *per se* (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1-2% w/v); citric acid and a salt (1-3% w/v); boric acid and a salt (0.5-2.5% w/v); and phosphoric acid and a salt (0.8-2% w/v). Suitable preservatives include benzalkonium chloride (0.003-0.03% w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

The pharmaceutical compositions of the invention contain an effective amount of a Lipophilic conjugate and optionally antigens and/or other therapeutic agents optionally included in a pharmaceutically-acceptable carrier. The term pharmaceutically-acceptable carrier means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal. The term carrier denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being commingled with the compounds of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

Examples

Materials and Methods Examples 1-5:

Oligodeoxynucleotides. All ODN were provided by Coley Pharmaceutical Group (Langenfeld, Germany) and had undetectable endotoxin levels (<0.1EU/ml) measured by the Limulus assay (BioWhittaker, Verviers, Belgium). ODN were suspended in sterile, endotoxin-free Tris-EDTA (Sigma, Deisenhofen, Germany), and stored and handled under aseptic conditions to prevent both microbial and endotoxin contamination. All dilutions were carried out using pyrogen-free phosphate-buffered saline (Life Technologies, Eggenstein, Germany).

TLR9 assay. HEK293 cells were transfected by electroporation with vectors expressing the human TLR9 and a 6xNFκB-luciferase reporter plasmid. Stable transfectants ($3x10^4$ cells/well) were incubated with ODN for 16h at 37°C in a humidified incubator. Each data point was done in triplicate. Cells were lysed and assayed for luciferase gene activity (using the Britlite kit from Perkin-Elmer, Ueberlingen, Germany). Stimulation indices were calculated in reference to reporter gene activity of medium without addition of ODN.

Cell purification. Peripheral blood buffy coat preparations from healthy human donors were obtained from the Blood Bank of the University of Düsseldorf (Germany) and PBMC were purified by centrifugation over Ficoll-Hypaque (Sigma). Cells were cultured in a humidified incubator at 37°C in RPMI 1640 medium supplemented with 5% (v/v) heat inactivated human AB serum (BioWhittaker) or 10% (v/v) heat inactivated FCS, 1.5mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin (all from Sigma).

Cytokine detection. PBMC were resuspended at a concentration of $5x10^6$ cells/ml and added to 48 well flat-bottomed plates (1ml/well) or 96 well round-bottomed plates (200µl/well), which had previously received nothing or ODN in different concentrations. Culture supernatants (SN) were collected after the indicated time points. If not used immediately, supernatants were frozen at –20°C until required. Amounts of cytokines in the supernatants were assessed using commercially available ELISA Kits (IL-6, IL-10; from Diaclone, Besancon, France) or an in-house ELISA developed using commercially available antibodies (from PBL, New Brunswick, NJ, USA for detection of multiple IFN-α species).

Materials and Methods Examples 6-9:

CpG ODN. The CpG ODN used were of sequences TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 116) and T-C-G-A-C-G-T-C-G-A-Cholesterol (SEQ ID NO: 13). The GpC analogue of SEQ ID NO: 116 was used as a non-CpG control. All ODN were supplied by Coley Pharmaceutical Group (Wellesley,

MA). All ODN were re-suspended in sterile, endotoxin free TE at pH 8.0 (OmniPer®; EM Science, Gibbstown, NJ) and stored and handled under aseptic conditions to prevent both microbial and endotoxin contamination. Dilution of ODNs for assays was carried out in sterile, endotoxin free PBS at pH 7.2 (Sigma Chemical Company, St. Lois, MO).

Animals. Female BALB/c mice (purchased from Charles River Canada; Montreal, Quebec, Canada, TLR9 knock out or their wild type counterparts (obtained from Dr. S. Akira at Osaka University, Japan and bred at the Coley Canada Animal Care Facility) all at 6-8 weeks of age were used for experiments. All animals were housed in micro-isolators at the Coley Canada Animal Care Facility and experiments were carried out with approval of the Animal Care Committee and under the guidelines of the Canadian Council on Animal Care.

In vitro assays. Naive BALB/c splenocytes (5 X 10^6 cells per ml) were stimulated with either CPG SEQ ID NO: 116, CpG SEQ ID NO: 13 or the non-CpG control at 0.3, 1, 3 or 10 μg/ml. Concanavalin A (10 μg/ml, Sigma Chemical Company) and/or LPS (10 μg/ml, Sigma Chemical Company) were used as positive controls and cells cultured with media alone were used as negative controls. Culture supernatants were collected at 6 hr (for TNF-α) or at 24 hr (for IL-6 and IL-12) and were tested for cytokines using commercial ELISA kits (mouse OptEIA kits; PharMingen, Mississauga, ON).

In vivo assays. Female BALB/c mice (n=3 or 5/group) were injected either subcutaneously or intravenously with 500 μg of ODN or 500 μl PBS (negative control) and bled at 3 or 8 hrs post ODN administration. Plasma was tested for IP-10, IL-6 or IL-12 by ELISA.

Example 1: Lipophilic Conjugates demonstrate enhanced IFN-α production.

Human PBMC were incubated with increasing concentrations of SEQ ID NO: 36 (CpG B-Class), SEQ ID NO: 39 (CpG C-Class), non-CpG control, SEQ ID NO: 40 (CpG

A-Class), non-CpG control A-Class or SEQ ID NO: 4 (CpG ODN with lipophilic conjugate) for 48h. Supernatants were harvested and IFN-α measured by ELISA. Shown is the Mean±SEM of three blood donors. The results are shown in Figure 1. The CpG ODN with lipophilic conjugate, the CpG C-Class oligonucleotide, and the CpG A-Class oligonucleotide all induced IFN-α production. The CpG ODN with lipophilic conjugate at a concentration of 0.5 μg/ml and 2 μg/ml was measured to induce IFN-α production of about 1000 pg/ml and 3250 pg/ml respectively. The CpG B-Class oligonucleotide, the non-CpG control oligonucleotide, and the non-CpG control A-Class oligonucleotide did not induce any measurable IFN-α response.

Example 2: Lipophilic Conjugates demonstrate potency in IL-6 production.

Human PBMC were incubated with increasing concentrations of SEQ ID NO: 36 (CpG B-Class), SEQ ID NO: 39 (CpG C-Class), non-CpG control, SEQ ID NO: 40 (CpG A-Class), non-CpG control A-Class or SEQ ID NO: 4 (CpG ODN with lipophilic conjugate) for 24h. Supernatant were harvested and IL-6 measured by ELISA. Shown is the Mean±SEM of three blood donors. The results are shown in Figure 2. In this assay the CpG ODN with lipophilic conjugate at a concentration of 2 μg/ml showed the highest measured induction of IL-6 in comparison to the other ODNs used, about 750 pg/ml. At lower concentrations (.031, .125 and .5) the CpG ODN with lipophilic conjugate induced IL-6 with reduced potency. The CpG B-Class, CpG C-Class and to certain extend the CpG A-Class, ODNs all demonstrated potency in IL-6 induction. The non-CpG control and the non-CpG control A-Class oligonucleotides showed no or low capacity for induction of IL-6.

Example 3: Lipophilic Conjugates demonstrate reduced potency in IL-10 production.

Human PBMC were incubated with increasing concentrations of SEQ ID NO: 36 (CpG B-Class), SEQ ID NO: 39 (CpG C-Class), non-CpG control, SEQ ID NO: 40 (CpG A-Class), non-CpG control A-Class or SEQ ID NO: 4 (CpG ODN with lipophilic conjugate) for 24h. Supernatants were harvested and IL-6 measured by ELISA. Shown is the Mean±SEM of three blood donors. The results are shown in Figure 3. The CpG ODN with lipophilic conjugate showed significantly reduced potency in stimulating IL-

10 production. Even at the highest concentration used 2 μg/ml the CpG ODN with lipophilic conjugate did not significantly induce IL-10 stimulation. Similar results were obtained with the A-Class ODN, while in contrast the B-Class ODN and the C-Class ODN showed high capacity for induction of IL-10 production. The non-CpG control and the non-CpG control A-Class oligonucleotides showed no or low capacity for induction of IL-10.

Example 4: Lipophilic conjugates induce TLR9-dependent NFκB signaling.

HEK293 cells expressing the human TLR9 were incubated with the indicated ODN concentrations. NFκB stimulation was measured through luciferase activity. Stimulation indices were calculated in reference to luciferase activity of medium without addition of CpG ODN (fold induction of luciferase activity). The results are shown in Figure 4. The CpG ODN with lipophilic conjugate at the highest used dose of 10 μg/ml induced a stimulation index of about 20. In comparison the B-Class ODN induced a stimulation index of 20 at a much lower concentration of .625 10 μg/ml. The A-Class ODN showed the lowest NFκB stimulation and the highest measured stimulation index was 5 for an ODN concentration of 10 μg/ml.

Example 5: Effect of sequence and lipophilic group of conjugate on IFN-α production.

Human PBMC of three donors were incubated for 48h with the indicated ODN. Supernatants were harvested and IFN-α measured by ELISA. Shown is the level of activation of each ODN by -: no; +: low; +/++: intermediate; +++/++++: strong, as well as the maximal IFN-α amount induced by each ODN. The results are shown in Table 2.

Table 2

ODN #	Sequence and Modification	IFN-α secretion	
SEQ ID NO: 36	T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*T*C*G*T*T	+	65/400
SEQ ID NO: 40	G*G*G_G_A_C_G_A_C_G_T_C_G_T_G_G*G*G*G*G*G	++++	3134
SEQ ID NO: 3	T*C_G_A_C_G_T_C_G*T-Chol	+	1126

SEQ ID NO: 4	T_C_G_A_C_G_T_C_G_T_Chol	+++	2134
SEQ ID NO: 5	Chol-T_C_G_A_C_G_T_C_G_T-Chol	+	456
SEQ ID NO: 6	Chol_T_C_G_A_C_G_T_C_G_T_teg	-	7
SEQ ID NO: 36	T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T	+	58
SEQ ID NO: 39	T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*C*G	+++	3198
SEQ ID NO: 40	G*G*G_G_A_C_G_A_C_G_T_C_G_T_G_G*G*G*G*G*G	++++	5018
SEQ ID NO: 4	T_C_G_A_C_G_T_C_G_T_Chol	+++	3439
SEQ ID NO: 7	T_C_G_T_C_G_A_C_G_T_G_Chol	+++	3395
SEQ ID NO: 8	T_C_G_A_C_G_T_C_G_T_T_Chol	+++	3383
SEQ ID NO: 9	G_T_C_G_A_C_G_T_C_G_T_Chol	+++	3408
SEQ ID NO: 10	G_T_C_G_A_C_G_T_C_G_T_T_Chol	+++	3511
SEQ ID NO: 11	T_C_G_T_C_G_A_C_G_T_T_Chol	+++	3468
SEQ ID NO: 19	T_C_G_A_C_G_T_C_G_A_C_G_T_C_G_T_Chol	++	3351
SEQ ID NO: 25	T*C*G*T_C_G_A_C_G_T_C_G_T_Chol	+	374
SEQ ID NO: 26	T*C*G*T*C*G*T*T*T*T_C_G_A_C_G_T_C_G_T_Chol	-	23
SEQ ID NO: 27	T_C_G_G_C_G_G_C_C_G_C_C_G_Chol	+++	3233
SEQ ID NO: 28	T*C*G*T_C_G_G_C_G_G_C_C_G_C_C_G_T_Chol	+	208
SEQ ID NO: 29	U_C_G_A_C_G_T_C_G_U-Chol	+++	2190
SEQ ID NO: 30	T_C_I_A_C_I_T_C_I_T-Chol	-	9
SEQ ID NO: 31	T_C_7_A_C_7_T_C_7_T-Chol	+++	2259
SEQ ID NO: 36	T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T	+	477
SEQ ID NO: 39	T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*C*G	+++	2329
SEQ ID NO: 40	G*G*G_G_A_C_G_A_C_G_T_C_G_T_G_G*G*G*G*G*G	++++	3667
SEQ ID NO: 12	A_C_G_A_C_G_T_C_G_T_Chol	+/-	71
SEQ ID NO: 13	T_C_G_A_C_G_T_C_G_A_Chol	++++	2894

SEQ ID NO: 14	G_A_C_G_A_C_G_T_C_G_T_T_Chol	++	3490
SEQ ID NO: 15	T*C*G*A*C*G*T*C*G*T_Chol	-	7
SEQ ID NO: 16	T*C_G_A_C_G_T_C_G_T_Chol	+	2717
SEQ ID NO: 17	T_C_G_A_C_G_T_C_G*T_Chol	+++	3600
SEQ ID NO: 18	T_C_G_A_C_G_T_C_G_T_teg	-	21
SEQ ID NO: 19	T_C_G_T_C_G_T_C_G_T_Chol	-	21
SEQ ID NO: 20	T_G_C_A_G_C_T_G_C_T-Chol	-	14
SEQ ID NO: 21	..C_G_A_C_G_T_C_G.._Chol	-	8
SEQ ID NO: 22	T_A_A_C_G_T_T_T_Chol	-	24
SEQ ID NO: 23	T_G_A_C_G_T_T_T_Chol	-	18
SEQ ID NO: 32	T_C_A_T_C_G_A_T_G_A_Chol	+	650
SEQ ID NO: 33	....G_A_C_G_A_T_C_G_T_C_Chol	+	877
SEQ ID NO: 34	T_C_A_C_C_G_G_T_G_A_Chol	-	7
SEQ ID NO: 35	G_A_C_G_T_T_A_A_C_G_T_C_Chol	-	0
SEQ ID NO: 105	T_C_A_A_C_G_T_T_G_A-Chol	+	418
SEQ ID NO: 36	T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T	+	315
SEQ ID NO: 39	T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*C*G*C*C*G	+++	3053
SEQ ID NO: 40	G*G*G_G_A_C_G_A_C_G_T_C_G_T_G_G*G*G*G*G*G	++++	4503
SEQ ID NO: 4	T_C_G_A_C_G_T_C_G_T_Chol	+++	
SEQ ID NO: 13	T_C_G_A_C_G_T_C_G_A_Chol	++++	3610
SEQ ID NO: 41	T_C_G_A_Chol	-	73
SEQ ID NO: 42	T_C_G_C_G_A_Chol	-	23
SEQ ID NO: 43	T_C_G_C_G_C_G_A_Chol	-	44
SEQ ID NO: 48	T_C_G_T_A_C_G_A_Chol	++(+)	2531
SEQ ID NO: 44	T_C_G_C_C_G_G_C_G_A_Chol	++	2060
SEQ ID NO: 45	T_C_G_G_C_G_C_C_G_A_Chol	+++	3654
SEQ ID NO: 46	T_C_G_C_G_C_G_C_G_A_Chol	+++	3573
SEQ ID NO: 47	T_C_G_T_C_G_A_C_G_A_Chol	-	40
SEQ ID NO: 49	T_C_G_A_A_T_T_C_G_A_Chol	++	2788
SEQ ID NO: 50	T_C_G_T_T_A_A_C_G_A_Chol	++++	4161

SEQ ID NO: 51	T_C_G_A_A_C_G_T_T_C_G_A_Chol	++(+)	2954
SEQ ID NO: 52	T_C_G_T_T_C_G_A_A_C_G_A_Chol	++++	4033
SEQ ID NO: 53	T_C_G_A_C_G_A_T_C_G_T_C_G_A_Chol	+(+)	3187
SEQ ID NO: 56	T_C_G_G_C_G_G_C_C_G_C_C_G_A_Chol	+	1385
SEQ ID NO: 54	T_C_G_G_A_C_G_A_T_C_G_T_C_C_G_A_Chol	++(+)	3391
SEQ ID NO: 36	T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T	+	776
SEQ ID NO: 39	T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*C*G*C*C*G	+++	3201
SEQ ID NO: 40	G*G*G_G_A_C_G_A_C_G_T_C_G_T_G_G*G*G*G*G*G	++++	3706
SEQ ID NO: 13	T_C_G_A_C_G_T_C_G_A_Chol	++++	3485
SEQ ID NO: 59	T_C_G_A_C_G_T_C*G*A_Chol	+++(+)	2744
SEQ ID NO: 60	T_C_G_A_C_G_T*C*G*A_Chol	+++(+)	3297
SEQ ID NO: 61	G_C_G_A_C_G_T_C_G_A_Chol	-	304
SEQ ID NO: 62	C_C_G_A_C_G_T_C_G_A_Chol	-	562
SEQ ID NO: 63	I_C_G_A_C_G_T_C_G_A_Chol	-	226
SEQ ID NO: 64	U_C_G_A_C_G_T_C_G_A_Chol	+(+)	1578
SEQ ID NO: 65	Z_C_G_A_C_G_T_C_G_A_Chol	-	272
SEQ ID NO: 66	T_T_C_G_A_C_G_T_C_G_A_Chol	+++(+)	2619
SEQ ID NO: 67	T_T_T_C_G_A_C_G_T_C_G_A_Chol	++	1800
SEQ ID NO: 68	T_C_G_T_C_G_A_C_G_T_C_G_A_Chol	+++	2593
SEQ ID NO: 69	T_C_G_A_A_T_A_T_A_T_A_T_T_A_C_G_A_chol	-	43
SEQ ID NO: 70	T_C_G_A_A_T_A_T_A_T_A_T_T_A_chol	-	96
SEQ ID NO: 71	T_C_A_T_C_G_A_T_G_A_Chol	-	293
SEQ ID NO: 106	T*C_G*T*C_G*T*T*T*C_G*T*C_G*T*T_chol	-	108
SEQ ID NO: 107	T_C_G_T_C_G_T_T_T_C_G_T_C_G_T_T_chol	-	48
SEQ ID NO: 36	T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T	+	177
SEQ ID NO: 39	T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*C*G*C*C*G	+++	1881

SEQ ID NO: 40	G*G*G_G_A_C_G_A_C_G_T_C_G_T_G_G *G*G*G*G*G	++++	2309
SEQ ID NO: 13	T_C_G_A_C_G_T_C_G_A_Chol	+++	2562
SEQ ID NO: 108	T_C_G_T_C_G_T_C_G_A_Chol	-	35
SEQ ID NO: 109	T_C_G_A_C_G_A_C_G_A_Chol	+	635
SEQ ID NO: 108 + SEQ ID NO: 109		+++	2340

A palindromic or partial palindromic sequence with at least one CpG motif was required but not sufficient for high IFN-α induction. SEQ ID NO: 27 having the palindrome CGGCGGCCGCCG (SEQ ID NO: 114) and an additional T at the 5'-end resulted in potent induction of IFN-α. Addition of a G residue to the 5'-end or a T residue to the 3'-end, such as in SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO: 8, resulted in similar biological activity. However, significant extension of the palindrome (SEQ ID NO: 19) resulted in slightly decreased IFN-α induction. These results suggest that with an oligonucleotide of this length and design the sequence of the ODN must be at least partially palindromic and that a TCG (e.g. SEQ ID NO: 4, SEQ ID NO: 13), GTCG (SEQ ID NO: 9), GACG (SEQ ID NO: 14), or a UCG (as in SEQ ID NO: 29) motif at the 5'-end or near the 5'-end is of particular advantage to obtaining high IFN-α induction.

The 3'-cholesterol modified SEQ ID NO: 4 shows high secretion of IFN-α but low induction of IL-10 secretion, a characteristic property of A-class CpG oligonucleotides.

Example 6: ***In vitro*** **mouse splenocyte stimulation.**

BALB/c mouse splenocytes were incubated for 24h (Figure 5a-b) or 6h (Figure 5c) with the indicated concentrations of SEQ ID NO: 13 or control SEQ ID NO: 117. SN were harvested and cytokines measured by ELISA. As shown in Figure 5a, the CpG ODN with lipophilic conjugate induced IL-6 production in adose dependent manner. At the highest ODN concentration tested of 10 μg/ml the measured IL-6 repsonse was approximately 900 pg/ml. The control non-CpG ODN did not stimulate any IL-6 induction. Figure 5b shows the induction of IL-12 by the CpG ODN with lipophilic

conjugate. The CpG ODN with lipophilic conjugate induced IL-12 production in a dose dependent matter, and at the highest ODN concentration used, 10 μg/ml, the measured IL-12 induction was about 3750 pg/ml. In contrast the control non-CpG ODN did not stimulate any IL-12 production. Figure 5c shows the induction of TNF-α by the CpG ODN with lipophilic conjugate in comparison to the control non-CpG ODN. At the concentration of 10 μg/ml, the CpG ODN with lipophilic conjugate induced about 140 pg/ml of TNF-α, while in contrast the control non-CpG ODN did not significantly induce any TNF-α production.

Example 7: ***In vitro*** **TLR9$^{+/+}$ and TLR9$^{-/-}$ splenocyte stimulation.**

Balb/c splenocytes from TLR9$^{+/+}$ (Figure 6a) or TLR9$^{-/-}$ (Figure 6b) mice were incubated for 24h with the indicated concentrations of SEQ ID NO: 13 or control SEQ ID NO: 117. SN were harvested and IL-12p40 measured by ELISA. Figure 6a shows that the CpG ODN with lipophilic conjugate induced IL-12 dose-response that was TLR-dependent. The highest concentration of CpG ODN with lipophilic conjugate used, 10 μg/ml, induced IL-12 concentration of 1200 pg/ml. In contrast, the control non-CpG ODN did not significantly induce any IL-12 production at any concentration used. Figure 6b shows that both, the CpG ODN with lipophilic conjugate and the control non-CpG ODN did not significantly induce any IL-12 production in TLR deficient cells even at concentration of 10 μg/ml.

Example 8: ***In vivo*** **time-dependent plasma IP-10 stimulation.**

Balb/c mice (n=5) were injected SC with 500μg of SEQ ID NO: 13 and bled at 1, 2, 3, 6, 8, 12 and 24 hr post ODN administration. Plasma was tested for IP-10 by ELISA (Figure 7). As shown in Figure 7, the CpG ODN with lipophilic conjugate stimulated production of IP-10 in time-dependent fashion. There was no detectable IP-10 induction during the first three hours post injection. At six hours post injection the IP-10 concentration was increased to 500 pg/ml. At eight hours post injection the IP-10 stimulation peaked at about 2000 pg/ml. At 12 hours post injection the IP-10 concentration decreased to about 500 pg/ml, equaling the stimulation measured at six hours post injection. At twenty-four hours post injection there was no detectable

stimulation of IP-10 production. The control PBS treatment showed no induction of IP-10 production at any of the time points examined.

Example 9: *In vivo* plasma cytokine and chemokine stimulation.

Balb/c mice (n=3) were injected IV with 500μg of SEQ ID NO: 13 or 500μl PBS (negative control) and bled at 3 and 8 hrs post ODN administration. Plasma was tested for cytokines or chemokine by ELISA (Figure 8). Solid bar=3hr; Hatched bar=8hr. Figure 8a shows that the CpG ODN with lipophilic conjugate stimulated production of IP-10 in time-dependent fashion, about 9000 and 4000 pg/ml of IP-10 were stimulated at 3 and 8 hrs respectively. In contrast the control non-CPG ODN (SEQ ID NO: 117) did not stimulate any IP-10 production at the same time points. Figure 8b shows that the stimulation of IL-12 production by the CpG ODN with lipophilic conjugate was lower at 3hrs, about 20,000 pg/ml of IL-12, than at 8hrs, about 25,000 pg/ml of IL-12 produced. The control non-CpG ODN did not induce any IL-12 production at either time point tested. Figure 8c shows that the the CpG ODN with lipophilic conjugate stimulated production of IL-6 in time-dependent fashion. At 3hrs post injection the IL-6 production ranged from 250 to 500 pg/ml, while at 8hrs post injection the IL-6 production was about 400 pg/ml. The control non-CpG ODN did not show significant induction of IL-6 production in comparison to the PBS control.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

We claim:

CLAIMS

1. A composition comprising;

$(N_1PN_2) \cdot L$

wherein N_1 and N_2 are independently nucleic acids of 0-100 nucleotides in length, P is a palindromic containing nucleic acid and comprising at least one YR dinucleotide, wherein Y is a cytosine or a modified cytosine and R is a guanine or a modified guanine, and wherein L is a lipophilic group.

2. The composition of claim 1, wherein N_1PN_2 is 3-14 nucleotides in length.

3. The composition of claim 1, wherein L is linked to the nucleotide at the 3' end of N_1PN_2.

4. The composition of claim 1, wherein L is linked by a linker to a 2'-position of a nucleotide in N_1PN_2, to a heterocyclic base of a nucleotide in N_1PN_2, or a phosphodiester linkage in N_1PN_2.

5. The composition of claim 4, wherein the nucleotide is selected from the group consisting of a nucleotide at the 3' end of N_1PN_2 and an internal nucleotide.

6. The composition of claim 1, wherein L is selected from the group consisting of a cholesteryl, a modified cholesteryl, a cholesterol derivative, a reduced cholesterol, and a substituted cholesterol.

7. The composition of claim 6, wherein the reduced cholesterol is cholestan.

8. The composition of claim 1, wherein L is selected from the group consisting of C16 alkyl chain, bile acids, cholic acid, taurocholic acid, deoxycholate, oleyl litocholic acid, oleoyl cholenic acid, glycolipids, phospholipids, sphingolipids, isoprenoids, such as steroids, vitamins, such as vitamin E, saturated fatty acids, unsaturated fatty acids, fatty acid esters, such as triglycerides, pyrenes, porphyrines, Texaphyrine, adamantane,

acridines, biotin, coumarin, fluorescein, rhodamine, Texas-Red, digoxygenin, dimethoxytrityl, t-butyldimethylsilyl, t-butyldiphenylsilyl, cyanine dyes (e.g. Cy3 or Cy5), Hoechst 33258 dye, psoralen, and ibuprofen.

9. The composition of claim 1, wherein the formula comprises N_1PN_2-L- N_3PN_4, wherein N_3 and N_4 are independently nucleic acids of 0-100 nucleotides in length.

10. The composition of claim 9, wherein L is linked to N_1PN_2 and N_3PN_4 though a linkage selected from the group consisting of -3'-L-3'-, -2'-L-2'-, -3'-L-2'- and -2'-L-3'- .

11. The composition of claim 9, wherein N_1PN_2 and N_3PN_4 are identical.

12. The composition of claim 9, wherein N_1PN_2 and N_3PN_4 are different.

13 The composition of claim 1, further comprising at least 2 L.

14. The composition of claim 1, wherein the formula comprises
$([N_1PN_2]_n- (X_3)_m) \cdot (L)_p$
wherein X_3 is a linker, m is an integer from 0 to 20 (preferably from 1-10), n is an integer from 0 to 20 (preferably from 1-10), p is an integer from 1 to 10 (preferably 1), and wherein the oligonucleotide N_1PN_2 has a length of 4 to 40 nucleotides.

15. The composition of claim 14, wherein X_3 is a non-nucleotidic linker selected from the group consisting of abasic residues (dSpacer), oligoethyleneglycol, such as triethyleneglycol (spacer 9) or hexaethylenegylcol (spacer 18), and alkane-diol, such as butanediol.

16. The composition of claim 14, wherein the linker is attached to the oligonucleotide through a linkage selected from the group consisting of phosphodiester, phosphorothioate, methylphosphonate, and amide linkages.

17. The composition of claim 14, wherein n is greater than 1 and the multiple $[N_1PN_2]$ are linked through 3'-ends.

18. The composition of claim 14, wherein N_1PN_2 is a branched ODN and wherein N_1 includes at least one CG dinucleotide.

19. The composition of claim 14, wherein L is attached to the 3' end of the oligonucleotide $[N_1PN_2]$.

20. The composition of claim 14, wherein P is X_1-Y-R-X_2, wherein X_1 and X_2 are independently from 0 to 4 nucleotides.

21. The composition of claim 20, wherein X_1 is 1 to 2 nucleotides.

22. The composition of claim 20, wherein X_1 is 1 nucleotide.

23. The composition of claim 20, wherein X_1 is a pyrimidine.

24. The composition of claim 20, wherein the pyrimidine is selected from the group consisting of a thymidine, deoxyuridine and a 5-substituted deoxyuridine.

25. The composition of claim 20, wherein X_2 is a palindrome or an inverted repeat.

26. The composition of claim 25, wherein the palindrome or inverted repeat contains at least one unmethylated CpG motif.

27. The composition of claim 20, wherein the oligonucleotide N_1PN_2 has a length of 4 to 20 nucleotides.

28. The composition of claim 20, wherein the oligonucleotide N_1PN_2 has a length of 6 to 14 nucleotides.

29. The composition of claim 1, wherein the oligonucleotide includes at least one linear or branched non-nucleoside linkage.

30. The composition of claim 1, further comprising an immune stimulatory molecule associated with the composition.

31. The composition of claim 30, wherein immune stimulatory molecule is a TLR9 ligand.

32. The composition of claim 1, wherein the internucleotide linkages of the oligonucleotide are all phosphodiester linkages.

33. The composition of claim 1, wherein the oligonucleotide includes at least one stabilized internucleotide linkage.

34. The composition of claim 33, wherein the stabilized internucleotide linkage is the linkage between Y and R and is a phosphorothioate linkage in an Rp configuration.

35. The composition of claim 1, wherein P is selected from the group consisting of C_G_A_C, C_G_T_C, T_C_G_A_C, C_G_A_C_G_T_C, C_G_G_C_G_G_and G_A_C_G_A.

36. The composition of claim 1, wherein at least one nucleotide in the oligonucleotide is a substituted or modified purine or pyrimidine.

37. The composition of claim 36, wherein the substituted pyrimidine is a C5 substitution.

38. The composition of claim 36, wherein the substituted purine is a C8 or C7 substitution.

39. The composition of claim 36, wherein the substituted or modified pyrimidine is a 5-substituted cytosine.

40. The composition of claim 1, wherein multiple oligonucleotides are linked by multiple doubler or trebler moieties and form a dendrimer.

41. The composition of claim 1, wherein the composition includes at least one amino acid residue linked by an amide linkage.

42. The composition of claim 1, wherein the oligonucleotide includes at least one internucleotide linkage selected from the group consisting of a 3'5'-, a 2'5'-, a 3'3'-and a 5'5'-linkage.

43. The composition of claim 1, wherein the linkage between L and N_1PN_2-is metabolically stable.

44. The composition of claim 1, wherein the linkage between L and N_1PN_2-is metabolically labile.

45. The composition of claim 1, wherein L is associated with a carrier.

46. The composition of claim 1, wherein the carrier is selected from the group consisting of a liposome, ISCOM, a hydrophobic bead, a hydrophobic formulation, a polymer, a peptide, a protein, and a nucleic acid.

47. The composition of claim 46, further comprising a therapeutic agent associated with the composition.

48. The composition of claim 1, further comprising:

a nucleic acid having at least one exposed 5' end; comprising, at least one YR dinucleotide, wherein Y is a cytosine or a modified cytosine and R is a guanine or a

modified guanine, at least one single stranded region, at least one double stranded region and wherein the nucleic acid is linked to at least one lipophilic group.

49. The composition of claim 48, wherein the nucleic acid is a single chain nucleic acid.

50. The composition of claim 49, wherein the nucleic acid forms a double stranded region involving base pairing of at least two nucleic acids on each side of the double stranded region.

51. The composition of claim 49, wherein the nucleic acid forms a double stranded region involving base pairing of at least three nucleic acids on each side of the double stranded region.

52. The composition of claim 48, wherein the nucleic acid is a branched nucleic acid.

53. The composition of claim 48, wherein the nucleic acid is two single chain nucleic acids at least partially hybridized to one another.

54. The composition of claim 48, wherein the nucleic acid is linked to at least two lipophilic groups.

55. The composition of claim 48, wherein the lipophilic group is linked to the nucleotide at the 3' end of the nucleic acid.

56. A method for modulating an immune response, comprising administering to a subject a composition of any one of claims 1-55, in an effective amount to modulate an immune response.

57. The method of claim 56, wherein the composition is delivered to the subject to treat asthma in the subject.

58. The method of claim 56, wherein the composition is delivered to the subject to treat allergy in the subject.

59. The method of claim 56, wherein the composition is delivered to the subject to treat cancer in the subject.

60. The method of claim 56, wherein the composition is delivered to the subject to treat an infectious disease in the subject.

61. The method of claim 56, wherein the composition is delivered to the subject to treat autoimmune disease in the subject.

62. The method of claim 56, wherein the composition is delivered to the subject to treat airway remodeling in the subject.

63. The method of claim 56, further comprising administering an antigen to the subject.

64. The method of claim 56, further comprising administering a therapeutic protocol to the subject.

65. The method of claim 64, wherein the therapeutic protocol is surgery.

66. The method of claim 64, wherein the therapeutic protocol is radiation.

67. The method of claim 64, wherein the therapeutic protocol is a medicament.

68. The method of claim 56, wherein the composition is formulated.

69. The method of claim 68, wherein the composition is associated with a targeting molecule.

70. The method of claim 56, wherein the composition is delivered by a route selected from the group consisting of oral, nasal, sublingual, intravenous, subcutaneous, mucosal, respiratory, direct injection, and dermally.

71. The composition of claim 33, wherein the at least one stabilized internucleotide linkage is a phosphorothioate linkage.

72. The composition of claim 33, wherein the oligonucleotide includes at least one phosphorothioate inter nucleotide linkage at the 5' end and the 3' end of the oligonucleotide.

73. A composition comprising;

$(N_1YRN_2) \cdot L$

wherein N_1 and N_2 are independently nucleic acids of 0-100 nucleotides in length, wherein Y is a cytosine or a modified cytosine and R is a guanine or a modified guanine, and N_1YRN_2 is at least 10 nucleotides in length,
wherein L is a lipophilic group, and
wherein L is linked by a linker to a 2'-position of a nucleotide in N_1YRN_2, or to a heterocyclic base of a nucleotide in N_1YRN_2.

74. The composition of claim 73, wherein L is selected from the group consisting of a cholesteryl, a modified cholesteryl, a cholesterol derivative, a reduced cholesterol, and a substituted cholesterol.

75. The composition of claim 74, wherein the reduced cholesterol is cholestan.

76. The composition of claim 73, wherein L is selected from the group consisting of C16 alkyl chain, bile acids, cholic acid, taurocholic acid, deoxycholate, oleyl litocholic acid, oleoyl cholenic acid, glycolipids, phospholipids, sphingolipids, isoprenoids, such as steroids, vitamins, such as vitamin E, saturated fatty acids, unsaturated fatty acids, fatty acid esters, such as triglycerides, pyrenes, porphyrines, Texaphyrine, adamantane, acridines, biotin, coumarin, fluorescein, rhodamine, Texas-Red, digoxygenin,

dimethoxytrityl, t-butyldimethylsilyl, t-butyldiphenylsilyl, cyanine dyes (e.g. Cy3 or Cy5), Hoechst 33258 dye, psoralen, and ibuprofen.

77. The composition of claim 73, wherein N_1YRN_2 is selected from the group consisting of 5'TCCG3', 5'TTCG3' and 5'TCGTCG3'.

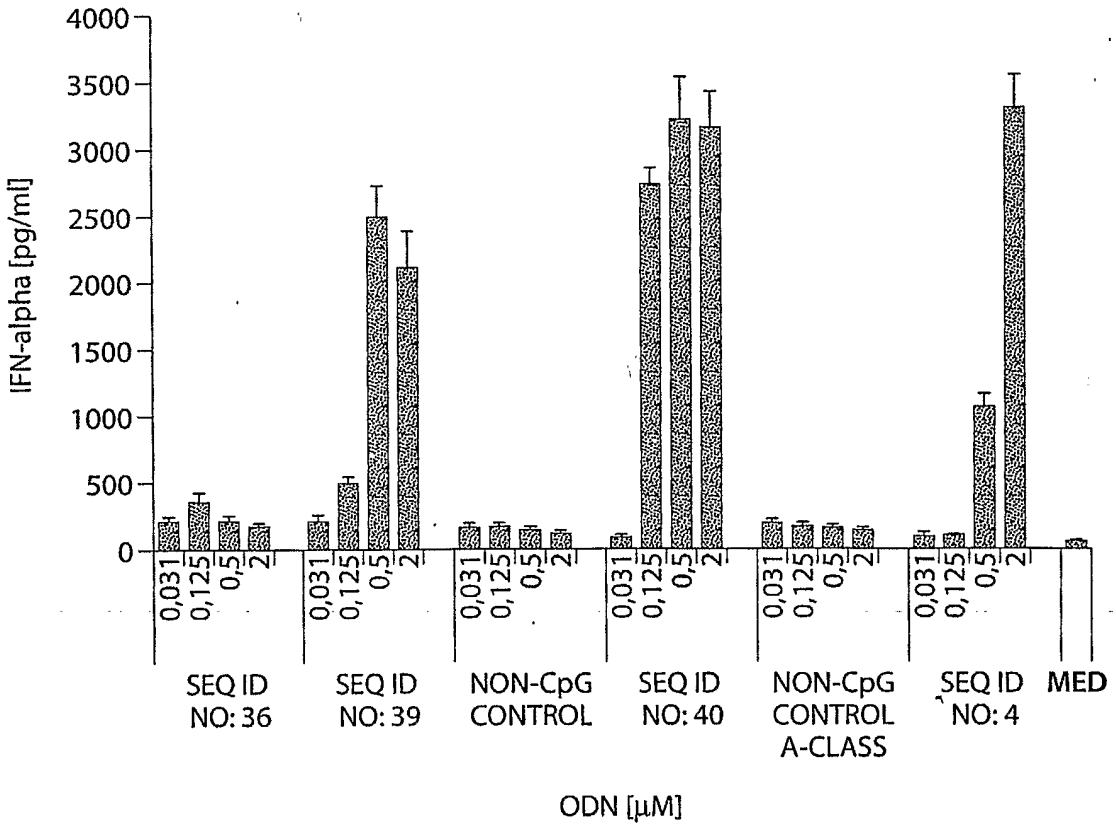
78. A composition comprising;

$(N_1PN_2) \cdot L$

wherein N_1 and N_2 are independently nucleic acids of 0-100 nucleotides in length, P is a palindromic containing nucleic acid and comprising at least one YR dinucleotide, wherein Y is a cytosine or a modified cytosine and R is a guanine or a modified guanine, and wherein L is cholesterol.

79. The composition of claim 78, wherein L is linked to the nucleotide at the 3' end of N_1PN_2.

80. The composition of claim 78, wherein N_1PN_2 is selected from the group consisting of 5'TCGACGTCGT3' (SEQ ID NO: 111) and 5'TCGACGTCGA3' SEQ ID NO: 112).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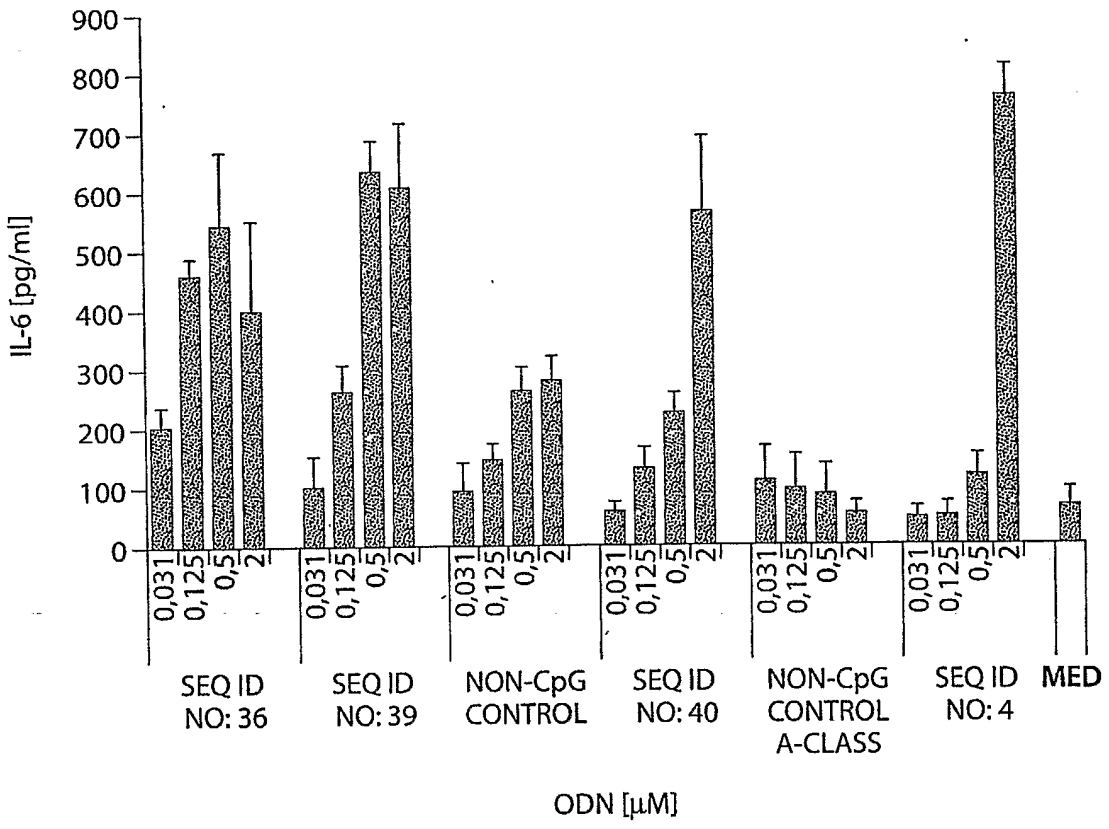
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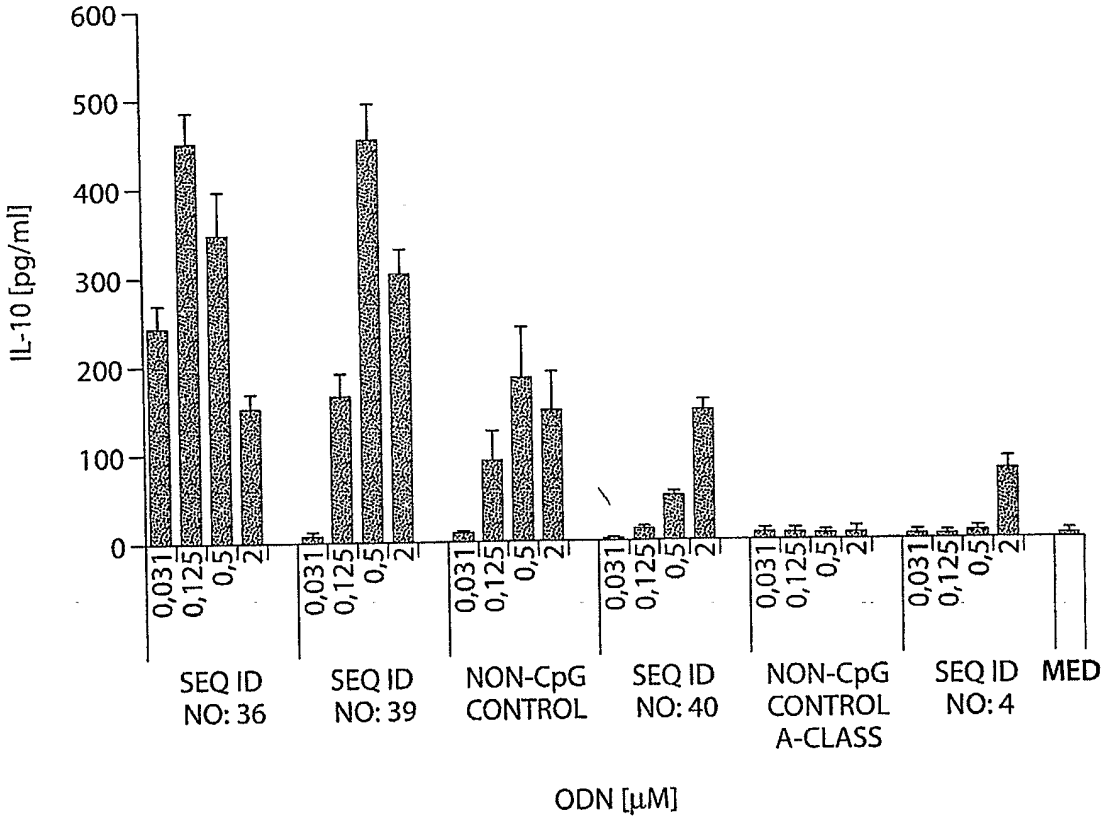
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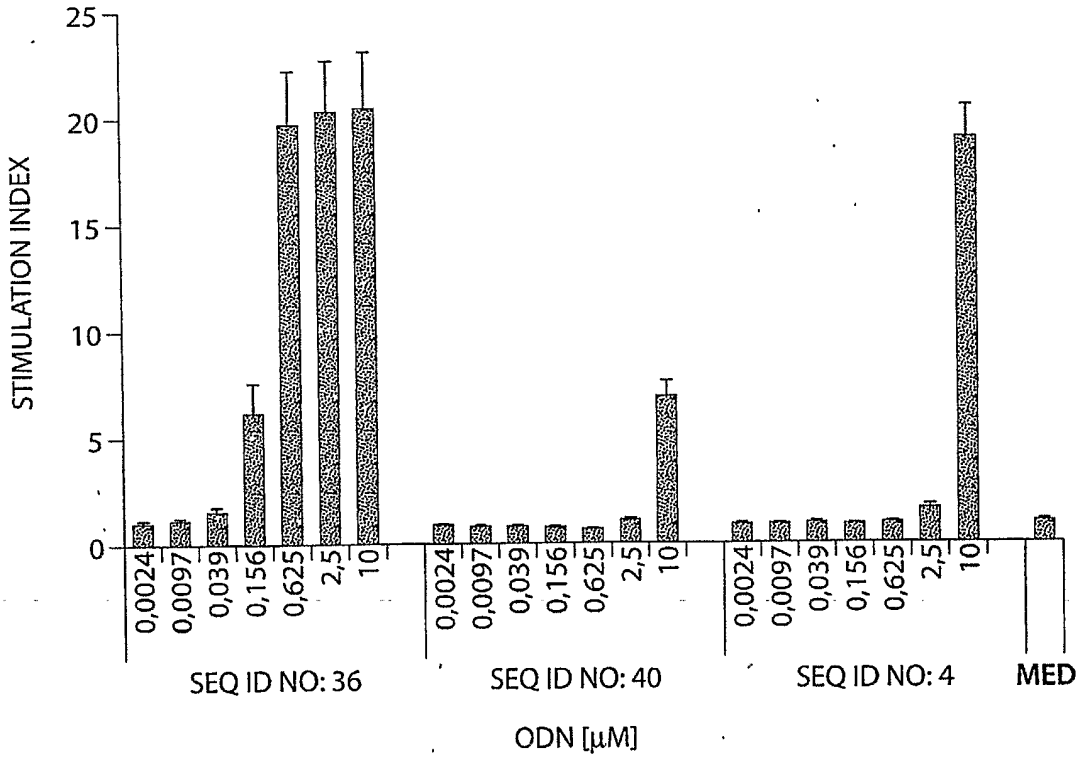
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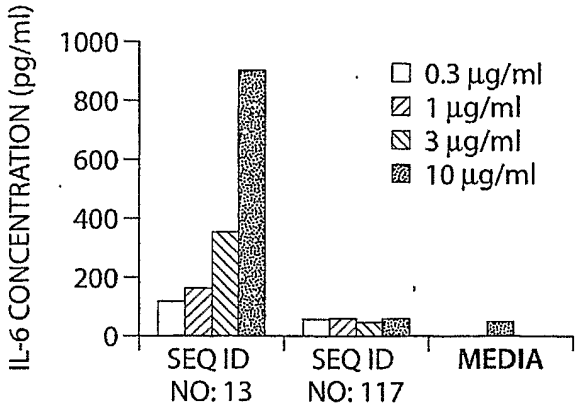

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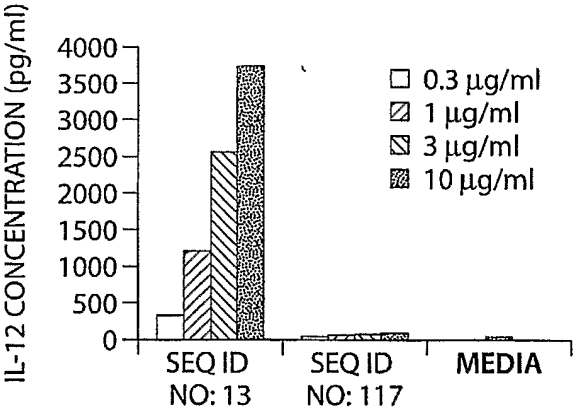

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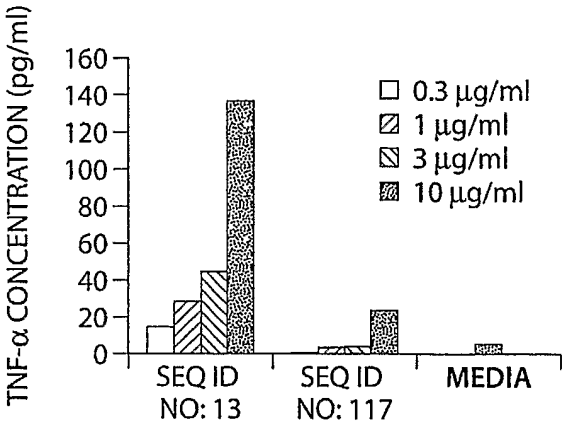

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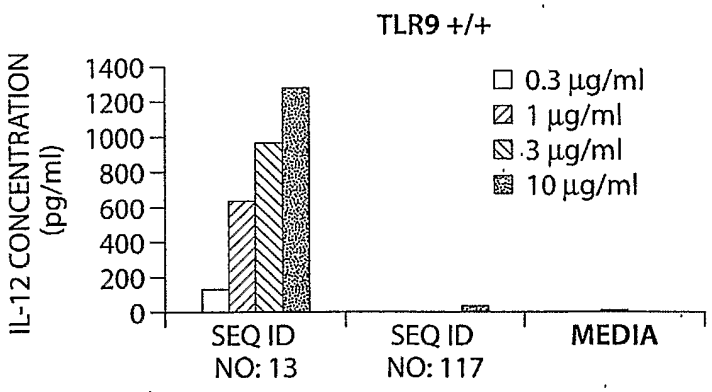
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Fig. 6A

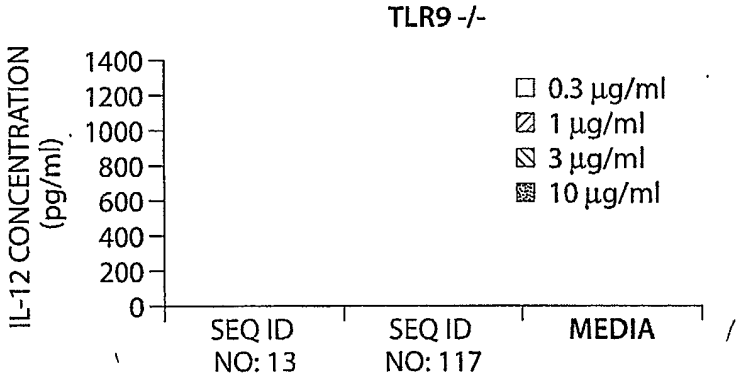
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Fig. 6B

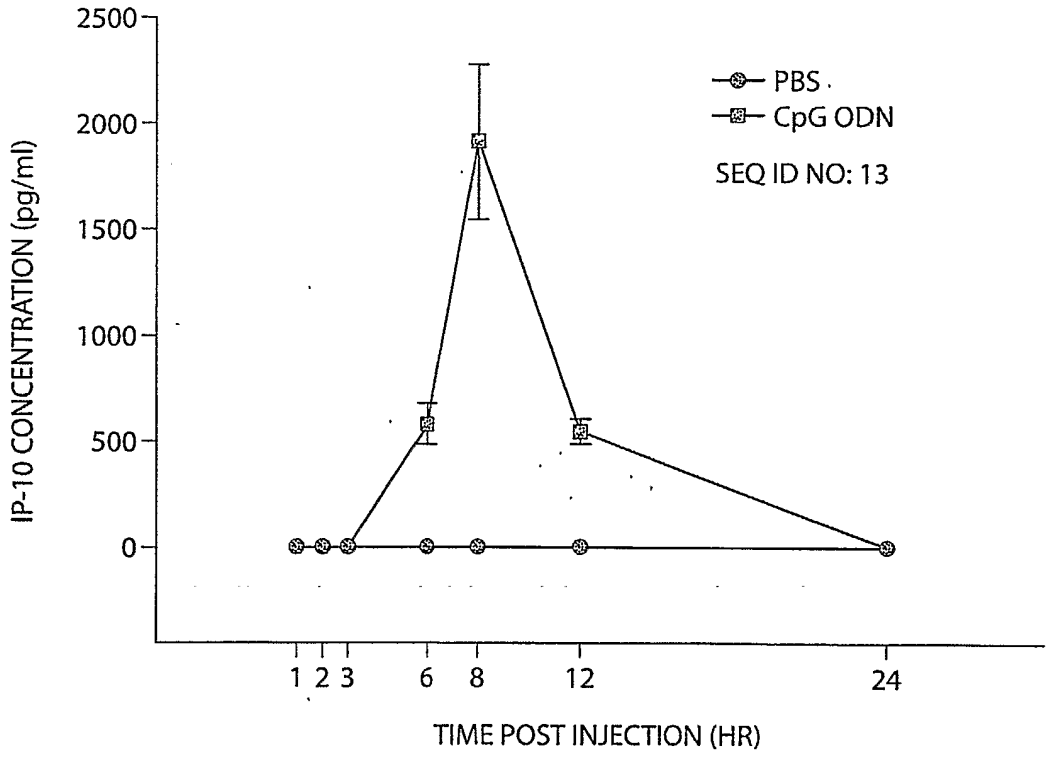
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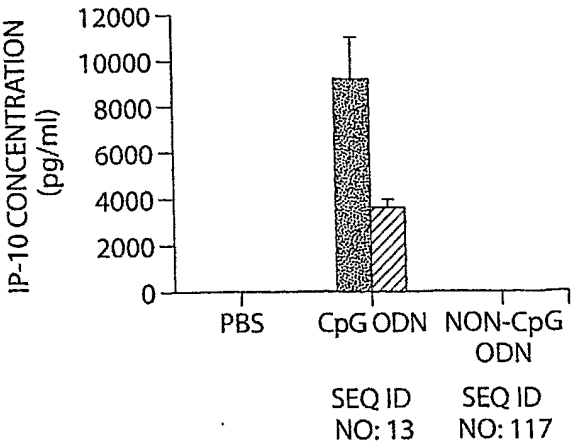
Fig. 7



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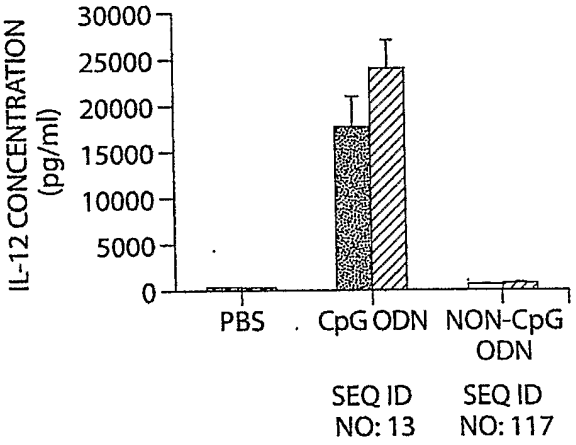

Fig. 8B

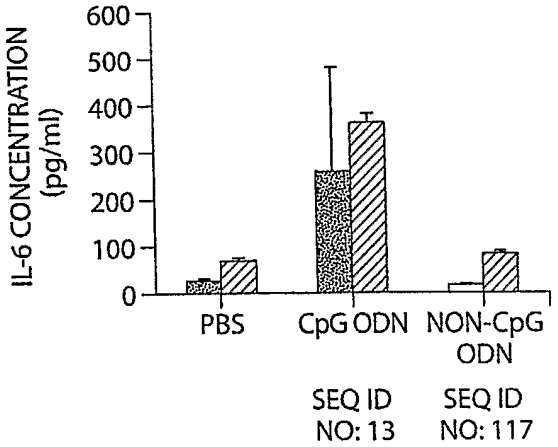

Fig. 8C

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<223> where linkage is phosphorothioate linkage

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 16
tcgacgtcgt 10

<210> 17
<211> 10
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<223> where linkage is phosphotrohioate linkage

<220>
<221> misc_feature
<222> (10)..(10)

<223> cholesterol

<400> 17
tcgacgtcgt 10

<210> 18
<211> 10
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<220>
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<220>
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<223> triethyleneglycol phosphate

<400> 18
tcgacgtcgt 10

<210> 19
<211> 16
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<220>
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<220>
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<222> (16)..(16)
<223> cholesterol

<400> 19
tcgacgtcga cgtcgt 16

<210> 20
<211> 10
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<220>
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<220>
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<222> (10)..(10)
<223> cholesterol

<400> 20
tcgtcgtcgt 10

<210> 21
<211> 10
<212> DNA
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<220>
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<220>
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<222> (10)..(10)
<223> cholesterol

<400> 21
tgcagctgct 10

<210> 22
<211> 8
<212> DNA
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<220>
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<220>
<221> misc_feature
<222> (8)..(8)
<223> cholesterol

<400> 22
cgacgtcg 8

<210> 23
<211> 8
<212> DNA
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<220>
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<220>
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<222> (8)..(8)
<223> cholesterol

<400> 23
taacgttt 8

<210> 24
<211> 8
<212> DNA
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<220>

<223> Synthetic oligonucleotide

<220>
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<222> (8)..(8)
<223> cholesterol

<400> 24
tgacgttt 8

<210> 25
<211> 13
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<220>
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<222> (13)..(13)
<223> cholesterol

<400> 25
tcgtcgacgt cgt 13

<210> 26
<211> 19
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<220>
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<222> (19)..(19)
<223> cholesterol

<400> 26
tcgtcgtttt cgacgtcgt 19

<210> 27
<211> 13
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<220>
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<220>
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<223> cholesterol

<400> 27
tcggcggccg ccg 13

<210> 28
<211> 17
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<220>
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<222> (17)..(17)
<223> cholesterol

<400> 28
tcgtcggcgg ccgccgt 17

<210> 29
<211> 10
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<220>
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<222> (1)..(10)
<223> where N is 2'-Deoxyuridine

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 29
ncgacgtcgt 10

<210> 30
<211> 10
<212> DNA
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<220>
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<220>
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<222> (1)..(10)
<223> where N is Inosine (deoxy)

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 30
tcnacntcnt 10

<210> 31
<211> 10
<212> DNA
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<220>
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<220>
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<223> where N is 7-Deaza-dG

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 31
tcnacntcnt 10

<210> 32
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 32
tcatcgatga 10

<210> 33
<211> 10
<212> DNA
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<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 33
gacgatcgtc 10

<210> 34
<211> 10
<212> DNA
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<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 34
tcaccggtga 10

<210> 35
<211> 12
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<222> (12)..(12)
<223> cholesterol

<400> 35
gacgttaacg tc 12

<210> 36
<211> 24
<212> DNA
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<220>
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<220>
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<222> (1)..(24)
<223> where all linkages are phosphorothioate linkages

<400> 36

tcgtcgtttt gtcgttttgt cgtt 24

<210> 37
<211> 24
<212> DNA
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<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (1)..(1)
<223> cholesterol

<400> 37
tcgtcgtttt gtcgttttgt cgtt 24

<210> 38
<211> 24
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (24)..(24)
<223> cholesterol

<400> 38
tcgtcgtttt gtcgttttgt cgtt 24

<210> 39
<211> 22
<212> DNA
<213> Artificial sequence

<220>
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<220>
<221> misc_feature
<222> (1)..(22)
<223> where all linkages are phosphorothioate linkages

<400> 39
tcgtcgtttt cggcgcgcgc cg 22

<210> 40
<211> 21
<212> DNA

<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<400> 40
ggggacgacg tcgtgggggg g 21

<210> 41
<211> 4
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (4)..(4)
<223> cholesterol

<400> 41
tcga 4

<210> 42
<211> 6
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (6)..(6)
<223> cholesterol

<400> 42
tcgcga 6

<210> 43
<211> 8
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (8)..(8)
<223> cholesterol

<400> 43

tcgcgcga 8

<210> 44
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 44
tcgccggcga 10

<210> 45
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 45
tcggcgccga 10

<210> 46
<211> 10
<212> DNA
<213> Artificial sequence

<220>
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<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 46
tctctctcta 10

<210> 47
<211> 10
<212> DNA

<213> Artificial sequence

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<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 47
tcgtcgacga 10

<210> 48
<211> 8
<212> DNA
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<220>
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<220>
<221> misc_feature
<222> (8)..(8)
<223> cholesterol

<400> 48
tcgtacga 8

<210> 49
<211> 10
<212> DNA
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<220>
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<222> (10)..(10)
<223> cholesterol

<400> 49
tcgaattcga 10

<210> 50
<211> 10
<212> DNA
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<220>
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<220>
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<222> (10)..(10)
<223> cholesterol

<400> 50
tcgttaacga 10

<210> 51
<211> 12
<212> DNA
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<220>
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<220>
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<222> (12)..(12)
<223> cholesterol

<400> 51
tcgaacgttc ga 12

<210> 52
<211> 12
<212> DNA
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<220>
<221> misc_feature
<222> (12)..(12)
<223> cholesterol

<400> 52
tcgttcgaac ga 12

<210> 53
<211> 14
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (14)..(14)
<223> cholesterol

<400> 53

tcgacgatcg tcga 14

<210> 54
<211> 16
<212> DNA
<213> Artificial sequence

<220>
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<220>
<221> misc_feature
<222> (16)..(16)
<223> cholesterol

<400> 54
tcggacgatc gtccga 16

<210> 55
<211> 16
<212> DNA
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<220>
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<220>
<221> misc_feature
<222> (16)..(16)
<223> cholesterol

<400> 55
tcgacgagct cgtcga 16

<210> 56
<211> 14
<212> DNA
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<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (14)..(14)
<223> cholesterol

<400> 56
tcggcggccg ccga 14

<210> 57
<211> 10
<212> DNA

<213> Artificial sequence

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<223> cholesterol

<220>
<221> misc_feature
<222> (10)..(10)
<223> where the linkage to cholesterol is a phosphorothioate linkage

<400> 57
tcgacgtcga 10

<210> 58
<211> 10
<212> DNA
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<222> (9)..(10)
<223> where the linkage is a phosphorothioate linkage

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 58
tcgacgtcga 10

<210> 59
<211> 10
<212> DNA
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<220>
<223> Synthetic oligonucleotide

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<222> (8)..(10)
<223> where the linkages are phosphorothioate linkages

<220>
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<222> (10)..(10)

<223> cholesterol

<400> 59
tcgacgtcga 10

<210> 60
<211> 10
<212> DNA
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<220>
<223> Synthetic oligonucleotide

<220>
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<222> (7)..(10)
<223> where the linkages are phosphorothioate linkages

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 60
tcgacgtcga 10

<210> 61
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 61
gcgacgtcga 10

<210> 62
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 62
ccgacgtcga 10

<210> 63
<211> 10
<212> DNA
<213> Artificial sequence

<220>
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<220>
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<222> (1)..(1)
<223> where n is Inosine (deoxy)

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 63
ncgacgtcga 10

<210> 64
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<222> (1)..(1)
<223> where n is 2'-Deoxyuridine

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 64
ncgacgtcga 10

<210> 65
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<222> (1)..(1)
<223> where n is 5-Methyl-deoxycytidine

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 65
ncgacgtcga 10

<210> 66
<211> 11
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<222> (11)..(11)
<223> cholesterol

<400> 66
ttcgacgtcg a 11

<210> 67
<211> 12
<212> DNA
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<222> (12)..(12)
<223> cholesterol

<400> 67
tttcgacgtc ga 12

<210> 68
<211> 13
<212> DNA
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<220>
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<220>

<221> misc_feature
<222> (13)..(13)
<223> cholesterol

<400> 68
tcgtcgacgt cga 13

<210> 69
<211> 17
<212> DNA
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<222> (17)..(17)
<223> cholesterol

<400> 69
tcgaatatat attacga 17

<210> 70
<211> 14
<212> DNA
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<222> (14)..(14)
<223> cholesterol

<400> 70
tcgaatatat atta 14

<210> 71
<211> 10
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<222> (10)..(10)
<223> cholesterol

<400> 71
tcatcgatga 10

<210> 72
<211> 10
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<222> (10)..(10)
<223> cholesterol

<400> 72
tcgacgttga 10

<210> 73
<211> 10
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<223> where n is 5-Fluoro-dU

<220>
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<223> cholesterol

<400> 73
ncgacgncga 10

<210> 74
<211> 10
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<223> where n is 5-Hydroxy-dC

<220>
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<222> (10)..(10)

<223> cholesterol

<400> 74
tngangtnga 10

<210> 75
<211> 10
<212> DNA
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<220>
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<223> where n is 5-Methyl-deoxycitidine

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 75
tngangtnga 10

<210> 76
<211> 10
<212> DNA
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<220>
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<220>
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<222> (1)..(10)
<223> where n is 2.6-Diaminopurine (deoxyribofuranoside)

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 76
tcgncgtcgn 10

<210> 77
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<222> (1)..(10)
<223> where n is 2.6-Diaminopurine (deoxyribofuranoside)

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 77
tcnacntcna 10

<210> 78
<211> 10
<212> DNA
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<220>
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<220>
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<222> (1)..(10)
<223> where n is 2-Aminopurine (deoxyribofuranoside)

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 78
tcnacntcna 10

<210> 79
<211> 10
<212> DNA
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<220>
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<220>
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<223> where n is 8-Oxo-dG

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 79
tcnacntcna 10

<210> 80
<211> 10
<212> DNA
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<220>
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<220>
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<223> where n is 5NI = 5-Nitroindol

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 80
tcnacntcna 10

<210> 81
<211> 10
<212> DNA
<213> Artificial sequence

<220>
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<220>
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<223> where n is 5NI = 5-Nitroindol

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 81
tcgncgtcgn 10

<210> 82
<211> 10
<212> DNA
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<220>
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<220>
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<222> (1)..(10)
<223> where n is 5NI = 5-Nitroindol

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 82
tngangtnga 10

<210> 83
<211> 10
<212> DNA
<213> Artificial sequence

<220>
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<220>
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<222> (1)..(10)
<223> where n is 5NI = 5-Nitroindol

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 83
tcgacgncga 10

<210> 84
<211> 10
<212> DNA
<213> Artificial sequence

<220>
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<220>
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<223> where g is 6-Thiodeoxyguanosine

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 84
tcgacgtcga 10

<210> 85
<211> 10
<212> DNA
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<220>
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<220>
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<222> (1)..(1)
<223> where n is Difluorotoluyldeoxyribonucleotide

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 85
ncgacgtcga 10

<210> 86
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<223> where t is 4-thiothymidine

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 86
tcgacgtcga 10

<210> 87
<211> 10
<212> DNA
<213> Artificial sequence

<220>
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<220>
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<222> (1)..(1)
<223> where n is Pseudodeoxyuridine

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 87
ncgacgtcga 10

<210> 88
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (1)..(1)
<223> where n is 5-Hydroxymethyldeoxyuridine

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 88
ncgacgtcga 10

<210> 89
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<222> (3)..(3)
<223> where n is dSpacer (a basic residue)

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 89
tcnacgtcga 10

<210> 90
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (5)..(5)
<223> where n is dSpacer (a basic residue)

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 90
tcgangtcga 10

<210> 91
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<223> where n is dSpacer (a basic residue)

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 91
tcgacgtcna 10

<210> 92
<211> 10
<212> DNA
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<220>
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<220>
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<222> (5)..(6)
<223> where n is dSpacer (a basic residue)

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 92
tcganntcga 10

<210> 93
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<222> (1)..(1)
<223> where t is 5-Methoxythymidine

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 93
tcgacgtcga 10

<210> 94
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<222> (1)..(1)
<223> where t is 2´3´- Dideoxythymidine

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 94
tcgacgtcga 10

<210> 95
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (1)..(10)
<223> where c is Ara-cytidine (5´5´- linked)

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 95
tcgacgtcga 10

<210> 96
<211> 10
<212> DNA
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<220>
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<223> where c is 2´- Fluoro-cytidine

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 96
tcgacgtcga 10

<210> 97
<211> 10
<212> DNA
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<220>
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<220>
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<222> (10)..(10)
<223> cholesterol

<400> 97
ncgacgncga 10

<210> 98
<211> 10
<212> DNA
<213> Artificial sequence

<220>
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<220>
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<223> 2'-O-Methyl Uridine

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 98
ncgacgtcga 10

<210> 99
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<222> (1)..(10)
<223> where all residues are 2'- or 3'-O-Methyl Ribonucleotides (A, C, G)

<220>
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<222> (1)..(10)
<223> where n is 2'-O-Methyl Uridine

<220>
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<222> (10)..(10)
<223> cholesterol

<400> 99
ncgacgncga 10

<210> 100
<211> 10
<212> DNA
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<223> where n is Ribo-uridine

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 100
ncgacgtcga 10

<210> 101
<211> 10
<212> DNA
<213> Artificial sequence

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<222> (1)..(10)
<223> where n is Ribo-uridine

<220>
<221> misc_feature
<222> (1)..(10)
<223> where c, g, and a are ribonucleotides

<220>
<221> misc_feature
<222> (10)..(10)
<223> cholesterol

<400> 101
ncgacgncga 10

<210> 102
<211> 10
<212> DNA
<213> Artificial sequence

<220>
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<220>
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<223> where c, g, and a are 3'-O-Methyl-A (C, G)

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<223> cholesterol

<400> 102
ncgacgncga 10

<210> 103
<211> 24
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

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<223> n is a, c, g, or t

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<222> (24)..(24)
<223> cholesterol

<400> 103
tcgacgtcga nnnntcgacg tcga 24

<210> 104
<211> 24
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<223> Spacer 9 (triethylenglycol phosphate)

<220>
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<222> (10)..(11)
<223> 5'5' link

<220>
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<222> (12)..(14)
<223> n is a, c, g, or t

<220>
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<222> (24)..(24)
<223> cholesterol

<400> 104
agctgcagct nnnntcgacg tcga 24

<210> 105
<211> 10
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<220>
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<220>
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<223> cholesterol

<400> 105
tcaacgttga 10

<210> 106
<211> 16
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<220>
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<222> (3)..(5)
<223> where the linkages are phosphorothioate linkages

<220>
<221> misc_feature
<222> (6)..(10)
<223> where the linkages are phosphorothioate linkages

<220>
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<222> (11)..(13)
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<222> (14)..(16)
<223> where the linkages are phosphorothioate linkages

<220>
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<223> cholesterol

<400> 106

tcgtcgtttc gtcgtt 16

<210> 107
<211> 16
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
<221> misc_feature
<222> (16)..(16)
<223> cholesterol

<400> 107
tcgtcgtttc gtcgtt 16

<210> 108
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<220>
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<223> cholesterol

<400> 108
tcgtcgtcga 10

<210> 109
<211> 10
<212> DNA
<213> Artificial sequence

<220>
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<220>
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<223> cholesterol

<400> 109
tcgacgacga 10

<210> 110
<211> 10
<212> DNA

<213> Artificial sequence

<220>
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<220>
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<223> where the linkages are phosphorothioate linkages

<400> 110
acgacgtcgt 10

<210> 111
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<400> 111
tcgacgtcgt 10

<210> 112
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<400> 112
tcgacgtcga 10

<210> 113
<211> 14
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<400> 113
cgacgtcgac gtcg 14

<210> 114
<211> 12
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<400> 114

cggcggccgc cg 12

<210> 115
<211> 10
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<400> 115
gacgatcgtc 10

<210> 116
<211> 24
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic oligonucleotide

<400> 116
tcgtcgtttt gtcgttttgt cgtt 24