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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 October 2012 (18.10.2012)

(10) International Publication Number
WO 2012/139628 A1

(51) International Patent Classification:
C12N 15/113 (2010.01) *C12Q 1/68* (2006.01)
A61K 31/713 (2006.01) *A61P 35/00* (2006.01)

(21) International Application Number:
PCT/EP2011/055647

(22) International Filing Date:
11 April 2011 (11.04.2011)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicants *(for all designated States except US)*: **LYKERA BIOMED SA** [ES/ES]; C/Innovació 2, E-08225 Terrasa (ES). **UNIVERSIDAD DE BARCELONA** [ES/ES]; Centro de Patentes, Baldiri i Reixac 4, E-08028 Barcelona (ES).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CIUDAD GÓMEZ, Carlos** [ES/ES]; Carrer Diputació 202. 2ª planta, E-08011 Barcelona (ES). **NOÉ MATA, Verónica** [ES/ES]; Carrer Diputació 202. 2ª planta, E-08011 Barcelona (ES). **HERNÁNDEZ MÍGUEZ, José Luis** [ES/ES]; Passeig Joan Salvat i Papasseit 56, Esc. A,1º1ª, E-08830 Sant Boi de Llobregat (ES). **MASA ÁLVAREZ, Marc** [ES/ES]; Passeig del Castell 97, E-08292 Esparreguera (ES).

(74) Agent: **ALCONADA RODRÍGUEZ, Agustín**; ABG Patentes, Avenida de Burgos, 16D, Edificio Euromor, E-28036 Madrid (ES).

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2012/139628 A1

(54) Title: CODING POLYPURINE HAIRPINS FOR THE TREATMENT OF CANCER

(57) Abstract: The present invention relates to polypurine hairpins, particularly to oligonucleotides comprising two polypurine regions connected by a thymidine-rich region wherein said regions are capable of forming an antiparallel hairpin maintained by reverse-Hoogsteen bonds that have the ability to bind to a polypyrimidine target sequence located in the coding strand of an intronic region of a target gene. The invention also relates to pharmaceutical compositions containing said oligonucleotides and to methods of treatment and/or the prevention of cancer, diseases associated with inflammation, Alzheimer's disease or atherosclerosis comprising the administration of said oligonucleotides. Additionally, the invention relates to diagnostic methods using said oligonucleotides.

CODING POLYPURINE HAIRPINS FOR THE TREATMENT OF CANCER

FIELD OF THE INVENTION

5 The present invention relates to polypurine hairpins, particularly to oligonucleotides that are able to bind to a polypyrimidine target sequence located in the coding strand of an intronic region of a target gene, thus causing a decrease in gene expression at the mRNA level. The invention also relates to pharmaceutical compositions containing said oligonucleotides and to methods of treatment and/or the prevention of cancer, diseases 10 associated with inflammation, Alzheimer's disease or atherosclerosis comprising the administration of said oligonucleotides. Additionally, the invention relates to diagnostic methods using said oligonucleotides.

BACKGROUND OF THE INVENTION

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The efficient and specific inhibition of gene expression is the basis of some gene therapy protocols and functional gene studies. The expression of certain genes is sometimes toxic for the cell, generating diseases such as the expression of viral genes in infected cells, which are dominant negative in hereditary diseases, or genes the 20 expression of which should be restricted, such as oncogenes. The inhibition of the expression of these toxic genes would allow curing the diseases they trigger. In addition, genomic and proteomic studies allow isolating a large number of genes, some of which have an unknown function. These techniques allow classifying these genes either according to their expression levels or according to the conditions regulating such 25 expression, but do not say much about their function. The systematic and specific inhibition of the expression of these genes would allow developing simple protocols to determine their function.

Nowadays, there exist a series of molecules developed to decrease gene expression, 30 mainly antisense oligonucleotides (aODNs), small interfering RNAs (siRNAs) and triplex-forming oligonucleotides (TFOs).

aODNs are single strands of DNA or RNA that are complementary to a chosen sequence. In the case of antisense RNA they prevent protein translation of certain messenger RNA strands by binding to them. Antisense DNA can be used to target a specific, complementary RNA. aODNs that bind to pre-mRNA sterically alter RNA processing. If binding takes places this DNA/RNA hybrid can be degraded by the enzyme RNAse H.

siRNAs consist of an RNA duplex made up of two complementary RNA strands with a length of 21-22 nucleotides with projecting 3' ends. (Elbashir, S.M. *et al.*, Genes Dev. 2001, 15:188-200). siRNA is involved in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene. Specifically, siRNA target mRNA and produce its degradation.

TFOs are molecules that bind in the major groove of duplex DNA and by so doing can produce triplex structures. They bind to the purine-rich strand of the duplex through Hoogsteen or reverse Hoogsteen hydrogen bonding. They exist in two sequence motifs, either pyrimidine or purine. Under experimental conditions, triplex DNA can inhibit DNA transcription and replication, generate site-specific mutations, cleave DNA, and induce homologous recombination.

There have been previous studies reporting the use of oligonucleotides to regulate splicing of specific genes with successful results. In those approaches, aODNs were implemented to interfere with translation and pre-mRNA splicing, changing levels of alternatively spliced genes, or skipping an exon in order to restore a disrupted reading frame (Aartsma-Rus, A. and van Ommen, G.J. 2007. RNA, 13:1609-1624; Marshall, N.B. *et al.* 2007. J Immunol Methods, 325:114-126). Antisense RNAs have also been studied. HeLa or K562 cells that stably expressed thalassemic genes, when treated with U7 and U1 small nuclear RNAs modified to contain sequences antisense to the aberrant splice sites, restored correct splicing and correct expression of ß-globin (Vacek, M. *et al.* 2003. Cell Mol Life Sci, 60:825-833).

However, each of these molecules presents disadvantages such as lack of stability or specificity. TFOs and circular oligonucleotides, which have been used with great

success, have found to have some stability and specificity problems (Casey, B.P. and Glazer, P.M. 2001. Prog Nucleic Acid Res Mol Biol, 67:163-92; Chan, P.P and Glazer, P.M. 1997. J Mol Med, 75:267-282; Giovannangeli, C. and Hélène, C. 1997. Antisense Nucleic Acid Drug Dev, 7:413-21; Ryan, K. and Kool, E.T. 1998. Chem Biol, 5:59-67).

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As an attempt to solve these problems, new molecules have been developed. For instance, patent application WO 2009/030440 A2 discloses short interfering DNA oligonucleotides (siDNA) which are oligodeoxynucleotides consisting of an antisense-
10 strand homologous to the target RNA and a second strand, partially complementary to the antisense-strand and wherein the two strands are preferably linked by a thymidine linker (eg. 4 thymidines). Said siDNAs have a hairpin structure and are capable of forming a triple-helix by non-Watson-Crick base pairing with the RNA target strand, thus silencing gene expression by the enzyme RNAse H. siDNAs are easier to
15 synthesize and more stable than siRNAs. However, said siDNAs are not stabilized by reverse-Hoogsteen bonds.

Other molecules named polypurine reverse-Hoogsteen hairpins (PPRHs) have been designed. PPRHs are non-modified DNA molecules formed by two polypurine stretches
20 linked by a five-thymidine loop (Aviñó, A. *et al.* 2002. Nucleic Acids Res, 30:2609-2619; Coma, S. *et al.* 2005. Oligonucleotides, 15:269-283). The hairpin structure is maintained by intramolecular reverse-Hoogsteen bonds between the two polypurine stretches, which are in antiparallel orientation. These molecules bind to their polypyrimidine target sequence by Watson-Crick bonds instead of by Hoogsteen bonds,
25 thus forming a triplex structure.

Recently, a type of PPRHs that have the ability to bind to a target sequence located in the template DNA strand have been described and named Template-PPRHs (de Almagro, M.C. *et al.* 2009. J Biol Chem, 284:11579-11589). The formation of the
30 triplex between these PPRHs and their double-stranded (ds) DNA target sequence interferes with the transcription process, thus producing an mRNA decrease.

However, there is still a need for further molecules useful for gene-targeting strategies, in particular, those capable of decreasing gene expression of genes involved in diseases such as cancer or inflammatory diseases.

5 **SUMMARY OF THE INVENTION**

In one aspect, the invention relates to an oligonucleotide comprising a first polypurine region and a second polypurine region connected by a thymidine-rich region
wherein said first polypurine region has the reverse sequence of said second polypurine
10 region,
wherein said first and second regions are capable of forming an antiparallel hairpin maintained by reverse-Hoogsteen bonds and
wherein the oligonucleotide is capable of forming a triplex with a polypyrimidine region which is substantially complementary to said first polypurine region and which is
15 located in the coding strand of an intronic region of a target gene.

In another aspect, the invention relates to a pharmaceutical composition comprising a pharmaceutically effective amount of at least one oligonucleotide according to the invention and at least one pharmaceutically acceptable excipient.

20

In yet another aspect, the invention relates to a method of treatment and/or the prevention in a subject of a disease selected from cancer, a disease associated with inflammation, Alzheimer's disease and atherosclerosis comprising the administration to said subject of a pharmaceutically effective amount of an oligonucleotide according to the invention
25 wherein the target gene is a gene selected, respectively, from the group consisting of a gene associated with cancer, inflammation, Alzheimer's disease and atherosclerosis.

In yet another aspect, the invention is related to an *in vitro* method for detecting gene amplification of a target gene in a sample containing genomic DNA comprising
30 measuring the number of copies of a target gene in said sample by using an oligonucleotide according to the invention.

In yet another aspect, the invention is related to an *in vitro* method for the diagnosis of cancer or of drug resistance to chemotherapy and/or to radiotherapy associated to the amplification of a given gene in a subject comprising:

(a) measuring in a sample from said subject the number of copies of said gene by using an oligonucleotide according to the invention wherein said oligonucleotide is specific for an intronic region of said gene and

(b) comparing said number of copies with a reference value

wherein an increase in the number of copies in said sample with respect to the reference value is indicative of the subject suffering from cancer or drug resistance.

In yet another aspect, the invention relates to a kit for detecting gene amplification in a sample from a subject which comprises at least one oligonucleotide according to the first aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Coding-PPRH binding to its target sequence in the *dhfr* gene. **(a)** Scheme of the *dhfr* gene and the PPRH target location. **(b)** Schematic representation of PPRHs binding to their polypyrimidine ssDNA target within *dhfr* intron-3. The sequences in parenthesis are not PPRH target sequences. Curved lines refer to Hoogsteen bonds, whereas straight lines correspond to Watson-Crick bonds. **(c)** Binding of Coding-PPRHs to [^{32}P]-dsDNA polypyrimidine/polypurine (Duplex) or to [^{32}P]-ssDNA (For). The mobility of the probes alone (For and Duplex) is shown in the two first lanes on the left. The following lanes represent the bindings using HpdI3-A-TA (A-TA), HpdI3-A-WC (A-WC), and HpdI3-A-NH (A-NH) with either the ssDNA (For) or dsDNA (Duplex) probes. **(d)** Binding of Coding-PPRHs to [^{32}P]-ssRNA. The mobility of the probe alone is shown in the first lane on the left. The following lanes correspond to the binding using HpdI3-A-TA (A-TA), HpdI3-A-NH (A-NH), or HpdI3-A-WC (A-WC). Arrows indicate the bands corresponding to the different species (ssDNA, dsDNA, RNA, or triplex).

Figure 2. Cytotoxicity caused by Coding-PPRHs. **(a)** The suitable nucleotide to place when a purine interruption is found in the target sequence. Two PPRHs, one with an adenine (HpdI3-A-TA) and another with a guanine (HpdI3-A-TG) were tested for cell survival in SKBR3 breast cancer cells. The effects of DOTAP (10μM) alone and that of
5 the aODN HATNL-24 are also shown. **(b)** Comparison of the cytotoxic effect caused by HpdI3-A-TA with that produced by HpdI3-A-NH or by the negative controls HpdI3-A-WC and HpdI3-Sc. Additional controls were DOTAP (10μM) alone, HpdI3-A-TA without DOTAP, and aODN HATNL-24. **(c)** Study of the optimal PPRH:DOTAP molar ratio to attain maximum cytotoxicity. HpdI3-A-TA was incubated at varying
10 concentrations in combination with a fixed concentration of 10 μM DOTAP. For comparative purposes HpdI3-B was used. **(d)** Minimum time needed for PPRH action. After transfection of 100 nM HpdI3-A-TA with 10 μM DOTAP, culture medium was changed at the indicated times. For all conditions, 10,000 SKBR3 cells were plated in -GHT DHFR-selective medium for a week, after which the 3-(4,5-dimethylthiazol-2-yl)-
15 2,5-diphenyltetrazolium bromide assay was performed. Data are mean ± SEM values of at least four experiments (a-c) or at least three experiments (d). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Figure 3. DHFR activity and mRNA levels in SKBR3 cells treated with Coding-
20 PPRHs. **(a)** Dose-response of the effect of Coding-PPRHs on DHFR activity. SKBR3 cells (10,000) were incubated in Ham's F-12 medium lacking thymidine with DOTAP alone (10μM) and increasing concentrations of HpdI3-A-TA. HpdI3-A-NH and HpdI3-A-WC were used at 100 nM. Two days after transfection, 6-[^{3}H]-deoxyuridine was added for 24 hr, and its incorporation into DNA was quantified to determine DHFR
25 activity. Data are mean ± SEM values of three experiments. **(b)** Dose-response of the effect of Coding-PPRHs on DHFR mRNA levels. SKBR3 cells (30,000) were incubated with HpdI3-A-TA at the indicated concentrations plus 10μM DOTAP. For comparative purposes the effect of the aODN HATNL-24 and Template HpdI3-B were also tested. HpdI3-A-NH and negative controls HpdI3-A-WC and HpdI3-Sc, as well as DOTAP
30 (10μM) and HpdI3-A-TA without DOTAP, were used. Three days after transfection, DHFR mRNA levels were determined by reverse transcription-real-time PCR using APRT to normalize the results. Data are mean ± SEM values of at least ten experiments. **(c)** Schematic representation of the primers used to measure *dhfr* pre-mRNA and

mRNA levels as well as the sizes of the possible species obtained with each pair. **(d)** Pre-mRNA and mRNA levels. 30,000 SKBR3 cells were incubated with HpdI3-A-TA or HpdI3-A-WC (100 nM) plus 10 μM DOTAP. For each incubation condition, three different fragments were analyzed: Exon3-Intron3 (Ex3-Int3), Exon3-Exon4 (Ex3-Ex4),
5 and Exon3-Exon5 (Ex3-Ex5). In all conditions, 3 days after transfection, DHFR RNA levels were determined by reverse transcription-real-time PCR using APRT mRNA levels to normalize the results. Data are mean ± SEM values of at least five experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

10 **Figure 4.** Effect of Coding-PPRHs on *in vitro* splicing. **(a)** and **(c)** Representative images of the splicing products obtained from *in vitro* splicing reactions using the DCHIP and DCHIP-Hp RNA probes, respectively, incubated with the different hairpin molecules. Arrows indicate the sizes of the different products. The first lane in each panel corresponds to the unspliced probes in the absence of HeLa nuclear extract (NE).
15 The second lane shows the spliced species after incubation with HeLa NEs. In the following lanes the effects on splicing of HpdI3-A-TA and HpdI3-A-NH and the negative controls HpdI3-A-WC and HpdI3-Sc are shown. **(b)** and **(d)** Quantitation of the spliced products formed after incubation of DCHIP and DCHIP-Hp probes with the indicated Coding-PPRHs. The results were normalized using the signal of the whole
20 lane as reference. Background correction was performed. Data are mean ± SEM values of at least three experiments. *** $p < 0.005$.

Figure 5. PPRH competition for U2AF65 binding. A radiolabeled probe corresponding to the HpdI3-A-TA target sequence in *dhfr* intron-3 was incubated with 1μg of HeLa
25 Nuclear Extract (NE) and tRNA as unspecific competitor. The first lane in each panel corresponds to the probe in the absence of NE. **(a)** Gel shift assay. Binding of HpdI3-A-TA target sequence to NE (lane 2) and competition with HpdI3-A-TA at different fold excess (lanes 3-6). HpdI3-A-NH, HpdI3-A-WC, and HpdI3-Sc were used at 200-fold excess. **(b)** Supershift assay. U2AF65 antibody was incubated with different amounts of
30 NE, 1 μg or 0.5 μg. Lanes 2 and 4 show the gel-shift caused after incubation with NE, and lanes 3 and 5 represents the binding pattern after incubation with the antibody. Bands are indicated by arrows.

Figure 6. Coding-PPRH specificity. *Dhfr*$^{-}$ DG44 cells (30,000) stably transfected with **(a)** pDCHIP (DCHM1 cells) or **(b)** pDCHIP-Hp (DCHM1-Hp cells) were incubated with Coding-PPRHs in –GHT DHFR-selective medium for a week, after which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed. The
5 hairpins used were HpdI3-A-TA, HpdI3-A-NH, HpdI3-A-WC, or HpdI3-Sc incubated at the indicated concentrations plus 10 μM DOTAP. Data are mean ± SEM values of four experiments. * $p < 0.05$, *** $p < 0.005$.

Figure 7. Effects of Coding-PPRHs in MCF7 cells resistant to 10^{-6} M methotrexate. **(a)**
10 Cytotoxicity. MCF7-R cells (10,000) were plated in –GHT DHFR-selective medium. Cells were incubated with hairpins HpdI3-A-TA, HpdI3-A-TG, or HpdI3-A-NH or the negative controls HpdI3-A-WC and HpdI3-Sc at the indicated concentrations plus 10 μM DOTAP. The effect of DOTAP (10 μM) alone and HpdI3-A-TA without DOTAP was also determined. To assess cell responsiveness and for comparison purposes, the
15 aODN HATNL-24 and the Template hairpin HpdI3-B were also tested. A week after treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed. Data are mean ± SEM values of at least four experiments. **(b)** DHFR mRNA levels. MCF7-R cells (30,000) were incubated with the indicated PPRHs, aODN HATNL-24, or 30 μM DOTAP. Three days after transfection, DHFR mRNA levels
20 were determined by reverse transcription-real-time PCR using the signal corresponding to APRT mRNA to normalize the results. Data are mean ± SEM values of at least four experiments. **(c)** DHFR protein levels. MCF7-R cells (30,000) were incubated with HpdI3-B, HpdI3-A-TA, HpdI3-A-NH, or HpdI3-A-WC (300 nM) or 30μm DOTAP alone. Three days after transfection, DHFR protein levels were determined by western
25 blot using tubulin to normalize the results. Data are mean ± SEM values of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

DETAILED DESCRIPTION OF THE INVENTION

30 The authors of the present invention have discovered that, surprisingly, polypurine reverse-Hoogsteen hairpins (PPRHs) directed towards an intronic region of the coding DNA strand of a target gene are capable of decreasing gene expression. Said PPRHs

target the pre-mRNA interfering with the mRNA splicing process and are named Coding-PPRHs.

The results provided in the examples of the present invention show that these molecules act by promoting an increase in the pre-mRNA levels followed by a decrease in spliced mRNA. The oligonucleotides of the invention compete with the spliceosome factor U2 auxiliary factor (U2AF) 65 for binding to the polypyrimidine sequences present in introns, and then they avoid pre-mRNA splicing. Moreover, as shown in the examples of the present invention, coding-PPRHs against the human *dhfr* gene showed a noticeable activity in decreasing DHFR expression. At the suitable PPRH:DOTAP molar ratio (1:100), 100nM HpdI3-A-TA caused more than 90% cell death, similar to that caused by Template HpdI3-B (targeting the template DNA strand). HpdI3-A-TA was more active than the aODN HATNL-24, when used at their optimal conditions. The time of action of Coding-PPRHs was very fast because 48 hours was enough to exert their action.

These Coding-PPRHs directly target the pre-mRNA and the coding strand, thus allowing the application of the PPRH technology to genes whose polypyrimidine sequences are located in the coding DNA strand. In addition, Coding-PPRHs achieve the same cytotoxicity at concentrations of 100 nM as Template-PPRHs and have the same advantages in front of other knocking down molecules: (a) a remarkable increase in stability (half-life of 5 days compared with the 2 days for modified aODNs) without the need to include modified oligonucleotides; (b) easier handling (because they are DNA molecules instead of RNA); (c) ease of synthesis and inexpensive cost; and (d) do not activate the immune response.

These results indicate that the oligonucleotides of the invention are useful for decreasing gene expression in mammalian cells, thus becoming an alternative to other molecules such as siRNAs, aODNs and TFOs for the treatment and/or prevention of those pathologies coursing with deregulated gene expression, such as cancer or diseases associated with inflammation.

CODING POLYPURINE HAIRPINS

Thus, in a first aspect, the invention relates to an oligonucleotide comprising a first polypurine region and a second polypurine region connected by a thymidine-rich region wherein said first polypurine region has the reverse sequence of said second polypurine region,
5 wherein said first and second regions are capable of forming an antiparallel hairpin maintained by reverse-Hoogsteen bonds and
wherein the oligonucleotide is capable of forming a triplex with a polypyrimidine region which is substantially complementary to said first polypurine region and which is located in the coding strand of an intronic region of a target gene.
10
The terms “oligonucleotide of the invention”, “coding polypurine hairpin” and “Coding-PPRH” are used here interchangeably.

The term “oligonucleotide”, as used herein, refers to a short nucleic acid polymer
15 composed of non-modified oligodeoxyribonucleotides, typically with two hundred or fewer bases, preferably with eighty or fewer bases. The oligonucleotide of the invention has two polypurine regions connected by a thymidine-rich region.

A “polypurine region”, as used herein, refers to a motif within a sequence of an
20 oligonucleotide of the invention defined by at least 19 nucleotides of purine in succession, preferably at least 20 nucleotides, more preferably at least 24 nucleotides, most preferably at least 35 nucleotides. The polypurine region usually has between 19 nucleotides and the maximum possible length without exceeding 3 purine interruptions in the polypyrimidine region in the target gene. The nucleotides of purine can be adenine
25 (A) or guanine (G) nucleotides. The two polypurine regions are connected by a thymidine-rich region.

A “thymidine-rich region”, as used herein, refers to a sequence formed exclusively by thymidine (T) nucleotides that directly connects the two polypurine regions of the
30 oligonucleotide of the invention. There are no other nucleotides between the polypurine regions and the thymidine-rich region. The minimum number of thymidines in said region is four. The length of the thymidine-rich region may vary between 4 and 12 thymidines, preferably between 4 and 10 thymidines, more preferably between 4 and 8

thymidines, most preferably 5 thymidines. In a preferred embodiment of the invention the thymidine-rich region is a five-thymidine region.

Said thymidine-rich region forms a loop when the oligonucleotide of the invention is in the form of hairpin. Said loop may be on both sides of the hairpin since the terms "first polypurine region" and "second polypurine region" do not refer to the order in which said polypurine regions are found in the linear oligonucleotide. We designate as "first polypurine region" the polypurine region of the oligonucleotide that forms Watson-Crick bonds with the polypyrimidine region of the target gene.

In a preferred embodiment the thymidine-rich region of the oligonucleotide of the invention connects the 3'-end of the first polypurine region and the 5'-end of the second polypurine region. In another preferred embodiment the thymidine-rich region of the oligonucleotide of the invention connects the 5'-end of the first polypurine region and the 3'-end of the second polypurine region.

The term "5'-end" designates the end of a nucleotide strand that has the fifth carbon in the sugar-ring of the deoxyribose at its terminus.

The term "3'-end" designates the end of a nucleotide strand that has the hydroxyl group of the third carbon in the sugar-ring of the deoxyribose at its terminus.

In the context of the present invention "reverse sequence" is understood to mean a nucleic acid sequence in which the order of nucleotides is reversed as compared to the original sequence. For example, reverse sequence to AAGAG is GAGAA.

The oligonucleotides of the present invention may be in different conformations. They may be linear oligonucleotides or hairpin oligonucleotides. The biologically active oligonucleotides of the invention are hairpin oligonucleotides.

The term "antiparallel hairpin", as used herein, refers to a stable duplex formed by the single strand oligonucleotide of the invention that doubles back on itself to form a duplex

maintained by intramolecular reverse-Hoogsteen bonds between the first and second regions of polypurine.

The term “reverse-Hoogsteen bonds”, as used herein, refers to hydrogen bonds between nucleic acids, with a different binding code than Watson-Crick bonds. For the usage of Hoogsteen structures the presence of polypyrimidine/polypurine stretches is required, as this type of binding can only happen when these sequences are present. Specifically, in reverse-Hoogsteen bonds a purine is bound to another purine identical to the first one, in which the two strands forming reverse-Hoogsteen bonds are in antiparallel orientation. Said kind of linkage allows the formation of triplex structures, in which one DNA strand can simultaneously form Watson-Crick bonds with one strand, and Hoogsteen bonds with the other (Praseuth, D. *et al.* 1999. Biochim Biophys Acta, 1489:181-206). Thus, when binding to DNA/RNA the oligonucleotides of the invention form antiparallel triplexes that need the formation of the triads d(A#A•T) and d(G#G•C) wherein # correspond to Hoogsteen bonds and • correspond to Watson-Crick bonds, and the orientation between the Watson-Crick polypurine strand and the Hoogsteen polypurine strand is antiparallel. The main benefit of using reverse-Hoogsteen bonds (or antiparallel Hoogsteen bonds) is that they can be formed at physiological pH, unlike parallel Hoogsteen bonds.

The expression “capable of forming a triplex”, as used herein, means that the oligonucleotide of the invention is able to form a triplex structure with the strand of pre-mRNA or with the DNA coding strand, wherein the first and second polypurine regions of the oligonucleotide are linked by reverse-Hoogsteen bonds and the first or the second polypurine region forms Watson-Crick bonds with the pre-mRNA strand or with the DNA coding strand.

A “polypyrimidine region”, as used herein, refers to a motif within a sequence of a strand of nucleic acid defined by at least 19 nucleotides of pyrimidine in succession wherein said succession is a pure pyrimidine succession or may have a maximum of three purine interruptions. The polypurine region usually has between 19 nucleotides and the maximum possible length without exceeding 3 purine interruptions, preferably at least 20 nucleotides, more preferably at least 24 nucleotides, most preferably at least 35

nucleotides. When the nucleic acid is DNA the nucleotides of pyrimidine can be cytosine (C) or thymine (T) nucleotides. When the nucleic acid is RNA the nucleotides of pyrimidine can be cytosine (C) or uracil (U) nucleotides.

5 In its broadest sense, the expression “substantially complementary”, when used herein with respect to a polypyrimidine region in relation to the first polypurine region of the oligonucleotide, means a polypyrimidine region having a percentage of identity between the substantially complementary polypyrimidine region and the exact complementary polypurine region of at least 60 percent, at least 70 percent, at least 80 percent, at least 85
10 percent, at least 90 percent, at least 93 percent, at least 95 percent, at least 96 percent, at least 97 percent, at least 98 percent, at least 99 percent or at least 100 percent. Identity is assessed over the entire length of the polypyrimidine region to the polypurine region. The degree of identity between two nucleotide regions is determined using algorithms implemented in a computer and methods which are widely known by the persons skilled
15 in the art. The identity between two nucleotide sequences is preferably determined using the BLASTN algorithm (BLAST Manual, Altschul, S. *et al.*, NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., *et al.*, J., 1990, Mol. Biol. 215:403-410). A polypyrimidine region “substantially complementary” to a polypurine region hybridizes to the polypurine region under low stringency conditions, preferably medium stringency conditions, most
20 preferably high stringency conditions.

In a preferred embodiment the first polypurine region of the oligonucleotide of the invention and the polypyrimidine region located in the coding strand of an intronic region of a target gene are 100% complementary.

25

When there are no pure polypyrimidine regions in the target gene and said regions have purine interruptions, it is possible to design oligonucleotides substantially complementary to the target gene by placing adenines or guanines, preferably adenines, in front of said purine interruptions.

30 The purine interruptions cannot be more than three. In a preferred embodiment the polypurine region substantially complementary to the polypyrimidine region in the target gene is interrupted by at least one non-complementary nucleotide and wherein said one or more non-complementary nucleotide is adenine.

The term “non-complementary nucleotide”, as used herein, refers to a nucleotide in the polypurine region of the oligonucleotide of the invention that lacks the ability to form intermolecularly a Watson-Crick base pair through specific hydrogen bonds with the polypyrimidine region of the target gene. In the context of the present invention said non-complementary nucleotide is adenine or guanine, preferably adenine.

The term “coding strand”, as used herein, refers to the DNA strand which is complementary to the template strand. Such coding strand has the same base sequence as the mRNA (although with thymine replaced by uracil) and corresponds to the codons that are translated into protein.

The term “template strand”, as used herein, refers to the DNA strand that is used as a template for the synthesis of mRNA. Such template strand has a complementary sequence to the mRNA sequence.

The term “intronic region”, as used herein, refers to a region located in an intron of a gene. “Intron”, as used herein, refers to a nucleotide sequence within a gene that is removed by RNA splicing to generate the final mature RNA product of a gene. The term intron refers to both the DNA sequence within a gene, and the corresponding sequence in pre-mRNA transcripts.

The skilled in the art will recognize if a polypyrimidine region is located in the coding strand of an intronic region of a target gene by performing the assay described in the Materials and Methods section “Electrophoretic mobility shift assay” and in the Results section “Effects of Coding-PPRHs on U2AF65 binding”. Said assay shows that the coding-PPRH (the oligonucleotide of the invention) is capable of inhibiting the interaction between an oligonucleotide that comprises the polypyrimidine region target sequence and the U2 auxiliary factor (U2AF) 65. Thus, the skilled in the art will recognize if an oligonucleotide of the invention or Coding-PPRH falls into the scope of the claims of the present invention by performing an assay consisting of synthesizing an oligonucleotide whose sequence corresponds to the target polypyrimidine sequence to be

analyzed and detecting if the oligonucleotide of the invention (or Coding-PPRH) is capable of preventing the binding of the synthesized oligonucleotide to U2AF65.

The term "target gene", as used herein, refers to any gene of interest present in an organism. A target gene may be endogenous or introduced. For example, a target gene is a gene of known function or is a gene whose function is unknown, but whose total or partial nucleotide sequence is known. A target gene can be a native gene of the eukaryotic cell or can be a heterologous gene which has previously been introduced into the eukaryotic cell or a parent cell of said eukaryotic cell, for example by genetic transformation. A heterologous target gene can be stably integrated in the genome of the eukaryotic cell or is present in the eukaryotic cell as an extrachromosomal molecule, e.g. as an autonomously replicating extrachromosomal molecule. A target gene can include polynucleotides comprising a region that encodes a polypeptide or polynucleotide region that regulates replication, transcription, translation, or other process important in expression of the target protein; or a polynucleotide comprising a region that encodes the target polypeptide and a region that regulates expression of the target polypeptide; or non-coding regions such as the 5' or 3' UTR or introns. A target gene may refer to, for example, an mRNA molecule produced by transcription of a gene of interest.

The target gene may be an artificial gene or may be a gene from any species, including but not being limited to domestic and farm animals (cows, horses, pigs, sheep, goats, dogs, cats or rodents), primates, humans, birds, fish, insects, worms, amphibians and reptiles. Preferably, the target gene is from mammals, more preferably is human.

Examples of genes that can be used as a target for designing the oligonucleotides of the invention include, but are not limited to, oncogenes, genes encoding transcription factors, receptors, enzymes, structural proteins, cytokines, cytokine receptors, lectins, selectins, immunoglobulins, kinases, phosphatases, prions, proangiogenic polypeptides, proteases and proteins involved in the apoptosis process, genes encoding adhesion molecules, genes encoding surface receptors, genes encoding proteins involved in metastasis or in invasive processes of tumor cells, genes encoding growth factors, the multiple drug resistance gene (MDR1), genes encoding lymphokines, cytokines, immunoglobulins, T-cell receptors, MHC antigens, DNA and RNA polymerases, genes

involved in metabolic processes such as the synthesis of amino acids, nucleic acids, tumor suppressing genes, 5-lipooxygenase, phospholipase A2, protein kinase C, p53, p16, p21, MMAC1, p73, zac1, C-CAM, BRCA1, Rb, Harakiri, Ad E1B, protease ICE-CED3, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, TNF, GMCSF, interferons, CFTR, EGFR, VEGFR, IL-2 receptor, estrogen receptor, members of the Bcl-2 family (Bcl-2 or Bcl-xL), ras, myc, neu, raf erb, src, fins, jun, trk, ret, gsp, hst and abl, amyloid protein precursor, angiostatin, endostatin, METH-1, METH-2, Factor IX, Factor VIII, collagen, cyclin dependent kinase, cyclin D1, cyclin E, WAF 1, cdk4 inhibitor, MTS1, IL-1, IL-16, IL-17, erythropoietin, G-CSF, GM-CSF, M-CSF, SCF, thrombopoietin, BNDF, BMP, GGRP, EGF, FGF, GDNF, GGF, HGF, IGF-1, IGF-2, KGF, myotrophin, NGF, OSM, PDGF, somatrophin, TGF-beta, TGF-alpha, VEGF, TNF-alpha, TNF-beta, cathepsin K, cytochrome P-450, farnesyltransferase, glutathione-S transferase, heparanase, HMG CoA synthetase, n-acetyltransferase, phenylalanine hydroxylase, phosphodiesterase, carboxy-terminal protease of ras, Notch, telomerase, TNF-converting enzyme, cadherin E, cadherin N, selectin, CD40, ANF, calcitonin, the corticotrophin-releasing factor, glucagon, gonadotropin, the gonadotropin-releasing hormone, growth hormone, the growth hormone-releasing factor, somatotropin, insulin, leptin, luteinising hormone, luteinizing hormone-releasing hormone, PTH, thyroid hormones, thyroid-stimulating hormone, immunoglobulin CTLA4, hemagglutinin, MHC, VLA-4, the kallikrein-kininogen- kinin CD4 system, sis, mos, fos, H-ras, ki-ras, c-fins, bcl-2, L-myc, c-myc, gip, HER-2, bombesin receptor, GABA receptor, PDGFR, FGFR, NGFR, interleukin receptors, ion channel receptors, leukotriene receptor antagonists, lipoprotein receptors, opioid receptors, substance P receptors, retinoic acid and retinoid receptors, steroid receptors, T-cell receptors, thyroid hormone receptors, TNF receptors, tPA receptors, calcium pump, proton pump, Na/Ca exchanger, MRP 1, MRP2, P170, LRP, cMOAT, transferrin, APC, brca1, brca2, DCC, MCC, MTS1, NF1, NF2, nm23, and Rb.

The oligonucleotides of the invention are capable of producing a decrease in the mature mRNA and protein levels of the target gene. A decrease in the gene expression is a reduction in the level of the target gene, included a decrease in the level of a protein or mRNA in a cell or organism. The oligonucleotides of the present invention are able to produce a decrease in the gene expression of at least 10%, preferably at least 40%, more

preferably at least 50% relative to a cell or organism lacking the oligonucleotide of the invention.

Strategies to decrease the mRNA and protein levels of genes whose overexpression cause a disease or of genes that encode mutant proteins that lead to the development of a disease have been used with success. This approach has been showed useful in diseases such as cancer (Gleave, M.E. and Monia, B.P. 2005. Nat Rev Cancer, 5: 468-479), Alzheimer's disease (Miller V. M. *et al.* 2004. Nucl Acids Res, 32 (2): 661-668; Orlacchio, A. *et al.* 2007. Mini Rev Med Chem, 7 (11): 1166-76), atherosclerosis (Tavridou, A. and Manolopoulos, V.G. 2008. Curr Med Chem, 15(8): 792-802; Brown, J.M. *et al.*, 2010. Arterioscler Thromb Vasc Biol, 30 (1): 24-30), Huntington disease (Sah, D.W. and Aronin, N. 2011. J Clin Invest, 121: 500-7) and epilepsy (Boison, D. 2010. Epilepsia, 51: 1659-68), amongst others.

The oligonucleotides of the invention showed a decrease in the mRNA and protein levels of DHFR, which is a gene involved in cancer. Previously, the polypurine reverse-Hoogsteen hairpins that bind to a target sequence located in the template DNA strand (Template-PPRHs) had been employed against telomerase and survivin genes involved in cell proliferation (de Almagro, M.C. *et al.* 2009. J Biol Chem, 284:11579-11589). Thus, the target gene of the oligonucleotide of the invention may be a gene involved in cancer development and progression.

In a preferred embodiment, the target gene of the oligonucleotide of the invention is selected from the group consisting of a gene related to cell proliferation, replication, apoptosis, angiogenesis, metastasis, inflammation, Alzheimer's disease, atherosclerosis or development of resistance to chemotherapy and/or radiotherapy; preferably is selected from the group consisting of a gene related to cell proliferation, replication, apoptosis, angiogenesis, metastasis, inflammation or development of resistance to chemotherapy and/or radiotherapy.

In the context of the present invention, a "gene related to cell proliferation" is a gene that promotes cell growth, particularly involved in progression of cancer and tumours. Non-limiting examples of genes related to cell proliferation and which can be the target gene

of the oligonucleotide of the invention are CAD, Cdc2, Cdc25 A, Cyclin A, Cyclin D1, Cyclin E, DHFR, insulin-like growth factor 1 receptor (IGF1R), LDH-A, ODC, α-prothymosin, p53, RCC1, Rcl, c-myb, PKA-I, c-myc, HRAS and mdm2.

5 In the context of the present invention, a “gene related to replication” is a gene able to promote the process in which one double-stranded DNA molecule produces two identical copies of said molecule. Genes related to replication are, without limitation, DNA polymerases, DNA primase, processivity accessory proteins, single strand binding proteins, helicase, DNA ligase, topoisomerases, uracil-DNA N-glycosylase and
10 telomerase.

In the context of the present invention, a “gene related to apoptosis” is a gene involved in the process of programmed cell death that may occur in multicellular organisms. The expression “gene related to apoptosis” includes pro-apoptotic genes and anti-apoptotic
15 genes. Examples of genes related to apoptosis are, without limitation, bcl-2, clusterin, survivin, c-myc, mdm2, heat shock protein hsp27, FAS, TNFSFIO (TRAIL), TRADD, TNFRSFIOB (TRAILR2), TNFSF14, PECAMI, BIRC4, TNFSFII (RANKL), DIABLO, XIAP, ATM, CD47, CASPlO, BNIP3, TNFRSF7, STK17A, SMAD7, BCL2, BCL2L11, FYN, FAIM3, MCL1, GALECTIN3, AATF, KIT (CDl 17), MYB, EphB4, and
20 TNFRSFlOD (CD264).

In the context of the present invention, a “gene related to angiogenesis” is a gene able to promote the formation of new blood vessels from existing blood vessels. Angiogenesis is also known as neovascularization. Examples of genes involved in angiogenesis are,
25 without limitation, vascular endothelial growth factor (VEGF), placental growth factor (PGF), VEGFR-I (FLT1), VEGFR-2 (KDR), TIMP-I, TIMP-2, TIMP-3, TIMP-4, VEGF-A, VEGF- B, VEGF-C, VEGF-D, VEGF-E, IL-8, TGF beta and TGF alpha.

In the context of the present invention, a “gene related to metastasis” includes genes
30 able to promote remote propagation, fundamentally by lymphatic or blood channels, of the cancer causing cells, and the growth of new tumours in the place of destination of said metastasis. Examples of genes involved in metastasis are, without limitation, APC,

CD44, CDH1 (cadherin-1 / E-cadherin), CDH11, CDH6, FAT, FXYD5, ITGA7, PNN, SYK, VEGF, ITGA7, ITGB3, RPSA (LAMR1), CTNNA1, FN1, MCAM, MGAT5 (acetylglucosaminyltransferase V), MTSS1, MMP10, MMP11, MMP13, MMP2, MMP3, MMP7, MMP9.TIMP2, TIMP3, TIMP4, COL4A2 (collagen α2(IV)), HPSE (heparanase), HRAS, IL1B, KRAS, TGFB1 (TGF-β1), VEGFA, BRMS1 (BrMS1), CDKN2A, NME1, NME2, PTEN, RB1, TP53, MYC (c-myc), CTBP1, GNRH1, MDM2, NF2, SSTR2, IGF1, IL18, TSHR, VEGFA, HGF (Scatter Factor), CCL7, CXCL12, TNFSF10, CXCR4, EPHB2, FGFR4, FLT4, KISS1R, IL8RB, MET, NR4A3, PLAUR (uPAR), RORB, SSTR2, TSHR, DENR, EWSR1, HRAS (c-hRas), SET, SRC (c-src), SYK, TRPM1, HTATIP2, HTATIP2, ETV4, MTA1, MYCL1, NME2, RB1, SMAD4, TCF20, CHD4, EWSR1, SMAD2, CST7, CTSK, CTSL1 (cathepsin L), CD82 (KAI1), KISS1 (KiSS-1), METAP2 and NME4.

In the context of the present invention, a “gene related to inflammation” includes genes related to an excessive or altered inflammatory response. Examples of genes related to inflammation are, without limitation, complement component 3 (C3), interleukin 12B (IL12B), LMP7, lymphocyte antigen 6 complex and macrophage colony stimulating factor 1 (M-CSF 1), CCL11, CCL13, CCL16, CCL17, CCL19, CCL2, CCL21, CCL22, CCL23, CCL24, CCL3, CCL4, CCL5, CCL7, CCL8, CXCL1, CXCL10, CXCL2, CXCL3, CXCL5, CXCL6, CXCL9, IL8, CD40LG, CSF1, FASLG, FLT3LG, IFNG, IL10, IL18, IL1A, IL1B, IL1F10, IL1RN, IL22, IL23A, IL6, LTA, LTB, TNF, TNFSF14, IL10RB, IL1R1, IL1RAP, IL22RA2, IL6R, IL8RA, IL8RB, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CXCR4, CCR1, CD40, IL18RAP, IL23R, CEBPB, CRP, BCL6, C3, C3AR1, C4A, CD40LG, CRP, FOS, HDAC4, IL9, ITGB2, KNG1, LY96, MYD88, NFATC3, NFKB1, NOS2A, NR3C1, RIPK2, TIRAP, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7 and TOLLIP.

In the context of the present invention, a “gene related to Alzheimer’s disease” includes genes related to the onset, development, and progression of Alzheimer’s disease. Examples of genes related to Alzheimer’s disease are, without limitation, ADAM9, APH1A, BACE1, BACE2, CTSB, NCSTN, PSEN1, PSEN2, IDE, PLAT, PLAU, PLG, APLP1, APP, LRP1, LRP6, LRP8, A2M, ACHE, APBB1, APBB2, APOE, BCHE, UBQLN1, MAP2, MAPT, PKP4, PRKCI, APBA1, CHAT, BDNF, ABCA1, APOA1,

LRP8, CLU, HADH2, INS, LPL, LRP8, SNCB, BACE2, CASP3, CASP4, ERN1, PRKCA, PRKCE, IL1A, MPO, PRKCZ, SNCA, APLP1, APPBP1, CLU, EP300, MAPT, CDC2, CDK5, CDKL1, GSK3A, GSK3B, INSR, PRKCB1, PRKCD, PRKCG, PRKCQ, PRKCZ, LRP6, APH1A, NCSTN, PSEN1, PSEN2, APLP2, GNAO1, GNAZ, 5 GNB1, GNB4, GNB5, GNG10, GNG11, GNG3, GNG4, GNG5, GNG7, GNG8, GNGT1, GNGT2, APBA3, GAP43, GNB2, IDE, IL1A, INSR, HADH2, MPO, UQCRC1, UQCRC2, CTSC, CTSD, CTSG, CTSL, APLP2 and SERPINA3.

In the context of the present invention, a "gene related to atherosclerosis" includes 10 genes involved in the processes of blood coagulation and circulation as well as genes involved in cell-adhesion and lipid transport and metabolism, and genes involved in the stress response, cell growth and proliferation, and apoptosis. Examples of genes related to atherosclerosis are, without limitation, CCL2, CCL5, CCR1, CCR2, IL1R1, IL1R2, ITGB2, NFKB1, NOS3, SELE, SPP1, TNF, SOD1, CSF2, FN1, IL4, ITGB2, IFNAR2, 15 CTGF, FN1, VWF, IFNG, PPARG, VEGFA, BCL2, BCL2A1, BCL2L1, BIRC3, CFLAR, FAS (TNFRSF6), IL1A, IL2, SERPINB2, SPP1, TGFB1, TNFAIP3, BAX, BID, CFLAR, FAS (TNFRSF6), ITGB2, FGA, ITGA2, LPA, SERPINE1, APOA1, APOB, APOE, COL3A1, ELN, ENG, LPA, LPL, NPY, PDGFA, PDGFB, PDGFRB, VWF, ACE, FGA, CD44, CDH5, ICAM1, ITGB2, SELE, SELL, VCAM1, CD44, 20 ITGA2, ITGA5, ITGAX, SPP1, CTGF, SELPLG, THBS4, TNC, MMP1, MMP3, COL3A1, FN1, ADFP, CSF2, CTGF, FGA, FGF2, HBEGF (DTR), IFNAR2, IFNG, IL3, IL4, IL5, LAMA1, LIF, VWF, ABCA1, LDLR, FABP3, PPARA, PTGS1, ABCA1, MSR1, NR1H3, PPARD, PPARG, RXRA, ADFP, CSF2, KDR, SPP1, BCL2, FABP3, CSF1,, HBEGF (DTR), IL6, IL7, LIF, TGFB2, CTGF, ELN, IFNG, EGR1, 25 KLF2 and TNFAIP3.

In an embodiment the target gene is selected from DHFR, survivin, telomerase, c-myc, topoisomerase I, Mdm 2 and Bcl-2; more preferably DHFR.

In a preferred embodiment of the present invention the target gene is a gene involved in the development of resistance to chemotherapy and/or radiotherapy. The expression 30 "gene related to the development of resistance to chemotherapy and/or radiotherapy" refers to genes which are up-regulated in response to cytotoxic treatment and/or

radiation and which are involved in lack of response to chemotherapy and/or radiotherapy. Examples of genes involved in drug resistance, are, without limitation, galectin-3, galectin-4, syndecan-1, CD44, ICAM2, HRAS, XIAP, protein kinase C α (PKCA), aldehyde dehydrogenase, BCL2, $BCLX_L$, dihydrofolate reductase (DHFR), clusterin, heat shock protein hsp27, STAT3, clathrin and ECM1. In a more preferred embodiment the gene involved in drug resistance is DHFR.

In a particular embodiment the target gene of the oligonucleotide of the invention is dihydrofolate reductase (DHFR).

"Dihydrofolate reductase" or "DHFR" (E.C. 1.5.1.3) is an enzyme that reduces dihydrofolic acid to tetrahydrofolic acid, using NADPH as electron donor. Said enzyme is involved in the synthesis of glycine, hypoxanthine, and thymidylate, thus its inhibition blocks DNA synthesis and cell growth. Additionally, DHFR is involved in resistance to cancer drugs and other drugs aimed at DHFR such as trimethoprim.

The term DHFR encompasses the DHFR of any species, preferably mammalian species, including but not being limited to domestic and farm animals (cows, horses, pigs, sheep, goats, dogs, cats or rodents), primates and humans. More preferably, the DHFR is of human origin.

The skilled in the art will identify the DHFR gene by determining its sequence identity with a previously recognized DHFR gene sequence such as human DHFR gene using the BLASTN algorithm. DHFR genes contemplated in the invention include polynucleotides showing at least 60%, 65%, 70%, 72%, 74%, 76%, 78%, 80%, 90%, 95%, 97%, 99% similarity or identity with the different natural variants of DHFR gene.

Additionally, the product of the DHFR gene must show DHFR activity which can be determined by performing the DHFR activity assay described in Ciudad *et al.* (Ciudad, J.C. *et al.* 1988. J Biol Chem, 263:16274-16282) with modifications (Noe, V. *et. al.* 1998. Oncogene, 16: 1931-1938).

In the present invention, "DHFR gene of human origin" is understood as the gene defined by the sequence of the NCBI GenBank database with accession number NG_023304.1, corresponding to the sequence of the genomic DNA. The invention contemplates also the natural variants of human DHFR gene. The natural variants of human DHFR gene suitable for use in the present invention can also be derived from the sequence NG_023304.1 by means of insertion, substitution or deletion of one or more nucleotides and include natural alleles. Alternatively, the DHFR gene may be produced by recombinant and/or synthetic means.

The oligonucleotide of the invention may be designed against the coding strand of any intronic region of the genomic DNA sequence NG_023304.1.

The human DHFR gene (Entrez Gene ID:1719) is found in the q11→q22 region of chromosome 5. It has 6 exons and 5 introns, the third intron being the longest, having 11 kb (Emine Abalia, N.L.S. *et al.* 2008. Vitamins & Hormones, 79:267-292).

In a preferred embodiment the oligonucleotide of the invention is designed to be substantially complementary to a polypyrimidine region located in the third intron of the coding DNA strand of the DHFR gene. Preferably, the third intron is from human DHFR gene, said third intron starting at the nucleotide position 10594 and ending at the nucleotide position 21972 of the genomic DNA sequence NG_023304.1. In a preferred embodiment the sequence of the intronic region in the coding strand targeted by the oligonucleotide of the invention is 5'-TTTTTCACCCCTCTCCCCC-3' (SEQ ID NO: 1).

In a preferred embodiment the oligonucleotide of the invention has the sequence 5'-AAAAAG**A**GGGGAGAGGGGG-$(T)_5$-GGGGGAGAGGGG**A**GAAAAA-3' (SEQ ID NO: 2), wherein bold letters indicate mismatches between the target sequence and the oligonucleotide.

The oligonucleotides of the present invention have demonstrated that they are capable of producing cytotoxicity in tumour cells. Therefore, in a particular embodiment, the invention relates to an oligonucleotide wherein the target gene is selected from the group consisting of a gene related to cell proliferation, replication, apoptosis, angiogenesis,

metastasis, inflammation or development of resistance to chemotherapy and/or radiotherapy which is capable of producing a cytotoxic effect in tumour cells.

"Producing a cytotoxic effect in tumour cells" is understood as the toxic effect produced in tumour cells that reduces their survival by at least 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, 100%; preferably at least 50%; more preferably at least 60%; still more preferably at least 65%. Said cytotoxic effect can be evaluated by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay described in the Materials and Methods section of the present invention.

"Tumour cell" is understood as a malignant cell, also known as a cancerous or carcinogenic cell which grows and divides beyond the normal limits, invading the surrounding tissue and sometimes causing metastasis. The tumour cells that can be treated with the oligonucleotides of the present invention are cells which over-express a gene related to cell proliferation, replication, apoptosis, angiogenesis, metastasis, inflammation or development of resistance to chemotherapy and/or radiotherapy. Said cells include tumour cells from known and established cell lines and tumour cells present in the organism of a patient suffering from cancer.

The tumour cells which express a target gene selected by a gene related to cell proliferation, replication, apoptosis, angiogenesis, metastasis, inflammation or development of resistance to chemotherapy and/or radiotherapy can be identified by means of conventional methods such as reverse transcription and real-time PCR or ELISA or Western blot.

In one embodiment the tumour cells suffering from a cytotoxic effect produced by the oligonucleotides of the invention are breast cancer cells.

The oligonucleotide of the invention can be designed for any gene of interest, since the polypyrimidine targets can be found for all genes, especially in intronic or promoter sequences (non-coding).

The design of the oligonucleotides of the invention for a determined gene is performed using a software tool to search for polypurine stretches and their complementary polypyrimidine sequences in the appropriate DNA strand. Suitable software can be the Triplex-Forming Oligonucleotide Target Sequence Search software (M.D. Anderson Cancer Center, Houston, TX) (spi.mdanderson.org/tfo/). Even in the case of the presence of polypurine interruptions in the polypyrimidine target sequence, PPRHs against that sequence can be designed and used efficiently by introducing an adenine in the PPRH in front of the interruption.

The oligonucleotides of the invention are synthesized in the form of linear non-modified oligonucleotides. The synthesis of the oligonucleotides of the invention may be performed according to conventional methods known in the state of the art (Methods in Molecular Biology, vol. 288, "Oligonucleotide synthesis: Methods and Applications". Piet Herdewijn, Ed., Humana Press, 2005). Although they can be formed by bond cleavage of longer segments, they are now more commonly synthesized, in a sequence-specific manner, from individual nucleoside phosphoramidites by using automated synthesizers.

The oligonucleotides of the invention must bind specifically to their target sequence. As it is used herein, the expression "binds specifically to" refers to the capacity of the oligonucleotides for binding specifically to their target sequence and not to other sequence. The specificity is validated by performing BLAST analysis, by the use of negative controls, and by determining changes in mRNA levels of unrelated genes and cytotoxicity in cells with or without the PPRH target sequence.

PHARMACEUTICAL COMPOSITIONS OF THE CODING POLYPURINE HAIRPINS

Another aspect of the invention is a pharmaceutical composition comprising a pharmaceutically effective amount of at least one oligonucleotide of the invention and at least one pharmaceutically acceptable excipient.

As used in the present invention, the expression "pharmaceutical composition" relates to a formulation which has been adapted to administer a predetermined dose of one or several therapeutically useful agents to a cell, a group of cells, an organ, a tissue or an animal where there is an overexpression of a target gene.

5

The pharmaceutical compositions containing the oligonucleotides of the invention may be administered by any appropriate route, such as oral, topical or parenteral route for which it will include the pharmaceutically acceptable excipients necessary for the formulation of the form of administration desired. The preferred administration route of
10 the pharmaceutical composition is endovenous route.

The invention contemplates pharmaceutical compositions especially prepared for the administration of the oligonucleotides of the invention in naked form, i.e., in the absence of compounds that protect the oligonucleotides from degradation by the nucleases of the
15 organism, which entails the advantage of eliminating the toxicity associated with the reagents used for the transfection. Suitable routes of administration for the naked oligonucleotides include the intravascular, intratumoral, intracranial, intraperitoneal, intrasplenic, intramuscular, subretinal, subcutaneous, mucous, topic or oral route (Templeton, 2002, DNA Cell Biol., 21:857-867). The initial concerns in regard to the
20 capacity of other interference molecules such as siRNAs to induce an immune response when administered naked have been investigated by Heidel *et al.* (Nat. Biotechnol., 2004, 22:1579-1582). By means of the determination of the plasma interleukin and interferon levels after the intraperitoneal and intravenous administration of siRNAs, no immune response was observed while, at the same time, it was observed that the
25 systematic administration of the siRNA was well tolerated.

In another embodiment, the oligonucleotides of the invention are administered by means of the so-called "hydrodynamic administration" in which the oligonucleotides are introduced intravascularly into the organism at high speed and volume, which
30 results in high transfection levels with a more diffuse distribution (Aliño, S.F. *et al.* 2010. J Gene Med, 12:920-6). A modified version of this technique has made it possible to obtain positive results for silencing through the naked siRNAs of exogenous genes (Lewis *et al.*, 2002, Nat. Gen., 32:107-108; McCaffrey *et al.*, 2002, Nature, 418:38-39)

and endogenous genes (Song *et al.*, 2003, Science, Nat. Med., 9:347-351) in multiple organs. It has been shown that the effectiveness of the intercellular access depends directly on the volume of the fluid administered and the speed of the injection (Liu *et al.*, 1999, Science, 305:1437-1441). In mice, the administration has been optimized at values of 1 ml/10 g of body weight in a period of 3-5 seconds (Hodges, *et al.*, 2003, Exp. Opin. Biol. Ther., 3:91-918). The exact mechanism allowing in vivo cell transfection with siRNAs after their hydrodynamic administration is not fully known. In the case of mice, it is thought that administration through the tail vein takes place at a rate that exceeds the heart rate and that the administrated fluid accumulates in the superior vena cava. This fluid subsequently accesses the vessels in the organs, and after that, through fenestrations in said vessels, accesses the extravascular space. In this way, the siRNA comes into contact with the cells of the target organism before it is mixed with the blood, thus reducing the possibilities of degradation through nucleases.

The oligonucleotides of the invention can be administered forming part of liposomes, conjugated to cholesterol or conjugated to compounds capable of causing the translocation through cell membranes such as the TAT peptide, derived from the HIV-1 TAT protein, the third helix of the homeodomain of the *D. melanogaster* Antennapaedia protein, the VP22 protein of the herpes simplex virus, arginine oligomers and peptides such as those described in WO07069090 (Lindgren, A. *et al.*, 2000, Trends Pharmacol. Sci., 21:99-103; Schwarze, S.R. *et al.*, 2000, Trends Pharmacol. Sci., 21:45-48, Lundberg, M. *et al.*, 2003, Mol. Therapy, 8:143-150 and Snyder, E.L. and Dowdy, S.F., 2004, Pharm. Res., 21:389-393).

Alternatively, the oligonucleotides of the invention may be administered forming part of polyplexes which are complexes of polymers with DNA. Most polyplexes consist of cationic polymers and their production is regulated by ionic interactions. One large difference between the methods of action of polyplexes and lipoplexes is that polyplexes cannot release their DNA load into the cytoplasm, so to this end, co-transfection with endosome-lytic agents (to lyse the endosome that is made during endocytosis, the process by which the polyplex enters the cell) such as inactivated adenovirus must occur. However, this is not always the case, polymers such as

polyethylenimine have their own method of endosome disruption as does chitosan and trimethylchitosan.

Alternatively, the oligonucleotides of the invention can be administered associated to
5 dendrimers which are repeatedly branched, roughly spherical large molecules capable of delivering the oligonucleotides.

The amount of oligonucleotide required for the therapeutic or prophylactic effect will naturally vary according to the elected oligonucleotide, the nature and the severity of the
10 illness that is going to be treated, and the patient.

The compositions of the invention are suitable for administration to any type of mammal, preferably a human being.

15 "Pharmaceutically effective amount" relates to a quantity capable of exercising a therapeutic effect, and which may be determined by the person skilled in the art using typically used means.

The compositions of the invention may contain one or more oligonucleotides according
20 to the invention. In a preferred embodiment, the composition of the invention comprises several oligonucleotides, all of them being targeted to different regions of one and the same target pre-mRNA.

The compositions of the invention may also contain one or several additional compounds
25 for the treatment of a pathology related to cell proliferation, replication, apoptosis, angiogenesis, metastasis, inflammation, Alzheimer`s disease, atherosclerosis or to treat the development of resistance to chemotherapy and/or radiotherapy; preferably for the treatment of a pathology related to cell proliferation, replication, apoptosis, angiogenesis, metastasis, inflammation or to treat the development of resistance to chemotherapy
30 and/or radiotherapy.

The pharmaceutical compositions are prepared by conventional means with pharmaceutically acceptable excipients.

“Pharmaceutically acceptable excipient” is understood to be an inactive substance therapeutically speaking, used to incorporate the active principle and which is acceptable for the patient from a pharmacological/toxicological viewpoint and for the
5 pharmaceutical chemist that manufactured it from a physical/chemical standpoint with respect to the composition, formulation, stability, acceptance by the patient and bioavailability.

The number and the nature of the pharmaceutically acceptable excipients depend on the
10 desired administration form. The pharmaceutically acceptable excipients are known by the person skilled in the art (Faulí and Trillo C. (1993) “Tratado de Farmacia Galénica”, Luzán 5, S.A. Ediciones, Madrid). Said compositions may be prepared by the conventional methods known in the state of the art (“Remington: The Science and Practice of Pharmacy”, 20th edition (2003) Genaro A.R., ed., Lippincott Williams &
15 Wilkins, Philadelphia, US).

The person skilled in the art will appreciate that the compositions of the invention can be used for the treatment and/or prevention of all those diseases or conditions that result from the over-expression of a certain gene or which require a decrease in the expression
20 of a given gene.

In a particular embodiment the pharmaceutical composition is for the prevention and/or the treatment of a disease selected from cancer, a disease associated to inflammation, Alzheimer’s disease and atherosclerosis; preferably selected from cancer and a disease
25 associated to inflammation. In a more preferred embodiment the disease is cancer, preferably breast cancer. In another preferred embodiment the cancer is resistant to chemotherapy and/or radiotherapy, preferably resistant to methotrexate.

Said preferred embodiments will later be described in the context of the therapeutic uses
30 of the coding polypurine hairpins.

THERAPEUTIC USES OF THE CODING POLYPURINE HAIRPINS

Coding-PPRHs show a noticeable ability to specifically decrease gene expression. Thus, they could have potential applications for diseases coursing with deregulated gene expression. Thus, in another aspect, the invention relates to an oligonucleotide according to the invention for use in medicine. In another aspect, the invention relates to the use of 5 an oligonucleotide according to the invention in the manufacture of a medicament. In another aspect, the invention relates to an oligonucleotide according to the invention in the manufacture of a medicament.

Previously, other types of oligonucleotides such as antisense oligonucleotides or antigene 10 oligonucleotides directed to genes involved in cancer had showed their usefulness in cancer gene therapy (El-Aneed, A. 2004. European Journal of Pharmacology, 498:1-8) and in inflammation. Also Alzheimer's disease (Miller V. M. *et al.* 2004. Nucl Acids Res, 32 (2): 661-668; Orlacchio, A. *et al.* 2007. Mini Rev Med Chem, 7 (11): 1166-76) and atherosclerosis (Tavridou, A. and Manolopoulos, V.G. 2008. Curr Med Chem, 15(8): 15 792-802; Brown, J.M. *et al.*, 2010. Arterioscler Thromb Vasc Biol, 30 (1): 24-30) may be treated with such kind of oligonucleotides.

Thus, in one aspect, the invention relates to the use of an oligonucleotide according to the invention wherein the target gene is a gene selected from the group consisting of a 20 gene associated with cancer, inflammation, Alzheimer's disease and atherosclerosis for the preparation of a medicament for the treatment and/or the prevention in a subject of a disease selected from cancer, a disease associated with inflammation, Alzheimer's disease and atherosclerosis. In a preferred embodiment the target gene is a gene associated with cancer or inflammation.

25

In another aspect, the invention relates to an oligonucleotide according to the invention wherein the target gene is a gene selected from the group consisting of a gene associated with cancer, inflammation, Alzheimer's disease and atherosclerosis for use in the preparation of a medicament for the treatment and/or the prevention in a subject of a 30 disease selected from cancer, a disease associated with inflammation, Alzheimer's disease and atherosclerosis. In a preferred embodiment the target gene is a gene associated with cancer or inflammation.

In another aspect, the invention relates to a method of treatment and/or the prevention in a subject of a disease selected from cancer, a disease associated with inflammation, Alzheimer's disease and atherosclerosis comprising the administration to said subject of a pharmaceutically effective amount of an oligonucleotide according to the invention wherein the target gene is a gene selected from the group consisting of a gene associated with cancer, inflammation, Alzheimer's disease and atherosclerosis. In a preferred embodiment the disease is selected from cancer and inflammation.

"Medicament" is understood to be a pharmaceutical composition comprising one of the oligonucleotides of the invention.

The term "treatment" is used to designate the administration of an oligonucleotide according to the invention or of a medicament containing it to control the progression of the disease before or after clinical signs have appeared. Control of the disease progression is understood to mean the beneficial or desired clinical results that include, but are not limited to, reduction of the symptoms, reduction of the duration of the disease, stabilization of pathological states (specifically to avoid additional deterioration), delaying the progression of the disease, improving the pathological state and remission (both partial and total). The control of progression of the disease also involves an extension of survival, compared with the expected survival if treatment was not applied.

"Prevention" is understood to mean the administration of an oligonucleotide according to the invention or of a medicament containing it in an initial or early stage of the disease, or also to avoid its appearance.

The term "target gene" has been defined previously in the context of the oligonucleotides of the invention.

In the context of the present invention, a "gene associated with cancer" is a gene involved in development and progression of cancer and tumours, particularly a gene related to cell proliferation, replication, apoptosis, angiogenesis, metastasis, inflammation or development of resistance to chemotherapy and/or radiotherapy.

Examples of such genes have been described previously in the context of the oligonucleotides of the invention.

A preferred embodiment of the invention is the use of a combination of two or more oligonucleotides of the invention as anticancer agents capable of binding to pre-mRNA target regions of different genes overexpressed in cancer.

In the context of the present invention, a "gene associated with inflammation" is a gene related to an excessive or altered inflammatory response. Examples of such genes have been described previously in the context of the oligonucleotides of the invention.

The terms "gene associated with Alzheimer's disease" and "gene associated with atherosclerosis" have been defined in the context of the first aspect of the invention.

In a particular embodiment the disease to be treated is cancer.

The terms "cancer" and "tumour" refer to the physiological condition in mammals characterized by unregulated cell growth. The oligonucleotides of the present invention are useful in the treatment of any cancer or tumour, such as, without limitation, breast, heart, lung, small intestine, colon, spleen, kidney, bladder, head, neck, ovarian, prostate, brain, pancreas, skin, bone, bone marrow, blood, thymus, uterus, testicles and liver tumours. In particular, tumours which may be treated with the oligonucleotides of the invention include adenoma, angiosarcoma, astrocytoma, epithelial carcinoma, germinoma, glioblastoma, glioma, hemangioendothelioma, hemangiosarcoma, hematoma, hepatoblastoma, leukaemia, lymphoma, medulloblastoma, melanoma, neuroblastoma, osteosarcoma, retinoblastoma, rhabdomyosarcoma, sarcoma, and teratoma. In particular, the tumour/cancer is selected from the group of acral lentiginous melanoma, actinic keratosis adenocarcinoma, adenoid cystic carcinoma, adenomas, adenosarcoma, adenosquamous carcinoma, astrocytic tumours, bartholin gland carcinoma, basal cell carcinoma, bronchial gland carcinoma, capillary carcinoid, carcinoma, carcinosarcoma, cholangiocarcinoma, cystadenoma, endodermal sinus tumour, endometrial hyperplasia, endometrial stromal sarcoma, endometrioid adenocarcinoma, ependymal sarcoma, Swing's sarcoma, focal nodular hyperplasia, germ

cell tumours, glioblastoma, glucagonoma, hemangioblastoma, hemangioendothelioma, hemangioma, hepatic adenoma, hepatic adenomatosis, hepatocellular carcinoma, insulinoma, intraepithelial neoplasia, interepithelial squamous cell neoplasia, invasive squamous cell carcinoma, large cell carcinoma, leiomyosarcoma, melanoma, malignant melanoma, malignant mesothelial tumour, medulloblastoma, medulloepithelioma, mucoepidermoid carcinoma, neuroblastoma, neuroepithelial adenocarcinoma, nodular melanoma, osteosarcoma, papillary serous adenocarcinoma, pituitary tumours, plasmacytoma, pseudosarcoma, pulmonary blastoma, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, sarcoma, serous carcinoma, small cell carcinoma, soft tissue carcinoma, somatostatin-secreting tumour, squamous carcinoma, squamous cell carcinoma, undifferentiated carcinoma, uveal melanoma, verrucous carcinoma, vipoma, Wilm's tumour. Even more preferably, the tumour/cancer to be treated and/or prevented with the oligonucleotides of the invention include intracerebral cancer, head and neck cancer, rectal cancer, astrocytoma, glioblastoma, small cell cancer, and non-small cell cancer, preferably non-small cell lung cancer, metastatic melanoma, androgen-independent metastatic prostate cancer, androgen-dependent metastatic prostate cancer and breast cancer. In a preferred embodiment the cancer that may be treated and/or prevented with the oligonucleotides of the invention is breast cancer.

"Breast cancer" or "malignant breast neoplasm" is a cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Cancers originating from ducts are known as ductal carcinomas; those originating from lobules are known as lobular carcinomas. The ducts and lobules of the breast are glandular tissue, thus cancers starting in these areas are often called adenocarcinomas. There are other types of breast cancers named medullary carcinomas, mucinous carcinomas and tubular carcinomas. Types of breast cancer to be treated and/or prevented by the oligonucleotides of the invention are, without limitation, ductal carcinoma in-situ, lobular carcinoma in-situ, infiltrating ductal carcinoma, medullary carcinoma, infiltrating lobular carcinoma, tubular carcinoma, mucinous carcinoma, inflammatory breast cancer, sarcoma, triple-negative breast cancer, mixed tumours, metaplastic carcinoma, Paget disease of the nipple, papillary carcinoma, adenocystic carcinoma, Phyllodes tumour and angiosarcoma. In a preferred embodiment the breast cancer is an adenocarcinoma.

A major obstacle to successful cancer treatment is the development of resistance to chemotherapy and/or radiotherapy. Innate resistance is the lack of response to a therapy; whereas acquired resistance occurs when cancers that have been responding to a therapy suddenly begin to grow.

In both cases the overexpression of some genes can confer chemotherapy and/or radiotherapy resistance to cancer cells. Strategies that target the transcription and/or translation of said genes can be useful to fight against cancer therapy resistance.

Cancer therapy resistance can arise through a number of different mechanisms, including alterations in drug pharmacokinetics and metabolism, modification of drug target expression or function (for example, gene amplification/over-expression, over-expression of β-tubulin isotypes, and topoisomerase II mutations), drug compartmentalization in cellular organelles, altered repair of drug-induced DNA damage, changes in apoptotic signaling pathways (for example, mutated p53), and expression of proteins directly affecting cellular drug transport (efflux pumps). The heterogeneity of cancer cells, coupled with their high mutation rate, contributes to rapid selection for drug-resistant clones.

One of said mechanisms is the gene amplification, when a cancer cell may produce hundreds of copies of a particular gene that triggers an overproduction of protein that renders the anticancer drug ineffective. The oligonucleotides of the invention directed to said amplified target gene are useful in treating and/or preventing the development of resistance to chemotherapy and/or radiotherapy caused by gene over-expression.

In a preferred embodiment the cancer to be treated and/or prevented is a cancer resistant to chemotherapy and/or radiotherapy.

A “cancer resistant” is a cancer that does not respond to treatment with conventional chemotherapy and/or radiotherapy, either due to intrinsic resistance or to acquired resistance.

The term “chemotherapy” refers to antineoplastic drugs used to treat cancer or the combination of these drugs into a cytotoxic standardized treatment regimen. In the context of the present invention, the term chemotherapy comprises any antineoplastic agent including small sized organic molecules, peptides, oligonucleotides and such like
5 used to treat any kind of cancer as well as related processes such as angiogenesis or metastasis. Drugs included in the definition of chemotherapy are, without limitation, alkylating agents such as nitrogen mustards/oxazaphosphorines (e.g. cyclophosphamide, ifosfamide), nitrosoureas (e.g. carmustine), triazenes (e.g.temozolamide), and alkyl sulfonates (e.g. busulfan); anthracycline antibiotics such as doxorubicin and
10 daunorubicin, taxans such as Taxol™ and docetaxel, vinca alkaloids such as vincristin and vinblastine, 5-fluorouracyl (5-FU), leucovorin, irinotecan, idarubicin, mitomycin C, oxaliplatin, raltitrexed, pemetrexed, tamoxifen, cisplatin, carboplatin, methotrexate, actinomycin D, mitoxantrone, blenoxane, mithramycin, methotrexate, paclitaxel, 2-methoxyestradiol, prinomastat, batimastat, BAY 12-9566, carboxyamidotriazole, CC-
15 1088, dextromethorphan acetic acid, dimethylxanthenone acetic acid, endostatin, IM-862, marimastat, penicillamine, PTK787/ZK 222584, RPI.4610, squalamine lactate, SU5416, thalidomide, combretastatin, tamoxifen, COL-3, neovastat, BMS-275291, SU6668, anti-VEGF antibodies, Medi-522 (Vitaxin II), CAI, Interleukin 12, IM862, amiloride, angiostatin, angiostatin Kl-3, angiostatin Kl-5, captopril, DL-alpha-
20 difluoromethylornithine, DL-alpha-difluoromethylornithine HCl, endostatin, fumagillin, herbimycin A, 4-hydroxyphenylretinamide, juglone, laminin, laminin hexapeptide, laminin pentapeptide, lavendustin A, medroxyprogesterone, minocycline, placental ribonuclease inhibitor, suramin, thrombospondin, antibodies targeted against proangiogenic factors (for example, Avastin, Erbitux, Vectibix, Herceptin);
25 topoisomerase inhibitors; antimicrotubule agents; low molecular weight tryrosine kinases inhibitors of proangiogenic growth factors (for example Tarceva, Nexavar, Sutent, Iressa); GTPase inhibitors; histone deacetylase inhibitors; AKT kinase or ATPase inhibitors; Wnt signaling inhibitors; inhibitors of the E2F transcription factor; mTOR inhibitors (for example Torisel); alpha, beta and gamma interferon, IL-12, matrix
30 metalloproteinase inhibitors (for example, COL3, Marimastat, Batimastat); ZD6474, SUl1248, vitaxin; PDGFR inhibitors (for example Gleevec); NM3 and 2- ME2; cyclic peptides such as cilengitide. Other chemotherapy agents suitable are described in detail in The Merck Index in CD-ROM, 13rd Edition.

It is known that DHFR is involved in the establishment of resistances to chemotherapy treatment, particularly to methotrexate. Therefore, in a particular embodiment the cancer to be treated and/or prevented is a cancer resistant to methotrexate.

5

"Methotrexate" or "MTX", formerly known as amethopterin, is an antimetabolite and antifolate drug used in the treatment of cancer and autoimmune diseases. It acts by inhibiting the metabolism of folic acid. Specifically, methotrexate competitively inhibits dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate
10 synthesis that is needed for the de novo synthesis of the nucleoside thymidine, for purine base synthesis. Methotrexate, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins.

Methotrexate is a common drug used in the treatment of lymphoma, choriocarcinoma
15 and acute lymphocytic leukaemia. In addition, methotrexate is an important component of many combination chemotherapy regimes used in metastatic breast, advanced bladder and gastric cancer. It also plays a role in the adjuvant regime with cyclophosphamide, methotrexate and 5-fluorouracil for operable breast cancer (Mellor, H. R and Callaghan, R. 2008. Pharmacology, 81:275-300). However, methotrexate therapy is associated with
20 drug resistance, and *in vitro* studies have revealed that numerous mechanisms may be responsible for the phenotype. One of said mechanisms is the amplification of the target gene DHFR. The gene amplification ultimately results in elevated expression of DHFR, and this is frequently observed in cells selected for drug resistance in the presence of methotrexate. Increased expression of the enzyme will necessitate administration of
25 higher methotrexate doses to ensure that the concentration of tetrahydrofolate is maintained at a sufficiently low level.

Genes that are known to be over-expressed in cancer cells resistant to methotrexate are cytochrome c oxidase (Alemany, C. *et al.* 2000. Biochim Biophys Acta, 1495(3):319-26), survivin (Peñuelas, S. *et al.* 2005. FEBS J. 272(3):696-710), caveolin-1, enolase-2
30 and PKCalpha (Selga, E. *et al.* 2008. BMC Med Genomics, 1:35), Dikkopf homolog-1 (DKK1), eukaryotic translation elongation factor-1 alpha-1 (EEF1A1) and genes from the UDP-glucuronosyltransferase 1A (UGT1A) family (Selga, E. *et al.* 2009. Genome

Med, 1(9):83). Inhibitors of the products of said genes, antisense or siRNAs against these genes effectively reduced cell viability and caused a decreased methotrexate resistance capacity.

In a preferred embodiment, the target gene of the oligonucleotide of the invention is a
5 gene involved in the resistance to methotrexate selected from cytochrome c oxidase, survivin, caveolin-1, enolase-2, PKCalpha, DKK1, EEF1A1, UGT1A (preferably, UGT1A6) and DHFR; more preferably DHFR.

In a more preferred embodiment the resistance to MTX is due to the amplification of the dhfr gene.

10

The term “radiotherapy”, as used herein, refers to the medical use of ionizing radiation as part of cancer treatment to control malignant cells. The term radiotherapy comprises any radiotherapy agent such as ^{131}I and beta particle emitters such as ^{90}Y.

15 Examples of genes that mediate chemotherapy and radiotherapy resistance to fluoropyrimidines, taxanes and platinum compounds are shown in Table 1.

Molecular Function	Gene or Biomarker of Resistance	Drugs Affected
ATP-binding transporters	ABCB1	Platinum, taxanes
	ABCC1-6	Taxanes
	ABCG2 (BCRP)	Fluorouracil
	ABCC11	
Drug metabolism	MTDHFR, TPMT	Fluorouracil
	CYP1A1-A2, CYP2B6, CYP2C8, CYP2C9-19, CYP2E1, CYP3A4-A5, NAT, SOD1, SULT1E1, GSTP1, GSK3A, EPHX1,	All three drug classes
Drug target	TS, TK	Fluorouracil
	MAPT, STMN1, MAP4	Taxanes
DNA repair	ERCC1, ATM, APC	Platinum, Fluorouracil
	BRCA1, BRCA2	All three drug classes
	ERCC3-4, XPA, XPC, MSH2	Platinum***
Cell cycle and checkpoints	CCND1, CCNE, CDK2, CDK4, CDKNs, TP53	All three drug classes
Apoptosis	Survivin, BAX, BLC2, BCL2L1, TOP1, TOP2A-2B, p53	All three drug classes
Transcription factors	NFKB1, Rel-b, TRIP9, TNFRSF11a (Rank), FOS, ELK1, HIF1A, MYC, AHR and AP1S1	All three drug classes
Growth factor receptors	ERBB1 (EGFR), ERBB2 (Her-2), ERBB3 and 4, IGF1R, IGF2R, MET	All three drug classes
Nuclear receptors	AR, ESR1, ESR2, PPARA, PPARD, PPARG, RAR, RARE	All three drug classes
Miscellaneous	SNGG	Taxanes

Table 1. Genes that mediate chemotherapy and radiotherapy resistance (Ajani, J. A. *et al.* 2009. Journal of Clinical Oncology, 27:162-163).

The methotrexate is used not only in the treatment of cancer but also to treat and/or
5 prevent other diseases such as rheumatoid arthritis, inflammatory bowel disease, graft-versus-host disease, aborts and psoriasis (Banerjee, D.E. *et al.*, 1995. Acta Biochim Pol, 42: 457-64; Schmiegelow, K. 2009. Br J Haematol, 146:489-503).

In another particular embodiment the disease to be treated and/or prevented is a disease
10 associated with inflammation.

The expression "disease associated with inflammation" relates to all those diseases where pathogenic inflammation occurs i.e. when said process is harmful or undesirable, whether cancerous or not. Diseases associated with inflammation include inflammatory
15 diseases, where there is an excessive or altered inflammatory response that leads to inflammatory symptoms. Said inflammatory diseases which may be treated by the oligonucleotides of the invention include, without limitation, Addison's disease, acne

vulgaris, alopecia areata, amyloidosis, ankylosing spondylitis, ulcerations, aphthous stomatitis, arthritis, arteriosclerosis, osteoarthritis, rheumatoid arthritis, bronchial asthma, Bechet's disease, Boeck's disease, intestinal inflammatory disease, Crohn's disease, choroiditis, ulcerative colitis, celiac's disease, cryoglobulinemia, macular degeneration, dermatitis, dermatitis herpetiformis, dermatomyositis, insulin dependent diabetes, juvenile diabetes, inflammatory demyelinating disease, Dupuytren contracture, encephalomyelitis, allergic encephalomyelitis, endophthalmia, allergic enteritis, autoimmune enteropathy syndrome, erythema nodosum leprosum, ankylosing spondylitis, idiopathic facial paralysis, chronic fatigue syndrome, rheumatic fever, cystic fibrosis, gingivitis, glomerulonephritis, Goodpasture syndrome, Graves syndrome, Hashimoto's disease, chronic hepatitis, histiocytosis, regional ileitis, iritis, disseminated lupus erythematous, systemic lupus erythematous, cutaneous lupus erythematous, lymphogranuloma, infectious mononucleosis, miastenia gravis, transverse myelitis, primary idiopathic myxedema, nephrosis, obesity, sympathetic ophthalmia, granulomatous orchitis, pancreatitis, panniculitis, pemphigus vulgaris, periodontitis, polyarteritis nodosa, chronic polyarthritis, polymyositis, acute polyradiculitis, psoriasis, chronic obstructive pulmonary disease, purpura, gangrenous pioderma, Reiter's syndrome, diabetic retinopathy, rosacea, sarcoidosis, ataxic sclerosis, progressive systemic sclerosis, scleritis, sclerodermia, multiple sclerosis, disseminated sclerosis, acute anterior uveitis, vitiligo, Whipple's disease, diseases associated with AIDS, severe combined immunodeficiency and Epstein Barr's virus such as Sjögren's syndrome, osteoarticular tuberculosis and parasitic diseases such as leishmaniasis. Preferred inflammatory diseases are rheumatoid arthritis, arteriosclerosis, psoriasis, inflammatory bowel disease and graft-versus-host disease.

The expression "Alzheimer's disease" refers to the most common form of dementia and is also called Alzheimer disease, senile dementia of the Alzheimer type, primary degenerative dementia of the Alzheimer's type or simply Alzheimer's. Alzheimer's disease (AD) is an age-related, non-reversible brain disorder that develops over a period of years. Initially, people experience memory loss and confusion, which may be mistaken for the kinds of memory changes that are sometimes associated with normal aging. However, the symptoms of AD gradually lead to behavior and personality changes, a decline in cognitive abilities such as decision-making and language skills, and

problems recognizing family and friends. AD ultimately leads to a severe loss of mental function. These losses are related to the worsening breakdown of the connections between certain neurons in the brain and their eventual death. AD is one of a group of disorders called dementias that are characterized by cognitive and behavioral problems. It is the most common cause of dementia among people age 65 and older. There are three major hallmarks in the brain that are associated with the disease processes of AD: amyloid plaques, neurofibrillary tangles (NFTs) and loss of connections between neurons responsible for memory and learning. The disease course is divided into 4 stages with progressive patterns of cognitive and functional impairments: pre-dementia, early, moderate and advanced. The oligonucleotides of the invention may be used in any stage of Alzheimer's disease.

The expression "atherosclerosis" refers to a condition in which an artery wall thickens as the result of a build-up of fatty materials such as cholesterol. It is a syndrome affecting arterial blood vessels, a chronic inflammatory response in the walls of arteries, in large part due to the accumulation of macrophage white blood cells and promoted by low-density lipoproteins. It is caused by the formation of multiple plaques within the arteries and is also known as arteriosclerotic vascular disease or ASVD.

The term "subject" in the present invention is understood as any animal classified as mammal and includes but is not limited to domestic and farm animals, primates and humans, for example human beings, non-human primates, cows, horses, pigs, sheep, goats, dogs, cats or rodents. Preferably, the subject is a female or male human being of any race or age. In the context of the present invention, the subject is a subject who potentially suffers from cancer, a disease associated with inflammation, Alzheimer's disease or atherosclerosis or has been previously diagnosed with cancer, a disease associated with inflammation, Alzheimer's disease or atherosclerosis.

The term "administration" refers to the delivery of a pharmaceutical drug to a patient. It can be performed in various dosage forms and routes of administration as has been described in the context of the pharmaceutical compositions of the invention.

The expression "pharmaceutically effective amount" has been described previously in the context of the pharmaceutical compositions of the invention.

In an embodiment of the present invention the medicament comprises one or more oligonucleotides according to the invention as sole therapeutic agent. Nevertheless, the medicament of the invention may also contain one or several additional compounds for the treatment of cancer or of a disease associated with inflammation or of Alzheimer's disease or of atherosclerosis; preferably for the treatment of cancer or of a disease associated with inflammation. Therefore, in another embodiment of the present invention, the medicament is prepared for the combined administration of an oligonucleotide according to the invention and one or more therapeutic agents useful in the treatment of said diseases.

The term "therapeutic agent useful in the treatment of said diseases" relates to an agent suitable for being used in the treatment of cancer or of a disease associated with inflammation or of Alzheimer's disease or of atherosclerosis.

Agents that may be used in combination with the oligonucleotides of the invention for the treatment of cancer include, without limitation, all the agents mentioned before as "chemotherapy". In a particular embodiment the agent with which the oligonucleotides of the invention are combined is methotrexate.

Agents that may be used in combination with the oligonucleotides of the invention for the treatment of a disease associated with inflammation are any anti-inflammatory drug known by the skilled in the art.

Agents that may be used in combination with the oligonucleotides of the invention for the treatment of Alzheimer's disease are, without limitation, acetylcholinesterase inhibitors such as tacrine, donepezil, rivastigmine, galantamine; or N-methtyl-D-aspartate receptor antagonists such as memantine.

Agents that may be used in combination with the oligonucleotides of the invention for the treatment of atherosclerosis are, without limitation, statins such as rosuvastatin,

atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, simvastatin; ezetimibe; niacin; amlodipine besylate; fibrates such as bezafibrate, ciprofibrate, clofibrate, gemfibrozil, fenofibrate; and acetylsalicylic acid.

5 “Combined administration” is understood to mean that the oligonucleotide according to the invention may be administered jointly or separately, simultaneously, at the same time or sequentially with a therapeutic agent useful in the treatment of the pathologies previously mentioned in any order. For example, the administration of the oligonucletotide of the invention may be performed first, followed by the administration
10 of one or more therapeutic agents useful in the treatment of said pathologies; or the administration of the oligonucleotide of the invention may be performed at the end, preceded by the administration of one or more therapeutic agents useful in the treatment of said pathologies; or the administration of the oligonucleotide of the invention may be performed at the same time as the administration of one or more therapeutic agents
15 useful in the treatment of said pathologies.

The person skilled in the art shall understand, in the context of the present invention, that the medicament for the combined administration of an oligonucleotide according to the invention and an additional therapeutic agent useful in the treatment of said diseases may
20 be prepared as a single dosing form or in separate dosing forms.

In a particular embodiment the oligonucleotide used is the oligonucleotide having a sequence SEQ ID NO: 2.

25 NON-THERAPEUTIC USES OF THE CODING POLYPURINE HAIRPINS

Another aspect of the invention is a non-therapeutic method for the decrease *in vitro* or *in vivo* of the expression of a target gene in a biological system comprising putting said system in contact with a composition according to the invention or with an
30 oligonucleotide according to the invention. The biological model is preferably a cell culture or an animal.

In the case of compositions and oligonucleotides suitable for their application on a cell

culture, there are different procedures for achieving that the oligonucleotides access the inside of the cell. On the one hand, it is possible to put the cells in contact with the naked oligonucleotides. Putting the cells in contact with the oligonucleotides, although it allows the access to the endosomal compartment, does not allowing reach the cytoplasm in functional state (Lingor, P. *et al.*, 2004, Biochem. Biophys. Res. Commun., 315:1123-1133). Therefore, the oligonucleotides are preferably administered by means of microinjection or electroporation. Alternatively, it is possible to use chemical transfection using suitable reagents for the transfection of DNA polynucleotides, particularly, cationic liposomes, peptides permeable to membranes, atelocollagen, hybrid organic-inorganic nanoparticles formed by polymers of polyaspartic polyethylene glycol with calcium phosphate (Kakizawa *et al.*, 2004; J. Control Rel., 97:345-356; Minakuchi *et al.*, 2004, Natl. Acids Res., 32:109 and Muratovska and Eccles, 2004, Fed. Euro. Biochem. Soc. Lett., 558:63-68).

Alternatively, it is possible to modify the oligonucleotides forming the composition of the invention with chemical moieties that allow an increase in their capture by cells. Thus, the oligonucleotides of the invention can be conjugated with different types of compounds such as peptides and organic compounds. The conjugation can be carried out by means of methods known to a person skilled in the art, including the methods of Lambert *et al.*, (Drug Deliv., 2001, Rev.: 47:99-112), in which nucleic acids are coupled to nanoparticles of polyalkylcyanoacrylate (PACA); Fattal *et al.*, (J. Control Release 1998, 53:137-143) which describes nucleic acids coupled to nanoparticles; Schwab *et al.*, (Ann. Oncol., 1994, 5 Suppl. 4:55-58) which describes nucleic acids unique to intercalating agents, hydrophobic groups, polycations or PACA nanoparticles and Godard *et al.*, (Eur. J. Biochem., 1995, 232:404-410) which describes nucleic acids coupled to nanoparticles.

In another embodiment, the oligonucleotides of the invention are coupled to lipophilic moieties which can include a cationic group and can be associated with the expression silencing-agent. Suitable lipophilic agents include cholesterol, vitamin E, vitamin K, vitamin A, folic acid, a cationic coloring (for example Cy3), cholic acid, acetic adamantine, 1-pyrenebutyric acid, dihydrotestosterone, 1,3-bis-O (hexadecyl) glycerol, a geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol,

heptadecyl, palmitic acid, myristic acid, O3-(oleoyl) lithocholic acid, 03-(oleoyl) cholenic acid, dimethoxytrityl or phenoxazine.

The effects of the decrease in expression can be observed by means of the detection of phenotype changes or by means of the use of biochemical techniques allowing the detection of changes in the expression levels of a certain mRNA or of the proteins encoded by it, such as RNA hybridization, nuclease protection, Northern hybridization, monitoring of genetic expression by means of DNA microarrays, Western blotting, RIA, ELISA and FACS. In animal or cultured cell models, it is possible to detect the modification of the expression of a reporter gene or a gene of resistance to a toxic agent the products of which are easily detectable. Suitable reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatise (AP), beta galactosidase (LacZ), beta glucuronidase (GUS), chloramphenicol acetyltransferase (CAT), fluorescent green protein (GFP), horse-radish peroxidise (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS) and the derivatives thereof. Suitable selection markers include genes encoding resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinotricin, puromycin and tetracycline.

Alternatively, the non-therapeutic method for decreasing gene expression can be applied to an animal model in which the gene the expression of which is to be decreased is expressed. In this case, it is necessary for the oligonucleotide of the invention to access the tissue or organ in the animal in which a decrease of gene expression is to be caused. To that end, any of the previously described methods for the therapeutic administration of the agents of the invention to a patient can be used. In a preferred embodiment, the invention contemplates the administration of compositions targeted against different target pre-mRNAs encoding proteins involved in the same functional pathway. Functional pathways that can be the object of inhibition by means of the use of combinations of compositions of the invention including, but not necessarily limited to, glucolysis, gluconeogenesis, Krebs cycle, the pentose phosphate pathway, synthesis of glycogen, Calvin’s cycle, degradation of triacylglycerides, activation of fatty acids, beta oxidation, de novo synthesis of fatty acids, synthesis of cholesterol, urea cycle, shikimate pathway, synthesis of amino acids, oxidative phosphorylation,

photosynthesis, synthesis of purines, synthesis of pyrimidines, histidine metabolism, porphyrin metabolism, inositol metabolism, and the like. A person skilled in the art will appreciate that the different steps of the aforementioned pathways as well as the enzymes responsible for each of said stages are known, therefore the development of
5 oligonucleotides specific for one or several genes involved in the steps of said process can be carried out by means of routine experiment.

USES OF THE CODING POLYPURINE HAIRPINS FOR DETERMINING GENE AMPLIFICATION

10

Since the oligonucleotides of the invention can bind to a polypirimidine target sequence located in the coding strand of an intronic region of a target gene and also to the pre-mRNA encoded by said gene, said oligonucleotides can be used for the determination of the number of copies of a gene of interest as well as for determining the level of
15 transcription of a gene of interest. Moreover, the oligonucleotides of the present invention may also be used for the diagnosis of said diseases where a target gene is over-expressed, either due to gene amplification or due to increased transcription, such as cancer, a disease associated with inflammation, Alzheimer's disease or atherosclerosis wherein an increase in the copy number of a gene occurs or wherein an
20 increase in the transcription of a given gene occurs.

Therefore, another aspect of the invention is related to an *in vitro* method for detecting gene amplification of a target gene in a sample containing genomic DNA comprising measuring the number of copies of a target gene in said sample by using an
25 oligonucleotide according to the invention.

Gene amplification is a common event in some type of cancers and the detection of gene copy number alterations has been used in cancer diagnosis (Horbinski, C. *et al.* 2011. Brain Pathol, 21:57-73) and also in the determination of drug resistance.

30

Therefore, another aspect of the invention is an *in vitro* method for the diagnosis of cancer or of drug resistance to chemotherapy and/or to radiotherapy associated to the amplification of a given gene in a subject comprising:

(a) measuring in a sample from said subject the number of copies of said gene by using an oligonucleotide according to the invention wherein said oligonucleotide is specific for an intronic region of said gene and

(b) comparing said number of copies with a reference value

5 wherein an increase in the number of copies in said sample with respect to the reference value is indicative of the subject suffering from cancer or drug resistance.

In the context of the present invention "*in vitro* method for detecting gene amplification" is understood as a method which allows showing the existence of 10 multiple copies of a particular gene in a subject by means of quantifying the copy number of said particular gene in a biological sample isolated from the patient.

As used herein, the term "gene amplification" refers to a process by which specific DNA sequences are disproportionately replicated such that the amplified gene becomes 15 present in a higher copy number than was initially present in the genome.

In the context of the present invention "*in vitro* method for the diagnosis of cancer or of drug resistance" is understood as a method which allows showing the existence of a cancer or of drug resistance in a subject by means of quantifying the number of copies 20 of a target gene associated with said diseases in a biological sample isolated from the patient.

The first step of the method of the invention comprises determining the number of copies of a target gene in a sample from the subject by using an oligonucleotide 25 according to the invention.

The term "sample" in the context of the present invention refers to any sample which can be obtained from the subject. The present method can be applied to any kind of biological sample from a subject, such as a biopsy sample, tissue, cell or fluid (serum, 30 saliva, semen, sputum, cerebral spinal fluid (CSF), tears, mucus, sweat, milk, brain extracts and the like). In a particular embodiment said sample is a tissue sample. Said samples can be obtained by conventional methods using processes known in the state of the art by the person skilled in the art, such as biopsy extraction. Methods for obtaining

the sample from the biopsy include gross apportioning of a mass, or microdissection or other art-known cell-separation methods. Cells can additionally be obtained from fine needle aspiration cytology. In order to simplify conservation and handling of the samples, these can be formalin-fixed and paraffin-embedded or first frozen and then embedded in a cryosolidifiable medium, such as OCT-compound, through immersion in a highly cryogenic medium that allows for rapid freeze.

In order to measure copy number of the target gene, the biological sample may be treated to physically or mechanically disrupt tissue or cell structure, to release intracellular components into an aqueous or organic solution to prepare nucleic acids for further analysis. The nucleic acids are extracted from the sample by procedures known to the skilled person and commercially available. DNA is then extracted from frozen or fresh samples by any of the methods typical in the art, for example, Sambrook, J., et al., 2001. Molecular cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, N.Y., Vol. 1-3.

Samples can also be formalin-fixed, paraffin-embedded tissue samples.

Suitable techniques for use in gene amplification detection are Southern blot, fluorescence *in situ* hybridization (FISH), chromogenic *in situ* hybridization (CISH), tissue microarrays, microarray-based comparative genomic hybridization (CGH) or microfluidic techniques for DNA analysis. The detection can be carried out in individual samples or in tissue microarrays.

In addition, the oligonucleotides used in the method of the invention may or may not be labeled with a detectable agent. In a particular embodiment the oligonucleotide used is conjugated to a detectable agent.

In the context of the present invention, the terms “detectable agent” and “labeling” are synonyms and they refer to an agent the nature of which allows its detection by means of enzymatic, radioactive or fluorescence methods. The detectable compound can be an enzyme, a radioactively labeled compound or a radioactive isotope, a fluorochrome, a

chemiluminescent reagent, an enzymatic substrate or cofactor, an enzymatic inhibitor, a particle, a dye, etc.

The compounds radioactively labeled by means of radioactive isotopes, also called
5 radioisotopes or radionuclides, may include, without limitation, ^{3}H, ^{14}C, ^{15}N, ^{35}S, ^{90}Y, ^{99}Tc, ^{111}In, ^{125}I, ^{131}I. The fluorescent labels may include, without limitation, rhodamine, phosphorus-lanthanides or FITC. The enzymatic labels may include, without limitation, horseradish peroxidase, β-galactosidase, luciferase or alkaline phosphatase. The preferred labeling include, but are not limited to, fluorescein, a
10 phosphatase such as alkaline phosphatase, biotin, avidin, a peroxidase such as horseradish peroxidase and compounds related to biotin or compounds related to avidin (for example, streptavidin or ImmunoPure® NeutrAvidin available from Pierce, Rockford, IL).

15 Once the number of copies in a sample has been determined, step (b) of the invention which consists of comparing said number with a reference value takes place.

The “reference value” derives from a sample collection formed preferably by a mixture of the sample to be analyzed from normal individuals not affected by the gene
20 amplification. Said reference value can be determined by means of techniques well known in the state of the art, for example, determining the mean of the number of gene copies measured in samples taken from healthy subjects.

Once the reference value is established, the gene copy number obtained in step (a) can be
25 compared with this reference value and, therefore, allows detecting alterations in the gene copy number of the subject with respect to the reference value. More specifically, in the method of the invention, an increase in the number of copies is indicative of gene amplification. If the method is an *in vitro* method for the diagnosis of cancer or of drug resistance, an increase in the number of copies of the gene tested with respect to the
30 reference value is indicative of the subject suffering from cancer or suffering from drug resistance.

In the context of the present invention, “an increase in the number of copies” with respect to the reference value is understood as a variation of the number of copies of the target gene tested above the reference value of at least 1 times, 2 times, 5 times, 10 times, 20 times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times, 100 times
5 or even more times as compared to the reference value.

Therefore, once said comparison has been performed, the method of the invention allows detecting gene amplification of a target gene or allows diagnosing if the subject suffers from cancer or from drug resistance.
10

Exemplary suitable genes for which copy number is determined or scored is a gene which is amplified (for example, has an increased copy number, such as copy number greater than about 2), for example, in a disease state, such as cancer. Examples of genes for which copy number may be scored by the methods disclosed herein include genes
15 which are known to be amplified in cancer (such as NSCLC, breast cancer, head and neck cancer, gastric cancer, or colorectal cancer). Examples include, but are not limited to IGFlR (15q26.3; e.g., GENBANK™ Accession No. NC_000015, nucleotides 97010284-97325282), EGFR (7pl2; e.g., GENBANK™ Accession No. NC_000007, nucleotides 55054219-55242525), HER2 (17q21.1; e.g., GENBANK™ Accession No.
20 NC_000017, nucleotides 35097919-35138441), C-MYC (8q24.21; e.g., GENBANK™ Accession No. NC_000008, nucleotides 128817498-128822856), TOP2A (17q21-q22; e.g., GENBANK™ Accession No. NC_000017, complement, nucleotides 35798321-35827695), MET (7q31; e.g., GENBANK™ Accession No. NC 000007, nucleotides 116099695-116225676), FGFRl (8pl 1.2-pl 1.1; e.g., GENBANK™ Accession No.
25 NC_000008, complement, nucleotides 38387813- 38445509), FGFR2 (10q26; e.g., GENBANK™ Accession No. NC_000010, complement, nucleotides 123227845-123347962), MDM2 (12ql4.3-ql5; e.g., GENBANK™ Accession No. NC_000012, nucleotides 67488247-67520481), KRAS (12pl2.1; e.g. GENBANK™ Accession No. NC_000012, complement, nucleotides 25249447-25295121), and TYMS (18pl 1.32; e.g.,
30 GENBANK™ Accession No. NC_000018, nucleotides 647651-663492).

Exemplary suitable genes for which copy number is determined or scored is a gene which is amplified (for example, has an increased copy number, such as copy number

greater than about 2), for example, in a subject showing resistance to a given therapy (chemotherapy or radiotherapy). Examples of genes for which copy number may be scored by the methods disclosed herein include those described above in Table 1.

5

KITS OF THE INVENTION AND USES THEREOF

In another aspect, the invention relates to a kit for detecting gene amplification in a sample from a subject which comprises at least one oligonucleotide according to the first
10 aspect of the invention.

In another aspect, the invention relates to the use of a kit as previously defined for detecting gene amplification in a sample from a subject which comprises at least one oligonucleotide according to the first aspect of the invention.

15

In another aspect, the invention relates to a kit for diagnosing a disease selected from cancer, a disease associated with inflammation, Alzheimer's disease and atherosclerosis or for diagnosing resistance to chemotherapy and/or radiotherapy in a sample from a subject which comprises at least one oligonucleotide according to the first aspect of the
20 invention. In a particular embodiment, the disease is cancer, preferably breast cancer. In another embodiment, the kit is suitable for diagnosing resistance to chemotherapy and/or radiotherapy.

In another aspect, the invention relates to the use of a kit as previously defined for
25 diagnosing a disease selected from cancer, a disease associated with inflammation, Alzheimer's disease and atherosclerosis or for diagnosing resistance to chemotherapy and/or radiotherapy in a sample from a subject. In a particular embodiment, the disease is cancer, preferably breast cancer. In another embodiment, the kit is suitable for diagnosing resistance to chemotherapy and/or radiotherapy.

30

The term "kit", as used in the present document, refers to a combination of a set of reagents suitable for detecting gene amplification of a target gene together with one or more types of elements or components (for example, other types of biochemical reagents,

containers, packaging suitable for its commercial sale, substrates to which the reagents are bound, electronic hardware components, etc.).

In the present invention, “reagent suitable for detecting gene amplification” is understood
5 as a specific oligonucleotide of the invention and, optionally, reagents for detecting one or more constitutive gene.

As it will be understood by the person skilled in the art, the oligonucleotides of the kit of the invention can be used in all the techniques for determining gene amplification known
10 to be suitable for the analysis of a sample, such as techniques based on the use of biochips, DNA microarrays, microfluidics, FISH,, Southern blot, etc.

The oligonucleotides can be fixed to a solid support such as a membrane, a plastic or a glass, optionally treated to facilitate the fixation of said oligonucleotides to the support.
15 Said solid support comprises, at least, a set of oligonucleotides which specifically recognize the DNA in study, and which can be used for detecting gene amplification.

The kits of the invention additionally comprise reagents for detecting a constitutive gene. The availability of said additional reagents allows normalizing the measurements
20 performed in different samples (for example, the sample to be analyzed and the control sample) to rule out that the differences in the expression of the biomarkers are due to a different quantity of genes in the sample more than the real differences in the relative levels of expression. The constitutive genes in the present invention are genes that are always active or being transcribed constantly and which encode for proteins that are
25 expressed constitutively and carry out essential cellular functions. Genes that are expressed constitutively and can be used in the present invention include, without limitation, β-2-microglobulin (B2M), ubiquitin, 18-S ribosomal protein, cyclophilin, GAPDH, PSMB4, tubulin and actin.

30 All the particular embodiments of the method of the present invention are applicable to the kits of the invention and to their uses.

The invention is described below by the following examples, which must be considered

as merely illustrative and in no case limiting of the scope of the present invention.

EXAMPLES

5 MATERIALS AND METHODS

Design and usage of PPRHs

The PPRHs used in this study were made up of two antiparallel polypurine domains, bound by intramolecular reverse-Hoogsteen bonds and linked by a pentathymidine loop. 10 The Triplex-Forming Oligonucleotide Target Sequence Search software (M.D. Anderson Cancer Center, Houston, TX) (spi.mdanderson.org/tfo/) was used to find the polypyrimidine stretches in the target sequence. BLAST analyses were performed to check for the specificity of all these sequences. PPRHs were synthesized as non-modified oligodeoxynucleotides by Sigma-Aldrich (Madrid, Spain) (0.05 μmol scale). 15 All concentrations were expressed in strand molarity. PPRHs were dissolved in sterile RNase-free Tris-EDTA buffer (1 mM EDTA and 10 mM Tris, pH 8.0) and stored at – 20°C until use.

Preparation of polypurine/ polypyrimidine duplexes

20 The duplexes to be targeted by the hairpins were formed by mixing 25 μg of each single-stranded (ss) polypurine and polypyrimidine oligodeoxynucleotide with 150 mM NaCl and incubated at 90°C for 5 minutes as described by de Almagro *et al.* (de Almagro, M.C. *et al.* 2009. J Biol Chem, 284:11579-11589).

25 **Oligodeoxynucleotide labeling**

One hundred nanograms of single or double-stranded oligodeoxynucleotides or RNA was 5'-end-labeled with [γ-^{32}P]-ATP as described by de Almagro *et al.* (de Almagro, M.C. *et al.* 2009. J Biol Chem, 284:11579-11589).

30 **DNA-PPRH and RNA-PPRH binding analysis**

Triplex formation was analyzed by incubating radiolabeled single or double-stranded DNA or RNA targets (2 nM strand concentration; 20,000 cpm) in the presence or absence of unlabeled PPRHs (250 nM strand concentration) in a buffer containing 10

mM $MgCl_2$, 100 mM NaCl and 50 mM HEPES, pH 7.2. Binding reactions (20 μl) were incubated for 45 minutes at 37°C before electrophoresis, which was performed on a nondenaturing 12% polyacrylamide gel containing 10 mM $MgCl_2$, 5% glycerol, and 50 mM HEPES, pH 7.2. Gels were electrophoresed for 3-4 hr at 190V at 4°C, dried, and
5 analyzed on a Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Quantification was performed using ImageQuant version 5.2 software (Molecular Dynamics). Binding specificity was tested by addition of 2 μg of tRNA (Sigma Aldrich) to the binding reaction.

10 **Plasmid construction**

The Chinese hamster *dhfr* minigene construct pDCHIP has been described previously (Ciudad, C.J. *et al.* 1988. J Biol Chem, 263:16274-16282). It contains the six exons of the gene, intron-1, about 900 bp of the 5' flank, and the first of the two major polyadenylation sites in exon-6. Plasmid pDCHIP-Hp was constructed by cloning the
15 Coding-PPRH target sequence (Fig. 1b or Table 1), present in the intron-3 of the human *dhfr* gene, into the unique *Pst*I site in intron-1 of pDCHIP. This 31-nucleotide sequence contained a *Not*I site close to its 5' end, to select the plasmids containing the insert. The number of inserts cloned was measured by polymerase chain reaction (PCR) product size, and a plasmid containing just one insert was selected.

20

Cell culture

SKBR3 breast cancer cells were grown in Ham's F-12 medium containing 7% fetal bovine serum (GIBCO, Invitrogen, Barcelona, Spain) and incubated at 37°C in a humidified 5% CO_2 atmosphere. MCF7-R breast cancer cells resistant to 10^{-6} M
25 methotrexate (MTX) were previously generated in our laboratory (Selga, E. *et al.* 2009. Genome Med, 1:83) and bear amplification of the *dhfr* locus. This cell line was grown in Ham's F-12 medium lacking the final products of DHFR activity (glycine, hypoxanthine and thymidine [-GHT medium]) (Chasin, L. 1985. The dihydrofolate reductase locus. In *Molecular Cell Genetics*. M. Gottesman, ed. J. Wiley, New York.
30 pp. 449-488) containing 7% dialyzed fetal bovine serum (GIBCO, Invitrogen). pDCHIP and pDCHIP-Hp plasmids were stably transfected into *dhfr*$^{-}$ DG44 Chinese hamster ovary cells to obtain the DCHM1 and DCHM1-Hp cell lines, respectively. In brief, 500,000 *dhfr*$^{-}$ DG44 cells were transfected with 1 μg of plasmid pDCHIP or pDCHIP-

Hp or with unspecific calf DNA. Five hours after the calcium phosphate transfection the medium was changed, and 24 hours later the selective medium -GHT was added. Transfectants were selected for survival in -GHT medium. These cell lines were used for specificity studies.

5

Transfection

SKBR3, MCF7-R, DCHM1, and DCHM1-Hp cells were plated in 35-mm-diameter dishes. PPRHs were mixed with N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) (Roche, Mannheim, Germany) at the

10 appropriate oligonucleotide-DOTAP molar ratio (1:10-1:100) for 15 min at room temperature before lipofecting the cells.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

SKBR3, MCF7-R, DCHM1, and DCHM1-Hp cells (10,000) were plated in 35-mm-

15 diameter dishes in -GHT medium. After 7 days, 500 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 5.6 mg of succinate (both from Sigma-Aldrich) were added to the culture medium and allowed to react for 3 hours at 37°C before addition of the solubilization reagent (0.57% acetic acid and 10% sodium dodecyl sulfate in dimethyl sulfoxide). Cell viability was measured at 570 nm in a WPA S2100

20 Diode Array spectrophotometer (Biochrom Ltd., Cambridge, UK).

mRNA analysis

At 72 hours after PPRH transfection, total RNA from 30,000 SKBR3 or MCF7-R cells was extracted using Ultraspec RNA (Biotech Laboratories, Houston) following the

25 manufacturer's specifications. Reverse transcription and real-time PCR were performed as described by de Almagro *et al.* (de Almagro, M.C. *et al.* 2009. J Biol Chem, 284:11579-11589).

The primer sequences for Sybr were as follows:

30 Dhfr-Ex3 forward, 5'-GAAGACCTGGTTCTCCATTCC-3' (SEQ ID NO: 3);

Dhfr-Int3 reverse, 5'-GCAGCTTCATCAATAGCTCCT-3' (SEQ ID NO: 4);

Dhfr-Ex5 reverse, 5'-GGCAGAAGTTTATATTTCTCCAAA-3' (SEQ ID NO: 5);

Dhfr-Ex4 reverse, 5'-TGCCACCAACTATCCAGACC-3' (SEQ ID NO: 6);

Tert-forward, 5'-AGGAGCTGACGTGGAAGATG-3' (SEQ ID NO: 7);
Tert-reverse, 5'-GCTCGACGACGTACACACTC-3' (SEQ ID NO: 8);
Surv-forward, 5'-AGCCAGATGACGACCCCATAG-3' (SEQ ID NO: 9);
Surv-reverse, 5'-CACAGGAAGGCTGGTGGCAC-3' (SEQ ID NO: 10);
5 Aprt-forward, 5'-GCAGCTGGTTGAGCAGCGGAT-3' (SEQ ID NO: 11); and
Aprt-reverse, 5'-AGAGTGGGGCCTGGCAGCTTC-3' (SEQ ID NO: 12).

S100A4, DHFR, UGT1A10, and adenine phosphoribosyltransferase (APRT) mRNA Taqman probes were used (Applied Biosystems, Barcelona). A 5% polyacrylamide gel
10 was used to analyze the size of the PCR products.

Western analysis

MCF7-R cells were used to detect DHFR protein. MCF7-R cells (30,000) were plated in 35-mm-diameter dishes and treated with PPRHs. At 72 hours after transfection, total
15 protein extracts were obtained, and western blot analysis to detect the levels of DHFR protein was performed as described by de Almagro *et al.* (de Almagro, M.C. *et al.* 2009. J Biol Chem, 284:11579-11589).

DHFR activity assay

20 At 72 hours after PPRH transfection, DHFR activity was determined by incorporation of tritiated deoxyuridine to cellular DNA (Ciudad, C.J. *et al.* 1988. J Biol Chem, 263:16274-16282) with modifications (Noe, V. *et al.* 1998. Oncogene, 16:1931-1938).

***In vitro* transcription**

25 Linear DNA templates for *in vitro* transcription assays were generated by PCR using pDCHIP and pDCHIP-Hp as templates and the following primers: T7Dex1 (5'-TAATACGACTCACTATAGCCAAACTTGGGGGAAGCA-3') (SEQ ID NO: 13) and Dex3U1Tail (5'-ATACTTA CCTGCGATTCTTCTCAGGAATGGAG-3') (SEQ ID NO: 14). PCR products were purified in a 5% polyacrylamide gel.

30

The *in vitro* transcription reactions were performed with the RiboScribe T7 Probe Synthesis Kit (Epicentre Biotechnologies, Madison, WI). Reactions contained 0.5 µg of template DNA, 1x Transcription Buffer, 0.5 mM each ATP, CTP, and GTP, 15 µM

unlabeled UTP, and 30 µCi of [α-^{32}P]-UTP (3,000 Ci/mmol, Perkin Elmer, Madrid), 10 mM dithiothreitol, 40 units of RNase inhibitor (Promega Biotech Ibérica, Madrid), and 750 µM Cap analog, in a final volume of 20 µl. The reaction was incubated for 3 hours at 37°C and then stopped by addition of formamide loading buffer (80% [v/v] formamide, 20% [v/v] 5x Tris-borate-EDTA, bromophenol blue, and xylene cyanol). The samples were heated at 85-95°C for 5 min, placed on ice, and resolved in 7 M urea/5% polyacrylamide gels using Tris-borate-EDTA as the electrophoresis buffer. The electrophoresis was carried out at 350 V and 55°C. Following electrophoresis, gels were subjected to autoradiography, and the transcription products were gel-extracted, precipitated with ethanol, and resuspended in diethylpyrocarbonate-water.

***In vitro* splicing**

PPRHs were incubated for 45 min at 37°C with DCHIP or DCHIP-Hp transcripts (20,000 cpm or 1 µg) in the presence of 4 units of RNase inhibitor and 5.8 mM magnesium acetate. After PPRH binding, splicing reaction was started by adding 1 mM ATP, 5 mM creatinine phosphate, 0.5 mM dithiothreitol, 80 mM potassium acetate, and HeLa nuclear extract specific for splicing (CilBiotech, Mons, Belgium) to the reaction mixture, in a final volume of 25 µL. The splicing reaction was carried out at 30°C for 2 hours. The reaction was stopped by addition of 100 mM Tris (pH 7.5), 10 mM EDTA, 1% sodium dodecyl sulfate, 150 mM NaCl, and 300 mM sodium acetate (AppliChem, Ecogen, Barcelona). Nucleic acids were extracted with phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v), and precipitated from the aqueous phase by adding 1 volume of isopropanol (AppliChem, Ecogen). The pellets were then dissolved in formamide loading buffer and heated for 5 min at 85-95°C, before electrophoresis in 7 M urea/5% polyacrylamide gels at 55°C and 350 V. The gels were dried and analyzed on a Storm 840 Phosphorimager, and the transcripts were quantified using ImageQuant version 5.2 software. Results were normalized by the signal of the whole lane after background correction.

Electrophoretic mobility shift assay (EMSA)

A single-stranded DNA probe corresponding to HpdI3-A-TA target sequence 5'-TACTTCATTTTTCACCCCTCTCCCCCAATG-3' (SEQ ID NO: 15), was end-labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-^{32}P]ATP

(3,000 Ci/mmol, Perkin Elmer), and used in the gel shift assays. A DNA probe was used because U2 auxiliary factor (U2AF) 65 is able to bind either ssDNA or ssRNA polypyrimidine sequences as reported by Singh *et al.* (Singh, R. *et al.* 2000. RNA, 6:901-911). One microgram of HeLa nuclear extract specific for splicing (CilBiotech)
5 was used. In competition experiments, the different PPRHs used were added to the reaction mixture in excess to the radiolabeled probe ranging from 10- to 200-fold. DNA binding assays were performed as described (Noe, V. *et al.* 2001. Eur J Biochem, 268:3163-3173) and were analyzed on a Storm 840 Phosphorimager. tRNA was used as a nonspecific competitor in a 1:1 nonspecific/specific ratio. In the supershift assays, 1
10 μg of rabbit polyclonal antibody U2AF65 (Santa Cruz, Heidelberg, Germany) was added to the reaction mixture in combination with 0.5 μg or 1 μg of nuclear extract and incubated overnight at 4°C.

Statistical analysis

15 Data are presented as mean±SEM values. Statistical analysis was performed using Student's *t* test using SPSS (Chicago, IL) version 13.0 software for Mac OS X (Apple Computer, Cupertino, CA). Results were considered significant if $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.005$ (***).

20 EXAMPLE 1

Design of Coding-PPRHs

We searched for polypyrimidine stretches in the coding DNA strand of the human dihydrofolate reductase (*dhfr*) gene. One polypyrimidine stretch was found in intron-3,
25 which contained a purine interruption (Fig.1a and b). Two PPRHs were designed against this polypyrimidine target, one with an adenine matching the adenine interruption (HpdI3-A-TA) of the target sequence and another with a guanine (HpdI3-A-TG) because we had determined that placing either a T or C in the PPRH sequence was less effective (de Almagro, M.C. *et al.* 2009. J Biol Chem, 284:11579-11589). To
30 study the importance of the hairpin structure and Hoogsteen bonds on PPRH activity, we used a polypurine structure that had the same target sequence as HpdI3-A-TA, but without the ability to form a hairpin structure due to its lack of intramolecular Hoogsteen bonds (HpdI3-A-NH). This PPRH would act as an antisense oligonucleotide

(aODN) with a hanging tail. As negative controls we used a PPRH with a scrambled sequence (HpdI3-Sc) and a PPRH with intramolecular Watson-Crick bonds instead of Hoogsteen bonds, thus displaying a structure able neither to form triplexes nor to bind DNA (HpdI3-A-WC). To test cell responsiveness, HATNL-24, a phosphorothioate aODN targeting *dhfr* translational start (5’-gatGcAGtttAGcGAAccAAccaT-3’ (SEQ ID NO: 16), [where lowercase letters indicate phosphorothioate bonds]) (Rodriguez, M. *et al.* 1999. Int J Cancer, 81:785-792) and the *dhfr* Template-PPRH HpdI3-B directed against intron-3 of the *dhfr* template DNA strand (de Almagro, M.C. *et al.* 2009. J Biol Chem, 284:11579-11589) were used. Blast analyses were performed to assess PPRH specificity. The PPRHs sequences are listed in Table 2, and their structures are depicted in Fig. 1b.

Table 2. Sequences of the PPRHs used in this study and their target sequences

Name	Sequence
dhfr	Target sequence Intron 3, reverse strand
Template	5’-(CATTCTCTTGATTG)CCTCCTCCCTCTCCCTCCTC-3’ (SEQ ID NO: 17)
HpdI3-B	5’-GGAGGAGGGAGAGGGAGGAG-$(T)_5$- GAGGAGGGAGAGGGAGGAGG-3’ (SEQ ID NO: 18)
dhfr	Target sequence Intron 3, forward strand
Coding	5’-(TACTTCA) TTTTTC**A**CCCCTCTCCCCC (AATG)-3’(SEQ ID NO: 15)
HpdI3-A-TA	5’-AAAAAG**A**GGGGAGAGGGGG-$(T)_5$-GGGGGAGAGGGG**A**GAAAAA-3’ (SEQ ID NO: 2)
HpdI3-A-TG	5’-AAAAAG**G**GGGGAGAGGGGG-$(T)_5$-GGGGGAGAGGGG**G**GAAAAA-3’ (SEQ ID NO: 19)
HpdI3-A-TA-WC	5’-TTTTTC**T**CCCCTCTCCCCC-$(T)_5$-GGGGGAGAGGGG**A**GAAAAA-3’ (SEQ ID NO: 20)
HpdI3-A-TA-NH	5’-GGGGGA**G**AAAAGAGAAAAA-$(T)_5$-GGGGGAGAGGGG**A**GAAAAA-3’ (SEQ ID NO: 21)
HpdI3-Sc	5’-GAGAGGAGGAGGGAGGGAGA-$(T)_5$-AGAGGGAGGGAGGAGGAGAG-3’ (SEQ ID NO: 22)

Bold letters indicate mismatches between the target sequence and the PPRH. The sequences in parentheses are not PPRH target sequences.

EXAMPLE 2

Binding of Coding-PPRHs to ssDNA, dsDNA and RNA

The ability of Coding-PPRHs to bind to their target sequence was studied by electrophoretic mobility shift assays. Three probes were used: the For sequence, containing the polypyrimidine ssDNA target; the Duplex probe, which corresponds to the polypyrimidine/polypurine dsDNA target sequence; and the RNA probe corresponding to the polypyrimidine target sequence. Figure 1b shows the different PPRH sequences and their corresponding binding structures. In Fig. 1c and 1d, the mobility of the For, the Duplex, and the RNA probes can be observed. The mobility shift due to PPRH binding to ssDNA (For) indicated that HpdI3-A-TA and HpdI3-A-NH (no Hoogsteen bonds) were able to bind to its target sequence. However, only HpdI3-A-TA was able to bind to its dsDNA target sequence (Duplex) and to form a triplex. To assure that PPRHs were also able to bind to its corresponding RNA target sequence, they were incubated in presence of the radiolabeled RNA probe (Fig. 1d). HpdI3-A-TA and HpdI3-A-NH bound to its RNA target sequence, whereas HpdI3-A-WC was unable to bind to it. Binding specificity was tested by addition of 2 μg of tRNA to the binding reactions. It is interesting to note that ssDNA probe incubated with HpdI3-A-WC suffered a shift in mobility corresponding to a duplex structure. If that shift were due to the formation of a duplex with the polypurine sequence of the Hp-A-WC after displacement of the polypyrimidine strand, it would form a structure similar to that of Hp-A-NH, which produced higher retardation in mobility. However, when testing Hp-A-WC with its real target, ssRNA, no retardation was observed.

EXAMPLE 3

Coding-PPRH cytotoxicity

We tested the cytotoxic effect of HpdI3-A-TA and HpdI3-A-TG in SKBR3 breast cancer cells. In Fig. 2a, it is shown that HpdI3-A-TA produced 67% cytotoxicity, whereas the cell death caused by HpdI3-A-TG was 55%; thus HpdI3-A-TA was selected for the following assays. The specificity of HpdI3-A-TA was studied using a series of different controls that did not cause a significant change in cell viability (Fig. 2b). On the other hand, HATNL-24 (at its optimal conditions, 1:10 aODN:DOTAP molar ratio) and HpdI3-A-NH caused a significant drop of cell survival, as they acted as aODNs. Additionally, an HpdI3-A-TA dose-response curve was performed (Fig. 2c). The highest cytotoxicity was 86% at 100 nM HpdI3-A-TA using a 1:100 PPRH:DOTAP molar ratio

(10 μM DOTAP), similar to that produced by the Template-PPRH HpdI3-B. The minimum time that HpdI3-A-TA needed to cause cell death was analyzed by removing the PPRH at different intervals (Fig. 2d). At 48 hr, almost the maximum activity was achieved.

5

EXAMPLE 4

Effects of Coding-PPRHs on DHFR activity

The 6-[^{3}H]deoxyuridine assay was used to determine DHFR activity in SKBR3 cells incubated with Coding-PPRHs (against DHFR mRNA) for 72 hr. HpdI3-A-TA (100
10 nM):DOTAP (10 μM) (1:100 ratio) showed the greatest inhibition of DHFR activity (60%)(Fig. 3a), in agreement with the cytotoxicity results. Lower PPRH:DOTAP molar ratios caused less DHFR inhibition, despite the increase in PPRH concentration. HpdI3-A-NH also inhibited DHFR activity but in a lesser extent than HpdI3-A-TA (100 nM), whereas DOTAP (10 μM) alone or HpdI3-A-WC did not provoke any decrease in DHFR
15 activity.

EXAMPLE 5

Effects of Coding-PPRHs on DHFR mRNA levels

SKBR3 cells were incubated with Coding-PPRHs for 72 hours. Surprisingly, HpdI3-A-
20 TA (100 nM) produced a decrease of only 20% in DHFR mRNA levels, whereas a reduction close to 50% was caused by the aODN HATNL-24 and the *dhfr* Template-PPRH HpdI3-B (Fig. 3b). HpdI3-A-NH also caused a very slight decrease in DHFR mRNA levels (17%). The different controls did not produce a drop in DHFR mRNA levels.

25

EXAMPLE 6

Effects of Coding-PPRHs on DHFR pre-mRNA levels

Taking into account that the PPRH target sequence was included in intron-3, we decided to study if there was any change in the pre-mRNA levels. Three different species were
30 analyzed by reverse transcription-real-time PCR: Exon3-Intron3, corresponding to pre-mRNA levels; Exon3-Exon4, corresponding to the splicing of intron-3; and Exon3-Exon5, to explore exon-4 skipping (Fig. 3c). As shown in Fig. 3d, when SKBR3 cells were incubated for 72 hr with HpdI3-A-TA, an increase of more than 200% was

observed in the pre-mRNA levels, whereas the amounts of the Exon3-Exon4 and Exon3-Exon5 products were reduced by 22% and 42%, respectively. The relative abundance of each RNA species was 3.8% for pre-mRNA, 50% for Exon3-Exon4, and 46.2% for Exon3-Exon5. The negative control HpdI3-WC did not produce a significant drop in
5 DHFR mRNA levels. No loss of exon-4 was observed (data not shown).

EXAMPLE 7

Effects of Coding-PPRHs on DHFR pre-mRNA splicing

The increase in the pre-mRNA DHFR levels, followed by a decrease of the spliced form,
10 suggested a possible alteration in the splicing process of intron-3 in the presence of Coding-PPRHs. To study this possibility, we generated the plasmids pDCHIP and pDCHIP-Hp. The insertion of the human *dhfr* HpdI3-A-TA target sequence in the intron sequence present in pDCHIP to generate pDCHIP-Hp did not affect the splicing of intron-1 and the functionality of the DHFR protein (data not shown), as DCHM1 and
15 DCHM1-Hp cells grew normally in -GHT medium. No other HpdI3-A-TA target sequence was found in the Chinese hamster ovary *dhfr* gene.

Next, we proceeded to perform *in vitro* splicing experiments with probes generated by *in vitro* transcription from pDCHIP and pDCHIP-Hp plasmids. Representative images of
20 the different species after DHFR pre-mRNA splicing from the DCHIP and DCHIP-Hp probes are shown in Fig. 4a and c. The upper band corresponded to the pre-mRNA probe, the second band to the spliced product, and the lower band to free exon-1. Figure 4a and c also include the effect of Coding-PPRHs on the *in vitro* splicing reaction from the DCHIP or DCHIP-Hp probes, respectively. No decrease in splicing was observed
25 when DCHIP was incubated with Coding-PPRHs (Fig. 4b). In contrast, when the DCHIP-Hp probe was incubated with HpdI3-A-TA, the decrease in splicing was dose dependent, reaching a 51% decrease at 400 nM (Fig. 4d). HpdI3-A-NH also caused a decrease in splicing (29%) but to a lesser degree than HpdI3-A-TA. The negative controls HpdI3-A-WC and HpdI3-Sc did not affect splicing.

30

EXAMPLE 8

Effects of Coding-PPRHs on U2AF65 binding

The effects of PPRHs on protein binding to HpdI3-A-TA target sequence were studied by electrophoretic mobility shift assays. A radiolabeled probe corresponding to the HpdI3-A-TA target sequence in *dhfr* intron-3 was incubated with HeLa nuclear extracts specific for splicing, as shown in Fig. 5a. A DNA probe was used because U2AF65 is
5 able to bind to either ssDNA or ssRNA polypyrimidine sequences (Singh, R. *et al.* 2000. RNA, 6:901-911). A shifted band was observed due to protein binding to the probe (gel shift). When HpdI3-A-TA was added as competitor, a dose-dependent decrease in the gel-shifted band was observed, as well as the appearance of a lower band due to the binding of the PPRH to the probe and an upper band due to PPRH-probe-protein binding.
10 The upper band seemed to be mainly due to albumin binding (data not shown). To test the involvement of U2AF65, an important splicing factor that recognizes intronic polypyrimidine sequences and recruits the splicing machinery, in the molecular mechanism of Coding-PPRHs splicing blockage, a super-shift assay using U2AF65 antibody was performed (Fig. 5b). When using 1 μg of nuclear extract in the presence of
15 U2AF65 antibody, a supershifted band and a decrease of 40% in the intensity of the shifted band were observed, whereas with 0.5 μg of nuclear extract, a major decrease of 88% in the gel-shifted band was observed, although the supershifted band was not visually detectable.

20 EXAMPLE 9

Specificity

The specificity of HpdI3-A-TA was assessed by BLAST analysis, by determining the mRNA levels of unrelated genes, and by cell survival analysis after its transfection in cells lacking the HpdI3-A-TA target sequence.

25

SKBR3 cells were transfected with HpdI3-A-TA, and a reverse transcription-real-time PCR was performed to determine the mRNA levels of unrelated genes. The mRNA levels of the *Telomerase*, *Survivin*, *UGT1A10,* and *S100A4* genes did not change significantly in SKBR3 cells incubated for 72 hr with HpdI3-A-TA, as shown in Table 3.

30

Table 3. Coding-PPRH specificity

	dhfr	Telomerase	Survivin	UGT1A10	S100A4
Control	100	100	100	100	100
HpdI3-A-TA	58.5 ± 9.7	143.7 ± 35.5	110 ± 6.1	127 ± 12.5	85.2 ± 14.7

Data are mean ± SEM values of at least four experiments.

5 DCHM1 or DCHM1-Hp cells were incubated with Coding-PPRHs in DHFR-selective medium (-GHT). Interestingly, after 1 week of incubation (Fig. 6b) only DCHM1-Hp (cells containing the HpdI3-A-TA target sequence) suffered a decrease in cell survival of 40% when treated with HpdI3-A-TA and of 21% when incubated with HpdI3-A-NH. Negative controls HpdI3-A-WC and HpdI3-Sc produced a relative cell death that was not 10 statistically significant in both transfected cell lines.

EXAMPLE 10

Effects of Coding-PPRHs on methotrexate-resistant cells

The effectiveness of Coding-PPRHs in MCF7 breast cancer cells resistant to the 15 chemotherapeutic drug methotrexate at 10^{-6} M was tested. These cells have the *dhfr* locus amplified (gene copy number 58) and overexpress DHFR mRNA (mRNA change 33-fold) (Selga, E. *et al.* 2009. Genome Med, 1:83). HpdI3-A-TA presented a higher cytotoxicity than HpdI3-A-TG and HpdI3-A-NH (58%, 30%, and 45%, respectively) in these cells (Fig. 7a). The aODN HATNL-24 (at its optimal conditions, 1:10 20 aODN:DOTAP molar ratio) scarcely showed any effect. DOTAP (10 μM) and HpdI3-Sc caused a certain degree of cell death, unlike HpdI3-A-TA without DOTAP and HpdI3-A-WC. Template HpdI3-B showed a greater effect than HpdI3-A-TA (71%). As previously noted in SKBR3 cells, MCF7-R cells did not present a large DHFR mRNA decrease after HpdI3-A-TA transfection (Fig. 7b). However, at the protein level, a 40% decrease 25 in DHFR level was observed upon incubation of MCF7-R cells with HpdI3-A-TA (Fig. 7c). HpdI3-B (Template-PPRH) and HpdI3-A-NH also caused a decrease in DHFR protein levels, whereas DOTAP and HpdI3-A-WC did not cause a significant effect.

CLAIMS

1. An oligonucleotide comprising a first polypurine region and a second polypurine region connected by a thymidine-rich region
5 wherein said first polypurine region has the reverse sequence of said second polypurine region,
wherein said first and second regions are capable of forming an antiparallel hairpin maintained by reverse-Hoogsteen bonds and
wherein the oligonucleotide is capable of forming a triplex with a polypyrimidine
10 region which is substantially complementary to said first polypurine region and which is located in the coding strand of an intronic region of a target gene.

2. An oligonucleotide according to claim 1 wherein the thymidine-rich region is a five-thymidine region.

15

3. An oligonucleotide according to claims 1 or 2 wherein the thymidine-rich region connects the 3'-end of the first polypurine region and the 5'-end of the second polypurine region or wherein the thymidine-rich region connects the 5'-end of the first polypurine region and the 3'-end of the second polypurine region.

20

4. An oligonucleotide according to claims 1 to 3 wherein the first polypurine region and the polypyrimidine region located in the coding strand of an intronic region of a target gene are 100% complementary.

25 5. An oligonucleotide according to any of claims 1 to 3 wherein the polypurine region substantially complementary to the polypyrimidine region in the target gene is interrupted by at least one non-complementary nucleotide and wherein said one or more non-complementary nucleotide is adenine.

30 6. An oligonucleotide according to any of the preceding claims wherein each polypurine region has at least 19 nucleotides.

7. An oligonucleotide according to any of the preceding claims wherein the target gene is selected from the group consisting of a gene related to cell proliferation, replication, apoptosis, angiogenesis, metastasis, inflammation, Alzheimer's disease, atherosclerosis or development of resistance to chemotherapy and/or radiotherapy.

5

8. An oligonucleotide according to any of the preceding claims wherein the target gene is DHFR.

9. An oligonucleotide according to claim 8 wherein the DHFR gene is of human
10 origin.

10. An oligonucleotide according to claims 8 or 9 wherein the intronic region is the third intron of the coding DNA strand of the DHFR gene.

15 11. An oligonucleotide according to claim 10 having a sequence SEQ ID NO: 2.

12. A pharmaceutical composition comprising a pharmaceutically effective amount of at least one oligonucleotide according to claims 1 to 11 and at least one pharmaceutically acceptable excipient.

20

13. A method of treatment and/or the prevention in a subject of a disease selected from cancer, a disease associated with inflammation, Alzheimer's disease and atherosclerosis comprising the administration to said subject of a pharmaceutically effective amount of an oligonucleotide according to claims 1 to 11 wherein the
25 target gene is a gene selected, respectively, from the group consisting of a gene associated with cancer, inflammation, Alzheimer's disease and atherosclerosis.

14. A method of treatment and/or the prevention according to claim 13 wherein the disease is cancer.

30

15. A method of treatment and/or the prevention according to claim 14 wherein the cancer is breast cancer.

16. A method of treatment and/or the prevention according to claims 14 or 15 wherein the cancer is resistant to chemotherapy and/or radiotherapy.

17. A method of treatment and/or the prevention according to claim 16 wherein the cancer is resistant to methotrexate.

18. An *in vitro* method for detecting gene amplification of a target gene in a sample containing genomic DNA comprising measuring the number of copies of a target gene in said sample by using an oligonucleotide according to claims 1 to 11.

19. An *in vitro* method for the diagnosis of cancer or of drug resistance to chemotherapy and/or to radiotherapy associated to the amplification of a given gene in a subject comprising:

    (a) measuring in a sample from said subject the number of copies of said gene by using an oligonucleotide according to any of claims 1 to 11 wherein said oligonucleotide is specific for an intronic region of said gene and
    (b) comparing said number of copies with a reference value

    wherein an increase in the number of copies in said sample with respect to the reference value is indicative of the subject suffering from cancer or drug resistance.

20. A kit for detecting gene amplification in a sample from a subject which comprises at least one oligonucleotide according to claims 1 to 11.

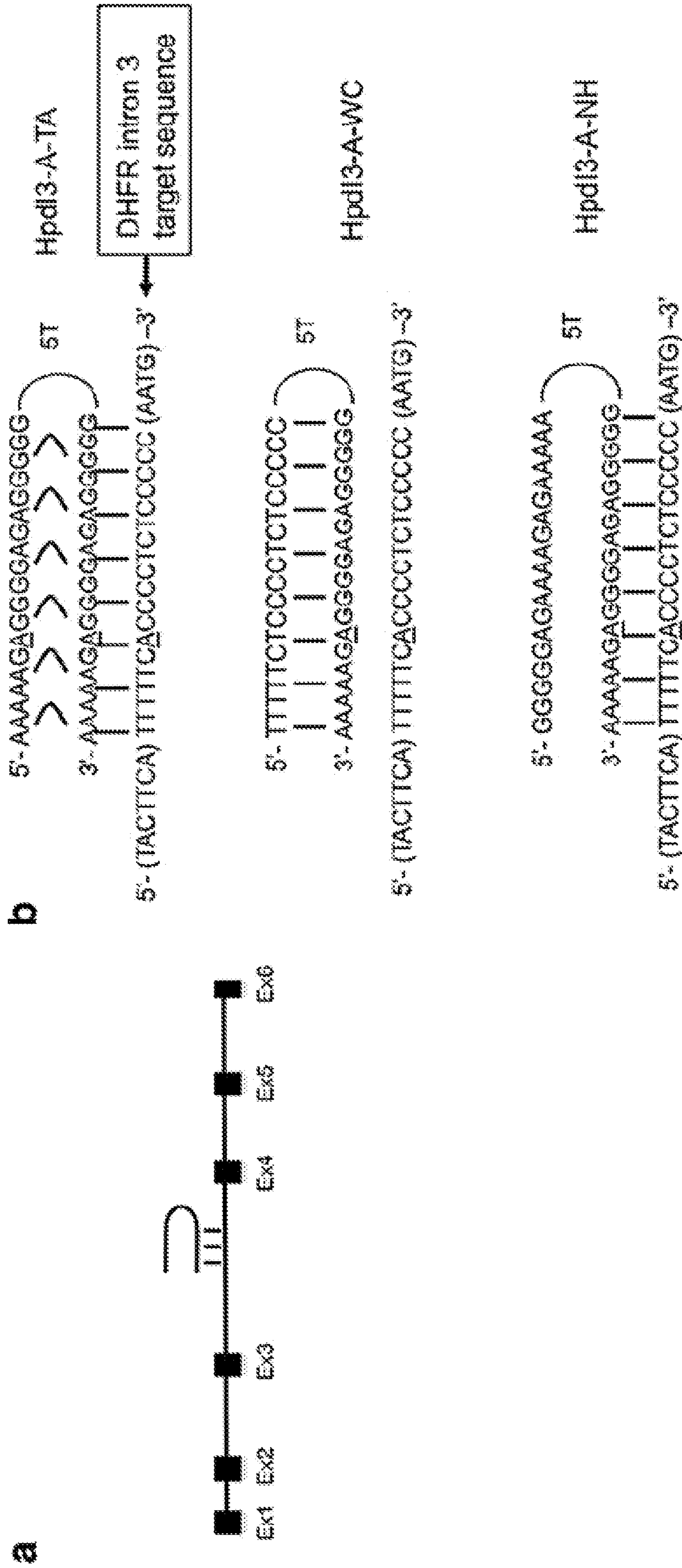
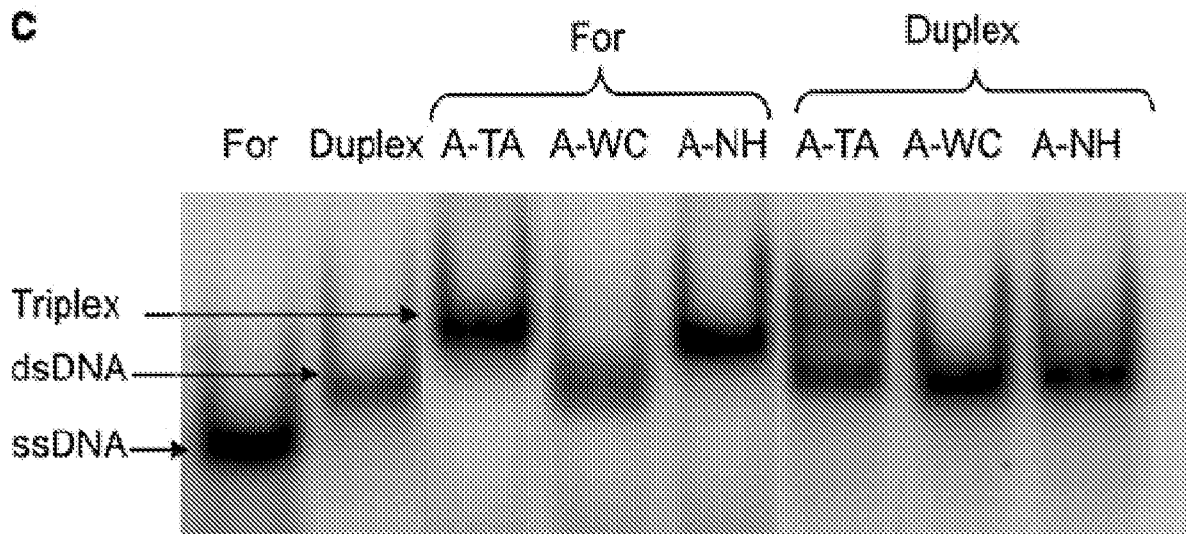


FIGURE 1



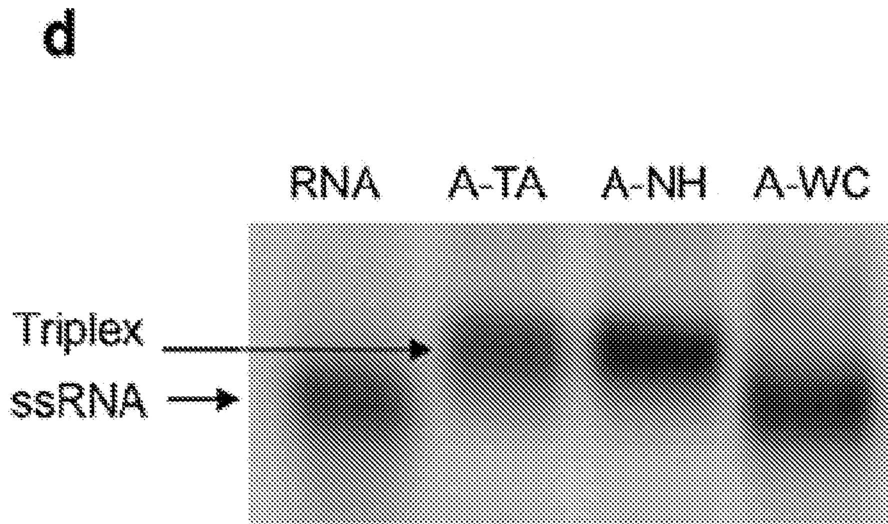


FIGURE 1

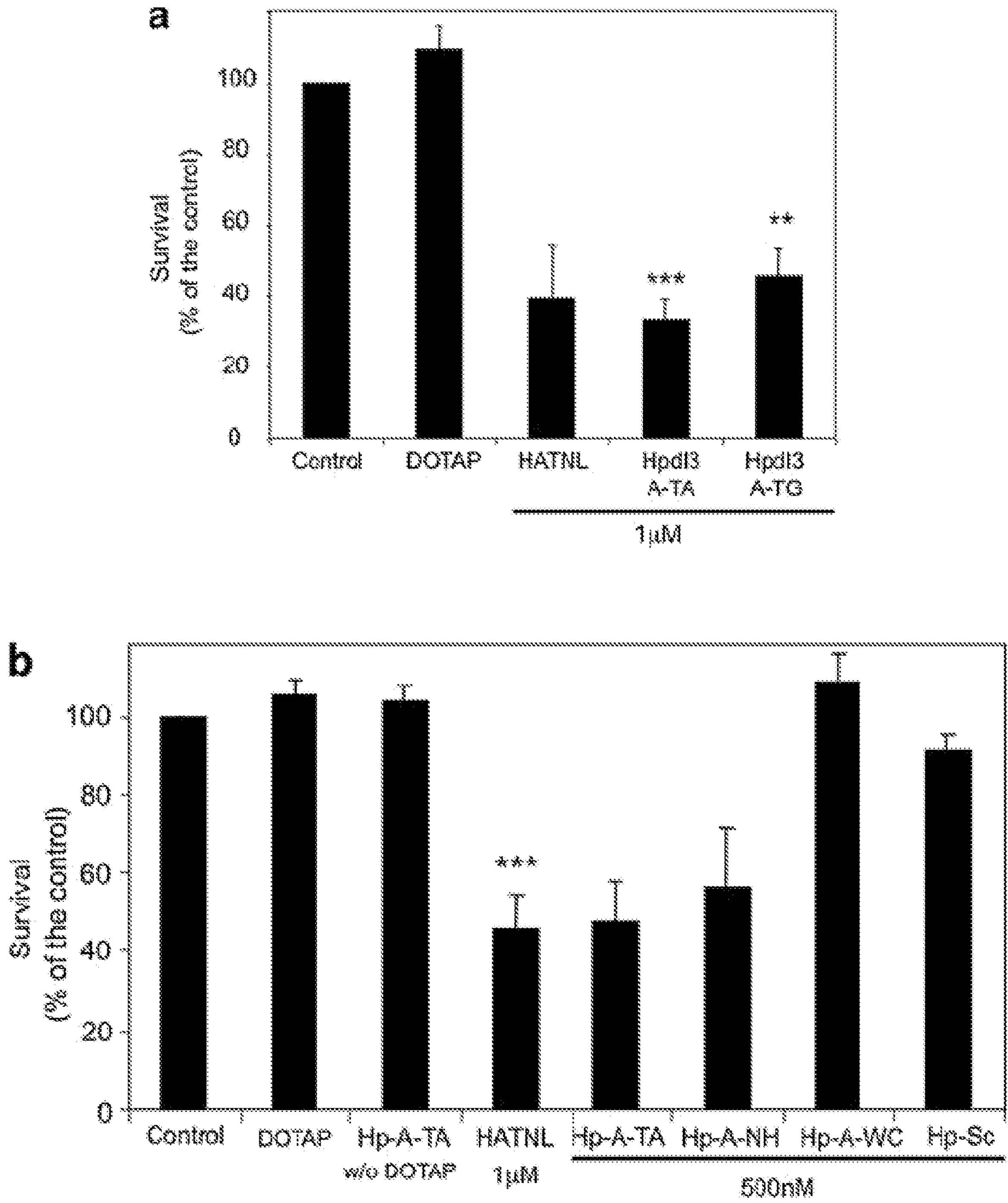
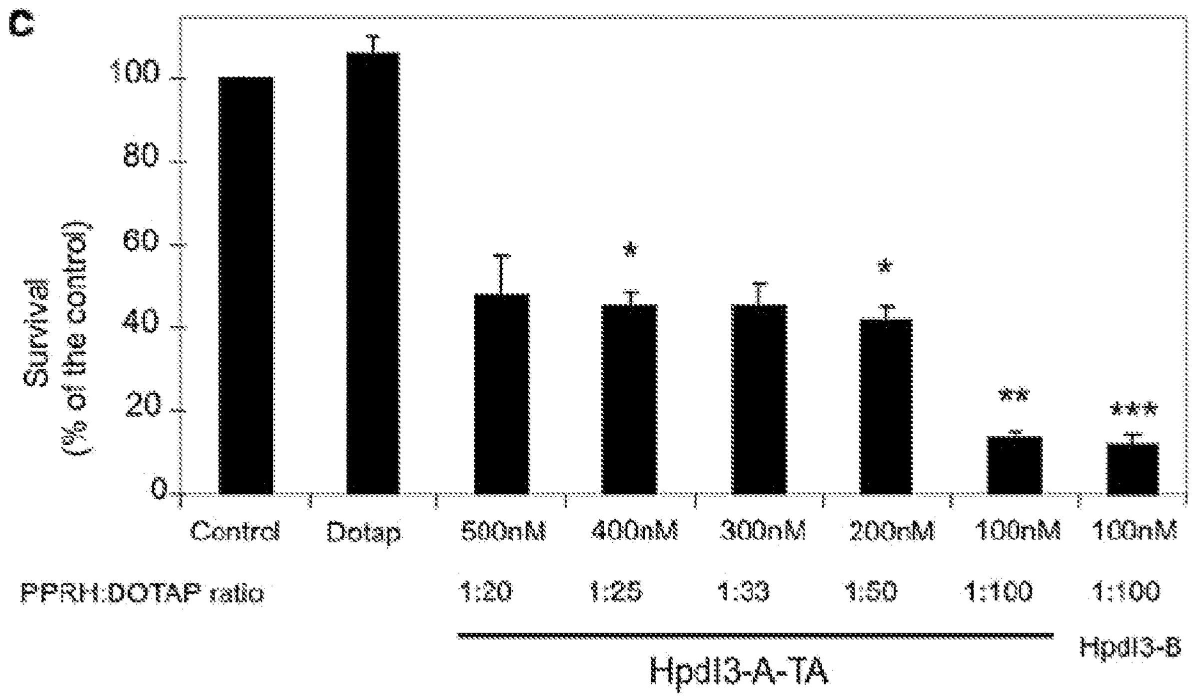


FIGURE 2



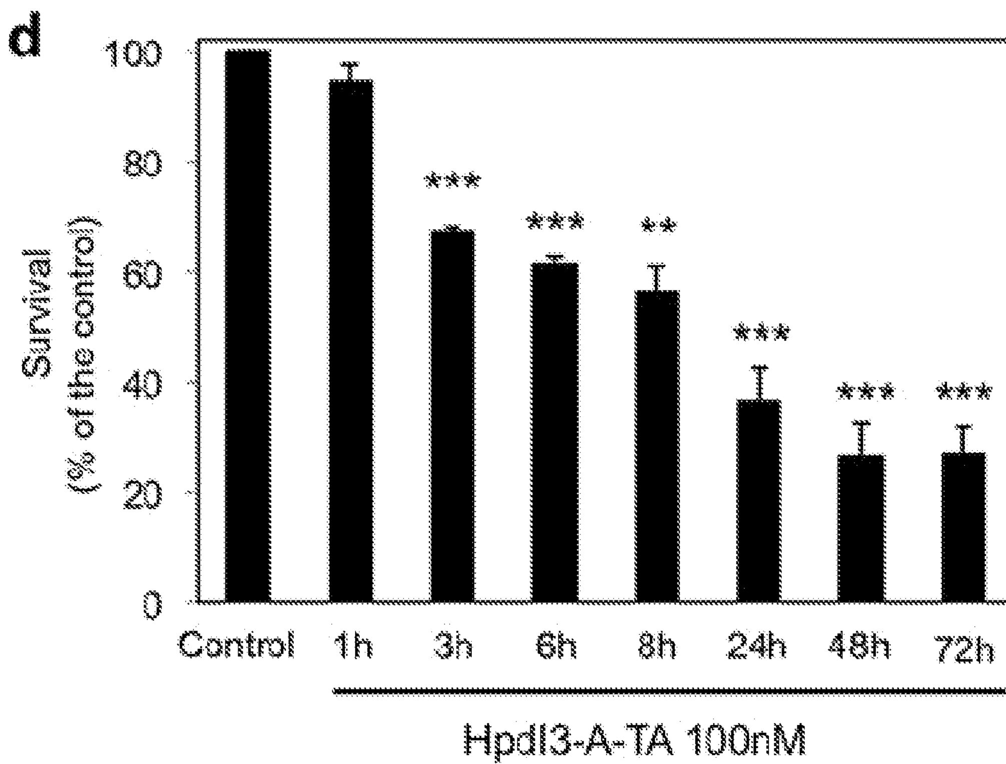


FIGURE 2

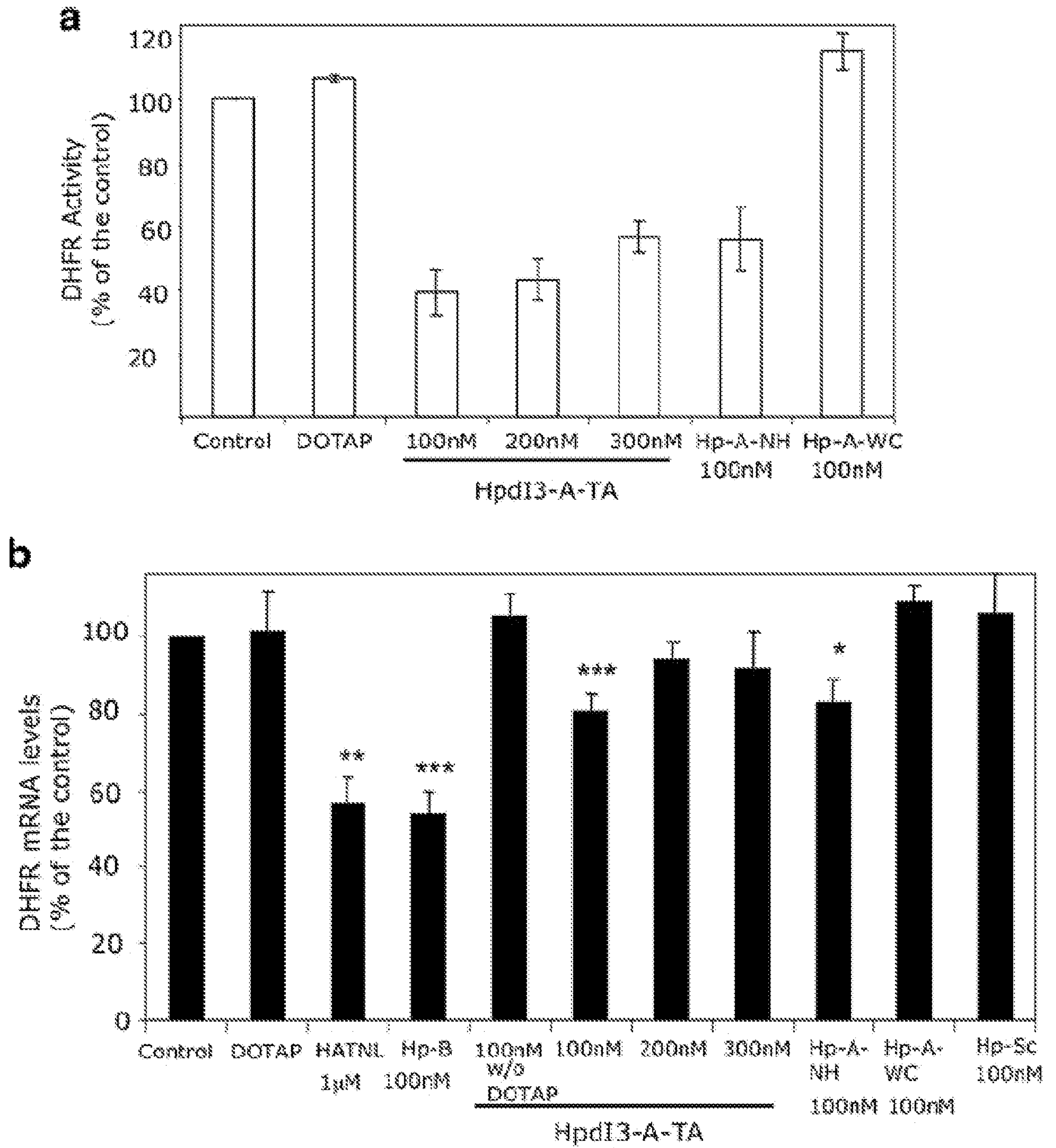
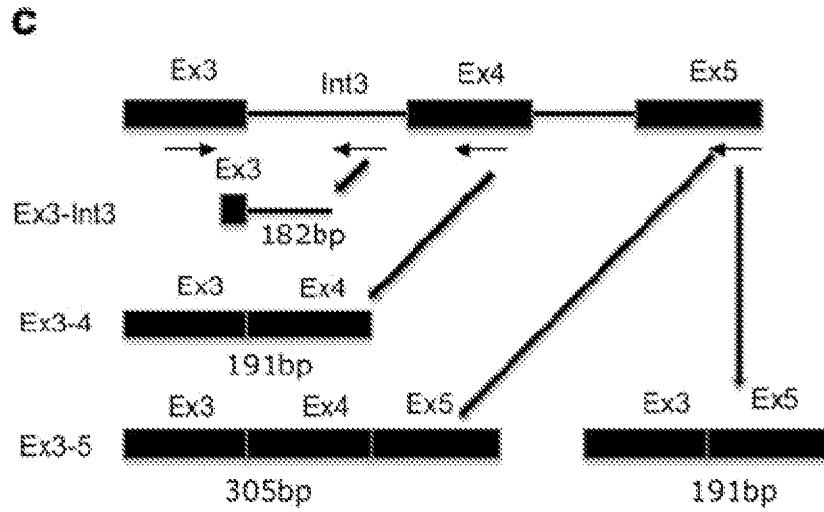


FIGURE 3



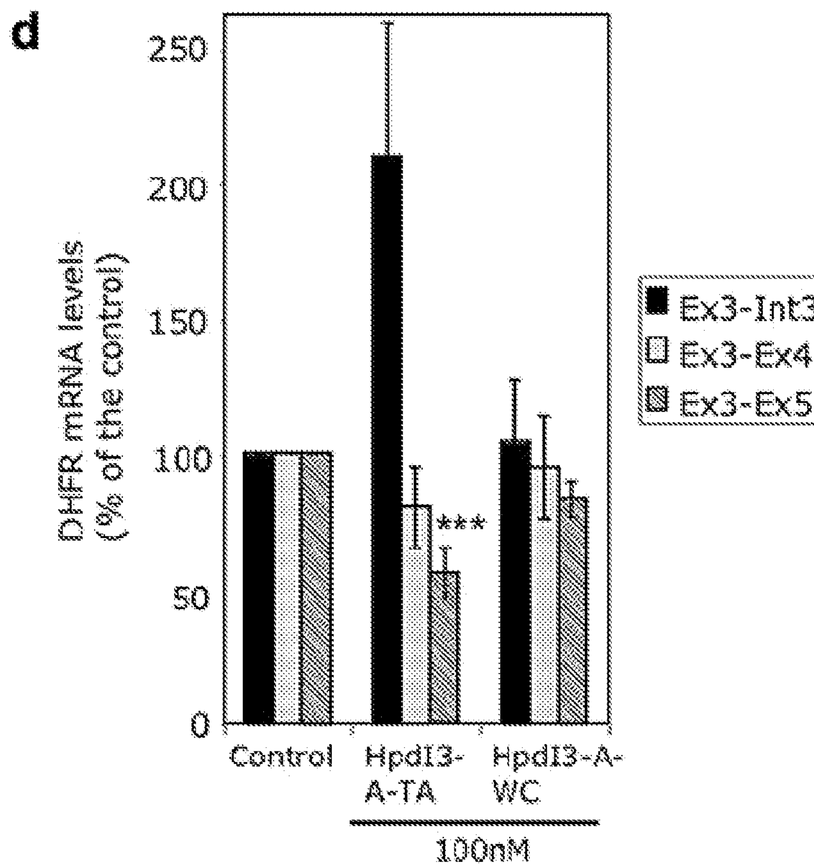


FIGURE 3

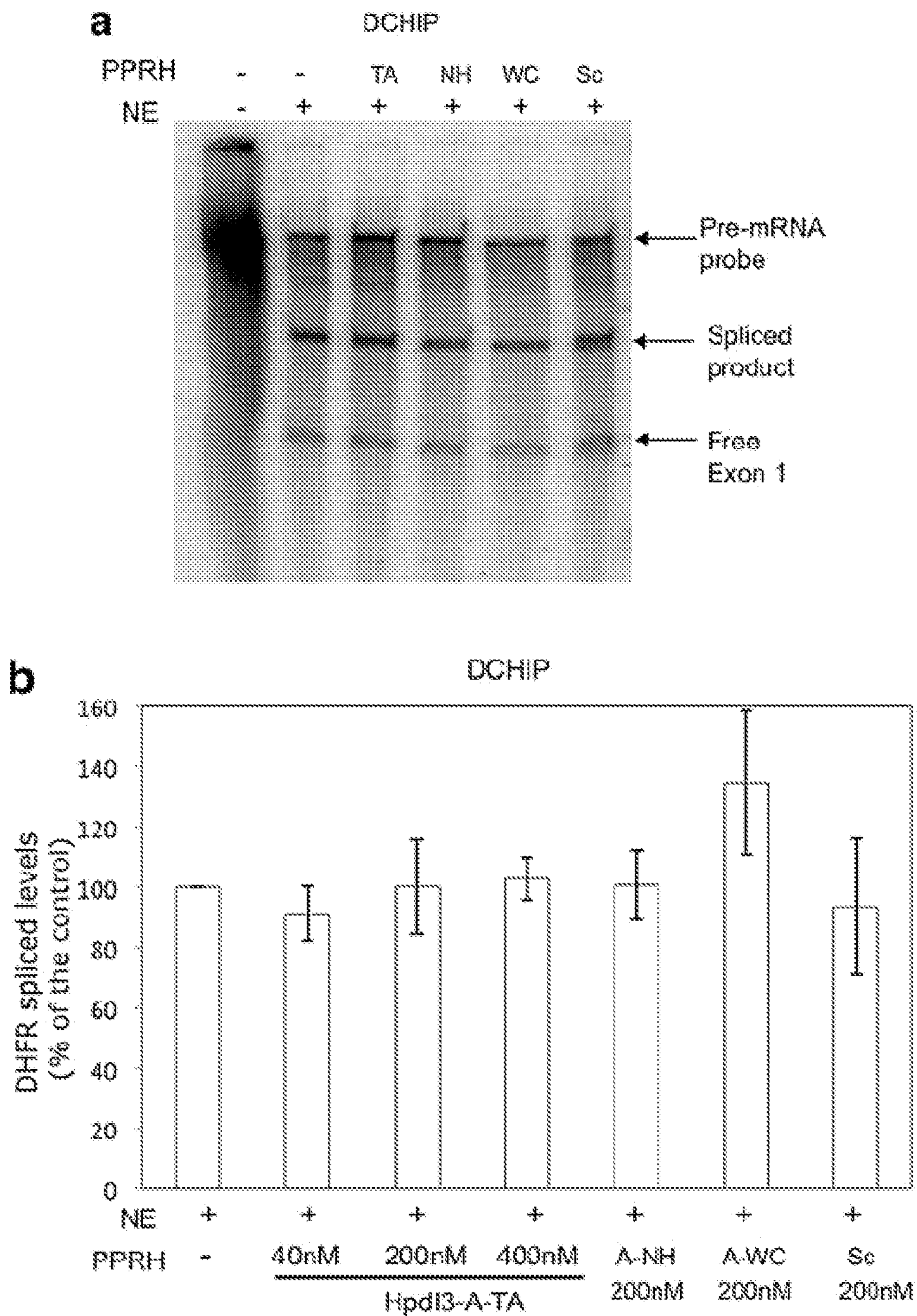
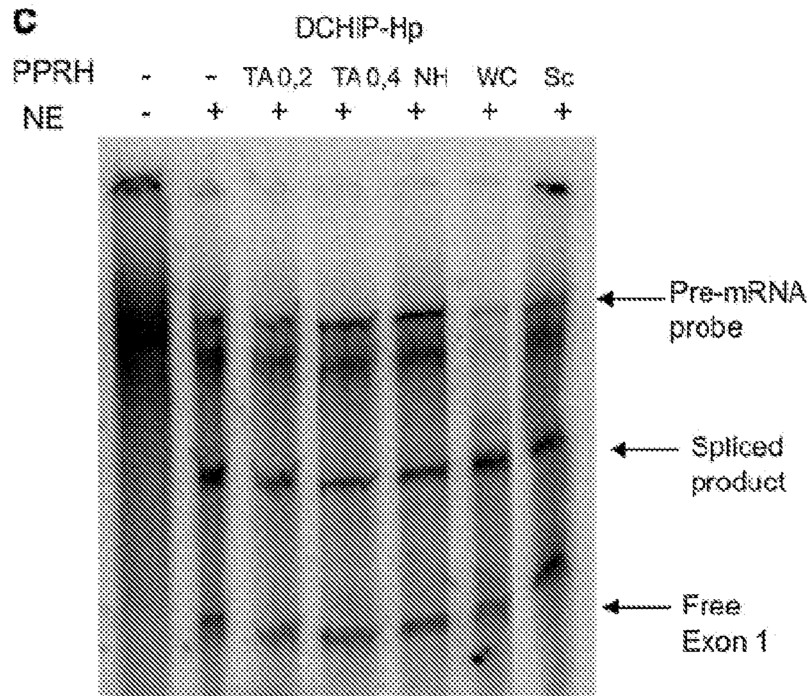


FIGURE 4



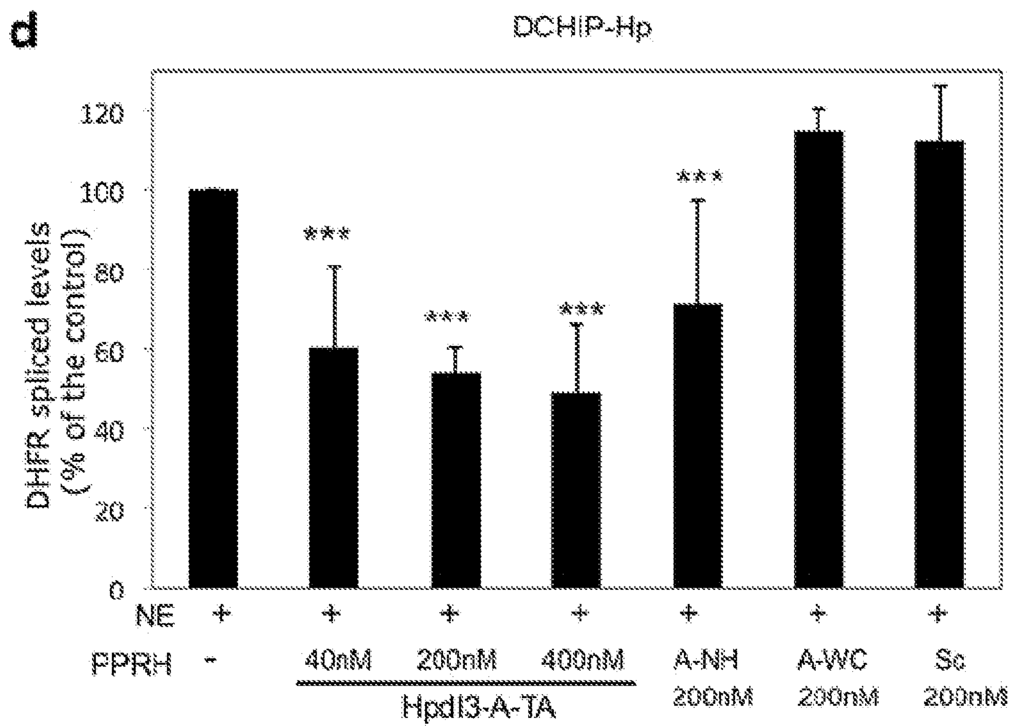


FIGURE 4

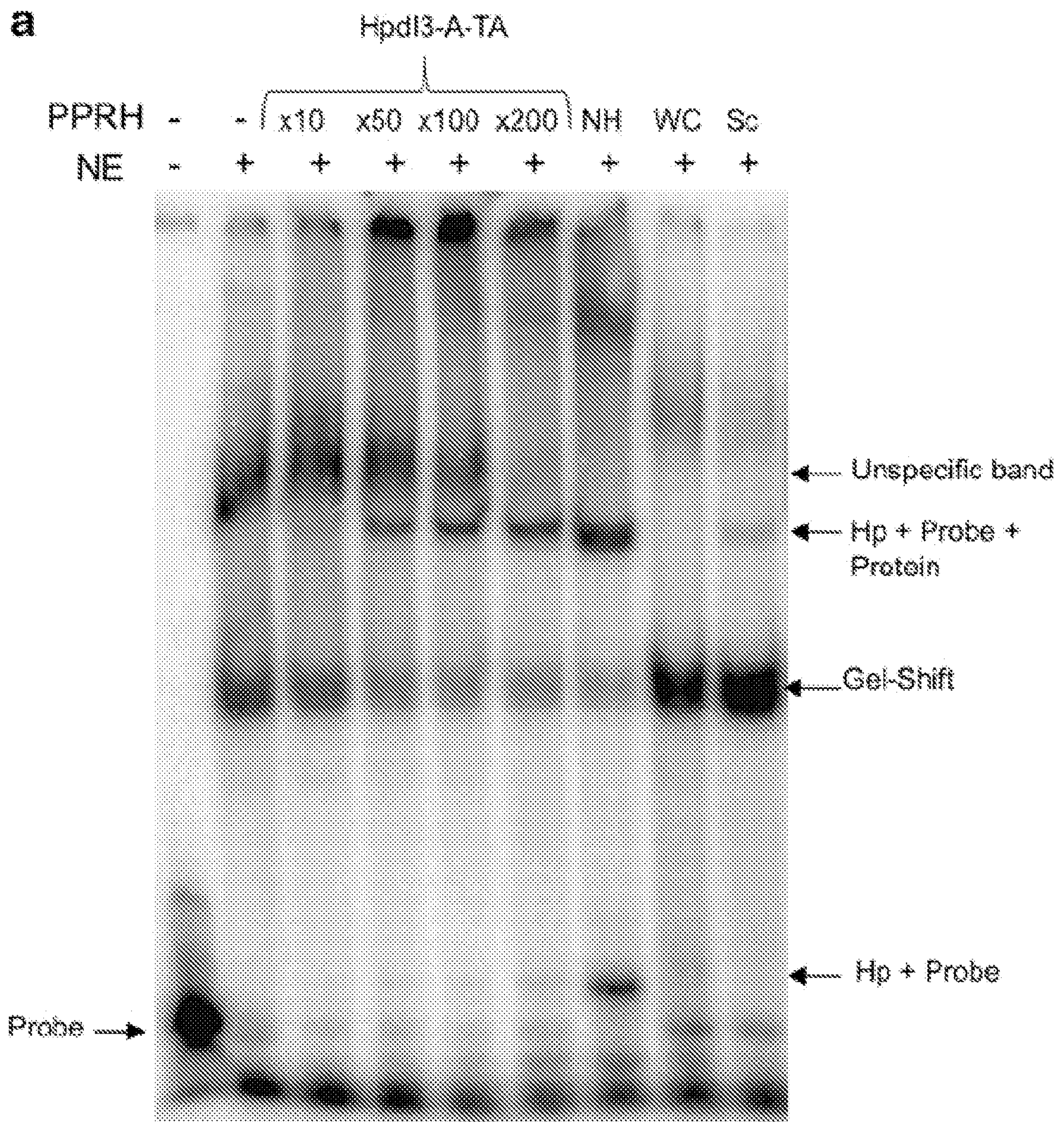


FIGURE 5

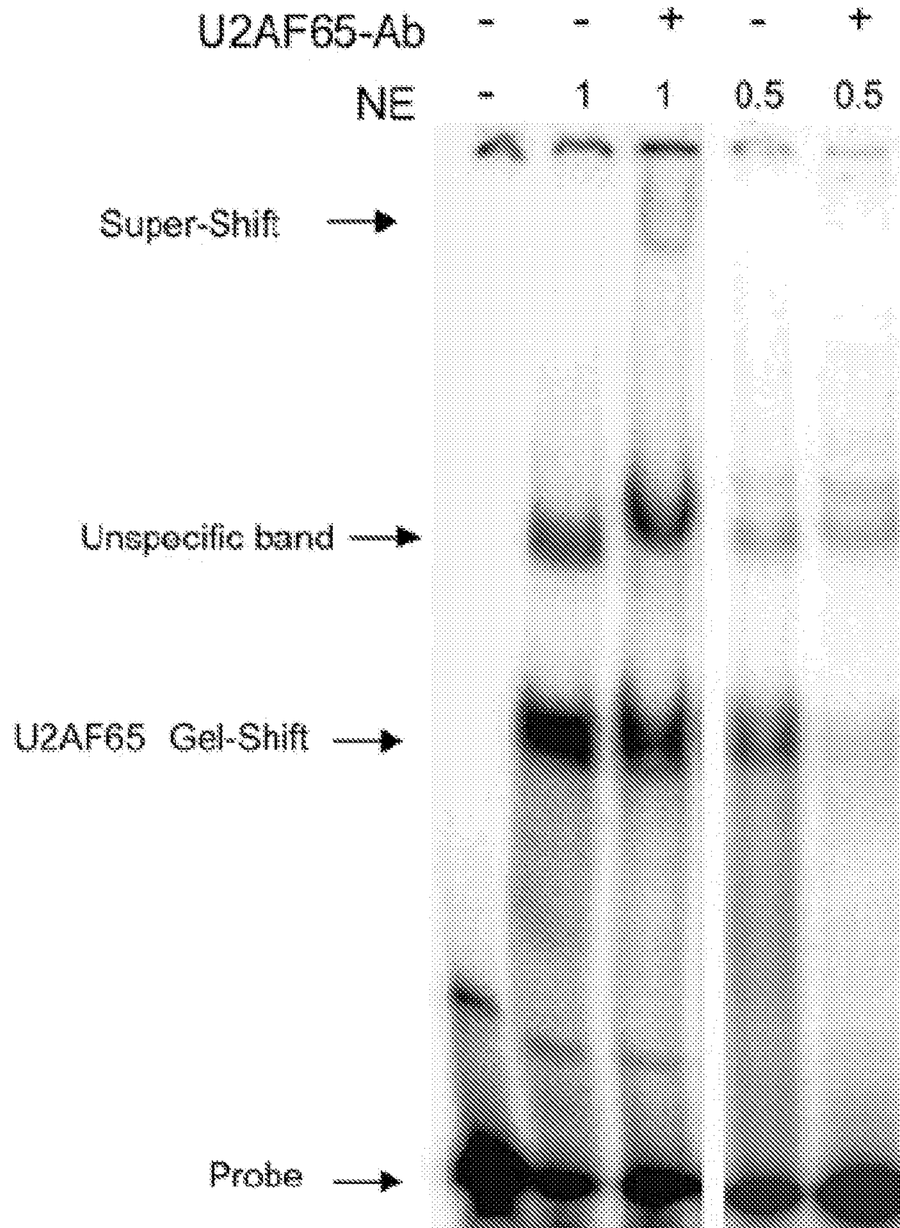


FIGURE 5

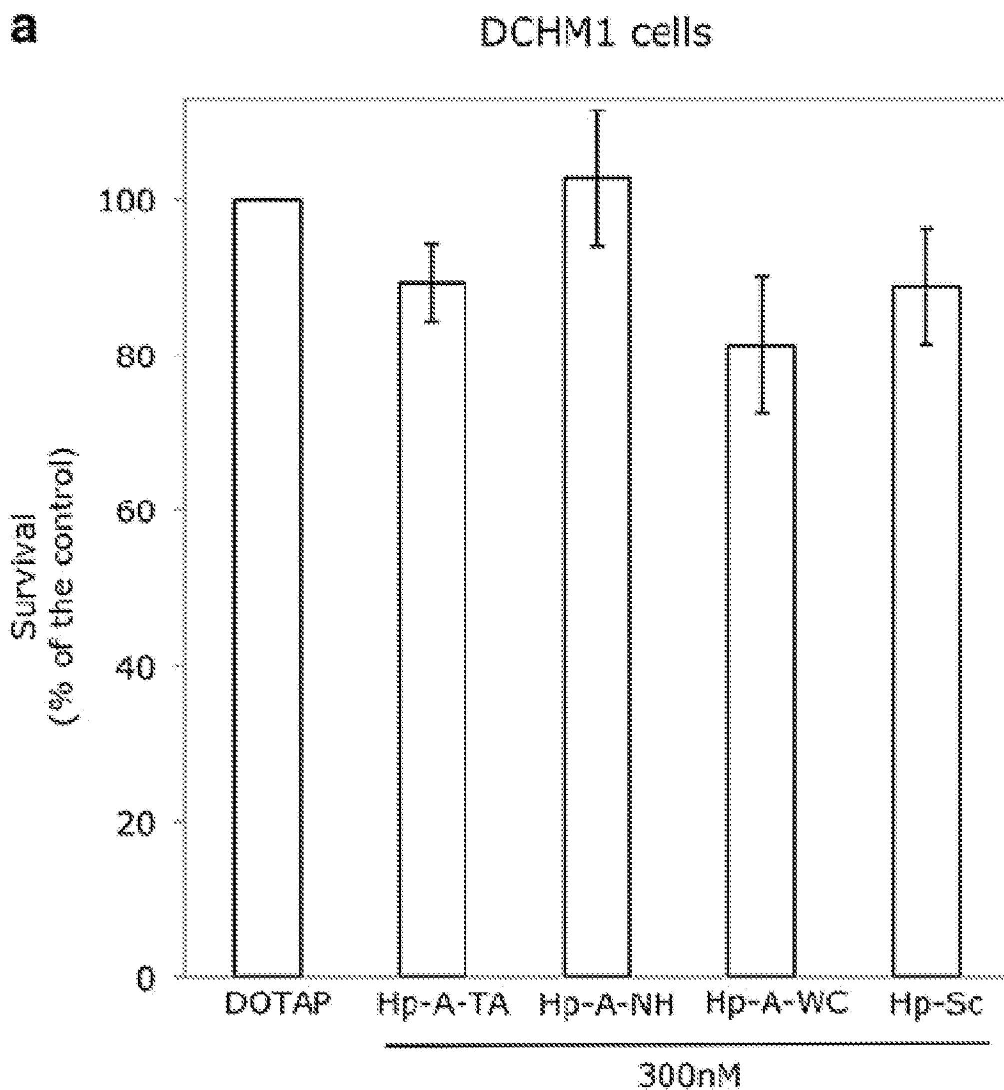


FIGURE 6

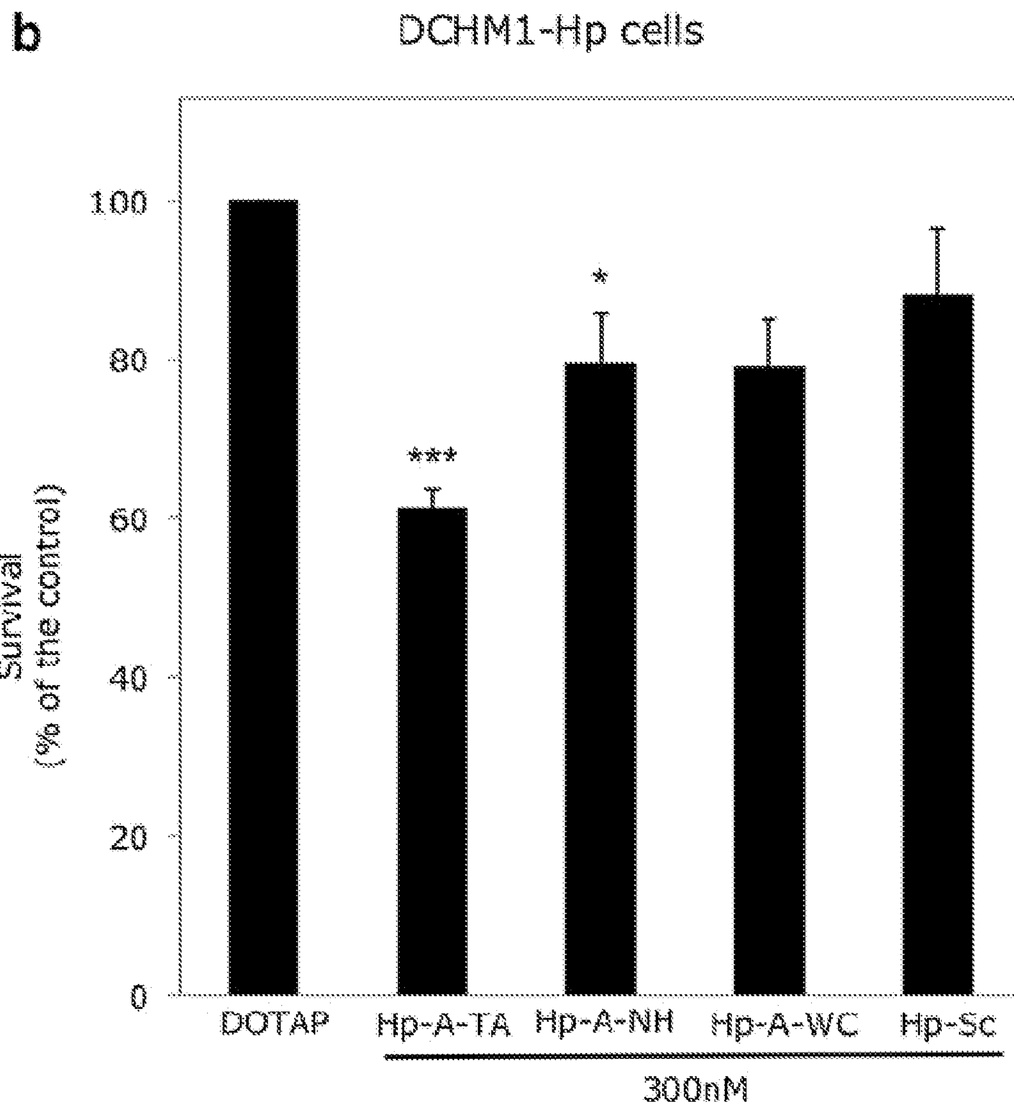


FIGURE 6

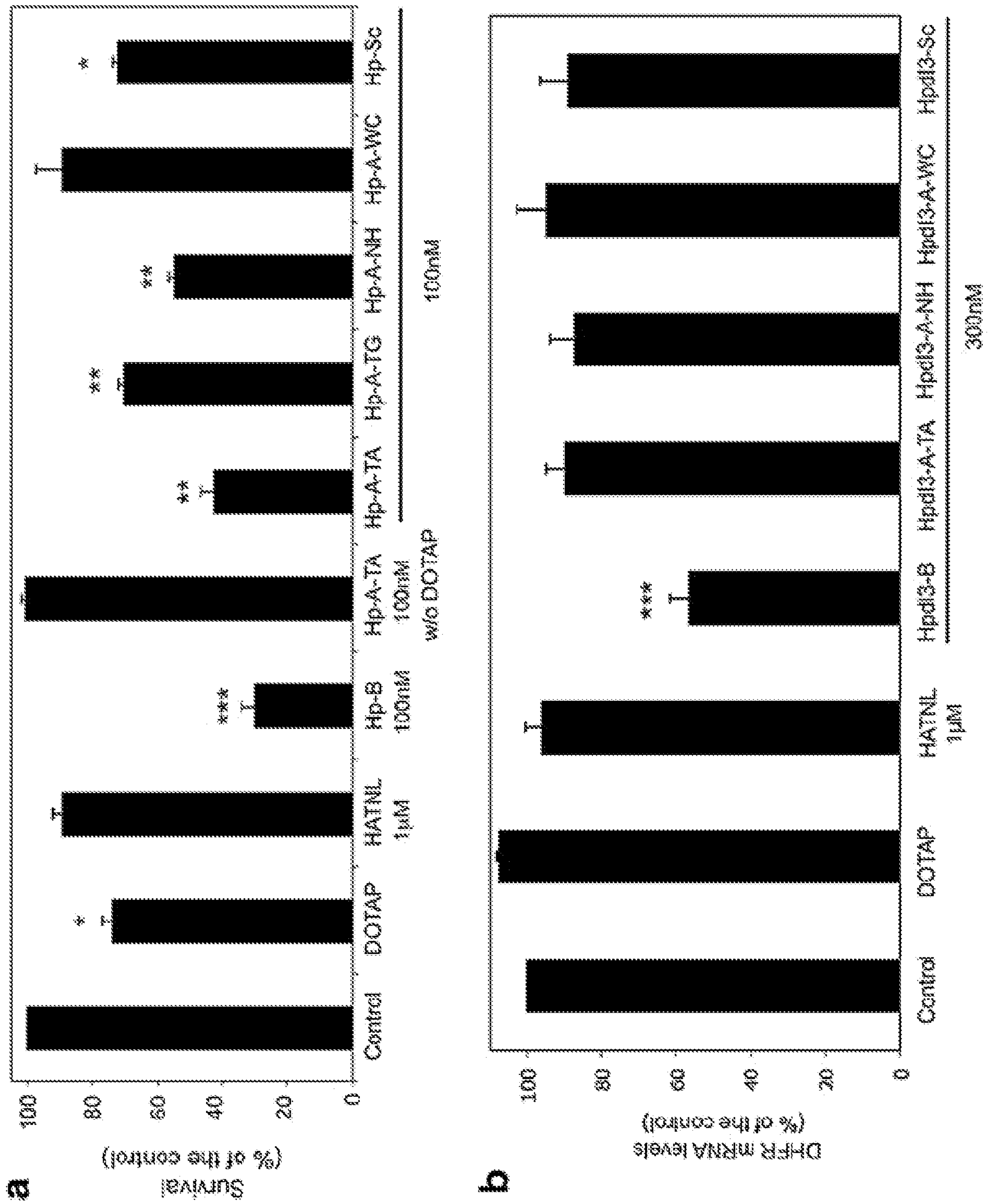


FIGURE 7

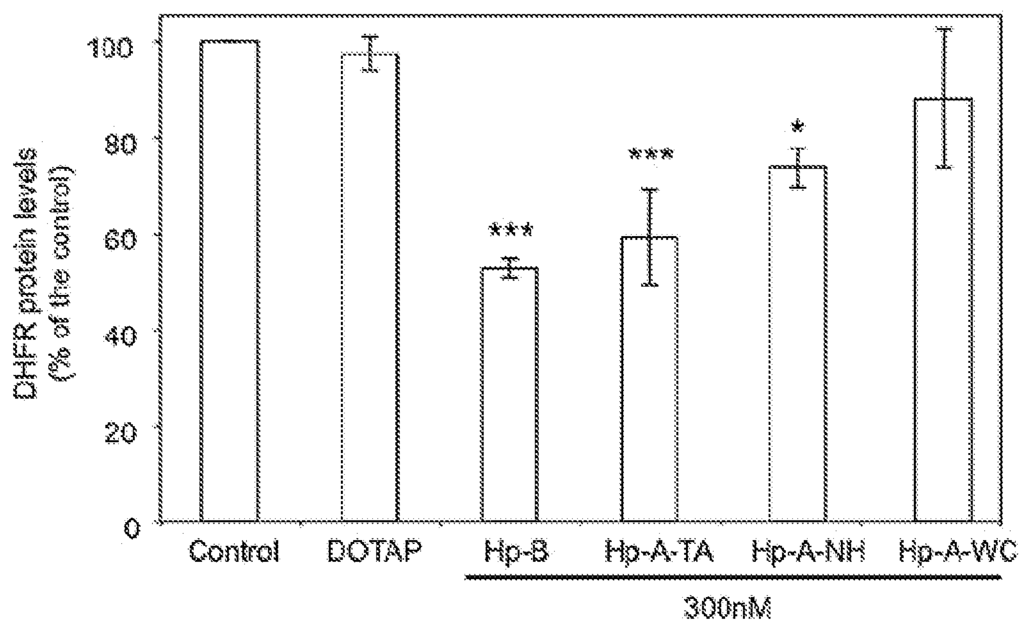


FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/055647

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113 A61K31/713 C12Q1/68
ADD. A61P35/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K C12Q

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. CRISTINA DE ALMAGRO ET AL: "Coding Polypurine Hairpins Cause Target-Induced Cell Death in Breast Cancer Cells", HUMAN GENE THERAPY, vol. 22, no. 4, April 2011 (2011-04), pages 451-463, XP55013893, ISSN: 1043-0342 **** Online publication 13.10.2010 ***** the whole document -----	1-20

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[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 December 2011	16/12/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Andres, Serge

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	almagro gracia cristina: "Paper de la UGT1A6 en la resistència al metotrexat: estudi dels hairpins de polipurines per disminuir l'expressió gènica", Universitat de Barcelona , 2010, XP55013960, Retrieved from the Internet: URL:http://tdx.cat/bitstream/handle/10803/974/MCAG_TESI.pdf?sequence=1 [retrieved on 2011-12-06] page 51; compounds HpdI3-A-TA page 87 - page 128 page 151	1-17
X	CRISTINA DE ALMAGRO M ET AL: "Polypurine Hairpins Directed against the Template Strand of DNA Knock Down the Expression of Mammalian Genes", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 284, no. 17, April 2009 (2009-04), pages 11579-11589, XP002665155, ISSN: 0021-9258 cited in the application the whole document	1-10, 12-17,20
A	C. MORALES ET AL: "Dihydrofolate reductase amplification and sensitization to methotrexate of methotrexate-resistant colon cancer cells", MOLECULAR CANCER THERAPEUTICS, vol. 8, no. 2, February 2009 (2009-02), pages 424-432, XP55013896, ISSN: 1535-7163 the whole document	1-20
A	CRAIG HORBINSKI ET AL: "Gone FISHing: Clinical Lessons Learned in Brain Tumor Molecular Diagnostics over the Last Decade", BRAIN PATHOLOGY, vol. 21, no. 1, January 2011 (2011-01), pages 57-73, XP55013871, ISSN: 1015-6305 cited in the application the whole document	18-20
T	NÚRIA MENCIA ET AL: "Underexpression of miR-224 in methotrexate resistant human colon cancer cells", BIOCHEMICAL PHARMACOLOGY, vol. 82, no. 11, 1 December 2011 (2011-12-01), pages 1572-1582, XP55013894, ISSN: 0006-2952	