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(54) Title: SENSITIZATION OF CANCER CELLS TO THERAPY USING SINA TARGETING GENES FROM THE 1P AND 19Q CHROMOSOMAL REGIONS

(57) Abstract: The invention relates to the identification of genes involved in resistance of cancer cells to therapy, to short nucleic acid molecules which inhibit the expression of these genes by RNA interference and to their use as adjuvant in cancer therapy, to sensitize cancer cells to conventional anticancer agents ; said short nucleic acid molecules are double-stranded short interfering nucleic acid molecules comprising a sense and an antisense region, wherein the sense region comprises a nucleotide sequence that is selected from the group consisting of: the sequences SEQ ID NO: 15, 11, 13, 14, 30, 31, 38, 46, 64 and 70 and the sequences having at least 70 % identity, preferably at least 80 % identity, more preferably at least 90 % identity with said sequences, and the antisense region comprises a nucleotide sequence that is complementary to the sense region.

## SENSITIZATION OF CANCER CELLS TO THERAPY USING siNA TARGETING GENES FROM THE 1p and 19q CHROMOSOMAL REGIONS

The invention relates to the identification of genes involved in resistance of cancer cells to therapy, to short nucleic acid molecules which inhibit the expression of these genes by RNA interference and to their use as adjuvant in cancer therapy, to sensitize cancer cells to conventional anticancer agents.

Gliomas are the most prevalent primary brain tumours. Among them, astrocytomas are a notable source of preoccupation in oncology because their incidence continually increases in industrialized countries (Ohgaki, H. and Kleihues, P., *Acta Neuropathol.*, 2005, 109, 93-108) and, above all, their prognosis is pessimistic because they are refractory to even the most aggressive therapy. For example, median survival time in a patient with glioblastoma multi-form (GBM), the worst grade of astrocytoma, is between 6 to 15 months after diagnosis (Chinot, O. et Martin, P.M., *Biologie des tumeurs cérébrales gliales*, 1996, Montpellier, France). Standard treatment is surgery followed by radiotherapy and chemotherapy. Recently, a large phase III study demonstrated that temozolomide (Temodal®) therapy associated to radiotherapy provided a modest 3 months increase survival in glioblastoma (Stupp et al., *N. Engl. J. Med.*, 2005, 352, 987-996).

Numerous works have addressed the chemoresistance as the principal cause of therapeutic fail (Harris, A.L., *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.*, 1985, 48, 675-680).

Contrasting with astrocytomas, oligodendrogliomas have a dramatic chemosensitivity (Perry et al., *Arch. Neurol.*, 1999, 56, 434-436) resulting in a median survival of 10 years after medical management. Oligodendroglioma response to chemotherapy has been correlated with the loss of heterozygosity (LOH) on 1p and 19q chromosomal arms (Cairncross et al., *J. Natl. Cancer Inst.*, 1998, 90, 1473-1479) with a common chromosomal area including 1p32-36 and 19q13.2-4 (Smith et al., *Oncogene*, 1999, 18, 4144-4152).

The major part of studies addressing the chemoresistance of astrocytomas concerned drug efflux mechanisms. However, the data are scattered and conflicting (Ashmore et al., *Anticancer drugs*, 1999, 10, 861-872). Expression of MDR-1 P-glycoprotein (P-gp), a protein associated with MDR, was not preferentially

detected in resistant gliomas (Demeule et al., *Int. J. Cancer*, 2001, 93, 62-66). Moreover, MDR-type drug resistance in glioma cell lines results of long-term culturing, and *in vivo*, only cerebral endothelium induces this mechanism (Bahr et al., *Brain Pathol.*, 2003, 13, 482-494). Finally, MDR-related genes were not located in the  
5 common 1p/19q deletion.

The correlation between 1p/19q LOH and oligodendrogliomas chemosensitivity strongly suggests that the reduction of global expression of some genes in the common region of deletion could be responsible for the observed chemosensitivity.

10 An adjuvant treatment targeting these genes could increase the chemosensitivity of cancer cells.

The completion of human genome sequencing provides the opportunity to investigate potential candidates among the 1,700 genes located in the  
1p/19q LOH region.

15 Some studies have focused the search on genes with potential oncogenic properties, which inactivation would lead to oligodendroglioma oncogenesis but no convincing results were obtained to date.

Numerous genes located in the 1p/19q chromosomal regions (LOH regions) could have potential impact on drug resistance, including those involved in  
20 drug efflux systems, metabolism, apoptosis, cell-cycle regulation and DNA-repair.

To date, the correlation between gene expression and drug resistance has been demonstrated for two genes of the Nucleotide Excision Repair (NER) system, extensively studied in cancer cells: the *ERCC1* and *ERCC2* genes. Furthermore, a functional involvement of these genes in drug resistance has been established for the  
25 *ERCC1* gene only.

Chemotherapy drugs commonly employed, such as PCV regimen (procarbazine, lomustine or CCNU, and vincristine), cisplatin, fotemustine, or temozolomide, are DNA-alkylating molecules. These drugs damage DNA leading cells to apoptosis. Cells can correct chemotherapy-induced alterations thanks to various  
30 DNA-repair mechanisms (Li et al., *Anticancer Res.*, 2000, 20, 645-652 ; Wu et al., *Clin. Cancer Res.*, 2003, 9, 5874-5879) and thus overcome treatment. This

phenomenon is responsible for chemoresistance (Bosken et al., *J. Natl. Cancer Inst.*, 2002, 94, 1091-1099).

A major role of ERCC1 in reparation of DNA alterations related to alkylating chemotherapy has been reported (Chaney S.G. & Sancar A., *J. Natl. Cancer Inst.*, 1996, 88, 1346-1360; Li et al., *Anticancer Res.*, 2000, 20, 645-652). Suppression of ERCC1 expression *in vitro* by antisense or siRNA technology, leads to a decreased repair activity and an increased sensitivity of cultured cell lines to platinum-based anticancer agents (Selvakumaran et al., *Cancer Res.*, 2003, 63, 1311-1316 ; Youn et al., *Cancer Res.*, 2004, 64, 4849-4857 ; Chang et al., *Biochem. Biophys. Res. Commun.*, 10 2005, 327, 225-233).

ERCC2 (XPD) expression has been correlated with resistance to alkylating compounds in numerous cell lines (Chen et al., *Ai Zheng*, 2002, 21, 233-239; Xu et al., *Anticancer drugs*, 2002, 13, 511-519), comprising glioma cell lines (Chen et al., *Neurosurgery*, 1998, 42, 1112-1119) and it has been reported that its 15 overexpression increased DNA repair in glioma cell lines (Chen et al., *Chin. Med. J.*, 2003, 116, 1171-1174). However, a functional involvement of ERCC2 in drug resistance has not been established.

RNAi interference is the process where the introduction of double-stranded RNA into a cell inhibits gene expression in a sequence dependent fashion 20 (reviewed in Shuey et al., *Drug Discovery Today*, 2002, 7, 1040-1046). RNAi has been observed in a number of organisms such as mammalian, *Drosophila*, nematodes, fungi and plants and is believed to be involved in anti-viral defense, modulation of transposon-activity and regulation of gene expression. RNAi is usually described as a post-transcriptional gene-silencing mechanism in which dsRNA triggers degradation of 25 homologous messenger RNA in the cytoplasm. Target recognition is highly sequence specific since one or two base pair mismatches between the siRNA and the target gene will greatly reduce silencing effect. The mediators of RNA interference are 21- and 23-nucleotide small interfering RNAs (siRNA). In a second step, siRNAs bind to a ribonuclease complex called RNA-induced silencing complex (RISC) that guides the 30 small siRNA to its homologous mRNA target. Consequently, RISC cuts the mRNA approximately in the middle of the region paired with the antisense siRNA, after which the mRNA is further degraded. Therefore, the use of exogenous siRNA holds great

promise as a new tool for mammalian functional genomics and may also have future applications as gene-specific therapeutics.

Using siRNA technology, the inventors have established that four other genes involved in DNA repair (*MUTYH*, *PNKP*, *POLD1*, and *RUVBL2*) and two  
5 genes encoding P450 cytochrom isoforms (*CYP2A6* and *CYP4B1*) are also involved in astrocytomas chemoresistance. In addition, the inventors have established, for the first time that ERCC2 is functionally involved in drug resistance.

*MUTYH* which belongs to the Base Excision Repair (BER) system is known to repair 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) caused by oxidation.  
10 *MUTYH* mutations and variants were associated with development of multiple colorectal adenomas and cancers (Chow et al., Lancet Oncol., 2004, 5, 600-606). *PNKP*, also belonging to the BER system, was shown to be involved in repairing DNA strand breaks caused by reactive oxygen species, ionizing radiations or alkylating agents (Whitehouse et al., Cell, 2001, 104, 107-117; Chappell et al., EMBO J., 2002,  
15 21, 2827-2832). It has been related to susceptibility to genotoxic agents but not to chemoresistance (Rasouli-Nia et al., P.N.A.S., 2004, 101, 6905-6910). *POLD1* is known to be involved in NER and NMR (Nucleotide Mismatch Repair) systems, while *RUVBL2* is known to be involved in homologous recombination; these proteins have neither been related to chemosensitivity.

20 The cytochrome P450 isoforms *CYP2A6* and *CYP4B1* are known as activators of carcinogenic aromatic amines, and represent possible risk factors for tobacco-related and bladder cancers in human (Kamatani et al., Biochem. Res. Comm., 2005, Sep 19; Imaoka et al., Biochem. Res. Comm., 2000, 277, 776-780): they have neither been related to chemosensitivity.

25 The inventors have engineered siRNA which efficiently inhibit the targeted genes expression and significantly sensitize astrocytoma cells to chemotherapy.

These siRNAs are useful as adjuvant in cancer therapy, to sensitize cancer cells to chemotherapy and radiotherapy.

30 Therefore, the invention relates to a double-stranded short interfering nucleic acid (siNA) molecule comprising a sense and an antisense region, wherein the sense region comprises a nucleotide sequence that is selected from the group consisting

of: the sequences SEQ ID NO: 11, 13, 14, 15, 30, 31, 38, 46, 64 and 70 and the sequences having at least 70 % identity, preferably at least 80 % identity, more preferably at least 90 % identity with said sequences, and the antisense region comprises a nucleotide sequence that is complementary to the sense region.

5           The siNA molecules according to the invention target seven genes (*ERCC2*, *MUTYH*, *PNKP*, *POLD1*, *RUVBL2*, *CYP2A6* and *CYP4B1*) of the chromosomal regions 1p32-36 and 19q13.2-4 (loss of heterozygosity regions or LOH region) which are all involved in resistance of cancer cells to chemotherapy and/or radiotherapy. The siNA molecules according to the invention are able to down regulate  
10 the expression of the *ERCC2*, *MUTYH*, *PNKP*, *POLD1*, *RUVBL2*, *CYP2A6* or *CYP4B1* genes (target genes) by RNA interference and thereby increase the sensitivity of cancer cells to conventional anticancer agents. Thus the siNA molecules according to the present invention potentiate the cytotoxic effect of chemotherapy/radiotherapy on cancer cells.

15           The resistance of cancer cells to an anticancer agent may be evaluated by a resistance index (RI) corresponding to the proportion of a cell population that survived to treatment with said anticancer agent. It is calculated as follows: cell number with anticancer agent treatment / cell number in control condition.

          The sensibilization effect mediated by the siNA according to the  
20 present invention, may be evaluated by the siNA-induced drug sensibilization index (DS) which corresponds to the cell population (%) that survived to a simple treatment with an anticancer agent but died in response to the same treatment with an siNA transfection. It is calculated as follows:  $(RI_{\text{control siNA}} - RI_{\text{target siNA}}) / RI_{\text{control siNA}} \times 100$ ; the target siNA is directed to the resistance gene and the control siNA is directed to a  
25 gene which is not involved in resistance to anticancer therapy.

          Both indexes may be determined by any assay that measures cell viability, which is well-known in the art, such as for example a MTT assay.

          Confirmation that the sensitization effect is mediated by inhibition of the target gene expression may be assayed by any RNA or protein analysis technique,  
30 which is well-known in the art (Northern-blot, Western-blot, quantitative RT-PCR).

          The siNA molecules according to the invention are defined by reference to the human *ERCC2*, *MUTYH*, *PNKP*, *POLD1*, *RUVBL2*, *CYP2A6* or

*CYP4B1* gene sequences (Table I); the target sequence corresponds to the portion of the mRNA which is complementary to the antisense region of the siNA molecule.

**Table I: Genes targeted with the siNA**

Target gene	GenBank accession number	Target sequence	positions
<i>ERCC2</i>	NM_000400	SEQ ID NO: 11	415-433
		SEQ ID NO: 13	1278-1296
		SEQ ID NO: 14	1719-1737
		SEQ ID NO: 15	1978-1996
<i>MUTYH</i>	NM_012222	SEQ ID NO: 30	1475-1493
<i>PNKP</i>	NM_007254	SEQ ID NO: 31	379-397
<i>POLD1</i>	NM_002691	SEQ ID NO: 38	1231-1249
<i>RUVBL2</i>	NM_006666	SEQ ID NO: 46	335-353
<i>CYP2A6</i>	NM_000762	SEQ ID NO: 64	591-609
<i>CYP4B1</i>	NM_000779	SEQ ID NO: 70	645-663

The invention encompasses the synthetic, semi-synthetic or recombinant siNAs which inhibit the expression of a target gene from any organism. Given the positions of the targets in the human mRNAs, one skilled in the art will easily find the corresponding positions in the homologous sequences of other organisms (eukaryotes, for example mammals) which are accessible in the databases such as the NCBI database (<http://www.ncbi.nlm.nih.gov/>). Such homologous sequences can be identified as is known in the art, for example using sequence alignment. In addition, the siNA molecule of the invention may inhibit the expression of target gene variants, for example polymorphic variants resulting from haplotype polymorphism.

siNA molecules can be designed to target such homologous sequences, for example using perfectly complementary sequences or by incorporating non-canonical base pairs, for example mismatches and/or wobble base pairs, including flipped mismatches, single hydrogen bond mismatches, trans-type mismatches, triple base interactions and quadruple base interactions, that can provide additional target sequences. For example, the siNA molecule can be designed to target a sequence that is unique to a specific target gene mRNA sequence (a single allele or single nucleotide polymorphism (SNP)) due to the high degree of specificity that the siNA molecule requires, to mediate RNA activity. Alternatively, when mismatches are identified, non-

canonical base-pairs (for example mismatches and/or wobble bases) can be used to generate siNA molecules that target more than one sequence. In a non-limiting example, non-canonical base-pairs such as uu and cc base pairs are used to generate siNA molecules that are capable of targeting homologous target gene sequences. In this approach, a single siNA can be used to inhibit expression of more than one gene instead of using more than one siNA molecule to target the different genes.

### ***Definitions***

- "short nucleic acid molecule" refers to a nucleic acid molecule no more than 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably, no more than 50 nucleotides in length.

- "interfering nucleic acid molecule" refers to a nucleic acid molecule capable of mediating RNA interference.

- "RNA interference" (RNAi) refers to the process of sequence specific post-transcriptional gene silencing, induced by introduction of duplexes of synthetic short nucleic acid molecule in cells, for example duplexes of synthetic 21-nucleotide RNAs, as first described by Elbashir et al., *Nature* 2001, 411, 494- and in the International PCT Application WO 01/75164.

- "nucleotide" refers to standard ribonucleotides and deoxyribonucleotides including natural bases (adenine, cytosine, guanine, thymine or uracil) and modified nucleotides that are modified at the sugar, phosphate, and/or base moiety.

- "Identity" refers to sequence identity between two nucleic acid molecules. Identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base, then the molecules are identical at that position. A degree of similarity or identity between nucleic acid or amino acid sequences is a function of the number of identical or matching nucleotides at positions shared by the nucleic acid sequences. Various alignment algorithms and/or programs may be used to calculate the identity between two sequences, including FASTA, or BLAST which are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default settings.

- "homologous" refers to a nucleic acid molecule having enough identity to another one to lead to RNAi activity, more particularly having at least 70 % identity, preferably 80 % identity and more preferably 90 %.

5 - "complementary" refers to the ability of a nucleic acid to form hydrogen bond(s) by either traditional Watson-Crick base-pairing or other non-traditional type base-pairing. In reference to the nucleic acid molecules of the present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well-known in the art (see, e.g., Turner et al., 1987, *CSH Symp. Quant. Biol.*, 1987, LII, pp 123-133, Frier et al., *P.N.A.S.*, 1986, 83, 9373-9377; Turner et al., *J. Am. Chem. Soc.*, 1987, 109, 3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base-pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, or 10 nucleotides out of a total of 10 nucleotides, in the first oligonucleotide being base-paired to a second nucleic acid sequence having 10 nucleotides represents 50 %, 60 %, 70 %, 80 %, 90 % and 100 % complementarity, respectively). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

15 - "target gene" refers to a gene whose expression is to be down-regulated, e.g. *ERCC2*, *MUTYH*, *PNKP*, *POLD1*, *RUVBL2*, *CYP2A6* or *CYP4B1* gene.

- "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked.

25 - "anticancer agent", "anticancer therapy" refers to both chemotherapy using cytotoxic agents and radiotherapy.

In one embodiment, the invention features an siNA molecule wherein each strand comprises or consists of 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, and each strand comprises at least 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) nucleotides that are complementary to the nucleotides of the other strand.

For example, the siNA molecule of the invention comprises or consists of a 19 to 21-nucleotide duplex (19 to 21 base pairs).

In another embodiment, the invention features an siNA molecule wherein the sense region comprises or consists of a nucleotide sequence having any of SEQ ID NO: 11, 13, 14, 15, 30, 31, 38, 46, 64 and 70 and the antisense region comprises or consists of a nucleotide sequence having any of SEQ ID NO: 93 to 102, respectively. These siNA target the human genes (Table II).

**Table II: siNA targeting the human genes**

Target gene	siNA sense strand identification number	siNA antisense strand	
		Identification number	sequence
<i>ERCC2</i>	SEQ ID NO: 11	SEQ ID NO: 93	5'-gcauuucccaucgacgucc-3'
	SEQ ID NO: 13	SEQ ID NO: 94	5'-uccaaagggcucgaugauga-3'
	SEQ ID NO: 14	SEQ ID NO: 95	5'-aauaaagagcagcuuguucc-3'
	SEQ ID NO: 15	SEQ ID NO: 96	5'-aucgaagguaagaaaguca-3'
<i>MUTYH</i>	SEQ ID NO: 30	SEQ ID NO: 97	5'-auauacuugauaugucagc-3'
<i>PNKP</i>	SEQ ID NO: 31	SEQ ID NO: 98	5'-auugaccaaauacagugug-3'
<i>POLD1</i>	SEQ ID NO: 38	SEQ ID NO: 99	5'-uggauguuguaaccgguga-3'
<i>RUVBL2</i>	SEQ ID NO: 46	SEQ ID NO: 100	5'-ucaucuccagggagaagau-3'
<i>CYP2A6</i>	SEQ ID NO: 64	SEQ ID NO: 101	5'-ugacaggaacucuuugucc-3'
<i>CYP4B1</i>	SEQ ID NO: 70	SEQ ID NO: 102	5'-aucgcugacugcaagguag-3'

In another embodiment of the invention, the siNA molecule comprises overhanging nucleotide(s) at one or both end(s), preferably, 1 to about 3 (e.g. about 1, 2, or 3) overhanging nucleotides. The overhanging nucleotides which are advantageously at the 3' end of each strand, are preferably 2'-deoxynucleotide(s), preferably 2'-deoxypyrimidine(s), such as a 2'-deoxythymidine(s). For example, the siNA molecule of the invention is a 19 to 21-nucleotide duplex with 3'-terminal tt overhang(s).

In another embodiment of the invention, the siNA molecule comprises blunt end(s), where both ends are blunt, or alternatively, where one of the ends is blunt.

In another embodiment of the invention, the siNA molecule is assembled from two separate oligonucleotide fragments or strands, wherein one fragment (sense strand) comprises the sense region and the second fragment (antisense strand) comprises the antisense region of the siNA molecule.

5 In another embodiment, the invention features an siNA molecule wherein the sense region is connected to the antisense region via a linker molecule, such as a nucleotide or non-nucleotide linker. A nucleotide linker can be a linker of at least 2 nucleotides in length, for example about 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. Examples of such siNA molecules include small hairpin nucleic acid (shNA)  
10 molecules.

A non-nucleotide linker comprises abasic nucleotides, aptamers, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds.

In another embodiment of the invention, the siNA molecule comprises  
15 mismatches, bulges, loops or wobble base pairs to modulate the activity of the siNA molecule to mediate RNA interference.

In another embodiment of the invention, the siNA molecule comprises or consists of ribonucleotide(s) (2'-OH nucleotides).

In addition, the siNA molecule may include one or more modifications  
20 which increase resistance to nuclease degradation *in vivo* and/or improve cellular uptake. The siNA may include nucleotides which are modified at the sugar, phosphate, and/or base moiety, and/or modifications of the 5' or 3' end(s), or the internucleotidic linkage.

In another embodiment of the invention, the siNA molecule comprises  
25 one or more modified pyrimidine and/or purine nucleotides, preferably on each strand of the double-stranded siNA. More preferably, said modified nucleotides are selected from the group consisting of : 2'-O-methylnucleotides, 2'-O-methoxyethylnucleotides, deoxynucleotides, such as 2'-deoxynucleotides and 2'-deoxy-2'-fluoronucleotides, universal base nucleotides, acyclic nucleotides and 5-C-methyl nucleotides. An siNA  
30 molecule of the invention can generally comprise about 5 % to about 100 % modified nucleotides (e.g., about 5 %, 10 %, 15 %, 20 %, 25 %, 30 %, 35 %, 40 %, 45 %, 50%, 55 %, 60 %, 65 %, 70 %, 75 %, 80 %, 85 %, 90 %, 95 % or 100 % modified

nucleotides). The actual percentage of modified nucleotides present in a given siNA molecule will depend on the total number of nucleotides present in the siNA molecule. The percent modification can be based upon the total number of nucleotides present in the sense strand, antisense strand or both the sense and the antisense strands.

5                    In another embodiment, the invention features an siNA molecule wherein the strand comprising the sense region (sense strand) includes a terminal cap moiety at the 5'-end, the 3'-end, or both the 5' and 3' ends of the strand, preferably a deoxy abasic moiety or glyceryl moiety.

                    In another embodiment, the invention features an siNA molecule  
10 wherein the strand comprising said antisense region (antisense strand) includes a phosphate group at the 5'-end.

                    In another embodiment of the invention, the siNA molecule comprises at least one modified internucleotidic linkage, such as a phosphorothioate linkage.

                    The siNA molecules according to the invention may be produced by  
15 chemical synthesis by using well-known oligonucleotides synthesis methods which make use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites, at the 3' end. The nucleic acid molecules of the present invention can be modified to enhance stability by modification with nuclease resistant groups, for example 2'-amino, 2'-C-allyl, 2'-  
20 fluoro, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, *TIBS*, 1992, 17, 34 and Usman et al., *Nucleic Acids Symp. Ser.*, 1994, 31, 163). Examples of such modified oligonucleotides include with no limitation: 2' F-CTP, 2' F-UTP, 2' NH<sub>2</sub>-CTP, 2' NH<sub>2</sub>-UTP, 2' N<sub>3</sub>-CTP, 2-thio CTP, 2-thio UTP, 4-thio UTP, 5-iodo CTP, 5-iodo UTP, 5-bromo UTP, 2-chloro ATP, adenosine 5'-(1-thiotriphosphate), cytidine  
25 5'-(1-thiotriphosphate),            guanosine-5'-(1-thiotriphosphate),            uridine-5'-(1-thiotriphosphate), pseudo-UTP, 5-(3-aminoallyl)-UTP and 5-(3-aminoallyl)-dUTP. siNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC) and re-suspended in water.

                    The chemically-synthesized siNA molecule according to the  
30 invention may be assembled from two distinct oligonucleotides which are synthesized separately. Alternatively, both strands of the siNA molecule may be synthesized in tandem using a cleavable linker, for example a succinyl-based linker.

Alternatively, the siNA molecules of the invention may be expressed (*in vitro* or *in vivo*) from transcription units inserted into DNA or RNA vectors known to those skilled in the art and commercially available.

The invention relates also to a transcription unit comprising: a  
5 transcription initiation region, a transcription termination region, and a nucleic acid sequence encoding at least one siNA molecule according to the present invention, wherein said nucleic acid sequence is operably linked to said initiation region in a manner that allows expression and/or delivery of the siNA molecule.

The nucleic acid sequence may encode one or both strands of the  
10 siNA molecule, or a single self-complementary strand that self-hybridizes into an siNA duplex.

The transcription initiation region may be from a promoter for a eukaryotic RNA polymerase I, II or III (pol I, II or III). Transcripts from pol II or pol III promoters are expressed at high levels in all cells. Alternatively, prokaryotic RNA  
15 polymerase promoters may be used, providing that prokaryotic RNA polymerase enzyme is expressed in the appropriate cells. Transcription units derived from genes encoding U6 small nuclear transfer RNA and adenovirus VA RNA are useful in generating high concentrations of desired siNA in cells.

The invention concerns also an expression vector comprising a  
20 nucleic acid encoding at least one siNA molecule of the instant invention. The expression vector may encode one or both strands of the siNA molecule, or a single self-complementary strand that self-hybridizes into an siNA duplex. The nucleic acid encoding the siNA molecule of the instant invention is preferably inserted in a transcription unit as defined above.

25 Large numbers of DNA or RNA vectors suitable for siNA molecule expression are known to those of skill in the art and commercially available. The recombinant vectors can be DNA plasmids or viral vectors. SiNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus or alphavirus. The recombinant vectors capable of expressing the  
30 siNA molecules can be delivered *in vivo*, and persist in target cells. Alternatively, viral vectors can be used to provide transient expression of siNA molecules.

The invention concerns also eukaryotic or prokaryotic cells which are modified by a vector as defined above.

The invention concerns also a pharmaceutical composition comprising at least an siNA molecule or an expression vector, as defined above, in an acceptable carrier, such as stabilizer, buffer and the like.

A pharmaceutical composition or formulation refers to a form suitable for administration, e.g., systemic or local administration, into a cell or subject, including for example a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, inhalation, or by injection. These compositions or formulations are prepared according to any method known in the art for the manufacture of pharmaceutical compositions.

In one embodiment, the invention features a composition wherein the siNA molecule or vector is associated to a compound that allows the delivery of the siNA/vector into cancer cells. The compound may be a membrane peptide, transporter, lipid, hydrophobic moiety, cationic polymer, PEI. Examples of membrane peptides include those able to cross the blood-brain barrier, such as with no limitation the Pep:Trans<sup>TM</sup> (<http://www.syntem.com/english/techpeptrans.html>). Preferably, the siNA and the compound are formulated in microspheres, nanoparticules or liposomes. Furthermore, the siNA molecule or vector may be associated with a compound that allows a specific targeting of the tumor, such as a ligand of a cell-surface antigen or receptor, for example a peptide or an antibody specific for said antigen/receptor (e.g., PS100, PDGFR, erb-B2).

In another embodiment, the invention features a composition comprising a combination of at least two different siNA molecules.

In another embodiment, the invention features a composition wherein the siNA molecule or vector is associated with at least one anticancer drug.

The invention also concerns an siNA molecule or a vector as defined above, as a medicament.

The invention concerns also the use of an siNA molecule or a vector as defined above, for the manufacture of a medicament for treating cancer.

The cancer may be of any type. Preferably, the cancer is a solid tumor, for example brain tumors such as astrocytomas, glioblastomas, oligodendrogliomas or mixed tumors.

In one embodiment of said use, the siNA molecule or vector is associated with an anticancer drug.

The invention concerns also a product containing at least one siNA molecule or vector as defined above, and an anticancer drug, as a combined preparation for simultaneous, separate or sequential use in anticancer therapy.

The anticancer drugs which are used in combination with the siNA molecule or the vector according to the invention are those commonly used in chemotherapy, and include cytotoxic agents, such as alkylating agents and antimetabolites.

Preferred anticancer drugs are alkylating agents, such as: cisplatin (cis-diaminedichloroplatinum, CDDP or DDP), temozolomide, fotemustine, procarbazine, lomustine and vincristine.

In addition, the siNA molecule according to the invention may be used in combination with other conventional anticancer therapies including radiotherapy, immunotherapy and surgery.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence or treat (alleviate a symptom to some extent, preferably all the symptoms) of a disease or state. The pharmaceutically effective dose of the siNA depends upon the type of cancer, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors, that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered.

The siNA of the invention may be administered by a single or multiple route(s) chosen from: local (intratumoral, for example intracerebral (intrathecal, intraventricular)), parenteral (percutaneous, subcutaneous, intravenous, intramuscular, intraperitoneal), oral, sub-lingual, or inhalation.

When the siNA molecule or vector is used in combination with chemotherapy or radiotherapy, it is preferably administered immediately prior to the anticancer agent or several hours (2 to 48 hours) before.

In addition to the preceding features, the invention further comprises other features which will emerge from the description which follows, which refers to examples illustrating the siNA molecules and their uses according to the invention, as well as to the appended drawings in which:

- Figure 1 illustrates the screening of DNA-repair genes in human glioma (GHD cells). Cells were seeded in 96-well plates, transfected with siRNA after 24 h and treated with Cisplatin (CDDP) after 48 h. 96 hours post treatment, alive cell number was evaluated with MTT. 1: *ERCC2*, 2: *RAD54L*, 3: *LIG1*, 4: *MUTYH* 5: *PNKP*, 6: *POLD1*, 7: *REV7(MAD2L2)*, 8: *MGC13170*, 9: *RUVBL2*, G: GFP. The proportion of a cell population that survives to chemotherapy is evaluated by the chemoresistance index (CI): cell number (OD) with chemotherapy / cell number (OD) in control condition. CI for each siRNA was compared with control CI (siRNA GFP). Data represented the mean of 3 independent experiments. CI is inferior to  $CI_{GFP}$  (0.5 or 50%) when siRNA transfection improves chemosensitivity and superior to  $CI_{GFP}$  (grey) when it increases chemoresistance. White square corresponds to siRNA with low toxicity (< 35 % cell death without cisplatin) and with chemosensitization properties.

- Figure 2 illustrates drug sensitization by siRNAs targeting four DNA repair genes : *ERCC1*, *ERCC2*, *MUTYH*, and *PNKP*. SiRNAs were transfected in 6 different astrocytoma derived cell lines treated with CDDP or temozolomide (TMZ). DS corresponds to chemosensitivity induced by siRNA. siRNA targeting *ERCC1* was included for comparison. Data represented the mean of 3 independent experiments. Only significant results were reported (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ).

- Figure 3 illustrates the effect of siRNAs targeting two cytochrome P450 isoforms (*CYP2A6* and *CYP4B1*) on the chemosensitization of astrocytoma cells. SiRNAs were transfected in U373 cell line treated with CDDP. The chemoresistance indice (CI) for each siRNA was compared with control CI (siRNA GFP). siRNA targeting *ERCC1* was included for comparison. Data represented the mean of 3 independent experiments.

**Example 1: siRNA directed to 1p/19q DNA repair genes are able to sensitize cancer cells to chemotherapy**

**1) Material and Methods**

a) Cell culture

5 U87, U373, U138, CCF and LN229 cells deriving from human astrocytoma were purchased from American Type Culture Collection (ATCC). GHD cell line derived from a human astrocytoma biopsy, was checked by fluorescence *in situ* hybridization, chromosome 7 polysomy, chromosome 10 monosomy and immuno-histo-chemistry. Cells were maintained in DMEM (CAMBREX BIOSCIENCES), 10 %  
10 FCS (v/v; ABCYS), and incubated in a humidified atmosphere with 5 % CO<sub>2</sub>, at 37 °C.

b) Inhibition of gene expression with siRNA

Three to five siRNA pairs were designed for each candidate gene and prepared into duplex form (EUROGENTEC). siRNAs targeting the Green Fluorescent Protein-(GFP) and the ERCC1 protein were used as control siRNAs. 24 hours after cell  
15 seeding (96 well plates), siRNA (150 nM) were transfected with Oligofectamine™ (Invitrogen), according to the manufacturer's instructions. Each condition (siRNA) was tested in 3 independent experiments, each time in hexaplicate.

c) Cell survival and chemoresistance

Cells were seeded in 96 well plates, transfected with siRNA after 24 h  
20 and treated with cisplatin (CDDP, MERCK, 5 µM final concentration) or temozolomide (TMZ, SCHERING-PLOUGH, 1 µM final concentration) after 48 h. Cell survival was determined 96 hours post-treatment, by measuring mitochondrial succinate dehydrogenase activity, with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, SIGMA) added to a final concentration of 0.5  
25 mg/ml into culture medium. Culture medium was discarded after 4 h of incubation with MTT and formazan crystals were dissolved in DMSO/ethanol (50/50, v/v). Optical density was read at 540 nm. Chemoresistance was related to an index (CI) corresponding to the proportion of a cell population that survived to chemotherapy. It was calculated as follows: cell number (OD) with chemotherapy / cell number (OD) in  
30 control condition. The benefit of siRNA transfection was represented by the siRNA-induced drug sensibilization index (DS) which corresponds to the cell population (%) that survived to a simple chemotherapy treatment but died in response to the same

treatment with a siRNA transfection. It was calculated as follows:  $(CI_{siRNA\ GFP} - CI_{siRNA\ X}) / CI_{siRNA\ GFP} \times 100$ . Significant differences between series were tested by ANOVA with Statview (SAS institute). Differences were considered significant when  $p < 0.05$  (\*) and very significant when  $p < 0.01$  (\*\*).

5 d) Q-RT PCR

10 RNAs were extracted on silica column (Nucleospin™, MACHEREY-NAGEL). RNAs were reverse transcribed using the M-MLV reverse transcriptase RNase H minus enzyme (Promega) and oligodT as a primer, at 40 °C during 1.5 hours. After reverse transcription, cDNAs were purified (Mini Elute, PCR Purification Kit™, QIAGEN) and assayed by quantitative PCR using the HotStar Reaction mix (ROCHE), using a pair of primers specific for the candidate gene and the reference gene *ERCC1* (Table III). Measurements were carried out on a Light Cycler thermo-cycler (ROCHE).

**Table III: qRT-PCR primers sequences**

Gene	Primer	Sequence (5'-3')	SEQ ID NO:	Annealing temperature (°C)	Product size (bp)
<i>Cyclophyline A</i>	Forward	ttcatctgcactgccaagac	1	61.8	158
	Reverse	tcgagttgtcacagtcagc	2		
<i>ERCC1</i>	Forward	ggcgacgtaattcccgacta	3	61.8	121
	Reverse	agttctccccaggctctgc	4		
<i>ERCC2</i>	Forward	cggaactatgggaacctcct	5	64.0	200
	Reverse	tactctccaggcgacact	6		
<i>MUTYH</i>	Forward	gtcctgacgtggaggagtgt	7	64.0	200
	Reverse	cctctgcaccagcagaattt	8		
<i>PNKP</i>	Forward	tcgagagatgacggactcct	9	64.0	206
	Reverse	tttattgtggaggggagctg	10		

15 Measures were performed twice from each cell sample, and results were expressed as the mean of at least three independent samples (originating from independent *in vitro* experiments).

**2) Results**

20 a) Identification of DNA repair genes potentially involved in chemoresistance of astrocytoma cells

Genes potentially responsible for chemoresistance of astrocytoma, were screened from the chromosomic area commonly considered as correlated with

oligodendroglioma chemosensitivity (1p36-1p32 and 19q13.2-19q13.4; Smith et al., Oncogene, 1999, 18, 4144-4152). Gene sequences were retrieved from data bases on the web (<http://www.ncbi.nlm.nih.gov/PubMed/> ; <http://www.ensembl.org> ; [http://www.cgal.icnet.uk/DNA\\_Repair\\_Genes.html](http://www.cgal.icnet.uk/DNA_Repair_Genes.html) ; Supplement to the paper by Wood et al., Mutat. Res., 2005, 577, 275-283) and published data (Harris, A.L., Int J Radiat Biol Relat Stud Phys Chem Med, 1985, 48, 675-90 ; Christmann et al., Toxicology, 2003, 193, 3-34 ; Wood et al., Mutat. Res., 2005, 577, 275-283). Eight genes belonging to different repair systems were selected (Table IV).

**Table IV : List of DNA repair-associated genes located on 1p/19q LOH regions**

DNA repair genes	DNA repair system	Accession number	Localization
<i>LIG1</i>	NER	NM_000234	19q13.2-3
<i>ERCC2 (XPD)</i>	NER	NM_000400	19q13.3
<i>POLD1</i>	NER and MMR**	NM_002691	19q13.3
<i>RUVBL2</i>	Homologous Recombination	NM_006666	19q13.3
<i>PNKP</i>	BER***	NM_007254	19q13.3-4
<i>RAD 54L</i>	Homologous Recombination	NM_003579	1p32
<i>MUTYH</i>	BER	NM_012222	1p34.3
<i>MAD2L2 (REV7)</i>	DNA polymerase	NM_006341	1p36

10

\* NER: Nucleotide Excision Repair

\*\* NMR: Nucleotide Mismatch repair

\*\*\* BER: Base excision repair

b) *In vitro* chemosensitivity assay

An *in vitro* assay for the siRNAs high-throughput screening, was developed to find out which genes (Table IV) are implicated in glioma chemoresistance mechanisms. The cell number after chemotherapy and siRNA transfection was measured to have a global chemosensitization evaluation, defined as a significant increased cell death compared with the effect of the drug alone. In a first series of experiments, the following conditions of the assay were established: (i) drug and siRNA treatment protocols, (ii) specific temporal sequence of cell seeding and drug treatment, (iii) siRNA transfection, (iii) cell viability measurement. CDDP was chosen because it is an alkylating agent harboring a very reproducible activity *in vitro*. Most, if not all, cell damages occurred during the first hour of drug treatment since one and 24 hour(s) incubations were equally efficient. Moreover, it was established that the mRNA amount was the lowest 24 and 48 hours after siRNA transfection. Altogether, these data suggested that siRNAs had to be added prior to the drug.

25

c) Screening of DNA-repair genes involved in chemoresistance of astrocytoma cells

The screening was performed in three steps. First siRNAs targeting candidate genes were screened on one cell line and results were confirmed at statistic level in further experiments. The study was then extended to six astrocytoma-derived cell lines and the siRNAs were validated at molecular level. Finally, the study was extended to a second chemotherapy agent.

The eight DNA-repair genes located in the 1p/19q (Table IV) were screened with five different siRNAs by gene, on the GHD cell line. The siRNAs sequences are presented in Table V. One siRNA specific for GFP and one siRNA specific for ERCC1 were used as controls.

**Table V: SiRNAs sequences**

Gene	siRNA sequence*(1) (2)	SEQ ID NO:
<b>ERCC2</b>	5'-ggacgucgaugggaaaugc-3'	11
	5'-agacggugcucaggaucaa-3'	12
	5'-ucaucaucgagccuuuga-3'	13
	5'-ggaacaagcugcucuuuau-3'	14
	5'-ugacuuucuuaccuucgau-3'	15
<b>LIG1</b>	5'-agacgcucagcagcuucuu-3'	16
	5'-gaagauagacaucaucaa-3'	17
	5'-agacagcagagcccagaaa-3'	18
	5'-gcagacguucugcgagguu-3'	19
	5'-gcagauccagccauuccaa-3'	20
<b>MAD2L2</b>	5'-gaagaugauguggagaaa-3'	21
	5'-gacucgcuguugucucaug-3'	22
	5'-cucgcaacaugcagaagau-3'	23
	5'-gaagauccaggucaucaag-3'	24
	5'-ugagcaggauuccacaug-3'	25
<b>MUTYH</b>	5'-gaagcaugcuaagaacaac-3'	26
	5'-ugggaugauugcugagugu-3'	27
	5'-gcacccuuguuuccagca-3'	28
	5'-gguuguccacccuucucu-3'	29
	5'-gcugacaucaaguauau-3'	30

**Table V: SiRNAs sequences (continued)**

Gene	siRNA sequence*(1) (2)	SEQ ID NO:
<b>PNKP</b>	<b>5'-cacacuguauuuggucaau-3'</b>	<b>31</b>
	5'-agagacccgcacaccagaa-3'	32
	5'-gaaucuuguaccagagau-3'	33
	5'-aguccaccuuucaagaa-3'	34
	5'-caaccgguuucgagagaug-3'	35
<b>POLD1</b>	5'-ggagauggaggcagaacac-3'	36
	5'-guuggagauugaccuuau-3'	37
	<b>5'-ucaccgguuacaacaacca-3'</b>	<b>38</b>
	5'-cuuagacuccaccagcugc-3'	39
	5'-auucagaugggauaccucc-3'	40
<b>RAD54L</b>	5'-ccagcauugugaauagaug-3'	41
	5'-ucaccucgcuaaagaagcu-3'	42
	5'-ggagcuguuuauccuggau-3'	43
	5'-ugaucugcuugaguauuuc-3'	44
	5'-gcagugagaccagaucca-3'	45
<b>RUVBL2</b>	<b>5'-aucuucuccuggagauga-3'</b>	<b>46</b>
	5'-acugaccucaagaccaca-3'	47
	5'-acgcaagguacagaagug-3'	48
<b>GFP</b>	5'-gacguaaacggccacaaguuc-3'	49
<b>ERCC1</b>	<b>5'-ggagcuggcuaagaugugu-3'</b>	<b>50</b>

(1) siRNA sequences are defined by the sequence of the sense strand. The antisense strand is perfectly complementary to the sense strand. SiRNAs responsible for a significant sensitization effect are in bold.

5

(2) Said sequences comprise a 3'-terminal tt overhang.

Cell viability was measured in absence and in presence of CDDP.

SiRNAs were selected on the basis of two criteria: the absence of basal toxicity and their efficiency to improve chemotherapy treatment. Toxic siRNAs inducing more than 35 % cell death after transfection (without CDDP) were discarded. In contrast, those increasing cell death were retained. An siRNA increased chemosensitivity when its chemoresistance index (CI) was lower than GFP siRNA CI, ie lower than 0.5. In figure 1, the white square corresponds to siRNA with low toxicity and with chemosensitization properties. Eight siRNAs out of 38 matched with these criteria, corresponding to 5 genes: *ERCC2*, *MUTYH*, *PNKP*, *POLD1*, and *RUVBL2* (figure 1).

Only one sequence of *MUTYH*, *PNKP*, *RUVBL2* and *POLD1* (SEQ ID NO: 30, 31, 38 and 46) siRNAs increased chemosensitivity while 4 (SEQ ID NO: 11, 13, 14 and 15) out of 5 sequences targeting *ERCC2* fulfilled these conditions.

The most efficient siRNA for each of these genes was transfected in 5 6 different glioma derived cell lines treated with CDDP or temozolomide (TMZ). CIs for each siRNA were compared with control CI (siRNA GFP). The results confirmed that three siRNAs targeting *ERCC2*, *MUTYH* and *PNKP*, respectively, had a significant chemo-sensitization effect on astrocytoma cells (figure 2). The three siRNAs were equally efficient (up to 17 %) on 3 (*ERCC2*) or 2 (*MUTYH* and *PNKP*) cell lines (figure 10 2). When combined by 2 or 3, siRNAs did not show any greater efficiency. By comparison, inhibition of *ERCC1* was more potent to sensitize cells to CDDP (up to 24.9 %) and its effect was the most widespread since 4 out of the 6 cell lines were sensitized.

The previously observed effects were validated at molecular level ; a 15 significant mRNA content decrease was confirmed by qRT-PCR for all siRNAs (Table VI).

**Table VI: Validation of siRNA –induced mRNA content down-regulation**

Cell line	mRNA	inhibition %	P ANOVA
GHD	<i>ERCC2</i>	61.6	0.011
	<i>MUTYH</i>	71.0	0.004
	<i>PNKP</i>	83.8	0.005
	<i>ERCC1</i>	58.6	0.019
U373	<i>ERCC2</i>	95.7	0.003
	<i>MUTYH</i>	72.9	0.007
	<i>PNKP</i>	44.2	0.013
	<i>ERCC1</i>	77.4	0.018

When a particular siRNA was transfected, the quantities in mRNA 20 corresponding to all the other non targetted genes were unchanged, confirming the target specificity of the selected siRNA. There was no obvious link between the efficiency of siRNA to decrease mRNA amounts (nor with final mRNA content) and functional impact on cell viability (Figure 2). This is reinforced by the observation that

*ERCC1* mRNA amount actually decreased (by 65 %) in U87 cells which were yet not chemosensitized.

d) Study for a link between DNA-repair genes expression and siRNA-induced chemotherapy

5 Expression of the 4 DNA-repair genes was studied by qRT-PCR in the 6 cell lines, with (induced level) or without cisplatin (basal level) to analyse the hypothesis that differences in expression could account for differences in siRNA responses. For each individual mRNA, the relative gene expression levels were roughly similar in all cell lines in absence of drug (Table VII).

10

**Table VII : DNA repair gene expression**

Cell line	<i>ERCC1</i>				
	Basal		Induced		
	Qty*	sem	Qty	sem	%**
GHD	33	13	35	15	104
LN229	33	13	78	30	233
U138	40	17	97	36	242
U373	11	5	12	6	110
U87	25	10	25	10	99
CCF	8	4	12	6	148
Cell line	<i>ERCC2</i>				
	Basal		Induced		
	Qty*	sem	Qty	sem	%**
GHD	10	3	17	5	163
LN229	4	1	11	1	268
U138	21	4	66	15	312
U373	14	1	17	4	122
U87	5	1	5	1	96
CCF	2	1	3	1	196
Cell line	<i>MUTYH</i>				
	Basal		Induced		
	Qty*	sem	Qty	sem	%**
GHD	108	19	114	14	105
LN229	137	52	439	44	320
U138	185	43	469	157	254
U373	49	33	135	41	278
U87	91	28	101	37	110
CCF	45	20	117	40	257
Cell line	<i>PNKP</i>				
	Basal		Induced		
	Qty*	sem	Qty	sem	%**
GHD	94	28	83	23	88
LN229	137	35	283	70	206
U138	550	116	1198	171	218
U373	115	31	149	4	130
U87	224	50	196	63	88
CCF	76	20	130	29	171

\* quantity in zeptomol ( $10^{-21}$ mol) in a 25 ng total RNA extract

\*\* percentage of basal level

*MUTYH* and *PNKP* mRNAs were the most abundant and *ERCC2* was generally the less one. Expression of the four genes was reinforced up to three times in response to chemotherapy, in at least two cell lines (Table VII). No gene was up-regulated in all cell lines, the four genes were up-regulated in two cell lines (LN229 and U138) and none in one other (U87). Thus, there was no common induction profile conserved among all cell lines. Finally, no correlation was found between chemosensitization by a siRNA (DS) and basal or CDDP-induced expression of corresponding gene or even global expression of the 4 genes.

e) Transposition of chemo-sensitization by siRNAs from CDDP to temozolomide.

Since temozolomide is becoming the chemotherapy gold standard for gliomas, the study was extended to this drug. *ERCC1* siRNAs were more efficient for sensitizing astrocytoma-derived cell lines to this second drug. However, down-regulating *MUTYH*, *PNKP* or *ERCC2* improved very significantly drug effects in U373 cells (figure 2).

**Example 2: siRNA directed to 1p/19q genes involved in drug metabolism are able to sensitize cancer cells to chemotherapy**

**1) Material and Methods**

The experimental procedures are described in example 1.

**2) Results**

a) Identification of genes potentially involved in chemoresistance of astrocytoma cells

Genes potentially responsible for chemoresistance of astrocytoma, were screened from the chromosomic area considered as commonly correlated with oligodendroglioma chemosensitivity, as described in example 1. Nine genes involved in drug metabolism (detoxification, cellular efflux, apoptosis) were selected (Table VIII).

**Table VIII: List of drug metabolism-associated genes located on 1p/19q LOH regions**

Genes	Function	Accession number	Localization
<i>CYP2B6</i> <i>CYP2F1</i> <i>CYP2A6</i> <i>CYP4B1</i>	Drug and lipid metabolism : Cytochrome P450 proteins (monooxygenases) catalyze reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids	NM_000767 NM_000774 NM_000762 NM_000779	19q13.2 19q13.2 19q13.2 1p34-p12
<i>FRAP1</i>	Signal transduction : The corresponding protein belongs to the family of phosphatidylinositol kinase-related kinases. These kinases mediate cellular responses to stresses such as DNA damage and nutrient deprivation	NM_004958	1p36.2
<i>MGC13170</i>	Multidrug resistance-related protein : Putative MDR-like function	NM_199249	19q13.33
<i>MLP</i>	Signal transduction : The myristoylated, alanine-rich protein MARCKS is a widely expressed, prominent substrate for protein kinase C, involved in brain development	NM_023009	1p35.1
<i>MSH4</i>	DNA metabolism : DNA mismatch repair and meiotic recombination	NM_002440	1p31
<i>RPS8</i>	Translation : Protein participating to translation complex	NM_001012	1p34.1-p32

b) Screening of DNA-repair genes involved in chemoresistance of astrocytoma cells

The eight genes located in the 1p/19q (Table VIII) were screened with five different siRNAs by gene, on two different cell-lines, U373 and GHD. The siRNAs sequences are presented in Table IX. A siRNA specific for GFP was used as control.

5

**Table IX: SiRNAs sequences\***

Gene	siRNA sequence	position	SEQ ID NO:	
<i>CYP2B6</i>	ccaccauccuccagaacuu	1359	51	
	ggaaaaucaaugcuuacauu	724	52	
	acaggugauuggcccacau	994	53	
	ugaccacacuaacuugaa	1198	54	
	acacgcucucgcucuucuu	879	55	
	ccagggagaggaguuuagu	326	91	
	gaagcauugaggagcgau	475	92	
	<i>CYP2F1</i>	ccacacauaaccugcucu	928	56
	gcauaagcacagccaucuu	64	57	
	ucaaugacaacuuccaaau	661	58	
	acacggaguucuacuugaa	857	59	
	ccaccgucaugcagaacuu	1370	60	
	<i>CYP2A6</i>	ugaccacguugaaccucu	887	61
	ccaaguucgggauuucuu	1142	62	
	gcaccagcaucguuguaga	1020	63	
	<b>ggacaaagaguuccuguca</b>	<b>591</b>	<b>64</b>	
	gcaagccugucaccuuugu	1175	65	
	<i>CYP4B1</i>	ugagccugacuaugccaaa	297	66
		ugaugugcugaagccuau	453	67
ggagucuaucgcucuucuu		253	68	
gcacgaucauucuuucuu		3228	69	
<b>cuaccuugcagucagcgau</b>		<b>645</b>	<b>70</b>	
<i>FRAP1</i>		agaacucgcugaucacaaa	1230	71
	ccagcagcauaagcaggaa	5287	72	
	caagcgacauccaugaaa	7670	73	
	gcaggcugcucuccauggu	969	74	
	ggcucaugcugggacccaa	1010	75	

**Table IX: SiRNAs sequences\*(continued)**

Gene	siRNA sequence	position	SEQ ID NO:
<b>MGC13170</b>	ggagcuguccauacgccac	1159	76
	ggagaagguggauaagugg	1068	77
	ccaggcucaugcugggacc	1007	78
	gugcagccucagaagaaga	674	103
	gaagaagaaaauucucuuc	453	104
<b>MLP</b>	cgagggcacugcucaggaa	597	79
	gaagaaaauucucuucaag	456	80
	aaagcaauggagacuuauc	293	81
	cgacuucguucuaauauau	1089	82
	ucaacuuccuucagaauuu	1694	83
<b>MSH4</b>	guagacgacuucguucuaa	1084	84
	agagcuuacuaugguuccu	1380	85
	agaagguauuggcauuugu	2345	86
	gcugacuccugaggaagaa	407	87
	ccaagaccucggugaagaa	301	105
<b>RPS8</b>	ccacaagaagcggaaguau	86	88
	agaguuggaguucuaucuu	602	89
	agagaaagcccuaccacaa	73	90

\*siRNA sequences are defined by the sequence of the sense strand.  
 The antisense strand is perfectly complementary to the sense strand.  
 SiRNAs responsible for a significant sensitization effect are highlighted in bold.

To select siRNAs specifically including a chemosensitive effect, those inducing a basal toxic effect were eliminated (more than 35 % cell death after transfection). A positive chemosensitive effect was considered for siRNAs inducing a cytotoxic effect compared to siRNA GFP/CDDP condition. 2 siRNAs out of 45, matched with these criteria, corresponding to 2 genes: *CYP2A6* and *CYP4B1* (Table IX).

The most efficient siRNA for each of these genes was transfected in 6 different glioma derived cell lines treated with CDDP or temozolomide (TMZ). CIs for each siRNA were compared with control CI (siRNA GFP). The results confirmed that two siRNAs (SEQ ID NO: 64 and 70) targeting *CYP2A6* and *CYP4B1*, respectively, had a significant chemosensitization effect on astrocytoma cells (figure 3).

## CLAIMS

1°) A double-stranded short interfering nucleic acid molecule comprising a sense and an antisense region, wherein the sense region comprises a nucleotide sequence that is selected from the group consisting of: the sequences SEQ  
5 ID NO: 15, 11, 13, 14, 30, 31, 38, 46, 64 and 70 and the sequences having at least 70% identity, preferably at least 80 % identity, more preferably at least 90 % identity with said sequences, and the antisense region comprises a nucleotide sequence that is complementary to the sense region.

2°) The double-stranded short interfering nucleic acid molecule  
10 according to claim 1, wherein each strand comprises 15 to about 30 nucleotides, and each strand comprises at least 15 to about 30 nucleotides that are complementary to the nucleotides of the other strand.

3°) The double-stranded short interfering nucleic acid molecule according to claim 2, which comprises a 19 to 21-nucleotide duplex.

4°) The double-stranded short interfering nucleic acid molecule  
15 according to anyone of claims 1 to 3, wherein the sense region comprises a nucleotide sequence having any of SEQ ID NO: 15, 11, 13, 14, 30, 31, 38, 46, 64 and 70 and the antisense region comprises a nucleotide sequence having any of SEQ ID NO: 96, 93, 94 and 95 to 102, respectively.

5°) The double-stranded short interfering nucleic acid molecule  
20 according to anyone of claims 1 to 4, which comprises 1 to about 3 overhanging nucleotides at the 3' end of each strand.

6°) The double-stranded short interfering nucleic acid molecule according to anyone of claims 1 to 4, which comprises blunt end(s).

7°) The double-stranded short interfering nucleic acid molecule  
25 according to anyone of claims 1 to 6, which is assembled from two separate oligonucleotide fragments, wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the short interfering nucleic acid molecule.

8°) The double-stranded short interfering nucleic acid molecule  
30 according to anyone of claims 1 to 6, wherein the sense region is connected to the antisense region via a linker molecule.

9°) The double-stranded short interfering nucleic acid molecule according to anyone of claims 1 to 8, which comprises ribonucleotides.

10°) The double-stranded short interfering nucleic acid molecule according to anyone of claims 1 to 9, which comprises one or more modified  
5 pyrimidine and/or purine nucleotides.

11°) The double-stranded short interfering nucleic acid molecule according to anyone of claims 1 to 10, wherein the strand comprising the sense region includes a terminal cap moiety at the 5' and/or 3' -end(s).

12°) The double-stranded short interfering nucleic acid molecule  
10 according to anyone of claims 1 to 11, wherein the strand comprising said antisense region includes a phosphate group at the 5'-end.

13°) The double-stranded short interfering nucleic acid molecule according to anyone of claims 1 to 12, which comprises at least one modified  
internucleotidic linkage.

14°) A transcription unit comprising : a transcription initiation  
15 region, a transcription termination region, and a nucleic acid sequence encoding at least one short interfering nucleic acid molecule according to anyone of claims 1 to 10 and 12, wherein said nucleic acid sequence is operably linked to said  
initiation region in a manner that allows expression and/or delivery of the short  
20 interfering nucleic acid molecule.

15°) An expression vector comprising a transcription unit according to claim 14.

16°) A cell which is modified by a vector according to claim 15.

17°) A pharmaceutical composition comprising at least a short  
25 interfering nucleic acid molecule according to anyone of claims 1 to 13 or an expression vector according to claim 15, in an acceptable carrier.

18°) The composition according to claim 17, which comprises a combination of at least two different short interfering nucleic acid molecules.

19°) The composition according to claim 17 or claim 18, wherein  
30 the short interfering nucleic acid molecule or vector is associated with at least one anticancer drug.

20°) The short interfering nucleic acid molecule according to anyone of claims 1 to 13 or the vector according to claim 15 as a medicament.

21°) Use of the short interfering nucleic acid molecule according to anyone of claims 1 to 13 or the vector according to claim 15, for the manufacture of a  
5 medicament for treating cancer.

22°) A product containing at least a short interfering nucleic acid molecule according to anyone of claims 1 to 13 or a vector according to claim 15, and an anticancer drug, as a combined preparation for simultaneous, separate or sequential use in anticancer therapy.

10 23°) The product according to claim 22, wherein said siNA molecule or vector is for use prior to the anticancer drug.

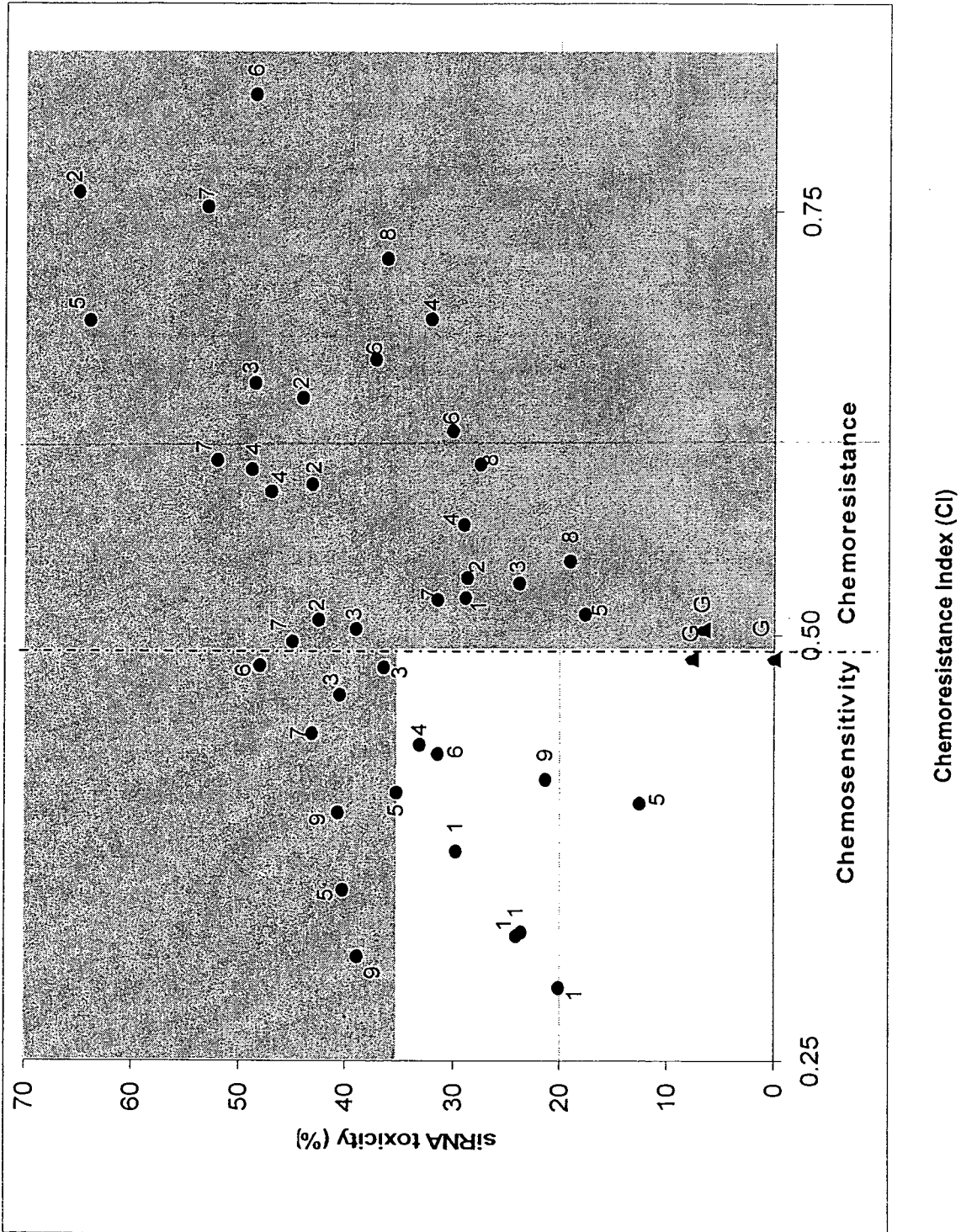


Figure 1

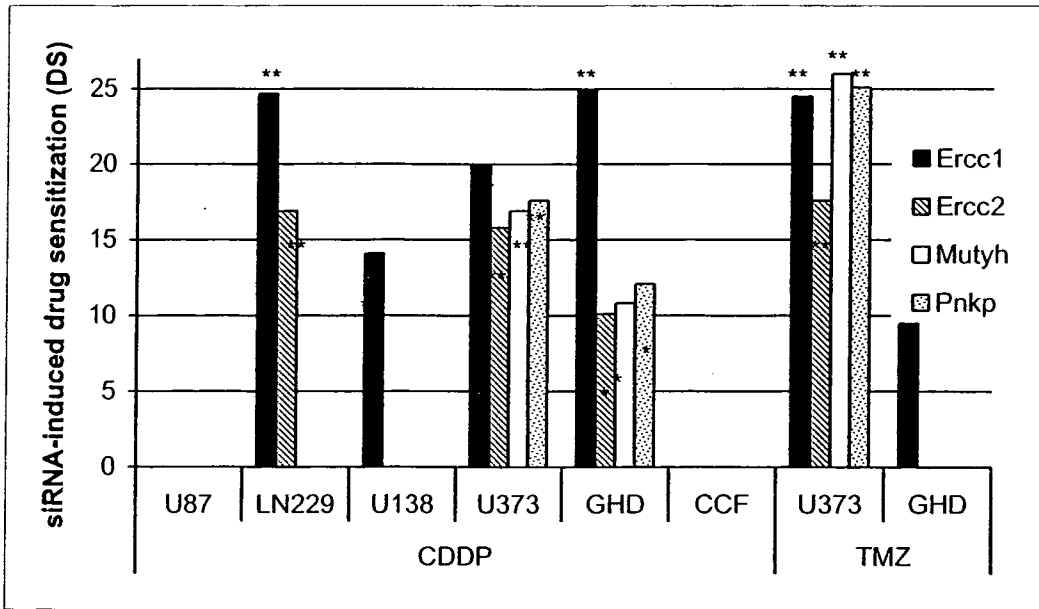


Figure 2

3/3

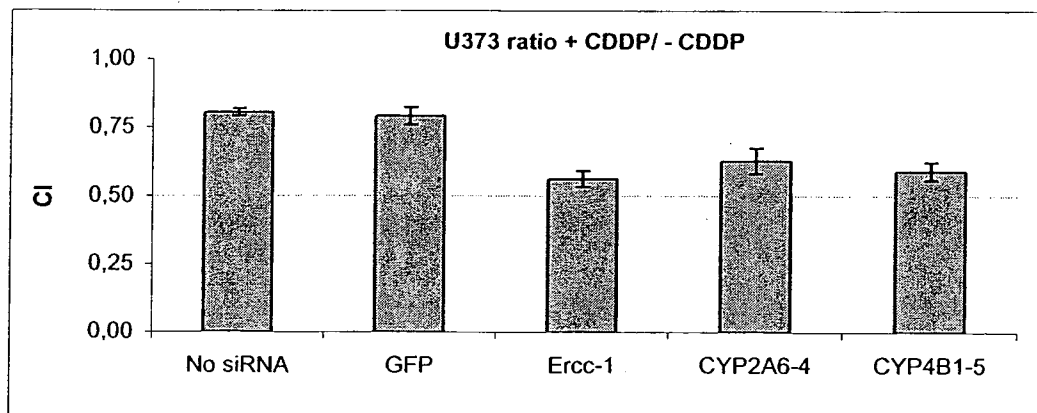


Figure 3