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(54) **Title**: OLIGONUCLEOTIDES FOR MODULATING GENE EXPRESSION AND USES THEREOF

(57) **Abstract**: The present invention regards oligonucleotides for modulating the expression of a gene, in particular for modulating a gene responsible for a pathology of genetic, tumoural or viral origin. Moreover, the present invention relates to the use of said oligonucleotides, possibly chemically modified, for the treatment and/or the diagnosis of said diseases.

"OLIGONUCLEOTIDES FOR MODULATING GENE EXPRESSION AND USES THEREOF"

DESCRIPTION

The present invention relates to oligonucleotides for modulating the expression of a gene, in particular for modulating a gene responsible for a pathology of genetic, tumoural or viral origin.

Furthermore, the present invention relates to the use of said oligonucleotides, possibly chemically modified, for the treatment and/or the diagnosis of said diseases.

Oligonucleotides are short sequences of natural RNA or DNA nucleic acids or of synthetic nucleic acids, e.g. PNA (Peptide Nucleic Acid), LNA (Locked Nucleic Acid) and morpholinos.

Oligonucleotides have experimentally demonstrated to be very effective in modulating the expression of a gene at the level of both transcription and translation. Because of this ability, oligonucleotides represent a valid means for the treatment of numerous pathologies, in particular diseases of genetic, tumoural or viral origin.

Modulating the expression of a gene can imply inhibiting or activating it.

For example, oligonucleotides are known which are capable of inhibiting the transcription of a gene by forming a complementary bond with the antisense strand of the gene (Hélène C, Bioch Bioph Acta 1990, 1049(2):99-125) or modifying the state of the chromatin in the regulatory regions of the gene of interest (Rossi JJ, Nat Chem Biol 2007, 3(3):136-7). Other oligonucleotides are capable of inhibiting gene translation, for example, via a complementary bond with the target Messenger RNA (mRNA). In the case of single-stranded oligonucleotides, this bond causes enzymatic degradation of the mRNA by the RNase H complex. In the case

in which the oligonucleotides are molecules of double-stranded "interfering" RNA, the complementary bond of the oligonucleotides with the target mRNA causes degradation of the messenger by the "slicer" enzyme of the RISC complex. In the latter case, the oligonucleotides can also be oligonucleotides equivalent to an endogenous microRNA capable of associating, by virtue of imperfect complementarity, with the 3'UTR region (3' untranslated region) of the target mRNA, causing the translation of that mRNA to be blocked.

Oligonucleotides can also induce the activation of a gene or an increase in the transcription thereof, for example via a complementary bond with long antisense non-coding RNA (Morris KV, Epigenetics, 2009, 4(5): 296-301), or by inhibiting the complementary microRNA, with a consequent increase in the translation of the target mRNA of the microRNA.

Oligonucleotides can be chemically modified with the aim of increasing their effectiveness in therapeutic and/or diagnostic terms. For example, an oligonucleotide can be modified in order to improve its specificity and/or the force with which it pairs with the complementary sequence, or else an oligonucleotide can be modified in order to make it less sensitive to enzymatic degradation, to improve its pharmacokinetic/pharmacodynamic profile, or to facilitate its passage through cell membranes.

In addition to oligonucleotides of natural nucleic acids (i.e. short sequences of DNA and/or RNA), there exist oligonucleotides of synthetic nucleic acids, e.g. peptide nucleic acids (PNA) and locked nucleic acids (LNA), which have been greatly studied and characterized, above all with the aim of modulating the expression of a gene by means of a an antigene strategy (i.e. designed to strike the gene directly). PNA and LNA oligonucleotides, like all modified oligonucleotides, are in general much more chemically stable

than DNA or RNA oligonucleotides. Their stability can be further improved by synthesizing chimera oligonucleotides. A chimera oligonucleotide is, for example, an oligonucleotide sequence in which both classic monomers (deoxyribonucleotides or ribonucleotides) and synthetic nucleobases (monomers), for example LNA monomers, are inserted.

LNAs are used with an antisense strategy to silence genes by inhibiting the transcript of the target gene (Braasch DA, Nucl Acids Res, 2002, 30(23):5160-7). Alternatively, LNA oligonucleotides can also be used in an antigene strategy, as was done by Smith and colleagues (Ge R, Faseb J, 2007, 1902-14), who designed the oligonucleotide sequence in such a way as to enable interaction on both strands of the nucleic acid by means of a "strand invasion" mechanism, so as to form a "Z-shaped" structure (defined as "Zorro" oligonucleotides).

PNA oligonucleotides are enzymatically more stable when compared with other oligonucleoside structures. PNAs can bind to double-stranded DNA (DNAds) through "strand invasion", or can pair, in a complementary manner, with a molecule of single-stranded DNA (DNAss), or else they can bind to strands of RNA, giving rise to a hybrid double-helix PNA/DNA or PNA/RNA structure which is thermodynamically very stable compared to "homoduplex" structures (such as a DNA/DNA double strand).

PNAs represent a highly advantageous system for modulating the expression of a gene, above all using an antigene strategy. In fact, it has been demonstrated that PNAs exhibit high specificity for the target sequences and thus enable the expression of the protein to be inhibited in an efficient manner.

Therefore, PNAs represent a promising therapeutic approach for the treatment of genetic or viral diseases.

The only disadvantage of PNAs is the fact that they have a

limited ability to pass through cell membranes. However, this limitation has been solved by conjugating oligonucleotides in general, and PNAs in particular, with molecules capable of rendering the passage through cell membranes more effective (carriers).

In fact, oligonucleotides, and PNAs specifically, can in general be administered using carriers (or "tags") conjugated to them, for example peptide sequences with a length varying from 1 to 30 amino acids.

One particular application of oligonucleotides regards modulating the expression of the genes which are activated or repressed in tumours.

It is well known that tumours are caused by the dysregulation of various genes. Usually, the damage affects proto-oncogenes (or also simply oncogenes) like the MYC genes (tra cui MYC, MYCN, MYCL1), survivin (BIRC5), BCL2, PLK4, ALK and PKM2, which are activated or overexpressed in the tumour. Moreover, in a tumour, antitumour or oncosuppressor genes such as caspase-8 and RASSF1 are usually also inactivated.

In particular, the oncogenes of the MYC family are involved in the development of numerous human tumours and are among the genes most responsible for the onset and progression of neoplasms. Amplification and/or overexpression of these genes is almost always associated with tumours, both of the paediatric type (e.g. neuroblastoma, medulloblastoma and rhabdomyosarcoma) and adult type (e.g. small cell lung cancer, or glioblastoma)(Pession A, Cur Cancer Drug Target, 2005, 5(4):273-83). In fact, they act by means of mechanisms which are fundamental for tumour growth, such as induction of cell proliferation, resistance to apoptosis, formation of metastasis and resistance to chemotherapy drugs.

Many oligonucleotides with an antitumour effect are known in the literature.

For example, there exist oligonucleotides which are directed against the MYC, MYCN, BCL2, BIRC5 genes in antisense strategies (EV Prochownik, Exp Rev Antic Ther 2004, 4(2):289-302; Felsher DW, Drug News Persp, 2003, 16(6):370-4; CF Bennet, Exp Opin Investig Drugs, 1999, 8(3):237-53) or oligonucleotides directed against MYCN and MYC with an antigene effect (LC Boffa, Oligonucleotides 2005, 15(2):85-93).

DNA-based phosphorothioate antisense oligonucleotides have also been generated with the aim of inhibiting the translation of the MYCN gene into neuroblastoma cells (Burkhart CA, JNCI, 2003, 95(18):1394-403) and antisense oligonucleotides have been generated through "small interfering RNA (siRNA)" to inhibit the translation of the MYCN gene into neuroblastoma cells (Kang JH, Bioch Bioph Res Com, 2006, 351(1):192-197).

However, there is still a strong felt need to identify further oligonucleotides capable of modulating the expression of a gene in an increasingly specific and selective manner so as to be able to have a potent therapeutic and/or antitumour effect.

In order to obtain oligonucleotides utilizable as therapeutic and/or diagnostic means, it is necessary to identify the sequences of a target gene, or a target messenger RNA and/or their respective regulatory sequences which are capable of determining a significant and selective modulation effect on the expression of the gene itself in terms of both transcription and translation.

Thus there is also a felt need to define the general rules governing the process of identifying - within the sequence of a gene, or the sequence of a messenger RNA or the regulatory sequences thereof - the oligonucleotide sequences that are most promising for the purposes of modulating the

transcription/translation of the gene itself.

The needs in this sector as just described are met by the present invention, which, according to a first aspect, relates to an oligonucleotide for modulating the expression of a gene, comprising 6-30 nucleotides (monomers), preferably 12-24 nucleotides, said oligonucleotide being characterized by a sequence comprising at least one group of at least two consecutive guanines. The oligonucleotide having the sequence SEQ ID NO: 1 is understood as being excluded from the definition just given.

In the case of natural nucleic acids (DNA and RNA), each monomer (nucleotide) consists of a nitrogenous base, a sugar and a triphosphate. The base is selected from among adenine, guanine, thymine, cytosine and uracil (only in RNA). The sugar is deoxyribose in the case of DNA and ribose in the case of RNA. The monomers are linked in the polymer by a phosphodiester bond.

Preferably, the oligonucleotide of the present invention comprises a sequence comprising at least one group of at least three consecutive guanines. The oligonucleotide having the sequence SEQ ID NO: 1 is understood as being excluded from this definition.

More preferably, the oligonucleotide of the present invention comprises a sequence comprising at least one group of at least four consecutive guanines.

Even more preferably, the oligonucleotide of the present invention comprises a sequence comprising at least one group of at least five consecutive guanines.

Particularly preferred for the purposes of the present invention is an oligonucleotide comprising a sequence comprising at least one group of at least six consecutive guanines.

In some embodiments of the invention, the oligonucleotide

comprises a sequence comprising at least one group consisting of two to six consecutive guanines.

In further embodiments the at least one group of guanines preferably comprises at least two groups of at least two consecutive guanines.

Alternatively the at least one group of guanines preferably comprises at least one group of at least two consecutive guanines and at least one group of at least three consecutive guanines.

In further embodiments the at least one group of guanines preferably comprises at least three groups of at least two consecutive guanines.

In further embodiments the at least one group of guanines preferably comprises at least four, five or six groups of at least two consecutive guanines.

Alternatively the at least one group of guanines preferably comprises at least one group of at least two consecutive guanines and at least two groups of at least three consecutive guanines.

Alternatively the at least one group of guanines preferably comprises at least one group of at least three consecutive guanines and at least two groups of at least two consecutive guanines.

Alternatively the at least one group of guanines preferably comprises at least one group of at least two consecutive guanines, at least one group of at least three consecutive guanines and/or at least one group of six consecutive guanines.

In general, the oligonucleotides of the present invention are perfectly complementary to the target sequence and, preferably, the groups of consecutive guanines can be consecutive to one another so that, for example, three groups of 2 consecutive guanines is a group of 6 consecutive

guanines. Alternatively, the groups of consecutive guanines can be spaced apart by at least one nucleotide.

In general, the at least one group of at least two consecutive guanines according to the present invention can be located near the 5′ end of the oligonucleotide, or near the 3′ end of the oligonucleotide, or else it can be located at the centre of the oligonucleotide sequence.

In preferred embodiments of the present invention said oligonucleotide is conjugated, preferably at its 3′ and/or 5′ end, with a carrier sequence, which is preferably a short amino acid sequence.

Said short amino acid sequence (carrier) preferably consists of a number of amino acids ranging from 1 to 30, preferably 1 to 10, even more preferably 1 to 7. The amino acids can be in L or D form, preferably in D form.

The carriers that are preferred for the purposes of the present invention are selected from the group consisting of: SEQ ID NO: 47 (PKKKRKV); SEQ ID NO: 48 (VKRKKKP); SEQ ID NO: 49 (KKKKKK); SEQ ID NO: 50 (PKRKRKV); SEQ ID NO: 51 (KRKRKRK); SEQ ID NO: 52 (KKKRKV); SEQ ID NO: 53 (PKKKRK); SEQ ID NO: 54 (KKKRK); SEQ ID NO: 55 (RRRR) and SEQ ID NO: 56 (PKKKRKVHHHHH).

The carrier that is particularly preferred for the purposes of the present invention is the peptide having the SEQ ID NO: 47.

In the context of the present invention, "carrier" means a peptide capable of favourably modifying the pharmacokinetic and/or pharmacodynamic profile and/or cellular and/or nuclear penetration of an oligonucleotide.

In the context of the present invention, "modulating the expression of a gene" means inhibiting or activating (increasing) the expression of a gene. Said inhibition or activation of (increase in) gene expression can occur on a

transcription or translation level.

The inhibition or activation of gene expression can be achieved on a transcription level by means of oligonucleotides which act with an antigene mechanism (or antigene oligonucleotide, i.e. directed against the antisense strand of the gene, that is, antigene strategy). Alternatively, the inhibition of gene expression can be achieved on a translation level using oligonucleotides which act through an antisense mechanism (or antisense oligonucleotide, i.e. directed against the messenger, that is, antisense strategy), whereas an increase in expression on a translation level can be achieved by inhibiting the microRNAs which degrade the messenger RNA.

The parameters or rules or prerequisites that an oligonucleotide must satisfy in order to effectively modulate the expression of a gene, identified by the Applicant and stated above, are valid for any gene whatsoever and can thus be applied, for example, for the purpose of identifying sequences of oligonucleotides which are capable of modulating the expression of the gene(s) responsible for diseases of genetic and/or viral origin or the genes involved in the onset of tumour pathologies.

The oligonucleotides thus identified can be used, preferably as drugs, in therapeutic approaches for the treatment of specific genetic, viral or tumoural diseases.

Alternatively, the oligonucleotides can be utilized for diagnostic purposes.

In fact, the subject matter of the present invention further relates to the use of the oligonucleotides of the invention, possibly chemically modified, for therapeutic and/or diagnostic purposes.

The oligonucleotides of the present invention are short oligonucleotides of 6-30, preferably 12-24 residues

(nucleotides or monomers). The oligonucleotides can consist of a natural nucleic acid base, for example DNA or RNA, or a synthetic nucleic acid base, for example PNA, LNA or morpholino. Alternatively, the oligonucleotides can comprise a combination of DNA, RNA and/or synthetic nucleic acids, preferably PNA or LNA (hybrid or chimeric oligonucleotides). Moreover, the oligonucleotides can be single- or double-stranded.

In some embodiments of the present invention, the oligonucleotides can be chemically modified, for example for the purpose of improving their therapeutic and/or diagnostic effectiveness.

In preferred embodiments of the present invention, the oligonucleotides can be PNA molecules with a backbone in which the carbon in the alpha position ($C_{alpha(\alpha)}$) is bound to substituents other than the typical hydrogen atom of glycine. For example, instead of the side chain of glycine the side chain of another amino acid of natural or synthetic origin can be used, preferably selected from the group consisting in: arginine, lysine, histidine, leucine, isoleucine, tyrosine, asparagine, serine, threonine, glutamine, valine, alanine, cysteine, methionine, phenylalanine, glutamate, aspartate, proline, tryptophan and ornithine. Said amino acid can be of the dextrorotatory configuration (D) or the levorotatory configuration (L).

In other preferred embodiments of the invention, the oligonucleotides are: mutually complementary single- or double-stranded RNA molecules (the mutually complementary double-stranded RNA molecules are defined as siRNA, acronym of "small interfering RNA").

In some embodiments, said "small interfering RNA" comprises RNA monomers (ribonucleotides) and at least one modified monomer in the ribose 2' position, preferably a 2'-O-

methoxyethyl, 2'-O-methyl or 2'-fluoro monomer; or said "small interfering RNA" comprises RNA monomers (ribonucleotides) and at least one monomer of a synthetic nucleic acid preferably selected from among: LNA, Methylphosphonate LNA, BNA (bridged nucleic acid), UNA (unlocked nucleic acid), ENA (ethylene-bridged nucleic acid), ANA (arabinose nucleic acid) and F-ANA (fluoro-arabinoside nucleic acid).

In preferred embodiments, said "small interfering RNA" is designed in such a way that at the ends of the complementary double strand, only one of the two strands has at least one, preferably two, monomers of natural or synthetic nucleic acids which protrude, i.e. are not paired. In further embodiments, natural or synthetic protruding nucleic acids are preferably selected from among: a 2'-O- methoxyethyl, 2'-O-methyl and 2'-fluoro monomer or a monomer of: LNA, Methylphosphonate LNA, BNA, UNA (unlocked nucleic acid), ENA (ethylene-bridged nucleic acid), ANA (arabinose nucleic acid) and F-ANA (fluoro-arabinoside nucleic acid).

In further embodiments, the oligonucleotides are defined as hybrid or chimeric and are preferably single- or double-stranded, comprising RNA monomers (ribonucleotides) and LNA monomers (this oligonucleotide is represented as RNA/LNA).

Alternatively, the hybrid oligonucleotides can comprise the RNA monomers (ribonucleotides) and at least one RNA monomer selected from among: a 2'-O-Methoxyethyl (MOE) monomer, a 2'-O-methyl monomer and a 2'-fluoro monomer; or the hybrid oligonucleotides can comprise RNA monomers (ribonucleotides) and at least one monomer of a synthetic nucleic acid preferably selected from among: LNA, methylphosphonate LNA, UNA (unlocked nucleic acid), BNA, ENA (ethylene nucleic acid), ANA (arabinose nucleic acid) and F-ANA (fluoro-arabinoside nucleic acid).

In a further embodiment, the oligonucleotides based on single- or double-stranded RNA can comprise classic ribonucleotides (i.e. not chemically modified) and ribonucleotides or deoxyribonucleotides which have been modified at the level of the phosphodiester bond, for example by means of a phosphorothioate bond, or DNG (deoxyribonucleic guanidine), RNG (ribonucleic guanidine), GNA (glycerol nucleic acid), G-PNA (gamma-PNA) or PMO (Morpholino).

In preferred embodiments of the invention, the oligonucleotides are chimeric single-stranded sequences comprising DNA monomers (deoxyribonucleotides) and LNA monomers.

For an antigene strategy, which means modulating the expression of a gene on the transcription level, one can preferably employ:

- PNA-based oligonucleotides, optionally conjugated with a carrier (generally consisting of 1 to 30 residues), preferably at the 3′ end and/or 5′; or
- PNA-based oligonucleotides, said PNA comprising at least one alpha carbon (C-alpha) of the backbone with a substituent other than the H atom of the canonical glycine; or
- single-stranded oligonucleotides comprising RNA monomers (the classic ribonucleotides) and optionally at least one modified nucleotide (monomer) (for example a 2′-O-Methyl RNA monomer, a 2′-Fluoro RNA monomer), or at least one monomer of a nucleic acid selected from among: LNA, methylphosphonate LNA, BNA, UNA, GNA, ANA, FANA, ENA, DNG and RNG, or a ribonucleotide modified at the level of the phosphodiester bond; or
- mutually complementary double-stranded RNA-based oligonucleotides (siRNA); or
- partially complementary double-stranded chimeric

oligonucleotides comprising RNA monomers (the classic ribonucleotides) and at least one LNA monomer; or

- double-stranded chimeric oligonucleotides comprising RNA monomers (the classic ribonucleotides) and at least one 2'-O-(2-Methoxyethyl) RNA monomer; or
- single-stranded chimeric oligonucleotides comprising DNA monomers (the classic deoxyribonucleotides) and at least one LNA monomer; or
- oligonucleotides comprising DNA monomers (the classic deoxyribonucleotides) and at least one 2'-Fluoro RNA monomer or at least one monomer of a nucleic acid selected from among: LNA, Methylphosphonate LNA, BNA, UNA, GNA, ENA, ANA, FANA, DNG and RNG.

For an antisense strategy, which means modulating the expression of a gene on a translation level, one can preferably utilize:

- mutually complementary double-stranded oligonucleotides comprising RNA monomers (the classic ribonucleotides) (siRNA); or
- single-stranded oligonucleotides comprising RNA monomers (the classic ribonucleotides) and at least one RNA monomer modified at the ribose level and/or at the level of the phosphodiester bond; or
- single-stranded chimeric oligonucleotides comprising DNA monomers (the classic deoxyribonucleotides) and at least one phosphorothioate DNA monomer; or
- double-stranded chimeric oligonucleotides comprising RNA monomers (the classic ribonucleotides) and at least one 2'-O-(2-Methoxyethyl) RNA monomer; or
- double-stranded chimeric oligonucleotides comprising RNA monomers (the classic ribonucleotides) and at least one 2'O-methylate RNA monomer; or

- double-stranded chimeric oligonucleotides comprising RNA monomers (the classic ribonucleotides) and at least one 2'-fluoro RNA monomer; or
- double-stranded chimeric oligonucleotides comprising RNA monomers (the classic ribonucleotides) and at least one LNA monomer; or
- double-stranded chimeric oligonucleotides comprising RNA monomers (the classic ribonucleotides) and at least one arabinoside RNA monomer; or
- single-stranded chimeric oligonucleotides comprising DNA monomers (the classic deoxyribonucleotides) and at least one LNA monomer; or
- single-stranded oligonucleotides comprising morpholino monomers; or
- PNA-based oligonucleotides, said PNA comprising at least one alpha carbon (C-alpha) of the backbone with a substituent other than the H atom of the canonical glycine; preferably the substituent is the side chain of arginine or lysine; or
- oligonucleotides comprising DNA monomers (the classic deoxyribonucleotides) and at least one monomer of a nucleic acid selected from among: PNA, LNA, LNA methylphosphonate, BNA, UNA, GNA, ENA, DNG and RNG.

Preferably, the oligonucleotides of the present invention are directed against a gene involved in the development of a disease of genetic and/or viral origin or a tumour. Said gene is preferably selected from the group consisting of: genes of the MYC family (preferably MYC, MYCN, MYCL1), survivin genes (BIRC5), BCL2, PLK4, ALK, PKM2, caspase-8 and RASSF1.

In particular, the oligonucleotides of the present invention are directed against the genes of the MYC family, preferably against MYCN.

Said oligonucleotides are preferably selected from the group

consisting of: SEQ ID NO: 2-15, 66-84, SEQ ID NO: 24, 25, 31 and 32, the pair of complementary oligonucleotides having SEQ ID NO: 26 and 57, the pair of complementary oligonucleotides having SEQ ID NO: 27 and 58, the pair of complementary oligonucleotides having SEQ ID NO: 28 and 59, the pair of complementary oligonucleotides having SEQ ID NO: 29 and 60, the pair of complementary oligonucleotides having SEQ ID NO: 30 and 61, the pair of complementary oligonucleotides having SEQ ID NO: 33 and 62, the pair of complementary oligonucleotides having SEQ ID NO: 34 and 63, the pair of complementary oligonucleotides having SEQ ID NO: 35 and 64 and the pair of complementary oligonucleotides having SEQ ID NO: 36 and 65.

In some embodiments, said oligonucleotides are PNA oligonucleotides, preferably said PNAs are directed against MYCN.

In preferred embodiments the PNAs are selected from the group consisting of: SEQ ID NO: 2-15.

In further preferred embodiments the PNAs are selected from the group consisting of: SEQ ID NO: 66-84.

In further preferred embodiments the PNAs are selected from the group consisting of: SEQ ID NO: 2-15, 66-84.

Preferably, the PNA oligonucleotides are selected from the group consisting of: SEQ ID NO: 2-13, more preferably SEQ ID NO: 2-8, even more preferably SEQ ID NO: 2-6. The PNA oligonucleotide that is particularly preferred for the purposes of the present invention is SEQ ID NO: 5.

Preferably, SEQ ID NO: 5 is conjugated at the 5′ or 3′ end with SEQ ID NO: 47. More preferably, SEQ ID NO: 47 consists of amino acids in D form.

The PNAs SEQ ID NO: 2-15 are preferably directed against MYCN and, more preferably, they modulate the expression of MYCN with an antigene strategy.

The PNAs SEQ ID NO: 66-69 are also preferably directed against MYCN and, more preferably, they modulate the expression of MYCN with an antigene strategy.

The PNAs SEQ ID NO: 70-74 are preferably directed against MYC and, more preferably, they modulate the expression of MYC with an antigene strategy.

The PNAs SEQ ID NO: 75, 76 are preferably directed against BIRC5 and, more preferably, they modulate the expression of BIRC5 with an antigene strategy.

The PNAs SEQ ID NO: 77-79 are preferably directed against ALK and, more preferably, they modulate the expression of ALK with an antigene strategy.

The PNAs SEQ ID NO: 80-82 are preferably directed against BCL2 and, more preferably, they modulate the expression of BCL2 with an antigene strategy.

The PNAs SEQ ID NO: 83, 84 are preferably directed against PLK4 and, more preferably, they modulate the expression of PLK4 with an antigene strategy.

In a further embodiment, said oligonucleotides are double-stranded and preferably comprise RNA monomers. Preferably said oligonucleotides are directed against MYCN.

Alternatively, said double-stranded RNA oligonucleotides are selected from the group consisting of: the pair of complementary oligonucleotides having SEQ ID NO: 26 and 57, the pair of complementary oligonucleotides having SEQ ID NO: 27 and 58, the pair of complementary oligonucleotides having SEQ ID NO: 28 and 59, the pair of complementary oligonucleotides having SEQ ID NO: 29 and 60 and the pair of complementary oligonucleotides having SEQ ID NO: 30 and 61. Said oligonucleotides are preferably directed against MYCN. More preferably, they modulate the expression of the gene through an antisense strategy.

In further embodiments, said oligonucleotides are DNA-LNA

chimeric oligonucleotides, preferably said oligonucleotides are directed against MYCN.

In preferred embodiments, said DNA-LNA chimeric oligonucleotides are selected from the group consisting of: SEQ ID NO: 24 and 25.

SEQ ID NO: 24 and 25 are preferably directed against the MYCN gene.

SEQ ID NO: 24 and 25 preferably modulate the gene's expression through an antigene strategy.

In further embodiments, said oligonucleotides are single-stranded chimeric oligonucleotides comprising DNA monomers and/or at least one phosphorothioate DNA monomer. Said oligonucleotides are preferably directed against MYCN.

Particularly preferred for the purposes of the present invention are the chimeric oligonucleotides selected from the group consisting of: SEQ ID NO: 31 and 32. SEQ ID NO: 31 and 32 and are preferably directed against the MYCN gene. SEQ ID NO: 31 and 32, preferably, modulate the expression of MYCN through an antisense strategy.

In further embodiments, said oligonucleotides are double-stranded chimeric oligonucleotides comprising RNA monomers and at least one monomer preferably of 2'-O-(2-Methoxyethyl) or 2'-methyl RNA.

Said oligonucleotides are preferably directed against MYCN.

Particularly preferred for the purposes of the present invention is the pair of complementary chimeric oligonucleotides having SEQ ID NO: 33 and 62. Said pair of oligonucleotides preferably modulates the expression of the gene through an antisense mechanism.

In further embodiments, said oligonucleotides are double-stranded chimeric oligonucleotides comprising RNA monomers and at least one monomer preferably of 2'-Fluoro RNA. Preferably, said oligonucleotides are directed against MYCN.

Particularly preferred for the purposes of the present invention is the pair of complementary chimeric oligonucleotides having SEQ ID NO: 34 and 63. Said pair of oligonucleotides preferably modulates the gene's expression through an antisense mechanism.

In further embodiments, said oligonucleotides are double-stranded chimeric oligonucleotides comprising RNA monomers and at least one LNA monomer.

Said oligonucleotides are preferably directed against MYCN.

Particularly preferred for the purposes of the present invention is the pair of complementary chimeric oligonucleotides having SEQ ID NO: 35 and 64. Said pair of oligonucleotides preferably modulates the gene's expression through an antisense mechanism.

In further embodiments, said oligonucleotides are double-stranded chimeric oligonucleotides, comprising RNA monomers and at least one arabinoside RNA monomer.

Preferably, said oligonucleotides are directed against MYCN.

Particularly preferred for the purposes of the present invention is the pair of complementary chimeric oligonucleotides having SEQ ID NO: 36 and 65. Said pair of oligonucleotides preferably modulates the gene's expression through an antisense mechanism.

A further aspect of the invention relates to the use of the above-described oligonucleotides for therapeutic and/or diagnostic purposes.

In particular, the oligonucleotides can be used individually or combined together for the treatment of diseases of genetic and/or viral origin, in particular for the treatment of genetic diseases caused either by overexpression or inhibition of a gene, i.e. genetic diseases which require the modulation of the expression of a gene which is overexpressed or inhibited.

The oligonucleotides of the invention are used for the therapeutic treatment of a genetic disease, preferably selected from the group consisting of: Gorlin syndrome, Down syndrome, Feingold syndrome, Hirschsprung's disease, Von Hippel Lindau syndrome, Ataxia Telangiectasia, Li-Fraumeni syndrome, Turcot syndrome, familial tumours and Parkinson's disease.

Furthermore, the oligonucleotides of the invention are used for the therapeutic treatment of tumoural pathologies in children or adults. In particular, the tumours to which reference is made are caused, preferably, by the overexpression of a gene or oncogene selected from the group consisting of: MYC, MYCN, MYCL1, survivin (BIRC5), BCL2, PLK4, ALK and PKM2. Alternatively, the tumours are caused, preferably, by the inhibition (inactivation) of an onco-suppressor or anti-tumour gene and preferably selected from the group consisting of: caspase-8 and RASSF1.

The tumours to which reference is made are preferably selected from the group consisting of: neuroblastoma, retinoblastoma, medulloblastoma, ependymoma, pheochromocytoma, embryonal carcinoma, germ cell tumour, alveolar rabdomyosarcoma, embryonal rabdomiosarcoma, Wilms' tumour, clear cell sarcoma of the kidney, synovial sarcoma, hepatoblastoma, acute lymphoid leukaemia, chronic lymphoid leukaemia, acute lymphoblastic leukaemia, chronic lymphoblastic leukaemia, Burkitt's lymphoma, acute myeloid leukaemia, chronic myeloid leukaemia, acute megakaryoblastic leukaemia, B chronic lymphoid leukaemia, T-cell leukaemia, lymphomas, small cell lung cancer (microcytoma), lung adenocarcinoma, squamous cell lung carcinoma, typical and atypical primary lung cancer, large cell lung carcinoma, large-cell neuroendocrine lung carcinoma, glioblastoma, hepatocarcinoma, basal cell carcinoma, ovarian tumour, breast

tumour and colon cancer.

Particularly preferred for the purposes of the present invention are the tumours selected from the group consisting of: neuroblastoma, retinoblastoma, rabdomyosarcoma, Wilms' tumour, medulloblastoma, small cell lung cancer and basal cell carcinoma.

The subject matter of the present invention further relates to a composition comprising at least one oligonucleotide according to the present invention and at least one pharmacologically accepted excipient. Preferably, said at least one oligonucleotide is a PNA, preferably selected from the group consisting of: SEQ ID NO: 2-15, 66-84, preferably SEQ ID NO: 2-13, more preferably SEQ ID NO: 2-8, even more preferably SEQ ID NO: 2-6. The oligonucleotide that is particularly preferred is SEQ ID NO: 5. Preferably, SEQ ID NO: 5 is conjugated at the 5′ or 3′ end with SEQ ID NO: 47. More preferably, SEQ ID NO: 47 consists of amino acids in the D form.

Said PNA is preferably conjugated at its 5′ or 3′ end with a carrier which is preferably selected from the group consisting of: SEQ ID NO: 47-56.

The subject matter of the present invention further relates to a combination comprising at least one oligonucleotide according to the present invention, including the oligonucleotide having SEQ ID NO: 1, at least one compound, preferably at least one compound with a pharmacological effect, more preferably a chemotherapeutic agent, and, optionally, at least one pharmacologically accepted excipient.

In preferred embodiments said at least one compound is at least one additional antigene and/or antisense oligonucleotide, or at least one pharmacological agent, or at least one compound of biological or biotechnological origin

or deriving from chemical synthesis or combinations thereof.

Said compound of biological or biotechnological origin or deriving from chemical synthesis is preferably selected from the group consisting of: a monoclonal antibody, a chemotherapeutic agent, an immunomodulating agent, a growth factor, a cytokine, a peptide, an angiogenesis inhibitor, a tumour growth inhibitor, a steroid hormone and/or a non-steroid hormone and vitamins.

Examples of compounds that are particularly preferred for the purposes of the present invention are selected from the group consisting of: nerve growth factor (NGF), somatostatin, retinoic acid, actinomycin D, asparaginase, bleomycin, busulphan capecitabine, carboplatin, cyclophosphamide, cyclosporine, cisplatin, cytarabine, clorambucil, dacarbazine, daunorubicin, docetaxel, doxorubicin hydrochloride, epirubicin hydrochloride, etoposide, fludarabine phosphate, fluorouracil, gemcitabine, idarubicin hydrochloride, hydroxyurea, ifophosphamide, irinotecan hydrochloride, melphalan, mercaptopurine, methotrexate, mitomycin, mitoxantrone, nutline, oxaliplatin, paclitaxel, procarbazine, raltitrexed, streptozocin, tegafur-uracil, temozolomide, thioguanine, thiotepe, topotecan, vinblastine, vincristine, vindesine and vinorelbine and combinations thereof.

More preferably, the compounds are selected from the group consisting of: carboplatin, cisplatin, etoposide, vincristine, cyclophosphamide and combinations thereof.

The Applicant has found that the administration of at least one oligonucleotide according to the invention in concomitance with at least one compound, preferably at least one chemotherapeutic agent, as described above, makes it possible to reduce the concentration of said compound to be administered, while at the same time guaranteeing an increase

in the therapeutic effectiveness and lower toxicity.

The Applicant has found that, under these conditions, the reduction in the concentration of said compound depends on the particular pathology; in particular, the concentration of the chemotherapeutic compound depends on the type of tumour.

For some tumours, such as: neuroblastoma, retinoblastoma, medulloblastoma, small cell lung cancer, Wilms' tumour, alveolar rabdomyosarcoma and embryonal rabdomiosarcoma, the concentration of the at least one chemotherapeutic agent, administered in combination with at least one oligonucleotide according to the present invention, can be reduced by up to 10 times while guaranteeing the same therapeutic effect as a normal dose of a chemotherapeutic agent.

Particularly effective as a pharmaceutical combination (in terms of improved therapeutic effect) is the combination of at least one PNA according to the present invention, preferably at least one PNA selected from the group consisting of: SEQ ID NO: 1-15, 66-84, preferably SEQ ID NO: 1-13, more preferably SEQ ID NO: 1-8, more preferably SEQ ID NO: 1-6, even more preferably SEQ ID NO: 1 and/or 5, and at least one compound, preferably a chemotherapeutic agent, more preferably selected from the group consisting of: etoposide (VP16), carboplatin, cisplatin or vincristine, cyclophosphamide and combinations thereof.

Particularly preferred for the purposes of the present invention is a combination selected from among: SEQ ID: NO 1 and carboplatin, or etoposide or cisplatin or vincristine; or SEQ ID: NO 5 and carboplatin or etoposide or cisplatin or vincristine.

Said PNA is preferably conjugated at its 3′ and/or 5′ end with a carrier, which is preferably selected from the group consisting in: SEQ ID NO: 47-56.

The improvement effect is preferably to be found both when

the combination is administered simultaneously and when said at least one compound is administered at successive times, preferably at intervals, more preferably at regular intervals of 3 hours, 6 hours, 12 hours, 24 hours, 48 hours or 72 hours.

In other preferred embodiments, at least one oligonucleotide of the invention, including the oligonucleotide having SEQ ID NO: 1, can be administered conjugated or complexed, preferably with at least one vehicle particle, at least one vehicle polymer or at least one self-assembled vehicle oligonucleotide (also known as aptamers).

In further preferred embodiments, at least one oligonucleotide of the invention, including the oligonucleotide having SEQ ID NO: 1, can be conjugated or complexed and administered with at least one liposomal micelle, at least one micro-particle or at least one nanoparticle such as to favour permeation of the target tissue.

Said particle, usually spherical in shape and used as a means of specific delivery, can be formulated with many different chemical compounds. For example, said particle can be a formulation or co-formulation of polymeric compounds such as: chitosan, hyaluronic acid, polyethylene glycol (PEG), polyethyleneimine (PEI), polylactic acid (PLA), poly(lactic-co-glycolic acid) (PLGA), hydroxyapatite (HAP), polyunsaturated fatty acids, saturated fatty acids, cationic lipids, HAP-PLA, HAP-PLA/PGA and derivatives thereof. In further preferred embodiments, at least one oligonucleotide of the invention can be administered conjugated or complexed, preferably with at least one particle of the previously described type, and at least one ligand or at least one portion of a ligand for a specific receptor of the target cells (such as, for example, GD2, folic acid, TRAIL, NGF),

either of chemical or biotechnological origin, may be present in the polymeric membranes as an adjuvant, useful for favouring the internalization of said oligonucleotide of the invention in the target cells.

In further preferred embodiments, at least one oligonucleotide of the invention can be conjugated with at least one ligand or a portion of a ligand (such as, for example, GD2 (ganglioside GD2), folic acid, TRAIL (TNF-RELATED APOPTOSIS-INDUCING LIGAND), NGF (Nerve Growth Factor) specific for a receptor of the target cells.

In further preferred embodiments, at least one oligonucleotide of the invention, including the oligonucleotide having SEQ ID NO: 1, can be administered, on its own or in a combination, in association with at least one further medical application in order to enhance their effectiveness, preferably also by facilitating permeation of the target cells and/or tissues.

Said medical application is preferably selected from the group consisting of: oxygen therapy, magnetotherapy, thermotherapy, electrostimulation, ultrasound, radiotherapy, chemotherapy and phototherapy.

Example 1

Chemical synthesis of the oligonucleotides.

The chemical synthesis of the oligonucleotides is based on the use of DNA nucleoside phosphoroamidites modified with a protecting group, 4,4'-dimethoxytrityl (DMTr) on 5'-OH and β-cyanoethyl on the 3'-phosphate group; protecting groups are also used for the primary amines (nucleobase heterocycles), which are otherwise too reactive.

The chemical synthesis of DNA oligonucleotides takes place in a 3'-5' direction. Use is made of a CPG (acronym of controlled pore glass) resin or a polystyrene support, functionalized with the first nucleotide base. The synthesis

begins with a step of deprotecting the 5'- dimethoxytrityl group using a solution of 3% trichloroacetic acid (TCA) in dichloromethane (DCM). This is followed by activation, using ethylthiotetrazole (ETT) or benzylthiotetrazole (BTT) 0,3M, of the phosphoroamidite corresponding to the second base to be inserted in sequence, which will then be coupled with the previously deprotected 5'OH, thereby forming a phosphodiester bond.

The next step is "capping", which serves to acetylate the 5'OH groups that have not reacted. Capping is carried out using 2 solutions, one containing tetrahydrofuran (THF)/lutidine/acetic anhydride (8:1:1) and the other containing a 10% solution of methylimidazole in THF. The unstable trivalent bond of the phosphite triesters is stabilized by iodine in a THF/pyridine solution which oxidizes them to pentavalent phosphodiesters.

After oxidization, the cycle is repeated, starting with detritylation of the second unit introduced and so forth. This cycle is repeated for the number of times necessary, depending on how many bases it is desired to insert in sequence. Finally, the final 5'-DMTr group is removed by means of a treatment with an acid at room temperature.

Depending on the protecting groups present on the bases (which in turn depends on the chemistry of the bases selected, PTO, 2'OMe, etc.) it can be left at 55 °C for 16 hours with ammonium hydroxide or at 55 °C for 35 minutes with an ammonium hydroxide/methylamine (AMA) solution in order to deprotect the phosphors by β-elimination of the cyanoethyl groups and remove the protecting groups on the nucleobase heterocycles.

Alternatively, the 5'-DMTr group can be maintained throughout the phase of analysis (HPLC, MS) and preparative chromatography in order to better purify the final product

from the by-products and finally removed by means of a treatment with acetic acid

The chemical synthesis of RNA oligonucleotides differs from that of DNA oligonucleotides because of the 2'OH group present on the ribose and thus the presence of an additional protecting group for each phosphoroamidite.

Consequently, the synthesis of RNA oligonucleotides requires a longer coupling time and further steps to deprotect that group.

The same protocol as described above is used for the synthesis of oligonucleotides with chemically modified monomers, such as phosphorothioates (PTO), 2'O-Methyl (2'OMe), 2'Fluoro (2'-F), arabinoside nucleic acid (ANA), and locked nucleic acid (LNA).

The details of the specific techniques for each modified base are provided by the company the monomer molecules are purchased from (Link Technologies Ltd.).

The morpholinos were purchased from the manufacturer (Gene Tools, LLC).

The synthesis of PNA oligonucleotides was carried out on a 10 micromole scale, and included a purification and characterization step.

The synthesis of the molecule was carried out in the solid phase, using a Rink Amide-Chemmatrix® resin and a Syro automatic synthesizer (MultiSynTech). The first monomer of the synthesis is manually bound to the resin. Each automatic synthesis cycle is divided into three steps. The first step is deprotection, carried out using a solution of 20% piperidine in DMF (dimethylformammide).

The second step is the coupling reaction between the entering monomer and the growing chain. This reaction is carried out by adding 5 0.22M equivalents (eq) of monomer (FMOC-PNA-G(Bhoc)-OH, FMOC-PNA-A(Bhoc)-OH, FMOC-PNA-C(Bhoc)-OH, FMOC-

PNA-T-OH) in NMP (N-methylpirrolidone) and 4.5 0.32M eq of an activator in DMF (in this case HATU) in an alkaline environment with an 8% solution of 2,6-lutidine and DIPEA (N,N-diisopropylethylamine) in DMF. The coupling reaction is repeated, in duplicate, at the point of attachment of the first and second monomer on the preloaded resin, in the passage from the peptide chain to the PNA one, and on the last monomer.

The third step is the "capping" reaction, which serves to block, by acetylation, the sites that have not reacted during the coupling step. The reaction is achieved using a solution containing 6% 2,6-lutidine and 5% acetic anhydride in DMF.

Upon completion of the synthesis, the molecules are removed from the solid support.

This reaction is obtained with a solution of TFA (trifluoroacetic acid) and meta-cresol in 4:1 ratios.

The molecule thus obtained is collected by precipitation in diethyl ether.

Once recovered in water, it is purified in HPLC. The column used for purification is a C18 300A 5u Jupiter (© Phenomenex, Inc.). Purification is carried out using a linear gradient from 100% A (water 95%; acetronitrile 5%; 0.1% TFA)- 0% B (water 60%; acetonitrile 40%; 0.1% TFA) to 60% A-40% B in 30 minutes. The complete gradient used is 0-5 minutes 0% B; 5-35 minutes 40% B; 35-37 100% B; 37-42 100% B; 42-44 0%B.

Finally, the purified product is analyzed by ESI mass spectroscopy (© Waters).

Antigene oligonucleotides for blocking gene transcription.

For the purpose demonstrating that the oligonucleotides of the invention, selected and designed according to the parameters described in the present invention, work to selectively block the transcription of a gene, PNA-based oligonucleotides directed against the MYCN gene were designed

and synthesized; they are shown in Table 1.

TABLE 1

SEQ ID NO	PNA SEQUENCE	% GC	mRNA Inhibition (%)	Cell prolif. (%)	Phoenix prolif. (%)	NIH-3T3 prolif. (%)
SEQ ID NO: 1	*ATGCCGGGCATGATCT*	*56.3*	*42*	*70*	*100*	*100*
SEQ ID NO: 2	GGGTGGATGCGGGGGG	81.3	75	32	100	100
SEQ ID NO: 3	GATGCGGGGGGCTCCT	75	68	41	100	100
SEQ ID NO: 4	GTCGGCGGGAGGTAAG	68.8	65	48	100	100
SEQ ID NO: 5	GCTGGGTGGATGCGGG	75	62	53	100	100
SEQ ID NO: 6	TGGACGCGCTGGGTGG	75	60	54	100	100
SEQ ID NO: 7	CGCGCTGGGTGGATGC	75	58	57	100	100
SEQ ID NO: 8	GTCTGGACGCGCTGGG	75	54	59	100	100
SEQ ID NO: 9	CCCTGCAGTCGGCGGG	81.3	51	64	100	100
SEQ ID NO: 10	CGGCCGCGGGCCGCCA	93.8	51	65	100	100
SEQ ID NO: 11	GGGAACTGTGTTGGAG	56.3	48	68	100	100
SEQ ID NO: 12	TGTCTGGACGCGCTGG	68.8	47	69	100	100
SEQ ID NO: 13	ACGCTCAGGGACCACG	68.8	48	66	100	100
SEQ ID NO: 14	CCCGGACGAAGATGAC	62.5	40	70	100	100
SEQ ID	ACTGTGTTGGAGCCGA	56.3	37	77	100	100

NO: 15						
SEQ ID NO: 16	CCTGTCGTAGACAGCT	56.3	14	90	100	100
SEQ ID NO: 17	TGTGACAGTCATCTGT	56.3	10	98	100	100
SEQ ID NO: 18	GTGACAGTCATCTGTC	50	10	98	100	100
SEQ ID NO: 19	GACAGTCATCTGTCTG	50	5	100	100	100
SEQ ID NO: 20	CGTCGATTTCTTCCTC	50	5	100	100	100
SEQ ID NO: 21	CTCGAGTTTGACTCGC	56.3	1	100	100	100
SEQ ID NO: 22	GCGCCTCCCCTGATTT	62.5	2	100	100	100
SEQ ID NO: 23	ATATCCCCCGAGCTTC	56.3	2	100	100	100

The PNA oligonucleotides were tested both individually and conjugated with a carrier at the 3′ and/or 5′ end with the aim of favouring cell membrane permeation. In particular, the oligonucleotides were conjugated to the carboxyl terminus at 3′ with the amino acid sequence SEQ ID NO: 43, i.e. proline-lysine-lysine-lysine-arginine-lysine-valine.

The oligonucleotide having SEQ ID NO: 1 is the sequence that was the subject of patent EP 1618195 and represents the control sequence and sequence used for comparison.

The oligonucleotides having SEQ ID NO: 2-15 contain a group of two consecutive guanines (SEQ ID NO: 14, 15), or a group of three consecutive guanines (SEQ ID NO: 13), or two groups of two consecutive guanines (SEQ ID NO: 12), or a group of two guanines and a group of three consecutive guanines (SEQ ID NO: 11, 10, 9, 8 and 7), or two groups of two consecutive guanines and a group of three consecutive guanines (SEQ ID NO: 6 and 4), or a group of two consecutive guanines and two groups of three consecutive guanines (SEQ ID NO: 5), or a

group of six consecutive guanines (SEQ ID NO: 3), or a group of six consecutive guanines, a group of three consecutive guanines and a group of two consecutive guanines (SEQ ID NO: 2).

The groups of consecutive guanines are shown underlined in Table 1.

The oligonucleotides having SEQ ID NO: 16-23 do not have groups of consecutive guanines, being negative controls.

Moreover, for the purpose of selectively modulating the transcription of a gene, oligonucleotides having SEQ ID NO: 24-25 were also designed and synthesized; these are shown in Table 2.

Table 2

SEQ ID NO: 24	DNA-LNA 25-1	ATGCCGGGCATGATC T
SEQ ID NO: 25	DNA-LNA 25-2	ATGCCGGGCATGATC T

In particular, single-stranded chimeric oligonucleotides, comprising DNA monomers and LNA monomers (SEQ ID NO: 24 and 25) were designed and synthesized.

The DNA bases in the oligonucleotide sequence are shown as bases in boldface type, whereas the LNA monomers are underlined. Each chimeric oligonucleotide molecule was designed and synthesized by inserting the LNA monomers spaced apart by 1, 2 or 3 DNA bases in order to avoid rapid degradation by the endogenous nucleases, as has been reported in the literature (Koch T, Biochem J 2001, 354 (Pt 3):481-4; Koji Nagahama, Bioorg Med Chem Lett, 2009, 19(10):2707-9).

Antisense oligonucleotides for blocking gene translation.

For the purpose demonstrating that the oligonucleotides of the invention, selected and designed according to the parameters described in the present invention, work to selectively block the translation of the target gene, antisense oligonucleotides having SEQ ID NO: 26-36, directed

against the MYCN gene, were designed, synthesized and experimentally analyzed in vitro. They are shown in Table 3.

Table 3

SEQ ID NO		SENSE SEQUENCE		ANTISENSE SEQUENCE
SEQ ID NO: 26	siMYCN (795)	UGAAGAUGAUGAAGAGGAA	SEQ ID NO: 57	UUCCUCUUCAUCAUC UUCA
SEQ ID NO: 27	siMYCN (799)	GAUGAUGAAGAGGAAGAUG	SEQ ID NO: 58	CAUCUUCCUCUUCAU CAUC
SEQ ID NO: 28	siMYCN (801)	UGAUGAAGAGGAAGAUGAA	SEQ ID NO: 59	UUCAUCUUCCUCUUC AUCA
SEQ ID NO: 29	siMYCN (808)	GAGGAAGAUGAAGAGGAAG	SEQ ID NO: 60	CUUCCUCUUCAUCUU CCUC
SEQ ID NO: 30	siMYCN (810)	GGAAGAUGAAGAGGAAGAA	SEQ ID NO: 61	UUCUUCCUCUUCAUC UUCC
SEQ ID NO: 31	MYCN-PTO (1) as			CGTGGAGCAGCTCGG CAT
SEQ ID NO: 32	MYCN-PTO (763) as			CAGGGTGTCCTCTCC GGA
SEQ ID NO: 33	siRNA-2'-OMe-RNA 808	GAGGAAGAUGAAGAGGAAG TT	SEQ ID NO: 62	CUUCCUCUUCAUCUU CCUCTT
SEQ ID NO: 34	siRNA-2'F-RNA 808	CAGGAAGAUGAAGAGGAAG UU	SEQ ID NO: 63	GUUCCUCUUCAUCUU CCUCUU
SEQ ID NO: 35	siRNA-LNA 808	GAGGAAGAUGAAGAGGAAG TT	SEQ ID NO: 64	CUUCCUCUUCAUCUU CCUCTT
SEQ ID NO: 36	siRNA-ANA	CAGGAAGAUGAAGAGGAAG AA	SEQ ID NO: 65	GUUCCUCUUCAUCUU CCUCAA

Complementary double-stranded oligonucleotides based on RNA (small interfering RNA (siRNA) (SEQ ID NO: 26-30) were produced to selectively modulate the translation of the target gene, MYCN.

Moreover, oligonucleotides based on DNA SEQ ID NO: 31 and 32, in which the phosphodiester bond was modified into phosphorothioate, were produced.

Double-stranded chimeric oligonucleotides: based on RNA monomers and monomers 2'O-Methyl (SEQ ID NO: 33); based on RNA monomers and 2'-Fluoro monomers (SEQ ID NO: 34); based on RNA monomers and LNA monomers (SEQ ID NO: 35) and based on RNA monomers and arabinoside RNA monomers (SEQ ID NO: 36), were also produced. In the oligonucleotide sequences SEQ ID NO: 33-36, the 2'O-Methyl, 2'-Fluoro, LNA and arabinoside (ANA) monomers are shown in boldface, whereas each group of at least two consecutive guanines is underlined.

As the siRNA of the oligonucleotides is double-stranded, the chimera was designed and synthesized in such a manner as to leave two 2'O-Me monomers, or two 2'-Fluoro monomers, or two LNA monomers, or two RNA arabinoside monomers, both in 3' and in 5', unpaired to the complementary strand and thereby avoid rapid degradation by the cell enzymes.

Treatments with Oligonucleotides - QT-PCR

For the purpose of assessing the ability of the oligonucleotides of the present invention to modulate the expression of the target genes, their ability to reduce the quantity of messenger RNA was analyzed using the Real-Time PCR technique.

For this purpose, use was made of 24-well plates, which were seeded with 5.0x104 cells with 0.3 ml of OPTI-MEM (GIBCO BRL) medium, 4% FBS and 2mM L-glutammine (experiments in

triplicate) per well.

The cells were incubated for 24 hours at 37 °C, in an atmosphere containing 5% CO_2 to permit adherence to the base of the wells.

Each oligonucleotide analyzed, except for the PNA oligonucleotides, was incubated beforehand with 2µl of Lipofectamine 2000 (Invitrogen), with 0.3 mL of serum-free OPTI-MEM (GIBCO BRL) medium.

For each well, the oligonucleotides were analyzed at the following final concentrations:

- the antisense oligonucleotides siRNA and siRNA gapmer (i.e. the oligonucleotides containing one or more monomers of chemically modified nucleic acids at the 3′ or 5′ end, while in the central portion they have monomers of nucleic acids that have not been modified or have been modified at the level of the phosphodiester bond as a phosphorothioate bond) were used at 200nM;
- the antisense oligonucleotides containing phosphorothioate DNA monomers, the RNA antigene oligonucleotides (agRNA) and the oligonucleotides containing DNA monomers and LNA monomers were used at 10 µM;
- the morpholino oligonucleotides were used at a concentration of 1 µM, and
- the PNA oligonucleotides were administered at a concentration of 1 µM.

The cells were treated with the oligonucleotides, adding FBS up to 4% 6 hours after administration of the same. After 24 hours the total RNA was extracted from each well and purified using the RNeasy Mini Kit (QIAGEN).

Assays were performed on 8 cell lines obtained from 5 different human tumours which are correlated with MYCN expression, i.e.:

- as a neuroblastoma model use was made of the cell lines Kelly, IMR-32 (where the MYCN gene is amplified and superexpressed) and SKNBE2c and LAN1 (where the MYCN gene is amplified and superexpressed and the p53 gene is mutated);
- as a rabdomiosarcoma model use was made of the cell line RH30, where the MYCN gene is amplified and superexpressed,;
- as a Wilms' tumour model use was made of the cell line WiT49, where the MYCN gene is amplified and superexpressed;
- as a retinoblastoma model use was made of the cell line Y79, where the MYCN gene is amplified and superexpressed; and
- as a model of small cell lung cancer use was made of the cell line H69 where the MYCN gene is amplified and superexpressed.

As a control, use was made of the same cell lines as listed above treated with sterile water instead of oligonucleotides. Each RNA sample was quantified (in duplicate) using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The first strand of cDNA was produced using the cDNA Synthesis Kit for RT-PCR (Roche). For the cDNA synthesis reaction a total of 1 µg of RNA was used. For the real-time PCR, 10 ng of cDNA in a final volume of 20 µl was used with SYBR Green Master Mix 2X (Applied Biosystems) (3 identical experiments were conducted in triplicate). The sequences and the concentrations of the primer used to carry out the Real-Time PCRs are shown in Table 4. Two housekeeping genes were used as positive controls: GAPDH and beta-actin (ACTB).
The conditions of the QT-PCR reaction were: 10 min at 95 °C, 20 sec at 95 °C, and 30 sec at 60 °C, for 50 cycles.

Table 4

Primer	Sequence	Concentration	SEQ ID NO
MYCN sense	CGACCACAAGGCCCTCAGT	300 nM	SEQ ID NO: 37
MYCN antisense	TGACCACGTCGATTTCTTCCT	300 nM	SEQ ID NO: 38
ACTB sense	GAGCACAGAGCCTCGCCTTTG	300 nM	SEQ ID NO: 39
ACTB antisense	ACCATCACGCCCTGGTGCCTG	300 nM	SEQ ID NO: 40
GAPDH sense	CCAATATGATTCCACCCATGGC	300 nM	SEQ ID NO: 41
GAPDH antisense	CTTGATTTTGGAGGGATCTCGC	300 nM	SEQ ID NO: 42

Treatments with oligonucleotides - Cellular proliferation assay.

For the purpose of assessing the gene modulation ability of the oligonucleotides of the present invention, the effect of their administration on cell viability was determined.

For this purpose $5x10^3$ cells per well were seeded into 96-well cell culture plates (experiments conducted in triplicate) with 100 µl of OPTI-MEM (GIBCO BRL) medium containing 4% FBS and 2mM of L-glutammine.

The PNA oligonucleotides were administered at different concentrations (1µM-2,5µM-5µM-10µM) in order to observe a dose-effect relationship.

As regards all the other oligonucleotides, the concentrations at which they administered are listed in the paragraph: Treatments with Oligonucleotides - QT-PCR.

The viability of the treated cells was determined at 48, 72, 96 and 168 hours after treatment.

Cell viability was assessed by means of an ATP-Lite assay (Luminescence ATP Detection Assay System, PerkinElmer) and is reported as the ratio between the average signal of the treated wells compared to the average value of the wells of

untreated control cells. The cells were processed following the instructions provided with the kit.

The assays were performed on the same cell lines used to determine the levels of the MYCN gene messenger and listed in the paragraph: Treatments with Oligonucleotides - QT-PCR.

RESULTS

As far as the PNA oligonucleotides are concerned, the results regarding their ability to inhibit the transcription of the MYCN gene and proliferation of Kelly cells are shown in Table 1. Table 6 shows the values (in percentage form) of the proliferating Kelly cells at the different concentrations of PNA analyzed.

Table 5

SEQ ID NO	Sequence	Cell Prolif (%) 1µM 72h	Cell Prolif (%)2,5µM 72h	Cell Prolif. (%)5µM 72h	Cell Prolif. (%)10µM 72h
SEQ ID NO: 1	*ATGCCGGGCATGATCT*	*89*	*66*	*50*	*24*
SEQ ID NO: 2	GGGTGGATGCGGGGGG	74	32	12	2
SEQ ID NO: 3	GATGCGGGGGGCTCCT	78	41	19	3
SEQ ID NO: 4	GTCGGCGGGAGGTAAG	78	48	21	3
SEQ ID NO: 5	GCTGGGTGGATGCGGG	79	53	27	5
SEQ ID NO: 6	TGGACGCGCTGGGTGG	82	59	35	13
SEQ ID NO: 7	CGCGCTGGGTGGATGC	80	54	31	8
SEQ ID NO: 8	GTCTGGACGCGCTGGG	80	57	32	11
SEQ ID NO: 9	CCCTGCAGTCGGCGGG	86	64	42	20
SEQ ID	TGTCTGGACGCGCT	84	60	40	16

NO: 12	GG				

The results demonstrate that the inhibition effect of these PNAs on the translated protein of the target genes is highly selective and specific. Moreover, it was observed that the arrest of growth of the tumour cells used as a model (characterized by amplification of the MYCN gene) was directly followed by apoptosis following the administration of the antigene PNAs.

In general, the PNA antigene oligonucleotides having SEQ ID: 2-SEQ ID NO: 13 (containing one or more groups of Gs) have greater antigene effectiveness (i.e. inhibition of MYCN mRNA and of the proliferation of tumour cells with MYCN expression) compared to PNA oligonucleotides devoid of groups of Gs (SEQ ID NO: 16-SEQ ID NO: 23).

In particular, the results shown in Table 1 and in Table 5 demonstrate that the sequences SEQ ID NO: 2-SEQ ID NO: 13 have greater antigene effectiveness than the sequence SEQ ID NO: 1, the subject of patent EP 1618195.

Sequences SEQ ID NO: 7-SEQ ID NO: 12 (containing two groups of two or three consecutive Gs) show greater antigene effectiveness than the sequences SEQ ID NO: 1, and SEQ ID NO: 13-SEQ ID NO: 15 (containing only one group of two or three consecutive Gs).

Sequences SEQ ID NO: 4-SEQ ID NO: 6 (containing three groups of two or three consecutive Gs) show greater antigene effectiveness than the sequences SEQ ID NO: 7-SEQ ID NO: 12 (containing two groups of two or three consecutive Gs).

SEQ ID NO: 3, containing a group of six consecutive Gs, shows greater antigene effectiveness than the sequences SEQ ID NO: 1 and sequences SEQ ID NO: 4-SEQ ID NO: 12 containing one or two or three groups of Gs (in which each group consists of at most two or three consecutive Gs).

Sequence SEQ ID NO: 2, containing three groups of consecutive Gs, which, in addition to two groups of two and three consecutive Gs, also comprises a group of six consecutive Gs, shows greater antigene effectiveness than both SEQ ID NO: 3, containing only one group of six Gs, and sequences SEQ ID NO: 1 and sequences SEQ ID NO: 4-SEQ ID NO: 12 containing one or two or three groups of Gs (in which each group consists of at most two or three consecutive Gs).

Moreover, the oligonucleotides having SEQ ID NO 1-23 were administered in lines of fibroblastoid-type cells (NIH-3T3 and Phoenix) and the results are shown in Table 1 (last two columns).

The results clearly demonstrate that the oligonucleotides analyzed are not specifically effective against and are not toxic for these cells (i.e. cells that do not express the target gene, which in this case is MYCN).

In fact, no changes were observed in cellular proliferation in these two lines of non-tumoural fibroblasts, and this result means that the PNA oligonucleotides act with a specific effect of inhibiting the expression of MYCN, whereas they do not have a non-specific, toxic effect in cells that do not express MYCN.

Therefore, it may be deduced that the PNA oligonucleotides designed on the basis of the parameters described in the present invention act specifically and effectively on the target gene and therefore on the cells which express/overexpress this gene.

The PNAs having SEQ ID NO: 1-12 were also tested on different cell lines which overexpress MYCN (Kelly, SKNBE2c, RH30, WiT49, WERI-Rb1 1 and H69). The results are shown in Table 6 and confirm the selectivity and specificity of the inhibition effect of the PNAs on the product of protein translation of the MYCN gene.

Table 6

SEQ ID NO	*SEQ ID NO: 1*	SEQ ID NO: 2	SEQ ID NO: 3	SEQ ID NO: 4	SEQ ID NO: 5	SEQ ID NO: 6	SEQ ID NO: 7	SEQ ID NO: 8	SEQ ID NO: 9	SEQ ID NO: 10	SEQ ID NO: 11	SEQ ID NO: 12
Kelly %mRNA	*48*	75	68	65	62	60	58	54	51	51	50	50
Kelly %cell prol.	*66*	32	41	48	53	54	57	59	60	64	64	65
SKNBE2c%mRNA	*41*	75	68	60	59	62	60	62	58	41	40	40
SKNBE2c%cell prol.	*72*	52	53	59	64	57	58	56	64	70	71	71
RH30 %mRNA	*39*	74	65	57	56	56	55	58	50	29	31	32
RH30 %cell prol.	*76*	56	59	62	64	64	63	60	71	80	78	77
WiT49 %mRNA	*62*	78	75	73	69	68	70	72	66	60	60	62
WiT49 %cell prol.	*74*	54	57	60	63	65	60	62	68	70	72	73
WERI-Rb1 %mRNA	*31*	46	43	41	38	37	38	40	36	30	30	30
WERI-Rb1 %cell prol.	*82*	79	78	79	80	80	79	78	79	85	84	84
H69 %mRNA	*40*	58	55	55	54	55	55	56	50	41	41	40

H69 %cell prol.	77	61	63	66	68	69	67	66	70	79	78	78

The antigene PNA oligonucleotide having SEQ ID NO: 5 was analyzed in a more detailed manner.

In particular, modified oligonucleotides, in which the groups of consecutive guanines present in the sequence were modified (the groups of consecutive guanines are underlined, whereas the modified nucleotides are in boldface type) so as to interrupt the consecutiveness of the guanines, were synthesized and analyzed in vitro on Kelly cells (which overexpress MYCN).

The results (summarized in Table 7) clearly demonstrate that the fact of the guanines being consecutive is of fundamental importance for the purposes of the activity of modulating the gene's expression. In fact, the PNAs having SEQ ID NO: 43, in which all the groups of consecutives guanine of the PNAs having SEQ ID NO: 5 were mutated, causes the inhibitory activity of the PNAs to be lost, whereas the mutation of one or two of the three groups of consecutive guanines considerably compromises, but does not completely suppress, the inhibitory activity of the molecule.

Table 7

SEQ ID NO	SEQUENCE	mRNA Inhibition (%) (Kelly)	(Kelly) Cell Prolif. %
SEQ ID NO: 5	GCTGGGTGGATGCGGG	62	53
SEQ ID NO: 43	GCTGAGTCGATGCGTG	0	100
SEQ ID NO: 44	GCTGAGTGGATGCGTG	25	89
SEQ ID NO: 45	GCTGGGTCGATGCGGG	47	69
SEQ ID NO: 46	GTTGGGTGGATGTGGG	58	60

Chimeric oligonucleotides comprising DNA monomers and LNA

monomers and having the MYCN gene as their target were tested in vitro on alveolar rabdomyosarcoma cells (RH30). The results are summarized in Table 8 and demonstrate these oligonucleotides have an intense, specific antigene activity.

Table 8

SEQ ID NO	NAME	SEQUENCE	mRNA Inhibition (%)	Cell Prolif. (%)
SEQ ID NO: 24	DNA-LNA 25-1	ATGCCGGGCATGA TCT	23	75
SEQ ID NO: 25	DNA-LNA 25-2	ATGCCGGGCATGA TCT	24	78

The antisense oligonucleotides designed and synthesized by the Applicant according to the parameters described in the present invention were analyzed in vitro on rabdomyosarcoma cells (RH30). The results are summarized in the Table 9 and show that the oligonucleotides are capable of inhibiting the MYCN transcript in a specific and effective manner; moreover, they are also capable of selectively inhibiting tumour cell proliferation in a more effective manner than the standard antisense oligonucleotides presently available for the MYCN gene (Chung DH, Bioch Bioph Res Commun, 2006, 351(1):192-7.
In particular, the siRNA identified by the Applicant and directed against MYCN mRNA, described in Table 11, exert an antisense activity with an inhibition of MYCN mRNA ranging from a minimum of 70% to a maximum of 85%.

Table 9

SEQ ID NO		SEQUENCE SENSE	SEQUENCE ANTISENSE	mRNA Inhibition (%)	Cell Prolif (%)
SEQ ID NO: 26	siMYCN (795)	UGAAGAUGAU GAAGAGGAA	UUCCUCUUCA UCAUCUUCA	82	28
SEQ ID	siMYCN	GAUGAUGAAG	CAUCUUCCUC	70	43

NO: 27	(799)	AGGAAGAUG	UUCAUCAUC		
SEQ ID NO: 28	siMYCN (801)	UGAUGAAGAG GAAGAUGAA	UUCAUCUUCC UCUUCAUCA	78	35
SEQ ID NO: 29	siMYCN (808)	GAGGAAGAUG AAGAGGAAG	CUUCCUCUUC AUCUUCCUC	85	28
SEQ ID NO: 30	siMYCN (810)	GGAAGAUGAA GAGGAAGAA	UUCUUCCUCU UCAUCUUCC	81	30
SEQ ID NO: 31	MYCN-PTO (1) as		CGTGGAGCAG CTCGGCAT	34	73
SEQ ID NO: 32	MYCN-PTO (763) as		CAGGGTGTCC TCTCCGGA	45	65
SEQ ID NO: 33	siRNA-2'-OMe-RNA 808	GAGGAAGAUG AAGAGGAAGT T	CUUCCUCUUC AUCUUCCUCT T	13	85
SEQ ID NO: 34	siRNA-2'F-RNA 808	CAGGAAGAUG AAGAGGAAGU U	GUUCCUCUUC AUCUUCCUCU U	59	52
SEQ ID NO: 35	siRNA-LNA 808	GAGGAAGAUG AAGAGGAAGT T	CUUCCUCUUC AUCUUCCUCT T	34	68
SEQ ID NO: 36	siRNA-ANA 808	CAGGAAGAUG AAGAGGAAGA A	GUUCCUCUUC AUCUUCCUCA A	69	35

For the purpose of verifying whether the oligonucleotides of the present invention are capable of lowering the concentrations of the chemotherapeutic agents presently used in the therapeutic protocols against cancer, the same were administered in concomitance with chemotherapy drugs.

Studies were conducted on the effect of associations between PNA and chemotherapy drugs (carboplatin, etoposide (VP16), cisplatin and vincristine) on different human and mouse

neuroblastoma tumour cell lines (SMS-KAN, LAN 1, IMR-32, SMS-KCN, Kelly, NHO2A, SKNBE2c).

The therapeutic schedule used in the treatments carried out provided for the cells to be treated with PNA and then afterwards, at a pre-established time (it could be 6 or 12 hours) the chemotherapeutic agent was administered.

The results demonstrate that the association of these compounds with the oligonucleotides of the invention determines, at specific concentration ranges, a greater therapeutic effect than individual treatments with the same compounds.

In particular, it may be observed that the treatment - be it concomitant and simultaneous or at different time intervals (3 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, etc.) - with a combination consisting of the oligonucleotides of the invention and other compounds with a pharmacological effect serves to enhance the desired therapeutic effect.

Therefore, combining one or more of the oligonucleotides identified by the Applicant with chemotherapy drugs makes it possible to reduce the chemotherapy drug concentration by as much as 10 times compared to the present standard therapeutic concentration while still obtaining the same effect.

In particular, the results of this study demonstrate that SEQ ID NO: 1 has a synergic effect, in terms of inhibiting cell proliferation, when it is administered in combination with vincristine, or etoposide, or carboplatin, or cisplatin, compared to the effects obtained in treatments with the oligonucleotide or with the aforesaid chemotherapeutic agents on their own.

EXAMPLE 2

For the purpose of supporting the present invention, the oligonucleotides summarized in Table 10 were also synthesized. In particular, Table 10 shows the

oligonucleotide sequences, the SEQ ID NO, the percentage value of the (G+C) content and the gene the synthesized oligonucleotides are directed against. The synthesis of the oligonucleotides was achieved as in example 1.

Table 10

	SEQ ID NO		% GC
MYCN	SEQ ID NO: 66	TCGGGAGCAGTGGGCA	68.8
	SEQ ID NO: 67	GCGGGTCGCGGGCACG	87.5
	SEQ ID NO: 68	TGGAGGTCGGCGCCGG	81.3
	SEQ ID NO: 69	TCGGCGGGAGGTAAGG	68.8
MYC	SEQ ID NO: 70	CTCAGAGGCTTGGCGG	68.8
	SEQ ID NO: 71	GCGGCCGGCTAGGGTG	81.3
	SEQ ID NO: 72	CGGCCGGCTAGGGTGG	81.3
	SEQ ID NO: 73	CGACGGCGGTGGCGGG	87.5
	SEQ ID NO: 74	GGACGGGGGCGGTGGA	81.3
BIRC5	SEQ ID NO: 75	GCGGCGGCATGGGTGC	81.3
	SEQ ID NO: 76	GGCGGCGGCATGGGTG	81.3
ALK	SEQ ID NO: 77	GCAGGAGAGGACGGTA	62.5
	SEQ ID NO: 78	CAGGAGAGGACGGTAC	62.5
	SEQ ID NO: 79	GGCAGGAGAGGACGGT	68.8
BCL2	SEQ ID NO: 80	GGATGGCGCACGCTGG	75.0
	SEQ ID NO: 81	GGGAAGGATGGCGCAC	68.8
	SEQ ID NO: 82	CCACGGTGGTGGAGGA	68.8
PLK4	SEQ ID NO: 83	ACGGCAAGCGGCGGGA	75.0
	SEQ ID NO: 84	GGACGGCAAGCGGCGG	81.3

The following, in particular, were synthesized: SEQ ID NO: 66-69 directed against MYCN, SEQ ID NO: 70-74 directed against MYC, SEQ ID NO: 75, 76 directed against BIRC5, SEQ ID NO: 77-79 directed against ALK, SEQ ID NO: 80-82 directed against BCL2 and SEQ ID NO: 83, 84 directed against PLK4.

The oligonucleotides were tested in vitro (at a concentration of 2.5 µM) to determine their ability to inhibit the transcription of the gene they are directed against by measuring the levels of the gene messenger in cellular models in which the gene of interest is overexpressed. The methods

used are the ones described in example 1.

In particular, SEQ ID NO: 66-69 (directed against MYCN) were tested in vitro in the Kelly and H69 cell lines; SEQ ID NO: 70-74 (directed against MYC) were tested in vitro in the H82 and RD cell lines, SEQ ID NO: 75, 76 (directed against BIRC5) were tested in vitro in the Kelly cell line, SEQ ID NO: 77-79 (directed against ALK) were tested in vitro in the Kelly cell line, SEQ ID NO: 80-82 (directed against BCL2) were tested in vitro in the Kelly cell line and SEQ ID NO: 83, 84 (directed against PLK4) were tested in vitro in the Kelly cell line.

The ability of the tested oligonucleotides to inhibit the messenger of the gene of interest was measured and the proliferative capacity of the cells following administration of the oligonucleotides was also assessed. The results are summarized in Table 11.

The data show a potent inhibitory activity against the mRNA of the target gene and an inhibition of cell proliferation which rises with increases in the number of groups of Gs present in the sequence of the oligonucleotide.

Table 11

	SEQ ID NO		mRna inhibit. (%)	Cell Prolif. (%)
MYCN Kelly	SEQ ID NO: 66	TCGGGAGCAGTGGGCA	57	60
	SEQ ID NO: 67	GCGGGTCGCGGGCACG	60	56
	SEQ ID NO: 68	TGGAGGTCGGCGCCGG	74	35
	SEQ ID NO: 69	TCGGCGGGAGGTAAGG	76	30
MYCN H69	SEQ ID NO: 66	TCGGGAGCAGTGGGCA	41	73
	SEQ ID NO: 67	GCGGGTCGCGGGCACG	42	71
	SEQ ID NO: 68	TGGAGGTCGGCGCCGG	62	49
	SEQ ID NO: 69	TCGGCGGGAGGTAAGG	67	45
MYC H82	SEQ ID NO: 70	CTCAGAGGCTTGGCGG	43	79
	SEQ ID NO: 71	GCGGCCGGCTAGGGTG	46	75
	SEQ ID NO: 72	CGGCCGGCTAGGGTGG	52	64
	SEQ ID NO: 73	CGACGGCGGTGGCGGG	56	65
	SEQ ID NO: 74	GGACGGGGGCGGTGGA	61	61
MYC RD	SEQ ID NO: 70	CTCAGAGGCTTGGCGG	34	73
	SEQ ID NO: 71	GCGGCCGGCTAGGGTG	42	65
	SEQ ID NO: 72	CGGCCGGCTAGGGTGG	58	53
	SEQ ID NO: 73	CGACGGCGGTGGCGGG	59	51
	SEQ ID NO: 74	GGACGGGGGCGGTGGA	64	47
BIRC5 Kelly	SEQ ID NO: 75	GCGGCGGCATGGGTGC	69	43
	SEQ ID NO: 76	GGCGGCGGCATGGGTG	78	35

ALK Kelly	SEQ ID NO: 77	GCAGGAGAGGACGGTA	57	55
	SEQ ID NO: 78	CAGGAGAGGACGGTAC	68	46
	SEQ ID NO: 79	GGCAGGAGAGGACGGT	79	39
BCL2 Kelly	SEQ ID NO: 80	GGATGGCGCACGCTGG	59	62
	SEQ ID NO: 81	GGGAAGGATGGCGCAC	62	58
	SEQ ID NO: 82	CCACGGTGGTGGAGGA	68	55
PLK4 Kelly	SEQ ID NO: 83	ACGGCAAGCGGCGGGA	62	59
	SEQ ID NO: 84	GGACGGCAAGCGGCGG	74	49

Finally, the oligonucleotides having SEQ ID NO 66-84 were administered in cell lines of a fibroblastoid type (Phoenix and NIH-3T3). In these cells the oligonucleotides tested did not show to be toxic.

These results indicate that the PNA oligonucleotides act through a specific inhibition effect on the expression of the gene of interest, whereas they do not have any non-specific, toxic effect in cells which do not express the gene.

It can thus be deduced that the PNA oligonucleotides designed on the basis of the parameters described in the present invention act specifically and effectively on the target gene and therefore on the cells which express/overexpress this gene.

CLAIMS

1. An oligonucleotide for modulating the expression of a gene through an anti-gene mechanism, comprising 6-30 nucleotides, preferably 12-24 nucleotides, said oligonucleotide being characterized by a sequence comprising at least three groups of at least two consecutive guanines.

2. The oligonucleotide according to claim 1, wherein the groups of at least two consecutive guanines are continuous with each other.

3. The oligonucleotide according to claim 1 or 2, wherein the groups of at least two consecutive guanines are spaced apart by at least one nucleotide, said nucleotide not being a guanine.

4. The oligonucleotide according to any one of claims 1-3, wherein the groups of at least two consecutive guanines are at least four, five or six in number.

5 The oligonucleotide according to any one of claims 1-4, wherein said oligonucleotide is conjugated with a carrier sequence at the 3′ and/or 5′ end of said oligonucleotide, preferably a carrier selected from the group consisting of: SEQ ID NO: 47-56.

6. The oligonucleotide according to any one of claims 1-5, wherein said oligonucleotide is at least one molecule of natural nucleic acid, preferably DNA or RNA, possibly chemically modified, or synthetic nucleic acid, preferably PNA, LNA or morpholino, possibly chemically modified, or a combination of said natural nucleic acid and said synthetic nucleic acid.

7. The oligonucleotide according to claim 6, wherein said natural or synthetic nucleic acid is single- or a double-stranded.

8. The oligonucleotide according to claim 6 or 7, wherein

said at least one DNA molecule comprises at least one LNA, methylphosphonate-LNA, BNA, RNG, DNG, GNA, UNA, ENA, ANA, F-ANA, PNA, G-PNA or morpholino nucleotide; or comprises a 2'O-Methyl, 2'O-Methoxyethyl or 2'-fluoro RNA nucleotide.

9. The oligonucleotide according to claim 6 or 7, wherein said at least one RNA molecule comprises at least one RNA nucleotide selected from among: a 2'O-Methyl nucleotide, a 2'O-Methoxyethyl nucleotide, a 2'-fluoro nucleotide; or at least one nucleotide of a nucleic acid selected from among: LNA, Methylphosphonate LNA, BNA, RNG, DNG, GNA, UNA, ENA, ANA, F-ANA, PNA, G-PNA or morpholino.

10. The oligonucleotide according to claim 6, wherein said at least one DNA molecule or said at least one RNA molecule comprises at least one nucleotide modified at the level of the phosphodiester bond as a phosphorothioate bond.

11. The oligonucleotide according to claim 6, wherein said PNA has a modified backbone wherein the alpha carbon has the side chain of arginine or lysine as a substituent.

12. The oligonucleotide according to any one of claims 1-11, wherein said oligonucleotide is directed against at least one gene responsible for a disease of genetic or viral origin or against at least one tumour gene.

13. The oligonucleotide according to claim 12, characterized in that said gene is selected from the group consisting of: at least one gene belonging to the MYC family, preferably MYC, MYCN or MYCL1, BIRC5, BCL2, PLK4, ALK, PKM2, CASP8 and RASSF1.

14. The oligonucleotide according to any one of claims 1-13, wherein said oligonucleotide is selected from among: SEQ ID NO: 2-15, SEQ ID NO: 24, 25; preferably it is selected from among: SEQ ID NO: 2, 4, 5 and 6.

15. The oligonucleotide according to any one of claims 1-13, wherein said oligonucleotide is selected from among: SEQ ID

NO: 66-84; preferably it is selected from among SEQ ID NO: 68-84.

16. The oligonucleotide according to claim 14 or 15, wherein SEQ ID NO: 2-15, 66-69, 24, 25, preferably SEQ ID NO: 2, 4, 5, 6, 68 and 69 are directed against the MYCN gene.

17. The oligonucleotide according to claim 15, wherein SEQ ID NO: 70-74 are directed against the gene MYC.

18. The oligonucleotide according to claim 15, wherein SEQ ID NO: 75, 76 are directed against the gene BIRC5.

19. The oligonucleotide according to claim 15, wherein SEQ ID NO: 77-79 are directed against the gene ALK.

20. The oligonucleotide according to claim 15, wherein SEQ ID NO: 80-82 are directed against the gene BCL2.

21. The oligonucleotide according to claim 15, wherein SEQ ID NO: 83, 84 are directed against the gene PLK4.

22. The oligonucleotide according to claim 14 or 16, wherein said oligonucleotide is a PNA selected from the group consisting of: SEQ ID NO: 2-15, preferably SEQ ID NO: 2-13, more preferably SEQ ID NO: 2-8, even more preferably SEQ ID NO: 2-6.

23. The oligonucleotide according to claim 22, wherein said PNA is SEQ ID NO: 5.

24. The oligonucleotide according to any one of claims 14-23, conjugated at the 5′ and/or 3′ end with SEQ ID NO: 47.

25. A composition comprising at least one oligonucleotide according to any one of claims 1 to 24 and at least one pharmacologically acceptable excipient.

26. The composition comprising at least one oligonucleotide according to any one of claims 1 to 24, including SEQ ID NO: 1, in combination with a selected compound with pharmacological action, preferably in the group consisting of: NGF, somatostatin, retinoic acid, actinomycin D, asparaginase, bleomycin, busulphan capecitabine, carboplatin,

cyclophosphamide, cyclosporine, cisplatin, cytarabine, clorambucil, dacarbazine, daunorubicin, docetaxel, doxorubicin hydrochloride, epirubicin hydrochloride, etoposide, fludarabine phosphate, fluorouracil, gemcitabine, idarubicin hydrochloride, hydroxyurea, ifophosphamide, irinotecan hydrochloride, melphalan, mercaptopurine, methotrexate, mitomycin, mitoxantrone, oxaliplatin, paclitaxel, procarbazine, raltitrexed, streptozocin, tegafur-uracil, temozolomide, thioguanine, thiotepe, topotecan, vinblastine, vincristine sulphate, vindesine and vinorelbine.

27. The composition according to claim 26, wherein said combination is: SEQ ID: NO 1 and carboplatin, or etoposide or cisplatin or vincristine; or SEQ ID: NO 5 and carboplatin, or etoposide or cisplatin or vincristine.

28. An oligonucleotide according to any one of claims 1 to 24 or a composition according to claims 25-27 for use as medicament or diagnostic agent.

29. The oligonucleotide or composition according to claim 28, for use in the treatment of a disease of genetic and/or viral origin or for treating a tumour, said disease of genetic and/or viral origin and said tumour being caused by an overexpression and/or inhibition of a gene.

30. The oligonucleotide or composition according to claim 28 or 29, wherein said disease genetic is selected from the group consisting of: Gorlin syndrome, Down syndrome, Feingold syndrome, Hirschsprung's disease, Von Hippel Lindau syndrome, ataxia telangiectasia, Li-Fraumeni syndrome, Turcot syndrome, familial tumours and Parkinson's disease; said tumour is an adult or pediatric tumour of adulto or pediatrico, preferably selected from the group consisting in: neuroblastoma, retinoblastoma, medulloblastoma, ependymoma, pheochromocytoma, embryonal carcinoma, germ cell tumour, alveolar rabdomyosarcoma, embryonal rabdomiosarcoma, Wilms'

tumour, clear cell sarcoma of the kidney, synovial sarcoma, hepatoblastoma, acute lymphoid leukaemia, chronic lymphoid leukaemia, acute lymphoblastic leukaemia, chronic lymphoblastic leukaemia, Burkitt's lymphoma, acute myeloid leukaemia, chronic myeloid leukaemia, acute megakaryoblastic leukaemia, B chronic lymphoid leukaemia, T-cell leukaemia, lymphomas, small cell lung cancer (microcytoma), lung adenocarcinoma, squamous cell lung carcinoma, typical and atypical primary lung cancer, large cell lung carcinoma, large-cell neuroendocrine lung carcinoma, glioblastoma, hepatocarcinoma, basal cell carcinoma, ovarian tumour, breast tumour and colon cancer.

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(54)发明名称

用于调节基因表达的寡核苷酸及其用途

(57)摘要

本发明涉及用于调节基因表达的寡核苷酸，特别是用于调节负责遗传、肿瘤或病毒来源的病状的基因。此外，本发明涉及所述寡核苷酸用于所述疾病的治疗和/或诊断的用途，所述寡核苷酸可能是化学修饰的。

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1. 一种通过反基因机制调节基因表达的寡核苷酸，其包含 6-30 个核苷酸，优选 12-24 个核苷酸，所述寡核苷酸的特征在于包含至少三组的至少两个连续鸟嘌呤的序列。

2. 根据权利要求 1 所述的寡核苷酸，其中所述至少两个连续鸟嘌呤的组是彼此连续的。

3. 根据权利要求 1 或 2 所述的寡核苷酸，其中所述至少两个连续鸟嘌呤的组被至少一个核苷酸隔开，所述核苷酸不是鸟嘌呤。

4. 根据权利要求 1-3 中任一项所述的寡核苷酸，其中所述至少两个连续鸟嘌呤的组的数量至少是四个、五个或六个。

5. 根据权利要求 1-4 中任一项所述的寡核苷酸，其中所述寡核苷酸在所述寡核苷酸的 3’和 / 或 5’端结合载体序列，载体优选是选自由 SEQ ID NO:47-56 组成的组。

6. 根据权利要求 1-5 中任一项所述的寡核苷酸，其中所述寡核苷酸是天然核酸或合成核酸中的至少一种分子，或所述天然核酸和所述合成核酸的组合；所述天然核酸优选是 DNA 或 RNA，可能是化学修饰的，所述合成核酸优选是 PNA、LNA 或吗啉代，可能是化学修饰的。

7. 根据权利要求 6 所述的寡核苷酸，其中所述天然核酸或合成核酸是单链的或双链的。

8. 根据权利要求 6 或 7 所述的寡核苷酸，其中所述至少一种 DNA 分子包含 LNA、甲基磷酸酯 -LNA、BNA、RNG、DNG、GNA、UNA、ENA、ANA、F-ANA、PNA、G-PNA 和吗啉代核苷酸中的至少一种；或包含 2’O- 甲基、2’O- 甲氧基乙基或 2’- 氟 RNA 核苷酸。

9. 根据权利要求 6 或 7 所述的寡核苷酸，其中所述至少一种 RNA 分子包含至少一种 RNA 核苷酸，所述 RNA 核苷酸选自 2’O- 甲基核苷酸、2’O- 甲氧基乙基核苷酸和 2’- 氟核苷酸；或包含至少一种核酸的核苷酸，所述核酸选自 LNA、甲基磷酸酯 LNA、BNA、RNG、DNG、GNA、UNA、ENA、ANA、F-ANA、PNA、G-PNA 和吗啉代。

10. 根据权利要求 6 所述的寡核苷酸，其中所述至少一种 DNA 分子或所述至少一种 RNA 分子包含至少一个在磷酸二酯键水平修饰为硫逐磷酸酯键的 DNA 分子。

11. 根据权利要求 6 所述的寡核苷酸，其中所述 PNA 具有修饰的主链，其中 α 碳具有精氨酸或赖氨酸作为取代基的侧链。

12. 根据权利要求 1-11 中任一项所述的寡核苷酸，其中所述寡核苷酸针对至少一种负责遗传或病毒来源的疾病的基因或对抗至少一种肿瘤基因。

13. 根据权利要求 12 所述的寡核苷酸，其特征在于所述基因选自由至少一种属于 MYC 家族的基因组成的组，所述组优选为 MYC、MYCN 或 MYCL1、BIRC5、BCL2、PLK4、ALK、PKM2、CASP8 和 RASSF1。

14. 根据权利要求 1-13 中任一项所述的寡核苷酸，其中所述寡核苷酸选自 :SEQ ID NO:2-15 和 SEQ ID NO:24、25；优选选自 :SEQ ID NO:2、4、5 和 6。

15. 根据权利要求 1-13 中任一项所述的寡核苷酸，其中所述寡核苷酸选自 :SEQ ID NO:66-84；优选选自 SEQ ID NO:68-84。

16. 根据权利要求 14 或 15 所述的寡核苷酸，其中 SEQ ID NO:2-15、66-69、24、25，优选 SEQ ID NO:2、4、5、6、68 和 69 针对 MYCN 基因。

17. 根据权利要求 15 所述的寡核苷酸，其中 SEQ ID NO:70-74 针对基因 MYC。

18. 根据权利要求 15 所述的寡核苷酸，其中 SEQ ID NO:75、76 针对基因 BIRC5。

19. 根据权利要求 15 所述的寡核苷酸，其中 SEQ ID NO:77-79 针对基因 ALK。

20. 根据权利要求 15 所述的寡核苷酸，其中 SEQ ID NO:80-82 针对基因 BCL2。

21. 根据权利要求 15 所述的寡核苷酸，其中 SEQ ID NO:83、84 针对基因 PLK4。

22. 根据权利要求 14 或 16 所述的寡核苷酸，其中所述寡核苷酸是选自由 SEQ ID NO:2-15，优选 SEQ ID NO:2-13，更优选 SEQ ID NO:2-8，甚至更优选 SEQ ID NO:2-6 组成的组中的 PNA。

23. 根据权利要求 22 所述的寡核苷酸，其中所述 PNA 是 SEQ ID NO:5。

24. 根据权利要求 14-23 中任一项所述的寡核苷酸，在 5’和 / 或 3’端结合 SEQ ID NO:47。

25. 一种组合物，其包含至少一种根据权利要求 1 至 24 中任一项所述的寡核苷酸和至少一种药物学上可接受的赋形剂。

26. 一种组合物，其包含至少一种根据权利要求 1 至 24 中任一项所述的寡核苷酸，包括 SEQ ID NO:1，结合具有药物作用的选定化合物，所述选定化合物优选选自由 NGF、生长激素抑制素、视磺酸、放线菌素 D、天门冬酰胺酶、博来霉素、白消安卡培他滨、卡铂、环磷酰胺、环孢霉素、顺铂、阿糖胞苷、clorambucil、氮烯唑胺、道诺霉素、多西他赛、盐酸阿霉素、盐酸表柔比星、足叶乙苷、磷酸氟达拉滨、氟脲嘧啶、吉西他滨、盐酸伊达比星、羟基脲、ifophosphamide、盐酸伊立替康、美法仑、巯基嘌呤、氨甲喋呤、丝裂霉素、米托蒽醌、奥沙利铂、紫杉醇、甲基苄肼、雷替曲塞、链脲霉素、替加氟 - 尿嘧啶、替莫唑胺、巯鸟嘌呤、thiotepe、拓扑替康、长春花碱、硫酸长春新碱、长春地辛和长春瑞滨组成的组。

27. 根据权利要求 26 所述的组合物，其中所述组合是 :SEQ ID NO:1 和卡铂，或足叶乙苷或顺铂或长春新碱 ;或 SEQ ID NO:5 和卡铂，或足叶乙苷或顺铂或长春新碱。

28. 根据权利要求 1 至 24 中任一项所述的寡核苷酸或根据权利要求 25-27 所述的组合物，用作药物或诊断剂。

29. 根据权利要求 28 所述的寡核苷酸或组合物，用于遗传和 / 或病毒来源的疾病的治疗中或用于治疗肿瘤，所述遗传和 / 或病毒来源的疾病和所述肿瘤由基因的超表达和 / 或抑制引起。

30. 根据权利要求 28 或 29 所述的寡核苷酸或组合物，其中所述遗传疾病选自由 Gorlin 综合症、唐氏综合症、Feingold 综合症、Hirschsprung’s 病、Von Hippel Lindau 综合症、共济失调毛细血管扩张、Li-Fraumeni 综合症、Turcot 综合症、家族性肿瘤和帕金森病 ;所述肿瘤是成人或儿科的成人或儿科肿瘤，优选选自 :成神经细胞瘤、成视网膜细胞瘤、成神经管细胞瘤、室鼓膜瘤、嗜铬细胞瘤、胚胎癌、生殖细胞肿瘤、腺泡状横纹肌肉瘤、胚胎横纹肌肉瘤、Wilms’肿瘤、肾脏的透明细胞肉瘤、滑膜肉瘤、肝胚细胞瘤、急性淋巴性白细胞、慢性淋巴性白血病、急性淋巴母细胞白血病、慢性淋巴母细胞白血病、Burkitt’s 淋巴瘤、急性骨髓性白血病、慢性骨髓性白血病、急性原巨核细胞白血病、B 慢性淋巴性白血病、T- 细胞白血病、淋巴瘤、小细胞肺癌（肺微小细胞瘤）、肺腺癌、鳞状上皮细胞肺癌、典型和非典型原发性肺癌、大细胞肺癌、大细胞神经内分泌性肺癌、成胶质细胞瘤、肝癌、基细胞癌、卵巢肿瘤、乳房肿瘤和结肠癌组成的组。

用于调节基因表达的寡核苷酸及其用途

[0001] 说明书

[0002] 本发明涉及用于调节基因表达的寡核苷酸，特别是用于调节负责遗传、肿瘤或病毒来源的病状的基因。

[0003] 此外，本发明涉及所述寡核苷酸用于所述疾病的治疗和 / 或诊断的用途，所述寡核苷酸可能是化学修饰的。

[0004] 寡核苷酸是天然 RNA 或 DNA 核酸或合成核酸的短序列，例如，PNA（肽核酸）、LNA（锁核酸）和吗啉代寡核苷酸。

[0005] 已经通过实验证明了寡核苷酸在转录和翻译水平上调节基因的表达中是非常有效的。由于这种能力，寡核苷酸代表了用于各种病状治疗的有效方法，特别是遗传、肿瘤或病毒来源的疾病。

[0006] 调节基因的表达意味着可以将其抑制或激活。

[0007] 例如，能够通过与基因的反义链形成互补键 (Hélène C，Bioch Bioph Acta1990，1049(2):99-125) 或改变目标基因调控区中的染色质状况 (Rossi JJ，Nat Chem Biol2007，3(3):136-7) 来抑制基因转录的寡核苷酸是已知的。其他寡核苷酸能够抑制基因翻译，例如，通过与靶标信使 RNA(mRNA) 的互补键。在单链寡核苷酸的情况中，这种键通过 RNase H 复合物引起 mRNA 的酶降解。在其中寡核苷酸是双链“干扰”RNA 分子的情况中，寡核苷酸与靶标 mRNA 的互补键通过 RISC 复合物的“沉默子”酶，引起信使的降解。在后一种情况中，寡核苷酸还可以是等于内源性 microRNA 的寡核苷酸，其依靠不完全的互补性，能够与靶标 mRNA 的 3’UTR(3’未翻译区）相连，引起 mRNA 的翻译被阻断。

[0008] 寡核苷酸还可以诱导基因的激活或其转录的提高，例如，经由与长的反义非编码 RNA 的互补键 (Morris KV，Epigenetics，2009，4(5):296-301)，或通过抑制互补微 RNA，结果是微 RNA 的靶标 mRNA 的翻译提高。

[0009] 为了提高它们在治疗和 / 或诊断条件中的有效性，可以将寡核苷酸进行化学修饰。例如，可以为了提高其特异性和 / 或与互补序列配对的效力，对寡核苷酸进行修饰，或为了使其对酶降解不太敏感、提高其药物动力学 / 药效谱，或促进其通过细胞膜，对寡核苷酸进行修饰。

[0010] 除了天然核酸的寡核苷酸（即，DNA 和 / 或 RNA 的短序列），还存在合成核酸的寡核苷酸，例如，肽核酸 (PNA) 和锁核酸 (LNA)，其已经得到了很大程度的研究和表征，以上全部的目的在于通过反基因策略来调节基因的表达（即，设计来直接冲击 (strike) 基因）。PNA 和 LNA 寡核苷酸，与全部修饰的寡核苷酸一样，通常在化学上比 DNA 或 RNA 寡核苷酸更稳定。可以通过合成嵌合寡核苷酸进一步提高它们的稳定性。嵌合寡核苷酸例如是其中插入了传统单体（脱氧核糖核苷酸或核糖核苷酸）和合成核碱基（单体）（例如，LNA 单体）两者的寡核苷酸序列。

[0011] 将 LNA 与反义策略一起使用，通过抑制靶标基因的转录产物来沉默基因 (Braasch DA，Nucl Acids Res，2002，30(23):5160-7)。或者，LNA 寡核苷酸也可以用于反义策略中，如 Smith 和同事所做的 (Ge R，Faseb J，2007，1902-14)，他们设计了以这样的方式能够通

过“链入侵”机理在核酸的两条链上相互作用的寡核苷酸序列，使得形成“Z- 型”结构（限定为“Zorro”寡核苷酸）。

[0012] 与其他寡核苷酸结构相比时，PNA 寡核苷酸对酶更稳定。PNA 可以通过“链入侵”结合双链 DNA(DNAds)，或可以以互补方式结合单链 DNA(DNAss) 的分子，或它们可以结合 RNA 链，产生杂合双螺旋 PNA/DNA 或 PNA/RNA 结构，其与“同质双链”结构（如，DNA/DNA 双链）相比，在热力学上非常稳定。

[0013] PNA 代表了用于调节基因表达的高度有利的系统，以上全部使用反基因策略。实际上，已经证明了 PNA 对靶标序列呈现出高特异性，并且因此能够以有效的方式抑制蛋白质的表达。

[0014] 因此，PNA 代表了用于遗传或病毒的疾病的有前景的治疗方法。

[0015] PNA 的唯一缺陷是它们具有有限的穿过细胞膜的能力的事实。然而，可以通过通常将寡核苷酸（并且特别是 PNA）与能够给予更有效地穿过细胞膜的分子（载体）结合解决了这一限制。

[0016] 实际上，寡核苷酸，并且特别是 PNA，通常可以使用与其结合的载体（或“标记物”）来给药，例如，具有 1 至 30 个氨基酸长度的肽序列。

[0017] 寡核苷酸的一个特别应用是调节基因的表达，其在肿瘤中被激活或抑制。

[0018] 公知肿瘤是由各种基因的失调引起的。通常，损害影响原癌基因（或也可能仅仅是致癌基因），如 MYC 基因（tra cui MYC、MYCN、MYCL1）、存活素 (BIRC5)、BCL2、PLK4、ALK 和 PKM2，其在肿瘤中被激活或超表达。此外，在肿瘤中，抗肿瘤或致癌抑制剂基因，如 caspase-8 和 RASSFI，通常也是被灭活的。

[0019] 特别地，MYC 家族的致癌基因涉及各种人肿瘤的产生，并且其中大部分基因引起肿瘤的发作和进展。这些基因的扩增和 / 或超表达几乎总是与肿瘤相关，儿科类型的（例如，成神经细胞瘤、成神经管细胞瘤和横纹肌肉瘤）和成人类型的（例如，小细胞肺癌或成胶质细胞瘤）(Pession A，Cur Cancer Drug Target，2005，5(4) :273-83)。实际上，它们通过对肿瘤生长是基础的机制来起作用，如诱导细胞增殖、抵抗凋亡、形成转移和抵抗化疗药物。

[0020] 许多具有抗肿瘤作用的寡核苷酸是文献中已知的。

[0021] 例如，在反义策略中存在针对 MYC、MYCN、BCL2、BIRC5 基因的寡核苷酸 (EV Prochownik，Exp Rev Antic Ther2004，16(6) :370-4 ；CF Bennet，Exp Opin Investig Drugs，1999，8(3) :237-53) 或具有反义作用的针对 MYCN 和 MYC 的寡核苷酸 (LC Boffa，Oligonucleotides2005，15(2) :85-93)。

[0022] 为了抑制 MYCN 基因翻译至成神经细胞瘤细胞中，还已经产生了基于 DNA 的硫逐磷酸酯反义寡核苷酸 (Burkhart CA，JNCI，2003，95(18) :1394-403)，并且通过“小干扰 RNA(siRNA)”产生了反义寡核苷酸，以抑制 MYCN 基因翻译至成神经细胞瘤细胞中 (Kang JH，Bioch Bioph Res Com，2006，351(1) :192-197)。

[0023] 然而，仍然强烈地感觉到需要鉴定更多的能够以提高的特异性和选择性方式调节基因表达的寡核苷酸，使得能够具有有效的治疗和 / 或抗肿瘤作用。

[0024] 为了获得可用作治疗和 / 或诊断方法的寡核苷酸，需要鉴定靶标基因，或靶标信使 RNA 的序列和 / 或其各自的调控序列，所述调控序列就转录和翻译而言，能够确定对基因自身表达的显著和选择性的调节作用。

[0025] 因此，还感觉到需要限定控制鉴定过程的一般规则－在基因的序列，或信使 RNA 的序列或其调控序列内－对于调节基因自身转录／翻译的目的最有前景的寡核苷酸序列。

[0026] 通过本发明满足了刚才所述的这部分中的需求，根据第一个方面，本发明涉及一种用于调节基因表达的寡核苷酸，其包含 6-30 个核苷酸（单体），优选 12-24 个核苷酸，所述寡核苷酸的特征在于包含至少一组的至少两个连续鸟嘌呤的序列。理解具有序列 SEQ ID NO:1 的寡核苷酸从刚才给出的限定中排除。

[0027] 在天然核酸 (DNA 和 RNA) 的情况中，每个单体（核苷酸）由含氮碱基、糖和三磷酸酯组成。碱基选自由腺嘌呤、鸟嘌呤、胸腺嘧啶、胞嘧啶和尿嘧啶（只有在 RNA 中）组成的组。糖在 DNA 的情况中是脱氧核糖，在 RNA 的情况中是核糖。单体在聚合物中通过磷酸二酯键连接。

[0028] 优选，本发明的寡核苷酸包含含有至少一组的至少三个连续鸟嘌呤的序列。理解具有序列 SEQ ID NO:1 的寡核苷酸从该限定中排除。

[0029] 更优选，本发明的寡核苷酸包含含有至少一组的至少四个连续鸟嘌呤的序列。

[0030] 再更优选，本发明的寡核苷酸包含含有至少一组的至少五个连续鸟嘌呤的序列。

[0031] 在本发明的一些实施方案中，寡核苷酸包含含有至少由两个至六个连续鸟嘌呤组成的一组的序列。

[0032] 在进一步的实施方案中，至少一组的鸟嘌呤优选包含至少两组的至少两个连续鸟嘌呤。

[0033] 或者，至少一组的鸟嘌呤优选包含至少一组的至少两个连续鸟嘌呤和至少一组的至少三个连续鸟嘌呤。

[0034] 在进一步的实施方案中，至少一组的鸟嘌呤优选包含至少三组的至少两个连续鸟嘌呤。

[0035] 在进一步的实施方案中，至少一组的鸟嘌呤优选包含至少四、五或六组的至少两个连续鸟嘌呤。

[0036] 或者，至少一组的鸟嘌呤优选包含至少一组的至少两个连续鸟嘌呤或至少两组的至少三个连续鸟嘌呤。

[0037] 或者，至少一组的鸟嘌呤优选包含至少一组的至少三个连续鸟嘌呤和至少两组的至少两个连续鸟嘌呤。

[0038] 或者，至少一组的鸟嘌呤优选包含至少一组的至少两个连续鸟嘌呤、至少一组的至少三个连续鸟嘌呤和／或至少一组的六个连续鸟嘌呤。

[0039] 通常，本发明的寡核苷酸优选与靶标序列互补，并且优选，连续鸟嘌呤的组可以是彼此连续的，使得，例如，三组 2 个连续的鸟嘌呤是一组 6 个连续的鸟嘌呤。或者，连续鸟嘌呤的组可以由至少一个核苷酸间隔开来。

[0040] 通常，根据本发明的至少一组的至少两个连续鸟嘌呤可以位于寡核苷酸 5’端附近，或在寡核苷酸 3’端附近，或可以位于寡核苷酸序列的中心。

[0041] 在本发明的优选实施方案中，优选在其 3’和／或 5’端使用载体序列结合所述寡核苷酸，所述载体序列优选是短氨基酸序列。

[0042] 所述短氨基酸序列（载体）优选由 1 至 30 个，优选 1 至 10 个，甚至更优选 1 至 7 个范围的多个氨基酸组成。氨基酸可以是 L 或 D 形式的，优选 D 形式。

[0043] 对于本发明的目的，优选的载体选自由 SEQ ID NO:47(PKKKRKV) ;SEQ ID NO:48(VKRKKKP) ;SEQ ID NO:49(KKKKKK) ;SEQ ID NO:50(PKRKRKV) ;SEQ ID NO:51(KRKRKRK) ;SEQ ID NO:52(KKKRKV) ;SEQ ID NO:53(PKKKRK) ;SEQ ID NO:54(KKKRK) ;SEQ ID NO:55(RRRR) 和 SEQ ID NO:56(PKKKRKVHHHHH) 组成的组。

[0044] 对于本发明的目的，特别优选的载体是具有 SEQ ID NO:47 的肽。

[0045] 在本发明的内容中，“载体”意思是能够有利地改变寡核苷酸的药物动力学 / 药效谱和 / 或细胞和 / 或核渗透的肽。

[0046] 在本发明的内容中，“调节基因的表达”意思是抑制或激活（提高）基因的表达。所述基因表达的抑制或激活（提高）可以在转录或翻译水平上发生。

[0047] 可以通过以反基因机制起作用的寡核苷酸（或反基因寡核苷酸，即，针对基因的反义链，也就是说，反基因策略），在转录水平上实现基因表达的抑制或激活。

[0048] 或者，可以使用通过反基因机制作用的寡核苷酸（或反基因寡核苷酸，即，针对信使，也就是说，反义策略），在翻译水平上实现基因表达的抑制，同时可以通过抑制降解信使 RNA 的微 RNA 来实现翻译水平上的表达提高。

[0049] 申请人鉴定的并且在以上描述的，为了有效调节基因的表达，寡核苷酸必须满足的参数或规则或必要条件适用于任何基因，并且因此可以例如用于鉴定能够调节基因表达的寡核苷酸序列的目的，所述基因负责遗传和 / 或病毒来源的疾病或涉及肿瘤病况发作的基因。

[0050] 由此鉴定的寡核苷酸可以用于治疗方法中，用于特定的遗传、病毒或肿瘤疾病，优选用作药物。

[0051] 或者，寡核苷酸可以用于诊断目的。

[0052] 实际上，本发明的主题进一步涉及本发明的寡核苷酸用于治疗和 / 或诊断目的的用途，所述寡核苷酸可能是化学修饰的。

[0053] 本发明的寡核苷酸是 6-30，优选 12-24 个残基（核苷酸或单体）的短寡核苷酸。寡核苷酸可以由天然核酸碱基组成，例如，DNA 或 RNA，或由合成的核酸碱基组成，例如，PNA、LNA 或吗啉代核苷酸。或者，寡核苷酸可以包含 DNA、RNA 和 / 或合成核酸的组合，优选 PNA 或 LNA（杂交或嵌合寡核苷酸）。此外，寡核苷酸可以是单链或双链的。

[0054] 在本发明的一些实施方案中，寡核苷酸可以是化学修饰的，例如，用于提高它们的治疗和 / 或诊断有效性的目的。

[0055] 在本发明的优选实施方案中，寡核苷酸可以是具有主链的 PNA 分子，其中主链中 α 位置 ($C_{alpha(\alpha)}$) 的碳结合取代基，而不是结合甘氨酸的典型氢原子。例如，替代甘氨酸的侧链，可以使用天然或合成来源的另一个氨基酸的侧链，所述氨基酸优选选自由精氨酸、赖氨酸、组氨酸、亮氨酸、异亮氨酸、酪氨酸、天冬氨酸、丝氨酸、苏氨酸、谷氨酰胺、缬氨酸、丙氨酸、半胱氨酸、甲硫氨酸、苯丙氨酸、谷氨酸盐、天冬氨酸盐、脯氨酸、色氨酸和鸟氨酸。所述氨基酸可以是右旋构造 (D) 或左旋构造 (L) 组成的组。

[0056] 在本发明的其他优选实施方案中，寡核苷酸是 :互相互补的单链或双链 RNA 分子（将互相互补的双链 RNA 分子限定为 siRNA，“小干扰 RNA”的首字母缩略词）。

[0057] 在一些实施方案中，所述“小干扰 RNA”包含 RNA 单体（核糖核苷酸）和至少一个在核糖 2’位置修饰的单体，优选 2’-O- 甲氧基乙基、2’-O- 甲基或 2’- 氟单体 ;或所述“小

干扰 RNA”包含 RNA 单体（核糖核苷酸）和至少一个合成核酸的单体，所述合成核酸选自由 LNA、甲基磷酸酯 LNA、BNA（桥接核酸）、UNA（未锁闭核酸）、ENA（乙烯－桥接核酸）、ANA（阿拉伯糖核酸）和 F-ANA（氟－阿拉伯糖苷核酸）组成的组。

[0058] 在优选的实施方案中，以如下的方式来设计所述“小干扰 RNA”：在互补双链的末端，两条链中只有一条具有至少一个，优选两个突出（即，未配对）的天然或合成核酸的单体。在进一步的实施方案中，天然或合成突出核酸优选选自由 2’-O- 甲氧基乙基、2’-O- 甲基或 2’- 氟单体，或 LNA、甲基磷酸酯 LNA、BNA、UNA（未锁闭核酸）、ENA（乙烯－桥接核酸）、ANA（阿拉伯糖核酸）和 F-ANA（氟－阿拉伯糖苷核酸）的单体组成的组。

[0059] 在进一步的实施方案中，将寡核苷酸限定为杂交或嵌合的，并且优选是单链或双链的，包含 RNA 单体（核糖核苷酸）和 LNA 单体（将这种寡核苷酸表示为 RNA/LNA）。

[0060] 或者，杂交寡核苷酸可以包含 RNA 单体（核糖核苷酸）和至少一个选自 2’-O- 甲氧基乙基 (MOE) 单体、2’-O- 甲基单体或 2’- 氟单体的 RNA 单体；或杂交寡核苷酸可以包含 RNA 单体（核糖核苷酸）和至少一个优选选自 LNA、甲基磷酸酯 LNA、UNA（未锁闭核酸）、BNA、ENA（乙烯－桥接核酸）、ANA（阿拉伯糖核酸）和 F-ANA（氟－阿拉伯糖苷核酸）的合成核酸的单体。

[0061] 在进一步的实施方案中，基于单链或双链 RNA 的寡核苷酸可以包含传统的核糖核苷酸（即，没有化学修饰）和已经在磷酸二酯键水平被修饰的核糖核苷酸或脱氧核糖核苷酸，例如，通过硫逐磷酸酯键或 DNG（脱氧核糖核胍）、RNG（核糖核胍）、GNA（甘油核酸）、G-PNA（γ-PNA）或 PMO（吗啉代）进行了修饰。

[0062] 在本发明的优选实施方案中，寡核苷酸是包含 DNA 单体（脱氧核糖核苷酸）和 LNA 单体的嵌合单链序列。

[0063] 对于反基因策略，其表示在转录水平调节基因的表达，可以优选使用：

[0064] • 基于 PNA 的寡核苷酸，可选与载体（通常由 1 至 30 个残基组成）结合，优选在 3’ 端和 / 或 5’；或

[0065] • 基于 PNA 的寡核苷酸，所述 PNA 包含至少一个主链的 α 碳 (C-α)，与取代基结合，而不是标准甘氨酸的 H 原子结合；或

[0066] •包含 RNA 单体（传统核糖核苷酸）和可选的至少一个修饰的核苷酸（单体）（例如，2’-O- 甲基 RNA 单体、2’- 氟 RNA 单体），或至少一个选自 LNA、甲基磷酸酯 LNA、BNA、UNA、GNA、ANA、FANA、ENA、DNG 和 RNG 的核酸的单体，或在磷酸二酯键水平上修饰的核糖核苷酸的单链寡核苷酸；或

[0067] • 互相互补的双链的基于 RNA 的寡核苷酸 (siRNA)；或

[0068] • 包含 RNA 单体（传统核糖核苷酸）和至少一个 LNA 单体的部分互补的双链嵌合寡核苷酸；或

[0069] •包含 RNA 单体（传统核糖核苷酸）和至少一个 2’-O-(2- 甲氧基甲基)RNA 单体的双链嵌合寡核苷酸；或

[0070] • 包含 DNA 单体（传统脱氧核糖核苷酸）和至少一个 LNA 单体的单链嵌合寡核苷酸；或

[0071] •包含 DNA 单体（传统脱氧核糖核苷酸）和至少一个 2’- 氟 RNA 单体或至少一个选自 LNA、甲基膦酸酯 LNA、BNA、UNA、GNA、ENA、ANA、FANA、DNG 和 RNG 的核酸的单体的寡核

苷酸。

[0072] 对于反义策略，其表示在翻译水平上调节基因的表达，可以优选利用：

[0073] • 包含 RNA 单体（传统核糖核苷酸）的互相互补的双链寡核苷酸（siRNA）；或

[0074] • 包含 RNA 单体（传统核糖核苷酸）和至少一个在核糖水平和 / 或在磷酸二酯键水平修饰的 RNA 单体的单链寡核苷酸；

[0075] • 包含 DNA 单体（传统脱氧核糖核苷酸）和至少一个硫逐磷酸酯 DNA 单体的单链嵌合寡核苷酸；或

[0076] •包含 RNA 单体（传统核糖核苷酸）和至少一个 2’-O-(2- 甲氧基乙基)RNA 单体的双链嵌合寡核苷酸；或

[0077] • 包含 RNA 单体（传统核糖核苷酸）和至少一个 2’O- 甲基化 RNA 单体的双链嵌合寡核苷酸；或

[0078] •包含 RNA 单体（传统核糖核苷酸）和至少一个 2’- 氟 RNA 单体的双链嵌合寡核苷酸；或

[0079] •包含 RNA 单体（传统核糖核苷酸）和至少一个 LNA 单体的双链嵌合寡核苷酸；或

[0080] • 包含 RNA 单体（传统核糖核苷酸）和至少一个阿拉伯糖苷 RNA 单体的双链嵌合寡核苷酸；或

[0081] • 包含 DNA 单体（传统脱氧核糖核苷酸）和至少一个 LNA 单体的单链嵌合寡核苷酸；或

[0082] • 包含吗啉代单体的单链寡核苷酸；或

[0083] • 基于 PNA 的寡核苷酸，所述 PNA 包含至少一个主链的 α 碳 (C-α)，与取代基结合，而不是标准甘氨酸的 H 原子结合；优选取代基是精氨酸或赖氨酸的侧链；或

[0084] •包含 DNA 单体（传统脱氧核糖核苷酸）和至少一个选自 PNA、LNA、LNA 甲基磷酸酯、BNA、UNA、GNA、ENA、DNG 和 RNG 的核酸的单体的寡核苷酸。

[0085] 优选，本发明的寡核苷酸针对涉及遗传和 / 或病毒来源的疾病或肿瘤的产生的基因。所述基因优选选自由 MYC 家族的基因（优选 MYC、MYCN、MYCL1）、存活素基因 (BIRC5)、BCL2、PLK4、ALK、PKM2、caspase-8 和 RASSF1 组成的组。

[0086] 特别地，本发明的寡核苷酸针对 MYC 家族的基因，优选针对 MYCN。

[0087] 所述寡核苷酸优选选自由 SEQ ID NO:2-15、66-84，SEQ ID NO:24、25、31 和 32 组成的组，具有 SEQ ID NO:26 和 57 的互补寡核苷酸对，具有 SEQ ID NO:27 和 58 的互补寡核苷酸对，具有 SEQ ID NO:28 和 59 的互补寡核苷酸对，具有 SEQ ID NO:29 和 60 的互补寡核苷酸对，具有 SEQ ID NO:30 和 61 的互补寡核苷酸对，具有 SEQ ID NO:33 和 62 的互补寡核苷酸对，具有 SEQ ID NO:34 和 63 的互补寡核苷酸对，具有 SEQ ID NO:35 和 64 的互补寡核苷酸对以及具有 SEQ ID NO:36 和 65 的互补寡核苷酸对。

[0088] 在一些实施方案中，所述寡核苷酸是 PNA 寡核苷酸，优选所述 PNA 针对 MYCN。

[0089] 在优选的实施方案中，PNA 选自由 SEQ ID NO:2-15 组成的组。

[0090] 在进一步优选的实施方案中，PNA 选自由 SEQ ID NO:66-84 组成的组。

[0091] 在进一步优选的实施方案中，PNA 选自由 SEQ ID NO:2-15、66-84 组成的组。

[0092] 优选，PNA 寡核苷酸选自由 SEQ ID NO:2-13，更优选 SEQ ID NO:2-8，再更优选 SEQ ID NO:2-6 组成的组。对于本发明的目的，特别优选的 PNA 寡核苷酸是 SEQ ID NO:5。

[0093] 优选，SEQ ID NO:5 在 5’或 3’端结合 SEQ ID NO:47。更优选，SEQ ID NO:47 由 D 形式的氨基酸组成。

[0094] PNA SEQ ID NO:2-15 优选针对 MYCN，并且更优选，它们以反基因策略调节 MYCN 的表达。

[0095] PNA SEQ ID NO:66-69 也优选针对 MYCN，并且更优选，它们以反基因策略调节 MYCN 的表达。

[0096] PNA SEQ ID NO:70-74 优选针对 MYC，并且更优选，它们以反基因策略调节 MYC 的表达。

[0097] PNA SEQ ID NO:75、76 优选针对 BIRC5，并且更优选，它们以反基因策略调节 BIRC5 的表达。

[0098] PNA SEQ ID NO:77-79 优选针对 ALK，并且更优选，它们以反基因策略调节 ALK 的表达。

[0099] PNA SEQ ID NO:80-82 优选针对 BCL2，并且更优选，它们以反基因策略调节 BCL2 的表达。

[0100] PNA SEQ ID NO:83、84 优选针对 PLK4，并且更优选，它们以反基因策略调节 PLK4 的表达。

[0101] 在进一步的实施方案中，所述寡核苷酸是双链的，并且优选包含 RNA 单体。优选，所述寡核苷酸针对 MYCN。

[0102] 或者，所述双链 RNA 寡核苷酸选自：具有 SEQ ID NO:26 和 57 的互补寡核苷酸对，具有 SEQ ID NO:27 和 58 的互补寡核苷酸对，具有 SEQ ID NO:28 和 59 的互补寡核苷酸对，具有 SEQ ID NO:29 和 60 的互补寡核苷酸对，具有 SEQ ID NO:30 和 61 的互补寡核苷酸对。

[0103] 所述寡核苷酸优选针对 MYCN。更优选，它们通过反义策略调节基因的表达。

[0104] 在进一步的实施方案中，所述寡核苷酸是 DNA-LNA 嵌合寡核苷酸，优选所述寡核苷酸针对 MYCN。

[0105] 在优选的实施方案中，所述 DNA-LNA 嵌合寡核苷酸选自由 SEQ ID NO:24 和 25 组成的组。

[0106] SEQ ID NO:24 和 25 优选针对 MYCN 基因。

[0107] SEQ ID NO:24 和 25 优选通过反基因策略调节基因的表达。

[0108] 在进一步的实施方案中，所述寡核苷酸是包含 DNA 单体和 / 或至少一个硫逐磷酸酯 DNA 单体的单链嵌合寡核苷酸。所述寡核苷酸优选针对 MYCN。

[0109] 对于本发明的目的，特别优选的是选自由 SEQ ID NO:31 和 32 组成的组的嵌合寡核苷酸。SEQ ID NO:31 和 32 优选针对 MYCN 基因。SEQ ID NO:31 和 32 优选通过反义策略调节 MYCN 的表达。

[0110] 在进一步的实施方案中，所述寡核苷酸是包含 RNA 单体和至少一个优选 2’-O-(2- 甲氧基乙基）或 2’- 甲基 RNA 单体的双链嵌合寡核苷酸。

[0111] 所述寡核苷酸优选针对 MYCN。

[0112] 对于本发明的目的，特别优选的是具有 SEQ ID NO:33 和 62 的互补嵌合寡核苷酸对。所述寡核苷酸对优选通过反义机制调节基因的表达。

[0113] 在进一步的实施方案中，所述寡核苷酸是包含 RNA 单体和至少一个优选 2’- 氟 RNA

单体的双链嵌合寡核苷酸。

[0114] 优选，所述寡核苷酸针对 MYCN。

[0115] 对于本发明的目的，特别优选的是具有 SEQ ID NO:34 和 63 的互补嵌合寡核苷酸对。所述寡核苷酸对优选通过反义机制调节基因的表达。

[0116] 在进一步的实施方案中，所述寡核苷酸是包含 RNA 单体和至少一个 LNA 单体的双链嵌合寡核苷酸。

[0117] 所述寡核苷酸优选针对 MYCN。

[0118] 对于本发明的目的，特别优选的是具有 SEQ ID NO:35 和 64 的互补嵌合寡核苷酸对。所述寡核苷酸对优选通过反义机制调节基因的表达。

[0119] 在进一步的实施方案中，所述寡核苷酸是双链嵌合寡核苷酸，包含 RNA 和至少一个阿拉伯糖苷 RNA 单体。

[0120] 优选，所述寡核苷酸针对 MYCN。

[0121] 对于本发明的目的，特别优选的是具有 SEQ ID NO:36 和 65 的互补嵌合寡核苷酸对。所述寡核苷酸对优选通过反义机制调节基因的表达。

[0122] 本发明的再一个方面涉及上述寡核苷酸用于治疗和 / 或诊断目的的用途。

[0123] 特别地，寡核苷酸可以单独地或结合在一起用于遗传和 / 或病毒来源的疾病的治疗，特别是用于由基因的超表达或抑制引起的遗传疾病的治疗，即，需要调节超表达或抑制的基因的表达的遗传疾病。

[0124] 本发明的寡核苷酸用于遗传疾病的治疗性处理，所述疾病优选选自由 Gorlin 综合症、唐氏综合症、Feingold 综合症、Hirschsprung’s 病、Li-Fraumeni 综合症、Turcot 综合症、家族性肿瘤和帕金森病组成的组。

[0125] 此外，本发明的寡核苷酸用于儿童或成人的肿瘤病况的治疗性处理。特别地，所述肿瘤优选由基因或致癌基因的超表达引起，所述基因选自由 MYC、MYCN、MYCL1、存活素 (BIRC5)、BCL2、PLK4、ALK 和 PKM2 组成的组。或者，肿瘤优选是由致癌抑制剂或抗肿瘤基因的抑制（失活）引起的并且优选选自由 caspase-8 和 RASSF1 组成的组。

[0126] 所述肿瘤优选选自由成神经细胞瘤、成视网膜细胞瘤、成神经管细胞瘤、室鼓膜瘤、嗜铬细胞瘤、胚胎癌、生殖细胞肿瘤、腺泡状横纹肌肉瘤、胚胎横纹肌肉瘤、Wilms’肿瘤、肾脏的透明细胞肉瘤、滑膜肉瘤、肝胚细胞瘤、急性淋巴性白细胞、慢性淋巴性白血病、急性淋巴母细胞白血病、慢性淋巴母细胞白血病、Burkitt’s 淋巴瘤、急性骨髓性白血病、慢性骨髓性白血病、急性原巨核细胞白血病、B 慢性淋巴性白血病、T- 细胞白血病、淋巴瘤、小细胞肺癌（肺微小细胞瘤）、肺腺癌、鳞状上皮细胞肺癌、典型和非典型原发性肺癌、大细胞肺癌、大细胞神经内分泌性肺癌、成胶质细胞瘤、肝癌、基细胞癌、卵巢肿瘤、乳房肿瘤和结肠癌组成的组。

[0127] 对于本发明的目的，特别优选的是选自由成神经细胞瘤、成视网膜细胞瘤、横纹肌肉瘤、Wilms’肿瘤、成神经管细胞瘤、小细胞肺癌和基细胞癌组成的组。

[0128] 本发明的主题进一步涉及包含至少一种根据本发明的寡核苷酸和至少一种药物学上接受的赋形剂的组合物。优选，所述至少一种寡核苷酸是 PNA，优选选自由 SEQ ID NO:2-15、66-84，优选 SEQ ID NO:2-13，更优选 SEQ ID NO:2-8，再更优选 SEQ ID NO:2-6 组成的组。特别优选的寡核苷酸是 SEQ ID NO:5。优选，SEQ ID NO:5 在 5’或 3’端结合 SEQ

ID NO:47。更优选，SEQ ID NO:47 由 D 型氨基酸组成。

[0129] 所述 PNA 优选在 5’或 3’端结合载体，所述载体优选选自由 SEQ ID NO:47-56 组成的组。

[0130] 本发明的主题进一步涉及包含至少一种根据本发明的寡核苷酸（包括具有 SEQ ID NO:1 的寡核苷酸）、至少一种化合物（优选，至少一种具有药物学作用的化合物，更优选化疗剂）和可选至少一种药学上接受的赋形剂的组合物。

[0131] 在优选的实施方案中，所述至少一种化合物是至少一种另外的反基因和 / 或反义寡核苷酸，或至少一种药物学制剂，或至少一种生物或生物技术来源的或源自化学合成或其组合的化合物。

[0132] 所述生物或生物技术来源或源自化学合成的化合物优选选自由单克隆抗体、化疗剂、免疫调节剂、生长因子、细胞因子、肽、血管生成抑制剂、肿瘤生长抑制剂、甾类激素和 / 或非甾类激素和维生素组成的组。

[0133] 对于本发明的目的，特别优选的化合物的实例选自由神经生长因子 (NGF)、生长激素抑制素、视磺酸、放线菌素 D、天门冬酰胺酶、博来霉素、白消安卡培他滨、卡铂、环磷酰胺、环孢霉素、顺铂、阿糖胞苷、clorambucil、氮烯唑胺、道诺霉素、多西他赛、盐酸阿霉素、盐酸表柔比星、足叶乙苷、磷酸氟达拉滨、氟脲嘧啶、吉西他滨、盐酸伊达比星、羟基脲、ifophosphamide、盐酸伊立替康、美法仑、巯基嘌呤、氨甲喋呤、丝裂霉素、米托蒽醌、nutline、奥沙利铂、紫杉醇、甲基苄肼、雷替曲塞、链脲霉素、替加氟 - 尿嘧啶、替莫唑胺、巯鸟嘌呤、thiotepe、拓扑替康、长春花碱、长春新碱、长春地辛和长春瑞滨，及其组合。

[0134] 更优选，所述化合物选自由卡铂、顺铂、足叶乙苷、长春新碱、环磷酰胺，及其组合组成的组。

[0135] 申请人已经发现了至少一种根据本发明的寡核苷酸结合至少一种化合物（优选至少一种化疗剂，如上所述）的给药使得可以降低待给药的所述化合物的浓度，同时确保治疗有效性的提高和较低的毒性。

[0136] 申请人已经发现了，在这些条件下，所述化合物浓度的降低取决于特定的病状；特别地，化疗组合物的浓度取决于肿瘤的类型。

[0137] 对于一些肿瘤，如：成神经细胞瘤、成视网膜细胞瘤、成神经管细胞瘤、小细胞肺癌、Wilms’肿瘤、腺泡状横纹肌肉瘤和胚胎横纹肌肉瘤，结合至少一种根据本发明的寡核苷酸给药的至少一种化疗剂的浓度可以降低高达 10 倍，同时确保治疗作用与正常剂量的化疗剂相同。

[0138] 作为药物组合（就提高的治疗作用而言），特别有效的是至少一种根据本发明的 PNA（优选，至少一种选自由 SEQ ID NO:1-15、66-84，优选 SEQ ID NO:1-13，更优选 SEQ ID NO:1-8，更优选 SEQ ID NO:1-6，甚至更优选 SEQ ID NO:1 和 / 或 5 的 PNA）和至少一种化合物的组合组成的组，所述化合物优选是化疗剂，更优选选自由足叶乙苷 (VP16)、卡铂、顺铂或长春新碱、环磷酰胺，及其组合组成的组。

[0139] 对于本发明的目的，特别优选的是选自以下组成的组的组合：SEQ ID NO:1 和卡铂，或足叶乙苷或顺铂或长春新碱；或 SEQ ID NO:5 和卡铂或足叶乙苷或顺铂或长春新碱。

[0140] 所述 PNA 优选在其 3’和 / 或 5’端结合载体，所述载体优选选自由 SEQ ID NO:47-56 组成的组。

[0141] 优选在同时给药组合物时和连续时间给药至少一种化合物时，发现提高的作用，所述连续时间给药优选以一定间隔给药，更优选以 3 小时、6 小时、12 小时、24 小时、48 小时或 72 小时的规律间隔给药。

[0142] 在其他优选实施方案中，本发明的至少一种寡核苷酸，包括具有SEQ ID NO:1 的寡核苷酸，可以优选与至少一种载体颗粒、至少一种载体聚合物或至少一种自体装配的载体寡核苷酸（也称为适体）结合或复合给药。

[0143] 在进一步优选的实施方案中，本发明的至少一种寡核苷酸，包括具有 SEQ ID NO:1 的寡核苷酸，可以与至少一种脂质体胶束、至少一种微粒或至少一种纳米颗粒结合或复合并给药，使得促进靶标组织的渗透。

[0144] 所述颗粒，通常是球形的，并且用作特定传送的工具，可以与许多不同的化合物一起配制。例如，所述颗粒可以是聚合化合物的配方或共同 - 配方，所述聚合化合物如 :壳聚糖、透明质酸、聚乙二醇 (PEG)、聚乙烯亚胺 (PEI)、聚乳酸 (PLA)、聚（乳酸 - 共 - 乙醇酸）(PLGA)、羟磷灰石 (HAP)、多不饱和脂肪酸、饱和脂肪酸、阳离子脂质、HAP-PLA、HAP-PLA/PGA 及其衍生物。在进一步优选的实施方案中，至少一种本发明的寡核苷酸可以优选与至少一种之前所述类型的颗粒和至少一种配体或至少一部分的配体结合或复合，所述配体针对靶标细胞的特定受体（如，例如，GD2、叶酸、TRAIL、NGF)，化学或生物技术来源的，可以作为佐剂存在于聚合膜中，用于促进所述本发明的寡核苷酸在靶标细胞中的内在化。

[0145] 在进一步优选的实施方案中，至少一种本发明的寡核苷酸可以结合至少一种配体或配体（如，例如，GD2（神经节苷脂 GD2)、叶酸、TRAIL(TNF- 相关凋亡诱导配体）、NGF（神经生长因子））的一部分，所述配体对于靶标细胞的受体是特异性的。

[0146] 在进一步优选的实施方案中，至少一种本发明的寡核苷酸，包括具有 SEQ ID NO:1 的寡核苷酸，可以自身单独给药，或在组合中，结合至少一种另外的医学应用来给药，以增强其有效性，优选还通过促进靶标细胞和 / 或组织的渗透来增强其有效性。

[0147] 所述医学应用优选选自由氧疗法、磁疗法、热疗法、电刺激、超声波、放疗、化疗和光线疗法组成的组。

[0148] 实施例 1

[0149] 寡核苷酸的化学合成。

[0150] 寡核苷酸的化学合成是基于在 5’-OH 用保护基团 4,4’- 二甲氧基三苯甲基 (DMTr) 和在 3’- 磷酸酯基团用 β - 氰基乙基修饰的 DNA 核苷酸亚磷酰胺的使用 ;保护基团还用于伯胺（核碱基杂环），其另外也是反应性的。

[0151] DNA 寡核苷酸的化学合成以 3’-5’ 方向进行。使用了 CPG（受控孔玻璃的首字母缩略词）树脂或聚苯乙烯支持物，用第一个核苷酸碱基功能化。合成从使用二氯甲烷 (DCM) 中的 3％三氯乙酸 (TCA) 溶液使 5’- 二甲氧基三苯甲基基团去保护的步骤开始。这接着是激活，使用对应于序列中待插入的第二个碱基的亚磷酰胺的乙基硫代四唑 (ETT) 或苄基硫代四唑 (BTT)0.3M，然后其将结合之前去保护的 5’ OH，由此形成磷酸二酯键。

[0152] 下一个步骤是“加帽”，其用于使未反应的 5’ OH 基团乙酰化。使用 2 种溶液来进行加帽，一种含有四氢呋喃 (THF)/ 卢剔啶 / 乙酸酐 (8:1:1)，而另一种含有 THF 中的 10％甲基咪唑溶液。通过 THF/ 吡啶溶液中的碘稳定亚磷酸三酯不稳定的三价键，碘将其氧化成五价磷酸二酯。

[0153] 氧化后，重复该循环，从第二个引入的单体的脱三苯甲基开始等等。将该循环重复需要的次数，这取决于希望多少碱基插入序列中。最后，通过在室温下用酸处理来除去最后的 5’-DMTr 基团。

[0154] 根据碱基上存在的保护基团（其随后取决于选定的碱基的化学性质，PTO、2’OMe 等），可以与氢氧化铵在 55℃下持续 16 小时，或与氢氧化铵 / 甲胺 (AMA) 溶液在 55℃下持续 35 分钟，使得通过氰基乙基基团的 β- 消除使磷去保护，并且除去核碱基杂环上的保护基团。

[0155] 或者，可以在整个分析 (HPLC、MS) 和制备性色谱期间保持 5’-DMTr 基团，以更好地从副产物中纯化出终产物，并且最终通过用乙酸处理来除去。

[0156] 由于核糖上存在的 2’OH 基团，并且因此用于每个亚磷酰胺的另外的保护基团的存在，RNA 寡核苷酸的化学合成不同于 DNA 寡核苷酸。

[0157] 因此，RNA 寡核苷酸的合成需要较长的耦合时间和更多的步骤，来使基团去保护。

[0158] 将如上所述的相同方案用于寡核苷酸的合成，使用化学修饰的单体，如硫逐磷酸酯 (PTO)、2’O- 甲基 (2’OMe)、2’氟 (2’-F)、阿拉伯糖苷核酸 (ANA) 和锁核酸 (LNA)。

[0159] 通过购买公司 (Link Technologies Ltd.) 的单体分子来提供用于每种修饰碱基的特定技术的详细内容。

[0160] 吗啉代核酸购自制造商 (Gene Tools，LLC)。

[0161] 以 10 微摩尔规模来进行 PNA 寡核苷酸的合成，并且包括纯化和表征步骤。

[0162] 分子的合成在固相进行，使用 Rink Amide-Chemmatrix®树脂和 Syro 自动化合成仪 (MultiSynTech)。将合成的第一个单体手动结合树脂。每个自动化合成循环分成三个步骤。第一个步骤是去保护，使用 DMF（二甲基甲酰胺）中的 20％哌啶溶液来进行。

[0163] 第二个步骤是进入的单体和生长中的链之间的耦合反应。通过加入 NMP(N- 甲基吡咯烷酮）中的5 0.22M 当量 (eq) 的单体 (FMOC-PNA-G(Bhoc)-OH、FMOC-PNA-A(Bhoc)-OH、FMOC-PNA-C(Bhoc)-OH、FMOC-PNA-T-OH) 和碱性环境中的 DMF 中的 4.50.32M eq 的激活剂（在这种情况中，为 HATU) 来进行这一反应，所述碱性环境含有 DMF 中的 2,6- 卢剔啶和 DIPEA(N,N- 二异丙基乙胺）的 8％溶液。在预先装载树脂上的第一个和第二个单体的连接点，从肽链通过至 PNA 链，并且最后一个单体上，重复耦合反应，一式两份。

[0164] 第三个步骤是“加帽”反应，其通过乙酰化用于阻断耦合步骤过程中未反应的位点。使用 DMF 中含有 6％ 2,6- 卢剔啶和 5％乙酸酐的溶液来实现这一反应。合成完成时，从固体支持物上取下分子。

[0165] 使用 4:1 比例的 TFA（三氟乙酸）和间酚紫的溶液来获得这一反应。

[0166] 通过在二乙醚中的沉淀来收集由此获得的分子。

[0167] 一旦收集在水中，在 HPLC 中纯化。用于纯化的柱子是 C18300A5u Jupiter（©Phenomenex，Inc.）。在 30 分钟内使用从 100％ A（水 95％；乙腈 5％；0.1％ TFA)-0％ B（水 60％；乙腈 40％；0.1％ TFA) 至 60％ A-40％ B 的线性梯度来进行纯化。所用的完整梯度为 0-5 分钟 0％ B；5-35 分钟 40％ B；35-37100％；37-42100％ B；42-440％ B。

[0168] 最后，通过 ESI 质谱（© Waters）来分析纯化的产品。

[0169] 用于阻断基因转录的反基因寡核苷酸

[0170] 为了证明根据本发明中所述的参数选择和设计的本发明的寡核苷酸能够选择性

地阻断基因的转录的目的，设计并合成了针对 MYCN 基因的基于 PNA 的寡核苷酸；它们显示于表 1 中。

[0171] 表 1

[0172]

SEQ ID NO	PNA 序列	% GC	mRNA 抑制 (%)	细胞增殖 (%)	Phoenix 增殖 (%)	NIH-3t3 增殖 (%)
SEQ ID NO: 1	*ATGCCGGGCATGATCT*	*56.3*	*42*	*70*	*100*	*100*
SEQ ID NO: 2	GGGTGGATGCGGGGGG	81.3	75	32	100	100
SEQ ID NO: 3	GATGCGGGGGGCTCCT	75	68	41	100	100
SEQ ID NO: 4	GTCGGCGGGAGGTAAG	68.8	65	48	100	100
SEQ ID NO: 5	GCTGGGTGGATGCGGG	75	62	53	100	100
SEQ ID NO: 6	TGGACGCGCTGGGTGG	75	60	54	100	100
SEQ ID NO: 7	CGCGCTGGGTGGATGC	75	58	57	100	100
SEQ ID NO: 8	GTCTGGACGCGCTGGG	75	54	59	100	100
SEQ ID NO: 9	CCCTGCAGTCGGCGGG	81.3	51	64	100	100
SEQ ID NO: 10	CGGCCGCGGGCCGCCA	93.8	51	65	100	100
SEQ ID NO: 11	GGGAACTGTGTTGGAG	56.3	48	68	100	100
SEQ ID NO: 12	TGTCTGGACGCGCTGG	68.8	47	69	100	100
SEQ ID NO: 13	ACGCTCAGGGACCACG	68.8	48	66	100	100
SEQ ID NO: 14	CCCGGACGAAGATGAC	62.5	40	70	100	100
SEQ ID	ACTGTGTTGGAGCCGA	56.3	37	77	100	100

[0173]

NO: 15						
SEQ ID NO: 16	CCTGTCGTAGACAGCT	56.3	14	90	100	100
SEQ ID NO: 17	TGTGACAGTCATCTGT	56.3	10	98	100	100
SEQ ID NO: 18	GTGACAGTCATCTGTC	50	10	98	100	100
SEQ ID NO: 19	GACAGTCATCTGTCTG	50	5	100	100	100
SEQ ID NO: 20	CGTCGATTTCTTCCTC	50	5	100	100	100
SEQ ID NO: 21	CTCGAGTTTGACTCGC	56.3	1	100	100	100
SEQ ID NO: 22	GCGCCTCCCCTGATTT	62.5	2	100	100	100
SEQ ID NO: 23	ATATCCCCCGAGCTTC	56.3	2	100	100	100

[0174] 针对促进细胞膜渗透的目的,单独地以及在 3’和 / 或 5’端结合载体,测试了 PNA 寡核苷酸。特别地,将寡核苷酸结合羧基端,在 3’具有氨基酸序列 SEQ ID NO:43,即,脯氨酸 - 赖氨酸 - 赖氨酸 - 赖氨酸 - 精氨酸 - 赖氨酸 - 缬氨酸。

[0175] 具有 SEQ ID NO:1 的寡核苷酸是专利 EP1618195 主题的序列,并且表示对照序列和用于比较的序列。

[0176] 具有 SEQ ID NO:2-15 的寡核苷酸含有一组两个连续鸟嘌呤 (SEQ ID NO:14、15),或一组三个连续鸟嘌呤 (SEQ ID NO:13),或两组两个连续鸟嘌呤 (SEQ ID NO:12),或一组两个鸟嘌呤和一组三个连续鸟嘌呤 (SEQ ID NO:11、10、9、8 和 7),或两组两个连续鸟嘌呤和一组三个连续鸟嘌呤 (SEQ ID NO:6 和 4),或一组两个连续鸟嘌呤和两组三个连续鸟嘌呤 (SEQ ID NO:5),或一组六个连续鸟嘌呤 (SEQ ID NO:3),或一组六个连续鸟嘌呤,一组三个连续鸟嘌呤和一组两个连续鸟嘌呤 (SEQ ID NO:2)。

[0177] 连续鸟嘌呤的组在表 1 中以下划线显示。

[0178] 具有 SEQ ID NO:16-23 的寡核苷酸不具有连续鸟嘌呤组,为负对照。

[0179] 此外,对于选择性调节基因转录的目的,还设计和合成了具有 SEQ ID NO:24-25 的寡核苷酸 ;这些显示于表 2 中。

[0180] 表 2

[0181]

SEQ ID NO: 24	DNA-LNA 25-1	ATGCCGGGCATGATC T
SEQ ID NO: 25	DNA-LNA 25-2	ATGCCGGGCATGATC T

[0182] 特别地,设计和合成了包含 DNA 单体和 LNA 单体 (SEQ ID NO:24 和 25) 的单链嵌合寡核苷酸。

[0183] 寡核苷酸序列中的 DNA 碱基显示为粗体字类型的碱基,而 LNA 单体是下划线的。通过间隔 1、2 或 3 个 DNA 碱基插入 LNA 单体来设计和合成每个嵌合寡核苷酸分子,间隔 DNA 碱基是为了避免受到内源性核酸酶的快速降解,如文献中已经报道的 (Koch T,Biochem J2001,354(Pt3) :481-4 ;Koji Nagahama,Bioorg Med Chem Lett,2009,19(10) :2707-9)。

[0184] 用于阻断基因翻译的反义寡核苷酸

[0185] 为了证明根据本发明中所述的参数选择和设计的本发明的寡核苷酸能够选择性地阻断靶标基因的翻译的目的，设计、合成并在体外实验分析了针对 MYCN 基因的具有 SEQ ID NO:26-36 的反义寡核苷酸。它们显示于表 3 中。

[0186] 表 3

[0187]

SEQ ID NO		正义序列		反义序列
SEQ ID NO: 26	siMYCN (795)	UGAAGAUGAUGAAGAGGAA	SEQ ID NO: 57	UUCCUCUUCAUCAUC UUCA
SEQ ID NO: 27	siMYCN (799)	GAUGAUGAAGAGGAAGAUG	SEQ ID NO: 58	CAUCUUCCUCUUCAU CAUC
SEQ ID NO: 28	siMYCN (801)	UGAUGAAGAGGAAGAUGAA	SEQ ID NO: 59	UUCAUCUUCCUCUUC AUCA
SEQ ID NO: 29	siMYCN (808)	GAGGAAGAUGAAGAGGAAG	SEQ ID NO: 60	CUUCCUCUUCAUCUU CCUC
SEQ ID NO: 30	siMYCN (810)	GGAAGAUGAAGAGGAAGAA	SEQ ID NO: 61	UUCUUCCUCUUCAUC UUCC
SEQ ID NO: 31	MYCN-PTO (1) as			CGTGGAGCAGCTCGG CAT
SEQ ID NO: 32	MYCN-PTO (763) as			CAGGGTGTCCTCTCC GGA
SEQ ID NO: 33	siRNA-2'-OMe-RNA 808	GAGGAAGAUGAAGAGGAAG TT	SEQ ID NO: 62	CUUCCUCUUCAUCUU CCUCTT
SEQ ID NO: 34	siRNA-2'F-RNA 808	CAGGAAGAUGAAGAGGAAG UU	SEQ ID NO: 63	GUUCCUCUUCAUCUU CCUCUU
SEQ ID NO: 35	siRNA-LNA 808	GAGGAAGAUGAAGAGGAAG TT	SEQ ID NO: 64	CUUCCUCUUCAUCUU CCUCTT
SEQ ID NO: 36	siRNA-ANA	CAGGAAGAUGAAGAGGAAG AA	SEQ ID NO: 65	GUUCCUCUUCAUCUU CCUCAA

[0188]

	808			

[0189] 生产了基于 RNA(小干扰 RNA(siRNA)(SEQ ID NO:26-30)) 的互补双链寡核苷酸，以选择性地调节靶标基因 MYCN 的翻译。

[0190] 此外，生产了基于 DNA SEQ ID NO:31 和 32 的寡核苷酸，其中将磷酸二酯键修饰成硫逐磷酸酯。

[0191] 还生产了基于以下的双链嵌合寡核苷酸 :基于 RNA 单体和单体 2'O- 甲基 (SEQ ID NO:33) ;基于 RNA 单体和 2' - 氟单体 (SEQ ID NO:34) ;基于 RNA 单体和 LNA 单体 (SEQ ID

NO:35) 和基于 RNA 单体和阿拉伯糖苷 RNA 单体 (SEQ ID NO:36)。在寡核苷酸序列 SEQ ID NO:33-36 中,2' O- 甲基、2' - 氟、LNA 和阿拉伯糖苷 (ANA) 单体以粗体字显示,而每组至少两个连续鸟嘌呤是下划线的。

[0192] 因为寡核苷酸的 siRNA 是双链的,以这样的方式设计和合成了嵌合体,使得在 3' 和 5' 留下两个 2' O-Me 单体,或两个 2' - 氟单体,或两个 LNA 单体,或两个 RNA 阿拉伯糖苷单体,与互补链未配对,并且由此避免受到细胞酶的快速降解。

[0193] 使用寡核苷酸的处理 -QT-PCR

[0194] 为了评价本发明的寡核苷酸调节靶标基因表达的能力的目的,使用实时 PCR 技术分析了它们降低信使 RNA 含量的能力。

[0195] 为此,使用了 24- 孔平板,将其接种 5.0×104 细胞,每孔含有 0.3ml OPTI-MEM(GIBCO BRL) 培养基,4% FBS 和 2mM L- 谷氨酰胺 (实验重复三份)。

[0196] 将细胞在 37℃下,在含有 5% CO_2 的气氛中,孵育 24 小时,以允许粘附壁的底部。

[0197] 预先用 2μl Lipofectamine2000(Invitrogen) 孵育每种分析的寡核苷酸,除了 PNA 寡核苷酸,使用 0.3mL 无血清 OPTI-MEM(GIBCO BRL) 培养基。

[0198] 对于每个孔,在以下终浓度下分析寡核苷酸 :

[0199] • 使用 200nM 的反义寡核苷酸 siRNA 和 siRNA gapmer(即,含有一个或多个在 3' 或 5' 端化学修饰的核酸的单体的寡核苷酸,同时在中央部分,它们具有未修饰的或在磷酸二酯键水平修饰为硫逐磷酸酯键的核酸的单体) ;

[0200] • 使用 10μM 的含有硫逐磷酸酯 DNA 单体的反义寡核苷酸,RNA 反基因寡核苷酸 (agRNA) 和含有 DNA 单体和 LNA 单体的寡核苷酸 ;

[0201] • 使用 1μM 浓度的吗啉代寡核苷酸,和

[0202] • 以 1μM 浓度给予 PNA 寡核苷酸。

[0203] 用寡核苷酸处理细胞,给予寡核苷酸后 6 小时,加入 FBS 至 4%。24 小时后,使用 RNeasy Mini 试剂盒 (QIAGEN),从每个孔提取总 RNA,并且纯化。

[0204] 对从 5 个不同的与 MYCN 表达相关的人肿瘤获得的 8 个细胞系进行了试验,即 :

[0205] • 作为成神经细胞瘤模型使用,由细胞系 Kelly、IMR-32(其中 MYCN 基因得到扩增和超表达) 以及 SKNBE2c 和 LAN1(其中 MYCN 基因得到扩增和超表达,并且 p53 基因突变) 组成 ;

[0206] • 作为横纹肌肉瘤使用,由细胞系 RH30 组成,其中 MYCN 基因得到扩增和超表达 ;

[0207] • 作为 Wilms' 肿瘤模型使用,由细胞系 WiT49 组成,其中 MYCN 基因得到扩增和超表达 ;

[0208] •作为成视网膜细胞瘤模型使用,由细胞系 Y79 组成,其中 MYCN 基因得到扩增和超表达 ;和

[0209] • 作为小细胞肺癌模型使用,由细胞系 H69 组成,其中 MYCN 基因得到扩增和超表达。

[0210] 作为对照,使用了用无菌水替代寡核苷酸处理的上述相同的细胞系。

[0211] 使用 NanoDrop ND-1000 分光光度计 (NanoDrop Technologies) 定量了每种 RNA 样品 (一式两份)。

[0212] 使用用于 RT-PCR 的 cDNA 合成试剂盒 (Roche) 生产了 cDNA 的第一条链。对于 cDNA

合成反应，总地使用了 1μg RNA。对于实时 PCR，使用了 20μl 终体积中的 10ng cDNA，使用 SYBR Green Master Mix2X(Applied Biosystems)(3 个相同的实验重复进行三份)。用于进行实时 PCR 的引物的序列和浓度显示于表 4 中。两个管家基因用作阳性对照：GAPDH 和 β-肌动蛋白 (ACTB)。

[0213] QT-PCR 反应的条件为：95℃ 10min，95℃ 20sec 和 60℃ 30sec，持续 50 个循环。

[0214] 表 4

[0215]

引物	序列	浓度	SEQ ID NO
MYCN 正义	CGACCACAAGGCCCTCAGT	300 nM	SEQ ID NO: 37
MYCN 反义	TCACCACGTCGATTTCTTCCT	300 nM	SEQ ID NO: 38
ACTB 正义	GAGCACAGAGCCTCGCCTTTG	300 nM	SEQ ID NO: 39
ACTB 反义	ACCATCACGCCCTGGTGCCTG	300 nM	SEQ ID NO: 40
GAPDH 正义	CCAATATGATTCCACCCATGGC	300 nM	SEQ ID NO: 41
GAPDH 反义	CTTGATTTTGGAGGGATCTCGC	300 nM	SEQ ID NO: 42

[0216] 使用寡核苷酸的处理 - 细胞增殖试验

[0217] 为了评价本发明的寡核苷酸的基因调节能力的目的，测定了它们的给予对细胞生活力的作用。

[0218] 为此，将每孔 $5x10^3$ 细胞接种于 96- 孔细胞培养平板中（实验重复进行三份），孔中含有 100μl 含有 4% FBS 和 2mM L- 谷氨酰胺的 OPTI-MEM(GIBCO BRL) 培养基。

[0219] 给予不同浓度 (1μM-2、5μM-5μM-10μM) 的 PNA 寡核苷酸，以观察剂量作用相关性。

[0220] 至于所有其他寡核苷酸，给予的浓度列于段落中：

[0221] 使用寡核苷酸的处理 -QT-PCR

[0222] 在处理后 48、72、96 和 168 小时时，测定了处理过的细胞的生活力。

[0223] 通过 ATP-Lite 试验（发光 ATP 检测试验系统，PerkinElmer）评价了细胞生活力，并且作为与未处理对照细胞的孔的平均值相比较的处理过的孔的平均信号之间的比例来记录。按照试剂盒提供的说明来处理细胞。

[0224] 在用于测定 MYCN 基因信使水平的相同细胞系上进行了试验并且列于以下段落中：使用寡核苷酸的处理 -QT-PCR。

[0225] 结果

[0226] 只要涉及 PNA 寡核苷酸，关于它们抑制 MYCN 基因转录和 Kelly 细胞增殖的能力显示于表 1 中。表 6 显示了在不同浓度的分析的 PNA 下，增殖 Kelly 细胞的值（以百分比的形式）。

[0227] 表 5

[0228]

SEQ ID NO	序 列	细胞增殖 (%) 1μM 72h	细胞增殖 (%) 2,5μM 72h	细胞增殖 (%) 5μM 72h	细胞增殖 (%) 10μM 72h
SEQ ID NO: 1	*ATGCCGGGCATGATCT*	*89*	*66*	*50*	*24*
SEQ ID NO: 2	GGGTGGATGCGGGGGG	74	32	12	2
SEQ ID NO: 3	GATGCGGGGGGCTCCT	78	41	19	3
SEQ ID NO: 4	GTCGGCGGGAGGTAAG	78	48	21	3
SEQ ID NO: 5	GCTGGGTGGATGCGGG	79	53	27	5
SEQ ID NO: 6	TGGACGCGCTGGGTGG	82	59	35	13
SEQ ID NO: 7	CGCGCTGGGTGGATGC	80	54	31	8
SEQ ID NO: 8	GTCTGGACGCGCTGGG	80	57	32	11
SEQ ID NO: 9	CCCTGCAGTCGGCGGG	86	64	42	20
SEQ ID	TGTCTGGACGCGCT	84	60	40	16
NO: 12	GG				

[0229] 结果证明了这些 PNA 对靶标基因翻译的蛋白的抑制作用是高度选择性和特异性的。此外，观察到了给予反基因 PNA 后，用作模型的肿瘤细胞的生长停止（通过 MYCN 基因的扩增来表征）直接接着就是凋亡。

[0230] 通常，具有 SEQ ID NO:2-SEQ ID NO:13（含有一组或多组 Gs）的 PNA 反基因寡核苷酸与不含 Gs 组 (SEQ ID NO:16-SEQ ID NO:23) 的 PNA 寡核苷酸相比，具有更高的反基因有效性（即，MYCN mRNA 和具有 MYCN 表达的肿瘤细胞增殖的抑制）。

[0231] 特别地，表 1 和表 5 中显示的结果证明了序列 SEQ ID NO:2-SEQ ID NO:13 具有比序列 SEQ ID NO:1（专利 EP1618195 的主题）更高的反基因有效性。

[0232] 序列 SEQ ID NO:7-SEQ ID NO:12（含有两组两个或三个连续 Gs）显示出高于序列 SEQ ID NO:1 和 SEQ ID NO:13-SEQ ID NO:15（只含有一组两个或三个连续 Gs）的反基因有效性。

[0233] 序列 SEQ ID NO:4-SEQ ID NO:6(含有三组两个或三个连续 Gs)显示出高于序列 SEQ ID NO:7-SEQ ID NO:12(含有两组两个或三个连续 Gs)的反基因有效性。

[0234] 含有一组六个连续 Gs 的 SEQ ID NO:3 显示出高于序列 SEQ ID NO:1 和含有一组或两组或三组 Gs(其中每组由至少两个或三个连续 Gs 组成)的序列 SEQ ID NO:4-SEQ ID NO:12 的反基因有效性。

[0235] 序列 SEQ ID NO:2,含有三组连续 Gs,其除了两组两个和三个连续 Gs 以外,还包含一组六个连续 Gs,显示出高于 SEQ ID NO:3(只含有一组六个 Gs)和序列 SEQ ID NO:1 和含有一组或两组或三组 Gs(其中每组由至少两个或三个连续 Gs 组成)的序列 SEQ ID NO:4-SEQ ID NO:12 的反基因有效性。

[0236] 此外,在成纤维样型细胞系(NTH-3T3 和 Phoenix)中给予了具有 SEQ ID NO:1-23 的寡核苷酸,并且结果显示于表 1 中(最后两栏)。

[0237] 结果清楚地证明了所分析的寡核苷酸针对这些细胞不是特异性地有效并且对这些细胞是无毒的(即,没有表达靶标基因的细胞,在这种情况中,是 MYCN)。

[0238] 实际上,观察到这两个非肿瘤成纤维细胞系的细胞增殖没有变化,并且这一结果表明 PNA 寡核苷酸以抑制 MYCN 表达的特异性作用来起作用,而它们在不表达 MYCN 的细胞中不具有非特异性的毒性作用。

[0239] 因此,可以推断基于本发明中所述的参数设计的 PNA 寡核苷酸特异性地且有效地作用于靶标基因,并且因此作用于表达 / 超表达这种基因的细胞。

[0240] 还对超表达 MYCN 的不同细胞系(Kelly、SKNBE2c、RH30、WiT90、WERI-Rb1 和 H69)测试了具有 SEQ ID NO:1-12 的 PNA。结果显示于表 6 中,并且证实了 PNA 对 MYCN 基因的蛋白质翻译产物的抑制作用的选择性和特异性。

[0241] 表 6

[0242]

SEQ ID NO	SEQ ID NO: 1	SEQ ID NO: 2	SEQ ID NO: 3	SEQ ID NO: 4	SEQ ID NO: 5	SEQ ID NO: 6	SEQ ID NO: 7	SEQ ID NO: 8	SEQ ID NO: 9	SEQ ID NO: 10	SEQ ID NO: 11	SEQ ID NO: 12
Kelly %mRNA	48	75	68	65	62	60	58	54	51	51	50	50
Kelly % 细胞增殖	66	32	41	48	53	54	57	59	60	64	64	65
SKNBE2c%mRNA	41	75	68	60	59	62	60	62	58	41	40	40
SKNBE2c% 细胞增殖	72	52	53	59	64	57	58	56	64	70	71	71
RH30 %mRNA	39	74	65	57	56	56	55	58	50	29	31	32
RH30 % 细胞增殖	76	56	59	62	64	64	63	60	71	80	78	77
WiT49 %mRNA	62	78	75	73	69	68	70	72	66	60	60	62
WiT49 % 细胞增殖	74	54	57	60	63	65	60	62	68	70	72	73
WERI-Rb1 %mRNA	31	46	43	41	38	37	38	40	36	30	30	30
WERI-Rb1 % 细胞增殖	82	79	78	79	80	80	79	78	79	85	84	84
H69 %mRNA	40	58	55	55	54	55	55	56	50	41	41	40
H69 % 细胞增殖	77	61	63	66	68	69	67	66	70	79	78	78

[0243] 以更详细的方式分析了具有 SEQ ID NO:5 的反基因 PNA 寡核苷酸。

[0244] 特别地，合成了修饰的寡核苷酸，其中对序列中存在的连续鸟嘌呤组进行了修饰（连续鸟嘌呤组是下划线的，而修饰的核苷酸是粗体类型的），使得打断了鸟嘌呤的连续性，并且在 Kelly 细胞（其超表达 MYCN）上进行了体外分析。

[0245] 结果（概括于表 7 中）清楚地证明了连续的鸟嘌呤对于调节基因表达活性的目的是基础的事实。实际上，具有 SEQ ID NO:43 的 PNA，其中具有 SEQ ID NO:5 的 PNA 的所有连续鸟嘌呤组被突变，引起 PNA 的抑制活性丢失，而三个连续鸟嘌呤组中的一个或两个的突变受到明显损害，但没有完全抑制分子的抑制活性。

[0246] 表 7

[0247]

SEQ ID NO	序列	mRNA 抑制 (%) (Kelly)	(Kelly) 细胞增殖 %
SEQ ID NO: 5	GCTGGGTGGATGCGGG	62	53
SEQ ID NO: 43	GCTGAGTCGATGCGTG	0	100
SEQ ID NO: 44	GCTGAGTGGATGCGTG	25	89
SEQ ID NO: 45	GCTGGGTCGATGCGGG	47	69
SEQ ID NO: 46	GTTGGGTGGATGTGGG	58	60

[0248] 在腺泡状横纹肌肉瘤细胞 (RH30) 上,在体外测试了包含 DNA 单体和 LNA 单体并且具有 MYCN 基因作为其靶标的嵌合寡核苷酸。结果概括于表 8 中,并且证明了这些寡核苷酸具有强烈的、特异性的反基因活性。

[0249] 表 8

[0250]

SEQ ID NO	名称	序列	mRNA 抑制 (%)	细胞增殖 (%)
SEQ ID NO: 24	DNA-LNA 25-1	ATGCCGGGCATGA TCT	23	75
SEQ ID NO: 25	DNA-LNA 25-2	ATGCCGGGCATGA TCT	24	78

[0251] 在腺泡状横纹肌肉瘤细胞 (RH30) 上,在体外分析了通过申请人根据本发明所述的参数设计并合成的反义寡核苷酸。结果概括于表 9 中,并且显示出寡核苷酸能够以特异性且有效的方式抑制 MYCN 转录产物 ;此外,它们还能够以比目前可用于 MYCN 基因的标准反义寡核苷酸 (Chung DH,Bioch Bioph Res Commun,2006,351(1):192-7) 更有效的方式选择性地抑制肿瘤细胞增殖。

[0252] 特别地,描述于表 11 中的通过申请人鉴定的并且针对 MYCN mRNA 的 siRNA 发挥出反义活性,具有最小 70% 至最大 85% 范围的 MYCN mRNA 的抑制。

[0253] 表 9

[0254]

SEQ ID NO		正义序列	反义序列	mRNA 抑制 (%)	细胞增殖 (%)
SEQ ID NO: 26	siMYCN (795)	UGAAGAUGAU GAAGAGGAA	UUCCUCUUCA UCAUCUUCA	82	28
SEQ ID	siMYCN	GAUGAUGAAG	CAUCUUCCUC	70	43

[0255]

NO: 27	(799)	AGGAAGAUG	UUCAUCAUC		
SEQ ID NO: 28	siMYCN (801)	UGAUGAAGAG GAAGAUGAA	UUCAUCUUCC UCUUCAUCA	78	35
SEQ ID NO: 29	siMYCN (808)	GAGGAAGAUG AAGAGGAAG	CUUCCUCUUC AUCUUCCUC	85	28
SEQ ID NO: 30	siMYCN (810)	GGAAGAUGAA GAGGAAGAA	UUCUUCCUCU UCAUCUUCC	81	30
SEQ ID NO: 31	MYCN-PTO (1) as		CGTGGAGCAG CTCGGCAT	34	73
SEQ ID NO: 32	MYCN-PTO (763) as		CAGGGTGTCC TCTCCGGA	45	65
SEQ ID NO: 33	siRNA-2'-OMe-RNA 808	GAGGAAGAUG AAGAGGAAGT T	CUUCCUCUUC AUCUUCCUCT T	13	85
SEQ ID NO: 34	siRNA-2'F-RNA 808	CAGGAAGAUG AAGAGGAAGU U	GUUCCUCUUC AUCUUCCUCU U	59	52
SEQ ID NO: 35	siRNA-LNA 808	GAGGAAGAUG AAGAGGAAGT T	CUUCCUCUUC AUCUUCCUCT T	34	68
SEQ ID NO: 36	siRNA-ANA 808	CAGGAAGAUG AAGAGGAAGA A	GUUCCUCUUC AUCUUCCUCA A	69	35

[0256] 为了证实本发明的寡核苷酸是否能够降低对抗癌症的治疗方案中目前所用的化疗剂的浓度的目的，将寡核苷酸结合化疗药物一起给药。

[0257] 在不同的人和小鼠成神经细胞瘤肿瘤细胞系 (SMS-KAN、LAN1、IMR-32、SMS-KCN、Kelly、NHO2A、SKNBE2c) 上针对 PNA 和化疗药物 (卡铂、足叶乙苷 (VP16)、顺铂和长春新碱) 之间的相关作用进行了研究。

[0258] 对于待使用 PNA 治疗的细胞提供了所进行的治疗中使用的治疗时间表，并且随后，在预定的时间 (可以是 6 或 12 小时)，给予化疗剂。

[0259] 结果证明了这些化合物与本发明的寡核苷酸的结合，在特定的浓度范围下，确定了比使用相同化合物的单独治疗更高的治疗作用。

[0260] 特别地，可以观察到治疗 - 伴随和同时的或以不同的时间间隔 (3 小时、6 小时、12 小时、24 小时、48 小时、72 小时等)- 使用由本发明的寡核苷酸和其他具有药物作用的化合物的组合，用来增强所需的治疗作用。

[0261] 因此，与目前标准的治疗浓度相比，结合一种或多种由申请人鉴定的寡核苷酸和

化疗药物使得可以将化疗药物浓度降低多如 10 倍，同时仍然获得相同的作用。

[0262] 特别地，这一研究的结果证明了就抑制细胞增殖而言，与使用单独的寡核苷酸或使用单独的上述化疗剂的治疗中获得的结果相比，结合长春新碱，或足叶乙苷，或卡铂，或顺铂给药时，SEQ ID NO:1 具有协同作用。

[0263] 实施例 2

[0264] 为了支持本发明的目的，还合成了表 10 中概括的寡核苷酸。特别地，表 10 显示了寡核苷酸序列、SEQ ID NO、(G+C) 含量的百分比值和合成的寡核苷酸所针对的基因。按照实施例 1，实现了寡核苷酸的合成。

[0265] 表 10

[0266]

	SEQ ID NO		% GC
MYCN	SEQ ID NO: 66	TCGGGAGCAGTGGGCA	68.8
	SEQ ID NO: 67	GCGGGTCGCGGGCACG	87.5
	SEQ ID NO: 68	TGGAGGTCGGCGCCGG	81.3
	SEQ ID NO: 69	TCGGCGGGAGGTAAGG	68.8
MYC	SEQ ID NO: 70	CTCAGAGGCTTGGCGG	68.8
	SEQ ID NO: 71	GCGGCCGGCTAGGGTG	81.3
	SEQ ID NO: 72	CGGCCGGCTAGGGTGG	81.3
	SEQ ID NO: 73	CGACGGCGGTGGCGGG	87.5
	SEQ ID NO: 74	GGACGGGGGCGGTGGA	81.3
BIRC5	SEQ ID NO: 75	GCGGCGGCATGGGTGC	81.3
	SEQ ID NO: 76	GGCGGCGGCATGGGTG	81.3
ALK	SEQ ID NO: 77	GCAGGAGAGGACGGTA	62.5
	SEQ ID NO: 78	CAGGAGAGGACGGTAC	62.5
	SEQ ID NO: 79	GGCAGGAGAGGACGGT	68.8
BCL2	SEQ ID NO: 80	GGATGGCGCACGCTGG	75.0
	SEQ ID NO: 81	GGGAAGGATGGCGCAC	68.8
	SEQ ID NO: 82	CCACGGTGGTGGAGGA	68.8
PLK4	SEQ ID NO: 83	ACGGCAAGCGGCGGGA	75.0
	SEQ ID NO: 84	GGACGGCAAGCGGCGG	81.3

[0267] 特别地，合成了以下的：针对 MYCN 的 SEQ ID NO:66-69，针对 MYC 的 SEQ ID NO:70-74，针对 BIRC5 的 SEQ ID NO:75、76，针对 ALK 的 SEQ ID NO:77-79，针对 BCL2 的 SEQ ID NO:80-82 和针对 PLK4 的 SEQ ID NO:83、84。

[0268] 在体外测试了寡核苷酸（以 2.5μM 的浓度），以通过测量其中目标基因超表达的细胞模型中基因信使的水平来确定它们抑制它们针对的基因的转录的能力。使用的这些方法是实施例 1 中描述的那些方法。

[0269] 特别地，在 Kelly 和 H69 细胞系中，体外测试了 SEQ ID NO:66-69（针对 MYCN）；在 H82 和 RD 细胞系中体外测试了 SEQ ID NO:70-74（针对 MYC），在 Kelly 细胞系中体外测试了 SEQ ID NO:75、76（针对 BIRC5），在 Kelly 细胞系中体外测试了 SEQ ID NO:77-79（针对 ALK），在 Kelly 细胞系中体外测试了 SEQ ID NO:80-82（针对 BCL2）和在 Kelly 细胞系中测试了 SEQ ID NO:83、84（针对 PLK4）。

[0270] 测量了所测试的寡核苷酸抑制目标基因信使的能力，并且还评价了给予寡核苷酸后细胞的增殖能力。结果概括于表 11 中。

[0271] 数据显示出对抗靶标基因的 mRNA 的有效抑制活性和细胞增殖的抑制，其随着寡核苷酸序列中存在的 Gs 组数量的增加而升高。

[0272] 表 11

[0273]

	SEQ ID NO		mRna 抑制 (%)	细胞增殖 (%)
MYCN Kelly	SEQ ID NO: 66	TCGGGAGCAGTGGGC A	57	60
	SEQ ID NO: 67	GCGGGTCGCGGGCAC G	60	56
	SEQ ID NO: 68	TGGAGGTCGGCGCCG G	74	35
	SEQ ID NO: 69	TCGGCGGGAGGTAAG G	76	30
MYCN H69	SEQ ID NO: 66	TCGGGAGCAGTGGGC A	41	73
	SEQ ID NO: 67	GCGGGTCGCGGGCAC G	42	71
	SEQ ID NO: 68	TGGAGGTCGGCGCCG G	62	49
	SEQ ID NO: 69	TCGGCGGGAGGTAAG G	67	45
MYC H82	SEQ ID NO: 70	CTCAGAGGCTTGGCG G	43	79
	SEQ ID NO: 71	GCGGCCGGCTAGGGT G	46	75
	SEQ ID NO: 72	CGGCCGGCTAGGGTG G	52	64
	SEQ ID NO: 73	CGACGGCGGTGGCGG G	56	65
	SEQ ID NO: 74	GGACGGGGGCGGTGG A	61	61
MYC RD	SEQ ID NO: 70	CTCAGAGGCTTGGCG G	34	73
	SEQ ID NO: 71	GCGGCCGGCTAGGGT G	42	65
	SEQ ID NO: 72	CGGCCGGCTAGGGTG G	58	53
	SEQ ID NO: 73	CGACGGCGGTGGCGG G	59	51
	SEQ ID NO: 74	GGACGGGGGCGGTGG A	64	47
BIRC5 Kelly	SEQ ID NO: 75	GCGGCGGCATGGGTG C	69	43
	SEQ ID NO: 76	GGCGGCGGCATGGGT G	78	35

[0274]

ALK Kelly	SEQ ID NO: 77	GCAGGAGAGGACGGTA	57	55
	SEQ ID NO: 78	CAGGAGAGGACGGTAC	68	46
	SEQ ID NO: 79	GGCAGGAGAGGACGGT	79	39
BCL2 Kelly	SEQ ID NO: 80	GGATGGCGCACGCTGG	59	62
	SEQ ID NO: 81	GGGAAGGATGGCGCAC	62	58
	SEQ ID NO: 82	CCACGGTGGTGGAGGA	68	55
PLK4 Kelly	SEQ ID NO: 83	ACGGCAAGCGGCGGGA	62	59
	SEQ ID NO: 84	GGACGGCAAGCGGCGG	74	49

[0275] 最后，在成纤维样型的细胞系中 (Phoenix 和 NIH-3T3) 中给予具有 SEQ ID NO:66-84 的寡核苷酸。在这些细胞中，测试的寡核苷酸没有显示出有毒。

[0276] 这些结果表明 PNA 寡核苷酸通过特定的抑制作用对目标基因的表达起作用，而它们在不表达这些基因的细胞中不具有任何非特异性的、毒性作用。

[0277] 因此，可以推断基于本发明中描述的参数设计的 PNA 寡核苷酸特异性地且有效地作用于靶标基因，并且因此作用于表达 / 超表达这种基因的细胞。

Abstract

The present invention regards oligonucleotides for modulating the expression of a gene, in particular for modulating a gene responsible for a pathology of genetic, tumoural or viral origin. Moreover, the present invention relates to the use of said oligonucleotides, possibly chemically modified, for the treatment and/or the diagnosis of said diseases.