Antisense compounds, compositions and methods are provided for modulating the expression of estrogen receptor alpha. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding estrogen receptor alpha. Methods of using these compounds for modulation of estrogen receptor alpha expression and for treatment of diseases associated with expression of estrogen receptor alpha are provided.
ANTISENSE MODULATION OF ESTROGEN RECEPTOR ALPHA EXPRESSION

INTRODUCTION


FIELD OF THE INVENTION

[0002] The present invention provides compositions and methods for modulating the expression of estrogen receptor alpha. In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding estrogen receptor alpha. Such compounds have been shown to modulate the expression of estrogen receptor alpha.

BACKGROUND OF THE INVENTION

[0003] Steroid, thyroid and retinoid hormones produce a diverse array of physiologic effects through the regulation of gene expression. Upon entering the cell, these hormones bind to a unique group of intracellular nuclear receptors which have been characterized as ligand-dependent transcription factors. This complex then moves into the nucleus where the receptor and its cognate ligand interact with the transcription preinitiation complex affecting its stability and ultimately, the rate of transcription of the target genes. Members of the nuclear receptor family share several structural features including a central, highly conserved DNA-binding domain which targets the receptor to specific DNA sequences known as hormone response elements (Kliewer et al., Science, 1999, 284, 757-760).

[0004] Estrogens are steroid hormones that exert a wide range of effects throughout the body. They are required for normal female sexual maturation and promote growth and differentiation of the breast, uterus, fallopian tubes, vagina and ovaries. Male reproductive tissues such as testis and prostate are also estrogen target tissues. Furthermore, estrogens are important for bone maintenance and have a protective role in the cardiovascular system. In the brain, estrogens appear to modulate the regulation and reproduction of autonomic and reproductive neuroendocrine systems, mood and cognition (Osterlund and Hurd, Prog. Neurobiol., 2001, 64, 251-267).

[0005] Similar to the other steroid hormone receptors, the estrogen receptors consist of several structural domains that exhibit different functional features. Both the estrogen receptor alpha and estrogen receptor beta consist of five different domains, the N-terminal domain (called A/B), the DNA-binding domain (C), a short hinge region (E) and a short C-terminal domain (F) (Osterlund and Hurd, Prog. Neurobiol., 2001, 64, 251-267).


[0008] The nucleic acid sequence of estrogen receptor alpha is disclosed in U.S. Pat. No. 6,235,872 (Bredesden and Rabizadeh, 2001).

[0009] Disclosed and claimed in PCT publications WO 01/00823 and WO 01/62969 are nucleic acid molecules encoding isoforms of human estrogen receptor alpha (Gannon et al., 2001; Kalush et al., 2001). Additionally disclosed and claimed in PCT publication WO 01/08023 are nucleic acid molecules hybridizing to said nucleic acid molecules encoding estrogen receptor alpha isoforms, under stringent conditions (Gannon et al., 2001).

[0010] The involvement of estrogen receptor alpha in cell proliferation indicates that its selective inhibition may prove to be a useful target for therapeutic intervention in a variety of cancers.

[0011] Because estrogens are thought to support breast cancer, estrogen antagonists have been developed which act through the estrogen receptor. Selective estrogen receptor modulators (SERMs) are small molecules which manifest variable agonist and antagonist properties when examined in the context of estrogen-dependent responses occurring in various tissues. Examples of SERMs include raloxifene and tamoxifen (DuFer and Smith, J. Pharmacol. Exp. Ther., 2000, 295, 431-437).

[0012] Disclosed and claimed in PCT publications WO 00/74485 and WO 99/54459 are catalytic nucleic acids (known as ribozymes), capable of modulating the expression of estrogen receptor alpha (Roy et al., 2000; Thompson et al., 1999).

[0013] Double-stranded DNA fragments containing 5' upstream sequences of the human estrogen receptor alpha gene have been used to modulate the transcriptional activity of estrogen receptor alpha (Penolazzi et al., Breast Cancer Res. Treat., 1998, 49, 227-235).


[0015] Antisense oligodeoxynucleotides have also been used to decrease levels of estrogen receptor alpha in rat brain (Inamdar et al., J. Appl. Physiol., 2001, 91, 1886-1892; McCarthy et al., Endocrinology, 1993, 133, 433-439) and in a mouse colon cancer cell line (Xu and Thomas, Mol. Cell Endocrinol., 1994, 105, 197-201).

[0016] Antisense 18-mer oligonucleotides targeting positions 117 to -99, -3 to 15 (encompassing the start codon), 302 to 320, 495 to 512, 539 to 557, 1140 to 1157 and 4134 to 4151, of estrogen receptor alpha have been used to inhibit its expression in MCF-7 human breast cancer cells (Santagati et al., Mol. Endocrinol., 1997, 11, 938-949; Taylor et al., Antisense Nucleic Acid Drug Dev., 2001, 11, 219-231). In a separate investigation, also using MCF-7 breast cancer cells, a 15-mer methylphosphonate-phosphodiester antisense oligonucleotide was used to target the start codon of estrogen receptor alpha (Madden et al., Eur. J. Cancer, 2000, 36, S34-S35).

[0017] To date, investigative strategies aimed at modulating estrogen receptor alpha expression have involved the use of small molecule SERMs, ribozymes, double-stranded DNA decoys, antisense RNA transfections and antisense oligonucleotides. There remains a long felt need for additional agents capable of effectively inhibiting estrogen receptor alpha function.

[0018] 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 expression of estrogen receptor alpha.

[0019] The present invention provides compositions and methods for modulating expression of estrogen receptor alpha, including modulation of spliced variants of estrogen receptor alpha.

SUMMARY OF THE INVENTION

[0020] The present invention is directed to compounds, particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding estrogen receptor alpha, and which modulate the expression of estrogen receptor alpha. Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of estrogen receptor alpha in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of estrogen receptor alpha 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

[0021] The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding estrogen receptor alpha, ultimately modulating the amount of estrogen receptor alpha produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding estrogen receptor alpha. As used herein, the terms “target nucleic acid” and “nucleic acid encoding estrogen receptor alpha” encompass DNA encoding estrogen receptor alpha, 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 is a 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, translation 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 estrogen receptor alpha. In the context of the present invention, “modulation” means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

[0022] 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 estrogen receptor alpha. 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'-UGU 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 estrogen receptor alpha, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or “stop codon”) of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms “start codon region” and “translation initiation codon region” refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 51 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 junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where overproduction of a particular mRNA splice product is implicated in disease. aberrant splicing due to rearrangements or deletions are also preferred targets. mRNA transcripts produced via the process of splicing of two (or more) mRNAs from different gene sources are known as “fusion transcripts. It has been also found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

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

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

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

Once one or more target sites have been identified, 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 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 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 that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, “specifically hybridizable” and “complementary” are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-
target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

[0031] 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.

[0032] 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.

[0033] 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.

[0034] 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.


[0036] 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.

[0037] 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 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.

[0038] 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.

[0039] 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 nucleotides 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 nucleotides 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.

[0040] Specific examples of preferred antisense compounds useful in this invention include oligonucleotides
containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the 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.

[0041] Preferred modified oligonucleotide backbones include, for example, phosphorothioate, chiral phosphorothioates, phosphorodithioates, phosphorothiester, amically phosphorothioester, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphonates, phosphoramidates including 3'-amino phosphoramidate and amically phosphoromimidates, thionophosphoramidates, thionalkyl phosphonates, thionalkylphosphothioesters, selenophosphates and borano-phosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleoside 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.

[0042] Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,867,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,276,302; 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; 5,194,595; 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.

[0043] 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, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholinolinkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sultide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; 2-methyleneimino and methylmethylenehydradine backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N₃-O₃-S and C₃-H₂ component parts.

[0044] Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,263,504; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,096; 5,602,240; 5,610,289; 5,625,050; of which are commonly owned with this application, and each of which is herein incorporated by reference.

[0045] 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 mimic 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 aminoethylyglycine backbone. The nucleobases are retained and are bound directly or indirectly to az 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. Pat. Nos. 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.

[0046] Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular 1-CH=NH-O-CH₂-N(CH₂-O-CH₂— [known as a methylene (methyleneimino) or MM backbone], 1-CH₂-O-N(CH₂)=CH₂—, 1-CH₂-N(CH₂) —N(CH₂)=CH₂— and 1-CH₂-O-N(CH₂)=CH₂— [wherein the native phosphodiester backbone is represented as —O—P—O—CH₂—] of the above referenced U.S. Pat. No. 5,489,677, and the amide backbone of the above referenced U.S. Pat. No. 5,602,240. Also preferred are oligonucleotides having morpholine backbone structures of the above-referenced United States Pat. No. 5,034,506.

[0047] Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; S— or N-alkyl; O—S— or N-alkenyl or N—O— alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₃ to C₁₀ alkyl or C₃ to C₁₀ alkenyl and alkynyl. Particularly preferred are O(CH₂)₃O, O(CH₂)CH₂OCH₂, O(CH₂)₂NH₂, O(CH₂)₂CH₂, O(CH₂)₂ONH₂, and O(CH₂)₂ON[(CH₂)₃CH₂], wherein r and m are from 1 to 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C₃ to C₁₀ lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkylaryl, aralkyl or O-alkylaryl or C₃ to C₁₀ alkenyl, alkynyl, alkylaryl, aralkyl or O-alkylaryl or C₃ to C₁₀ alkenyl, alkynyl, alkylaryl, aralkyl or O-alkylaryl or C₃ to C₁₀ alkenyl, alkynyl, alkylaryl, aralkyl or O-alkylaryl or C₃ to C₁₀ alkenyl, alkynyl, alkylaryl, aralkyl or O-alkylaryl. Further preferred modifications include 2'-dimethylaminoxyethoxy, i.e., a (CH₂)₃ON(CH₂)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.

[0048] A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methane (—CH₂—) 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.

[0049] Other preferred modifications include 2'-methoxy (2—O—CH₃), 2-aminoxypropoxy (2'-OCH₃CH₂CH₂NH₂), 2'-allyl (2'-CH₂—CH═CH₂) and 2'-fluoro (2'-F). The 2'-modification may be in the arabinho (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 cyclcobutil 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. Pat. Nos. 4,981,957; 5,118,800; 5,319,060; 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, each of which is commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

[0050] 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-amino- nadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiouracil and 2-thiouracil, 5,6-diuracil and cytosine, 5-propynyl (—C≡C—CH₂) uracil and cytosine and other alkylnucleosides. Further modified nucleobases include 6-azauracil, 6-azacytosine, 6-uracil (psuedouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxy and other 8-substituted adenines and guanines, 5-halo particularly 5-bromos, 5-fluorosulfonyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2'-F-adenine, 2'-amino-adenine, 8-azaguanine and 8-azadeguanine, 7-deazaadenine and 7-deaza guanine and 3-deazaadenine and 3-deazaguanine. 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]julolid-2-one), pyridoindole cytidine (1H-pyridino [3,2-c:2,3-5]pyrrole[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-aminopyri dine and 2-pyridine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,508, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, I. J., ed. John Wiley & Sons, 1990, those disclosed by English 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., 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-methoxymethyl sugar modifications.

[0051] Representative United States patents that teach the preparation of various of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,508, as well as U.S. Pat. Nos. 4,985,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, each of which is commonly owned with the instant application, and each of which is herein incorporated by reference, and U.S. Pat. No. 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

[0052] Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxy groups. Conjugate groups of the invention include intercalators, reporter molecules, polymers, polyanides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phanathanthidine, anthraquinone, acidine, fluorescein, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/01996, filed Oct. 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Lester et al., Proc. Natl Acad. Sci. USA, 1989, 86, 6553-6556), cholic acid (Manoharan et al., Bioorg. Med. Chem. Let., 1994, 4, 1053-1060), a thioether,

**[0053]** Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Pat. Nos. 4,826,979; 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; 4,702,729; 4,789,737; 4,824,944; 4,835,263; 4,876,335; 4,904,582; 4,958,015; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

**[0054]** 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 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 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, 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 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 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.

**[0055]** Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Pat. Nos. 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 with the instant application, and each of which is herein incorporated by reference in its entirety.

**[0056]** 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 such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for 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 phosphorothioates and alkylated derivatives.

**[0057]** 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.

**[0058]** 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, 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. Pat. Nos. 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; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,359,619; 5,416,016; 5,417,978; 5,468,854; 5,469,348; 5,512,295; 5,529,526; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

**[0059]** 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 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.

[0060] 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. Acidic, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published Dec. 9, 1993 or in WO 94/26764 and U.S. Pat. No. 5,770,713 to Imbach et al.

[0061] 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.

[0062] 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'-dibenzyethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," J. of Pharma Sci., 1977, 66, 1-19). The base addition salts of acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the composition of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sufox or phospho acids or N-substituted sulfamic acids, for example acetic acid, propanoic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, glacial 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.

[0063] 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, oxalic acid, tartaric acid, succinic acid, malic 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-toluensulfonic acid, naphthalenesulfonilic acid, polygalacturon酸ic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

[0064] 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 estrogen receptor alpha 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.

[0065] The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding estrogen receptor alpha, 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 estrogen receptor alpha 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 estrogen receptor alpha in a sample may also be prepared.

[0066] The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal,
epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2′-O-methoxyethyl modification are believed to be particularly useful for oral administration.

[0067] Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroylchelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (e.g. dimyristoylphosphtidyl glycerol DMPG) and cationic (e.g. dioleoyltrimethylammoniumpropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monolein, dilaurin, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylamine, an acylcholine, or a C10-12 alkyl ester (e.g. isoproplmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in U.S. patent application Ser. No. 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

[0068] 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 mini-tablets. 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 ursodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucolcholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycocolhydrofusidate. 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, monolein, dilaurin, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylamine, 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 spray dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include poly-amino acids, polylamines, polyacrylates, polyalkylacrylates, polyoxethanes, polyalkylacyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethylene glycols (PEG) and starches; polyalkylacryloacrylates; DEA-derivatized polylamines, polihulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polysperamines, protamine, polyvinylpyridine, polyglycylidylaminoo-methylplthylene (PDTDMA), polyaminostyrene (e.g. p-amino, poly(methylenacyrlate), poly(ethylencyanoacrylate), poly(2-butylcyanoacrylate), poly(isobutylenacyanoacrylate), poly(isohexylenacyanoacrylate), DEA-methacyrlate, DEA-hexylacrylate, DEA-crylaldehyde, DEA-alumina and DEA-dextran, polyoctylacrylate, polyethylene glycol, poly(DL-lactic acid), poly(DL-lactic-co-glycolic acid) (PLGA), alginates, and polyethylene glycol (PEG). Oral formulations for oligonucleotides and their preparation are described in detail in U.S. application Ser. No. 08/886,829 (filed Jul. 1, 1997), Ser. No. 09/108,673 (filed Jul. 1, 1997), Ser. No. 09/256,515 (filed Feb. 23, 1999), Ser. No. 09/082,624 (filed May 21, 1998) and Ser. No. 09/315,298 (filed May 20, 1999) each of which is incorporated herein by reference in its entirety.

[0069] Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

[0070] Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and 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.

[0071] The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the 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.

[0072] 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 heterogeneous 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 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 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. 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 (w/o/w) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

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

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 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 hydrophilic/lipophilic 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: non-ionic, 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 glycercyl tristearate.

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

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carbboxypropylcellulose), 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 include in emulsion formulations
include methyl paraben, propyl paraben, quaternary amm
omium 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.


[0082] The application of emulsion formulations via dermato logical, 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.

[0083] In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pa., 1985, p. 271).

[0084] The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

[0085] Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monooleate (M1310), tetraglycerol monostearate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaricale (PO500), decaglycerol monocaprate (MCA750), decaglycerol monostearate (MO750), decaglycerol sequioate (S0750), decaglycerol decaoleate (DA0750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Capso 300, Captop 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylene glycerol fatty acid esters, fatty alcohols, polyglycerolcasters, saturated polyglycerolized C8-C10 glycerides, vegetable oils and silicone oil.

[0086] Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid-based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J. Pharm. Sci.*, 1996, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

[0087] 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.

[0088] Liposomes

[0089] 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.

[0090] 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.

[0091] In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

[0092] Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

[0093] Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

[0094] 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 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.

[0095] Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

[0096] Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., Biochem. Biophys. Res. Commun., 1987, 147, 980-985).

[0097] Liposomes which are pH-sensitive or negatively charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., Journal of Controlled Release, 1992, 19, 269-274).

[0098] One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

[0099] Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g., as a solution or as an emulsion) were ineffective (Weiner et al., Journal of Drug Targeting, 1992, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., Antiviral Research, 1992, 18, 259-265).

[0100] Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. S.T.P. Pharma. Sci., 1994, 4, 6, 466).

[0101] 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 GM1, 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, 5765).

[0102] 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 GM1, 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, 6940). U.S. Pat. No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside GM1, or a galactocerebroside sulfate ester. U.S. Pat. No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-anhydromyristolethylphosphatidylcholine are disclosed in WO 97/13499 (Lin et al.).

[0103] 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, 2Cl15G, that contains a PEG moiety. Ihlem 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 polylkylene glycols (e.g., PEG) are described by Sears (U.S. Pat. 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 (DSPPE) 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. Pat. Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Pat. 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. Pat. No. 5,225,212 (both to Martin et al.) and in WO 94/20075 (Zalipsky et al.). Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi et al.). U.S. Pat. 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.

[0104] 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. Pat. No. 5,264,221 to Tagawa et al. discloses enzyme-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Pat. No. 5,665,710 to Rahman et al. describes certain methods of encapsulating oligodeoxy-nucleotides in liposomes. WO 97/04787 to Love et al. discloses liposomes comprising antisense oligonucleotides targeted to the ras gene.

[0105] Transferrosomes are yet another type of liposome, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transferrosomes 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. Transferrosomes 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 transferrosomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transferrosomes have been used to deliver serum albumin to the skin. The transferrosome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

[0106] 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 hydrophilic/lipophilic balance (HLB). The nature of the hydrophilic group (also known as the “head”) provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

[0107] 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 others such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propanoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

[0108] 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 sulfonic acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfostrucuates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

[0109] 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.

[0110] 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 alkylamines, N-alkylbetaines and phosphatides.


[0112] Penetration Enhancers

[0113] In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell 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.

[0114] 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.

[0115] 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.

[0116] 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, monoonoate (1-monooxoyleyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-mononaprate, 1-dodecylazacycloheptan-2-one, acylurinines, acylholines, Cl-12 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).


[0118] 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 homovanillic), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; Buur et al., *J. Control Rel.*, 1990, 14, 43-51).

[0119] Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacycloalkane derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, 1987, 39, 621-626).
Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al., U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

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

Carriers

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

Excipients

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

Pharmaceutically acceptable organic or inorganic 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 limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylene, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

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

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

Other Components

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

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

Certain embodiments of the invention provide pharmaceutical 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, dactinomycin, doxorubicin, etoposide, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylaminosporan, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogestosterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylenemelamine, pentamethylenemelamine, miloxantone, amscarine, chlorambucil, methylcyclclohexylaminosporan, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUddR), methotrexate (MTX), colchicine, taxol, vincristine, vinblas-
tine, 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. 1206-1228, 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 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 ribavirin, 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 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 repeated rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC50, 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.

**EXAMPLES**

**Example 1**

- **0136** Nucleoside Phosphoramidites for Oligonucleotide Synthesis Deoxy and 2'-alkoxy Amides
- **0137** 2'-Deoxy and 2'-methoxy beta-cyanethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling Va.). Other 2'-O-alkoxy substituted nucleoside amides are prepared as described in U.S. Pat. No. 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amides, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazelate and base was increased to 360 seconds.
- **0138** 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 Research, Sterling Va. or ChemGenes, Needham Mass.).
- **0139** 2'-Fluoro Amides
- **0140** 2'-Fluorodeoxyadenosine Amides
- **0141** 2'-flouro oligonucleotides were synthesized as described previously [Kawasaki, et. al., J. Med. Chem., 1993, 36, 831-841] and U.S. Pat. No. 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-arabinofuranosyl-adenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S2,2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9beta-D-arabinofuranosyl-adenine was selectively protected in moderate yield as the 3',5'-diterahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodology and standard methods were used to obtain the 5'-dimethoxytrityl(DMT) and 5'-DMT-3'-phosphoramidite intermediates.
- **0142** 2'-Fluorodeoxyguanosine
- **0143** The synthesis of 2'-deoxy-2'-fluorguanosine was accomplished using tetrasopropylsiloxoanilyl (TPPS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate disobutryl-arabinofuranosylguanine. Deprotection of the TPPS group was followed by protection of the hydroxyl group with THP to give disobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacetylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.
- **0144** 2'-Fluorouridine
- **0145** 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.
- **0146** 2'-Fluorodeoxycytidine
- **0147** 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 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 Martin, P., *Helvetic Chemist 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-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 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 (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 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 222-2°C).

2'-O-Methoxymethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxymethyl)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 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 CHCl₃ (600 mL) and evaporated. A silica gel column (3 kg) was packed in CHCl₃:acetone:MeOH (20:5:3) containing 0.5% Et₃N. The residue was dissolved in CHCl₃ (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 material was obtained by reworking impure fractions.

2'-O-Methoxymethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxymethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue 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 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 CH₂CN (200 mL). The residue was dissolved in CH₂CN (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%).

3'-O-Acetyl-2'-O-methoxymethyl-5'-O-dimethoxytrityl-5-methyluridine

3'-O-Acetyl-2'-O-methoxymethyl-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.

3'-O-Acetyl-2'-O-methoxymethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxymethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH₂CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH₂CN (1 L), cooled to ~50°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.

3'-O-Acetyl-2'-O-methoxymethyl-5'-O-dimethoxytrityl-5-methyluridine

A solution of 3'-O-acetyl-2'-O-methoxymethyl-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 NH₄ 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-dimethoxymethyltrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxymethyltrityl-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 (2x300 mL), dried over MgSO4 and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica gel column using EtOAc/hexane (1:1) containing 0.5% Et3NH 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-dimethoxymethyltrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxymethyltrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CHCl3 (1 L) Tetrzole disopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) was 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 NaHCO3 (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH2Cl2 (300 mL), and the extracts were combined, dried over MgSO4 and concentrated. The residue obtained was chromatographed on a 1.5 kg silica gel 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-(Aminoxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) Nucleoside Amidites

2'-O-(Dimethylaminooxyethyl) Nucleoside Amidites

2'-O-(Dimethylaminooxyethyl) 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-2'-O-2-anhydro-5-methyluridine

3'-O-(Hexyl-2'-O-2-anhydro-5-methyluridine (Pro. Bio. Sint., Varesco, Italy, 100.0 g, 0.416 mmol), dimethylaminopyridine (0.66 g, 0.013 eq, 0.0054 mmol) were dissolved in dry pyridine (500 mL) at ambient temperature under an argon atmosphere and with mechanical stirring. Tert-Butyldiphenylsilyl chloride (125.8 g, 119.0 mL, 1.1 eq, 0.485 mmol) 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 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 1:1 mixture of ethyl acetate and ethyl ether (600 mL) 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, 1 mm 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-hydroxy-ethyl)-5-methyluridine

5'-O-Tert-Butyldiphenylsilyl-2'-O-(2-hydroxy-ethyl)-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 1 mm 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 (2 kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to produce as a white crisp foam (84g, 50%), contaminated starting material (17.4 g) and pure reusable starting material 20 g. The yield based on starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-[2-phthalimidoxyethyl]-5'-t-butyldiphenylsilyl-5-methyluridine

2'-O-[2-phthalimidoxyethyl]-5'-t-butyldiphenylsilyl-5-methyluridine (20g, 36.98 mmol) was mixed with triphenylphosphine (11.63g, 44.36 mmol) and N-hydroxyphthalimide (7.24g, 44.36 mmol). It was then dried over PO4 under high vacuum for two days at 40° C. The reaction mixture was mixed with argon and dry THF (369.8 mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-acetocarboxylate (6.98 mL, 44.36 mmol) was added drop wise 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 (ethyliclate-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-phthalimidoxyethyl]-5'-t-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

5'-O-Tert-butyldiphenylsilyl-2'-O-[2-formamidoiminoxyethyl]-5-methyluridine

2'-O-[2-phthalimidoxyethyl]-5'-t-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5 mmol) was dissolved in dry CH2Cl2 (4.5 mL) and methylhydrazine (300 mL, 4.64
mmol) was added dropwise at -100°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CHCl₃, and the combined organic phase was washed with water, brine and dried over anhydrous Na₂SO₄. The solution was concentrated to get 2'-O-(dimethoxyethyl) thymidine, which was then dissolved in MeOH (67.5 mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-tert-butyldiphenylsilyl-2'-O-[2-formamidinooxy]ethyl]-5-methyluridine as white foam (1.95 g, 78%).

[0177] 5'-O-tert-Butyldiphenylsilyl-2'-O-[N,N-dimethy laminoxyethyl]-5-methyluridine

[0178] 5'-O-tert-butyldiphenylsilyl-2'-O-[2-formamidinoxyethyl]-5-methyluridine (1.77 g, 3.12 mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6 mL). Sodium cyanoborohydride (0.39 g, 6.13 mmol) 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%, 10 mL) was added and extracted with ethyl acetate (2x20 mL). Ethyl acetate phase was dried over anhydrous Na₂SO₄ evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6 mL). Formaldehyde (20% w/w, 30 mL, 3.37 mmol) 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 (0.39 g, 6.13 mmol) 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 (25 mL) solution was added and extracted with ethyl acetate (2x25 mL). 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-dimethy laminoxyethyl]-5-methyluridine as a white foam (14.6 g, 80%).

[0179] 2'-O-(dimethy laminoxyethyl)-5-methyluridine

[0180] Triethylamine trihydrofluoride (3.91 mL, 24.0 mmol) was dissolved in dry THF and triethylamine (1.67 mL, 12 mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethy laminoxyethyl]-5-methyluridine (1.40 g, 2.4 mmol) 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-(dimethy laminoxyethyl)-5-methyluridine (766 mg, 92.5%).

[0181] 5'-O-DMT-2'-O-(dimethy laminoxyethyl)-5-methyluridine

[0182] 2'-O-(dimethy laminoxyethyl)-5-methyluridine (750 mg, 2.17 mmol) was dried over P₂O₅ under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20 mL). The residue was then dissolved in pyridine (11 mL) under argon atmosphere. 4-dimethylaminopyridine (26.5 mg, 2.60 mmol), 4',4'-dimethoxytrityl chloride (880 mg, 2.60 mmol) 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-(dimethy laminoxyethyl)-5-methyluridine (1.13 g, 80%).

[0183] 5'-O-DMT-2'-O-(2',N,N-dimethy laminoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

[0184] 5'-O-DMT-2'-O-(dimethy laminoxyethyl)-5-methyluridine (1.08 g, 1.67 mmol) was co-evaporated with toluene (20 mL). To the residue N,N-diisopropylamine tetrazonide (0.29 g, 1.67 mmol) was added and dried over P₂O₅ under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetone/titrile (8.4 mL) and 2-cyanoethyl-NN,N¹,N¹-tetraisopropylphosphoramidite (2.12 mL, 6.08 mmol) 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 (70 mL) and washed with 5% aqueous NaHCO₃ (40 mL). Ethyl acetate layer was dried over anhydrous Na₂SO₄ and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2',N,N-dimethy laminoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04 g, 74.9%).

[0185] 2'-Aminooxyethoxy) Nucleoside Amidites

[0186] 2'-Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethy l) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

[0187] N2-isobutyl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

[0188] The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of dianinopurine riboside. Multigram quantities of dianinopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) dianinopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) dianinopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McCle, D. P. C., Cock, P. D., Guinocco, C. J., W0 94/02501 A1 940203.) Standard protection procedures should afford 2'-O(2-ethylacetyl)-5'-O(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyl-6-O-diphenylcarbamoyl-2'-O(2-ethylacetyl)-5'-O(4,4'-dimethoxytrityl)guanosine which may be reduced to 2'-N-isobutyl-6-O-diphenylcarbamoyl-2'-O(2-hydroxyethyl)-5'-O(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may be phosphorylated as usual to yield 2-N-isobutyl-6-O-diphenylcarbamoyl-2'-O(2-phthalimidoxyl)ethyl]-5'-O(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

[0189] 2'-dimethy laminoxyethoxy (2'-DMAEOE) Nucleoside Amidites
2'-O-[2-(N,N-dimethylaminoethoxy)ethyl]-5-methyl Uridine

20 Nov. 13, 2003

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 phosphate linkages. The thiation 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. Phosphonate oligonucleotides are prepared as described in U.S. Pat. No. 5,508,270, herein incorporated by reference.

Alkylphosphonate oligonucleotides are prepared as described in U.S. Pat. No. 4,469,863, herein incorporated by reference.

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

Phosphoramidite oligonucleotides are prepared as described in U.S. Pat. No. 5,256,775 or U.S. Pat. No. 5,366,878, herein incorporated by reference.

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

3'-Deoxy-3'-amino phosphoramidite oligonucleotides are prepared as described in U.S. Pat. No. 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Pat. No. 5,023,243, herein incorporated by reference.

Boranophosphate oligonucleotides are prepared as described in U.S. Pat. Nos. 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleotide Synthesis

Methylenemethylnlin linked oligonucleosides, also identified as MMI linked oligonucleosides, methylene-dimethyl-hydrazo linked oligonucleosides, also identified as MIII linked oligonucleosides, and methylene-carbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, and identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=S linkages are prepared as described in U.S. Pat. Nos. 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. Pat. Nos. 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Pat. No. 5,223,618, herein incorporated by reference.
Example 4

[0211] PNA Synthesis


Example 5

[0213] Synthesis of Chimeric Oligonucleotides

[0214] 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 nucleo-
sides 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 oligonucle-
tides. Oligonucleotides of the second type are also known in the art as “heminmers” or “wingmers”.

[0215] [2′-O-Me][2′-deoxy][2′-O-Me] Chimeric Phosphorothioate Oligonucleotides

[0216] Chimeric oligonucleotides having 2′-O-alkyl phosphorothioate and 2′-deoxy phosphorothioate oligo-nucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. oligo-
nucleotides are synthesized using the automated synthesizer and 2′-deoxy-5′-dimethoxytrityl-3′-O-phosphor-amidite for the DNA portion and 5′-dimethoxytrityl-2′-O-methyl-3′-O-
phosphomamide for 51 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 ½ volume by rotovac before being 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.

[0217] [2′-O-(2-Methoxyethyl)][2′-deoxy][2′-O-
(Methoxymethyl)] Chimeric Phosphorothioate Oligonucleotides

[0218] [2′-O-(2-methoxyethyl)][2′-deoxy][2′-O-
(methoxy-ethyl)] chimeric phosphorothioate oligonucle-
teides were prepared as per the procedure above for the 2′-O-methyl chimeric oligonucleotide, with the substitution of 2′-O-(methoxymethyl) amidites for the 2′-O-methyl amid-
ites.

[0219] [2′-O-(2-Methoxyethyl)Phosphodiester][2′-
deoxy Phosphorothioate][2′-O-(2-Methoxyethyl) Phos-
phodiester] Chimeric Oligonucleotides

[0220] [2′-O-(2-methoxyethyl phosphodiester)][2′-
deoxy phosphorothioate][2′-O-(methoxymethyl) phos-
phodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2′-O-methyl chimeric oligo-
nucleotide with the substitution of 2′-O-(methoxymethyl) amidites for the 2′-O-methyl amidites, as described above, except for the use of iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfuration utilizing 3H-1,2 benzodithiole-3-one 1,1 diox-
ide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Example 6

[0221] Other chimeric oligonucleotides, chimeric oligo-
nucleo-sides and mixed chimeric oligonucleotides/oligo-
nucleotides are synthesized according to U.S. Pat. No. 5,623,065, herein incorporated by reference.

Example 7

[0222] Oligonucleotide Isolation

[0223] After cleavage from the controlled pore glass col-
umn (Applied Biosy stems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucle-
otide or oligonucleoside are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel ele-
trophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothio-
ate and phosphodiester linkages obtained in synthesis were periodically checked by 31P 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.

Example 7

[0224] Oligonucleotide Synthesis—96 Well Plate Format

[0225] Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthe-
sizer 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 sulfuration utilizing 3H-1,2 benzodithiole-3-one 1,1 diox-
ide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethylidisopropyl phosphoram-
idies were purchased from commercial vendors (e.g. PE-2Applied Biosystems, Foster City, Calif., or Pharmacia, Piscataway, N.J.). Non-standard nucleosides are synthesized as per known literature or patented methods. They are then utilized as base protected beta-cyanoethylidisopropyl phosphoramidites.

[0226] Oligonucleotides were cleaved from support and deprotected with concentrated NH4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-sus-
pended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.
Example 8

[0227] Oligonucleotide Analysis—96 Well Plate Format

[0228] The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDO) or, for individually prepared 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 spectrometry. 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

[0229] Cell Culture and Oligonucleotide Treatment

[0230] 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 5 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.

[0231] T-24 Cells:

[0232] 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 SA basal media (Invitrogen Corporation, Carlsbad, Calif.), supplemented with 10% fetal calf serum ([Invitrogen Corporation, Carlsbad, Calif.]), penicillin 100 units per ml, and streptomycin 100 micrograms per ml (Invitrogen Corporation, Carlsbad, Calif.). 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.

[0233] For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

[0234] A549 Cells:

[0235] 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 (Invitrogen Corporation, Carlsbad, Calif.) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, Calif.), penicillin 100 units per ml, and streptomycin 100 micrograms per ml (Invitrogen Corporation, Carlsbad, Calif.). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

[0236] NHDF Cells:

[0237] 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.

[0238] HEK Cells:

[0239] Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville, Md.). HEKs were routinely maintained 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.

[0240] MCF7:

[0241] The human breast carcinoma cell line MCF-7 was obtained from the American Type Culture Collection (Manassas, Va.). MCF-7 cells were routinely cultured in DMEM low glucose (Gibco/Life Technologies, Gaithersburg, Md.) supplemented with 10% fetal calf serum (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.

[0242] For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

[0243] Treatment with Antisense Compounds:

[0244] When cells reached 70% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 100 µL OPTI-MEM™-1 reduced-serum medium (Invitrogen Corporation, Carlsbad, Calif.) and then treated with 130 µL of OPTI-MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Invitrogen Corporation, Carlsbad, Calif.) 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.

[0245] 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 with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, TCCGTCATCGTCTCT- CAGGG, SEQ ID NO: 1, a 2’-O-methoxethyl gapper (2’-O-methoxethyl gapper 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, ATGCATTCTGCCCCCAAAGGA, SEQ ID NO: 2, a 2’-O-methoxethyl gapper (2’-O-methoxethyl gapper in bold) with a phosphorothioate backbone which is targeted to both mouse and rat craf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or craf (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-ras 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 unsuitable for oligonucleotide transfection experiments.

Example 10

[0246] Analysis of Oligonucleotide Inhibition of Estrogen Receptor Alpha Expression

[0247] Antisense modulation of estrogen receptor alpha expression can be assayed in a variety of ways known in the art. For example, estrogen receptor alpha 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. The preferred method of RNA analysis of the present invention is the use of total cellular RNA as described in other examples herein. Methods of RNA isolation are taught in, for example, Ausubel, F. M. et al., Current Protocols in Molecular Biology, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993.

Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F. M. et al., Current Protocols in Molecular Biology, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE- Applied Biosystems, Foster City, Calif. and used according to manufacturer’s instructions.

[0248] Protein levels of estrogen receptor alpha 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 estrogen receptor alpha can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, Mich.), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antiserum are taught in, for example, Ausubel, F. M. et al., Current Protocols in Molecular Biology, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997.

Preparation of monoclonal antibodies is taught in, for example, Ausubel, F. M. et al., Current Protocols in Molecular Biology, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

[0249] Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F. M. et al., Current Protocols in Molecular Biology, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F. M. et al., Current Protocols in Molecular Biology, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F. M. et al., Current Protocols in Molecular Biology, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

Example 11

[0250] Poly(A)+mRNA Isolation

[0251] Poly(A)+mRNA was isolated according to Miura et al., Clin. Chem., 1996, 42, 1758-1764. Other methods for poly(A)+mRNA isolation are taught in, for example, Ausubel, F. M. et al., Current Protocols in Molecular Biology, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993.

Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μL cold PBS. 60 μL 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 μL of lysis was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine Calif.). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 μL 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 μL 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.

[0252] Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Example 12

[0253] Total RNA Isolation

[0254] Total RNA was isolated using an RNEASY™ kit and buffers purchased from Qiagen Inc. (Valencia, Calif.) 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 μL cold PBS. 150 μL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 150 μL 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™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 1 minute. 500 μL of Buffer RW1 was added to each well of the RNEASY™ plate and incubated for 1.5 minutes and the vacuum was again applied for 1 minute. An additional 500 μL of Buffer RW1 was added to each well of the RNEASY™ plate and the vacuum was applied for 2 minutes. 1 mL of Buffer RPE was then added to each well of the RNEASY™ plate and the vacuum applied for a period of 90 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 3 minutes. The plate was then removed from the QIAVAC™ 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 170 μL water into each well, incubating 1 minute, and then applying the vacuum for 3 minutes.

[0255] The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia Calif.). 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

[0256] Real-Time Quantitative PCR Analysis of Estrogen Receptor Alpha mRNA Levels

[0257] Quantitation of estrogen receptor alpha mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, Calif.) 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., FAM, obtained from either Operon Technologies Inc., Alameda, Calif. or Integrated DNA Technologies Inc., Coralville, Iowa) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, Calif. or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

[0258] 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.

[0259] PCR reagents were obtained from Invitrogen, Carlsbad, Calif. RT-PCR reactions were carried out by adding 20 μL PCR cocktail (2.5× PCR buffer (—MgCl2), 6.6 mM MgCl2, 375 μM each of dATP, dCTP, dGTP, dTTP, 375 nM each of forward primer and reverse primer, 125 nM of probe, 4 Units RNase inhibitor, 1.25 Units PLATINUM™ Taq, 5 Units MuLV reverse transcriptase, and 2.5x ROX dye) to 96 well plates containing 50 μL 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 PLATINUM™ Taq, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

[0260] 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, Oreg.). 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.

[0261] In this assay, 170 nL of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:350 in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 30 μL purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 480 nm and emission at 520 nm.

[0262] Probes and primers to human estrogen receptor alpha were designed to hybridize to a human estrogen receptor alpha sequence, using published sequence information (GenBank accession number NM_000125.1, incorporated herein as SEQ ID NO:3). For human estrogen receptor alpha the PCR primers were:

[0263] forward primer: GTAGAGGGCATGGTGGAGAIC(TT (SEQ ID NO: 4)

[0264] reverse primer: CAAACTCTCTCCGGCATGGATT (SEQ ID NO: 5) and the PCR probe was: FAM-TGCTGCTAGCTATCGTGTTCCG-C TAMRA (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, Calif.) is the fluorescent reporter dye and TAMRA (PE-Applied Biosystem, Foster City, Calif.) is the quencher dye. For human GAPDH the PCR primers were:

[0265] forward primer: GAAGGCTAAGGGTGGGCTG (SEQ ID NO:7)

[0266] reverse primer: GAAGATGTGATGGAATCTGTCG (SEQ ID NO:8) and the PCR probe was: JOE-CAAGCTTCCGTTCTCAGCC-C TAMRA (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster City, Calif.) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, Calif.) is the quencher dye.

Example 14

[0267] Northern Blot Analysis of Estrogen Receptor Alpha mRNA Levels

[0268] Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RAZO™ (TEL-TEST “B” Inc., Friendswood, Tex.). Total RNA was prepared following manufacturer’s
recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, Ohio). RNA was transferred from the gel to HYBOND™-N+nylon membranes (Amersham Pharmacia Biotech, Piscataway, N.J.) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST “B” Inc., Friendswood, Tex.). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKERTM UV Crosslinker 2400 (Stratagene, Inc., La Jolla, Calif.) and then probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, Calif.) using manufacturer’s recommendations for stringent conditions.

[0269] To detect human estrogen receptor alpha, a human estrogen receptor alpha specific probe was prepared by PCR using the forward primer GTAGAGGCTAGGTCAGAGATCTTT (SEQ ID NO: 4) and the reverse primer CAAAATCTTCTCCCTGCAAGT (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, Calif.).

[0270] Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, Calif.). Data was normalized to GAPDH levels in untreated controls.

Example 15

[0271] Antisense Inhibition of Human Estrogen Receptor Alpha Expression by Chimeric Phosphorothiolate Oligonucleotides Having 2'-MOE Wings and a Deoxy Gap

[0272] In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human estrogen receptor alpha RNA, using published sequences (GenBank accession number NM_000125.1, representing the main mRNA of estrogen receptor alpha, incorporated herein as SEQ ID NO: 3; a concatenated sequence of exons 1A, 2, 1B, 1C, and 4-12 from GenBank accession number NT_007587.3, a segment sequence of chromosome 6; residues 491001-833000 of GenBank accession number NT_007587.3, representing a partial genomic sequence of estrogen receptor alpha, incorporated herein as SEQ ID NO: 11; GenBank accession number AF258449.1, representing the estrogen receptor alpha variant herein designated ESR-alpha-V, incorporated herein as SEQ ID NO: 12; GenBank accession number AF258451.1, representing the estrogen receptor alpha variant herein designated ESR-alpha-VI, incorporated herein as SEQ ID NO: 13; GenBank accession number AB608241.1, representing the estrogen receptor alpha variant herein designated ESR-alpha-VII, incorporated herein as SEQ ID NO: 14; GenBank accession number M69297.1, representing the estrogen receptor alpha variant herein designated ESR-alpha-IX, incorporated herein as SEQ ID NO: 15; GenBank accession number U68067.1, representing the estrogen receptor alpha variant herein designated ESR-alpha-IX, incorporated herein as SEQ ID NO: 16; GenBank accession number U68068.1, representing the estrogen receptor alpha variant herein designated ESR-alpha-IX, incorporated herein as SEQ ID NO: 17; and GenBank accession number X73087.1, representing the estrogen receptor alpha variant herein designated ESR-alpha-IX, incorporated herein as SEQ ID NO: 18). The oligonucleotides are shown in Table 1. “Target site” indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 1 are chimeric oligonucleotides (“gapmers”) 20 nucleotides in length, composed of a central “gap” region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide “wings”. The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5'-methylcytidines. The compounds were analyzed for their effect on human estrogen receptor alpha 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”.

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| 192246 | 5'TUR   | 10 196          | tcctgctgctgctgctg | 0        | 20  
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| 192248 | 5'TUR   | 10 818          | tcccatctgctgctgctg | 55       | 24  
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| 192250 | 5'TUR   | 10 908          | gccctgccggctgctgctg | 56      | 26  
| 192251 | 5'TUR   | 10 1080         | cgccgctgctgctgctgctg | 71     | 27  
| 192252 | 5'TUR   | 10 1139         | cgccgctgctgctgctgctg | 75     | 28  
| 192253 | 5'TUR   | 10 1180         | cgccgctgctgctgctgctg | 67     | 29  
| 192254 | 5'TUR   | 10 1153         | cgccgctgctgctgctgctg | 57     | 30  
| 192255 | 5'TUR   | 10 1158         | gggctgctgctgctgctg | 78      | 31  
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Inhibition of human estrogen receptor alpha mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap.
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[0273] As shown in Table 1, SEQ ID NOs 19, 20, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 34, 35, 37, 39, 40, 42, 43, 44, 45, 46, 47, 48, 49, 50, 52, 53, 54, 56, 60, 74, 77, 88, 91 and 95 demonstrated at least 40% inhibition of human estrogen receptor alpha 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

[0274] Western Blot Analysis of Estrogen Receptor Alpha Protein Levels

[0275] 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 primary antibody directed to estrogen receptor alpha is used, with a radiolabeled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale Calif.).

Example 17

[0276] Targeting of Individual Oligonucleotides to Specific Variants of Estrogen Receptor Alpha

[0277] It is advantageous to selectively inhibit the expression of one or more variants of estrogen receptor alpha. Consequently, in one embodiment of the present invention are oligonucleotides that selectively target, hybridize to, and specifically inhibit one or more, but fewer than all of the variants of estrogen receptor alpha. A summary of the target sites of the variants is shown in Table 2 and includes Genbank accession number NM_000215.1, representing estrogen receptor alpha main mRNA (ESR-alpha), incorporated herein as SEQ ID NO: 3; Genbank accession number AF258449.1, representing ESR-alpha-V, incorporated herein as SEQ ID NO: 12; Genbank accession number AF258451.1, representing ESR-alpha-VI, incorporated herein as SEQ ID NO: 13; Genbank accession number AI630824.1, representing ESR-alpha-VIII, incorporated herein as SEQ ID NO: 14; Genbank accession number M69297.1, representing ESR-alpha-IX, incorporated herein as SEQ ID NO: 15; Genbank accession number U68067.1, representing ESR-alpha-IV, incorporated herein as SEQ ID NO: 16; Genbank accession number U68067.1, representing ESR-alpha-III, incorporated herein as SEQ ID NO: 17; Genbank accession number X73067.1, representing ESR-alpha-II, incorporated herein as SEQ ID NO: 18; Genbank accession number S80316.1, representing the estrogen
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<400> SEQUENCE: 13

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720
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<210> SEQ ID NO 13
<211> LENGTH: 1128
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE: CDS
<221> NAME/KEY: 5CDR
<222> LOCATION: (233)...(409)

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<210> SEQ ID NO: 14
<211> LENGTH: 530
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: exon/exon junction
<222> LOCATION: (260)...(261)
<223> OTHER INFORMATION: exon 1/exon 4

<400> SEQUENCE: 14

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cgaccccgcc tgcactcacc gcaacccgcc coccgatggt ctgcttttctt gcacccgaacc 180
ggcccagacg gcccttacta ccgtggagac gcggccgagt gcgttacaggt gcggcgggcc 240
ggcgcggcc gccttcagtc cagtctgtgg ctcgggtgca gcgcctccag ggtgtcaccac 300
tgatgttccs gagaattttgc gttgagtgta gctcactagg catggtagtg cttcggttac 360
cctccctct ctcgctgctc ataggcagcc actctatgtc gggagatgg tttatagatca 420
aaaattctct ggacgcttac tattgttga agagggttgc cattcgtcag ccacagttta 480	tttactaaa ttttacctgc gcacagctga gattttgcac gattttactc 530

<210> SEQ ID NO: 15
<211> LENGTH: 2315
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (241)...(903)
<223> OTHER INFORMATION: exon 5/exon 6

<400> SEQUENCE: 15

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atg acc atg acc ctc cac acc gaa tca tgg atg ggc cta cta cat
Met Thr Met Thr Leu His Thr Ala Ser Gly Met Ala Leu Leu His
1 5 10 15

cag atc cca ggg aac gag cag ggc ccc ctc cag ctc cag ccc ctc aag
Gln Ile Gin Gin Gin Glu Leu Pro Leu Asn Gin Gin Leu Lys
20 25 30

cgg ccc ctc gag cgg ccc ctc cgg gag tgc tac cag aac aac aag
Ile Pro Leu Gin Gin Pro Gin Leu Tyr Leu Val Leu Ser Ser Lys
35 40 45

cgg ggc ggt tac aac tac ccc ggg ggc ggc ggc tac ggg ttc aac ggc
Pro Ala Val Tyr Asn Tyr Pro Gin Gin Gin Gin Ala Tyr Glu Asn Ala
50 55 60

cgg ggc ggc ggc ggc ggc cgg gtc tac ggt cag acc ggc ctc ccc tac
Ala Ala Ala Ala Ala Gin Val Tyr Gin Thr Gin Gin Thr Gin Pro Tyr
65 70 75 80

ggc ccc cgg tct gag ggt ggc ttc ggc ccc ggc cgg ggt ggg gtt
Gly Pro Gin Gin Gin Gin Ala Ala Pro Ser Gin Gin Gin Gin Gly
90 95

ttc ccc cca ctc aac gag gtt tgt ctc cgg cgc ctc atg ctc ctc cac
Phe Pro Pro Leu Asn Ser Val Ser Pro Ser Gin Pro Leu Met Leu His
105 110

cgg ccc cgg cag ctc tcc cct tct ctt ctc cag ccc ccc ggc cag cag gtt
Pro Pro Pro Gin Pro Gin Gin Pro Pro Pro Gin Gin Gin Val
115 120 125

cgc tac tac ctc gag aac gag ccc aac ggc tac acc acc ggc ggc ggg cag
Pro Tryr Tyr Leu Gin Gin Gin Glu Gin Gin Gin Gin Glu Val Tryr
130 135

ggc cgc cgc gca ttc tac aag cca aat tca gat aat cga cgc cag gtt
Gly Pro Pro Ala Phe Tryr Arg Pro Asn Ser Arg Arg Gin Gin Gly
145 150 155 160

ggc aag gga aag tgt gcc aat acc aat gag aag aag atg gct gtt
Gly Arg Glu Arg Leu Ala Ser Thr Asn Gin Gin Gin Gin Gin Gin
165 170 175

gaa tct gcc aag gag acc ctc aag gag ggg gaa ggt tgc aat gac tat gtt
Glu Ser Ala Leu Gin Thr Tyr Gin Ala Cys Ala Val Cys Asn Arg Tyr Ala
180 185 190

tca gcc tac cat tat gga gtc tgt tcc tgt gag ggc egc ggc tgc
Ser Gin Gin Gin Gin Gin Gin Gin Ser Gin Gin Gin Gin Gin Gin
195 200 205

ttc aag aag aat att caa cca ctt cca aca ctc tgt gta tagagtgt
Phe Lys Arg Ser Ile Gin Gin Gin Leu Pro Thr Leu Cys
210 215 220

dctgacca aacagagat atagcatat ggagcagac aagagcgcta gaaatatac
973

cacactta caacactctg atcctgtgaca aacgtgacaa aacagaaaga tggggaagg
1033

tgttgttaggt ggagtgtgaggt cagagcgtaa acaagctgaa
1093

tggatgacac ctctacacac tataacaaat ttataacag atggatattat gacttagaatg
1153

tgacacacata cacacataagc cacatgacttg gataactaca gacatcagac
1213

gactggagc gactgactag tctaaacacac caaagacag ggggagac gggggaga
1273

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1333

937

993

1093
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1573
catcagaga atgacataa aaacacctat gagatccact ctatcaccagn ttaagatgt
1633
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1753
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1813
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1933
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1993
catatatgg tagggactcg gtaagaagctg aaccactcata ctctcagcaca actattgca
2053
gcgacaaaa ccaacatcgct aatgttctcac tctagtggtg gcattcgacca taagaacac
2113
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2173
aggtattaga atagggatgta taactatgtat aatgtgatag ttaagtggtt ccagccacaca
2233
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2293
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<210> SEQ ID NO 16
<211> LENGTH: 330
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (262)...(330)
<220> FEATURE:
<221> NAME/KEY: exonic:exon junction
<222> LOCATION: (191)...(192)
<223> OTHER INFORMATION: exon 1:exon 3

<400> SEQUENCE: 16
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cagatgcct gcggccocco cgcggcccttc ccggggtactt ccagacgcctc 180
cgacacatga aagttggccct ccggggtctct gagctctgc gctgggggac acgttgctga 240
cctgcgcccc ggggacagcag c cag ac atg acc ctc cac acc aa aca tct
291
Met Thr Met Thr Leu His Thr Lys Ala Ser 1 5 10

<210> SEQ ID NO 17
<211> LENGTH: 355
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (287)...(355)
<220> FEATURE:
<221> NAME/KEY: exonic:exon junction
<222> LOCATION: (85)...(86)
<223> OTHER INFORMATION: exon 1A:exon 2
<220> FEATURE:
<221> NAME/KEY: exonic:exon junction
<222> LOCATION: (216)...(217)
<223> OTHER INFORMATION: exon 2:exon 3

<400> SEQUENCE: 17
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Gly Met Ala Leu Leu His Gln Ile Gln Gly Aan Glu Leu
15 20
<400> SEQUENCE: 17
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Met  Thr  Met
1
acc  ctc  csc  acc  aca  gca  tct  ggg  atg  gcc  cta  ctg  cat  cag  atc  cca
Thr  Leu  His  Thr  Lys  Ala  Ser  Gly  Met  Ala  Leu  Leu  His  Gin  Ile  Gin 5 10 15
343
355
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Gly  Asn  Glu  Leu
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<210> SEQ ID NO 18
<211> LENGTH: 323
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: exon1 exon junction
<222> LOCATION: (115)...(116)
<223> OTHER INFORMATION: exon 7 exon 9
<220> FEATURE:
<221> NAME/KEY: exon1 exon junction
<222> LOCATION: (254)...(255)
<223> OTHER INFORMATION: exon 9 exon 10

<400> SEQUENCE: 18
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ctcgactgga  cactcatctgc  gtt
323

<210> SEQ ID NO 19
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 19
gaatgtgct  tgtagaatgc
20

<210> SEQ ID NO 20
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 20
cgattgctcc  ttgaactctt
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<210> SEQ ID NO 21
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 21

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<210> SEQ ID NO 22
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 22
tcagtgatgc ttcaagagtc

<210> SEQ ID NO 23
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 23
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<210> SEQ ID NO 24
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 24
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<210> SEQ ID NO 25
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 25
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<210> SEQ ID NO 26
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 26
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<210> SEQ ID NO 27
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide
<400> SEQUENCE: 27
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<210> SEQ ID NO 20
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide
<400> SEQUENCE: 20
tagggcactg gctggcctgtg

<210> SEQ ID NO 29
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide
<400> SEQUENCE: 29
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<210> SEQ ID NO 30
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide
<400> SEQUENCE: 30
cgttctcag gtgtaggggc

<210> SEQ ID NO 31
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide
<400> SEQUENCE: 31
gggctcgatt tccaggtattg

<210> SEQ ID NO 32
<211> LENGTH: 20
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<210> SEQ ID NO 33
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<212> TYPE: DNA
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<400> SEQUENCE: 34
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<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 37
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 38
ttcaccctcg ttggtgatgg

<210> SEQ ID NO 39
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<400> SEQUENCE: 47

ttgccagtc tcatgtctcc 20

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<400> SEQUENCE: 54
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<400> SEQUENCE: 65

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<400> SEQUENCE: 66

tggtccagas ctcggtgsts  

<210> SEQ ID NO 67
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<400> SEQUENCE: 68

asgtgtctgt gcsttgctcc  

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<223> OTHER INFORMATION: Antisense Oligonucleotide

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gctttgta catagtcgcc

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<400> SEQUENCE: 78

atctctttat gacatcagc 20

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ttggaagtcc tgatagtctt 20

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<400> SEQUENCE: 81

acaaaagctg tcacagagca 20

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cgtttatgtcc tgtagatgac 20

<210> SEQ ID NO 83
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<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 83

cocgtggtcc acctttgtatc 20

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<220> FEATURE:
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gggcccact ttaacctgagc 20

<210> SEQ ID NO: 85
<211> LENGTH: 20
<212> TYPE: DNA
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<400> SEQUENCE: 85
ttcctctctc tgaagagagc 20

<210> SEQ ID NO: 86
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<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 86

gggcccacc tcaagtgagc 20

<210> SEQ ID NO: 87
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<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 87

ccacaagcc acctttctac 20

<210> SEQ ID NO: 88
<211> LENGTH: 20
<212> TYPE: DNA
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<400> SEQUENCE: 89

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<400> SEQUENCE: 90
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<400> SEQUENCE: 91

cggggtacc tgtgaatagc

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<400> SEQUENCE: 92
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<210> SEQ ID NO 93
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<400> SEQUENCE: 93
gtcacttacc tgtccagagc

<210> SEQ ID NO 94
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<400> SEQUENCE: 94
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<210> SEQ ID NO 95
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<400> SEQUENCE: 95
catttcacct agcatrgtgt

<210> SEQ ID NO 96
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense Oligonucleotide

<400> SEQUENCE: 96
cottttgta cgtaggtggg

<210> SEQ ID NO 97
<211> LENGTH: 144
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:

<400> SEQUENCE: 97
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cotgcctgc gcgcagagac acacatgctg gcgcagagac 144

<210> SEQ ID NO 98
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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
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<400> SEQUENCE: 98
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agcgctgcgc gcgcagagac acacatgctg gcgcagagac 240
cotgcgtgc gcgcagagac acacatgctg gcgcagagac 300
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ttcgcgcgac gcgcagagac acacatgctg gcgcagagac 1218
What is claimed is:

1. A compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding estrogen receptor alpha, wherein said compound specifically hybridizes with said nucleic acid molecule encoding estrogen receptor alpha and inhibits the expression of estrogen receptor alpha.

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: 19, 20, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 34, 35, 37, 39, 40, 42, 43, 44, 45, 46, 47, 48, 49, 50, 52, 53, 54, 56, 60, 74, 77, 88, 91 or 95.

4. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.

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 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-methycytosine.

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 estrogen receptor alpha.

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.

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

15. A method of inhibiting the expression of estrogen receptor alpha in cells or tissues comprising contacting said cells or tissues with the compound of claim 1 so that expression of estrogen receptor alpha is inhibited.

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

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

18. The compound of claim 1 targeted to a nucleic acid molecule encoding an estrogen receptor alpha/fatty acid synthase fusion transcript, wherein said compound specifically hybridizes with and differentially inhibits the expression of said fusion transcript relative to variants of estrogen receptor alpha.

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

20. The compound of claim 19 targeted to a nucleic acid molecule encoding estrogen receptor alpha, wherein said compound hybridizes with and specifically inhibits the expression of a variant of estrogen receptor alpha, wherein said variant is selected from the group consisting of ESR-alpha, ESR-alpha-II, ESR-alpha-III, ESR-alpha-IV, ESR-alpha-V, ESR-alpha-VI, ESR-alpha-VII, ESR-alpha-VIII and ESR-alpha-IX.

* * * * *