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**WO 2004/012745 A1**

(54) Title: ANTISENSE MODULATION OF PERILIPIN EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of perilipin. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding perilipin. Methods of using these compounds for modulation of perilipin expression and for treatment of diseases associated with expression of perilipin are provided.

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**ANTISENSE MODULATION OF PERILIPIN EXPRESSION****FIELD OF THE INVENTION**

10           The present invention provides compositions and methods for  
modulating the expression of perilipin. In particular, this  
invention relates to compounds, particularly oligonucleotides,  
specifically hybridizable with nucleic acids encoding perilipin.  
Such compounds have been shown to modulate the expression of  
15           perilipin.

**BACKGROUND OF THE INVENTION**

          All types of eukaryotic cells contain intracellular lipid  
droplets which consist of a highly hydrophobic core of  
20           triacylglycerols and/or steroyl esters, surrounded by a  
phospholipid monolayer. These lipid droplets store neutral  
lipids as an energy source or a source of membrane components  
(Zweytick et al., *Biochim. Biophys. Acta*, **2000**, 1469, 101-120).  
The lipolytic reaction in adipocytes is one of the most  
25           important reactions in the management of bodily energy reserves  
and dysregulation of this important reaction may contribute to  
the symptoms of type 2 diabetes mellitus (Londos et al., *Ann. N.*  
*Y. Acad. Sci.*, **1999**, 892, 155-168; Londos et al., *Semin. Cell*  
*Dev. Biol.*, **1999**, 10, 51-58).

30           Perilipin (also known as PLIN and Peri) is a hormonally-  
regulated phosphoprotein that coats the surface of lipid  
droplets. The participation of perilipin in lipolysis has been  
indicated by findings that this protein can protect neutral  
lipids within droplets from hydrolysis (Londos et al., *Ann. N.*  
35           *Y. Acad. Sci.*, **1999**, 892, 155-168; Londos et al., *Semin. Cell*  
*Dev. Biol.*, **1999**, 10, 51-58).

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Human perilipin was first cloned in 1998 from an adipose tissue cDNA library and mapped to chromosome 15q26 (Nishiu et al., *Genomics*, **1998**, *48*, 254-257). The gene encodes a 522-amino acid polypeptide that is 79% homologous to the previously cloned rat homolog (Greenberg et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1993**, *90*, 12035-12039; Nishiu et al., *Genomics*, **1998**, *48*, 254-257). Northern blot analysis revealed a 3.0 kb mRNA expressed in visceral adipose tissue and mammary gland (Nishiu et al., *Genomics*, **1998**, *48*, 254-257). Alternative splicing of the rat perilipin gene results in two protein isoforms which are denoted perilipin A and perilipin B (Greenberg et al., *Proc. Natl. Acad. Sci. U. S. A.*, **1993**, *90*, 12035-12039). A hypothetical human perilipin B has been identified, based on comparison to the perilipin B rat sequence and includes exons 1-8, intron 8 and exon 9.

Nucleic acid sequences encoding human perilipin are disclosed and claimed in US Patent 6,074,842 and PCT publication WO 92/22638 (Londos et al., **1992**; Londos et al., **2000**). Additionally disclosed and claimed in PCT publication WO 92/22638 are vectors containing the perilipin nucleic acid sequence and antibodies having binding affinity to perilipin (Londos et al., **1992**).

Martinez-Botas et al. have shown that targeted disruption of the perilipin gene results in healthy mice with constitutively activated fat cell hormone-sensitive lipase. Perilipin null mice consumed more food than control mice, but had normal body weight. They were much leaner and more muscular than controls, had 62% smaller white adipocytes, showed elevated basal lipolysis that was resistant to beta-adrenergic agonist stimulation, and were resistant to diet-induced obesity. The results demonstrated a role for perilipin in reducing hormone sensitive lipase activity and regulating lipolysis and suggest that inactivation of perilipin may prove useful in the development of anti-obesity medications (Martinez-Botas et al., *Nat. Genet.*, **2000**, *26*, 474-479).

Additionally, Tansey et al. have created a perilipin knockout mouse and found that homozygous null and wild type mice consumed equal amounts of food, but the adipose tissue mass in

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the null animals was reduced to approximately 30% of that in wild type animals. Isolated adipocytes of perilipin null mice exhibited elevated basal lipolysis because of the loss of the protective function of perilipin. They also exhibited

5 dramatically attenuated stimulated lipolytic activity, indicating that perilipin was required for maximal lipolytic activity. Plasma leptin concentrations in null animals were greater than expected for the reduced adipose mass. The null animals had a greater lean body mass and increased metabolic

10 rate but they also showed an increased tendency to develop glucose intolerance and peripheral insulin resistance. When fed a high-fat diet, the perilipin null animals were resistant to diet-induced obesity but not to glucose intolerance. The data demonstrated a major role for perilipin in adipose lipid

15 metabolism and suggested that perilipin may be a potential target for therapeutic intervention in pathological conditions associated with obesity (Tansey et al., *Proc. Natl. Acad. Sci. U. S. A.*, **2001**, *98*, 6494-6499).

Souza et al. have found that tumor necrosis factor-alpha regulates lipolysis by decreasing perilipin protein levels at the lipid droplet surface, a finding that suggests a possible role for perilipin and tumor necrosis factor-alpha-induced lipolysis in the pathogenesis of the obese-diabetic state (Souza et al., *J. Biol. Chem.*, **1998**, *273*, 24665-24669).

25 The perilipin gene has been identified in human specimens of ruptured atherosclerotic lesions, indicating that reduced rates of lipolysis may lead to increased lipid retention and plaque destabilization (Faber et al., *Circ. Res.*, **2001**, *89*, 547-554).

30 Currently, there are no known therapeutic agents that effectively inhibit the synthesis of perilipin. To date, investigative strategies aimed at modulating perilipin expression have involved the use of gene knock-outs in mice. Consequently, there remains a long felt need for additional

35 agents capable of effectively inhibiting perilipin function.

Antisense technology is emerging as an effective means for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of

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therapeutic, diagnostic, and research applications for the modulation of expression of perilipin.

The present invention provides compositions and methods for modulating expression of perilipin, including modulation of  
5 variants of perilipin.

#### **SUMMARY OF THE INVENTION**

The present invention is directed to compounds,  
10 particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding perilipin, and which modulate the expression of perilipin. Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of  
15 perilipin in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression  
20 of perilipin by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

#### **DETAILED DESCRIPTION OF THE INVENTION**

25 The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding perilipin, ultimately modulating the amount of perilipin produced. This is accomplished by providing antisense compounds which specifically  
30 hybridize with one or more nucleic acids encoding perilipin. As used herein, the terms "target nucleic acid" and "nucleic acid encoding perilipin" encompass DNA encoding perilipin, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of

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an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translocation of the RNA to sites within the cell which are distant from the site of RNA synthesis, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of perilipin. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding perilipin. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present

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invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding perilipin, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50

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contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. mRNA transcripts produced via the

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process of splicing of two (or more) mRNAs from different gene sources are known as "fusion transcripts". It has also been found that introns can be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

It is also known in the art that alternative RNA transcripts can be produced from the same genomic region of DNA. These alternative transcripts are generally known as "variants". More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in either their start or stop position and contain both intronic and extronic regions.

Upon excision of one or more exon or intron regions or portions thereof during splicing, pre-mRNA variants produce smaller "mRNA variants". Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA variants are also known as "alternative splice-variants". If no splicing of the pre-mRNA variant occurs then the pre-mRNA variant is identical to the mRNA variant.

It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more than one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites.

Once one or more target sites have been identified,

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oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means  
5 hydrogen bonding, which may be Watson-Crick, Hoogsteen or  
reversed Hoogsteen hydrogen bonding, between complementary  
nucleoside or nucleotide bases. For example, adenine and  
thymine are complementary nucleobases which pair through the  
formation of hydrogen bonds. "Complementary," as used herein,  
10 refers to the capacity for precise pairing between two  
nucleotides. For example, if a nucleotide at a certain position  
of an oligonucleotide is capable of hydrogen bonding with a  
nucleotide at the same position of a DNA or RNA molecule, then  
the oligonucleotide and the DNA or RNA are considered to be  
15 complementary to each other at that position. The  
oligonucleotide and the DNA or RNA are complementary to each  
other when a sufficient number of corresponding positions in  
each molecule are occupied by nucleotides which can hydrogen  
bond with each other. Thus, "specifically hybridizable" and  
20 "complementary" are terms which are used to indicate a  
sufficient degree of complementarity or precise pairing such  
that stable and specific binding occurs between the  
oligonucleotide and the DNA or RNA target. It is understood in  
the art that the sequence of an antisense compound need not be  
25 100% complementary to that of its target nucleic acid to be  
specifically hybridizable.

An antisense compound is specifically hybridizable when  
binding of the compound to the target DNA or RNA molecule  
interferes with the normal function of the target DNA or RNA to  
30 cause a loss of activity, and there is a sufficient degree of  
complementarity to avoid non-specific binding of the antisense  
compound to non-target sequences under conditions in which  
specific binding is desired, i.e., under physiological  
conditions in the case of in vivo assays or therapeutic

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treatment, and in the case of in vitro assays, under conditions in which the assays are performed. It is preferred that the antisense compounds of the present invention comprise at least 80% sequence complementarity to a target region within the target nucleic acid, moreover that they comprise 90% sequence complementarity and even more comprise 95% sequence complementarity to the target region within the target nucleic acid sequence to which they are targeted. For example, an antisense compound in which 18 of 20 nucleobases of the antisense compound are complementary, and would therefore specifically hybridize, to a target region would represent 90 percent complementarity. Percent complementarity of an antisense compound with a region of a target nucleic acid can be determined routinely using basic local alignment search tools (BLAST programs) (Altschul et al., *J. Mol. Biol.*, **1990**, 215, 403-410; Zhang and Madden, *Genome Res.*, **1997**, 7, 649-656).

Antisense and other compounds of the invention, which hybridize to the target and inhibit expression of the target, are identified through experimentation, and representative sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The sites to which these preferred antisense compounds are specifically hybridizable are hereinbelow referred to as "preferred target regions" and are therefore preferred sites for targeting. As used herein the term "preferred target region" is defined as at least an 8-nucleobase portion of a target region to which an active antisense compound is targeted. While not wishing to be bound by theory, it is presently believed that these target regions represent regions of the target nucleic acid which are accessible for hybridization.

While the specific sequences of particular preferred target regions are set forth below, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention.

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Additional preferred target regions may be identified by one having ordinary skill.

Target regions 8-80 nucleobases in length comprising a stretch of at least eight (8) consecutive nucleobases selected from within the illustrative preferred target regions are considered to be suitable preferred target regions as well.

Exemplary good preferred target regions include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative preferred target regions (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the target region and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly good preferred target regions are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred target regions (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the target region and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). One having skill in the art, once armed with the empirically-derived preferred target regions illustrated herein will be able, without undue experimentation, to identify further preferred target regions. In addition, one having ordinary skill in the art will also be able to identify additional compounds, including oligonucleotide probes and primers, that specifically hybridize to these preferred target regions using techniques available to the ordinary practitioner in the art.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various

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members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000**, 480, 17-24; Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16), SAGE (serial analysis of gene expression) (Madden, et al., *Drug Discov. Today*, **2000**, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999**, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.*, **2000**, 97, 1976-81), protein arrays and proteomics (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16; Jungblut, et al., *Electrophoresis*, **1999**, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16; Larsson, et al., *J. Biotechnol.*, **2000**, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, et al., *Anal. Biochem.*, **2000**, 286, 91-98; Larson, et al., *Cytometry*, **2000**, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and

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Belmont, *Curr. Opin. Microbiol.*, **2000**, 3, 316-21), comparative genomic hybridization (Carulli, et al., *J. Cell Biochem. Suppl.*, **1998**, 31, 286-96), FISH (fluorescent *in situ* hybridization) techniques (Going and Gusterson, *Eur. J. Cancer*, **1999**, 35, 1895-5 904) and mass spectrometry methods (reviewed in To, *Comb. Chem. High Throughput Screen*, **2000**, 3, 235-41).

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic 10 moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that 15 can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or 20 mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are 25 often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form 30 of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 80 nucleobases (i.e. from about 8

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to about 80 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides from about 8 to about 50 nucleobases, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds  
5 include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

Antisense compounds 8-80 nucleobases in length comprising a  
10 stretch of at least eight (8) consecutive nucleobases selected from within the illustrative antisense compounds are considered to be suitable antisense compounds as well.

Exemplary preferred antisense compounds include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases  
15 from the 5'-terminus of one of the illustrative preferred antisense compounds (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and  
20 continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly preferred antisense compounds are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred antisense compounds (the remaining  
25 nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). One having skill in the art,  
30 once armed with the empirically-derived preferred antisense compounds illustrated herein will be able, without undue experimentation, to identify further preferred antisense compounds.

Antisense and other compounds of the invention, which hybridize

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to the target and inhibit expression of the target, are identified through experimentation, and representative sequences of these compounds are herein identified as preferred embodiments of the invention. While specific sequences of the antisense compounds are set forth herein, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention. Additional preferred antisense compounds may be identified by one having ordinary skill.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. In addition, linear structures may also have internal nucleobase complementarity and may therefore fold in a manner as to produce a double stranded structure. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the

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backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone  
5 can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates,  
10 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked  
15 analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside  
20 residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include,  
25 but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799;  
30 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not

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include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or  
5 heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl  
10 backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts.

Representative United States patents that teach the  
15 preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240;  
20 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar  
25 and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent  
30 hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza

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nitrogen atoms of the amide portion of the backbone.

Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.:

5 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et al.*, *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular -  
10  $\text{CH}_2\text{-NH-O-CH}_2\text{-}$ ,  $\text{-CH}_2\text{-N(CH}_3\text{)-O-CH}_2\text{-}$  [known as a methylene (methylimino) or MMI backbone],  $\text{-CH}_2\text{-O-N(CH}_3\text{)-CH}_2\text{-}$ ,  $\text{-CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2\text{-}$  and  $\text{-O-N(CH}_3\text{)-CH}_2\text{-CH}_2\text{-}$  [wherein the native phosphodiester backbone is represented as  $\text{-O-P-O-CH}_2\text{-}$ ] of the above referenced U.S. patent 5,489,677, and the amide backbones  
15 of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise  
20 one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C<sub>1</sub> to C<sub>10</sub> alkyl or C<sub>2</sub> to C<sub>10</sub> alkenyl and alkynyl. Particularly preferred are  $\text{O}[(\text{CH}_2)_n\text{O}]_m\text{CH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{OCH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{NH}_2$ ,  
25  $\text{O}(\text{CH}_2)_n\text{CH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{ONH}_2$ , and  $\text{O}(\text{CH}_2)_n\text{ON}[(\text{CH}_2)_n\text{CH}_3]_2$ , where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C<sub>1</sub> to C<sub>10</sub> lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl,  
aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>,  
30 OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic

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properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, **1995**, 5 78, 486-504) *i.e.*, an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxy, *i.e.*, a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-  
10 DMAEOE), *i.e.*, 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>3</sub>)<sub>2</sub>, also described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), 2'-allyl (2'-CH<sub>2</sub>-CH=CH<sub>2</sub>), 2'-O-allyl (2'-O-CH<sub>2</sub>-CH=CH<sub>2</sub>) and 2'-fluoro (2'-F). The 2'-  
15 modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides  
20 and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957;  
25 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant  
30 application, and each of which is herein incorporated by reference in its entirety.

A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic

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sugar moiety. The linkage is preferably a methylene (-CH<sub>2</sub>)<sub>n</sub> group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

5 Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and  
10 uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-  
15 thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH<sub>3</sub>) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted  
20 adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further  
25 modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-  
30 b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or

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pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, 5 Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. 10 and Lebleu, B. , ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5- 15 propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base 20 substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not 25 limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 30 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated

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by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem. Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO*

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*J.*, **1991**, *10*, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al., *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate  
5 (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654), a  
10 palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937). Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin,  
15 warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepam, indomethacin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an  
20 antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the  
25 preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718;  
30 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241,

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5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667;  
5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481;  
5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and  
5,688,941, certain of which are commonly owned with the instant  
5 application, and each of which is herein incorporated by  
reference.

It is not necessary for all positions in a given compound  
to be uniformly modified, and in fact more than one of the  
aforementioned modifications may be incorporated in a single  
10 compound or even at a single nucleoside within an  
oligonucleotide. The present invention also includes antisense  
compounds which are chimeric compounds. "Chimeric" antisense  
compounds or "chimeras," in the context of this invention, are  
antisense compounds, particularly oligonucleotides, which  
15 contain two or more chemically distinct regions, each made up of  
at least one monomer unit, i.e., a nucleotide in the case of an  
oligonucleotide compound. These oligonucleotides typically  
contain at least one region wherein the oligonucleotide is  
modified so as to confer upon the oligonucleotide increased  
20 resistance to nuclease degradation, increased cellular uptake,  
increased stability and/or increased binding affinity for the  
target nucleic acid. An additional region of the  
oligonucleotide may serve as a substrate for enzymes capable of  
cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H  
25 is a cellular endonuclease which cleaves the RNA strand of an  
RNA:DNA duplex. Activation of RNase H, therefore, results in  
cleavage of the RNA target, thereby greatly enhancing the  
efficiency of oligonucleotide inhibition of gene expression.  
The cleavage of RNA:RNA hybrids can, in like fashion, be  
30 accomplished through the actions of endoribonucleases, such as  
interferon-induced RNaseL which cleaves both cellular and viral  
RNA. Consequently, comparable results can often be obtained  
with shorter oligonucleotides when chimeric oligonucleotides are  
used, compared to phosphorothioate deoxyoligonucleotides

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hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

5 Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers.

10 Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned

15 with the instant application, and each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for

20 such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the

25 phosphorothioates and alkylated derivatives.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor-targeted molecules, oral, rectal,

30 topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption-assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127;

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5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721;  
4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804;  
5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016;  
5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528;  
5 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each  
of which is herein incorporated by reference.

The antisense compounds of the invention encompass any  
pharmaceutically acceptable salts, esters, or salts of such  
esters, or any other compound which, upon administration to an  
10 animal, including a human, is capable of providing (directly or  
indirectly) the biologically active metabolite or residue  
thereof. Accordingly, for example, the disclosure is also drawn  
to prodrugs and pharmaceutically acceptable salts of the  
compounds of the invention, pharmaceutically acceptable salts of  
15 such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is  
prepared in an inactive form that is converted to an active form  
(i.e., drug) within the body or cells thereof by the action of  
endogenous enzymes or other chemicals and/or conditions. In  
20 particular, prodrug versions of the oligonucleotides of the  
invention are prepared as SATE [(S-acetyl-2-thioethyl)  
phosphate] derivatives according to the methods disclosed in WO  
93/24510 to Gosselin et al., published December 9, 1993 or in WO  
94/26764 and U.S. 5,770,713 to Imbach et al.

25 The term "pharmaceutically acceptable salts" refers to  
physiologically and pharmaceutically acceptable salts of the  
compounds of the invention: i.e., salts that retain the desired  
biological activity of the parent compound and do not impart  
undesired toxicological effects thereto.

30 Pharmaceutically acceptable base addition salts are formed  
with metals or amines, such as alkali and alkaline earth metals  
or organic amines. Examples of metals used as cations are  
sodium, potassium, magnesium, calcium, and the like. Examples  
of suitable amines are N,N'-dibenzylethylenediamine,

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chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge *et al.*, "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid,

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and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, 5 naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically 10 acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of 15 pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts 20 formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, 25 polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be 30 utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of perilipin is treated by administering antisense compounds in accordance

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with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding perilipin, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding perilipin can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of perilipin in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

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Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C<sub>1-10</sub> alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-

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aqueous media, capsules, gel capsules, sachets, tablets or minitabets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Preferred fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pullulans, celluloses and starches. Particularly preferred

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complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate),  
5 poly(ethylcyanoacrylate), poly(butylcyanoacrylate),  
poly(isobutylcyanoacrylate), poly(isohexylcyanoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate,  
10 poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA),  
alginate, and polyethyleneglycol (PEG). Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 08/886,829 (filed July 1, 1997), 09/108,673 (filed July 1, 1998), 09/256,515 (filed February 23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298 (filed  
15 May 20, 1999), each of which is incorporated herein by reference in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other  
20 suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and  
25 liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention,  
30 which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the

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formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

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

          In one embodiment of the present invention the  
15           pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product.  
20           The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

## 25   Emulsions

          The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1  $\mu\text{m}$  in diameter (Idson, in  
30           *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y.,

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Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, 5 p. 301). Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets 10 into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional 15 components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed.

20 Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not.

25 Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion.

30 Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the

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emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may  
5 broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

10 Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p.  
15 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., **1988**, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has  
20 been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in  
25 *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin  
30 and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with

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surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal  
5 magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty  
10 acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger  
15 and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

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

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often  
30 incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration

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of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear

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dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., **1989**, VCH Publishers, New York, pages 185-215).

5 Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and

10 on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 271).

The phenomenological approach utilizing phase diagrams has

15 been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical*

20 *Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

25 Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate

30 (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprinate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-

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propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, **1993**, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385; Ho et al., *J. Pharm. Sci.*, **1996**, *85*, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal

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delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

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

#### Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be

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delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

5 In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

10 Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff,  
15 in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

20 Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes and as the merging of the liposome  
25 and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes  
30 present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to

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administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

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

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, **1992**, 19, 269-274).

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

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such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, **1992**, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., *Antiviral Research*, **1992**, 18, 259-265).

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

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the

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vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G<sub>M1</sub>, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound  
5 by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the  
10 reticuloendothelial system (RES) (Allen *et al.*, *FEBS Letters*, **1987**, 223, 42; Wu *et al.*, *Cancer Research*, **1993**, 53, 3765).

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

25 Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (*Bull. Chem. Soc. Jpn.*, **1980**, 53, 2778) described liposomes comprising a nonionic detergent, 2C<sub>12</sub>15G, that contains a PEG moiety. Illum *et al.*  
30 (*FEBS Lett.*, **1984**, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S.

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Patent Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (*FEBS Lett.*, **1990**, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood  
5 circulation half-lives. Blume *et al.* (*Biochimica et Biophysica Acta*, **1990**, 1029, 91) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their  
10 external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S.  
15 Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described  
20 in WO 96/10391 (Choi *et al.*). U.S. Patent Nos. 5,540,935 (Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are  
25 known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No.  
30 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

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

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

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

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ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

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

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

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

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

#### Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell

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membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

5 Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92). Each of the above mentioned classes of penetration  
10 enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface  
15 tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate,  
20 polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, *J. Pharm. Pharmacol.*, **1988**, 40, 252).

Fatty acids: Various fatty acids and their derivatives  
25 which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-  
30 monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C<sub>1-10</sub> alkyl esters thereof (*e.g.*, methyl, isopropyl and *t*-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier*

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

5 Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural  
10 bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically  
15 acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid  
20 (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92;  
25 Swinyard, Chapter 39 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; Yamamoto et al., *J. Pharm. Exp. Ther.*, 1992, 263, 25; Yamashita et al., *J. Pharm. Sci.*, 1990,  
30 79, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes

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therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, **1993**, 618, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; Buur et al., *J. Control Rel.*, **1990**, 14, 43-51).

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

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic

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molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

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

### Carriers

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

### Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable

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

Pharmaceutically acceptable organic or inorganic excipient  
20 suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene  
25 glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-  
30 aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or

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inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

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

#### 10 Other Components

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

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

Certain embodiments of the invention provide pharmaceutical

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compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, 5 dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, 10 pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramidate, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, 15 taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. **1987**, pp. 1206-20 1228, Berkow et al., eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g., 5-FU and oligonucleotide), sequentially (e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more 25 other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, 30 vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., **1987**, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope

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of this invention. Two or more combined compounds may be used together or sequentially.

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

10 The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC<sub>50</sub>s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with

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specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

## 5 **EXAMPLES**

### **Example 1**

#### **Nucleoside Phosphoramidites for Oligonucleotide Synthesis Deoxy and 2'-alkoxy amidites**

10 2'-Deoxy and 2'-methoxy beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by  
15 reference. For oligonucleotides synthesized using 2'-alkoxy amidites, optimized synthesis cycles were developed that incorporate multiple steps coupling longer wait times relative to standard synthesis cycles.

The following abbreviations are used in the text: thin  
20 layer chromatography (TLC), melting point (MP), high pressure liquid chromatography (HPLC), Nuclear Magnetic Resonance (NMR), argon (Ar), methanol (MeOH), dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), triethylamine (TEA), dimethyl formamide (DMF), ethyl acetate (EtOAc), dimethyl sulfoxide (DMSO), tetrahydrofuran (THF).

25 Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-dC) nucleotides were synthesized according to published methods (Sanghvi, et. al., *Nucleic Acids Research*, **1993**, *21*, 3197-3203) using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA) or prepared as  
30 follows:

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**Preparation of 5'-O-Dimethoxytrityl-thymidine intermediate for 5-methyl dC amidite**

To a 50 L glass reactor equipped with air stirrer and Ar gas line was added thymidine (1.00 kg, 4.13 mol) in anhydrous pyridine (6 L) at ambient temperature. Dimethoxytrityl (DMT) chloride (1.47 kg, 4.34 mol, 1.05 eq) was added as a solid in four portions over 1 h. After 30 min, TLC indicated approx. 95% product, 2% thymidine, 5% DMT reagent and by-products and 2% 3',5'-bis DMT product ( $R_f$  in EtOAc 0.45, 0.05, 0.98, 0.95 respectively). Saturated sodium bicarbonate (4 L) and  $\text{CH}_2\text{Cl}_2$  were added with stirring (pH of the aqueous layer 7.5). An additional 18 L of water was added, the mixture was stirred, the phases were separated, and the organic layer was transferred to a second 50 L vessel. The aqueous layer was extracted with additional  $\text{CH}_2\text{Cl}_2$  (2 x 2 L). The combined organic layer was washed with water (10 L) and then concentrated in a rotary evaporator to approx. 3.6 kg total weight. This was redissolved in  $\text{CH}_2\text{Cl}_2$  (3.5 L), added to the reactor followed by water (6 L) and hexanes (13 L). The mixture was vigorously stirred and seeded to give a fine white suspended solid starting at the interface. After stirring for 1 h, the suspension was removed by suction through a 1/2" diameter teflon tube into a 20 L suction flask, poured onto a 25 cm Coors Buchner funnel, washed with water (2 x 3 L) and a mixture of hexanes-  $\text{CH}_2\text{Cl}_2$  (4:1, 2x3 L) and allowed to air dry overnight in pans (1" deep). This was further dried in a vacuum oven (75°C, 0.1 mm Hg, 48 h) to a constant weight of 2072 g (93%) of a white solid, (mp 122-124°C). TLC indicated a trace contamination of the bis DMT product. NMR spectroscopy also indicated that 1-2 mole percent pyridine and about 5 mole percent of hexanes was still present.

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**Preparation of 5'-O-Dimethoxytrityl-2'-deoxy-5-methylcytidine intermediate for 5-methyl-dC amidite**

To a 50 L Schott glass-lined steel reactor equipped with an electric stirrer, reagent addition pump (connected to an addition funnel), heating/cooling system, internal thermometer and an Ar gas line was added 5'-O-dimethoxytrityl-thymidine (3.00 kg, 5.51 mol), anhydrous acetonitrile (25 L) and TEA (12.3 L, 88.4 mol, 16 eq). The mixture was chilled with stirring to -10°C internal temperature (external -20°C).

Trimethylsilylchloride (2.1 L, 16.5 mol, 3.0 eq) was added over 30 minutes while maintaining the internal temperature below -5°C, followed by a wash of anhydrous acetonitrile (1 L). Note: the reaction is mildly exothermic and copious hydrochloric acid fumes form over the course of the addition. The reaction was allowed to warm to 0°C and the reaction progress was confirmed by TLC (EtOAc-hexanes 4:1;  $R_f$  0.43 to 0.84 of starting material and silyl product, respectively). Upon completion, triazole (3.05 kg, 44 mol, 8.0 eq) was added the reaction was cooled to -20°C internal temperature (external -30°C). Phosphorous oxychloride (1035 mL, 11.1 mol, 2.01 eq) was added over 60 min so as to maintain the temperature between -20°C and -10°C during the strongly exothermic process, followed by a wash of anhydrous acetonitrile (1 L). The reaction was warmed to 0 °C and stirred for 1 h. TLC indicated a complete conversion to the triazole product ( $R_f$  0.83 to 0.34 with the product spot glowing in long wavelength UV light). The reaction mixture was a peach-colored thick suspension, which turned darker red upon warming without apparent decomposition. The reaction was cooled to -15°C internal temperature and water (5 L) was slowly added at a rate to maintain the temperature below +10°C in order to quench the reaction and to form a homogenous solution. (Caution: this reaction is initially very strongly exothermic). Approximately one-half of the reaction volume (22 L) was transferred by air pump to another vessel, diluted with EtOAc (12 L) and extracted

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with water (2 x 8 L). The combined water layers were back-extracted with EtOAc (6 L). The water layer was discarded and the organic layers were concentrated in a 20 L rotary evaporator to an oily foam. The foam was coevaporated with anhydrous  
5 acetonitrile (4 L) to remove EtOAc. (note: dioxane may be used instead of anhydrous acetonitrile if dried to a hard foam). The second half of the reaction was treated in the same way. Each residue was dissolved in dioxane (3 L) and concentrated ammonium hydroxide (750 mL) was added. A homogenous solution formed in a  
10 few minutes and the reaction was allowed to stand overnight (although the reaction is complete within 1 h).

TLC indicated a complete reaction (product  $R_f$  0.35 in EtOAc-MeOH 4:1). The reaction solution was concentrated on a rotary evaporator to a dense foam. Each foam was slowly redissolved in  
15 warm EtOAc (4 L; 50°C), combined in a 50 L glass reactor vessel, and extracted with water (2 x 4L) to remove the triazole by-product. The water was back-extracted with EtOAc (2 L). The organic layers were combined and concentrated to about 8 kg total weight, cooled to 0°C and seeded with crystalline product.  
20 After 24 hours, the first crop was collected on a 25 cm Coors Buchner funnel and washed repeatedly with EtOAc (3 x 3L) until a white powder was left and then washed with ethyl ether (2 x 3L). The solid was put in pans (1" deep) and allowed to air dry overnight. The filtrate was concentrated to an oil, then  
25 redissolved in EtOAc (2 L), cooled and seeded as before. The second crop was collected and washed as before (with proportional solvents) and the filtrate was first extracted with water (2 x 1L) and then concentrated to an oil. The residue was dissolved in EtOAc (1 L) and yielded a third crop which was  
30 treated as above except that more washing was required to remove a yellow oily layer.

After air-drying, the three crops were dried in a vacuum oven (50°C, 0.1 mm Hg, 24 h) to a constant weight (1750, 600 and 200 g, respectively) and combined to afford 2550 g (85%) of a

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white crystalline product (MP 215-217°C) when TLC and NMR spectroscopy indicated purity. The mother liquor still contained mostly product (as determined by TLC) and a small amount of triazole (as determined by NMR spectroscopy), bis DMT product and unidentified minor impurities. If desired, the mother liquor can be purified by silica gel chromatography using a gradient of MeOH (0-25%) in EtOAc to further increase the yield.

10 **Preparation of 5'-O-Dimethoxytrityl-2'-deoxy-N4-benzoyl-5-methylcytidine penultimate intermediate for 5-methyl dC amidite**

Crystalline 5'-O-dimethoxytrityl-5-methyl-2'-deoxycytidine (2000 g, 3.68 mol) was dissolved in anhydrous DMF (6.0 kg) at ambient temperature in a 50 L glass reactor vessel equipped with an air stirrer and argon line. Benzoic anhydride (Chem Impex not Aldrich, 874 g, 3.86 mol, 1.05 eq) was added and the reaction was stirred at ambient temperature for 8 h. TLC (CH<sub>2</sub>Cl<sub>2</sub>-EtOAc; CH<sub>2</sub>Cl<sub>2</sub>-EtOAc 4:1; R<sub>f</sub> 0.25) indicated approx. 92% complete reaction. An additional amount of benzoic anhydride (44 g, 0.19 mol) was added. After a total of 18 h, TLC indicated approx. 96% reaction completion. The solution was diluted with EtOAc (20 L), TEA (1020 mL, 7.36 mol, ca 2.0 eq) was added with stirring, and the mixture was extracted with water (15 L, then 2 x 10 L). The aqueous layer was removed (no back-extraction was needed) and the organic layer was concentrated in 2 x 20 L rotary evaporator flasks until a foam began to form. The residues were coevaporated with acetonitrile (1.5 L each) and dried (0.1 mm Hg, 25°C, 24 h) to 2520 g of a dense foam. High pressure liquid chromatography (HPLC) revealed a contamination of 6.3% of N4, 3'-O-dibenzoyl product, but very little other impurities.

The product was purified by Biotage column chromatography (5 kg Biotage) prepared with 65:35:1 hexanes-EtOAc-TEA (4L). The crude product (800 g), dissolved in CH<sub>2</sub>Cl<sub>2</sub> (2 L), was applied

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to the column. The column was washed with the 65:35:1 solvent mixture (20 kg), then 20:80:1 solvent mixture (10 kg), then 99:1 EtOAc:TEA (17kg). The fractions containing the product were collected, and any fractions containing the product and  
5 impurities were retained to be resubjected to column chromatography. The column was re-equilibrated with the original 65:35:1 solvent mixture (17 kg). A second batch of crude product (840 g) was applied to the column as before. The column was washed with the following solvent gradients: 65:35:1  
10 (9 kg), 55:45:1 (20 kg), 20:80:1 (10 kg), and 99:1 EtOAc:TEA (15 kg). The column was reequilibrated as above, and a third batch of the crude product (850 g) plus impure fractions recycled from the two previous columns (28 g) was purified following the procedure for the second batch. The fractions containing pure  
15 product combined and concentrated on a 20L rotary evaporator, co-evaporated with acetontirile (3 L) and dried (0.1 mm Hg, 48 h, 25°C) to a constant weight of 2023 g (85%) of white foam and 20 g of slightly contaminated product from the third run. HPLC indicated a purity of 99.8% with the balance as the diBenzoyl  
20 product.

**[5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-deoxy-N<sup>4</sup>-benzoyl-5-methylcytidin-3'-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (5-methyl dC amidite)**

25 5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-deoxy-N<sup>4</sup>-benzoyl-5-methylcytidine (998 g, 1.5 mol) was dissolved in anhydrous DMF (2 L). The solution was co-evaporated with toluene (300 ml) at 50°C under reduced pressure, then cooled to room temperature and 2-cyanoethyl tetraisopropylphosphorodiamidite (680 g, 2.26 mol)  
30 and tetrazole (52.5 g, 0.75 mol) were added. The mixture was shaken until all tetrazole was dissolved, N-methylimidazole (15 ml) was added and the mixture was left at room temperature for 5 hours. TEA (300 ml) was added, the mixture was diluted with DMF (2.5 L) and water (600 ml), and extracted with hexane (3 x 3

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L). The mixture was diluted with water (1.2 L) and extracted with a mixture of toluene (7.5 L) and hexane (6 L). The two layers were separated, the upper layer was washed with DMF-water (7:3 v/v, 3 x 2 L) and water (3 x 2 L), and the phases were  
5 separated. The organic layer was dried ( $\text{Na}_2\text{SO}_4$ ), filtered and rotary evaporated. The residue was co-evaporated with acetonitrile (2 x 2 L) under reduced pressure and dried to a constant weight (25 °C, 0.1mm Hg, 40 h) to afford 1250 g an off-white foam solid (96%).

10

## 2'-Fluoro amidites

### 2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, 36, 831-841]  
15 and United States patent 5,670,633, herein incorporated by reference. The preparation of 2'-fluoropyrimidines containing a 5-methyl substitution are described in US Patent 5,861,493. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available  
20 9-beta-D-arabinofuranosyladenine as starting material and whereby the 2'-alpha-fluoro atom is introduced by a  $\text{S}_{\text{N}}2$ -displacement of a 2'-beta-triflate group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP)  
25 intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

30

### 2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate isobutyryl-

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arabinofuranosylguanosine. Alternatively, isobutyryl-  
arabinofuranosylguanosine was prepared as described by Ross *et al.*, (Nucleosides & Nucleosides, 16, 1645, 1997). Deprotection  
of the TPDS group was followed by protection of the hydroxyl  
5 group with THP to give isobutyryl di-THP protected  
arabinofuranosylguanine. Selective O-deacylation and triflation  
was followed by treatment of the crude product with fluoride,  
then deprotection of the THP groups. Standard methodologies  
were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

10

### **2'-Fluorouridine**

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by  
the modification of a literature procedure in which 2,2'-  
anhydro-1-beta-D-arabinofuranosyluracil was treated with 70%  
15 hydrogen fluoride-pyridine. Standard procedures were used to  
obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

### **2'-Fluorodeoxycytidine**

2'-deoxy-2'-fluorocytidine was synthesized via amination of  
20 2'-deoxy-2'-fluorouridine, followed by selective protection to  
give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures  
were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

### **2'-O-(2-Methoxyethyl) modified amidites**

25 2'-O-Methoxyethyl-substituted nucleoside amidites  
(otherwise known as MOE amidites) are prepared as follows, or  
alternatively, as per the methods of Martin, P., (Helvetica  
Chimica Acta, 1995, 78, 486-504).

### **30 Preparation of 2'-O-(2-methoxyethyl)-5-methyluridine intermediate**

2,2'-Anhydro-5-methyl-uridine (2000 g, 8.32 mol), tris(2-  
methoxyethyl)borate (2504 g, 10.60 mol), sodium bicarbonate (60  
g, 0.70 mol) and anhydrous 2-methoxyethanol (5 L) were combined

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in a 12 L three necked flask and heated to 130 °C (internal temp) at atmospheric pressure, under an argon atmosphere with stirring for 21 h. TLC indicated a complete reaction. The solvent was removed under reduced pressure until a sticky gum formed (50-  
5 85°C bath temp and 100-11 mm Hg) and the residue was redissolved in water (3 L) and heated to boiling for 30 min in order the hydrolyze the borate esters. The water was removed under reduced pressure until a foam began to form and then the process was repeated. HPLC indicated about 77% product, 15% dimer (5'  
10 of product attached to 2' of starting material) and unknown derivatives, and the balance was a single unresolved early eluting peak.

The gum was redissolved in brine (3 L), and the flask was rinsed with additional brine (3 L). The combined aqueous  
15 solutions were extracted with chloroform (20 L) in a heavier-than continuous extractor for 70 h. The chloroform layer was concentrated by rotary evaporation in a 20 L flask to a sticky foam (2400 g). This was coevaporated with MeOH (400 mL) and EtOAc (8 L) at 75°C and 0.65 atm until the foam dissolved at  
20 which point the vacuum was lowered to about 0.5 atm. After 2.5 L of distillate was collected a precipitate began to form and the flask was removed from the rotary evaporator and stirred until the suspension reached ambient temperature. EtOAc (2 L) was added and the slurry was filtered on a 25 cm table top  
25 Buchner funnel and the product was washed with EtOAc (3 x 2 L). The bright white solid was air dried in pans for 24 h then further dried in a vacuum oven (50°C, 0.1 mm Hg, 24 h) to afford 1649 g of a white crystalline solid (mp 115.5-116.5°C).

The brine layer in the 20 L continuous extractor was  
30 further extracted for 72 h with recycled chloroform. The chloroform was concentrated to 120 g of oil and this was combined with the mother liquor from the above filtration (225 g), dissolved in brine (250 mL) and extracted once with chloroform (250 mL). The brine solution was continuously

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extracted and the product was crystallized as described above to afford an additional 178 g of crystalline product containing about 2% of thymine. The combined yield was 1827 g (69.4%). HPLC indicated about 99.5% purity with the balance being the dimer.

**Preparation of 5'-O-DMT-2'-O-(2-methoxyethyl)-5-methyluridine penultimate intermediate**

In a 50 L glass-lined steel reactor, 2'-O-(2-methoxyethyl)-5-methyl-uridine (MOE-T, 1500 g, 4.738 mol), lutidine (1015 g, 9.476 mol) were dissolved in anhydrous acetonitrile (15 L). The solution was stirred rapidly and chilled to -10°C (internal temperature). Dimethoxytriphenylmethyl chloride (1765.7 g, 5.21 mol) was added as a solid in one portion. The reaction was allowed to warm to -2°C over 1 h. (Note: The reaction was monitored closely by TLC (EtOAc) to determine when to stop the reaction so as to not generate the undesired bis-DMT substituted side product). The reaction was allowed to warm from -2 to 3°C over 25 min. then quenched by adding MeOH (300 mL) followed after 10 min by toluene (16 L) and water (16 L). The solution was transferred to a clear 50 L vessel with a bottom outlet, vigorously stirred for 1 minute, and the layers separated. The aqueous layer was removed and the organic layer was washed successively with 10% aqueous citric acid (8 L) and water (12 L). The product was then extracted into the aqueous phase by washing the toluene solution with aqueous sodium hydroxide (0.5N, 16 L and 8 L). The combined aqueous layer was overlaid with toluene (12 L) and solid citric acid (8 moles, 1270 g) was added with vigorous stirring to lower the pH of the aqueous layer to 5.5 and extract the product into the toluene. The organic layer was washed with water (10 L) and TLC of the organic layer indicated a trace of DMT-O-Me, bis DMT and dimer DMT.

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The toluene solution was applied to a silica gel column (6 L sintered glass funnel containing approx. 2 kg of silica gel slurried with toluene (2 L) and TEA(25 mL)) and the fractions were eluted with toluene (12 L) and EtOAc (3 x 4 L) using vacuum applied to a filter flask placed below the column. The first EtOAc fraction containing both the desired product and impurities were resubjected to column chromatography as above. The clean fractions were combined, rotary evaporated to a foam, coevaporated with acetonitrile (6 L) and dried in a vacuum oven (0.1 mm Hg, 40 h, 40°C) to afford 2850 g of a white crisp foam. NMR spectroscopy indicated a 0.25 mole % remainder of acetonitrile (calculates to be approx. 47 g) to give a true dry weight of 2803 g (96%). HPLC indicated that the product was 99.41% pure, with the remainder being 0.06 DMT-O-Me, 0.10 unknown, 0.44 bis DMT, and no detectable dimer DMT or 3'-O-DMT.

**Preparation of [5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-5-methyluridin-3'-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (MOE T amidite)**

5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-5-methyluridine (1237 g, 2.0 mol) was dissolved in anhydrous DMF (2.5 L). The solution was co-evaporated with toluene (200 ml) at 50°C under reduced pressure, then cooled to room temperature and 2-cyanoethyl tetraisopropylphosphorodiamidite (900 g, 3.0 mol) and tetrazole (70 g, 1.0 mol) were added. The mixture was shaken until all tetrazole was dissolved, N-methylimidazole (20 ml) was added and the solution was left at room temperature for 5 hours. TEA (300 ml) was added, the mixture was diluted with DMF (3.5 L) and water (600 ml) and extracted with hexane (3 x 3L). The mixture was diluted with water (1.6 L) and extracted with the mixture of toluene (12 L) and hexanes (9 L). The upper layer was washed with DMF-water (7:3 v/v, 3x3 L) and water (3x3 L). The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated. The residue was co-evaporated with acetonitrile (2

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x 2 L) under reduced pressure and dried in a vacuum oven (25°C, 0.1mm Hg, 40 h) to afford 1526 g of an off-white foamy solid (95%).

5 **Preparation of 5'-O-Dimethoxytrityl-2'-O-(2-methoxyethyl)-5-methylcytidine intermediate**

To a 50 L Schott glass-lined steel reactor equipped with an electric stirrer, reagent addition pump (connected to an addition funnel), heating/cooling system, internal thermometer  
10 and argon gas line was added 5'-O-dimethoxytrityl-2'-O-(2-methoxyethyl)-5-methyl-uridine (2.616 kg, 4.23 mol, purified by base extraction only and no scrub column), anhydrous acetonitrile (20 L), and TEA (9.5 L, 67.7 mol, 16 eq). The mixture was chilled with stirring to -10°C internal temperature  
15 (external -20°C). Trimethylsilylchloride (1.60 L, 12.7 mol, 3.0 eq) was added over 30 min. while maintaining the internal temperature below -5°C, followed by a wash of anhydrous acetonitrile (1 L). (Note: the reaction is mildly exothermic and copious hydrochloric acid fumes form over the course of the  
20 addition). The reaction was allowed to warm to 0°C and the reaction progress was confirmed by TLC (EtOAc, R<sub>f</sub> 0.68 and 0.87 for starting material and silyl product, respectively). Upon completion, triazole (2.34 kg, 33.8 mol, 8.0 eq) was added the reaction was cooled to -20°C internal temperature (external -  
25 30°C). Phosphorous oxychloride (793 mL, 8.51 mol, 2.01 eq) was added slowly over 60 min so as to maintain the temperature between -20°C and -10°C (note: strongly exothermic), followed by a wash of anhydrous acetonitrile (1 L). The reaction was warmed to 0°C and stirred for 1 h, at which point it was an off-white  
30 thick suspension. TLC indicated a complete conversion to the triazole product (EtOAc, R<sub>f</sub> 0.87 to 0.75 with the product spot glowing in long wavelength UV light). The reaction was cooled to -15°C and water (5 L) was slowly added at a rate to maintain the temperature below +10°C in order to quench the reaction and

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to form a homogenous solution. (Caution: this reaction is initially very strongly exothermic). Approximately one-half of the reaction volume (22 L) was transferred by air pump to another vessel, diluted with EtOAc (12 L) and extracted with water (2 x 8 L). The second half of the reaction was treated in the same way. The combined aqueous layers were back-extracted with EtOAc (8 L) The organic layers were combined and concentrated in a 20 L rotary evaporator to an oily foam. The foam was coevaporated with anhydrous acetonitrile (4 L) to remove EtOAc. (note: dioxane may be used instead of anhydrous acetonitrile if dried to a hard foam). The residue was dissolved in dioxane (2 L) and concentrated ammonium hydroxide (750 mL) was added. A homogenous solution formed in a few minutes and the reaction was allowed to stand overnight

TLC indicated a complete reaction ( $\text{CH}_2\text{Cl}_2$ -acetone-MeOH, 20:5:3,  $R_f$  0.51). The reaction solution was concentrated on a rotary evaporator to a dense foam and slowly redissolved in warm  $\text{CH}_2\text{Cl}_2$  (4 L, 40°C) and transferred to a 20 L glass extraction vessel equipped with a air-powered stirrer. The organic layer was extracted with water (2 x 6 L) to remove the triazole by-product. (Note: In the first extraction an emulsion formed which took about 2 h to resolve). The water layer was back-extracted with  $\text{CH}_2\text{Cl}_2$  (2 x 2 L), which in turn was washed with water (3 L). The combined organic layer was concentrated in 2 x 20 L flasks to a gum and then recrystallized from EtOAc seeded with crystalline product. After sitting overnight, the first crop was collected on a 25 cm Coors Buchner funnel and washed repeatedly with EtOAc until a white free-flowing powder was left (about 3 x 3 L). The filtrate was concentrated to an oil recrystallized from EtOAc, and collected as above. The solid was air-dried in pans for 48 h, then further dried in a vacuum oven (50°C, 0.1mm Hg, 17 h) to afford 2248 g of a bright white, dense solid (86%). An HPLC analysis indicated both crops to be

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99.4% pure and NMR spectroscopy indicated only a faint trace of EtOAc remained.

**Preparation of 5'-O-dimethoxytrityl-2'-O-(2-methoxyethyl)-N4-benzoyl-5-methyl-cytidine penultimate intermediate:**

5 Crystalline 5'-O-dimethoxytrityl-2'-O-(2-methoxyethyl)-5-methyl-cytidine (1000 g, 1.62 mol) was suspended in anhydrous DMF (3 kg) at ambient temperature and stirred under an Ar atmosphere. Benzoic anhydride (439.3 g, 1.94 mol) was added in one portion. The solution clarified after 5 hours and was  
10 stirred for 16 h. HPLC indicated 0.45% starting material remained (as well as 0.32% N4, 3'-O-bis Benzoyl). An additional amount of benzoic anhydride (6.0 g, 0.0265 mol) was added and after 17 h, HPLC indicated no starting material was present.  
15 TEA (450 mL, 3.24 mol) and toluene (6 L) were added with stirring for 1 minute. The solution was washed with water (4 x 4 L), and brine (2 x 4 L). The organic layer was partially evaporated on a 20 L rotary evaporator to remove 4 L of toluene and traces of water. HPLC indicated that the bis benzoyl side  
20 product was present as a 6% impurity. The residue was diluted with toluene (7 L) and anhydrous DMSO (200 mL, 2.82 mol) and sodium hydride (60% in oil, 70 g, 1.75 mol) was added in one portion with stirring at ambient temperature over 1 h. The reaction was quenched by slowly adding then washing with aqueous  
25 citric acid (10%, 100 mL over 10 min, then 2 x 4 L), followed by aqueous sodium bicarbonate (2%, 2 L), water (2 x 4 L) and brine (4 L). The organic layer was concentrated on a 20 L rotary evaporator to about 2 L total volume. The residue was purified by silica gel column chromatography (6 L Buchner funnel  
30 containing 1.5 kg of silica gel wetted with a solution of EtOAc-hexanes-TEA(70:29:1)). The product was eluted with the same solvent. (30 L) followed by straight EtOAc (6 L). The fractions containing the product were combined, concentrated on a rotary evaporator to a foam and then dried in a vacuum oven (50°C, 0.2

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mm Hg, 8 h) to afford 1155 g of a crisp, white foam (98%). HPLC indicated a purity of >99.7%.

**Preparation of [5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N<sup>4</sup>-benzoyl-5-methylcytidin-3'-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (MOE 5-Me-C amidite)**

5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N<sup>4</sup>-benzoyl-5-methylcytidine (1082 g, 1.5 mol) was dissolved in anhydrous DMF (2 L) and co-evaporated with toluene (300 ml) at 50 °C under reduced pressure. The mixture was cooled to room temperature and 2-cyanoethyl tetraisopropylphosphorodiamidite (680 g, 2.26 mol) and tetrazole (52.5 g, 0.75 mol) were added. The mixture was shaken until all tetrazole was dissolved, N-methylimidazole (30 ml) was added, and the mixture was left at room temperature for 5 hours. TEA (300 ml) was added, the mixture was diluted with DMF (1 L) and water (400 ml) and extracted with hexane (3 x 3 L). The mixture was diluted with water (1.2 L) and extracted with a mixture of toluene (9 L) and hexanes (6 L). The two layers were separated and the upper layer was washed with DMF-water (60:40 v/v, 3 x 3 L) and water (3 x 2 L). The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated. The residue was co-evaporated with acetonitrile (2 x 2 L) under reduced pressure and dried in a vacuum oven (25 °C, 0.1mm Hg, 40 h) to afford 1336 g of an off-white foam (97%).

**Preparation of [5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N<sup>6</sup>-benzoyl-adenosin-3'-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (MOE A amidite)**

5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N<sup>6</sup>-benzoyl-adenosine (purchased from Reliable Biopharmaceutical, St. Lois, MO), 1098 g, 1.5 mol) was dissolved in anhydrous DMF (3 L) and co-evaporated with toluene (300 ml) at 50 °C. The mixture was cooled to room temperature and 2-cyanoethyl tetraisopropylphosphorodiamidite (680 g, 2.26 mol) and tetrazole (78.8 g, 1.24 mol) were added. The mixture was shaken until all

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tetrazole was dissolved, N-methylimidazole (30 ml) was added, and mixture was left at room temperature for 5 hours. TEA (300 ml) was added, the mixture was diluted with DMF (1 L) and water (400 ml) and extracted with hexanes (3 x 3 L). The mixture was  
5 diluted with water (1.4 L) and extracted with the mixture of toluene (9 L) and hexanes (6 L). The two layers were separated and the upper layer was washed with DMF-water (60:40, v/v, 3 x 3 L) and water (3 x 2 L). The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated to a sticky foam. The residue was co-  
10 evaporated with acetonitrile (2.5 L) under reduced pressure and dried in a vacuum oven (25 °C, 0.1mm Hg, 40 h) to afford 1350 g of an off-white foam solid (96%).

**Preparation of [5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N<sup>4</sup>-isobutyrylguanosin-3'-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (MOE G amidite)**

5'-O-(4,4'-Dimethoxytriphenylmethyl)-2'-O-(2-methoxyethyl)-N<sup>4</sup>-isobutyrylguanosine (purchased from Reliable Biopharmaceutical, St. Louis, MO, 1426 g, 2.0 mol) was dissolved in anhydrous DMF  
20 (2 L). The solution was co-evaporated with toluene (200 ml) at 50 °C, cooled to room temperature and 2-cyanoethyl tetraisopropylphosphorodiamidite (900 g, 3.0 mol) and tetrazole (68 g, 0.97 mol) were added. The mixture was shaken until all tetrazole was dissolved, N-methylimidazole (30 ml) was added,  
25 and the mixture was left at room temperature for 5 hours. TEA (300 ml) was added, the mixture was diluted with DMF (2 L) and water (600 ml) and extracted with hexanes (3 x 3 L). The mixture was diluted with water (2 L) and extracted with a mixture of toluene (10 L) and hexanes (5 L). The two layers  
30 were separated and the upper layer was washed with DMF-water (60:40, v/v, 3x3 L). EtOAc (4 L) was added and the solution was washed with water (3 x 4 L). The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and evaporated to approx. 4 kg. Hexane (4 L) was added, the mixture was shaken for 10 min, and the

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supernatant liquid was decanted. The residue was co-evaporated with acetonitrile (2 x 2 L) under reduced pressure and dried in a vacuum oven (25 °C, 0.1mm Hg, 40 h) to afford 1660 g of an off-white foamy solid (91%).

5

**2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites**

**2'-(Dimethylaminooxyethoxy) nucleoside amidites**

2'-(Dimethylaminooxyethoxy) nucleoside amidites (also known  
10 in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites) are prepared as described in the following paragraphs.

Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case  
15 of adenosine and cytidine and with isobutyryl in the case of guanosine.

**5'-O-tert-Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine**

O<sup>2</sup>-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese,  
20 Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The  
25 reaction was stirred for 16 h at ambient temperature. TLC (R<sub>f</sub> 0.22, EtOAc) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between CH<sub>2</sub>Cl<sub>2</sub> (1 L) and saturated sodium bicarbonate (2 x 1 L) and brine (1 L). The organic layer was dried over  
30 sodium sulfate, filtered, and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of EtOAc and ethyl ether (600mL) and cooling the solution to -10°C afforded a white crystalline solid which was collected by filtration, washed with ethyl ether (3 x2 00 mL) and dried (40°C,

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1mm Hg, 24 h) to afford 149g of white solid (74.8%). TLC and NMR spectroscopy were consistent with pure product.

**5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine**

5 In the fume hood, ethylene glycol (350 mL, excess) was added cautiously with manual stirring to a 2 L stainless steel pressure reactor containing borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). (Caution : evolves hydrogen gas). 5'-O-tert-  
10 Butyldiphenylsilyl-0<sup>2</sup>-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel  
15 was cooled to ambient temperature and opened. TLC (EtOAc, R<sub>f</sub> 0.67 for desired product and R<sub>f</sub> 0.82 for ara-T side product) indicated about 70% conversion to the product. The solution was concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to  
20 remove the ethylene glycol. (Alternatively, once the THF has evaporated the solution can be diluted with water and the product extracted into EtOAc). The residue was purified by column chromatography (2kg silica gel, EtOAc-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined,  
25 evaporated and dried to afford 84 g of a white crisp foam (50%), contaminated starting material (17.4g, 12% recovery) and pure reusable starting material (20g, 13% recovery). TLC and NMR spectroscopy were consistent with 99% pure product.

**30 2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine**

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol)

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and dried over P<sub>2</sub>O<sub>5</sub> under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dissolved in dry THF (369.8mL, Aldrich, sure seal bottle). Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture with the rate of addition maintained such that the resulting deep red coloration is just discharged before adding the next drop. The reaction mixture was stirred for 4 hrs., after which time TLC (EtOAc:hexane, 60:40) indicated that the reaction was complete. The solvent was evaporated in vacuo and the residue purified by flash column chromatography (eluted with 60:40 EtOAc:hexane), to yield 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%) upon rotary evaporation.

15           **5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine**

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate washed with ice cold CH<sub>2</sub>Cl<sub>2</sub>, and the combined organic phase was washed with water and brine and dried (anhydrous Na<sub>2</sub>SO<sub>4</sub>). The solution was filtered and evaporated to afford 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL).  
25 Formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. The solvent was removed under vacuum and the residue was purified by column chromatography to yield 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%) upon rotary evaporation.  
30

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**5'-O-tert-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine**

5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinoxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL) and cooled to 10°C under inert atmosphere. Sodium cyanoborohydride (0.39g, 6.13mmol) was added and the reaction mixture was stirred. After 10 minutes the reaction was warmed to room temperature and stirred for 2 h. while the progress of the reaction was monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Aqueous NaHCO<sub>3</sub> solution (5%, 10mL) was added and the product was extracted with EtOAc (2 x 20 mL). The organic phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated to dryness. This entire procedure was repeated with the resulting residue, with the exception that formaldehyde (20% w/w, 30 mL, 3.37 mol) was added upon dissolution of the residue in the PPTS/MeOH solution. After the extraction and evaporation, the residue was purified by flash column chromatography and (eluted with 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) to afford 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine as a white foam (14.6g, 80%) upon rotary evaporation.

**2'-O-(dimethylaminoxyethyl)-5-methyluridine**

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and TEA (1.67mL, 12mmol, dry, stored over KOH) and added to 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine (1.40g, 2.4mmol). The reaction was stirred at room temperature for 24 hrs and monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). The solvent was removed under vacuum and the residue purified by flash column chromatography (eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) to afford 2'-O-(dimethylaminoxyethyl)-5-methyluridine (766mg, 92.5%) upon rotary evaporation of the solvent.

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**5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine**

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750 mg, 2.17 mmol) was dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C, co-evaporated with anhydrous pyridine (20 mL), and dissolved in pyridine (11 mL) under argon atmosphere. 4-dimethylaminopyridine (26.5 mg, 2.60 mmol) and 4,4'-dimethoxytrityl chloride (880 mg, 2.60 mmol) were added to the pyridine solution and the reaction mixture was stirred at room temperature until all of the starting material had reacted. Pyridine was removed under vacuum and the residue was purified by column chromatography (eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> containing a few drops of pyridine) to yield 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%) upon rotary evaporation.

**5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]**

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08 g, 1.67 mmol) was co-evaporated with toluene (20 mL), N,N-diisopropylamine tetrazonide (0.29 g, 1.67 mmol) was added and the mixture was dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C. This was dissolved in anhydrous acetonitrile (8.4 mL) and 2-cyanoethyl-N,N,N<sup>1</sup>,N<sup>1</sup>-tetraisopropylphosphoramidite (2.12 mL, 6.08 mmol) was added. The reaction mixture was stirred at ambient temperature for 4 h under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:EtOAc 1:1). The solvent was evaporated, then the residue was dissolved in EtOAc (70mL) and washed with 5% aqueous NaHCO<sub>3</sub> (40mL). The EtOAc layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The residue obtained was purified by column chromatography (EtOAc as eluent) to afford 5'-O-DMT-

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2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%) upon rotary evaporation.

5           **2'-(Aminooxyethoxy) nucleoside amidites**

2'-(Aminooxyethoxy) nucleoside amidites (also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites) are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

10

**N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]**

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-hydroxyethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may be phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-([2-phthalamidoxy]ethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

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**2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites**

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>2</sub>)<sub>2</sub>, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

**2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine**

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) was slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. (Caution: Hydrogen gas evolves as the solid dissolves). O<sup>2</sup>-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) were added and the bomb was sealed, placed in an oil bath and heated to 155°C for 26 h. then cooled to room temperature. The crude solution was concentrated, the residue was diluted with water (200 mL) and extracted with hexanes (200 mL). The product was extracted from the aqueous layer with EtOAc (3 x 200 mL) and the combined organic layers were washed once with water, dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified by silica gel column chromatography (eluted with 5:100:2 MeOH/CH<sub>2</sub>Cl<sub>2</sub>/TEA) as the eluent. The appropriate fractions were combined and evaporated to afford the product as a white solid.

**5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine**

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine in anhydrous pyridine (8 mL), was added TEA (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) and the reaction was stirred for 1 h. The reaction mixture was poured into water (200 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 x 200 mL). The combined CH<sub>2</sub>Cl<sub>2</sub> layers were washed with saturated NaHCO<sub>3</sub> solution, followed by saturated NaCl

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solution, dried over anhydrous sodium sulfate, filtered and evaporated. The residue was purified by silica gel column chromatography (eluted with 5:100:1 MeOH/CH<sub>2</sub>Cl<sub>2</sub>/TEA) to afford the product.

5

**5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite**

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) were added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) under an atmosphere of argon. The reaction mixture was stirred overnight and the solvent evaporated. The resulting residue was purified by silica gel column chromatography with EtOAc as the eluent to afford the title compound.

15

**Example 2**

**20 Oligonucleotide synthesis**

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 394) using standard phosphoramidite chemistry with oxidation by iodine.

25 Phosphorothioates (P=S) are synthesized similar to phosphodiester oligonucleotides with the following exceptions: thiation was effected by utilizing a 10% w/v solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the oxidation of the phosphite linkages. The thiation reaction step time was increased to 180 sec and preceded by the normal capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (12-16 hr), the oligonucleotides were recovered by precipitating with >3 volumes

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of ethanol from a 1 M NH<sub>4</sub>OAc solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

### **Example 3**

#### **Oligonucleoside Synthesis**

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides,

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as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated  
5 by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as  
10 described in U.S. Patent 5,223,618, herein incorporated by reference.

#### **Example 4**

##### **PNA Synthesis**

15 Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, **1996**, 4, 5-23. They may also be prepared in accordance with U.S. Patents  
20 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

#### **Example 5**

##### **Synthesis of Chimeric Oligonucleotides**

25 Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end"  
30 type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

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**[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides**

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 394, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by incorporating coupling steps with increased reaction times for the 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite. The fully protected oligonucleotide is cleaved from the support and deprotected in concentrated ammonia (NH<sub>4</sub>OH) for 12-16 hr at 55°C. The deprotected oligo is then recovered by an appropriate method (precipitation, column chromatography, volume reduced *in vacuo* and analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

**[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides**

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

**[2'-O-(2-Methoxyethyl) Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides**

[2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of

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2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidation with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

#### **Example 6**

##### **Oligonucleotide Isolation**

After cleavage from the controlled pore glass solid support and deblocking in concentrated ammonium hydroxide at 55°C for 12-16 hours, the oligonucleotides or oligonucleosides are recovered by precipitation out of 1 M NH<sub>4</sub>OAc with >3 volumes of ethanol. Synthesized oligonucleotides were analyzed by electrospray mass spectroscopy (molecular weight determination) and by capillary gel electrophoresis and judged to be at least 70% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in the synthesis was determined by the ratio of correct molecular weight relative to the -16 amu product (+/-32 +/-48). For some studies oligonucleotides were purified by HPLC, as described by Chiang *et al.*, *J. Biol. Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

30

#### **Example 7**

##### **Oligonucleotide Synthesis - 96 Well Plate Format**

Oligonucleotides were synthesized via solid phase P(III)

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phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a 96-well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,4-dihydro-2H-benzodithiole-3-one 1,1-dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per standard or patented methods. They are utilized as base protected beta-cyanoethyl-diisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated  $\text{NH}_4\text{OH}$  at elevated temperature ( $55\text{-}60^\circ\text{C}$ ) for 12-16 hours and the released product then dried *in vacuo*. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

20

**Example 8****Oligonucleotide Analysis - 96-Well Plate Format**

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96-well format (Beckman P/ACE<sup>TM</sup> MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE<sup>TM</sup> 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the

30

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compounds on the plate were at least 85% full length.

### Example 9

#### Cell culture and oligonucleotide treatment

5           The effect of antisense compounds on target nucleic acid  
expression can be tested in any of a variety of cell types  
provided that the target nucleic acid is present at measurable  
levels. This can be routinely determined using, for example,  
PCR or Northern blot analysis. The following cell types are  
10 provided for illustrative purposes, but other cell types can be  
routinely used, provided that the target is expressed in the  
cell type chosen. This can be readily determined by methods  
routine in the art, for example Northern blot analysis,  
ribonuclease protection assays, or RT-PCR.

15

T-24 cells:

          The human transitional cell bladder carcinoma cell line T-  
24 was obtained from the American Type Culture Collection (ATCC)  
(Manassas, VA). T-24 cells were routinely cultured in complete  
20 McCoy's 5A basal media (Invitrogen Corporation, Carlsbad, CA)  
supplemented with 10% fetal calf serum (Invitrogen Corporation,  
Carlsbad, CA), penicillin 100 units per mL, and streptomycin 100  
micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells  
were routinely passaged by trypsinization and dilution when they  
25 reached 90% confluence. Cells were seeded into 96-well plates  
(Falcon-Primaria #3872) at a density of 7000 cells/well for use  
in RT-PCR analysis.

          For Northern blotting or other analysis, cells may be  
seeded onto 100 mm or other standard tissue culture plates and  
30 treated similarly, using appropriate volumes of medium and  
oligonucleotide.

A549 cells:

          The human lung carcinoma cell line A549 was obtained from

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the American Type Culture Collection (ATCC) (Manassas, VA).

A549 cells were routinely cultured in DMEM basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, CA),

5 penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

10 NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville, MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville, MD) supplemented as recommended by  
15 the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the  
20 Clonetics Corporation (Walkersville, MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville, MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

25

Differentiated human adipocytes:

Human adipocytes were obtained from Zen-Bio (Research Triangle Park, NC) and plated (4k/well) in pre-adipocyte media (DME/hams F-10 medium (1:1), 10% FBS, 15 mM HEPES, 100 u/ml  
30 penicillin, 100 ug/ml streptomycin and 0.25 ug/ml amphotericin B. Cells reached confluence after 3 days and were then put on diff media (pre-adipocyte basal media (above) + 2% more FBS to a total of 12%, amino acids, 100 nM insulin, 0.5 mM ibmx, 1 uM dexamethasone and 1 uM BRL49653). Cells were left in diff media

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for 3-5 days and then re-fed with adipocyte media (same as pre-adipocyte media but including: 33 uM biotin, 17 uM pantothenate, 100 nM insulin and 1 uM dexamethasone. Cells were differentiated within one week. Cells were then treated with lipofectin (10  
5 ul/ml) at 250 nM for 4 hours and the media was exchanged for basal adipocyte media. Cells were lysed 24 hours later.

#### Differentiated 3T3-L1 cells:

The mouse embryonic adipocyte cell line 3T3-L1 was obtained  
10 from the American Type Culture Collection (Manassas, VA). 3T3-L1 cells were differentiated by culturing for three days in the presence of 400nM insulin, 250nM dexamethasone and 0.5mM IBMX (Sigma). Differentiated 3T3L1 cells were then routinely cultured in DMEM, high glucose (Gibco/Life Technologies,  
15 Gaithersburg, MD) supplemented with 10% fetal calf serum, 100 units per ml penicillin, 100 micrograms per ml streptomycin (Gibco/Life Technologies, Gaithersburg, MD), 400nM bovine insulin (Sigma), 125mM dexamethasone , 0.5mM IBMX, and fungizone. Cells were routinely passaged by trypsinization and  
20 dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 10000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and  
25 treated similarly, using appropriate volumes of medium and oligonucleotide. Treatment of these cells with oligonucleotide was accomplished via electroporation as follows: 2.5 ml of 0.25% trypsin in a solution of 1 mM EDTA was added to a cell solution in a flask until the cells were released from the surface of the  
30 flask, at which point, 20 ml of complete medium was added to neutralize the trypsin activity. The suspended cells were then mixed to obtain a homogeneous solution which was then centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in OPTI-MEM™ (Gibco BRL/Invitrogen, Carlsbad, CA) at

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1 x 10<sup>7</sup> cells/ml. 10 ul of oligonucleotide was mixed with 90 ul of cell suspension and the mixture was electroporated in a single pulse in a 0.1 cm cuvette at 75 V for 6 milliseconds. The mixture was then transferred to a 24-well plate and incubated  
5 for 24 hours prior to harvest.

Treatment with antisense compounds:

When cells reached 70% confluency, they were treated with  
10 oligonucleotide. For cells grown in 96-well plates, wells were washed once with 100 µL OPTI-MEM™-1 reduced-serum medium (Invitrogen Corporation, Carlsbad, CA) and then treated with 130 µL of OPTI-MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Invitrogen Corporation, Carlsbad, CA) and the desired concentration of  
15 oligonucleotide. After 4-7 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide  
20 concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is selected from either ISIS 13920 (**TCCGTCATCGCTCCTCAGGG**, SEQ ID NO: 1) which is targeted to human  
25 H-ras, or ISIS 18078, (**GTGCGCGAGCCCCGAAATC**, SEQ ID NO: 2) which is targeted to human Jun-N-terminal kinase-2 (JNK2). Both controls are 2'-O-methoxyethyl gapmers (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone. For differentiated mouse 3T3-L1 cells, the positive control oligonucleotide is ISIS  
30 165422, (**TGCTTGTGTGTGGATTCGCG**, SEQ ID NO: 3) a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to mouse resistin. The concentration of positive control oligonucleotide that

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results in 80% inhibition of human H-ras (for ISIS 13920) or mouse resistin (for ISIS 165422) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not  
5 achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of human H-ras or mouse resistin mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell  
10 line is deemed as unsuitable for oligonucleotide transfection experiments. The concentrations of antisense oligonucleotides used herein are from 50 nM to 300 nM.

#### 15 **Example 10**

##### **Analysis of oligonucleotide inhibition of perilipin expression**

Antisense modulation of perilipin expression can be assayed in a variety of ways known in the art. For example, perilipin mRNA levels can be quantitated by, e.g., Northern blot analysis,  
20 competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. The preferred method of RNA analysis of the present invention is the use of total cellular RNA as described in other  
25 examples herein. Methods of RNA isolation are taught in, for example, Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. *et al.*, *Current*  
30 *Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied

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Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of perilipin can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to perilipin can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., (*Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997). Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., (*Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997).

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., (*Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998). Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., (*Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997). Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., (*Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991).

### **Example 11**

#### **30 Poly(A)+ mRNA isolation**

Poly(A)+ mRNA was isolated according to Miura et al., (*Clin. Chem.*, 1996, 42, 1758-1764). Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., (*Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-

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4.5.3, John Wiley & Sons, Inc., 1993). Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200  $\mu$ L cold PBS. 60  $\mu$ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM  
5 vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55  $\mu$ L of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times  
10 with 200  $\mu$ L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60  $\mu$ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C, was added to each well, the plate was  
15 incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

## 20 **Example 12**

### **Total RNA Isolation**

Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia, CA) following the manufacturer's recommended procedures. Briefly, for cells grown  
25 on 96-well plates, growth medium was removed from the cells and each well was washed with 200  $\mu$ L cold PBS. 150  $\mu$ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 150  $\mu$ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The  
30 samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 1

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minute. 500  $\mu$ L of Buffer RW1 was added to each well of the RNEASY 96<sup>TM</sup> plate and incubated for 15 minutes and the vacuum was again applied for 1 minute. An additional 500  $\mu$ L of Buffer RW1 was added to each well of the RNEASY 96<sup>TM</sup> plate and the vacuum was applied for 2 minutes. 1 mL of Buffer RPE was then added to each well of the RNEASY 96<sup>TM</sup> plate and the vacuum applied for a period of 90 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 3 minutes. The plate was then removed from the QIAVAC<sup>TM</sup> manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC<sup>TM</sup> manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 170  $\mu$ L water into each well, incubating 1 minute, and then applying the vacuum for 3 minutes.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

### Example 13

#### Real-time Quantitative PCR Analysis of perilipin mRNA Levels

Quantitation of perilipin mRNA levels was determined by real-time quantitative PCR using the ABI PRISM<sup>TM</sup> 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are

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quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., FAM or JOE, obtained from either PE-Applied Biosystems, Foster City, CA, Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either PE-Applied Biosystems, Foster City, CA, Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the internal standard gene GAPDH are amplified concurrently in a

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single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following  
5 PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their  
10 corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from Invitrogen Corporation, (Carlsbad, CA). RT-PCR reactions were carried out by adding 20  
15  $\mu$ L PCR cocktail (2.5x PCR buffer (-MgCl<sub>2</sub>), 6.6 mM MgCl<sub>2</sub>, 375  $\mu$ M each of dATP, dCTP, dGTP and dTTP, 375 nM each of forward primer and reverse primer, 125 nM of probe, 4 Units RNase inhibitor, 1.25 Units PLATINUM® Taq, 5 Units MuLV reverse transcriptase, and 2.5x ROX dye) to 96-well plates containing 30  $\mu$ L total RNA  
20 solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the PLATINUM® Taq, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

25 Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA using RiboGreen™ (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run  
30 simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent from Molecular Probes. Methods of RNA quantification by

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RiboGreen™ are taught in Jones, L.J., et al, (Analytical Biochemistry, 1998, 265, 368-374).

In this assay, 170  $\mu$ L of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:350 in 10mM Tris-HCl, 1 mM EDTA, 5 pH 7.5) is pipetted into a 96-well plate containing 30  $\mu$ L purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 480nm and emission at 520nm.

Probes and primers to human perilipin were designed to 10 hybridize to a human perilipin sequence, using published sequence information (GenBank accession number AB005293.1, incorporated herein as SEQ ID NO:4). For human perilipin the PCR primers were:

forward primer: GCCTCTGTGTGCAATGCCTAT (SEQ ID NO: 5)

15 reverse primer: AGCTCATTTGGCAGCTGTGAA (SEQ ID NO: 6) and the PCR probe was: FAM-AAGGGCGTGCCAGAGCGCCAG-TAMRA

(SEQ ID NO: 7) where FAM is the fluorescent dye and TAMRA is the quencher dye. For human GAPDH the PCR primers were:

forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO:8)

20 reverse primer: GAAGATGGTGATGGGATTTTC (SEQ ID NO:9) and the PCR probe was: 5' JOE-CAAGCTTCCCCTTCTCAGCC-TAMRA 3' (SEQ ID NO: 10) where JOE is the fluorescent reporter dye and TAMRA is the quencher dye.

Probes and primers to mouse perilipin were designed to 25 hybridize to a mouse perilipin sequence, using published sequence information (a consensus sequence assembled from GenBank accession numbers BF300643 and AI573604, incorporated herein as SEQ ID NO: 11). For mouse perilipin the PCR primers were:

30 forward primer: GGGAGGTCTGAGAGGCATTG (SEQ ID NO:12)

reverse primer: GTGGAAGGTCTCCTCCTCAGAA (SEQ ID NO: 13) and the

PCR probe was: FAM-CAAGATTCCTGGAGTGGCTGCAAGT-TAMRA

(SEQ ID NO: 14) where FAM is the fluorescent reporter dye and

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TAMRA is the quencher dye. For mouse GAPDH the PCR primers were:

forward primer: GGCAAATTCAACGGCACAGT (SEQ ID NO:15)

reverse primer: GGGTCTCGCTCCTGGAAGAT (SEQ ID NO:16) and the PCR

5 probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTCATC-TAMRA 3' (SEQ ID NO: 17) where JOE is the fluorescent reporter dye and TAMRA is the quencher dye.

#### 10 **Example 14**

##### **Northern blot analysis of perilipin mRNA levels**

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared  
15 following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham  
20 Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La  
25 Jolla, CA) and then probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

To detect human perilipin, a human perilipin specific probe was prepared by PCR using the forward primer  
30 GCCTCTGTGTGCAATGCCTAT (SEQ ID NO: 5) and the reverse primer AGCTCATTTGGCAGCTGTGAA (SEQ ID NO: 6). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate

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dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

To detect mouse perilipin, a mouse perilipin specific probe was prepared by PCR using the forward primer GGGAGGTCTGAGAGGCATTG (SEQ ID NO: 12) and the reverse primer  
5 GTGGAAGGTCTCCTCCTCAGAA (SEQ ID NO: 13). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Hybridized membranes were visualized and quantitated using  
10 a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

#### 15 **Example 15**

##### **Antisense inhibition of human perilipin expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap**

In accordance with the present invention, a series of  
20 oligonucleotides were designed to target different regions of the human perilipin RNA, using published sequences (GenBank accession number AB005293.1, incorporated herein as SEQ ID NO: 4, GenBank accession number NM\_002666.1, incorporated herein as SEQ ID NO: 18; a consensus sequence assembled from contigs of  
25 GenBank accession number AC013787.5, incorporated herein as SEQ ID NO: 19; and a hypothetical sequence representing human perilipin B which includes exons 1-8, intron 8 and exon 9 from SEQ ID NO: 19, incorporated herein as SEQ ID NO: 20). The oligonucleotides are shown in Table 1. "Target site" indicates  
30 the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten

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2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human perilipin mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments in which differentiated human adipocytes were treated with the oligonucleotides of the present invention. The positive control for each datapoint is identified in the table by sequence ID number. If present, "N.D." indicates "no data".

Table 1

Inhibition of human perilipin mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO	CONTROL SEQ ID NO
165207	Coding	4	150	aggagggtctccatccagca	60	21	1
165208	Coding	4	155	tgctcagggagggtctccatc	71	22	1
165214	Coding	4	271	tgcacacagaggccaccagg	43	23	1
165215	Coding	4	276	ggcattgcacacagaggcca	25	24	1
165217	Coding	4	287	cccttctcataggcattgca	65	25	1
165222	Coding	4	332	accggctccatgctccaggc	77	26	1
165223	Coding	4	338	cggaccaccggctccatgct	42	27	1
165224	Coding	4	360	tgtgaactgggtggacagcc	63	28	1
165225	Coding	4	365	gcagctgtgaactgggtgga	90	29	1
165226	Coding	4	372	ctcattggcagctgtgaact	84	30	1
165229	Coding	4	408	ctttcctccagggtggcca	68	31	1
165230	Coding	4	413	gggatctttcctccagggtg	55	32	1
165242	Coding	4	1256	gatagggacatggccctccc	65	33	1
165249	Coding	4	1349	tcgctctcgggctccatcag	0	34	1
165250	Coding	4	1354	ggaattcgctctcgggctcc	5	35	1
165255	Coding	4	1619	acgctgggcccgaagaagct	60	36	1
165257	Coding	4	1667	ttcttgcgagctggctgta	78	37	1
165280	5'UTR	18	4	ctcagtctcacagagctcgt	76	38	1
165281	5'UTR	18	52	taaggcagggtgccccaggac	33	39	1
165282	5'UTR	18	65	taaacaagccatgtaaggca	0	40	1
165283	5'UTR	18	104	ctcgctcctcaagcttaac	47	41	1
165284	Start	18	111	tgccatcctcgctcctcaag	58	42	1

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165285	Codon Start Codon	18	117	gttgactgccatcctcgctc	52	43	1
165286	Coding	18	143	tctccatccagcaaggtgag	32	44	1
165287	Coding	18	171	ctgcagcacattctcctgct	76	45	1
165288	Coding	18	392	tccaagcctcggcaggccag	74	46	1
165289	Coding	18	611	gctcagtggttggcagcaaa	76	47	1
165290	Coding	18	764	ctcaagaggcttggcttggc	73	48	1
165291	Coding	18	802	tgtatcgagagaggggtgttg	60	49	1
165292	Coding	18	901	aggcaccctctggccagg	0	50	1
165293	Coding	18	988	gatcctcctcctggcggct	56	51	1
165294	Coding	18	1078	gggctgctacctcactgaac	24	52	1
165295	Coding	18	1315	gcggcagcgaatgcaccact	76	53	1
165296	Stop Codon	18	1682	gcggcgactcagctcttctt	54	54	1
165297	3'UTR	18	1867	gccccaaaaggatgctaaaa	51	55	1
165298	3'UTR	18	1897	tgtcccttaaaaactggctc	65	56	1
165299	3'UTR	18	1902	tctgggtgctcccttaaaaact	65	57	1
165300	3'UTR	18	2035	cagaggcagaatctgaattt	26	58	1
165301	3'UTR	18	2052	tggcaaatatcttccgcag	16	59	1
165302	3'UTR	18	2088	tggaccttcagagtgggtgac	56	60	1
165303	3'UTR	18	2133	atcacagaggagttcagtgc	52	61	1
165304	3'UTR	18	2145	agatcatcctagatcacaga	74	62	1
165305	3'UTR	18	2211	attgttcccttcaaagtagc	54	63	1
165306	3'UTR	18	2256	gtgacactagtattttaaact	64	64	1
165307	3'UTR	18	2268	ggtactcagaaagtgacact	21	65	1
165308	3'UTR	18	2304	aagcacacaggcctggactc	6	66	1
165309	3'UTR	18	2347	atgcaaatggaaatgtggct	24	67	1
165310	3'UTR	18	2500	aattgtatgaatgcattttc	36	68	1
165311	3'UTR	18	2613	caggtgcataagccctgcata	68	69	1
165312	3'UTR	18	2634	gagtgacatacacacgtgcct	19	70	1
165313	3'UTR	18	2660	ccacagcttgtgtaaacaca	36	71	1
165314	3'UTR	18	2871	ttatagcatcgtttgcagta	65	72	1
165315	3'UTR	18	2882	aaggacatttattatagcat	41	73	1
165316	Exon: Intron Junction	19	4507	aggcccttaccttcaacttc	63	74	1
165317	Intron: Exon Junction	19	5922	gctcctcaagctgcaaaaca	1	75	1
165318	Intron	19	7356	gtcagattccgatgctcagg	64	76	1
165319	Intron	19	9699	ctgatacctactggtagaga	24	77	1
165320	Exon: Intron Junction	19	10223	aaggactcacactgggtgga	53	78	1
165321	Intron	19	11197	gggcatgcatcagagatgca	41	79	1
165322	Intron: Exon Junction	19	15640	ccaggctgctctgaggagg	83	80	1
165323	Intron	19	15891	accctatgcctctgcttctc	17	81	1
165324	Coding	20	1324	tggtaactaccggcacgtaa	49	82	1
165325	Coding	20	1353	cccttgggacactaacagtt	0	83	1
165326	Coding	20	1473	tgttaaaatggtgccagggc	46	84	1
165327	Stop Codon	20	1568	agactcctctaccagcaggt	59	85	1
165328	3'UTR	20	1790	aaagggatggcattgggtatc	26	86	1
165329	3'UTR	20	1833	ccaggcctgcataatctgta	49	87	1
165330	3'UTR	20	1913	atgctgcttggtagagtgac	60	88	1

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165331	3'UTR	20	2000	cacacagtgacctggccagg	0	89	1
165332	3'UTR	20	2033	ggatcatcagctttcctaact	45	90	1
165333	3'UTR	20	2122	cttctccatggaccaggctg	37	91	1
165334	3'UTR	20	2137	cctgccactctgagcttct	32	92	1
165335	3'UTR	20	2293	gccgccttagagtcttgct	52	93	1

As shown in Table 1, SEQ ID NOs 21, 22, 23, 25, 26, 27, 28, 29, 30, 31, 32, 33, 36, 37, 38, 41, 42, 43, 45, 46, 47, 48, 49, 51, 53, 54, 55, 56, 57, 60, 61, 62, 63, 64, 69, 72, 73, 74, 76, 78, 79, 80, 82, 84, 85, 87, 88, 90 and 93 demonstrated at least 40% inhibition of human perilipin expression in this assay and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "preferred target regions" and are therefore preferred sites for targeting by compounds of the present invention. These preferred target regions are shown in Table 3. The sequences represent the reverse complement of the preferred antisense compounds shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number of the corresponding target nucleic acid. Also shown in Table 3 is the species in which each of the preferred target regions was found.

#### 20 **Example 16**

**Antisense inhibition of mouse perilipin expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap.**

In accordance with the present invention, a second series of oligonucleotides were designed to target different regions of the mouse perilipin RNA, using published sequences (a consensus sequence assembled from GenBank accession numbers BF300643 and AI573604, incorporated herein as SEQ ID NO: 11; a consensus sequence assembled from GenBank accession numbers AI019721 and AI154591, incorporated herein as SEQ ID NO: 94; and a consensus sequence representing mouse perilipin B, assembled

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from GenBank accession numbers I014299, AI019721, AI182139, incorporated herein as SEQ ID NO: 95). The oligonucleotides are shown in Table 2. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on mouse perilipin mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments in which differentiated 3T3-L1 cells were treated with the oligonucleotides of the present invention. The positive control for each datapoint is identified in the table by sequence ID number. If present, "N.D." indicates "no data".

**Table 2**

**Inhibition of mouse perilipin mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap**

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO	CONTROL SEQ ID NO
165206	Start Codon	11	175	ggcccttgttcattgacatc	78	96	3
165209	Coding	11	212	ttctcctgctcagggaggtc	44	97	3
165213	Coding	11	278	taggtcttctggaagcactc	80	98	3
165216	Coding	11	332	tcataggcattgcacacaga	86	99	3
165219	Coding	11	355	tgctggcaccctgtacaccc	94	100	3
165221	Coding	11	378	ctccatgctccaggcagcca	85	101	3
165228	Coding	11	444	gtccaggcctctgcaggcca	51	102	3
165254	Coding	94	534	cggaagaagctgtcgctgac	28	103	3
165256	Coding	94	559	caggatgggctccatgacgc	73	104	3

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165258	Stop Codon	94	599	ctcagctcttcttgcgagc	65	105	3
165262	Coding	95	300	tgggagtcagaaggtggccc	67	106	3
165265	3'UTR	95	337	aaaggagggttatactcc	52	107	3
165267	3'UTR	95	393	tgtataagaaggttctgaac	38	108	3
165270	3'UTR	95	483	ctcacaacaatgattcta	39	109	3
165273	3'UTR	95	805	actgccatcataaggacaca	0	110	3

As shown in Table 2, SEQ ID NOS 96, 97, 98, 99, 100, 101,  
 102, 104, 105, 106 and 107 demonstrated at least 44% inhibition  
 5 of mouse perilipin expression in this experiment and are  
 therefore preferred. The target sites to which these preferred  
 sequences are complementary are herein referred to as "preferred  
 target regions" and are therefore preferred sites for targeting  
 by compounds of the present invention. These preferred target  
 10 regions are shown in Table 3. The sequences represent the  
 reverse complement of the preferred antisense compounds shown in  
 Table 1. "Target site" indicates the first (5'-most) nucleotide  
 number of the corresponding target nucleic acid. Also shown in  
 Table 3 is the species in which each of the preferred target  
 15 regions was found.

**Table 3**  
**Sequence and position of preferred target regions identified in**  
**perilipin.**

SITEID	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	REV COMP OF SEQ ID	ACTIVE IN	SEQ ID NO
80666	4	150	tgctggatggagacctcct	21	<i>H. sapiens</i>	111
80667	4	155	gatggagacctccctgagca	22	<i>H. sapiens</i>	112
80673	4	271	cctggtggcctctgtgtgca	23	<i>H. sapiens</i>	113
80676	4	287	tgcaatgcctatgagaaggg	25	<i>H. sapiens</i>	114
80681	4	332	gcctggagcatggagccggt	26	<i>H. sapiens</i>	115
80682	4	338	agcatggagccggtggtccg	27	<i>H. sapiens</i>	116
80683	4	360	ggctgtccaccagttcaca	28	<i>H. sapiens</i>	117
80684	4	365	tccaccagttcacagctgc	29	<i>H. sapiens</i>	118
80685	4	372	agttcacagttgccaatgag	30	<i>H. sapiens</i>	119
80688	4	408	tggaccacctggaggaaaag	31	<i>H. sapiens</i>	120
80689	4	413	cacctggaggaaaagatccc	32	<i>H. sapiens</i>	121
80701	4	1256	gggagggccatgtccctatc	33	<i>H. sapiens</i>	122

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80714	4	1619	agcttctctccggcccagcgt	36	<i>H. sapiens</i>	123
80716	4	1667	tacagccagctgcgcaagaa	37	<i>H. sapiens</i>	124
80739	18	4	acgagctctgtgagactgag	38	<i>H. sapiens</i>	125
80742	18	104	gttgaagcttgaggagcgag	41	<i>H. sapiens</i>	126
80743	18	111	cttgaggagcgaggatggca	42	<i>H. sapiens</i>	127
80744	18	117	gagcgaggatggcagtcaac	43	<i>H. sapiens</i>	128
80746	18	171	agcaggagaatgtgctgcag	45	<i>H. sapiens</i>	129
80747	18	392	ctggcctgccgaggcttgga	46	<i>H. sapiens</i>	130
80748	18	611	tttgctgccaacactcgagc	47	<i>H. sapiens</i>	131
80749	18	764	gccaaagccaagcctcttgag	48	<i>H. sapiens</i>	132
80750	18	802	caacaccctctctcgataca	49	<i>H. sapiens</i>	133
80752	18	988	agccgcccaggaggaggatc	51	<i>H. sapiens</i>	134
80754	18	1315	agtgggtgcattacgtgccgc	53	<i>H. sapiens</i>	135
80755	18	1682	aagaagagctgagtcgccgc	54	<i>H. sapiens</i>	136
80756	18	1867	ttttagcatccttttggggc	55	<i>H. sapiens</i>	137
80757	18	1897	gagccagttttaagggaca	56	<i>H. sapiens</i>	138
80758	18	1902	agtttttaagggacaccaga	57	<i>H. sapiens</i>	139
80761	18	2088	gtcaccactctgaaggtcca	60	<i>H. sapiens</i>	140
80762	18	2133	gcactgaactcctctgtgat	61	<i>H. sapiens</i>	141
80763	18	2145	tctgtgatctaggatgatct	62	<i>H. sapiens</i>	142
80764	18	2211	gctactttgaagggacaact	63	<i>H. sapiens</i>	143
80765	18	2256	atntaaaatactagtgtcac	64	<i>H. sapiens</i>	144
80770	18	2613	tatgcagggctatgcacctg	69	<i>H. sapiens</i>	145
80773	18	2871	tactgcaaacgatgtataaa	72	<i>H. sapiens</i>	146
80774	18	2882	atgctataataaatgtcctt	73	<i>H. sapiens</i>	147
80775	19	4507	gaagttgaaggtaagggcct	74	<i>H. sapiens</i>	148
80777	19	7356	cctgagcatcggaatctgac	76	<i>H. sapiens</i>	149
80779	19	10223	tccaccagtgtagtcctt	78	<i>H. sapiens</i>	150
80780	19	11197	tgcactctctgatgcagccc	79	<i>H. sapiens</i>	151
80781	19	15640	cctccctcagagcagcctgg	80	<i>H. sapiens</i>	152
80783	20	1324	ttacgtgccggtagtacc	82	<i>H. sapiens</i>	153
80785	20	1473	gccctggcaacattttaaca	84	<i>H. sapiens</i>	154
80786	20	1568	acctgctggtagaggagctc	85	<i>H. sapiens</i>	155
80788	20	1833	tacagattatgcaggcctgg	87	<i>H. sapiens</i>	156
80789	20	1913	gtcactctaccaagcagcat	88	<i>H. sapiens</i>	157
80791	20	2033	agttaggaaagctgatgacc	90	<i>H. sapiens</i>	158
80794	20	2293	agccaggactctaaggcgcc	93	<i>H. sapiens</i>	159
80665	11	175	gatgtcaatgaacaagggcc	96	<i>M. musculus</i>	160
80668	11	212	gacctccctgagcaggagaa	97	<i>M. musculus</i>	161
80672	11	278	gagtgtctccagaagaccta	98	<i>M. musculus</i>	162
80675	11	332	tctgtgtgcaatgcctatga	99	<i>M. musculus</i>	163
80678	11	355	gggtgtacaggtgcccagca	100	<i>M. musculus</i>	164
80680	11	378	tggctgcctggagcatggag	101	<i>M. musculus</i>	165
80687	11	444	tggcctgcagaggcctggac	102	<i>M. musculus</i>	166
80715	94	559	gcgtcatggagcccacctg	104	<i>M. musculus</i>	167
80717	94	599	gctgcgcaagaagagctgag	105	<i>M. musculus</i>	168
80721	95	300	ggccacctctgactcca	106	<i>M. musculus</i>	169
80724	95	337	ggagatataagccctcctt	107	<i>M. musculus</i>	170

As these "preferred target regions" have been found by  
 5 experimentation to be open to, and accessible for, hybridization  
 with the antisense compounds of the present invention, one of

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skill in the art will recognize or be able to ascertain, using no more than routine experimentation, further embodiments of the invention that encompass other compounds that specifically hybridize to these sites and consequently inhibit the expression  
5 of perilipin.

In one embodiment, the "preferred target region" may be employed in screening candidate antisense compounds. "Candidate antisense compounds" are those that inhibit the expression of a nucleic acid molecule encoding perilipin and which comprise at  
10 least an 8-nucleobase portion which is complementary to a preferred target region. The method comprises the steps of contacting a preferred target region of a nucleic acid molecule encoding perilipin with one or more candidate antisense compounds, and selecting for one or more candidate antisense  
15 compounds which inhibit the expression of a nucleic acid molecule encoding perilipin. Once it is shown that the candidate antisense compound or compounds are capable of inhibiting the expression of a nucleic acid molecule encoding perilipin, the candidate antisense compound may be employed as  
20 an antisense compound in accordance with the present invention.

According to the present invention, antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic  
25 acid and modulate its expression.

### **Example 17**

#### **Western blot analysis of perilipin protein levels**

30 Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and

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transferred to membrane for western blotting. Appropriate primary antibody directed to perilipin is used, with a radiolabeled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are  
 5 visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

**Example 18**

10 **Targeting of individual oligonucleotides to specific variants of human perilipin**

It is advantageous to selectively inhibit the expression of one or more variants of human perilipin. Consequently, in one embodiment of the present invention are oligonucleotides that  
 15 selectively target, hybridize to, and specifically inhibit human perilipin B relative to human perilipin. A summary of the target sites of human perilipin B (SEQ ID NO: 20) is shown in Table 4. Human perilipin B can be specifically inhibited using the oligonucleotides in Table 3, relative to SEQ ID NO: 4 which  
 20 represents the main mRNA sequence of human perilipin.

**Table 4**

25 **Targeting of individual oligonucleotides specifically to human perilipin B, a variant of human perilipin**

ISIS #	OLIGO SEQ ID NO.	TARGET SITE	VARIANT	VARIANT SEQ ID NO.
165324	86	1324	perilipin B	20
165325	87	1353	perilipin B	20
165326	88	1473	perilipin B	20
165327	89	1568	perilipin B	20
165328	90	1790	perilipin B	20
165329	91	1833	perilipin B	20
165330	92	1913	perilipin B	20
165331	93	2000	perilipin B	20
165332	94	2033	perilipin B	20
165333	95	2122	perilipin B	20
165334	96	2137	perilipin B	20
165335	97	2293	perilipin B	20

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**What is claimed is:**

1. A compound 8 to 80 nucleobases in length targeted to  
5 a nucleic acid molecule encoding perilipin, wherein said  
compound specifically hybridizes with said nucleic acid molecule  
encoding perilipin and inhibits the expression of perilipin.
2. The compound of claim 1 which is an antisense  
oligonucleotide.
- 10 3. The compound of claim 2 wherein the antisense  
oligonucleotide comprises at least one modified internucleoside  
linkage.
4. The compound of claim 3 wherein the modified  
internucleoside linkage is a phosphorothioate linkage.
- 15 5. The compound of claim 2 wherein the antisense  
oligonucleotide comprises at least one modified sugar moiety.
6. The compound of claim 5 wherein the modified sugar  
moiety is a 2'-O-methoxyethyl sugar moiety.
7. The compound of claim 2 wherein the antisense  
20 oligonucleotide comprises at least one modified nucleobase.
8. The compound of claim 7 wherein the modified  
nucleobase is a 5-methylcytosine.
9. The compound of claim 2 wherein the antisense  
oligonucleotide is a chimeric oligonucleotide.
- 25 10. A compound 8 to 80 nucleobases in length which  
specifically hybridizes with at least an 8-nucleobase portion of  
a preferred target region on a nucleic acid molecule encoding  
perilipin.
11. A composition comprising the compound of claim 1 and  
30 a pharmaceutically acceptable carrier or diluent.
12. The composition of claim 11 further comprising a  
colloidal dispersion system.
13. The composition of claim 11 wherein the compound is  
an antisense oligonucleotide.

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14. A method of inhibiting the expression of perilipin in cells or tissues comprising contacting said cells or tissues with the compound of claim 1 so that expression of perilipin is inhibited.

5 15. A method of treating an animal having a disease or condition associated with perilipin comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of perilipin is inhibited.

10 16. A method of screening for an antisense compound, the method comprising the steps of:

a. contacting a preferred target region of a nucleic acid molecule encoding perilipin with one or more candidate antisense compounds, said candidate  
15 antisense compounds comprising at least an 8-nucleobase portion which is complementary to said preferred target region, and

b. selecting for one or more candidate antisense compounds which inhibit the expression of a nucleic  
20 acid molecule encoding perilipin.

17. The method of claim 15 wherein the disease or condition is a metabolic disorder.

18. The method of claim 17 wherein the metabolic disorder is selected from the group consisting of obesity, diabetes and  
25 atherosclerosis.

19. The compound of claim 1 targeted to a nucleic acid molecule encoding perilipin, wherein said compound specifically hybridizes with and differentially inhibits the expression of a nucleic acid molecule encoding one of the variants of perilipin  
30 relative to the remaining variants of perilipin.

20. The compound of claim 18 targeted to a nucleic acid molecule encoding perilipin, wherein said compound hybridizes with and specifically inhibits the expression of a nucleic acid molecule encoding a variant of perilipin, wherein said variant  
35 is perilipin B.

## SEQUENCE LISTING

<110> Isis Pharmaceuticals, Inc.  
Sanjay Bhanot  
Susan M. Freier

<120> ANTISENSE MODULATION OF PERILIPIN EXPRESSION

<130> RTS-0355WO

<150> 10/213,796

<151> 2002-08-06

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 gagg atg gca gtc aac aaa ggc ctc acc ttg ctg gat gga gac ctc cct 169  
 Met Ala Val Asn Lys Gly Leu Thr Leu Leu Asp Gly Asp Leu Pro  
 1 5 10 15

gag cag gag aat gtg ctg cag cgg gtc ctg cag ctg ccg gtg gtg agt 217  
 Glu Gln Glu Asn Val Leu Gln Arg Val Leu Gln Leu Pro Val Val Ser  
 20 25 30

ggc acc tgc gaa tgc ttc cag aag acc tac acc agc act aag gaa gcc 265  
 Gly Thr Cys Glu Cys Phe Gln Lys Thr Tyr Thr Ser Thr Lys Glu Ala  
 35 40 45

cac ccc ctg gtg gcc tct gtg tgc aat gcc tat gag aag ggc gtg cag 313  
 His Pro Leu Val Ala Ser Val Cys Asn Ala Tyr Glu Lys Gly Val Gln  
 50 55 60

agc gcc agt agc ttg gct gcc tgg agc atg gag ccg gtg gtc cgc agg 361  
 Ser Ala Ser Ser Leu Ala Ala Trp Ser Met Glu Pro Val Val Arg Arg  
 65 70 75

ctg tcc acc cag ttc aca gct gcc aat gag ctg gcc tgc cga ggc ttg 409  
 Leu Ser Thr Gln Phe Thr Ala Ala Asn Glu Leu Ala Cys Arg Gly Leu  
 80 85 90 95

gac cac ctg gag gaa aag atc ccc gcc ctc cag tac ccc cct gaa aag 457  
 Asp His Leu Glu Glu Lys Ile Pro Ala Leu Gln Tyr Pro Pro Glu Lys  
 100 105 110

att gct tct gag ctg aag gac acc atc tcc acc cgc ctc cgc agt gcc 505  
 Ile Ala Ser Glu Leu Lys Asp Thr Ile Ser Thr Arg Leu Arg Ser Ala  
 115 120 125

aga aac agc atc agc gtt ccc atc gcg agc act tca gac aag gtc ctg 553  
 Arg Asn Ser Ile Ser Val Pro Ile Ala Ser Thr Ser Asp Lys Val Leu  
 130 135 140

ggg gcc gct ttg gcc ggg tgc gag ctt gcc tgg ggg gtg gcc aga gac 601  
 Gly Ala Ala Leu Ala Gly Cys Glu Leu Ala Trp Gly Val Ala Arg Asp  
 145 150 155

act gcg gaa ttt gct gcc aac act cga gct ggc cga ctg gct tct gga 649  
 Thr Ala Glu Phe Ala Ala Asn Thr Arg Ala Gly Arg Leu Ala Ser Gly

3

160	165	170	175	
ggg gcc gac ttg gcc ttg ggc agc att gag aag gtg gtg gag tac ctc				697
Gly Ala Asp Leu Ala Leu Gly Ser Ile Glu Lys Val Val Glu Tyr Leu	180	185	190	
ctc cct gca gac aag gaa gag tca gcc cct gct cct gga cac cag caa				745
Leu Pro Ala Asp Lys Glu Glu Ser Ala Pro Ala Pro Gly His Gln Gln	195	200	205	
gcc cag aag tct ccc aag gcc aag cca agc ctc ttg agc agg gtt ggg				793
Ala Gln Lys Ser Pro Lys Ala Lys Pro Ser Leu Leu Ser Arg Val Gly	210	215	220	
gct ctg acc aac acc ctc tct cga tac acc gtg cag acc atg gcc cgg				841
Ala Leu Thr Asn Thr Leu Ser Arg Tyr Thr Val Gln Thr Met Ala Arg	225	230	235	
gcc ctg gag cag ggc cac acc gtg gcc atg tgg atc cca ggc gtg gtg				889
Ala Leu Glu Gln Gly His Thr Val Ala Met Trp Ile Pro Gly Val Val	245	250	255	
ccc ctg agc agc ctg gcc cag tgg ggt gcc tca gtg gcc atg cag gcg				937
Pro Leu Ser Ser Leu Ala Gln Trp Gly Ala Ser Val Ala Met Gln Ala	260	265	270	
gtg tcc cgg cgg agg agc gaa gtg cgg gta ccc tgg ctg cac agc ctc				985
Val Ser Arg Arg Arg Ser Glu Val Arg Val Pro Trp Leu His Ser Leu	275	280	285	
gca gcc gcc cag gag gag gat cat gag gac cag aca gac acg gag gga				1033
Ala Ala Ala Gln Glu Glu Asp His Glu Asp Gln Thr Asp Thr Glu Gly	290	295	300	
gag gac acg gag gag gag gaa gaa ttg gag act gag gag aac aag ttc				1081
Glu Asp Thr Glu Glu Glu Glu Glu Leu Glu Thr Glu Glu Asn Lys Phe	305	310	315	
agt gag gta gca gcc ctg cca ggc cct cga ggc ctc ctg ggt ggt gtg				1129
Ser Glu Val Ala Ala Leu Pro Gly Pro Arg Gly Leu Leu Gly Gly Val	320	325	330	335
gca cat acc ctg cag aag acc ctc cag acc acc atc tcg gct gtg aca				1177
Ala His Thr Leu Gln Lys Thr Leu Gln Thr Thr Ile Ser Ala Val Thr	340	345	350	
tgg gca cct gca gct gtg ctg ggc atg gca ggg agg gtg ctg cac ctc				1225
Trp Ala Pro Ala Ala Val Leu Gly Met Ala Gly Arg Val Leu His Leu	355	360	365	
aca cca gcc ccc gct gtc tcc tca acc aag ggg agg gcc atg tcc cta				1273
Thr Pro Ala Pro Ala Val Ser Thr Lys Gly Arg Ala Met Ser Leu	370	375	380	
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c

2904

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cacagctggg ctgtctgaga ctgaggtggc ggtctgctgc acgtggagag taagg atg 178  
Met  
1

tca atg aac aag ggc cca acc ctg ctg gat gga gac ctc cct gag cag 226  
Ser Met Asn Lys Gly Pro Thr Leu Leu Asp Gly Asp Leu Pro Glu Gln  
5 10 15

gag aac gtg ctc cag aga gtt ctg cag ctg cct gtg gtg agc ggg acc 274  
Glu Asn Val Leu Gln Arg Val Leu Gln Leu Pro Val Val Ser Gly Thr  
20 25 30

tgt gag tgc ttc cag aag acc tac aac agc acc aaa gaa gcc cac ccc 322  
Cys Glu Cys Phe Gln Lys Thr Tyr Asn Ser Thr Lys Glu Ala His Pro  
35 40 45

7

ctg	gtg	gcc	tct	gtg	tgc	aat	gcc	tat	gag	aag	ggt	gta	cag	ggt	gcc	370
Leu	Val	Ala	Ser	Val	Cys	Asn	Ala	Tyr	Glu	Lys	Gly	Val	Gln	Gly	Ala	
50					55					60					65	

agc	aac	ctg	gct	gcc	tgg	agc	atg	gag	ccg	gtg	gtc	cgt	cgg	ctg	tcc	418
Ser	Asn	Leu	Ala	Ala	Trp	Ser	Met	Glu	Pro	Val	Val	Arg	Arg	Leu	Ser	
				70					75						80	

acc	cag	ttc	aca	gct	gcc	aat	gag	ttg	gcc	tgc	aga	ggc	ctg	gac	cac	466
Thr	Gln	Phe	Thr	Ala	Ala	Asn	Glu	Leu	Ala	Cys	Arg	Gly	Leu	Asp	His	
			85					90						95		

ctg	gag	gaa	aag	atc	ccg	gct	ctt	caa	tac	cct	cca	gaa	aag	atc	gcc	514
Leu	Glu	Glu	Lys	Ile	Pro	Ala	Leu	Gln	Tyr	Pro	Pro	Glu	Lys	Ile	Ala	
		100					105						110			

tct	gaa	ctg	aag	ggc	acc	atc	tct	acc	cgc	ctt	cga	agc	gcc	agg	aac	562
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

agc	atc	agt	gtg	ccc	att	gca	agc	acc	tct	gac	aag	gtt	ctg	ggg	gca	610
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gagg atg gca gtc aac aaa ggc ctc acc ttg ctg gat gga gac ctc cct 169
    Met Ala Val Asn Lys Gly Leu Thr Leu Leu Asp Gly Asp Leu Pro
      1                5                10                15

gag cag gag aat gtg ctg cag cgg gtc ctg cag ctg ccg gtg gtg agt 217
Glu Gln Glu Asn Val Leu Gln Arg Val Leu Gln Leu Pro Val Val Ser
      20                25                30

ggc acc tgc gaa tgc ttc cag aag acc tac acc agc act aag gaa gcc 265
Gly Thr Cys Glu Cys Phe Gln Lys Thr Tyr Thr Ser Thr Lys Glu Ala
      35                40                45

cac ccc ctg gtg gcc tct gtg tgc aat gcc tat gag aag ggc gtg cag 313
His Pro Leu Val Ala Ser Val Cys Asn Ala Tyr Glu Lys Gly Val Gln
      50                55                60

agc gcc agt agc ttg gct gcc tgg agc atg gag ccg gtg gtc cgc agg 361
Ser Ala Ser Ser Leu Ala Ala Trp Ser Met Glu Pro Val Val Arg Arg
      65                70                75

ctg tcc acc cag ttc aca gct gcc aat gag ctg gcc tgc cga ggc ttg 409
Leu Ser Thr Gln Phe Thr Ala Ala Asn Glu Leu Ala Cys Arg Gly Leu
      80                85                90

gac cac ctg gag gaa aag atc ccc gcc ctc cag tac ccc cct gaa aag 457
Asp His Leu Glu Glu Lys Ile Pro Ala Leu Gln Tyr Pro Pro Glu Lys
      100                105                110

att gct tct gag ctg aag gac acc atc tcc acc cgc ctc cgc agt gcc 505
Ile Ala Ser Glu Leu Lys Asp Thr Ile Ser Thr Arg Leu Arg Ser Ala
      115                120                125

aga aac agc atc agc gtt ccc atc gcg agc act tca gac aag gtc ctg 553
Arg Asn Ser Ile Ser Val Pro Ile Ala Ser Thr Ser Asp Lys Val Leu
      130                135                140

ggg gcc gct ttg gcc ggg tgc gag ctt gcc tgg ggg gtg gcc aga gac 601
Gly Ala Ala Leu Ala Gly Cys Glu Leu Ala Trp Gly Val Ala Arg Asp
      145                150                155

act gcg gaa ttt gct gcc aac act cga gct ggc cga ctg gct tct gga 649
Thr Ala Glu Phe Ala Ala Asn Thr Arg Ala Gly Arg Leu Ala Ser Gly
      160                165                170                175

ggg gcc gac ttg gcc ttg ggc agc att gag aag gtg gtg gag tac ctc 697
Gly Ala Asp Leu Ala Leu Gly Ser Ile Glu Lys Val Val Glu Tyr Leu
      180                185                190
    
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gct ctg acc aac acc ctc tct cga tac acc gtg cag acc atg gcc cgg Ala Leu Thr Asn Thr Leu Ser Arg Tyr Thr Val Gln Thr Met Ala Arg 225 230 235	841
gcc ctg gag cag ggc cac acc gtg gcc atg tgg atc cca ggc gtg gtg Ala Leu Glu Gln Gly His Thr Val Ala Met Trp Ile Pro Gly Val Val 240 245 250 255	889
ccc ctg agc agc ctg gcc cag tgg ggt gcc tca gtg gcc atg cag gcg Pro Leu Ser Ser Leu Ala Gln Trp Gly Ala Ser Val Ala Met Gln Ala 260 265 270	937
gtg tcc cgg cgg agg agc gaa gtg cgg gta ccc tgg ctg cac agc ctc Val Ser Arg Arg Arg Ser Glu Val Arg Val Pro Trp Leu His Ser Leu 275 280 285	985
gca gcc gcc cag gag gag gat cat gag gac cag aca gac acg gag gga Ala Ala Ala Gln Glu Glu Asp His Glu Asp Gln Thr Asp Thr Glu Gly 290 295 300	1033
gag gac acg gag gag gag gaa gaa ttg gag act gag gag aac aag ttc Glu Asp Thr Glu Glu Glu Glu Glu Leu Glu Thr Glu Glu Asn Lys Phe 305 310 315	1081
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Pro	Leu	Ser	Ser	Leu	Ala	Gln	Trp	Gly	Ala	Ser	Val	Ala	Met	Gln	Ala			
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gtg	tcc	cgg	cgg	agg	agc	gaa	gtg	cgg	gta	ccc	tgg	ctg	cac	agc	ctc	985		
Val	Ser	Arg	Arg	Arg	Ser	Glu	Val	Arg	Val	Pro	Trp	Leu	His	Ser	Leu			
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21

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 Pro Pro Thr Cys Trp \*  
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22

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aac act ctc cgg aac acc atc tcc gca gtg acc tgg gca cct gcg gct 143  
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 35 40 45

gtg ctg ggc acg gtg gga agg atc ctg cac ctc aca cca gcc cag gct 191  
 Val Leu Gly Thr Val Gly Arg Ile Leu His Leu Thr Pro Ala Gln Ala  
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**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US03/23760

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : A61K 31/70; A01N 43/04; C07H 21/04  
 US CL : 536/23.1, 24.3, 24.5; 435/6, 325, 375; 514/44

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
 U.S. : 536/23.1, 24.3, 24.5; 435/6, 325, 375; 514/44

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

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

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/028410 A1 (BAYLOR COLLEGE OF MEDICINE) 11 April 2002 (11.04.2002), see pages 28-33.	1-14, 16, 19-20
---		-----
A		15, 17-18

Further documents are listed in the continuation of Box C.

See patent family annex.

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

Date of the actual completion of the international search 21 November 2003 (21.11.2003)	Date of mailing of the international search report <b>15 DEC 2003</b>
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Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer <i>Telicia D. Roberts for</i> Janet L. Epps-Ford, Ph.D. Telephone No. 703-308-0196
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**INTERNATIONAL SEARCH REPORT**

PCT/US03/22760

**Continuation of B. FIELDS SEARCHED Item 3:**

Registry, USPATfull, Pctfull, Wpids, CAplus, Biosis

search terms: perilipin, antisense or aptamer, triplex, ribozyme, RNAi, dsRNA, siRNA.