



Office de la Propriété
Intellectuelle
du Canada

Un organisme
d'Industrie Canada

Canadian
Intellectual Property
Office

An agency of
Industry Canada

CA 2463521 A1 2003/04/17

(21) **2 463 521**

(12) **DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION**

(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2002/10/07

(87) Date publication PCT/PCT Publication Date: 2003/04/17

(85) Entrée phase nationale/National Entry: 2004/04/07

(86) N° demande PCT/PCT Application No.: US 2002/032060

(87) N° publication PCT/PCT Publication No.: 2003/030826

(30) Priorité/Priority: 2001/10/09 (09/975,123) US

(51) Cl.Int.⁷/Int.Cl.⁷ C12Q 1/68, A01N 47/40, C12N 15/63,
C07H 21/02, C12P 19/34, C07H 21/04

(71) Demandeur/Applicant:
ISIS PHARMACEUTICALS, INC., US

(72) Inventeur/Inventor:
FREIER, SUSAN M., US

(74) Agent: BORDEN LADNER GERVAIS LLP

(54) Titre : MODULATION ANTISENS DE L'EXPRESSION DE LA PROTEINE 5 DE LIAISON DES FACTEURS DE
CROISSANCE DE TYPE INSULINE

(54) Title: ANTISENSE MODULATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 5 EXPRESSION

(57) **Abrégé/Abstract:**

Antisense compounds, compositions and methods are provided for modulating the expression of insulin-like growth factor binding protein 5. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding insulin-like growth factor binding protein 5. Methods of using these compounds for modulation of insulin-like growth factor binding protein 5 expression and for treatment of diseases associated with expression of insulin-like growth factor binding protein 5 are provided.



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

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
17 April 2003 (17.04.2003)

PCT

(10) International Publication Number
WO 03/030826 A3

(51) International Patent Classification⁷: **C12Q 1/68**, C12P
19/34, C07H 21/02, 21/04, C12N 15/63, A01N 47/40

(21) International Application Number: PCT/US02/32060

(22) International Filing Date: 7 October 2002 (07.10.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/975,123 9 October 2001 (09.10.2001) US

(71) Applicant: **ISIS PHARMACEUTICALS, INC.**
[US/US]; 2292 Faraday Avenue, Carlsbad, CA 92008
(US).

(72) Inventor: **FREIER, Susan, M.**; 2946 Renault Street, San
Diego, CA 92122 (US).

(74) Agents: **LICATA, Jane, Massey** et al.; Licata & Tyrrell
P.C., 66 E. Main Street, Marlton, NJ 08053 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,

CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,
SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN,
YU, ZA, ZM, ZW.

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

Published:

- *with international search report*
- *before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments*

(88) Date of publication of the international search report:
7 August 2003

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: ANTISENSE MODULATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 5 EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of insulin-like growth factor binding protein 5. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding insulin-like growth factor binding protein 5. Methods of using these compounds for modulation of insulin-like growth factor binding protein 5 expression and for treatment of diseases associated with expression of insulin-like growth factor binding protein 5 are provided.



WO 03/030826 A3

-1-

5 **ANTISENSE MODULATION OF INSULIN-LIKE GROWTH FACTOR BINDING**
 PROTEIN 5 EXPRESSION

10 **FIELD OF THE INVENTION**

 The present invention provides compositions and methods for modulating the expression of insulin-like growth factor binding protein 5. In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with
15 nucleic acids encoding insulin-like growth factor binding protein 5. Such compounds have been shown to modulate the expression of insulin-like growth factor binding protein 5.

BACKGROUND OF THE INVENTION

20 The insulin-like growth factor (IGF) family includes insulin-like growth factors type I and II (IGF-I and IGF-II) which are structurally related to pro-insulin. IGFs act as potent mitogens to regulate somatic growth, cellular proliferation, and cellular differentiation. Their action is
25 determined by the availability of free IGFs to interact with IGF receptors. The level of free IGFs in a cell is modulated by the rate of IGF production and clearance, and also by their interaction with insulin-like growth factor binding proteins (IGFBPs) and IGFBP-related proteins (IGFBP-rPs) (Wetterau et
30 al., *Mol. Genet. Metab.*, **1999**, 68, 161-181).

 The superfamily of insulin-like growth factor binding proteins comprises six IGFBPs that bind to IGFs with high affinity, and an emerging group of nine IGFBP-related proteins which bind to IGFs with lower affinity. Cleavage of IGFBPs by
35 proteases also modulates the levels and the action of free IGFs and IGFBPs. IGFBPs not only regulate IGF action and bioavailability, but also appear to mediate IGF-independent actions including inhibition or enhancement of cell growth and induction of apoptosis. The growth inhibitory effects of

-2-

sequestration by IGFBPs are evidenced when they competitively bind IGFs and modulate their interactions with type I IGF cell surface receptors (Wetterau et al., *Mol. Genet. Metab.*, **1999**, 68, 161-181).

5 Using the amino terminal sequence of the purified rat insulin-like growth factor binding protein 5 (also known as insulin-like growth factor binding protein 5 precursor, IGF-binding protein 5, IGFBP5, igfbp-5, and IBP5) to prepare degenerate oligonucleotide probes, the rat and human genes were
10 cloned from rat ovary and human placental cDNA libraries (Shimasaki et al., *J. Biol. Chem.*, **1991**, 266, 10646-10653). The human genes for insulin-like growth factor binding protein 5 and insulin-like growth factor binding protein 2 are physically linked, as are the homologous mouse genes, which colocalize on
15 mouse chromosome 1 in a region of synteny with the human chromosomal locus 2q33-q36 (Kou et al., *Genomics*, **1994**, 21, 653-655). The human gene was mapped to chromosome 2 using a panel of somatic cell hybrids, and by fluorescence *in situ* hybridization to the 2q33-q34 locus (Allander et al., *J. Biol. Chem.*, **1994**,
20 269, 10891-10898).

Disclosed and claimed in the PCT publications WO 92/03470 and WO 92/03471 are recombinant DNA molecules encoding insulin-like growth factor binding protein 5 and subsequences thereof, microorganisms and cell lines containing said molecules, methods
25 for preparing insulin-like growth factor binding protein 5 from these, and the purified protein itself, as well as antibodies to the protein and derivatives thereof (Kiefer and Masiarz, **1992**; Kiefer et al., **1992**).

Fetal tissues express high levels of insulin-like growth
30 factor binding protein 5 during rapid growth, but tissue levels vary in adults. Insulin-like growth factor binding protein 5 is the main IGFBP expressed in the kidney and it is found in substantial amounts in connective tissues and cerebrospinal fluid. Serum levels of insulin-like growth factor binding
35 protein 5 also decrease with age, starting after puberty. Levels in older women are 30% of those of teenagers (Wetterau et al., *Mol. Genet. Metab.*, **1999**, 68, 161-181).

Insulin-like growth factor binding protein 5 has several

-3-

unique features that suggest it is a key component of the IGF system in bone. Bone cells produce large amounts of insulin-like growth factor binding protein 5, but levels decrease during maturation due to protease activity. Unlike other IGFBPs,

5 insulin-like growth factor binding protein 5 has a high specific binding affinity for hydroxyapatite and extracellular matrix proteins, and thus it is stored in abundance within bone. It appears to act as a growth factor, stimulating bone formation via a mechanism independent of IGF-I, an effect specific for
10 insulin-like growth factor binding protein 5 and not other IGFBPs. It is the only IGFBP shown to stimulate osteoblast cell proliferation *in vitro*. A single local injection of insulin-like growth factor binding protein 5 into the outer periosteum of the parietal bone of IGF-I knockout mice increased alkaline
15 phosphatase and osteocalcin levels of calvarial bone extracts. A significant age-related decrease in insulin-like growth factor binding protein 5 could contribute in part to the age-related impairment in the coupling of bone formation to resorption (Miyakoshi et al., *J. Clin. Invest.*, **2001**, 107, 73-81).

20 Insulin-like growth factor binding protein 5 is a component of a ternary complex in human serum. The other two components of this complex are an insulin-like growth factor and a glycoprotein known as the acid labile subunit (ALS). Formation of this ternary complex stabilizes insulin-like growth factor
25 binding protein 5 levels and is hypothesized to provide a circulating reservoir of insulin-like growth factor binding protein 5 and IGFs, implying a previously unrecognized form of IGF transport and delivery in the circulation and a novel mechanism for regulating its bioavailability (Twigg and Baxter, *J. Biol. Chem.*, **1998**, 273, 6074-6079). A basic carboxyl-terminal
30 domain of the protein is the principal site in insulin-like growth factor binding protein 5 responsible for ALS binding (Twigg et al., *J. Biol. Chem.*, **1998**, 273, 28791-28798).

Specific antisera against insulin-like growth factor
35 binding protein 5 have been generated. Mouse embryos as well as tissues from mice with a targeted disruption of insulin-like growth factor binding protein 5 were used to confirm histochemically that insulin-like growth factor binding protein 5 is expressed in various tissues, such as kidney, liver,

-4-

endothelium of the gut, tubules and mesenchyme of the lung, meninges, notochord, muscle and tongue. Differences between mRNA and protein localization suggest that the protein is secreted and transported (van Kleffens et al., *Endocrinology*, **1999**, *140*, 5944-5952).

Levels of insulin-like growth factor binding protein 5 mRNA are increased in inflamed colon smooth muscle cells in an experimental model of colitis in rat (Zeeh et al., *J. Recept. Signal Transduct. Res.*, **1998**, *18*, 265-280).

Insulin-like growth factor binding protein 5 has also been implicated in prostate cancer progression (Miyake et al., *Cancer Res.*, **2000**, *60*, 3058-3064), is expressed with high frequency in neuroendocrine tumors (Wulbrand et al., *Eur. J. Clin. Invest.*, **2000**, *30*, 729-739), and has been shown to be induced upon treatment of breast cancer cells with antiestrogens used to abolish tamoxifen resistant proliferation (Huynh et al., *Cell Growth Differ.*, **1996**, *7*, 1501-1506; Parisot et al., *Breast Cancer Res. Treat.*, **1999**, *55*, 231-242).

The pharmacological modulation of insulin-like growth factor binding protein 5 activity and/or expression is therefore believed to be an appropriate point of therapeutic intervention in developmental or growth disorders, and in pathological conditions such as cancer, colitis, Duchenne muscular dystrophy, diabetes and other metabolic diseases, such as osteoporosis and osteopetrosis.

Antisense mediated inhibition of insulin-like growth factor binding protein 5 has been utilized as an investigative tool to elucidate its mitogenic action in IGF signal transduction pathways and as a therapeutic agent for prostate cancer progression in a mouse model (Miyake et al., *Cancer Res.*, **2000**, *60*, 3058-3064).

A phosphorothioate antisense oligonucleotide, 18 nucleotides in length and complementary to the translation initiation site of the mouse insulin-like growth factor binding protein 5 mRNA, was used to inhibit its expression and show that the mouse insulin-like growth factor binding protein 5, which is up-regulated after castration, potentiates IGF-I activity and accelerates progression to androgen independence. Mice bearing androgen-dependent Shionogi prostate tumors were temporally

-5-

delayed in their progression to androgen independence and recurrence of androgen independent tumors was inhibited when this antisense oligonucleotide was administered systemically (Miyake et al., *Cancer Res.*, **2000**, 60, 3058-3064).

5 Approximately 1.2 kilobase pairs of the insulin-like growth factor binding protein 5 cDNA was cloned in the antisense orientation with respect to the Moloney sarcoma virus promoter in an expression vector. This construct was used to produce antisense sequences complementary to the mRNA for investigation
10 of the role of insulin-like growth factor binding protein 5 in autocrine secretion by C2 myoblasts. Myoblasts expressing the insulin-like growth factor binding protein 5 antisense transcript differentiate prematurely and more extensively than control cells, consistent with a model in which insulin-like
15 growth factor binding protein 5 blocks IGF-stimulated myogenesis. A possible mechanism of action involves sequestration of IGFs in the extracellular matrix (James et al., *J. Cell Biol.*, **1996**, 133, 683-693).

 A phosphorothioate antisense oligonucleotide, 15
20 nucleotides in length and complementary to a region of the mRNA encompassing the start codon, was used to inhibit expression of insulin-like growth factor binding protein 5 and show that insulin-like growth factor binding protein 5 acts as a diffusible factor to sequester IGFs and/or other growth factors
25 to downregulate Duchenne muscular dystrophy (DMD) myoblast proliferation (Melone et al., *J. Cell Physiol.*, **2000**, 185, 143-153).

 A phosphorothioate antisense oligonucleotide, 21
nucleotides in length and complementary to nucleotides 813-834
30 of Genbank Accession NM_000599, has been used in two studies to inhibit the expression of insulin-like growth factor binding protein 5. In the first study, expression of insulin-like growth factor binding protein 5 is induced by the antiestrogen ICI 182780 and that insulin-like growth factor binding protein 5
35 assists the drug in inhibiting proliferation of MCF-7 breast cancer cells (Huynh et al., *Cell Growth Differ.*, **1996**, 7, 1501-1506). The same antisense oligonucleotide was later used to demonstrate that the effect of a vitamin D analogue EB1089, which also inhibits proliferation and induce apoptosis of MCF-7

-6-

breast carcinoma cells, is attenuated when insulin-like growth factor binding protein 5 is not expressed (Rozen and Pollak, *Int. J. Oncol.*, **1999**, 15, 589-594).

Disclosed and claimed in PCT Publication WO 01/05435 is a method for treating hormone-regulated tumors (for example breast and prostatic tumors) in mammals including humans, by administration of antisense oligonucleotides. The antisense oligonucleotides disclosed are designed to target the murine and human insulin-like growth factor binding protein 5 genes (Gleave, **2001**).

Currently, there are no known therapeutic agents which effectively inhibit the synthesis of human insulin-like growth factor binding protein 5. Consequently, there remains a long felt need for additional agents capable of effectively inhibiting insulin-like growth factor binding protein 5 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 therapeutic, diagnostic, and research applications for the modulation of insulin-like growth factor binding protein 5 expression.

The present invention provides compositions and methods for modulating insulin-like growth factor binding protein 5 expression.

SUMMARY OF THE INVENTION

The present invention is directed to compounds, particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding insulin-like growth factor binding protein 5, and which modulate the expression of insulin-like growth factor binding protein 5. Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of insulin-like growth factor binding protein 5 in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention.

-7-

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 of insulin-like growth factor binding protein 5 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

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding insulin-like growth factor binding protein 5, ultimately modulating the amount of insulin-like growth factor binding protein 5 produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding insulin-like growth factor binding protein 5. As used herein, the terms "target nucleic acid" and "nucleic acid encoding insulin-like growth factor binding protein 5" encompass DNA encoding insulin-like growth factor binding protein 5, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of 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, 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 insulin-like growth factor binding protein 5. 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

-8-

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 insulin-like growth factor binding protein 5. 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 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 insulin-like growth factor binding protein 5, regardless of the sequence(s) of such codons.

-9-

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 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

-10-

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.

- 5 Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified,
10 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 hydrogen bonding, which may be Watson-Crick, Hoogsteen or
15 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, refers to the capacity for precise pairing between two
20 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 complementary to each other at that position. The
25 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 "complementary" are terms which are used to indicate a
30 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 100% complementary to that of its target nucleic acid to be
35 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 cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific

-11-

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 treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the invention encompasses compounds which hybridize to these active sites.

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 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.

-12-

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 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, 1895-904) 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 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 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 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

-13-

similarly. Such modified or substituted oligonucleotides are 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 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 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds 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.

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. 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

-14-

backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the 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 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, 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 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 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, 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; 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 include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages,

-15-

mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the 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; 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 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 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 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.: 5,539,082; 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

-16-

oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular -CH₂-NH-O-CH₂-, -CH₂-N(CH₃)-O-CH₂- [known as a methylene (methylinino) or MMI backbone], -CH₂-O-N(CH₃)-CH₂-, -CH₂-N(CH₃)-N(CH₃)-CH₂- and -O-N(CH₃)-CH₂-CH₂- [wherein the native phosphodiester backbone is represented as -O-P-O-CH₂-] of the above referenced U.S. patent 5,489,677, and the amide backbones 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 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 C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxyethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH₂-O-CH₂-N(CH₃)₂, also described in examples hereinbelow.

A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3'

-17-

or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene $(-\text{CH}_2-)_n$ 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.

Other preferred modifications include 2'-methoxy $(2'-\text{O}-\text{CH}_3)$, 2'-aminopropoxy $(2'-\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}_2)$, 2'-allyl $(2'-\text{CH}_2-\text{CH}=\text{CH}_2)$, 2'-O-allyl $(2'-\text{O}-\text{CH}_2-\text{CH}=\text{CH}_2)$ and 2'-fluoro $(2'-\text{F})$. The 2'-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 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; 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 application, and each of which is herein incorporated by reference in its entirety.

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 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-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl $(-\text{C}\equiv\text{C}-\text{CH}_3)$ uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-

-18-

amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further 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-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 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, 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. 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-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 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

-19-

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 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and
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
10 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.
15 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
20 enhance the pharmacodynamic properties of oligomers, and groups
that enhance the pharmacokinetic properties of oligomers.
Typical conjugates groups include cholesterol, lipids, phospho-
lipids, biotin, phenazine, folate, phenanthridine, anthra-
quinone, acridine, fluoresceins, rhodamines, coumarins, and
25 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
30 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.
35 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. Lett.*, **1994**, 4, 1053-1060), a thioether,
e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad.*

-20-

Sci., **1992**, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, **1993**, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, 259, 327-330; Svinarchuk et al., *Biochimie*, **1993**, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, 1264, 229-237), or an octadecylamine or hexylamino-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, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an 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 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; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667;

-21-

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
application, and each of which is herein incorporated by
5 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
compound or even at a single nucleoside within an
10 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
contain two or more chemically distinct regions, each made up of
15 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
resistance to nuclease degradation, increased cellular uptake,
20 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 is a cellular endonuclease
which cleaves the RNA strand of an RNA:DNA duplex. Activation
25 of RNase H, therefore, results in cleavage of the RNA target,
thereby greatly enhancing the efficiency of oligonucleotide
inhibition of gene expression. Consequently, comparable results
can often be obtained with shorter oligonucleotides when
chimeric oligonucleotides are used, compared to phosphorothioate
30 deoxyoligonucleotides 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.

Chimeric antisense compounds of the invention may be formed
35 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.
Representative United States patents that teach the preparation

-22-

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
5 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
10 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
15 phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in
vitro and do not include antisense compositions of biological
origin, or genetic vector constructs designed to direct the in
vivo synthesis of antisense molecules.

20 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,
topical or other formulations, for assisting in uptake,
25 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;
5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721;
30 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804;
5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016;
5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528;
5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each
of which is herein incorporated by reference.

35 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
animal including a human, is capable of providing (directly or
indirectly) the biologically active metabolite or residue

-23-

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 such prodrugs, and other bioequivalents.

5 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 particular, prodrug versions of the oligonucleotides of the
10 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.

15 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.

20 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, chlorprocaine, choline, diethanolamine, dicyclohexylamine,
25 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
30 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
35 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

-24-

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, methylemaleic 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, 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, 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 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 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 formed with organic acids such as, for example, acetic acid,

-25-

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, 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 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 insulin-like growth factor binding protein 5 is treated by administering antisense compounds in accordance 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 insulin-like growth factor binding protein 5, 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 insulin-like growth factor binding protein 5 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 insulin-like growth factor binding protein 5 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

-26-

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; 5 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 10 least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and 15 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 20 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. 25 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, 30 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, dicaprate, 35 tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C₁₋₁₀ alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United

-27-

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-aqueous media, capsules, gel capsules, sachets, tablets or minitables. 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, 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, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 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, pollulans, celluloses and starches. Particularly preferred

-28-

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,
poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA),
10 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, which may conveniently be presented in unit dosage form, may be
30 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
35 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.

-29-

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-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 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. 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.

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 μm in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a

-30-

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

5 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.

10 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.

15 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 provides an o/w/o emulsion.

20 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
25 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 broadly be classified into four categories: synthetic
30 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).

35 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. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger

-31-

and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

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

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

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example,

-32-

carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

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

-33-

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
5 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
10 dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., **1989**, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three
15 to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and
20 hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive
25 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 Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel
30 Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions
35 include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol

-34-

monocaprates (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-
5 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
10 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
15 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.

20 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**,
25 *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
30 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
35 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
delivery of active components in both cosmetic and

-35-

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 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*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter

-36-

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.

Further advantages of liposomes include; liposomes obtained
5 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, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker
10 (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.

Liposomes are useful for the transfer and delivery of
15 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. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the
20 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 present several advantages over other formulations. Such
25 advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to
30 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
35 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

-37-

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).

5 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
10 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
15 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,
20 while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine
25 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
30 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
35 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

-38-

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

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

-39-

dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

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, 2C₁₂15G, that contains a PEG moiety. Illum et al. (*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. 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 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 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. 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 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 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

-40-

protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 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.

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

-41-

fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

5 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
10 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.

15 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.

20 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.

25 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

30 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
35 discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

-42-

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 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 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, 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 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-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C₁₋₁₀ 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 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).

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

-43-

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 acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Swinyard, Chapter 39 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, **1990**, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; Yamamoto et al., *J. Pharm. Exp. Ther.*, **1992**, 263, 25; Yamashita et al., *J. Pharm. Sci.*, **1990**, 79, 579-583).

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

-44-

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-
5 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*
10 *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
15 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
20 lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

25 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.

30 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
35 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

-45-

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

5. 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

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

35 Pharmaceutically acceptable organic or inorganic excipient 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

-46-

limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

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

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

Other Components

20 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
25 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
30 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,
35 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.

-47-

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.

5 Certain embodiments of the invention provide pharmaceutical 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, 10 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, 15 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, 20 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. 1206-25 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 30 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, 35 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

-48-

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.

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_{50} 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 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.

-49-

EXAMPLES**Example 1****5 Nucleoside Phosphoramidites for Oligonucleotide Synthesis
Deoxy and 2'-alkoxy amidites**

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA).

10 Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole
15 and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, **1993**, 21, 3197-3203] using commercially available phosphoramidites (Glen
20 Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites**2'-Fluorodeoxyadenosine amidites**

2'-fluoro oligonucleotides were synthesized as described
25 previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, 36, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material
30 and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP
35 and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

-50-

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and
5 conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment
10 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.

2'-Fluorouridine

15 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% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

20

2'-Fluorodeoxycytidine

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

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

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of
30 Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenyl-
35 carbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced

-51-

pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether
5 (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The
10 material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

15 **2'-O-Methoxyethyl-5-methyluridine**

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at
20 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL)
25 and evaporated. A silica gel column (3 kg) was packed in CH₂Cl₂/acetone/MeOH (20:5:3) containing 0.5% Et₃NH. The residue was dissolved in CH₂Cl₂ (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional
30 material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue
35 dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added

-52-

and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH₃CN (200 mL). The residue was dissolved in CHCl₃ (1.5 L) and extracted with 2x500 mL of saturated NaHCO₃ and 2x500 mL of saturated NaCl. The organic phase was dried over Na₂SO₄, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et₃NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

15 **3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl₃ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl₃. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

35 **3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine**

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH₃CN (700 mL) and set aside. Triethylamine (189 mL, 1.44

-53-

M) was added to a solution of triazole (90 g, 1.3 M) in CH₃CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl₃ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO₃ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH₃ gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl₃ (700 mL) and extracted with saturated NaHCO₃ (2x300 mL) and saturated NaCl

-54-

(2x300 mL), dried over MgSO_4 and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl)-phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO_3 (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over MgSO_4 and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

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

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known 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 of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl- O^2 -2'-anhydro-5-methyluridine

O^2 -2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at

-55-

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 reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

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

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-*tert*-Butyldiphenylsilyl-2'-O-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 was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel,

-56-

ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

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). It was then dried over P₂O₅ under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

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 CH₂Cl₂ (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 was washed with ice cold CH₂Cl₂ and the combined organic phase was washed with water, brine and dried over anhydrous Na₂SO₄. The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting

-57-

mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5

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

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH₂Cl₂). Aqueous NaHCO₃ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na₂SO₄, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO₃ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH₂Cl₂ to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

35

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

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then

-58-

added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH₂Cl₂). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH₂Cl₂ to get 2'-O-(dimethylaminoxyethyl)-5-methyluridine (766mg, 92.5%).

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

2'-O-(dimethylaminoxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P₂O₅ under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH₂Cl₂ (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

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

5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P₂O₅ under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N¹,N¹-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous NaHCO₃ (40mL). Ethyl acetate layer was dried over anhydrous Na₂SO₄ and concentrated. Residue

-59-

obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminoethoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

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
10 thymidine nucleoside amidites are prepared similarly.

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

15 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-
20 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., Guinasso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-
25 (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
30 may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may 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].

35

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

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-

-60-

CH₂-O-CH₂-N(CH₂)₂, 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

5 2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O²-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg)
10 are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The
15 aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column
20 fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

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

25 To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH₂Cl₂
30 (2x200 mL). The combined CH₂Cl₂ layers are washed with saturated NaHCO₃ solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH₂Cl₂:Et₃N (20:1, v/v, with 1% triethylamine) gives the title compound.

35

-61-

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.) are 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 CH₂Cl₂ (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

15 Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

20 Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphate 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.

35 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

-62-

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, 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 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 described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide

-63-

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 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

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" 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".

[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 380B, 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 increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being

-64-

desalted on a G25 size exclusion column. The oligo recovered is then 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 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 3,4-dihydro-2H-benzodithiole-3-one 1,1 dioxides (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 column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on

-65-

denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and 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.

10 **Example 7**

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 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-cyanoethyldiisopropyl 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 known literature or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°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.

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/ACETM MDQ) or, for individually prepared

-66-

samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 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 compounds on the plate were at least 85% full length.

Example 9**10 Cell culture and oligonucleotide treatment**

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 4 cell types are 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.

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 McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they 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 treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

-67-

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

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 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 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.

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 μ L OPTI-MEMTM-1 reduced-serum medium (Gibco BRL) and then treated with 130 μ L of OPTI-MEMTM-1 containing 3.75 μ g/mL LIPOFECTINTM (Gibco BRL) and the desired concentration of 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 concentration for a particular cell line, the cells are treated

-68-

with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, **TCCGTCATCGCTCCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCATTCTGCCCCCAAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf 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 line is deemed as unsuitable for oligonucleotide transfection experiments.

Example 10

Analysis of oligonucleotide inhibition of insulin-like growth factor binding protein 5 expression

Antisense modulation of insulin-like growth factor binding protein 5 expression can be assayed in a variety of ways known in the art. For example, insulin-like growth factor binding protein 5 mRNA levels can be quantitated by, e.g., Northern blot analysis, 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. 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 Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be

-69-

conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

5 Protein levels of insulin-like growth factor binding protein 5 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 insulin-like growth factor
10 binding protein 5 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,
15 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.
20 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
25 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.
30 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

Example 11

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al.,
35 *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. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and

-70-

each well was washed with 200 μ L cold PBS. 60 μ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 μ 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 with 200 μ 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 μ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was 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.

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 on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 100 μ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 μ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The 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 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the

-71-

QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 µL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.

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 insulin-like growth factor binding protein 5 mRNA Levels

Quantitation of insulin-like growth factor binding protein 5 mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 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 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., JOE, FAM, or VIC, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) 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,

-72-

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 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 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 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 PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM MgCl₂, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse

-73-

transcriptase) to 96 well plates containing 25 μ L total RNA 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 AMPLITAQ GOLD™, 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).

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 simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent from Molecular Probes. Methods of RNA quantification by RiboGreen™ are taught in Jones, L.J., et al, *Analytical Biochemistry*, **1998**, 265, 368-374.

In this assay, 175 μ L of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:2865 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 25uL 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 insulin-like growth factor binding protein 5 were designed to hybridize to a human insulin-like growth factor binding protein 5 sequence, using published sequence information (GenBank accession number M65062, incorporated herein as SEQ ID NO:3). For human insulin-like growth factor binding protein 5 the PCR primers were: forward primer: CCAAACACACCCGCATCTC (SEQ ID NO: 4) reverse primer: TTGGACTGGGTCAGCTTCTTTC (SEQ ID NO: 5) and the PCR probe was: FAM-AGGCTGAAGCAGTGAAGAAGGACCGC-TAMRA (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For human GAPDH the PCR primers were: forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 7) reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 8) and the PCR

-74-

probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

5

Example 14**Northern blot analysis of insulin-like growth factor binding protein 5 mRNA levels**

10 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 following manufacturer's recommended protocols. Twenty
15 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 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
20 UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA) and then probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's
25 recommendations for stringent conditions.

To detect human insulin-like growth factor binding protein 5, a human insulin-like growth factor binding protein 5 specific probe was prepared by PCR using the forward primer CCAAACACACCCGCATCTC (SEQ ID NO: 4) and the reverse primer
30 TTGGACTGGGTCAGCTTCTTTC (SEQ ID NO: 5). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

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

- 75 -

RTSP-0423**Example 15****Antisense inhibition of human insulin-like growth factor binding protein 5 expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap**

5 In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human insulin-like growth factor binding protein 5 RNA, using published sequences (GenBank accession number M65062, incorporated herein as SEQ ID NO: 3, GenBank accession number

10 NM_000599, incorporated herein as SEQ ID NO: 10, residues 135001-156000 of Genbank accession number AC007563, incorporated herein as SEQ ID NO: 11, and GenBank accession number AF147308, incorporated herein as SEQ ID NO: 12). The oligonucleotides are shown in Table 1. "Target site" indicates the first (5'-most)

15 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 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-

20 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 insulin-like growth factor

25 binding protein 5 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 1

30 Inhibition of human insulin-like growth factor binding protein 5 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
154897	Start Codon	3	36	gagcaacaccatcttctctt	87	13
154898	Coding	3	65	taggcggccagcagcaggag	72	14
154899	Coding	3	299	gcgtgcagcggcttctcctc	69	15
154900	Coding	3	306	cagcagggcgtgcagcggct	78	16
154901	Coding	3	333	ttcgttgaggcaaaccgccgc	50	17

- 76 -

RTSP-0423

154902	Coding	3	338	ctcttttcggttgaggcaaac	67	18
154903	Coding	3	342	gtagctcttttcggttgaggc	87	19
154904	Coding	3	497	tttctgcggtccttcttcac	12	20
154905	Coding	3	503	agcttcttttctgcggtcctt	90	21
154906	Coding	3	511	actgggtcagcttctttctg	43	22
154907	Coding	3	518	aacttggtactgggtcagctt	79	23
154908	Coding	3	580	cctgtctcatctcaggtgca	62	24
154909	Coding	3	651	catgcgtgggtcgtggtttga	78	25
154910	Coding	3	656	ggcaccatgcgtgggtcgtgc	76	26
154911	Coding	3	676	tgggcaggtacacagcacgg	82	27
154912	Coding	3	698	tagaatcctttgcggtcaca	72	28
154913	Coding	3	703	tcttgtagaatcctttgcgg	56	29
154914	Coding	3	709	gctttctcttgtagaatcct	57	30
154915	3'UTR	3	986	gtcctcagtttctcaaata	74	31
154916	3'UTR	3	991	ccgaggtcctcagtttctc	89	32
154917	3'UTR	3	996	agattccgaggtcctcagtt	87	33
154918	3'UTR	3	1001	gctagagattccgaggtcct	89	34
154919	3'UTR	3	1006	cccttgctagagattccgag	94	35
154920	3'UTR	3	1186	cctaactctattcgtctttc	61	36
154921	3'UTR	3	1394	ggagaggtcctggagtgttt	0	37
154922	3'UTR	3	1579	gatctgcttccgaaaacctc	86	38
154923	5'UTR	10	11	ctttgcagctctttcctagc	80	39
154924	5'UTR	10	584	cttgcaaaaatgtagagaga	73	40
154925	Intron 1	11	4214	gctttggctgcctgccacct	79	41
154926	Intron 1: Exon 2	11	16817	ctgcaggagaaggagccgga	72	42
154927	3'UTR	12	197	actgtagtcaccgtggaaga	88	43

As shown in Table 1, SEQ ID NOs 13, 14, 15, 16, 17, 18, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 38, 39, 40, 41, 42 and 43 demonstrated at least 50% inhibition of human insulin-like growth factor binding protein 5 expression in this assay and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of the present invention.

Example 16

Western blot analysis of insulin-like growth factor binding protein 5 protein levels

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 transferred to membrane for western blotting. Appropriate

- 77 -

primary antibody directed to insulin-like growth factor binding protein 5 is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™

5 (Molecular Dynamics, Sunnyvale CA).

1

SEQUENCE LISTING

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

<120> ANTISENSE MODULATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN
5 EXPRESSION

<130> RTSP-0423

<150> 09/975,123

<151> 2001-10-09

<160> 43

<210> 1

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 1

tccgtcatcg ctcctcaggg

20

<210> 2

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 2

atgcattctg cccccaagga

20

<210> 3

<211> 1612

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (44) ... (862)

<400> 3

ctctcctgcc ccaccccgag gttaaaggggg cgactaagag aag atg gtg ttg ctc
Met Val Leu Leu

55

1

acc gcg gtc ctc ctg ctg ctg gcc gcc tat gcg ggg ccg gcc cag agc	103
Thr Ala Val Leu Leu Leu Leu Ala Ala Tyr Ala Gly Pro Ala Gln Ser	
5 10 15 20	
ctg ggc tcc ttc gtg cac tgc gag ccc tgc gac gag aaa gcc ctc tcc	151
Leu Gly Ser Phe Val His Cys Glu Pro Cys Asp Glu Lys Ala Leu Ser	
25 30 35	
atg tgc ccc ccc agc ccc ctg ggc tgc gag ctg gtc aag gag ccg ggc	199
Met Cys Pro Pro Ser Pro Leu Gly Cys Glu Leu Val Lys Glu Pro Gly	
40 45 50	
tgc ggc tgc tgc atg acc tgc gcc ctg gcc gag ggg cag tcg tgc ggc	247
Cys Gly Cys Cys Met Thr Cys Ala Leu Ala Glu Gly Gln Ser Cys Gly	
55 60 65	
gtc tac acc gag cgc tgc gcc cag ggg ctg cgc tgc ctc ccc cgg cag	295
Val Tyr Thr Glu Arg Cys Ala Gln Gly Leu Arg Cys Leu Pro Arg Gln	
70 75 80	
gac gag gag aag ccg ctg cac gcc ctg ctg cac ggc cgc ggg gtt tgc	343
Asp Glu Glu Lys Pro Leu His Ala Leu Leu His Gly Arg Gly Val Cys	
85 90 95 100	
ctc aac gaa aag agc tac cgc gag caa gtc aag atc gag aga gac tcc	391
Leu Asn Glu Lys Ser Tyr Arg Glu Gln Val Lys Ile Glu Arg Asp Ser	
105 110 115	
cgt gag cac gag gag ccc acc acc tct gag atg gcc gag gag acc tac	439
Arg Glu His Glu Glu Pro Thr Thr Ser Glu Met Ala Glu Glu Thr Tyr	
120 125 130	
tcc ccc aag atc ttc cgg ccc aaa cac acc cgc atc tcc gag ctg aag	487
Ser Pro Lys Ile Phe Arg Pro Lys His Thr Arg Ile Ser Glu Leu Lys	
135 140 145	
gct gaa gca gtg aag aag gac cgc aga aag aag ctg acc cag tcc aag	535
Ala Glu Ala Val Lys Lys Asp Arg Arg Lys Lys Leu Thr Gln Ser Lys	
150 155 160	
ttt gtc ggg gga gcc gag aac act gcc cac ccc cgg atc atc tct gca	583
Phe Val Gly Gly Ala Glu Asn Thr Ala His Pro Arg Ile Ile Ser Ala	
165 170 175 180	
cct gag atg aga cag gag tct gag cag ggc ccc tgc cgc aga cac atg	631
Pro Glu Met Arg Gln Glu Ser Glu Gln Gly Pro Cys Arg Arg His Met	
185 190 195	
gag gct tcc ctg cag gag ctc aaa gcc agc cca cgc atg gtg ccc cgt	679
Glu Ala Ser Leu Gln Glu Leu Lys Ala Ser Pro Arg Met Val Pro Arg	
200 205 210	
gct gtg tac ctg ccc aat tgt gac cgc aaa gga ttc tac aag aga aag	727
Ala Val Tyr Leu Pro Asn Cys Asp Arg Lys Gly Phe Tyr Lys Arg Lys	
215 220 225	
cag tgc aaa cct tcc cgt ggc cgc aag cgt ggc atc tgc tgg tgc gtg	775
Gln Cys Lys Pro Ser Arg Gly Arg Lys Arg Gly Ile Cys Trp Cys Val	

3

230	235	240	
gac aag tac ggg atg aag ctg cca ggc atg gag tac gtt gac ggg gac			823
Asp Lys Tyr Gly Met Lys Leu Pro Gly Met Glu Tyr Val Asp Gly Asp			
245	250	255	260
ttt cag tgc cac acc ttc gac agc agc aac gtt gag tga tgcgtcccc			872
Phe Gln Cys His Thr Phe Asp Ser Ser Asn Val Glu			
	265	270	
cccaaccttt ccctcacccc ctcccacccc cagccccgac tccagccagc gcctccctcc			932
accccaggac gccactcatt tcattctcatt taagggaata atatatatct atctatttga			992
ggaaactgag gacctcggaa tctctagcaa gggctcaact tcgaaaatgg caacaacaga			1052
gatgcaaaaa gctaaaaaga ccccccccc ctttaaattgg ttttcttttt gaggcaagtt			1112
ggatgaacag agaagggaag agaggaagaa cgagaggaag agaagggaag gaagtgtttg			1172
tgtagaagag agagaaagac gaatagagtt aggaaaagga agacaagcag gtgggcagga			1232
aggacatgca ccgagaccag gcaggggccc aactttcacg tccagccctg gcctggggtc			1292
gggagaggtg ggcgctagaa gatgcagccc aggatgtggc aatcaatgac actattgggg			1352
tttcccagga tggattggtc agggggagaa aggaaaaggc aaaacactcc aggacctctc			1412
ccggatctgt ctctctctct agccagcagt atggacagct ggacccctga acttcctctc			1472
ctcttacctg ggcagagtgt tgtctctccc caaatattata aaaactaaaa tgcattccat			1532
tcctctgaaa gcaaaacaaa ttcataattg agtgatatta aatagagagg ttttcggaag			1592
cagatctgtg aatatgaaat			1612

<210> 4

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR Primer

<400> 4

ccaaacacac ccgcatctc

19

<210> 5

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR Primer

<400> 5
ttggactggg tcagcttctt tc 22

<210> 6
<211> 26
<212> DNA
<213> Artificial Sequence

<220>

<223> PCR Probe

<400> 6
aggctgaagc agtgaagaag gaccgc 26

<210> 7
<211> 19
<212> DNA
<213> Artificial Sequence

<220>

<223> PCR Primer

<400> 7
gaaggtgaag gtcggagtc 19

<210> 8
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> PCR Primer

<400> 8
gaagatggtg atgggatttc 20

<210> 9
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> PCR Probe

<400> 9
caagcttccc gttctcagcc 20

<210> 10
<211> 1722
<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (752) ... (1570)

<400> 10

```

ggggaaaaga gctaggaaag agctgcaaag cagtgtgggc tttttccctt tttttgctcc      60
ttttcattac ccctcctccg ttttcaccct tctccggact tcgcgtagaa cctgcgaatt      120
tcgaagagga ggtggcaaag tgggagaaaa gaggtgttag ggtttggggg ttttttgttt      180
ttgtttttgt tttttaattt cttgatttca acattttctc ccaccctctc ggctgcagcc      240
aacgcctctt acctgttctg cggcgccgcg caccgctggc agctgagggt tagaaagcgg      300
ggtgtatttt agattttaag caaaaatttt aaagataaat ccatttttct ctcccacccc      360
caacgccatc tccactgcat ccgatctcat tatttcgggtg gttgcttggg ggtgaacaat      420
tttgtggcct tttttccctt ataattctga cccgctcagg cttgagggtt tctccggcct      480
ccgctcactg cgtgcacctg gcgctgccct gcttccccca acctgttgca aggctttaat      540
tcttgcaact gggacctgct cgcaggcacc ccagccctcc acctctctct acatttttgc      600
aagtgtctgg gggagggcac ctgctctacc tgccagaaat tttaaaacaa aaacaaaaac      660
aaaaaaatct ccggggggccc tcttgggccc tttatccctg cactctcgct ctctgcccc      720
accccgaggt aaagggggcg actaagagaa g atg gtg ttg ctc acc gcg gtc      772
                               Met Val Leu Leu Thr Ala Val
                               1                               5

ctc ctg ctg ctg gcc gcc tat gcg ggg ccg gcc cag agc ctg ggc tcc      820
Leu Leu Leu Leu Ala Ala Tyr Ala Gly Pro Ala Gln Ser Leu Gly Ser
                               10                               15                               20

ttc gtg cac tgc gag ccc tgc gac gag aaa gcc ctc tcc atg tgc ccc      868
Phe Val His Cys Glu Pro Cys Asp Glu Lys Ala Leu Ser Met Cys Pro
                               25                               30                               35

ccc agc ccc ctg ggc tgc gag ctg gtc aag gag ccg ggc tgc ggc tgc      916
Pro Ser Pro Leu Gly Cys Glu Leu Val Lys Glu Pro Gly Cys Gly Cys
                               40                               45                               50                               55

tgc atg acc tgc gcc ctg gcc gag ggg cag tcg tgc ggc gtc tac acc      964
Cys Met Thr Cys Ala Leu Ala Glu Gly Gln Ser Cys Gly Val Tyr Thr
                               60                               65                               70

gag cgc tgc gcc cag ggg ctg cgc tgc ctc ccc cgg cag gac gag gag      1012
Glu Arg Cys Ala Gln Gly Leu Arg Cys Leu Pro Arg Gln Asp Glu Glu
                               75                               80                               85

aag ccg ctg cac gcc ctg ctg cac ggc cgc ggg gtt tgc ctc aac gaa      1060
Lys Pro Leu His Ala Leu Leu His Gly Arg Gly Val Cys Leu Asn Glu

```

6

90	95	100	
aag agc tac cgc gag caa gtc aag atc gag aga gac tcc cgt gag cac Lys Ser Tyr Arg Glu Gln Val Lys Ile Glu Arg Asp Ser Arg Glu His 105 110 115			1108
gag gag ccc acc acc tct gag atg gcc gag gag acc tac tcc ccc aag Glu Glu Pro Thr Thr Ser Glu Met Ala Glu Glu Thr Tyr Ser Pro Lys 120 125 130 135			1156
atc ttc cgg ccc aaa cac acc cgc atc tcc gag ctg aag gct gaa gca Ile Phe Arg Pro Lys His Thr Arg Ile Ser Glu Leu Lys Ala Glu Ala 140 145 150			1204
gtg aag aag gac cgc aga aag aag ctg acc cag tcc aag ttt gtc ggg Val Lys Lys Asp Arg Arg Lys Lys Leu Thr Gln Ser Lys Phe Val Gly 155 160 165			1252
gga gcc gag aac act gcc cac ccc cgg atc atc tct gca cct gag atg Gly Ala Glu Asn Thr Ala His Pro Arg Ile Ile Ser Ala Pro Glu Met 170 175 180			1300
aga cag gag tct gag cag ggc ccc tgc cgc aga cac atg gag gct tcc Arg Gln Glu Ser Glu Gln Gly Pro Cys Arg Arg His Met Glu Ala Ser 185 190 195			1348
ctg cag gag ctc aaa gcc agc cca cgc atg gtg ccc cgt gct gtg tac Leu Gln Glu Leu Lys Ala Ser Pro Arg Met Val Pro Arg Ala Val Tyr 200 205 210 215			1396
ctg ccc aat tgt gac cgc aaa gga ttc tac aag aga aag cag tgc aaa Leu Pro Asn Cys Asp Arg Lys Gly Phe Tyr Lys Arg Lys Gln Cys Lys 220 225 230			1444
cct tcc cgt ggc cgc aag cgt ggc atc tgc tgg tgc gtg gac aag tac Pro Ser Arg Gly Arg Lys Arg Gly Ile Cys Trp Cys Val Asp Lys Tyr 235 240 245			1492
ggg atg aag ctg cca ggc atg gag tac gtt gac ggg gac ttt cag tgc Gly Met Lys Leu Pro Gly Met Glu Tyr Val Asp Gly Asp Phe Gln Cys 250 255 260			1540
cac acc ttc gac agc agc aac gtt gag tga tgcgtcccc cccaaccttt His Thr Phe Asp Ser Ser Asn Val Glu 265 270			1590
ccctcacccc ctcccacccc cagccccgac tccagccagc gcctccctcc accccaggac			1650
gccactcatt tcattctcatt taagggaata atatatatct atctatttga ggaaaaaaaa			1710
aaaaaaaaaa aa			1722

<210> 11

<211> 21000

<212> DNA

<213> Homo sapiens

<220>

<400> 11

```

ggaggggaagg ggagacgaag ttactctcct cattgtgttc accctgctcc gaagaactct      60
gtcttccact ggccctcca cctcctcccc attctcggta gcccagcct gtcccccttg      120
cccccttctt acattccggg gggaggagg cgctgttcag aggggaggag ggcgctgttc      180
agggagcgaa ggggagcccc cttgtgtcta gaaggcctct cccaccccc acccgtgtg      240
agtttgtact gcaaagctcc ttggcatcct tgcctgagtt ggggtgttggg aagctcaaatt      300
tgcagctaca aactggctgg cagccagggg ccggctatctt aaaagcgcct gctctcccgg      360
agccccgtag tctctttgga aacttctgca ggggaaaaga gctaggaaag agctgcaaag      420
cagtgtgggc tttttccctt ttttgcctct tttcattacc cctcctccgt tttcaccctt      480
ctccggactt cgcgtagaac ctgcgaattt cgaagaggag gtggcaaagt gggagaaaag      540
aggtgttagg gtttgggggt tttttgtttt tgtttttgtt ttttaatttc ttgatttcaa      600
cattttctcc caccctctcg gctgcagcca acgcctctta cctgttctgc ggcgccgcgc      660
accgctggca gctgagggtt agaaagcggg gtgtatttta gattttaagc aaaaatttta      720
aagataaatc catttttctc tcccaccccc aacgccatct cactgcac ccatctcatt      780
atttcgggtg ttgcttgggg gtgaacaatt ttgtggcttt ttttccccta taattctgac      840
ccgctcaggc ttgagggttt ctccggcctc cgctcactgc gtgcacctgg cgctgccctg      900
cttcccccaa cctgttgcaa ggctttaatt cttgcaactg ggacctgctc gcaggcaccc      960
cagccctcca cctctctcta catttttgca agtgtctggg ggagggcacc tgctctacct      1020
gccagaaatt ttaaaacaaa aacaaaaaca aaaaaatctc cgggggcccct cttggcccct      1080
ttatccctgc actctcgctc tcctgccccca ccccgaggta aagggggcga ctaagagaag      1140
atgggtgttg tcaccgcggg cctcctgctg ctggccgcct atgcgggggc ggcccagagc      1200
ctgggctcct tcgtgcactg cgagccctgc gacgagaaag ccctctccat gtgccccccc      1260
agccccctgg gctgcgagct ggtcaaggag ccgggctgcg gctgctgcat gacctgcgcc      1320
ctggccgagg ggcagtcgtg cggcgtctac accgagcgct gcgcccaggg gctgcgctgc      1380
ctcccccggc aggacgagga gaagccgctg cacgccctgc tgcacggccg cggggtttgc      1440
ctcaacgaaa agagctaccg cgagcaagtc aagatcgggtg agcgcgctca gtgtgccagt      1500
cagttacgcg gcgcacgggc gggggacacg agaccggctg ggcccgcgcg ctttgcgag      1560
caagtggctt cgagctgggg tgcagctcgg gagtagtcgg ggagggtcct tgcaccccg      1620
aattggagcc ctggaggatc ctgctacccg ggggaactgt ctagccgagt ctatcccagc      1680

```

tttcgctctt tctccttcct cctacccaca cgttgcccgc cccctcccc tactctgtcc 1740
gaatactgat tctgagctct tattcgggtca caagggtccaa atcccctggg accctatcta 1800
cagtctgaaa gagtgtatgt tacattatca actccctcct ccacccccct cgcgccccgc 1860
atagggttttt ttctgaactt ggaaaaaaaa tctcttttgt taaaatatta ttttaattgc 1920
ggcctggaga gagaggcatt acctatcttc tgagctaatt ccacctttgt ccctcttggc 1980
ccctccaccc cctgcctcc agacgtttct atccctccac ccctacacac acaccacaaa 2040
caccacacat ccttggtagc taatgccttt cgggtgggag ccctgaagcc cctcccatgt 2100
gcataaccat tgctttttat tgaagatgat tgcctgtggg agatggtaaa acatatttaa 2160
ctgcaaaatg actttttatt tatcccagga agggaaaaaa atactttagg agtgcggggg 2220
tgcggggtgg ggggtggggaa gagtagaaag gaagggtttg gtattctgtg cctggtgttc 2280
gtttctgaat ctaattcctt ttcccctct ccaattcaga ttttacctag gatgaaagg 2340
ggacataggg ctgggaggca aaaggggatg tgagattcag agctttcaac ttctcgcct 2400
atgcagattc ctggcaccac caccacctcc acccccgccc cgcaattcct ccctccgcaa 2460
tccccccacc ccgcctccc caatctctta aaccagatca caagtgggtg tggattaagt 2520
gcatgagggg ccctggaaac gcagaatgca aaattcaggg ggcggagaca gaaaagccac 2580
gcactgggag cctcgcctcc gcttgccccg ccccggggtt gcgccgcccc caaattctcg 2640
gcgccaggag tctagggttg aaatgattga cacagctact tgttcaatca gaacagttct 2700
acccccttag actgagtttc tttttggaaa gagttacctg atcttttagt aaaaaaaaaa 2760
aaagttaa ataaactacata aaagtctggg ttctaagacg tcaaactggc tattgattct 2820
ctccaaaagg ggttaaaact tggctaaaca atgtttattt ggcgtagttt agttacggag 2880
acagtttagt tacgggggca atccccttct ccctccctgt ccctttccgt tactcacgag 2940
caccctaaca tgagttttct gaagtgc aaa tttcagtcac tatagtgcag gagaggagt 3000
cgtccgcgct tgccaggagg ccggagagct tcctgtgttc agcctcagtc cccgcaaacc 3060
ctgcagggtt tgagtggcgc agggggccagc ccctctagct ttgcaagagg tagtcgatct 3120
ccaaacctgc aaaaagtcga tctttttgca gggtgcagcc tgcaaaaaga ggaaaagggg 3180
aggatgtgca ggctctggga accccagaga ccggcttgtg agattatttt tattaagatc 3240
cacttttcaa aggcggtcct gccgccttc atcttggggg atgtggattt ggaaaagcct 3300
agcggagaa gaaaacatta attgatttcc cggcttggga gccaccgccc tgggtaacaa 3360
tccagtcaca ccgaaagaac gtaagggtgc actcagccgc atcctggctc cacttattat 3420
ttaccaagcg tgtagtgtga tgtggctagt gtgaagggtg tacacgcaga gcgcacgcgc 3480

gcgcaattgc taggcgagca cggagggcgc atcacacaca cacacacaca cacacacaca 3540
 cacacacaca cagcactct cagtcccaag caagatcccc tgtagaattc cctgccgccc 3600
 cttgctcaac cccaccttc tgagtgcctt tgggccaacc cgaagccgcc tcttctaccc 3660
 gccccctccc cagcactcct ctccgactcc accccgccga aggcagggtgc ccggccggct 3720
 gctggtgagg gttggcgagg ctccaccgct gctggtgccg acatcttctt cccgaacaga 3780
 tgggatggga gtgggcgctc ctggaaaggg cgttttagcc ggacaaccac tgggcgggtc 3840
 cccaaaaagt tggagatggt gtagagaccg ttcttaagga gagagaaatg gacagagaga 3900
 gggagagtgc ctttttggtc ctttagccaa aattcctgct tcccaacaca cctcctacct 3960
 cctcctctc cccaccccc gccaaaatct gagcttgagc atatggattt gccccctccc 4020
 tcccgagtt gcagcatata cacacacaca cacacacaca cacacacaca cacacacag 4080
 acatattata tctatataat tatatattgt atatatggta tatactatat tatatatatt 4140
 tatatgtaac caaacatgta gaacccaaaa aaggtgcatt tctggaatca gaaatgggga 4200
 gcagaagaga gtgaggtggc aggcagccaa agcctctggg agggaaagga gagggcggag 4260
 gagaggggtg tttgctaggc tggtgctgag caactggaga gggagtgggc ccgagagcag 4320
 ggtgctgaga gcgagcctgg ctctgcattt cattctggcc agttcaggag cagagataga 4380
 tacggaaaaa agaatgtgtg tgaggctgga gggagatgaa agacgggagt tatttgatgg 4440
 gcaaagctca gtaaatagcg gtgagtggag ggagtcaagg aagtactggg tttgctttcg 4500
 ggcatttttg gtggggaatg aggtttgatg ggctgggtccc cagcatagat accgggggttc 4560
 gggtaggtct cctctgtgct ggtgagacat tcagtttaag atgtagggag ccgtgggttc 4620
 tggctttcgg ttctgtcatt aagaaatgag acaccgtggg acaaaaaata ggcttctgga 4680
 aaagaaagca aaagcattac ctaaataaaa gtgaattcag cttttgcttc tttgaggggt 4740
 ttggcctcca tcaccctggc ttcaactcca agcctgggtc aaaatgccat gttgaagtcg 4800
 agttccagag ttaggtttat tggccaaatt tatgtctggg ttgagcaaga tcatggaaaa 4860
 ctgaaggagc caaatcctt tcccaccgc accccctccc tctgaaacaa gtctttgaga 4920
 ggatggcgtg ccctgagcca accaaaacgg gagttgggaa aacgagatgg ggctttaact 4980
 ggacttttct cgccacctc gccaccgcc ccgcccccg cccaccgcg cccccctccc 5040
 ccacctttct gctgtgtcag ctgagactgg aagaacaagc agagttagcc ctagtgagag 5100
 ctcatattct ttccatttct ctttttcccc ttctcacc ctcctccta ctcttcctc 5160
 cgaccttctc catccacccc ccgaccccc accccaactc taaggcatcc cctgaggggt 5220

tgagtttttaa gtcagagcag ctgtgagtta gtcccggggc ttgggctgga tttgagcagg 5280
caggtggggc tgcagctgca gaggggtggac cagcctgacc ctaggagcca gaggtcatcg 5340
tgaggggttac agcaaagggc atgggagcag gaattccctg cagaagccct ggccccctta 5400
atggagcga gaagccagcg ggaagggaag gtaatacttg tctaaagttt ctactttgta 5460
cctgagcaat gcctatgaaa cttttaagat atttagtgtg tggaaaataa aactctggag 5520
aagagatcac cacactccag acccactaca tccagaaact ggaagaagat cataaagagc 5580
aggtaccaac cagggggccag agcagaaggg aagaggggtg gagaatggct gcgttgagtc 5640
aagcaggcga tttggagggg tttgtttagt ttacatttg ctttttctct acagttggtc 5700
caaaagctgc aattcccaga gactatcctt aaattgcaat caggacatgt tattcaaac 5760
agtaagactg attacacatt gattacatac ttgcaaaaat aaataataaa ataaacaagg 5820
atggcagtgt aggcttctac tcccctcccc caataccagt gaaatcccc ccttttatth 5880
ccagtttcat ttctttccat aaattctgtt atgatgtcat ttctctctct ctctctctth 5940
ttaaaaaata taaatccaga ggagtgactg aaaatctctg tgaggaggag aggttatatt 6000
cccaccttgg ccactatgct gttggtttag agcacctaac caggttagtt agttagtgcc 6060
tgggcactca gcttagctca gtccatgtgt ctctccagga attgtctggg tattggccaa 6120
ataatgttaa agctggaagg aaccatagc catcatcaca gtcaagggtc tagttttaga 6180
atgaggatac tgaggccctg agtgggaagt cacttgcca agatgtggca gcagggttag 6240
agacagacaa ggggctggga ctgagctctt tccgcaggg gctccagcac tgcattcagc 6300
cattcctggc tttctcgta gtactgggg ctcatgcctg gacctgggct aacctgcagt 6360
aaggcctttg gcaaacactg ccagatgcta tcactccatg acataagaac ccataatctg 6420
aaggcactct gttttagggg cagtaggaaa aggagtgaag cggccccaaa gtggagaggg 6480
ccacactctc ttattttcac actcacttct gtcccttttt ctacacagtc ttgcatacaa 6540
gcctggctta ggctagtggg ttggtcagag aaagaggacc aagaggggtg gaaggagggg 6600
tgttcacccc agcttttcagg tccatgcgcc aaacctcatt ttctacatga caggaagctc 6660
tcctgatgtg gagaggcagg caggcctggc tgcctctgag ctgtgcagcg gcctgtagtc 6720
ttgaagaaca tgggtgcagaa gtggcccgt tccaacagca cagtcctcac gtggtgcca 6780
cagaaagcag agatcgagag aggggtgtggg tggatggggg gagtgcagta actaggaagt 6840
tctgcatccg aaggagagaa ggaaaaccat ataccaccaa agctgataag gaagagagca 6900
tgagggcccc ctggctttct tcataggca gatgtgtctc agatctttca gcaggagagg 6960
agagatgtgc ttccagaggg cagctgggga cttctgggtg tgctgcagga agtgctgaga 7020

cccattctaa tacatcctgg aggccgtggc agtcctcct tctgggatgc ttaggaagga 7080
 ggcagataag ataggcccct ttctccggta agtcagacct gccagggtgaa ctataggaac 7140
 attttaaacg aactcagtta tctcatgttt gatccctcaa cctaaaccat caggcccctt 7200
 tccctggcct aaaataaggt cccactgaat ctgcactcag agccaggcct cagatgacct 7260
 ctgctgattc tctgagttgt ttaatgtggg ttccagttat cccgaactgg gactgcgaca 7320
 cccctctgtc acctcagcca ggatgactct tcttccagat catttagaac agcacttcca 7380
 attgaaacat aatacaagcc atatttgtca tttttaattt tctggtagcc acatcttttt 7440
 taaaaaggta aaaaaaacag ggaaattaat tgtaataata cattttaact gaatatatcc 7500
 aaaatatcct ttcaccacac gctcaaaata aaacaattat aatgagatat ttacattct 7560
 ttccttcata tttagccttt gaaatccggg gttttttaca cttacagctc atctcaattc 7620
 agacactaaa ttttcattgg aaatacttga tctgtattta gatttcataa aatttacagt 7680
 tgaaaaagca gagtcacata cctgaggcat tccaaacata ctttaaaatc tttccaataa 7740
 ctgagttgag tatctgtttt taaatttaaa ctaattaaaa tttaaataaat attaaataaa 7800
 ttttaaaatg tggttcctca gttccccggg ccacatttca gatgttccgt atgtatctgc 7860
 acatgggttaa tggctaccag caaacccctc tctcacctga gaaggcaact gctgtcccca 7920
 ctctttcctt ttgctcccaa agactcaaaa aaggggagtc tcagctttcg ttgacagaca 7980
 gggctcccctt gtttggtccc tagcagagcc tgggcacctt cttcggggcc tcaggagaga 8040
 agcgtaaacc ctacttcctc cctgccgccc ccttctgggtc atgcagaact ttgggggggat 8100
 gtggctgtgg ctggcaatgt tctgaactgg ctgtgatggg tcatgatggg aaagtggcca 8160
 ggagaaaagc tgcccagtg cttctccaga ggctgctccc cacctatccc cccaccatta 8220
 ccttctcatt tggggaatgt atgaggaaca gacagacact tctactaagc atgctcccc 8280
 ttccccaact ccaaagaag cttggctgga gattctcagc cagggacttg tgcacagtg 8340
 tgtatttcct atgtgtacag ttatcgccag ctgcttccca gggaccaaga aatgtgaacc 8400
 agagtcacaa ccccaaatcc tagtttggca tcagaaaaca acaaggagcc tctctttacc 8460
 tctggctcct ggacagtggg gcagatgtaa gggggacctc agccccttac tctgtgaggt 8520
 gtcaccttec cacctgtggc atctacactg tggagggcgg tgggccccct tttctctata 8580
 tatcttcagg gaaggaggct aagtgcctt aacgcagcat gaaggaatgg ggtaggcag 8640
 aggaaggact cccaggggga gctctctaag tggagacggg tccggagagc acctgtgcca 8700
 agtcctcacc tgggtgtggc caacagaccc ccagagacaa aggtcgagcc aacactttct 8760

ggcagacctt cctgcagtcc aaggagcctc agatcttgtg gtctacaagt aggcgtctgt 8820
caaccaggtg tcacagcaac caaatTTTTa gttgatgatt cgaactaggg aggtggtagg 8880
aaacagcact acaaaggcag agacctgttc tgagagtga ttagcatgca tcaccctgat 8940
taatcctcag aatataatgg agaaggggcc attgccatcc tccccataac acaggtgaga 9000
aaactgaatt ggaaagagga agaagaaagt aaggtctttg ggaggctgag gtgggtggat 9060
cacgagctca agagatcaag accatcctgg gcgtgggtgct gcatgccttt agtcccagct 9120
actcaagagg ctgaggcagg agaatcattt gaacctggga ggagaggggt gcagtgagcc 9180
aagatcatgc cactgcactc cagcctggcg acagagtga actccgtctc aaaaaaaaaa 9240
aaaaaaaaag aaaggtcaaa gtaaactctgt accaagggtt gtttatttat ttatttttag 9300
aaacaaggtc tcaactctgtc acccaggctg gagtgcagtg gcacaatcat ggctcactgc 9360
agcctccaac tcttgggttc aagccatcct cccttctcag actccccatt gctaggacta 9420
taggtgcaca ctgccacgcc cggctaatat ttcattttat gtagagatgg ggtcttgctg 9480
tgttgcaaaa gctgcatcaa ggtttactga gctattgtca acagcttcat cttcctccca 9540
gaaaggacag ctgaagggtt aggggtggcac agtttgctgt acctattcag tagtggagct 9600
gggatttgaa cccaggtggg ctgggttcctg attctgaatg gttatccact atactacatg 9660
gtgtctgtat ttctctggta aggatagaga tatattccca gcttggccag ctttgctgtt 9720
ggtttagagt acctaacat gctatttctc ccggcctaaa atggggtagg ggggctcagc 9780
tggggtacacc tatagccctc atcatccagt cagtagttct ctaagtctgc tcaactcccc 9840
tccgggggtct tcctcctggc tttcctccat tcttacagct gcttttttagg atgacagctt 9900
tggggtttttt ttttttctta aaagggttagt gctcatttcc tcctcagccc ataattcagg 9960
ttaaaaagaa cccggaacat gcatgcctat ttctgacgat caagtaaaaa caaaaacaaa 10020
caaacaaaaa acaaaaacaa aaaaaaatgt cttctgagag agactggcgc caggcagctg 10080
ctgggtcagag atgggatggg taggaaaaga aatgatgttg ccacttccct ggaagtgtcc 10140
acagcacctt cccctgcca caaccactgt ggcctttctc cttctgcaaa tgcacacaca 10200
caagcgcaga aaagccattt gacatccaca gctgggaaac agacttcaag agactgagac 10260
atgtcttaca ttttttcaaa cagtgaacag ccaatccctc atgcttccac cagccttgtg 10320
ttgtagggag gggctgggtg ctgcaagttg gctagggacc tagggttggc aaggaggag 10380
ggggactgca gaacattcta gactgggcct cagtattttc atccataccc aggcacaagt 10440
cagtgccgtc aggcagcac tgaccaggac tttctgcaat gatggaagcg ttgcaggtct 10500
gcaatgtcca tatgtggcca cgaagtcctt gaaatatggc tagagtgact gaggagctga 10560

atttttcatt ttatttaata tttatttaaa tagccacatt ggctagtggc taccatatta 10620
aatatatact tttttttttt tttttgtcac actattgccc a jgggggaat gtagtggcac 10680
gatcttggct cactgcaacc tccgcctccc aggttcaagt gattctcctg cctcagcttc 10740
ccgagcagct gggattacag gcacacacca ccatgcctgg ctgatttttt atttttagta 10800
gagacaggggt tttaccatgt tggccaggct ggtctcgaac tcctgacctt gtgatccaac 10860
ctccttggcc tcccaaagtg ctgggattac aggcgtgagc caccatgcct ggcctaagta 10920
gtacattaat atatagtagc cttaggccaa agagaagccc agtgaatcca ggagcacccc 10980
tgctgtttct cacacccatg acgctcatct gtctgcttcc ctgtgccttg cggcaggggt 11040
atcttgctcc agggcaggag catggatgag ataacctccc aaggcatgca ctgggctcta 11100
agccccagct tatattacag aaatacttcc agacctactt ctctttgcct cttgggcaga 11160
tttacacatc tctccattta aaggttatth attgaatact atatgctggg gagacaaaga 11220
gaaatgacaa agctctgccc gtaagaagtt ggctgtttta attccacagg ggggactaga 11280
atgaaacca gaacaaattc agctcccttt ttcattcttt gtagagagat tctaggcgtc 11340
ctcatcttat cttctgtcta gaaaaggaga tgggagaatc tccccaggtc tgggtggcct 11400
ggcaaagccc gacactcttc ctggactgtg aatttcaagg atgcctctgt ttcttcgatt 11460
tttgtaagct aaggcacaat atctgggtca cctagttttc tcttacaatt gattattagg 11520
tcaagtgaga tagagtgata aactaacacc agcccaggaa caggctgtta atagcaccgg 11580
ggtgtggcta agagggtccgg gggcgggggg tttagatttt aggcaggcag gtctcctgga 11640
tccatccatt gctgtgacca gcaagcaagg tggagtcaga acccttcaga ctctactgga 11700
agagaacagc cgtggcaaaa gggcagggtt ggagaaagt gttgagcttg ggcagggtac 11760
atgcgttgtg caattgtgca gcatggagta tttctcacc taagatgcct ctgggtctgt 11820
agcagctggg cttgcatcat gacactctct gtgtttgcca ggacatagag gatggtgcaa 11880
tcctcaaaca cgtgttcatt ttaccatggg gagaggctga tgtactgtgt gtgcctctgt 11940
ggatgctgct gcactgggggt gtatggggaa ctcttatga atgaaagagg actgagttgg 12000
ccacccaaat gtggcagggg ccagaggcat tggagagcca gtgggaagga ctggaggat 12060
tatatagaag ccagagattt agatcccacc ccaaaggtaa atactgtatc cagataatat 12120
tatcatcatc ccctccctta gtcattgaat agggttatga aggtaaggtc tggcctccat 12180
agatcccagc aggagaaatg gatgctgagc attccacatt tcccatcctc tgcaaaaacc 12240
tctcagaaca gcatctcatg ggcggggaag tctggaatat gtctctcttc cttcctggat 12300

cattttccag ttggagggtta cagaaacagc agaatgccat gcacaaacaa gccctgttcc 12360
cctctccctc acctgccttc actctccac acatgggttc tctccgggcc tcgcttggtt 12420
gtctccctga ctctttaaat atatcctgca gacacacaaa cagaaggaag ggatttat 12480
gaatgattat tgcagaagta ggagaggag gcacccaaat tccagattta ttttttcttt 12540
gagacagggt ctactctgt cccccaggct agagtgcagt ggagctatca aagtggagca 12600
atcatggctc acctcagcct cagcctcaac ctccaatctc caacctccta cctccaacct 12660
cccaccttcc gggctcaagc aatcctccgg tctcagcctc ctgagtttct cagactagag 12720
gcgacgcct ctatgccag ctaatttttg tgtttttttt gtagagacgg ggtttacta 12780
tgttgccag gctggctctg aactcctggg ctcaggcaat ccactcacct tggcctccca 12840
aagtgctggg attagagata tgagccaccg ctctcaattc caaattccag attttaaaaa 12900
cacttccagg ccgggcttgg tggctcacgc ctgtaatccc agctctttgg gaggccaagg 12960
cgggoggatc acgaggtcag gagatcgaga ccctcctggc taacacgggtg aaaccccatc 13020
tctactaaaa atacaaaaaa ttagccgggc atgggtggcg gcgcctgtag tcccagctac 13080
tcgggaggat gaggcaggag aatggcgtaa acccaggagg cggagcttac agtgagccga 13140
gatcgcgcca ctgcactcca gcctgggcca cagaaggaga ctctgtctca aaaaaaaaaa 13200
acaaaaaacg cttccagtcc ctagaagtgt gcccatgctg tgtgggtgtc agtctccac 13260
gccctctgct ggccacatgg agatattgac ctgtttgtca aacagggttc agagcagact 13320
gttgagttct tttgctgttc acgtgcagaa caatcttcac caggaagtcc tcccggtat 13380
cctgcctaga tcccttctgc aacctcagtt cattttctct tgatgaaata gctccagagt 13440
agccagtcta cttgttcctt cttgttttat ctaaacgca aagccttcca caattgaaag 13500
cctatttatg actcagccac agagcagctg agtcgggctg gctgcttctg gcactcagct 13560
ccagtcttac tttccagccc tttaatctcc atggctctgc cctggctccc ctccaagttt 13620
ccgctgtctc tctggagcct acaggtgacc acaagaccct gattagtatc ttcgacactg 13680
gactctgctg ctgcctaagg aataggctcc atgggggttaa gtggcaccag aaaccaggag 13740
gcacatcaaa atgttaagcc tgctcttcag aagcagccaa aaagcttgca aagctgggga 13800
aggtgtgccc tcaggagact tgccttgggg aggctgggaa aaggagagag tttgcaaaag 13860
cattttgagt tccttaaatg aagagagata caagaatcta cattttgtat gccagtatca 13920
tggttgcttg gcccggggc agtacttgaa cactttcaaa gaaaagagta agagagggtca 13980
aaataaattt accccaaggt ttatcaaacc gttgtcaaca gcccatccc cctcccagaa 14040
agggtgactc aaggtaagg acagcccaca gcacttctta gaaaaagtcc caaaaggtga 14100

aaggtcttga gaagagctgt tgaggggaagg ctgtgggcca ccatgcttct gtgtccccc 14160
aggccaaaga gagaataagc taaaatgaca gctggaaaag ggcaccctct gaccaaatgg 14220
acagagcctc cccaacagg tagccagagg aggatggaat ggcaccccca tttcagatgt 14280
ccttaagaac tagagagata gatgcttatt tctgcttgga cacaggggga tggactaaat 14340
ggcctacgga aaggattcca ggaactgcta caccagccac aggagagaag ccagcttccc 14400
aggctgtctc cccttgaaga ggtgacaatg tgttggctct ccaggccagg ctggggcagc 14460
ccactgaagg agacgcatag acgcatgcmc tggtcagcag ccagcccact ccccccactg 14520
ccctcacttt atccagttac aatcttgatt gaggaagggg tccaggcttc tccaatctga 14580
acccctcca ggctttcagt agccaccata tctgtctgc ccctccccct acagtagtca 14640
actacttttt gaggattaaa atgcgtggtg atgctgaggt tgagtggcac ggctccagtc 14700
cctccataag acgtgcccaa aagcccttct gtgcaatggt tagcactccc tctccctact 14760
ccctcttctc caagatctga gccttggcgt gaggcctgtg ttactagata acggagcccc 14820
agcctttcaa tttgtacaaa gtccccaac cagccatgct tctcccttcc tctgccaat 14880
ctcacttgcg ctacaaccaa gaaaagtcaa ttaccccag aacatgacct ttttaactaa 14940
tccctagaat cctgcattcc acctaccaga gatgtcacc tattgatgag tcgtcacagc 15000
agggtctgat agagcagaat cgaggctactg ggacatcaag acatgactgg gaagaggggg 15060
tgagagtcca gaaggggtac caggattctc aggttgaatt ttatttaggg agagcccacc 15120
caccaccttg ctatttgctt tccccaagtc atcatcttcc tctccagtaa ttacgtccct 15180
tcctttcctg tgaataaata ccaaagcaa ctcaccacca ggaacgctgc gaacctttgg 15240
aggcttcttt agggcaggga ccttctctgc caaggcctta aagaccagat acctatttca 15300
ttagacttga caaatcctga gccaaagagag aaacagatgc ttgggtgctt cctgccacct 15360
gccatcctgc ccaggtagg ccagggtgcc aaatgccttt cctaactcac acctggaact 15420
tgcccagggc tgcaagctca gaagtcaggt tctataacctg ccagaggca taaaacagcg 15480
tctttctcaa aaagcttctc tttccttgca cccaccccc acttcctgga attctacca 15540
cttcttctgt tccccatccc agatgtctgg cggtcactgt tctggacat ccagtgatc 15600
atggagtaga tggagaacag aattttcttt cacctcattt cagttattta aggtcactca 15660
aagctctatt ttctctttcc agtatttgaa aaaagaggga ggcaatgaag acagggcccc 15720
ccacctccac tcaccctgc atctgggtccc actgtgaggc atcctggcca cacgctgccc 15780
gcctggaaac aacaatctaa agttcccca ttctgggtcc tctgaggctg ggggaggtca 15840

aggatctgag aggagaaggg gacccagggg tggatgccta tgagatcaga atttagggga 15900
aaactgcgca ccaagaaaag gccggacggg tggaattaag gatcaacaaa tacatcaaca 15960
aagtctgact tctctaaagg tcaaaagcca gctaagtata gactgtcagc aagccactcc 16020
tatatataac tgtcccgggc tcagccagct gtccggcctct ctgagtcagc agctgagtaa 16080
cgtcccagcc tcccaggccc ccgagcaccg cgccagttct gagccagagc aggagcaagg 16140
cctccgactt ccacttgccc ccacgccggc cccctccggg cctacatttc tggctctccc 16200
ctccccctct cctgccgctt atgaagggtc tcaccaccct gctgctccag accccaggac 16260
cccctcctcc taggttcccc aggtccaaag agacctcaac atccagcagc tctgaaagtg 16320
caaagatctc actaacagga cagcctttgc ctttctcgtc cacgccctga ctgtgtcatc 16380
tgctccatg ggcccagatt cccgggttcc tacttctcag cccacctcc agccatgtcc 16440
catctacttg gccatcccc agcaagcaaa ccgctcgctg ccaccttcac ccttgagag 16500
gccattttcc ttgcccttcc tccttactgc cccagaagg cctgctccgt cctcacccat 16560
cagtctgtca gtctctcgat tctcctctc tgtgaagcct ccctgcctgc actctctgct 16620
tgactccaag catgccagaa acttcctgcc acttaaata gcattccctc tgcttccctg 16680
ttcttatccc cctgctttca ggggtgtgctc caggagggca gagacacatc tgcttcagcc 16740
tttattactc ccctcacccc aaactgccc gccctgggct ctggccgtgt gcaccttggc 16800
ccacgggggc tgacctccg gctccttctc ctgcagagag agactccgt gagcacgagg 16860
agcccaccac ctctgagatg gccgaggaga cctactcccc caagatcttc cggcccaaac 16920
acaccgcat ctccgagctg aaggctgaag cagtgaagaa ggaccgcaga aagaagctga 16980
cccagtccaa gtttgtcggg ggagccgaga aactgccc ccccggtatc atctctgcac 17040
ctgagatgag acaggagtct gagcaggtgc atctcgggga ttccctcatt ccctcccagc 17100
ttttgacctt ttctgcatc taaatggaca ttcattggcg caggaggtgc tagactagac 17160
ccagcagagg acctacagcc caccatgtgg ctgagcttag gacgtgggag tagagtttcc 17220
agagcataca cttgtcaggg ccaagggtt gtgcaagtaa gtgggaatag aggcggtgct 17280
catttgagga gagtgtgtgc cagcccagcg gccacgtacc aggtacctgg gccctgcagc 17340
tgtgtccagt ccaaggcaga gactgaatat tctcctagag catctagaaa tgggcacctc 17400
tggaacattgt tactcagcag aagagaaaag ctgcagggga gggggtggac tggaacacaa 17460
aagagaaagt atgactccgt acctggccaa aggcaggctt tgtatgatcc cagcgcacgt 17520
gacaggtgac agggaggaaa gatctttggt agcagatgat tagaaccaaa ggtggtgact 17580
ttcctccaga gaccttcag ccccccaccc ctgggccatt cgtcagcgca gctgggtggt 17640

18

cccccttta aatgggttttc tttttgaggc aagttggatg aacagagaag ggaagagagg 19440
aagaacgaga ggaagagaag ggaaggaagt gtttgtgtag aagagagaga aagacgaata 19500
gagttaggaa aaggaagaca agcaggtggg caggaaggac atgcaccgag accaggcagg 19560
ggcccaactt tcacgtccag ccctggcctg gggtcgggag aggtgggagc tagaagatgc 19620
agcccaggat gtggcaatca atgacactat tgggggtttcc caggatggat tggtcagggg 19680
gagaaaggaa aaggcaaaac actccaggac ctctcccga tctgtctcct cctctagcca 19740
gcagtatgga cagctggacc cctgaacttc ctctcctctt acctgggcag agtggtgtct 19800
ctccccaaat ttataaaaac taaaatgcat tccattcctc tgaaagcaaa acaaattcat 19860
aattgagtga tattaaatag agaggttttc ggaagcagat ctgtgaatat gaaatacatg 19920
tgcatatttc attccccagg cagacatttt ttagaaatca atacatgccc caatattgga 19980
aagacttggt cttccacggg gactacagta catgctgaag cgtgccgttt cagccctcat 20040
ttaattcaat ttgtaagtag cgcagcagcc tctgtggggg aggataggct gaaaaaaaaa 20100
agtgggctcg tatttatcta caggactcca tatagtcata tataggcata taaatctatt 20160
ctttttcttt gtttttttct ttcttccttt ctttcaaagg ttgcatata cttttcaaag 20220
tagttcctat aggggcattg aggagcttcc tcattctggg aaaactgaga aaacccatat 20280
tctcctaata caaccgtaa tagcattttt gcctgcctcg aggcagagtt tcccgtgagc 20340
aataaactca gcttttttgt ggggcacagt actggatttg acagtgttc cccacgtgtg 20400
ttcatctgca cccaccgagc caggcagagg ccagccctcc gtggtgcaca cagcacgcgc 20460
ctcagtccat cccatttttag tcttttaaacc ctcaggaagt cacagtctcc ggacaccaca 20520
ccacatgagc ccaacaggtc cacgatggat ccaccagtcc caccacagcc ttttcctttc 20580
atctgaacag aatgtgcatt tttggaagcc tccctcactc tccatgctgg cagagcagga 20640
gggagactga agtaagagat ggcagaggga gatggtggca aaaagggtta gatgcaggag 20700
aacagtaaga tggatggttc cggccagagt cgatgtgggg aggaacagag ggctgaaggg 20760
agagggggct gactgttcca ttctagcttt ggcacaaagc agcagaaagg gggaaaagcc 20820
aatagaaatt tccttagctt cccaccata tgtattttct aggatttgag aggaaagaga 20880
ggaaaatggg ggaatgggtt gcaaaataga aatgagctta atccaggccg cagagccagg 20940
gaaggtgagt aacttttaga ggggtgctaga ctttagaagc cagataggaa gaatcagtct 21000

<210> 12

<211> 374

<212> DNA

<213> Homo sapiens

<220>

<400> 12

gttgtctctc cccaaattta taaaaactaa aatgcattcc attcctctga aagcaaaaca 60
aattcataat tgagtgatat taaatagaga ggtttttcgga agcagatctg tgaatatgaa 120
atacatgtgc atattttcatt ccccaggcag acatttttta gaaatcaata catgccccaa 180
tattggaaag acttggttctt ccacgggtgac tacagtacat gctgaagcgt gccgtttcag 240
ccctcattta attcaatttg taagtagcgc agcagcctct gtgggggagg ataggctgaa 300
aaaaaaaaagt gggctcgtat ttatctacag gactccatat agtcatatat aggcatataa 360
atctaaaaaa aaaa 374

<210> 13

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 13

gagcaacacc atcttctctt 20

<210> 14

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 14

taggcggcca gcagcaggag 20

<210> 15

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 15

gcgtgcagcg gcttctcctc 20

20

<210> 16
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 16
cagcagggcg tgcagcggct

20

<210> 17
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 17
ttcgttgagg caaaccccgc

20

<210> 18
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 18
ctcttttcgt tgaggcaaac

20

<210> 19
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 19
gtagctcttt tcgttgaggc

20

<210> 20
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

21

<223> Antisense Oligonucleotide

<400> 20

tttctgcggt ccttcttcac

20

<210> 21

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 21

agcttctttc tgcggtcctt

20

<210> 22

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 22

actgggtcag cttctttctg

20

<210> 23

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 23

aacttggact gggtcagctt

20

<210> 24

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 24

cctgtctcat ctcaggtgca

20

<210> 25

22

<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 25
catgcgtggg ctggctttga

20

<210> 26
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 26
ggcaccatgc gtgggctggc

20

<210> 27
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 27
tgggcaggta cacagcacgg

20

<210> 28
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 28
tagaatcctt tgcggtcaca

20

<210> 29
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 29
tcttgtagaa tcctttgcgg 20

<210> 30
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 30
gctttctctt gtagaatcct 20

<210> 31
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 31
gtcctcagtt tcctcaaata 20

<210> 32
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 32
ccgaggtcct cagtttcctc 20

<210> 33
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 33
agattccgag gtcctcagtt 20

<210> 34
<211> 20

<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 34
gctagagatt ccgaggtcct 20

<210> 35
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 35
cccttgctag agattccgag 20

<210> 36
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 36
cctaactcta ttcgtctttc 20

<210> 37
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 37
ggagaggtcc tggagtgttt 20

<210> 38
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 38
gatctgcttc cgaaaacctc 20

<210> 39
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 39
ctttgcagct ctttcctagc 20

<210> 40
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 40
cttgcaaaaa thtagagaga 20

<210> 41
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 41
gctttggctg cctgccacct 20

<210> 42
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 42
ctgcaggaga aggagccgga 20

<210> 43
<211> 20
<212> DNA

WO 03/030826

PCT/US02/32060

26

<213> Artificial Sequence

<220>

<223> Antisense Oligonucleotide

<400> 43

actgtagtca ccgtggaaga

20

- 78 -

What is claimed is:

1. A compound 8 to 50 nucleobases in length targeted to the start codon, the coding region, the 3' untranslated region,
5 an intron or an intron/exon junction of a nucleic acid molecule encoding insulin-like growth factor binding protein 5, wherein said compound specifically hybridizes with said nucleic acid molecule encoding insulin-like growth factor binding protein 5 and inhibits the expression of insulin-like growth factor
10 binding protein 5.
2. The compound of claim 1 which is an antisense oligonucleotide.
3. The compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 13, 14, 15,
15 16, 17, 18, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 38, 39, 40, 41, 42 or 43.
4. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.
- 20 5. The compound of claim 4 wherein the modified internucleoside linkage is a phosphorothioate linkage.
6. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.
7. The compound of claim 6 wherein the modified sugar
25 moiety is a 2'-O-methoxyethyl sugar moiety.
8. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.
9. The compound of claim 8 wherein the modified nucleobase is a 5-methylcytosine.
- 30 10. The compound of claim 2 wherein the antisense oligonucleotide is a chimeric oligonucleotide.
11. A compound 8 to 50 nucleobases in length which specifically hybridizes with at least an 8-nucleobase portion of an active site on a nucleic acid molecule encoding insulin-like
35 growth factor binding protein 5.
12. A composition comprising the compound of claim 1 and a pharmaceutically acceptable carrier or diluent.
13. The composition of claim 12 further comprising a colloidal dispersion system.

- 79 -

14. The composition of claim 12 wherein the compound is an antisense oligonucleotide.

15. A method of inhibiting the expression of insulin-like growth factor binding protein 5 in cells or tissues comprising
5 contacting said cells or tissues with the compound of claim 1 so that expression of insulin-like growth factor binding protein 5 is inhibited.

16. A method of treating an animal having a disease or condition associated with insulin-like growth factor binding
10 protein 5 comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of insulin-like growth factor binding protein 5 is inhibited.

17. The method of claim 16 wherein the disease or
15 condition is a hyperproliferative disorder.

18. The method of claim 17 wherein the hyperproliferative disorder is cancer.

19. The method of claim 18 wherein the cancer is of the breast, prostate, pancreas, or neuroendocrine system.

20. The method of claim 16 wherein the disease or
20 condition is an inflammatory, developmental or metabolic disorder.