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(54) Title: INHIBITION OF DNA DAMAGE REPAIR BY ARTIFICIAL ACTIVATION OF PARP WITH OLIGONUCLEOTIDE MOLECULES

(57) Abstract: The present application relates to a new class of PARP inhibitors and their uses as a drug, especially as for use in a cancer therapy. These new PARP inhibitors include a double stranded portion with a nick or a gap on one strand and the tethered 5' and 3' ends of two extremities of the double stranded portion.

INHIBITION OF DNA DAMAGE REPAIR BY ARTIFICIAL ACTIVATION OF PARP WITH OLIGONUCLEOTIDE MOLECULES

FIELD OF THE INVENTION

The present invention relates to molecules, compositions and methods of inhibiting the DNA damage repair in mammalian cells. Accordingly, the invention relates to compositions and methods for treating proliferative disorders.

BACKGROUND OF THE INVENTION

To overcome DNA damage, cells have evolved mechanisms to detect DNA lesions, signal their presence and promote their repair. The wide diversity of types of DNA lesion generally necessitates multiple and specialized DNA-repair mechanisms. Although responses to different types of DNA lesions differ, most occur via signal transduction cascades involving post-translational modifications, such as ubiquitination, phosphorylation, acetylation and poly(ADP-ribosy)lation (PAR also called PARylation). Key regulators within these signaling cascades such as the phosphatidylinositol 3-kinase-related kinases (PI3K) ATM, ATR or DNA-PK, and the poly(ADP-ribose) polymerase (PARP), are activated via direct or indirect interaction with double-strand breaks (DSB) and single-strand breaks (SSB).

The most toxic DNA damage to the cell are DSB, which, if left unrepaired, lead to loss of chromosome fragments and cell death. Cells have two major pathways to repair DSB: homologous recombination (HR) and non-homologous end joining (NHEJ). These pathways are complementary and operate optimally during the S and G2 phases of the cell cycle for HR and throughout all cell cycle for NHEJ pathway. Thus, during S and G2 phases of the cell cycle, DSB are preferentially repaired by homologous recombination (HR) between sister chromatins. An important regulatory step that determines the choice between the NHEJ and HR pathways is the process of DSB by the MRE11-RAD50-NBS1 complex (MRN), in conjunction with other factors. After resection of DSB ends, the resulting single-strand DNA ends are coated with Replication Protein A (RPA) and then RAD51 with the help of RAD52, BReast CAncer 2 (BRCA2) and Fanconi anemia (FANC) proteins; these proteins promote invasion and strand exchange with the homologous region on the sister chromatin. Thereafter, repair proceeds either via the double Holliday junction model DSB repair (DSBR) pathway or via the synthesis-dependent strand-annealing (SDSA) pathway. In mammalian cells, NHEJ is the major pathway for repairing breaks not associated with replication. NHEJ involves the

direct rejoining of two damaged DNA ends in a sequence-independent manner: DNA ends are first bound by the Ku70/ku80 heterodimer, which recruits and activates the catalytic subunit, DNA-PKcs, to form the DNA-PK holoenzyme. Broken DNA ends are then processed and ligated by a set of enzymes including Artemis, polynucleotide kinase (PNK), X-ray cross-complementing 4 (XRCC4), and Ligase IV. If the classical mechanism of NHEJ is impeded, an alternative end-joining pathway operates that involves factors of HR and SSB repair, including MRN complex, PARP-1, X-ray cross-complementing 1 (XRCC1) and DNA Ligase I or III.

Although less harmful than DSB, SSB are toxic. One of the most common sources of SSB is oxidative attack by endogenous reactive oxygen species (ROS). In the case of free radicals from hydrogen peroxide (H_2O_2), a physiologically relevant source of ROS, SSB occur three orders of magnitude more frequently than DSB. Following exposure to ionizing radiation, SSB are 25 times more abundant than DSB. They are primarily detected by PARP-1 although other members of the PARP super family may contribute. Binding of PARP to SSB triggers poly(ADP-ribosy)lation of numerous nuclear proteins including PARP itself. These modifications in turn promote the binding of XRCC1, which acts as a molecular scaffold for SSB repair components. Therefore PARP, which binds to DSB with a greater affinity than that for its binding to SSB is involved in repair of both, whereas the recruitment of DNA-PK by Ku is strictly dependent on DSB, and seems to be involved in DSB repair only.

The outcome of DNA damage signaling is, literally, a matter of life or death. Depending on the severity of the DNA damage, the cell will either repair the damage to enable it to continue dividing, or enter apoptosis. The understanding of the dynamics of the repair proteins has been greatly advanced through the use of various types of DNA substrates in biochemical assays.

In particular, short interferring double-stranded DNA molecules (siDNA) that mimic DSB damage (called Dbait) induce a partial damage response in cells and can be used to analyze the early steps of repair protein recruitment *in vivo*. Dbait molecules activate DNA-PK kinase and have no significant effect on other PI3K kinases. In the course of this response, several nuclear DNA-PK targets, such as p53, Rpa32 or H2AX, are extensively phosphorylated. The activation of DNA-PK prevents recognition of further DSB and inhibits not only the NHEJ, which directly depends upon DNA-PK, but also HR pathways. Such Dbait molecules have been described and developed as new drugs for treating proliferative disorders, in particular cancers (WO 2005/040378; WO 2008/034866; WO 2008/084087).

Even if the Dbait molecules confirm their very promising status, there is a permanent need to improve the cancer therapy and to provide new targets and tools for treating proliferative disorders.

SUMMARY OF THE INVENTION

The present invention provides a new class of PARP inhibitors called Pbait.

The present invention relates to a molecule comprising a deoxyribonucleotide double-stranded portion of 12 to 200 bp, wherein it has preferably less than 80% sequence identity to any gene in a human genome, it has a single strand break or a gap on one strand, preferably, in the middle part of said double-stranded portion and the 5' and 3' ends at both extremities of said double-stranded portion are tethered by a linker. Preferably, the double-stranded portion is from 28 to 100 bp, preferably from 30 to 50 bp. Preferably, the single strand break or the gap is located at least 6 nucleotides for the extremities of said double-stranded portion, preferably at least 8, 12 or 15 nucleotides. Preferably, the gap is a gap of less than 7 nucleotides, preferably less than 5, more preferably a gap of 1-3 nucleotides, still more preferably a gap of one nucleotide. Preferably, the linker is selected from the group consisting of a polyethyleneglycol chain, an oligonucleotide and any hydrocarbon chain, optionally interrupted and/or substituted by one or more heteroatoms e.g., oxygen, sulfur, or nitrogen, or heteroatomic or heterocyclic groups, comprising one or several heteroatoms. More preferably, it is selected from the group consisting of hexaethyleneglycol, tetradeoxythymidylate (T4) and 2,19-bis(phosphor)-8-hydraza-1-hydroxy-4-oxa-9-oxo-nonadecane.

Optionally, the molecule further comprises a moiety facilitating endocytosis selected from a lipophilic molecule or a ligand which targets cell receptor enabling receptor mediated endocytosis and/or specific cells and tissues targeting, said moiety facilitating endocytosis being preferably covalently linked to the linker. Preferably, said moiety facilitating endocytosis and/or cell/tissue targeting is selected from the group consisting of single or double chain fatty acids such as octodecyl and dioleoyl, tocopherol, folates or folic acid, cholesterol, sugar such as galactose and mannose and their oligosaccharide, peptide such as RGD and bombesin, and protein such as integrin, preferably of dioleoyl, octadecyl, folic acid, and cholesterol, more preferably cholesterol.

The present invention further relates to a pharmaceutical composition comprising a molecule according to the present invention and a pharmaceutically acceptable carrier. Optionally, the pharmaceutical composition further comprises a DNA damaging antitumoral agent, preferably selected from the group consisting of an inhibitor of topoisomerases I or II, a

DNA crosslinker, a DNA alkylating agent, an anti-metabolic agent and inhibitors of the mitotic spindles. Optionally, the pharmaceutical composition further comprises an oxidative antitumoral agent, preferably selected from the group consisting of Oxygen, Ozone, superoxyde and Hydrogen peroxide (used in the so-called oxidative therapy). It can comprise a DNA damaging antitumoral agent and an oxidative antitumoral agent.

In addition, the present invention relates to a molecule according to the present invention for use a drug. More specifically, it relates to a molecule according to the present invention for use in the treatment of cancer, optionally either in combination with DNA damaging or oxidative therapies, or as a standalone treatment in the genetically instable cancer. Optionally, the molecule is combined with a radiotherapy, hyperthermia or chemotherapy, preferably with a DNA damaging antitumoral agent, more preferably selected from the group consisting of an inhibitor of topoisomerases I or II, a DNA crosslinker, a DNA alkylating agent, an anti-metabolic agent and inhibitors of the mitotic spindles. Optionally, the molecule is combined with oxidative therapy, more preferably with oxygen, ozone, superoxyde or hydrogen peroxide. Optionally, the molecule is administered by oral route or by intravenous, intra-tumoral or sub-cutaneous injection, intracranial or intra artery injection or infusion, preferably by intravenous, intra-tumoral or sub-cutaneous injection. Preferably, the cancer is characterized by cells deficient in homologous recombination, in particular caused by BRCA mutations, especially BRCA1 and/or BRCA2. Preferably, the cancer is characterized by cells with a high level of spontaneous damage, in particular caused by defects in replication or repair pathways. In particular, the cancer can be selected from the group consisting of breast cancer, ovarian cancer, prostate cancer, melanoma, lung cancer (e.g. squamous cell lung cancer and non-small-cell lung cancer (NSCLC)), colorectal cancer, glioblastoma, larynx and cervix cancer, sarcoma, soft tissue sarcoma, peritoneal carcinomatosis, myelodysplastic syndrome and hematological malignancies such as acute myeloid leukemia (AML), chronic lymphoma and multiple myeloma..

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a new class of PARP inhibitors called Pbait. The principle of Pbait is to mimic a single-strand break (SSB) in order to recruit the SSB repair components, primarily PARP. These molecules are very specific of PARP.

Therefore, Pbait molecules present the following advantages:

- They are well-tolerated in vitro and in vivo.

- They act by trapping PARP and rendering it unavailable to repair true SSB on chromatin rather than inhibiting the SSB repair system.
- This mechanism of action should avoid the commonly occurred resistance to the treatment due to the mutation on the inhibitor binding site.

Accordingly, the present invention relates to a molecule that mimics SSB and binds to PARP. Therefore, the molecules needs to present a double-stranded portion of at least 12 bp preferably 24 bp, whatsoever its sequence is, with a single stranded break or a gap on one of the strand of said double-stranded portion and with extremities of said double-stranded portion blocked by tethering the 5' and 3' ends in order to increase the stability of the molecule and avoid any free extremity, thereby avoid to recruit the DSB repair system.

Pbait molecules

As the nucleotide sequence of Pbait molecules does not impact on their activity (for instance, see Pbait32 and Pbait 32L activities). and in order to avoid any interaction with the human genome, Pbait molecules have preferably no significant degree of sequence homology or identity to known genes, promoters, enhancers, 5'- or 3'- upstream sequences, exons, introns, and the like of the human genome. In other words, Pbait molecules have less than 80% or 70%, even less than 60% or 50% sequence identity to any gene in a human genome. Methods of determining sequence identity are well known in the art and include, e.g., BLASTN 2.2.25. By "human genome", it is preferably considered for determining the identity percentage the Human Genome Build 37 (reference GRCh37.p2 and alternate assemblies). Pbait molecules do not hybridize, under stringent conditions, with human genomic DNA. Typical stringent conditions are such that they allow the discrimination of fully complementary nucleic acids from partially complementary nucleic acids.

The length of Pbait molecules may be variable, as long as it is sufficient to allow appropriate binding of PARP. It has been showed that the length of Pbait molecules must be at least 12 bp. Optionally, the length of Pbait molecules can be at least 12, 16, 20, 24 or 28 bp. Preferably, Pbait molecules comprise between 12-200 bp, preferably 28-100, and most preferably between 30-50 bp. For instance, Pbait molecules comprise between 12-160, 16-150, 20-140, 30-100, or 32-50 bp. In a very specific embodiment, the Pbait molecules comprise between 25-40, 28-38, 30-36 or 31-33 bp. By "bp" is intended that the molecule comprise a double stranded portion of the indicated length.

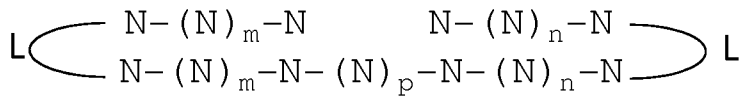
The Pbait molecule has a single strand break (also called nick) or a gap on one strand in the middle part of said double-stranded portion. It is important to note that only one strand of said double-stranded portion has a SSB or a gap. A SSB is a break between two

consecutive nucleotides of one strand of the double-stranded portion. A gap is a loss of one or several nucleotides on one strand of the double-stranded portion. Preferably, the gap is less than 7 nucleotides, preferably less than 5, 4 or 3 nucleotides. More preferably, the gap is of 1-3 nucleotides or 1 or 2 nucleotides. Still more preferably, the gap is of one nucleotide.

The choice of the gap size or of a nick is also directed to the length of the double-stranded portion. Indeed, if the double-stranded portion is short (e.g., 12 15 bp), then a nick or a 1 nucleotide gap will be preferred. If the double-stranded portion is longer, then a longer gap can be used, without any impact on the stability of the double-stranded portion of the molecule.

The SSB or gap is present on one strand of the double-stranded portion, preferably, in the middle part thereof. Preferably, the single strand break or the gap is located at least 6 nucleotides for the extremities of said double-stranded portion, preferably at least 8, 12 or 15 nucleotides.

Therefore, in a preferred embodiment of the present invention, the Pbait molecule has one of the following formulae:



(I)

wherein p is an integer from 0 to 6, and L is a linker, N is a nucleotide, preferably a deoxyribonucleotide, m and n are, independently from each other, an integer from 4 to 192, m+n being comprised between 8 and 196. Preferably, p is 0, 1 or 2.

Preferably, m and n are, independently from each other, an integer from 4 to 92, m+n being comprised between 24 and 96. More preferably, m and n are, independently from each other, an integer from 4 to 42, m+n being comprised between 26 and 46.

Optionally, N may refer to a nucleotide having or not a modified phosphodiester backbone. N is preferably selected from the group consisting of A (adenine), C (cytosine), T (thymine) and G (guanine).

At the SSB or gap site, the interrupted strand may present in either 3'-phosphate or 3'-OH end, and either 5'-phosphate or 5'-OH end. Optionally, one of the 5' and 3' end can be linked to a blocking group, for instance, 3'-dideoxy, 3'-5'-phosphate, 3'-3'nucleotide linkage.

The linker can be a nucleic acid, or other chemical groups known by skilled person or a mixture thereof. A nucleotide linker may include from 2 to 10 nucleotides, preferably, 3, 4 or 5 nucleotides. Preferably, the linker could be a polydeoxythymidylate, especially a tri-, tetra- or pinta- deoxythymidylate. Non-nucleotide linkers non exhaustively include abasic

nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (e. g. oligoethylene glycols such as those having between 2 and 10 ethylene glycol units, preferably 4, 5, 6, 7 or 8 ethylene glycol units). For instance, non-nucleotide linkers may be any hydrocarbon chain, optionally interrupted and/or substituted by one or more heteroatoms e.g., oxygen, sulfur, or nitrogen, or heteroatomic or heterocyclic groups, comprising one or several heteroatoms. A preferred linker is selected from the group consisting of tetradeoxythymidylate (T4), hexaethyleneglycol and other linkers such as 2,19-bis(phosphor)-8-hydraza-1-hydroxy-4-oxa-9-oxo-nonadecane. It could be noted that the two linkers can be the same or can be different. In particular, one of the two linkers may be covalently bound to a moiety facilitating endocytosis, cellular uptake, cellular/tissue targeting and/or improving pharmacokinetic properties. Optionally, both linkers may be covalently bound to a moiety facilitating endocytosis, cellular uptake, cellular/tissue targeting and/or improving pharmacokinetic properties.

The moiety facilitating endocytosis or cellular uptake may be lipophilic molecules such as cholesterol, single or double chain fatty acids, or ligands which target cell receptor enabling receptor mediated endocytosis, such as folic acid and folate derivatives or transferrin (Goldstein et al. Ann. Rev. Cell Biol. 1985 1:1-39; Leamon & Lowe, Proc Natl Acad Sci USA. 1991, 88: 5572–5576.). Fatty acids may be saturated or unsaturated and be in C_4-C_{28}, preferably in C_{14}-C_{22}, still more preferably being in C_{18} such as oleic acid or stearic acid. In particular, fatty acids may be octadecyl or dioleoyl. Fatty acids may be found as double chain form linked with in appropriate linker such as a glycerol, a phosphatidylcholine or ethanolamine and the like or linked together by the linkers used to attach on the Pbait molecule. As used herein, the term "folate" is meant to refer to folate and folate derivatives, including pteroic acid derivatives and analogs. The analogs and derivatives of folic acid suitable for use in the present invention include, but are not limited to, antifolates, dihydrofolates, tetrahydrofolates, folinic acid, pteropolyglutamic acid, 1-deza, 3-deaza, 5-deaza, 8-deaza, 10-deaza, 1,5-deaza, 5,10 dideaza, 8,10-dideaza, and 5,8-dideaza folates, antifolates, and pteroic acid derivatives. Additional folate analogs are described in US2004/242582. The molecule facilitating endocytosis may be tocopherol, sugar such as galactose and mannose and their oligosaccharide, peptide such as RGD and bombesin, and proteins such as integrin. Accordingly, the molecule facilitating endocytosis may be selected from the group consisting of single or double chain fatty acids, folates and cholesterol. More preferably, the molecule facilitating endocytosis is selected from the group consisting of

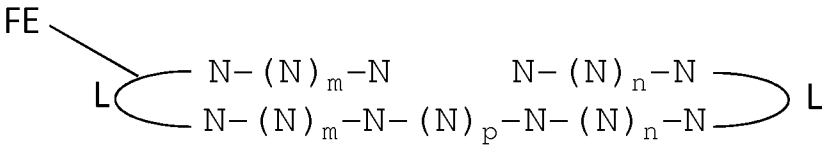
dioleoyl, octadecyl, folic acid, and cholesterol. In a most preferred embodiment, the Pbait molecule is conjugated to a cholesterol.

The moieties facilitating endocytosis are conjugated to Pbait molecules, preferably through a linker. Any linker known in the art may be used to covalently attach the moiety facilitating endocytosis to Pbait molecules. For instance, WO09/126933 provides a broad review of convenient linkers pages 38-45. The linker can be non-exhaustively, aliphatic chain, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (e.g. oligoethylene glycols such as those having between 2 and 10 ethylene glycol units, preferably 3, 4, 5, 6, 7 or 8 ethylene glycol units, still more preferably 6 ethylene glycol units), as well as incorporating any bonds that may be break down by chemical or enzymatical way, such as a disulfide linkage, a protected disulfide linkage, an acid labile linkage (e.g., hydrazone linkage), an ester linkage, an ortho ester linkage, a phosphonamide linkage, a biocleavable peptide linkage, an azo linkage or an aldehyde linkage. Such cleavable linkers are detailed in WO2007/040469 pages 12-14, in WO2008/022309 pages 22-28.

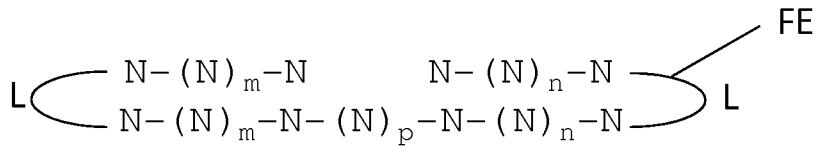
In a specific embodiment, the linker between the moiety facilitating endocytosis, in particular cholesterol, and Pbait molecule is CO-NH-$(CH_2$-CH_2-$O)_n$, wherein n is an integer from 1 to 10, preferably n being selected from the group consisting of 3, 4, 5 and 6. In a very particular embodiment, the linker is CO-NH-$(CH_2$-CH_2-$O)_4$ (carboxamido triethylene glycol). The linker can be linked to Pbait molecules at any convenient position which does not modify the activity of the Pbait molecules. In particular, the linker can be linked at an extremity of the double-stranded portion.

In another specific embodiment, the linker between the moiety facilitating endocytosis, in particular cholesterol, and Pbait molecule is dialkyl-disulfide [e.g., $(CH_2)_p$-S-S-$(CH_2)_q$ with p and q being integer from 1 to 10, preferably from 3 to 8, for instance 6].

Therefore, in a preferred embodiment of the present invention, the Pbait molecule with a moiety facilitating endocytosis and/or cell/tissue targeting has one of the following formulae:



(II)



(III)

wherein L, N, m, n and p have the same definition than for the formula (I) as above detailed and FE is a moiety facilitating endocytosis, and/or cell/tissue targeting as detailed above.

In a very particular embodiment, the Pbait molecules having a double stranded portion of at least 12, 14, 15, 16, 20, 30 pb, or of about 32 bp, comprise the same nucleotide sequence than Pbait12, Pbait14, Pbait15sd3, Pbait16, Pbait16S, Pbait16s3, Pbait16sd3, Pbait16p5, Pbait20, Pbait30, Pbait32, Pbait32b, or Pbait32L. Optionally, the Pbait molecules have the same nucleotide composition than Pbait12, Pbait14, Pbait15sd3, Pbait16, Pbait16S, Pbait16s3, Pbait16sd3, Pbait16p5, Pbait20, Pbait 30, Pbait32, Pbait32b or Pbait32L but their nucleotide sequence is different.

In a very specific embodiment, the molecule is selected from the group consisting of Pbait12, Pbait14, Pbait15sd3, Pbait16, Pbait16S, Pbait16s3, Pbait16sd3, Pbait16p5, Pbait20, Pbait30, Pbait32, Pbait32b, Pbait32s and Pbait32L.

Pbait molecules preferably comprise a 2'-deoxynucleotide backbone, and optionally comprise one or several (2, 3, 4, 5 or 6) modified nucleotides and/or nucleobases other than adenine, cytosine, guanine and thymine. In particular, the double-stranded portion of the Pbait molecules is made of deoxyribonucleotides.

Optionally, Pbait molecules comprise one or several chemically modified nucleotide(s) or group(s) at the end surrounding the SSB or gap, in particular in order to protect them from degradation. In a particular preferred embodiment, the end(s) surrounding the SSB or gap of the Pbait molecules is(are) protected by one, two or three modified phosphodiester backbones at the end of one or of each end surrounding the SSB or gap. Preferred chemical groups, in particular the modified phosphodiester backbone, comprise phosphorothioates. In particular, the two or three last nucleotides surrounding the SSB or gap may have phosphorothioate backbone. Alternatively, Pbait molecules may have 3'-3' nucleotide linkage at the 3' end surrounding the SSB or gap, or nucleotides with methylphosphonate backbone, or 3'-didexoynucleotide.

Other modified backbones are well known in the art and comprise phosphoramidates, morpholino nucleic acid, 2'-0,4'-C methylene/ethylene bridged locked nucleic acid, peptide nucleic acid (PNA), and short chain alkyl, or cycloalkyl intersugar linkages or short chain

heteroatomic or heterocyclic intrasugar linkages of variable length, or any modified nucleotides known by skilled person.

In a first preferred embodiment, the Pbait molecules have the end(s) surrounding the SSB or gap protected by one, two or three modified phosphodiester backbones at one or both ends surrounding the SSB or gap, more preferably by three modified phosphodiester backbones (in particular phosphorothioate or methylphosphonate) at least at the 3'end, but still more preferably at both 5' and 3' ends surrounding the SSB or gap.

Said Pbait molecules are made by chemical synthesis, semi-biosynthesis or biosynthesis, any method of amplification, followed by any extraction and preparation methods and any chemical modification. Linkers are provided so as to be incorporable by standard nucleic acid chemical synthesis.

More preferably, Pbait molecules are manufactured by specially designed convergent synthesis: two complementary strands with the nick or gap are prepared by standard nucleic acid chemical synthesis with the incorporation of appropriate linker precursor, after their purification, they are covalently coupled together.

Pharmaceutical compositions and Therapeutic Uses of Pbait molecules

The present invention relates to a pharmaceutical composition comprising a Pbait molecule as defined above, and optionally a pharmaceutically acceptable carrier or excipient.

In addition, the present invention also relates to a pharmaceutical composition further comprising an additional therapeutic agent. Preferably, the additional active ingredient is an antitumoral agent, more preferably a DNA-damaging anti-tumoral agent.

Optionally, when the Pbait molecule comprises a moiety facilitating endocytosis, the composition may further comprise an endosomolytic agent.

Accordingly, the present invention relates to a pharmaceutical composition comprising:

- a Pbait molecule as defined above, and optionally a pharmaceutically acceptable carrier or excipient; or
- a) a Pbait molecule as defined above, b) a DNA-damaging anti-tumoral agent and/or an oxidative agent, and optionally a pharmaceutically acceptable carrier or excipient; or
- a) a Pbait molecule as defined above, b) an endosomolytic agent, and optionally a pharmaceutically acceptable carrier or excipient; or

- a) a Pbait molecule as defined above, b) a DNA-damaging anti-tumoral agent and/or an oxidative agent, c) an endosomolytic agent, and optionally a pharmaceutically acceptable carrier or excipient.

Alternatively, the present invention also relates to a product, kit or combined preparation, comprising:

- a) a Pbait molecule as defined above, and b) a DNA-damaging anti-tumoral agent and/or an oxidative agent, preferably a DNA-damaging anti-tumoral agent, as a combined preparation for simultaneous, separate or sequential use; or

- a) a Pbait molecule as defined above, and b) an endosomolytic agent, as a combined preparation for simultaneous, separate or sequential use; or

- a) a Pbait molecule as defined above, b) a DNA-damaging anti-tumoral agent and/or an oxidative agent, preferably a DNA-damaging anti-tumoral agent, c) an endosomolytic agent, as a combined preparation for simultaneous, separate or sequential use.

The terms “kit”, “product” or "combined preparation", as used herein, defines especially a "kit of parts" in the sense that the combination partners (a) and (b), and optionally (c), as defined above can be dosed independently or by use of different fixed combinations with distinguished amounts of the combination partners (a) and (b), and optionally (c), i.e. simultaneously or at different time points. The parts of the kit of parts can then, e.g., be administered simultaneously or chronologically staggered, that is at different time points and with equal or different time intervals for any part of the kit of parts. The ratio of the total amounts of the combination partner (a) to the combination partner (b), and optionally (c), to be administered in the combined preparation can be varied. The combination partners (a) and (b) and optionally (c), can be administered by the same route or by different routes.

The present invention also relates to the Pbait molecule as defined above as a drug. More particularly, it relates to the Pbait molecule, a pharmaceutical composition, or a product, kit or combined preparation as defined above for use in the treatment of a cancer. Optionally, it is used in combination with radiotherapy and/or hyperthermia and/or a DNA-damaging anti-tumoral agent and/or an endosomolytic agent and/or an oxidative agent. Preferably, it is used in combination with radiotherapy and/or a DNA-damaging anti-tumoral agent.

The present invention also relates to the use of a Pbait molecule or a pharmaceutical composition as defined above for the manufacture of a medicament for treating a cancer. Optionally, it is used in combination with radiotherapy and/or hyperthermia and/or a DNA-damaging anti-tumoral agent and/or an endosomolytic agent and/or an oxidative agent.

Preferably, it is used in combination with radiotherapy and/or a DNA-damaging anti-tumoral agent.

The present invention further relates to a method for treating a cancer in a subject in need thereof, comprising administering an effective amount of a Pbait molecule or a pharmaceutical composition as defined above. In particular, it relates to a method for treating a cancer in a subject in need thereof, comprising administering an effective amount of a Pbait molecule as disclosed above, an effective amount of a DNA-damaging anti-tumoral agent and/or an oxidative agent, , preferably a DNA-damaging anti-tumoral agent, and optionally an effective amount of an endosomolytic agent. Optionally, the method further comprises radiotherapy, before, simultaneously or after administration of the Pbait molecule.

The present invention relates to a method for increasing the efficiency of a treatment of a cancer with radiotherapy and/or a DNA-damaging anti-tumoral agent or for enhancing tumor sensitivity to radiotherapy and/or to treatment with a DNA-damaging anti-tumoral agent in a subject in need thereof, comprising administering an effective amount of a Pbait molecule as disclosed above, and optionally an effective amount of an endosomolytic agent.

Within the context of the invention, the term treatment denotes curative, symptomatic, and preventive treatment. Pharmaceutical compositions, kits, products and combined preparations of the invention can be used in humans with existing cancer or tumor, including at early or late stages of progression of the cancer. The pharmaceutical compositions, kits, products and combined preparations of the invention will not necessarily cure the patient who has the cancer but will delay or slow the progression or prevent further progression of the disease, improving thereby the patients' condition. In particular, the pharmaceutical compositions, kits, products and combined preparations of the invention reduce the development of tumors, reduce tumor burden, produce tumor regression in a mammalian host and/or prevent metastasis occurrence and cancer relapse. In treating the cancer, the pharmaceutical composition of the invention is administered in a therapeutically effective amount.

The subject to be treated is a mammal, preferably a human being or an animal, such as a cat, dog, or horse. Therefore, by "pharmaceutical" is also intended "veterinary".

By "effective amount" it is meant the quantity of the pharmaceutical composition of the invention which prevents, removes or reduces the deleterious effects of cancer in mammals, including humans, alone or in combination with the other active ingredients of the pharmaceutical composition, kit, product or combined preparation. It is understood that the

administered dose may be adapted by those skilled in the art according to the patient, the pathology, the mode of administration, etc.

Whenever within this whole specification "treatment of a cancer" or the like is mentioned with reference to the pharmaceutical composition of the invention, there is meant: a) a method for treating a cancer, said method comprising administering a pharmaceutical composition of the invention to a subject in need of such treatment; b) the use of a pharmaceutical composition of the invention for the treatment of a cancer; c) the use of a pharmaceutical composition of the invention for the manufacture of a medicament for the treatment of a cancer; and/or d) a pharmaceutical composition of the invention for use in the treatment a cancer.

The pharmaceutical compositions contemplated herein may include a pharmaceutically acceptable carrier in addition to the active ingredient(s). The term "pharmaceutically acceptable carrier" is meant to encompass any carrier (e.g., support, substance, solvent, etc.) which does not interfere with effectiveness of the biological activity of the active ingredient(s) and that is not toxic to the host to which it is administered. For example, for parental administration, the active compounds(s) may be formulated in a unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

The pharmaceutical composition can be formulated as solutions in pharmaceutically compatible solvents or as emulsions, suspensions or dispersions in suitable pharmaceutical solvents or vehicle, or as pills, tablets or capsules that contain solid vehicles in a way known in the art. Formulations of the present invention suitable for oral administration may be in the form of discrete units as capsules, sachets, tablets or lozenges, each containing a predetermined amount of the active ingredient; in the form of a powder or granules; in the form of a solution or a suspension in an aqueous liquid or non-aqueous liquid; or in the form of an oil-in-water emulsion or a water-in-oil emulsion. Formulations suitable for parental administration conveniently comprise a sterile oily or aqueous preparation of the active ingredient which is preferably isotonic with the blood of the recipient. Every such formulation can also contain other pharmaceutically compatible and nontoxic auxiliary agents, such as, e.g. stabilizers, antioxidants, binders, dyes, emulsifiers or flavouring substances. The formulations of the present invention comprise an active ingredient in association with a pharmaceutically acceptable carrier therefore and optionally other therapeutic ingredients. The carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulations and not deleterious to the recipient thereof. The pharmaceutical

compositions are advantageously applied by injection or intravenous infusion of suitable sterile solutions or as oral dosage by the digestive tract. Methods for the safe and effective administration of most of these chemotherapeutic agents are known to those skilled in the art. In addition, their administration is described in the standard literature.

Pbait molecules comprising a moiety facilitating endocytosis are preferably used here in combination with an endosomolytic agent (ex. chloroquine, fusogenic lipids or peptides, etc.). Indeed, the treatment by an endosomolytic agent facilitates the release of such Pbait molecules from endosomes. In particular, the endosomolytic agents are capable of inducing the lysis of the endosome in response to a change in pH, and an encapsulating, or packaging, component capable of packaging a therapeutic agent to be delivered to cellular or subcellular components. Endosomolytic substance that includes, but is not limited to, quinoline compounds, especially 4-aminoquinoline and 2-phenylquinoline compounds and amino, thio, phenyl, alkyl, vinyl and halogen derivatives thereof, fusogenic lipids, peptides or proteins. In a preferred embodiment, the endosomolytic agent is chloroquine. Other suitable endosomolytic agents are disclosed in WO2011/161075.

In addition to the Pbait molecules, the treatment may also further comprise an antitumoral treatment, preferably a DNA damaging treatment agent or an oxidative agent. The DNA-damaging treatment can be radiotherapy or chemotherapy with a DNA-damaging antitumoral agent, or hyperthermia, or a combination thereof.

DNA strand breakage can be achieved by ionizing radiation (radiotherapy). Radiotherapy includes, but is not limited to, γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other radiotherapies include microwaves, surface plasmon resonance and UV-irradiation. Other approaches to radiation therapy are also contemplated in the present invention.

The DNA-damaging antitumoral agent is preferably selected from the group consisting of an inhibitor of topoisomerases I or II, a DNA crosslinker, a DNA alkylating agent, an anti-metabolic agent and inhibitors of the mitotic spindles.

Inhibitors of topoisomerases I and/or II include, but are not limited to, etoposide, topotecan, camptothecin, irinotecan, amsacrine, intoplicine, anthracyclines such as doxorubicine, epirubicine, daunorubicine, idanrubicine and mitoxantrone. Inhibitors of Topoisomerase I and II include, but are not limited to, intoplecin.

DNA crosslinkers include, but are not limited to, cisplatin, carboplatin and oxaliplatin.

Anti-metabolic agents block the enzymes responsible for nucleic acid synthesis or become incorporated into DNA, which produces an incorrect genetic code and leads to

apoptosis. Non-exhaustive examples thereof include, without limitation, folic acid antagonists, pyrimidine analogs, purine analogs and adenosine deaminase inhibitors, and more particularly Methotrexate, Floxuridine, Cytarabine, 6-Mercaptopurine, 6- Thioguanine, Fludarabine phosphate, Pentostatine, 5-fluorouracil, gemcitabine and capecitabine.

The DNA-damaging anti-tumoral agent can be alkylating agents including, without limitation, nitrogen mustards, ethylenimine derivatives, alkyl sulfonates, nitrosoureas, metal salts and triazenes. Non-exhaustive examples thereof include Uracil mustard, Chlormethine, Cyclophosphamide (CYTOXAN(R)), Ifosfamide, Melphalan, Chlorambucil, Pipobroman, Triethylenemelamine, Triethylenethiophosphoramine, Busulfan, Carmustine, Lomustine, Fotemustine, cisplatin, carboplatin, oxaliplatin, thiotepa, Streptozocin, Dacarbazine, and Temozolomide.

Inhibitors of the mitotic spindles include, but are not limited to, paclitaxel, docetaxel, vinorelbine, larotaxel (also called XRP9881; Sanofi-Aventis), XRP6258 (Sanofi-Aventis), BMS-184476 (Bristol-Meyer-Squibb), BMS-188797 (Bristol-Meyer-Squibb), BMS-275183 (Bristol-Meyer-Squibb), ortataxel (also called IDN 5109, BAY 59-8862 or SB-T-101131 ; Bristol-Meyer-Squibb), RPR 109881A (Bristol-Meyer-Squibb), RPR 116258 (Bristol-Meyer-Squibb), NBT-287 (TAPESTRY), PG-paclitaxel (also called CT-2103, PPX, paclitaxel poliglumex, paclitaxel polyglutamate or Xyotax^{TM}), ABRAXANE® (also called Nab-Paclitaxel ; ABRAXIS BIOSCIENCE), Tesetaxel (also called DJ-927), IDN 5390 (INDENA), Taxoprexin (also called docosahexanoic acid-paclitaxel ; PROTARGA), DHA-paclitaxel (also called Taxoprexin®), and MAC-321 (WYETH). Also see the review of Hennenfent & Govindan (2006, *Annals of Oncology*, 17, 735-749).

The oxidative agents include oxygen, ozone, superoxide, or/and hydrogen peroxide.

The pharmaceutical compositions and the products, kits or combined preparation described in the invention can be used for treating cancer in a subject.

The terms "cancer", "cancerous", or "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth.

The pharmaceutical compositions and the products, kits or combined preparations described in the invention may be useful for inhibiting the growth of solid tumors, decreasing the tumor volume, preventing the metastatic spread of tumors and the growth or development of micrometastases. The pharmaceutical compositions and the products, kits or combined preparations described in the invention are in particular suitable for the treatment of poor prognosis patients or of radio- or chemo-resistant tumors.

As cancer at the advanced stage generally has spontaneous DNA damage due to genetic instability, therefore the Pbait molecules can be used as a standalone treatment of these advanced malignancies.

Examples of cancer include, for example, leukemia, lymphoma, blastoma, carcinoma and sarcoma. More particular examples of such cancers include chronic myeloid leukemia, acute lymphoblastic leukemia, Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ ALL), squamous cell carcinoma, small-cell lung cancer, non-small cell lung cancer, glioma, gastrointestinal cancer, renal cancer, ovarian cancer, liver cancer, colorectal cancer, endometrial cancer, kidney cancer, prostate cancer, thyroid cancer, neuroblastoma, pancreatic cancer, glioblastoma multiforme, cervical cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon carcinoma, and head and neck cancer, gastric cancer, germ cell tumor, pediatric sarcoma, sinonasal natural killer, multiple myeloma, acute myelogenous leukemia (AML), chronic lymphocytic leukemia, mastocytosis and any symptom associated with mastocytosis.

"Leukemia" refers to progressive, malignant diseases of the blood-forming organs and is generally characterized by a distorted proliferation and development of leukocytes and their precursors in the blood and bone marrow. Leukemia is generally clinically classified on the basis of (1) the duration and character of the disease—acute or chronic; (2) the type of cell involved; myeloid (myelogenous), lymphoid (lymphogenous), or monocytic; and (3) the increase or non-increase in the number of abnormal cells in the blood—leukemic or aleukemic (subleukemic). Leukemia includes, for example, acute nonlymphocytic leukemia, chronic lymphocytic leukemia, acute granulocytic leukemia, chronic granulocytic leukemia, acute promyelocytic leukemia, adult T-cell leukemia, aleukemic leukemia, a leukocythemic leukemia, basophylic leukemia, blast cell leukemia, bovine leukemia, chronic myelocytic leukemia, leukemia cutis, embryonal leukemia, eosinophilic leukemia, Gross' leukemia, hairy-cell leukemia, hemoblastic leukemia, hemocytoblastic leukemia, histiocytic leukemia, stem cell leukemia, acute monocytic leukemia, leukopenic leukemia, lymphatic leukemia, lymphoblastic leukemia, lymphocytic leukemia, lymphogenous leukemia, lymphoid leukemia, lymphosarcoma cell leukemia, mast cell leukemia, megakaryocyte leukemia, micromyeloblastic leukemia, monocytic leukemia, myeloblasts leukemia, myelocytic leukemia, myeloid granulocytic leukemia, myelomonocytic leukemia, Naegeli leukemia, plasma cell leukemia, plasmacytic leukemia, promyelocytic leukemia, Rieder cell leukemia, Schilling's leukemia, stem cell leukemia, subleukemic leukemia, and undifferentiated cell leukemia. In certain aspects, the present invention provides treatment for chronic myeloid

leukemia, acute lymphoblastic leukemia, and/or Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ ALL).

Various cancers are also encompassed by the scope of the invention, including, but not limited to, the following: carcinoma including that of the bladder (including accelerated and metastatic bladder cancer), breast, colon (including colorectal cancer), kidney, liver, lung (including small and non-small cell lung cancer and lung adenocarcinoma), ovary, prostate, testes, genitourinary tract, lymphatic system, rectum, larynx, pancreas (including exocrine pancreatic carcinoma), esophagus, stomach, gall bladder, cervix, thyroid, and skin (including squamous cell carcinoma); hematopoietic tumors of lymphoid lineage including leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T-cell lymphoma, Hodgkins lymphoma, non-Hodgkins lymphoma, hairy cell lymphoma, histiocytic lymphoma, and Burketts lymphoma; hematopoietic tumors of myeloid lineage including acute and chronic myelogenous leukemias, myelodysplastic syndrome, myeloid leukemia, and promyelocytic leukemia; tumors of the central and peripheral nervous system including astrocytoma, neuroblastoma, glioma, and schwannomas; tumors of mesenchymal origin including fibrosarcoma, rhabdomyosarcoma, and osteosarcoma; other tumors including melanoma, xenoderma pigmentosum, keratoactanthoma, seminoma, thyroid follicular cancer, and teratocarcinoma; melanoma, unresectable stage III or IV malignant melanoma, squamous cell carcinoma, small-cell lung cancer, non-small cell lung cancer, glioma, gastrointestinal cancer, renal cancer, ovarian cancer, liver cancer, colorectal cancer, endometrial cancer, kidney cancer, prostate cancer, thyroid cancer, neuroblastoma, pancreatic cancer, glioblastoma multiforme, cervical cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon carcinoma, and head and neck cancer, retinoblastoma, gastric cancer, germ cell tumor, bone cancer, bone tumors, adult malignant fibrous histiocytoma of bone; childhood malignant fibrous histiocytoma of bone, sarcoma, pediatric sarcoma, sinonasal natural killer, neoplasms, plasma cell neoplasm; myelodysplastic syndromes; neuroblastoma; testicular germ cell tumor, intraocular melanoma, myelodysplastic syndromes; myelodysplastic/myeloproliferative diseases, synovial sarcoma. In addition, disorders include urticaria pigmentosa, mastocytosises such as diffuse cutaneous mastocytosis, solitary mastocytoma in human, as well as dog mastocytoma and some rare subtypes like bullous, erythrodermic and teleangiectatic mastocytosis, mastocytosis with an associated hematological disorder, such as a myeloproliferative or myelodysplastic syndrome, or acute leukemia, myeloproliferative disorder associated with mastocytosis, mast cell leukemia, in addition to other cancers. Other cancers are also included within the scope of disorders

including, but are not limited to, the following: carcinoma, including that of the bladder, urothelial carcinoma, breast, colon, kidney, liver, lung, ovary, pancreas, stomach, cervix, thyroid, testis, particularly testicular seminomas, and skin; including squamous cell carcinoma; gastrointestinal stromal tumors ("GIST"); hematopoietic tumors of lymphoid lineage, including leukemia, acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T-cell lymphoma, Hodgkins lymphoma, non-Hodgkins lymphoma, hairy cell lymphoma and Burketts lymphoma; hematopoietic tumors of myeloid lineage, including acute and chronic myelogenous leukemias and promyelocytic leukemia; tumors of mesenchymal origin, including fibrosarcoma and rhabdomyosarcoma; other tumors, including melanoma, seminoma, teratocarcinoma, neuroblastoma and glioma; tumors of the central and peripheral nervous system, including astrocytoma, neuroblastoma, glioma, and schwannomas; tumors of mesenchymal origin, including fibrosarcoma, rhabdomyosarcoma, and osteosarcoma; and other tumors, including melanoma, xenoderma pigmentosum, keratoactanthoma, seminoma, thyroid follicular cancer, teratocarcinoma, chemotherapy refractory non-seminomatous germ-cell tumors, and Kaposi's sarcoma, and any metastasis thereof.

In a preferred embodiment of the present invention, the cancer is a solid tumor. The term "solid tumor" especially means breast cancer, ovarian cancer, cancer of the colon and generally the GI (gastro-intestinal) tract, cervix cancer, lung cancer, in particular small- cell lung cancer, and non-small-cell lung cancer, head and neck cancer, bladder cancer, cancer of the prostate or Kaposi's sarcoma.

In a preferred embodiment, the cancer is characterized by cells deficient in homologous recombination, in particular caused by BRCA mutations, especially BRCA1 and/or BRCA2.

Accordingly, in a very preferred embodiment, the cancer is selected from the group consisting of breast cancer, ovarian cancer, prostate cancer, melanoma, lung cancer (e.g. squamous cell lung cancer and non-small-cell lung cancer (NSCLC)), colorectal cancer, glioblastoma, larynx and cervix cancer, sarcoma, soft tissue sarcoma, peritoneal carcinomatosis, myelodysplastic syndrome and hematological malignancies such as acute myeloid leukemia (AML), chronic lymphoma and multiple myeloma.

The effective dosage of each of the combination partners employed in the combined preparation of the invention may vary depending on the particular compound or pharmaceutical composition employed, the mode of administration, the condition being treated, the severity of the condition being treated. Thus, the dosage regimen of the combined preparation of the invention is selected in accordance with a variety of factors including the

route of administration and the patient status. A physician, clinician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the single active ingredients required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentration of the active ingredients within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the active ingredients' availability to target sites.

The Pbait molecules could be administered by any suitable route, for instance intramuscularly, intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, topically, intratumorally, peritoneally, subcutaneously, subconjunctival, intravesicularlly, mucosally, intrapericardially, intraumbilically, intraocularally, orally, topically.

When a DNA-damaging antitumoral agent is used in combination with the Pbait molecule, the DNA-damaging antitumoral agent and the Pbait molecules may be administered by the same route or by distinct routes. The administration route for the DNA-damaging antitumoral agent may be oral, parenteral, intravenous, intratumoral, subcutaneous, intracranial, intraartery, topical, rectal, transdermal, intradermal, nasal, intramuscular, intraosseous, and the like.

In a particular embodiment, the DNA-damaging antitumoral agent is to be administered by oral route, and the Pbait molecules may be administered, simultaneously, separately or sequentially, by intratumoral injection, by sub-sutaneous injection, by intraperitoneal injection, by intravenous injection, or by oral route, preferably by intratumoral, sub-sutaneous or intravenous injection or by sub-sutaneous or intravenous injection.

When an endosomolytic agent, preferably chloroquine or hydroxychloroquine, more preferably chloroquine, is to be administered, it will be administered by oral route or by intraperitoneal route, preferably by oral route. The endosomolytic agent, preferably chloroquine or hydroxychloroquine, more preferably chloroquine, is to be administered 2 hours before and/or simultaneously with and/or after Pbait molecules. For details dosage, it can be referred to WO2011/161075.

For Pbait molecules, the effective dosage of the DNA-damaging antitumoral agent employed in the combined preparation, kit or product of the invention may vary depending on the mode of administration, the condition being treated, the severity of the condition being treated. Thus, the dosage regimen of the Pbait molecules is selected in accordance with a variety of factors including the route of administration and the patient status. A physician,

clinician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the Pbait molecules required to prevent, counter or arrest the progress of the cancer, in particular in combination with the selected DNA damaging treatment.

For instance, for local administration (e.g., when the intratumoral or sub-cutaneous administration is used), the efficient amount of the Pbait molecules is at least 0.01 mg per 1 cm^3 of tumor, preferably 0.1 – 40 mg per 1 cm^3 of tumor, most preferably 1 - 20 mg per 1 cm^3 of tumor. The efficient amount can be administered in a daily treatment protocol (e.g., 5 days per week for 3 to 6 consecutive weeks or 3 times a week for 3 to 6 consecutive weeks). Alternatively, an efficient amount of at least 0.1 mg per 1 cm^3 of tumor, preferably 0.1 - 40 mg per 1 cm^3 of tumor, most preferably 1 - 20 mg per 1 cm^3 of tumor, can be administered in a weekly treatment protocol for 3-6 consecutive weeks, for instance. When other administration routes are used, the one skilled in the art can adapt the amount in order to obtain an efficient amount of the Pbait molecules in the tumor of at least 0.01 mg per 1 cm^3 of tumor, preferably 0.1 - 40 mg per 1 cm^3 of tumor, most preferably 1 - 20 mg per 1 cm^3 of tumor, in particular in a daily treatment protocolor in a weekly treatment protocol. For instance, for a systemic route, the efficient amount or unit dosage of the Pbait molecules may be of 1 to 1000 mg, preferably of 60 to 600 mg. Accordingly, for a systemic route, the efficient amount or unit dosage of the Pbait molecules may be of 0.016 to 16 mg/kg of patient. Of course, the dosage and the regimen can be adapted by the one skilled in art in consideration of the chemotherapy and/or radiotherapy regimen.

For radiotherapy, any radiotherapy regimen known in the art may be used, in particular stereotactic irradiation (e.g., 15 Gy) or a fractionated irradiation. The use of a fractionated irradiation may be particularly efficient, for instance irradiation may applied every day or every 2-5 days, preferably every 3-4 days, in a period of one, two, three, four, five or six weeks. The irradiation may be from 1 to 10 Gy, preferably from 2 to 5 Gy, in particular 2, 3, 4 or 5 Gy. For instance, fractionated irradiation of 15x 2Gy in six weeks, or of 4 to 6x 5Gy in two weeks may be contemplated. In a preferred embodiment, the contemplated radiotherapy is a protocol with 4 irradiations of 5 Gy in two weeks. Different regimens or conditions of combined treatments of cancer with irradiation and Pbait molecules have been tested and allowed to demonstrate the radio-sensibilization of tumors by Pbait molecules depends on the doses of Pbait molecules but not of the irradiation doses.

For chemotherapy, the effective dosage of the DNA-damaging antitumoral agent employed in the combined preparation, kit or product of the invention or in combination with the composition of the invention may vary depending on the particular DNA-damaging

antitumoral agent employed, the mode of administration, the condition being treated, the severity of the condition being treated. Thus, the dosage regimen of the DNA-damaging antitumoral agent is selected in accordance with a variety of factors including the route of administration and the patient status. A physician, clinician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the DNA-damaging antitumoral agent required to prevent, counter or arrest the progress of the cancer.

The treatment may include one or several cycles, for instance two to ten cycles, in particular two, three, four or five cycles. The cycles may be continued or separated. For instance, each cycle is separated by a period of time of one to eight weeks, preferably three to four weeks.

Generally, in the present application, "comprise" or "comprising" includes an embodiment in which "comprise" or "comprising" is replaced by "consist in" or "consisting in".

Further aspects and advantages of the present invention will be disclosed in the following experimental section, which should be regarded as illustrative and not limiting the scope of the present application. A number of references are cited in the present specification; each of these cited references is incorporated herein by reference.

DRAWINGS

Figure 1. PARP and DNA-PK activation induced by siDNA. PARylation and DNA-PK kinase activities were measured either with purified enzymes (Fig 1A, Fig 1B, Fig 1E) or nuclear extracts from MRC5 cells (Fig 1C, Fig 1D, Fig 1F): kinase activity was estimated by measuring P53 peptide phosphorylation after addition of 0.5 µM siDNA (Fig 1A, Fig 1C). PARP activation was estimated by measuring H1 histone parylation after addition of 0.2 µM siDNA (Fig 1B, Fig 1D) or increasing amounts of siDNA (Fig 1E, Fig 1F). Values were normalized to the maximal Dbait32Hc activity for each condition. Reported values represent the mean value and standard deviation of at least three independent experiments. Two 32bp-long Pbait molecules with different sequences were tested (Pbait32 and Pbait32L, noted 32a and 32b, respectively). The Dbait8H and Bait32C were used as negative control. Panel E,F: Pbait32 (triangle); Pbait12 (square); Dbait8H (diamond).

Figure 2. SiDNA molecules induce PARylation. (Fig 2A) Western blot of proteins from MRC5 cells treated with hydroxide peroxide (H_2O_2), Dbait8H, Dbait32Hc or Pbait32. Cell extracts were prepared 1h after the end of transfection or 10min after H_2O_2 treatment (500µM). (Fig 2B) Kinetics of PARylation induced by Pbait32 (light gray) and Dbait32Hc (dark gray) in MRC5 cells, 1h to 96h after the end of transfection. (Fig 2C)

Immunofluorescence of PAR and γH2AX in MO59J (DNA-PK deficient) cells and MO59K (DNA-PK wild-type) cells transfected with Dbait8H, Dbait32Hc or Pbait32 molecules or treated with H_2O_2. Scale bar: 20μM.

Figure 3. Kinetic of NAD consumption induced by Dbait and Pbait molecules. Kinetic of NAD consumption induced by Pbait32 (light gray), Dbait32Hc (dark gray) and Dbait8H in MRC5 cells during 4 days. All experiments were done in triplicate. Absorbance measurement (Fig 3A) and ratio measurement (Fig 3B) in treated samples and untreated. NAD quantification was determined by the CCKit8 test (Dojindo, Maryland, USA). Cells grown in 24-well plate were treated with 0.2 ug siDNA molecules for 5h in 200μl complete medium. WST-8 [2 - (2-methoxy-4-nitrophenyl) - 3 - (4-nitrophenyl) - 5 - (2,4-disulfophenyl) - 2H - tetrazolium, monosodium salt] was added and the water-soluble formazan dye produced by reduction of WST-8 during NAD consumption was monitored by absorbance at 450 nm. Samples were measured at different times during 4 days. All experiments were done in triplicate. Since cells growth was not affected by siDNA treatment, the difference observed during the 24 hours is probably due to the induction of PARP activity in the treated cells.

Figure 4. PARylation induced by Dbait and Pbait in several cell types. Quantification of PAR positive cells transfected with Dbait32Hc (dark gray) and Pbait32 (light gray), in glioblastoma (U87, T98G, SF763, SF767, MO59K, MO59J, U251, FOG), melanoma (SK28) and cervix/larynx cancer (HeLa and HEp2) cell lines.

Figure 5. PARylation induced by siDNA is mostly not associated with chromosomes. (Fig 5A) Immunodetection of PAR polymers and γH2AX in MRC5 cell lines after transfection with siDNA or H_2O_2 treatment. White squares are magnified 3.5 times. Pearson coefficients of colocalisation between DAPI and PAR (R values) or DAPI and γH2AX (R* value) were determined with *ImageJ software*. (Fig 5B) Immunofluorescence of PAR and γH2AX in MRC5 pre-treated (+) or not (-) with a pre-extraction buffer. (Fig 5C) DNA damage after H_2O_2 or siDNA treatments analyzed by alkaline comet assay. The moment value reported is the mean value from three independent measurements of comet tails estimated from 100 nuclei per condition. Scale bar: 5μM.

Figure 6. Proteins accumulation on siDNA. (Fig 6A) Immunodetection of PAR and XRCC1-EYFP 6 hours after beginning of siDNA transfection. (Fig 6B) Immunodetection of PARP, PCNA, DNA-PK and Ku70 proteins and XRCC1-EYFP after siDNA transfection. (Fig 6C) Pull-down assay with biotinylated siDNA. Western blotting reveals proteins trapped by biotinylated siDNA. Scale bar: 5μM.

Figure 7. Pbait prevent the relocalization of SSB but not DSB repair proteins. Numbers of foci of RAD51 (Fig 7A), XRCC1 (Fig 7B) and PCNA (Fig 7C) proteins formed in cells transfected with Pbait32, Dbait32Hc or Dbait8H and irradiated with 10 Gy (grey) or non-irradiated (black). (Fig 7D) Distribution of γH2AX in 10 Gy irradiated cells with (grey) or without P3bait32 treatment (black). (Fig 7E) Kinetics of XRCC1-EYFP relocalisation to laser damage sites (white squares) after siDNA treatment. White squares are magnified 5.5 times: (Fig 7F, Fig 7G) microcopy quantifications of XRCC1-EYFP foci at various times after laser damage:(Fig 7F) mean value of 100 laser treated cells; (Fig 7G) inverse correlation between numbers of XRCC1-EYFP foci in cells transfected with Pbait or Dbait at time zero and maximal recruitment at laser induced damage. Black square: Dbait 8H; Gray diamond: Dbait32Hc; White triangle: Pbait32. Scale bar: 5μM.

Figure 8. Pbait prevent the relocalisation of PARP but not NBS1 and MRE11. repair proteins after irradiation. Numbers of foci of NBS1 (Fig 8A), MRE11 (Fig 8B) and PARP (Fig 8C) proteins formed in cells transfected with Pbait32, Dbait32Hc or Dbait8H and irradiated (grey) or not (black) with 10 Gy.

Figure 9. SiDNA are synthetic lethal with BRCA mutations. DNA damage was monitored in Pbait32 or Dbait32Hc treated cells by comet assay (Fig 9A, Fig 9B) and survival was estimated by Trypan blue cell counting (Fig 9C, Fig 9D). Analyses were performed in breast cancer cell lines (panels A and C: MDA-MB-231 ($BRCA^{+/+}$), black; HCC1937 ($BRCA1^{-/-}$), grey), and in HeLa cells (panels B and D: HeLa, black; HeLa shBRCA1, dark grey; HeLa shBRCA2, grey). Values are mean value of at least three independent experiments.

Figure 10. SiDNA act as PARP inhibitor and induce a synthetic lethal effect in BRCA deficient cell lines. (Fig 10A 10B) Cellular survival was monitored in Pbait32 or Dbait32Hc treated cells by Trypan blue cell counting. Analyses were performed in breast cancer cell lines (panels A: MDA-MB-231 (BRCA+/+), black; HCC1937 (BRCA1-/-), grey), and in HeLa cells (panels B: HeLa, black; HeLa shBRCA1, dark grey; HeLa shBRCA2, grey).

EXAMPLES

One of the major early steps of repair is the recruitment of repair proteins at the damage site, and this is coordinated by a cascade of modifications controlled by phosphatidylinositol 3-kinase-related kinases and/or poly (ADP-ribose) polymerase (PARP). The inventors used short interfering DNA molecules mimicking double-strand breaks (called Dbait) or single-strand breaks (called Pbait), to promote DNA-dependent protein kinase

(DNA-PK) and PARP activation. Dbait bound and induced both PARP and DNA-PK activities, whereas Pbait acts only on PARP. Therefore, comparative study of the two molecules allows analysis of the respective roles of the two signaling pathways: both recruit proteins involved in single-strand break repair (PARP, XRCC1 and PCNA) and prevent their recruitment at chromosomal damage. Dbait, but not Pbait, also inhibits recruitment of proteins involved in double-strand break repair (53BP1, NBS1, RAD51 and DNA-PK).

By these ways, Pbait and Dbait disorganize DNA repair, thereby sensitizing cells to various treatments. SSB repair inhibition depends upon direct trapping of the main proteins on both molecules. DSB repair inhibition may be indirect, resulting from the phosphorylation of DSB repair proteins and chromatin targets by activated DNA-PK. The DNA repair inhibition by both molecules is confirmed by their synthetic lethality with BRCA mutations.

Results

Design of siDNA molecules mimicking SSB.

The siDNA used were short double-strand DNA that carries a single model lesion. Dbait molecules, that mimic DSB, were constructed by tethering two complementary oligonucleotides with an hexaethyleneglycol linker at one extremity of the duplex and protecting the ends at the other extremity by adding 3 phosphorotioate modifications (See Table 1).

The inventors designed new siDNA molecules mimicking SSB (called Pbait). These constructs form a duplex with no free end and an interruption in the middle of one strand (Table 1). Each end of the Pbait molecules was tethered by a hexaethyleneglycol loop to prevent DNA-PK binding. The duplex length in Pbait molecules was between 8 and 32 base pairs (bp).

Table 1: SiDNA Molecules

SiDNA Molecules	Sequences	SEQ ID No
Pbait12		1
Pbait20		2
Pbait30		

TG GTC TCG ATG ACC $A_{5',\ 3'}$TCC CGA CTG TCC GGT
AC CAG AGC TAC TGG TTA AGG GCT GAC AGG CCA 3

Pbait32

TGGTCTCGATGACCAA5'3'TTCCCGACTGTCCGGT
ACCAGAGCTACTGGTTAAGGGCTGACAGGCCA 4

Pbait32s

TAG TCT CGG TGAC*A*C*$G_{5'3'}$G*G*T*CT GAC TGT ACT GA
ATC AGA GCC ACT G T G C C C A G A CTG ACA TGA CT 5

Pbait32b

TAG TCT CGG TGA CAC $G_{5'3'}$GGTCTGACTGT ACT GA
ATC AGA GCC ACT GTG CCC AGA CTG ACA TGA CT 6

Pbait32L

CGACACACGTCACGAA5'3'CCTCGTACTGGGATCT
GCTGTGTGCAGTGCTTGGAGCATGACCCTAGA 7

Dbait8H

GACCT*A*G*C-3'
CTGGA*T*C*G-5' 8

Dbait32Hc

AGATCCGAACAAACGACCCAACACCCGTG*T*C*G-5'
TCTAGGCTTGTTTGCTGGGTTGTGGGCAC*A*G*C-3' 9

Bait32C

TGGTCTCGATGACCAATTCCCGACTGTCCGGT
ACCAGAGCTACTGGTTAAGGGCTGACAGGCCA 10

Pbait14

A TGA $CCA_{5',\ 3'}$T CCC GAC
T ACT GGT TAA GGG CTG 11

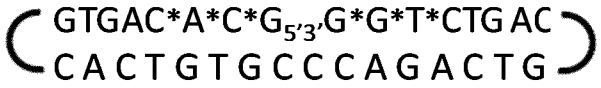
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Pbait16

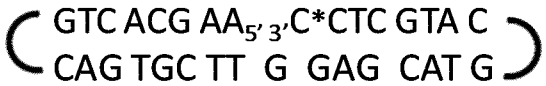
A TGA CCA $A_{5'3'}$TTCCCGAC
T ACT GGT TAA GGG CTG 13

Pbait16S



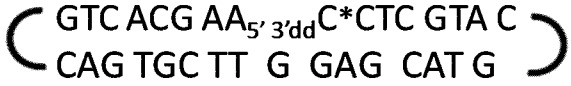
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Pbait16s3



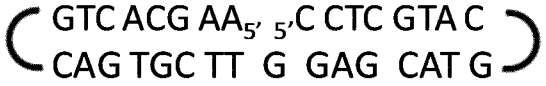
15

Pbait16sd3



16

Pbait16p5,



17

Pbait molecules are linear duplex DNAs with an interruption in the middle of one strand and a hexaethyleneglycol linker at each end.

Dbait molecules are linear duplex DNAs with one hexaethyleneglycol linker (black bracket) at one end and three phosphorotioate modifications (*) at the other end. dd refers to a dideoxynucleotide. 5'5' in Pbait16p5 refers to a modification of the 3'end by adding a 5'phosphate.

Bait32C is a linear duplex DNA with a hexaethyleneglycol linker at each end.

SiDNA molecules were screened for their ability to recruit PARP and induce the synthesis of a poly (ADP-ribose) chain (PAR) referred to as PARylation (Figure 1). PARP activity assays were performed using purified PARP enzyme (Fig 1B, Fig 1E) and MRC5 cell extracts (Fig 1D, Fig 1F).

Dbait32Hc molecules (32bp) efficiently activated both purified PARP and PARP in crude extracts. These results confirm previous observations that PARP binds to DSB with a high affinity. Shorter Dbait or Pbait molecules did not efficiently activate PARP in crude extract. Analysis of PARP activation as a function of siDNA concentration showed that maximal activation required 10-fold more Pbait12 than Pbait32 (Fig 1E-F). As expected, Bait32C molecules that have no nick or free ends did not activate PARP.

Pbait32L and Pbait32 that have the same structure but differ in DNA sequence (Table 1) had similar PARP activation activities. PARP activation thus did not depend upon the DNA sequence.

Moreover, only Dbait and not Pbait activated DNA-PK whether as a purified enzyme (Fig 1A) or in MRC5 cell extracts (Fig 1C). This result is in agreement with previous observation that 34-32bp long dumbbell form with no free ends does not bind DNA-PK.

The inventors chose the 32bp-long Pbait and Dbait molecules (hereafter called Pbait32 and Dbait32Hc), which activated PARP and PARP/DNA-PK, respectively, for further studies in cell cultures. They used an 8bp-long Dbait (Dbait8H), which does not activate DNA-PK or PARP, as a transfection control (Figure 1).

Pbait and Dbait molecules induce PARylation in cells.

The inventors confirmed PARP activation in cells by monitoring PAR-modified proteins in cells transfected with Dbait32Hc or Pbait32. Substantial PAR-modification of proteins was observed in cells after transfection with both siDNA (Fig 2A), consistent with the enzymatic assays. The extent of PARylation in cells with PARP activated by Dbait32Hc and Pbait32 was similar to that observed after oxidative stress induced by H_2O_2 treatment (Fig 2A). Kinetics of PARylation by the two siDNA was very similar with an activity maximum 4 hours after beginning of treatment (Fig 2B). Following NAD consumption, as NAD is used as a substrate by PARP for PARylation of its target proteins, can monitor PARP activity in the cell. As expected, the NAD concentration decreased as the PAR signal increased in cells transfected with Pbait32 and Dbait32Hc, but not with the control Dbait8H (Figure 3). The kinetics of NAD consumption coincided closely with the kinetics of PARP activation (Fig 2B).

SiDNA activated PARP in various tumor cell lines derived from melanoma, larynx and cervix (Figure 4), glioblastoma (Figure 2C), and in MRC5-transformed fibroblasts (Figure 5). In agreement with the enzymatic assay results, only Dbait32Hc treatment induced DNA-PK activation, as revealed by testing for the phosphorylated form of the histone variant H2AX, called γH2AX (Figure 2C). H2AX phosphorylation but not PARylation activity was affected by the DNA-PK defect in the MO59J cell line (Figure 2C). These results indicate that H2AX phosphorylation by Dbait is strictly dependent upon DNA-PK, but that in contrast PARP activation does not require DNA-PK.

Pbait and Dbait recruitment of DNA repair proteins.

Foci of PAR modification were observed in nucleus of siDNA (Dbait or Pbait)-treated cells. The localization of the foci induced by Dbait differed from those formed after H_2O_2

treatment to DNA damage. PAR foci induced by H_2O_2 perfectly colocalized with heterochromatin stained with DAPI, but those induced by Dbait32Hc and Pbait32 were distributed across the nuclei independently of chromatin condensation (Figure 5A). A pre-extraction treatment to remove soluble molecules before fixing the cells (Figure 5B) suppressed the PAR signal in Dbait treated cells but not in H_2O_2 treated cells. This confirms that PAR modifications induced by siDNA molecules are mainly on themself rather on chromatin. Numerous damages were detected by alkaline comet assay on chromosomes after H_2O_2 treatment and probably correspond to the sites of PARP activation in the cell; no chromosome damage was detected after siDNA treatments (Figure 5C). Thus PAR foci seem to form where the DNA damage is detected: on chromosomes after H_2O_2 and on Dbait32Hc or Pbait after siDNA treatments.

Self PARylation of PARP is an early event at SSB sites. XRCC1 is then rapidly recruited to the sites of PAR synthesis (Fig 6A) whereas PCNA (Proliferating Cell Nuclear Antigen) is recruited more slowly. After transfection with siDNA, PARP, XRCC1 and PCNA accumulate in foci that colocalized (Fig 6B). The inventors first demonstrated that XRCC1 tagged with an EYFP peptide colocalized with PAR foci (Fig 6A) then used this same construct to demonstrate colocalization of both PARP and PCNA with EYFP-XRCC1 (Fig 6B). In contrast, DNA-PK and Ku did not form foci in Dbait32Hc-treated cells, although the activation of the kinase activity revealed their interaction with the DNA bait (Figure 6B). They show a uniform distribution in nucleus after siDNA treatment indicating that their binding to Dbait32Hc did not lead to their aggregation in foci.

To confirm that all these repair proteins bind directly or indirectly to the siDNA, the inventors performed pull-down assays with biotinylated Dbait and Pbait molecules. Cells were transformed with biotinylated siDNA and proteins bound to these baits were retrieved on streptavidin beads. Both Pbait32 and Dbait32Hc recruited the SSB repair proteins (PARP, XRCC1 and PCNA), but only Dbait32Hc, and not Pbait32, recruited the NHEJ repair proteins such as DNA-PK and Ku70 (Figure 6C).

SiDNA inhibit XRCC1 and PCNA recruitment at damage sites.

The inventors previously demonstrated that Dbait32Hc treatment prevents recruitment of DSB repair proteins such as 53BP1, RAD51 and NBS1 at sites of damage induced by irradiation (Quanz et al, 2009, *Clin Cancer Res*, **15**, 1308-1316; Quanz et al, 2009, *PlosOne*, **4**, e6298). This inhibition could be a consequence of the activation of PARP as well as DNA-PK signaling enzymes by Dbait. To determine the role of PARP activation in the recruitment of these DSB repair proteins, the inventors analyzed the recruitment of the RAD51 protein at

site of DNA damage induced by irradiation in siDNA-transfected cells. As previously shown, they found that Dbait significantly reduced recruitment of RAD51 (Figure 7A), NBS1 or MRE11 (Figure 8). In contrast, Pbait had no effect on RAD51 recruitment, suggesting that the inhibition of DSB repair proteins by Dbait is probably specific to DNA-PK activation. In agreement with this result, the inventors observed that the number of γH2AX foci induced by irradiation was similar in untreated cells and Pbait32 treated cells (Figure 7B). They investigated whether PARP activation by the siDNA inhibited recruitment of the proteins involved in SSB repair at damage sites. In the absence of siDNA treatment, 10 Gy irradiation induced about 10-fold more XRCC1 and PCNA foci than RAD51 foci (Fig 7A, 7C-D); this reflects the greater number of SSB than DSB caused by irradiation. In contrast to RAD51, MRE11 or NBS1, the number of XRCC1 and PCNA foci did not increase after irradiation of cells transfected with Dbait32Hc or Pbait32 (Fig 7C-D) indicating that both siDNA prevent recruitment of SSB repair proteins to damage site on chromosomes.

To confirm this result, the inventors followed the movements of EYFP-XRCC1 in real time after laser-induced damage (Fig 7E-F). The rate of recruitment of EYFP-XRCC1 at sites of laser-induced damage was significantly lower in all cells treated with Dbait or Pbait than controls; the maximal amount of recruited proteins for siDNA-treated cells was half that for controls. The extent of inhibition as assessed from the amount of XRCC1 recruited in the 70 seconds after laser treatment directly correlated with the number of XRCC1 foci present before treatment. This indicates that as more XRCC1 protein was trapped in siDNA-induced foci, less XRCC1 protein was localized at damage sites (Figure 7G). These results show that the substantial damage caused by laser irradiation was not sufficient to displace proteins from siDNA to sites of chromosome damage.

Dbait and Pbait are synthetic lethal with BRCA mutations.

The inhibition of PARP, XRCC1 and PCNA foci formation at damage sites suggests that Dbait and Pbait, like PARP inhibitors, inhibit SSB repair. PARP inhibitors are lethal to cells that are already deficient in DSB repair but have less effect on DSB repair-proficient cells. PARP inhibition in recombination deficient BRCA mutants is synthetic lethal. To analyze if PARP activation by Dbait and Pbait has similar consequence as PARP inhibition, the inventors tested siDNA toxicity in various BRCA mutant cell lines. They used breast cancer cell lines HCC1937 ($BRCA1^{-/-}$) and MDAMB231 ($BRCA^{+/+}$) as controls, and HeLa cell lines silenced or not for BRCA1 and BRCA2 (Figure 9). They found that Dbait and Pbait had toxic effects on BRCA mutant cell lines but not on wild-type controls (Figure 9). In BRCA mutant cells, treatment with 0.1 μM Pbait gave similar survival than treatment with 10

μM ABT-888 PARP inhibitor (Fig 10A-B). Microscopy monitoring of living cells after siDNA treatment showed that more than 50% of the $BRCA^{-/-}$ cells treated by Pbait32 or Dbait32Hc undergo apoptosis within the 6 hours following treatment, whereas this event is extremely rare in $BRCA^{+/+}$ control cell lines (data not shown).

Discussion

The inventors have developed a new class of small molecules (called siDNA) that mimic one kind of damage each and that are not degraded, replicated or repaired in the cell. In this work, they used two kinds of siDNA molecules: Dbait molecules which mimic a DSB, and Pbait molecules which mimic a SSB. Both Pbait and Dbait activate the PARP polymerase activity. They differ in that Pbait activates only PARP, whereas Dbait activates both PARP and the kinase DNA-PK.

In mammalian cells, the main pathway for the repair of DNA double-strand breaks (DSB) is NHEJ that depends upon DNA-PK; however, when NHEJ is impaired, an alternative or back-up NHEJ (B-NHEJ) pathway dependent of PARP operates. Possibly, accessory proteins control a hierarchy in which DNA-PK-dependent regular NHEJ repair is privileged over PARP-dependent B-NHEJ. Most DNA damaging treatments causes many more other forms of lesions than DSB (e.g., SSB, base damages, etc.), and these lesions presumably compete for PARP-1; this is consistent with PARP-1 not being the principal actor in the repair of DSB despite its higher affinity for DNA ends than Ku. In cells treated with Dbait, there are molecules mimicking “DSB” and no other forms of DNA lesion, allowing the two pathways to be efficiently activated in the same cell: this was demonstrated by most of the cells displaying H2AX phosphorylation by DNA-PK also having substantial protein PARylation.

The first event observed after Pbait treatment is the formation of PAR foci. PARP-1 is one of the first proteins to recognize damaged DNA and its interaction with DNA lesions triggers the PARylation of a variety of proteins, with PARP-1 itself being the main PAR acceptor. After DNA damage, the modification of PARP is estimated to represent 90% of the total PAR synthesis. There is evidence that PARylation may affect the chromatin structure to facilitate DNA repair processes. Interestingly, pre-extraction of soluble compounds from cells before PAR detection revealed that very few chromatin components are PARylated after siDNA treatments. PAR act not only as covalent protein modifications but also as protein-binding matrices. This property could explain the formation of foci by XRCC1, PCNA and PARP that colocalize with PAR. Since all these proteins are precipitated by pull-down with siDNA, it is likely that PARylated complexes built on the PAR (itself synthesized by the PARP bound to the siDNA) form aggregates.

It has been suggested that Ku80 and DNA-PK bind to PAR. However, in contrast to PARP1, XRCC1 and PCNA, they did not form foci in Dbait32H-treated cells, and therefore it is unlikely that in our conditions they were recruited on the PARylated complexes. The lack of association of Ku and DNA-PK with PAR induced by Pbait32 is an interesting observation and suggests that the detection of the affinity of various proteins to PAR or PARylated PARP-1 on large damaged DNA should be re-examined. Alternatively, the polymers formed in response to Pbait may be different (in length or complexity) from those formed in response to chromatin DNA damage.

PARylation is transient, and the polymer is quickly degraded by PARG enzymes [poly(ADP-ribose) glycohydrolases] and poly (ADP-ribose) hydrolase 3 (ARH3) activities. The persistence of the PAR signal in cells treated with siDNA suggests that in these experiments, the siDNA remained in the cells for at least two days. NAD continued to be consumed during this period, ruling out the possibility that PAR persisted because of defective PARG and ARH3. The continuous consumption of NAD implies that PARP dissociates from the siDNA, the polymer is degraded and the native PARP re-binds the Dbait and synthesises new polymers as previously proposed. However this consumption, which represent an increase of 50% as compared to untreated cells was not sufficient to induce ATP depletion and subsequent cell death in $BRCA^{+/+}$ cells.

How Dbait and Pbait inhibit DNA repair? Here, the inventors demonstrate that the general phosphorylation and PARylation following siDNA treatment prevents the recruitment of DNA repair proteins at the damaged locus on chromosomes. The SSB repair proteins associating with Dbait formed foci, and it is likely that they are trapped in these structures and consequently cannot move to, and contribute to the repair of, the damaged chromosomal DNA. In contrast, the DSB repair proteins do not present such specific aggregation away from chromatin. The enzymes MRN and 53BP1 bind to damage independently of PARP and DNA-PK, so any inhibition of their recruitment is not likely to be a consequence of the trapping of the signaling enzymes on the siDNA. These proteins were not pulled-down with Dbait32Hc or Pbait32. The chromatin in cells treated with siDNA is extensively modified, as revealed by histone H2AX phosphorylation. It has long been known that all core histones are targets for phosphorylation following DNA damage. The resulting higher-order chromatin structure may be essential for facilitating the access of factors required for repairing DNA damage. After siDNA treatment, phosphorylated H2AX spread along all the chromosomes, and the organization of repair foci was impaired even following the localized accumulation of damage induced by laser. This inhibition may be due to a diffuse recruitment of the repair proteins

over all the modified chromatin, which would considerably decrease the probability of a repair protein being at the damage site with all its partners. Also, the possibility that the unscheduled phosphorylation of most of the repair proteins involved prevents appropriate organization of the repair process by modifying the interactions both between proteins and with the DNA cannot be excluded.

Pbait act as PARP inhibitors, and are lethal in cells deficient in homologous recombination. Clinical applications of PARP inhibitors has attracted much attention over the last 10 years: approximately 65 clinical trials testing eight different PARP inhibitors are currently in progress or have already been completed in various part of the world. Although PARP inhibitors have unprecedented therapeutic potential for the treatment of cancers, there is accumulating evidence that tumor resistance to these drugs develops in both preclinical and clinical settings. Therefore, there is a strong need of new kind of PARP inhibitors.

Materials and Methods

Cell culture, Dbait molecules and treatments.

The cell lines used in this study were: SV40-transformed MRC-5 (ATCC number CCL-171), HeLa (ATCC number CCL-2), DNA-PK-defective M059J (ATCC number CRL-2366), M059K (ATCC number CRL-2365) and HeLa Silencix cells (Tebu-Bio number 00301-00041 for BRCA1 HeLa Silencix and number 00301-00028 for BRCA1 HeLa Silencix). Cells were grown according to the supplier's instructions (ATCC, Molsheim, France & Tebu-Bio, Le-Perray-en-Yvelines, France).

SiDNA molecules were obtained by automated solid-phase oligonucleotide synthesis from Eurogentec (Seraing, Belgium).

Cultures were transfected with siDNA molecules with jetPEI (Polyplus-transfection, Illkirch, France) at a N/P ratio of six according to the manufacturer's instructions. Unless otherwise indicated, cells were transfected at 80% confluence, with 2 μg siDNA in 1.3 ml of culture medium (0.1μM) without FCS (in 60 mm diameter plates) for 5 h. Hydrogen peroxide solution (H_2O_2; Sigma-Aldrich, St Louis, USA) was used as a DNA damaging agent. PARP inhibitors ABT-888 was purchased to Selleckchem (Euromedex, Souffelweyersheim, France).

Plasmids and transfection.

The plasmid XRCC1-EYFP was a gift from P. Radiccella (DSV, CEA, Fontenay-aux-roses, France). Cells were transfected with XRCC1-EYFP (2 μg) in Superfect reagent (20μl; Qiagen, Courtaboeuf, France) in 1.2 ml medium (in 60 mm diameter plates) for 5 h, and then grown to express recombinant protein for at least 48 h.

Antibodies and immunological techniques.

The following antibodies were used: polyclonal rabbit anti-poly (ADP-ribose), purified mouse anti-poly (ADP-ribose) (BD Pharmigen, Le Pont de Claix, France), monoclonal mouse anti-PARP C2-10 (Trevigen, Gaithersburg, USA), polyclonal rabbit anti-PCNA (Cell Signaling, Boston, USA), monoclonal mouse anti-DNA-PK (NeoMarker, Fremont, USA), polyclonal rabbit anti-Ku70 (Santa Cruz Biotechnologie, Heidelberg, Germany), polyclonal rabbit anti-NBS1 (Novus Biologicals, Cambridge, UK), polyclonal rabbit anti-MRE11 (Novus Biologicals, Cambridge, UK), polyclonal rabbit anti-phospho H2AX (Millipore, Billerica, France) and monoclonal mouse anti-phospho-H2AX (Millipore, Billerica, France). For immunostaining, cells grown on cover slips (Menzel, Braunschweig, Germany) were fixed for 20 minutes in 4% formaldehyde/PBS 1X, permeabilized in 0.5% Triton X-100 for 10 minutes, blocked with 2% BSA/PBS 1X (or 2% nonfat milk/PBS 1X) and incubated with primary antibody for 1 hour at room temperature (RT) or overnight at 4°C. All secondary antibodies conjugated with Alexa-488 or Alexa-633 (Molecular Probes, Eugene, OR, USA) were used at a dilution of 1/200 for 30 min at RT and DNA was stained with DAPI. For immunoblotting, cells were boiled in SDS sample buffer (50mM Tris-HCl, pH 6.8; 1% β-mercaptoethanol; 2% SDS; 0.1% bromophenol blue; 10% glycerol). Proteins were separated by electrophoresis in 5% or 12% acrylamide/bisacrylamide (35.5/1) gels, transferred to nitrocellulose membranes, blocked with Odyssey buffer for 1 hour and hybridized overnight at 4°C with primary antibody. Blots were then incubated with IRD secondary antibodies at 1/10 000 dilution (A700 or A800) and protein–antibody complexes were revealed on Odyssey (LI-COR Biotechnology, Bad Homburg, Germany).

PARP and DNA-PK activity assay.

DNA-PK and PARP activities were monitored using the SignaTECT DNA-dependent Protein Kinase Assay System kit (Promega, Madison, USA) and Universal Chemiluminescent PARP Assay Kit with Histone-Coated Strip Wells (Trevigen, number 4676-096-K, Gaithersburg, USA). Phosphorylation reactions were performed with 50 units of DNA-Dependent Protein Kinase and 0.25 µg (500 nM) Dbait, or 1.5 µg of DEAE-purified MRC5 cell extract and 0.01 µg (20 nM) Dbait. The ribosylation reaction included 0.10 µg (200 nM) of siDNA and 0.5 unit/well PARP-HSA or 40 µg of MRC5 cell extract. Negative controls were Dbait8H and Bait32C.

Trypan blue survival test.

Cells were seeded in 6-well plates at concentration of 10^5 cells per well. Triplicate wells were processed for each experimental point. Treatments were performed the day

following seeding. Cells were then allowed to grow for one or 24 hours after treatment, treated with 0.025% trypsin and stained with 0.4 % Trypan blue (Sigma Aldrich, Saint-Louis, USA). Cells were counted under microscope using a Bürker counting chamber. Survival is estimated as a percentage of blue cells on the total number of cells.

Single-cell gel electrophoresis comet assay.

Cells, transfected or not transfected with siDNA were analyzed for DNA damage by an alkaline "comet assay" as described in Quanz *et al.,* 2009 (*PlosOne*, **4**, e6298). Duplicate slides were processed for each experimental point. The tail moment is defined as the product of the percentage of DNA in the tail and the displacement between the head and the tail of the comet (Olive, 2002, *Methods Mol Biol*, **203**, 179-194).

Inducing photo-damage.

These experiments were performed with a Leica SP5 confocal system, attached to a DMI6000 stand using a 63×/1.4 objective, under a controlled environment (37°C, 5% CO_2). All recordings were made using the appropriate sampling frequency (512 × 512 images, line average of four and zooming set to eight) and an argon laser line (514 nm for YFP) adapted to the fluorescent protein of interest. In the first step, two images were acquired within a time period of 2–3 sec at a laser energy setting sufficiently low not to induce any photodynamic damage. The 405 nm laser line (diode) was then set to maximum output for 100ms and focused onto a single spot of constant size (176 nm) within the nucleus to cause a point of photo damage with a reproducible amount of energy. Recruitment of the protein of interest was then monitored by fluorescence using the same setting as for the pre damage sequence. Laser damage was induced 6 hours after the beginning of siDNA transfection. Images were captured at 2–5 sec intervals for the following 70 seconds (Godon et al, 2008, *Nucleic Acids Res*, **36**, 4454-4464).

CLAIMS

1- A molecule comprising a deoxyribonucleotide double-stranded portion of 12 to 200 bp, wherein it has preferably less than 80% sequence identity to any gene in a human genome, it has a single strand break or a gap on one strand, preferably, in the middle part of said double-stranded portion and the 5' and 3' ends at both extremities of said double-stranded portion are tethered by a linker.

2- The molecule according to claim 1, wherein the double-stranded portion is from 28 to 100 bp, preferably from 30 to 50 bp.

3- The molecule according to claim 1 or 2, wherein the single strand break or the gap is located at least 6 nucleotides for the extremities of said double-stranded portion, preferably at least 8, 12 or 15 nucleotides.

4- The molecule according to any one of claims 1-3, wherein the gap is a gap of less than 7 nucleotides, preferably less than 5, more preferably a gap of 1-3 nucleotides, still more preferably a gap of one nucleotide.

5- The molecule according to any one of claims 1-4, wherein the linker is selected from the group consisting of a polyethyleneglycol chain, an oligonucleotide and any hydrocarbon chain, optionally interrupted and/or substituted by one or more heteroatoms e.g., oxygen, sulfur, or nitrogen, or heteroatomic or heterocyclic groups, comprising one or several heteroatoms.

6- The molecule according to claim 5, wherein the linker is selected from the group consisting of hexaethyleneglycol, tetradeoxythymidylate (T4) and 2,19-bis(phosphor)-8-hydraza-1-hydroxy-4-oxa-9-oxo-nonadecane.

7- The molecule according to any one claims 1-5, wherein the molecule further comprises a moiety facilitating endocytosis selected from a lipophilic molecule or a ligand which targets cell receptor enabling receptor mediated endocytosis and/or specific cells and tissue targeting, said moiety facilitating endocytosis being preferably covalently linked to the linker.

8- The molecule according to claim 7, wherein said moiety facilitating endocytosis and/or cell/tissue targeting is selected from the group consisting of single or double

chain fatty acids such as octodecyl and dioleoyl, tocopherol, folates or folic acid, cholesterol, sugar such as galactose and mannose and their oligosaccharide, peptide such as RGD and bombesin, and protein such as integrin, preferably of dioleoyl, octadecyl, folic acid, and cholesterol, more preferably cholesterol.

9- A pharmaceutical composition comprising a molecule according to any one of claims 1-8 and a pharmaceutically acceptable carrier.

10- The pharmaceutical composition according to claim 9, wherein the pharmaceutical composition further comprises a DNA damaging antitumoral agent, preferably selected from the group consisting of an inhibitor of topoisomerases I or II, a DNA crosslinker, a DNA alkylating agent, an anti-metabolic agent and inhibitors of the mitotic spindles, and/or oxidative agents, such as oxygen, ozone, superoxide, or/and hydrogen peroxide.

11- A molecule according to any one of claims 1-8 for use as a drug.

12- A molecule according to any one of claims 1-8 for use in the treatment of cancer, optionally either in combination with DNA damaging therapies, or as a standalone treatment in the genetically instable cancer.

13- The molecule for use in the treatment of cancer according to claim 12, wherein it is combined with a radiotherapy, chemotherapy, or hyperthermia, preferably with a DNA damaging antitumoral agent, more preferably selected from the group consisting of an inhibitor of topoisomerases I or II, a DNA crosslinker, a DNA alkylating agent, an anti-metabolic agent and inhibitors of the mitotic spindles, and/or oxidative agents, such as oxygen, ozone, superoxide, or/and hydrogen peroxide.

14- The molecule for use for treating cancer according to claim 12 or 13, wherein the molecule is administered by oral route or by intravenous, intra-tumoral or sub-cutaneous injection, intracranial or intra artery injection or infusion, preferably by intravenous, intra-tumoral or sub-cutaneous injection.

15- The molecule for use for treating cancer according to any one of claims 12 to 14, wherein the cancer is characterized by cells deficient in homologous

recombination, in particular caused by BRCA mutations, especially BRCA1 and/or BRCA2.

16- The molecule for use for treating cancer according to any one of claims 12 to 15, wherein the cancer is selected from the group consisting of breast cancer, ovarian cancer, prostate cancer, melanoma, lung cancer (e.g. squamous cell lung cancer and non-small-cell lung cancer (NSCLC)), colorectal cancer, glioblastoma, larynx and cervix cancer, sarcoma, soft tissue sarcoma, peritoneal carcinomatosis, myelodysplastic syndrome and hematological malignancies such as acute myeloid leukemia (AML), chronic lymphoma and multiple myeloma.

Fig 1A

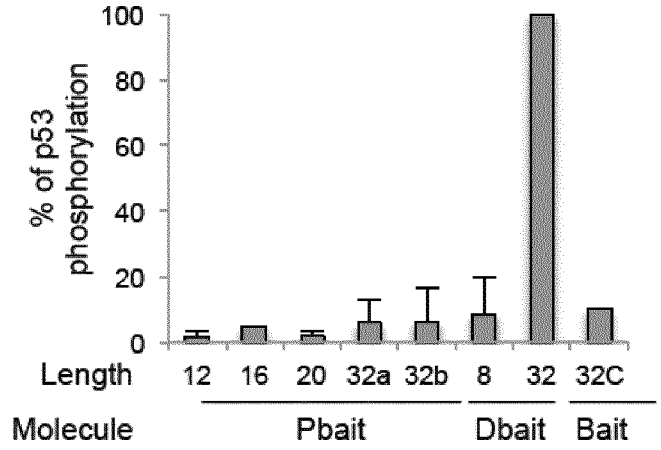


Fig 1B

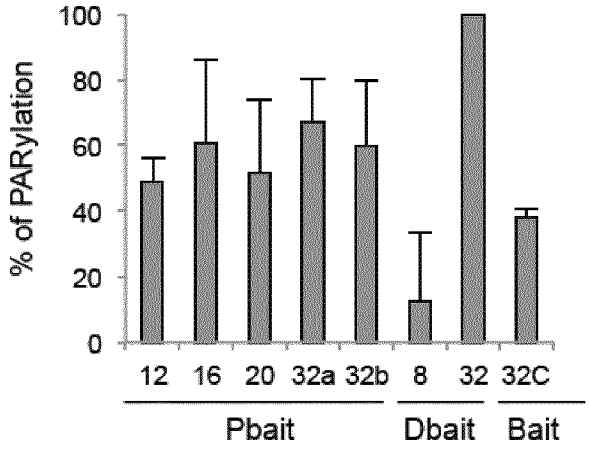


Fig 1C

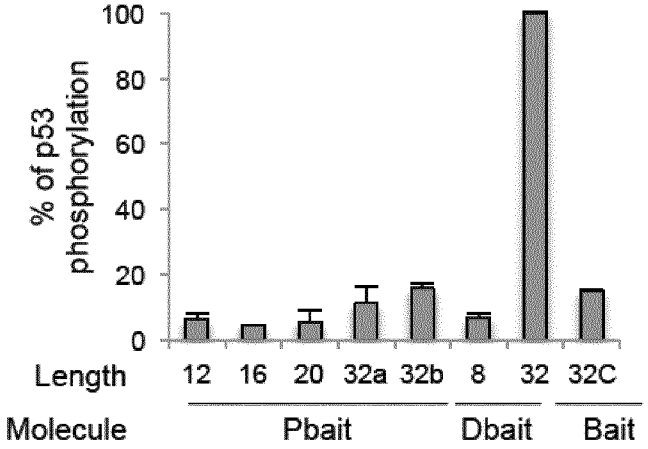


Fig 1D

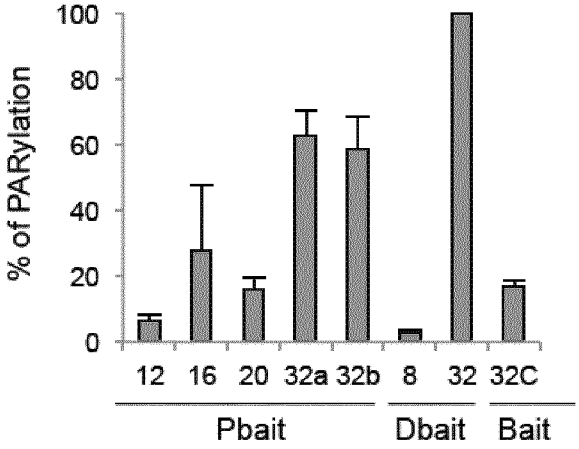


Fig 1E

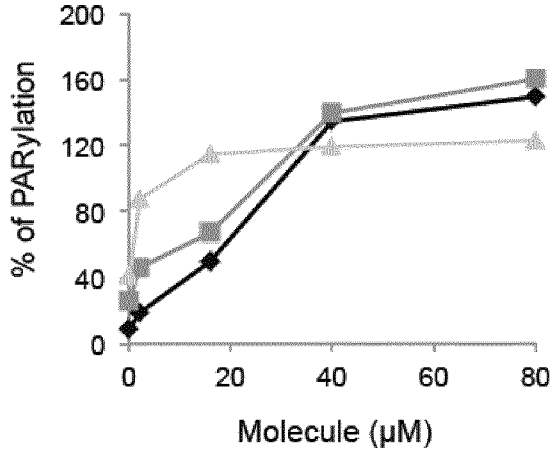


Fig 1F

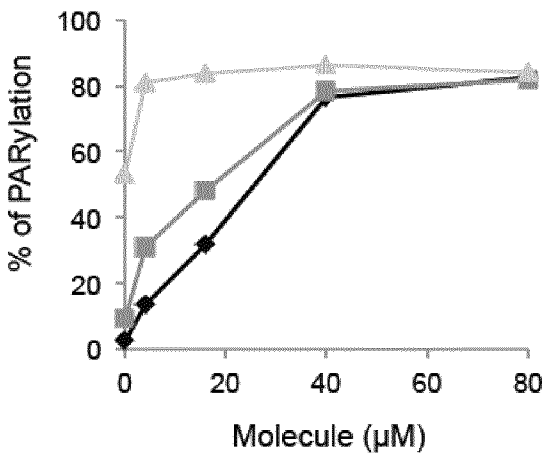


Fig 2A

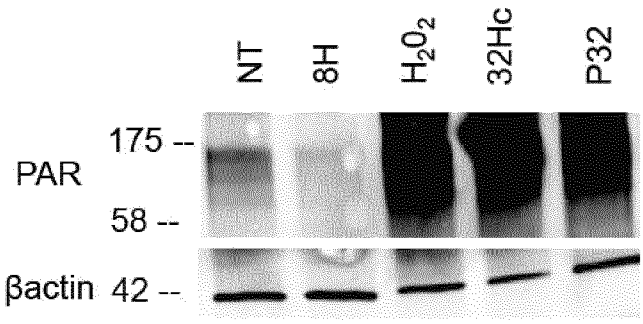


Fig 2B

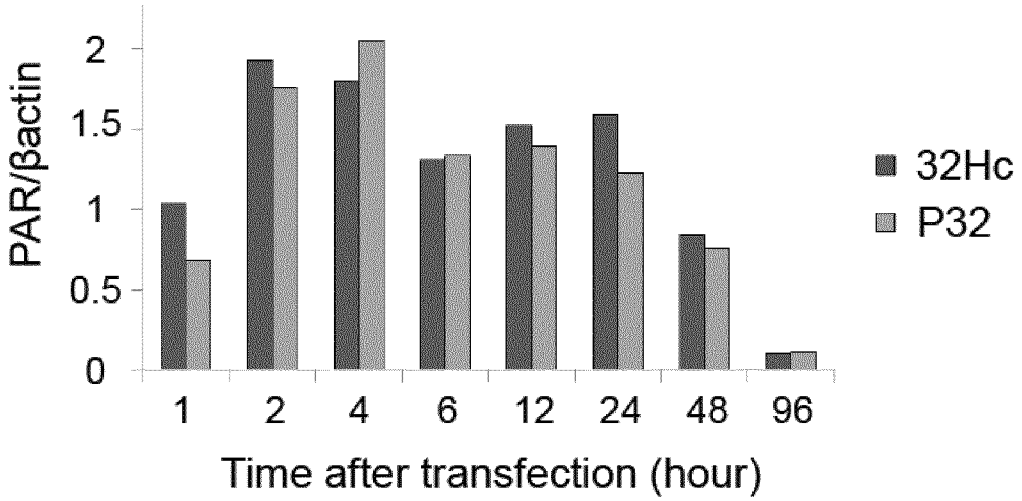


Fig 2C

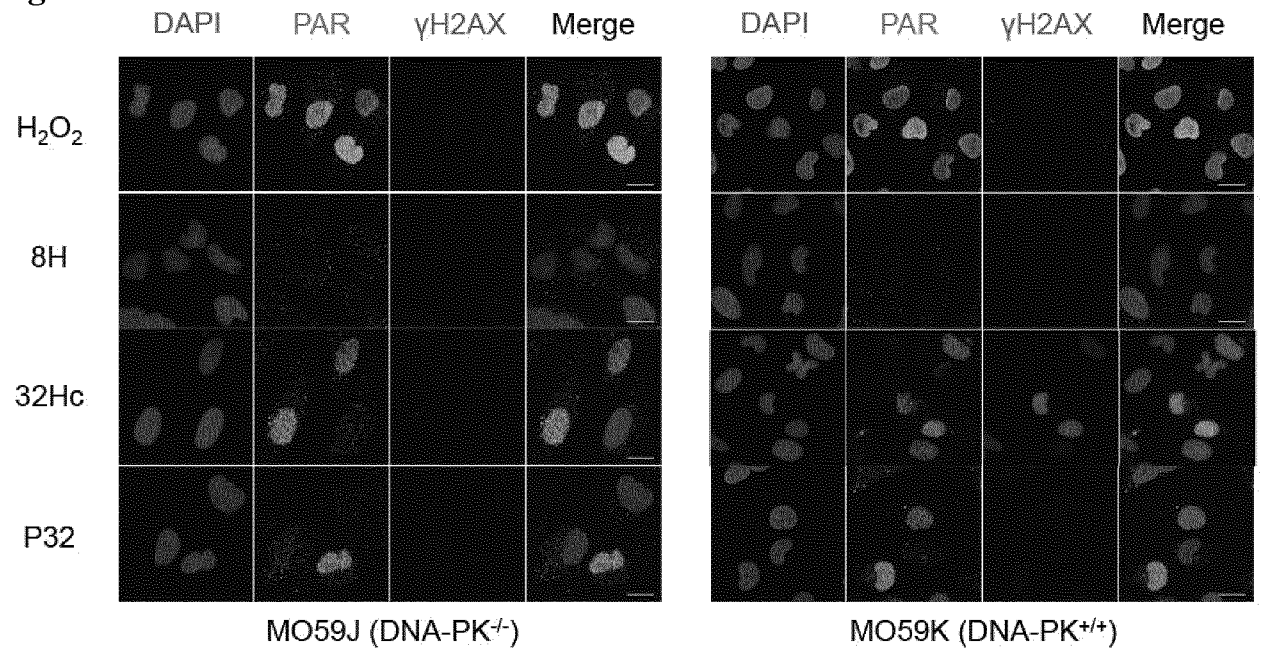


Fig 3A

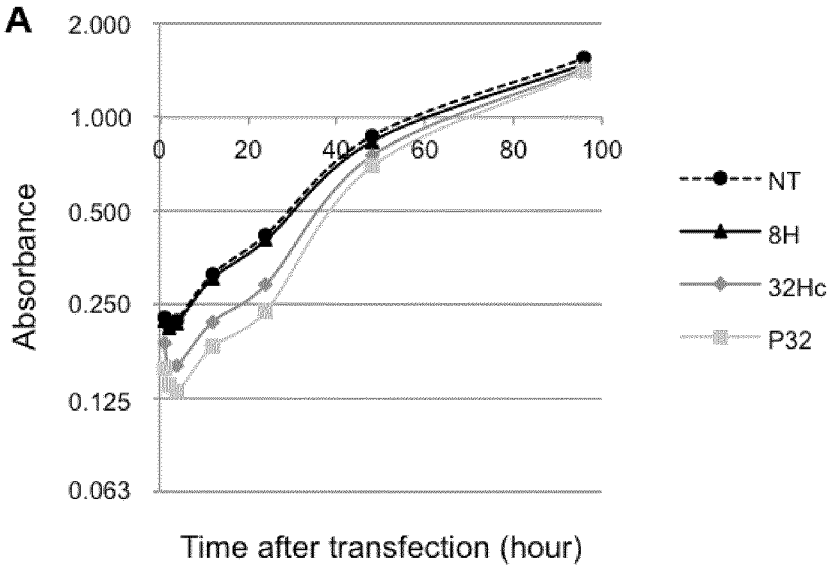


Fig 3B

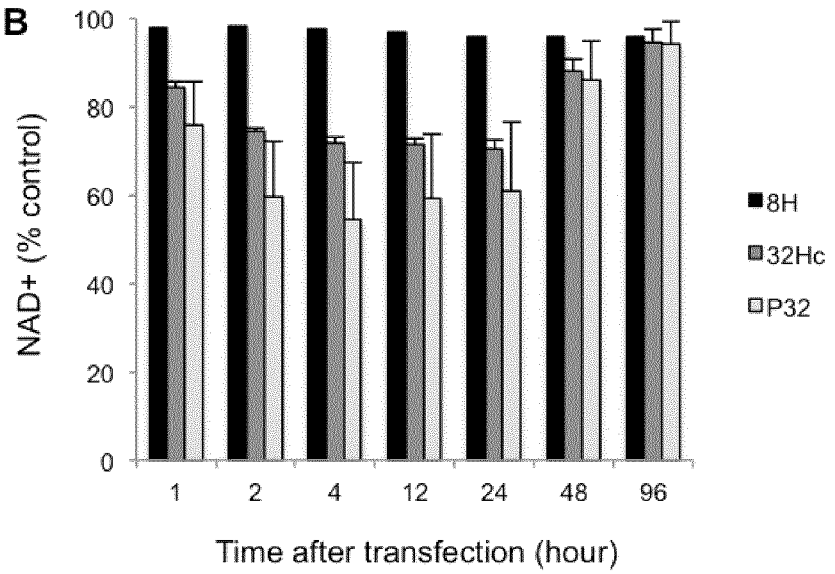


Fig 4

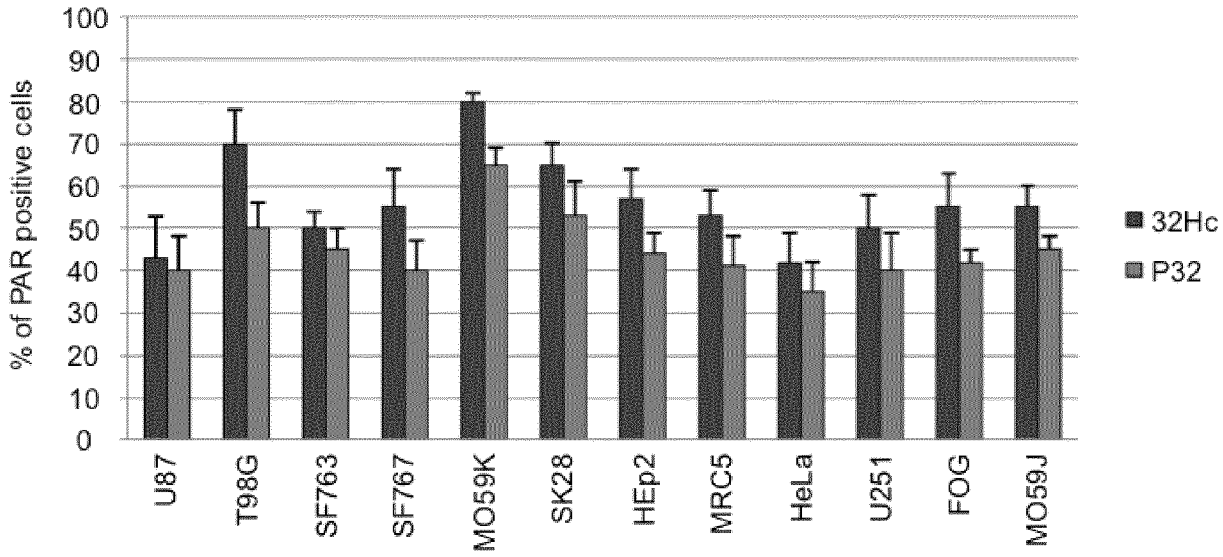


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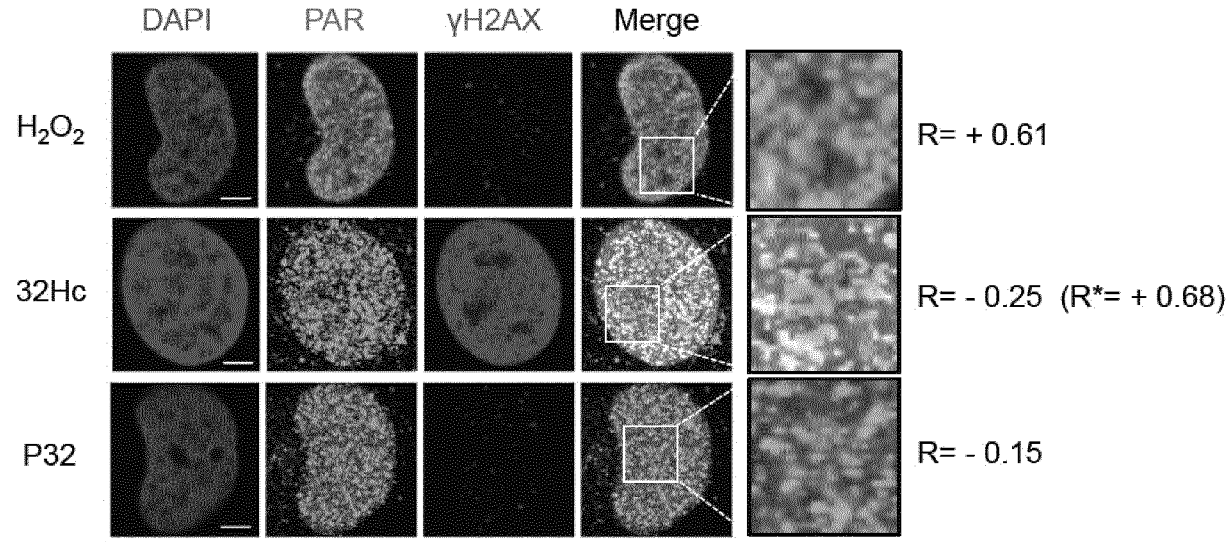


Fig 5B

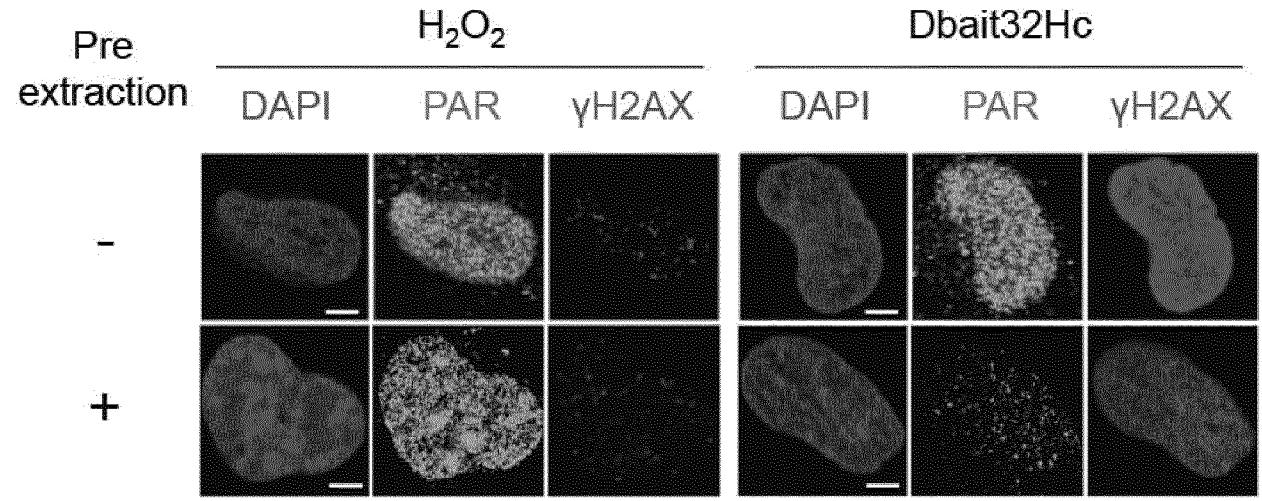


Fig 5C

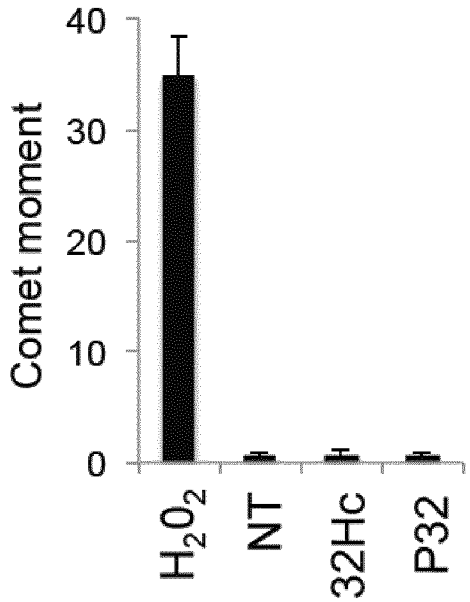


Fig 6A

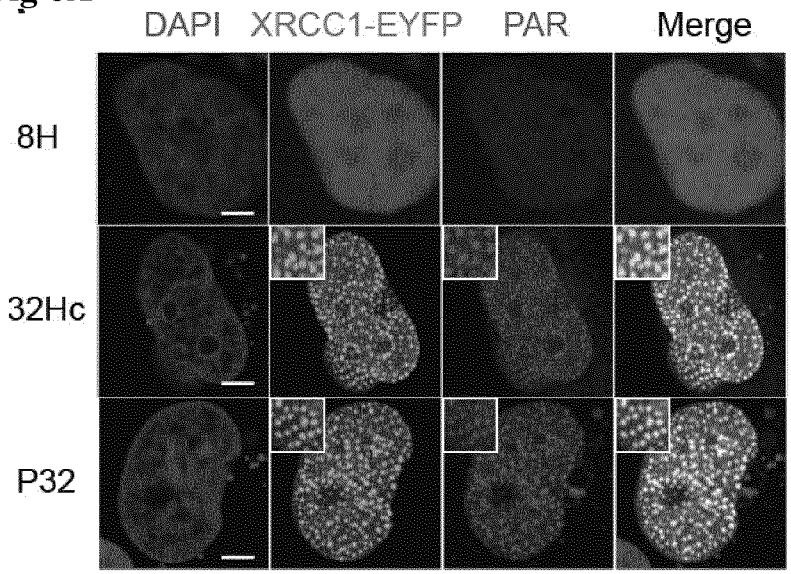


Fig 6C

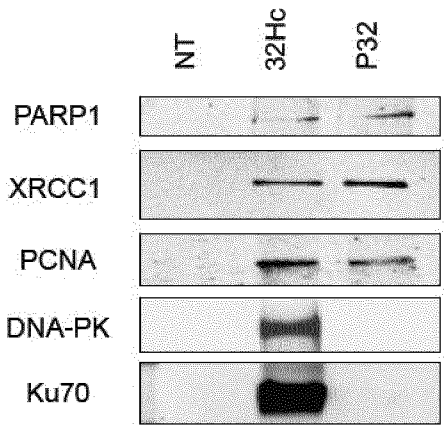


Fig 6B

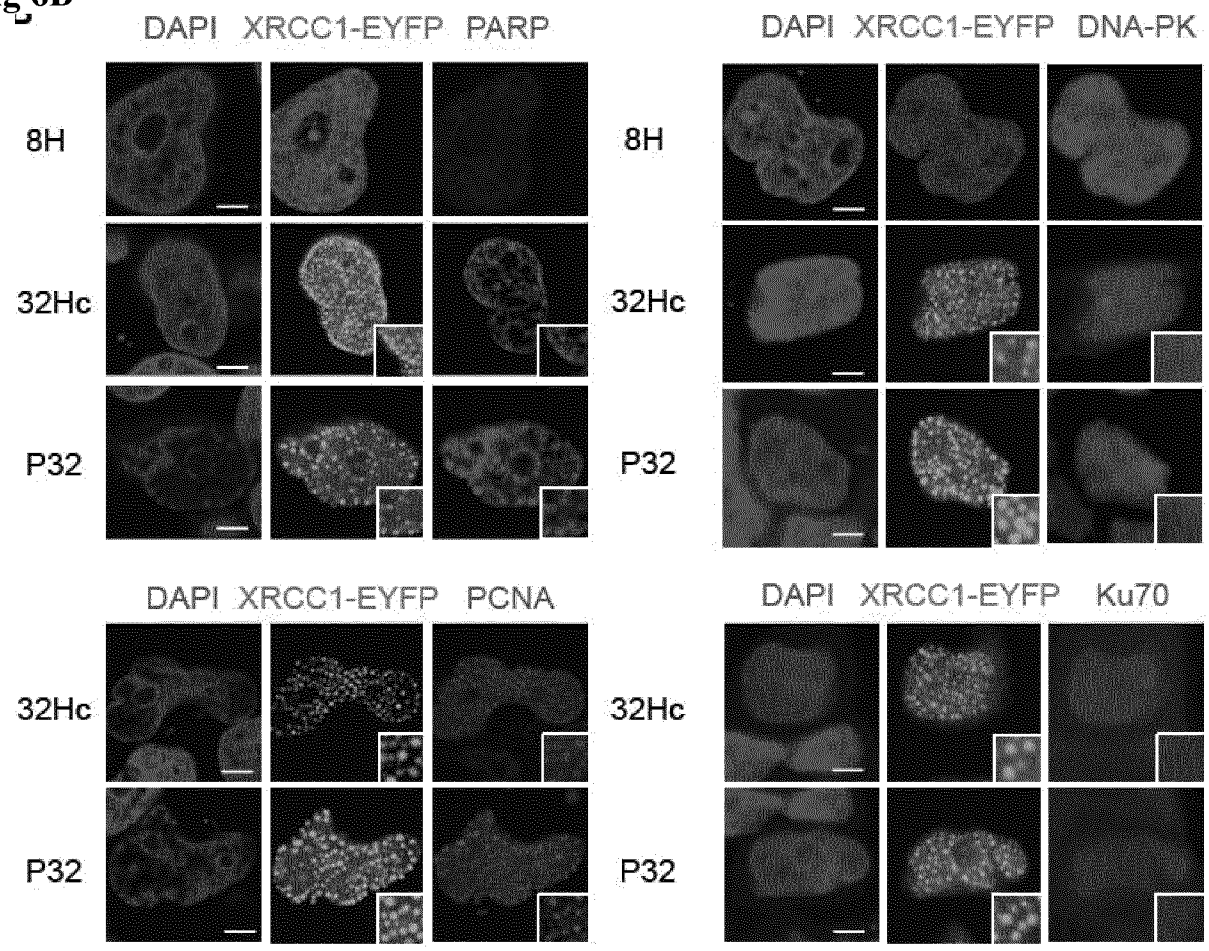


Fig 7A

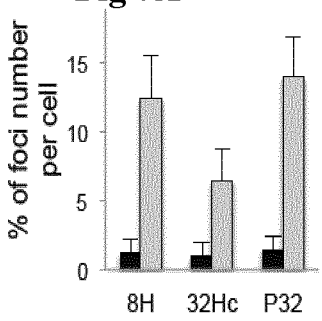


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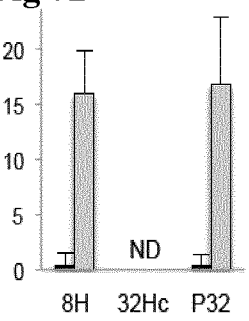


Fig 7C

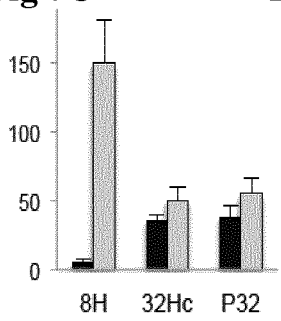


Fig 7D

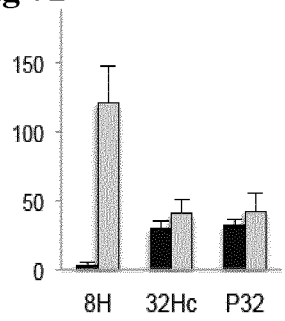


Fig 7E

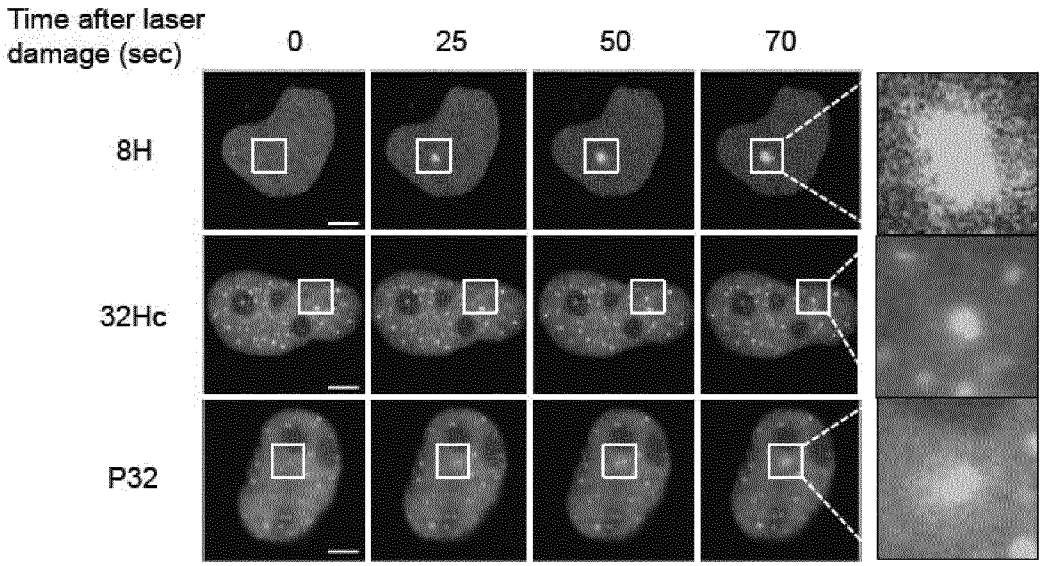


Fig 7F

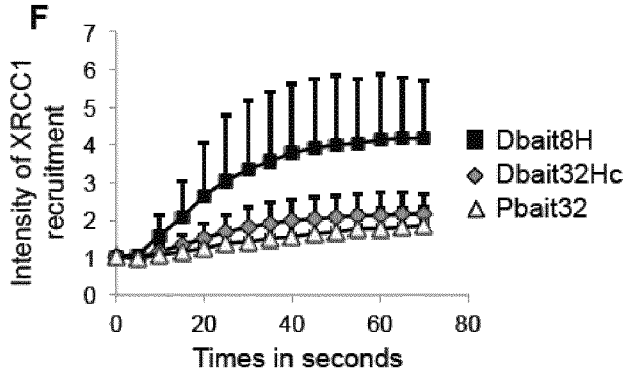


Fig 7G

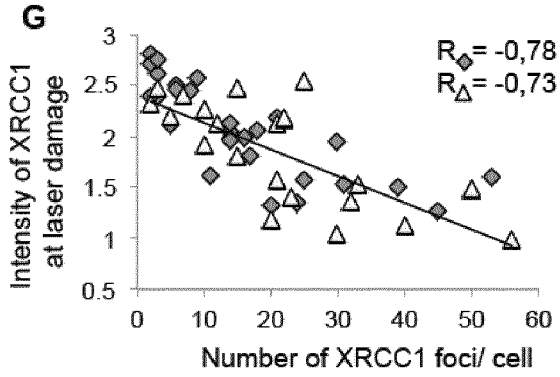


Fig 8A

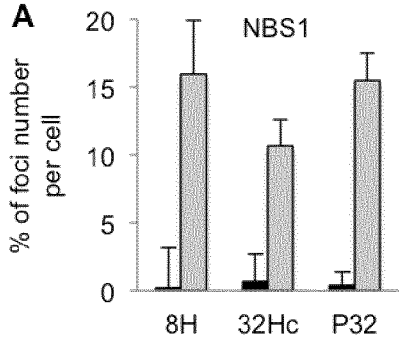


Fig 8B

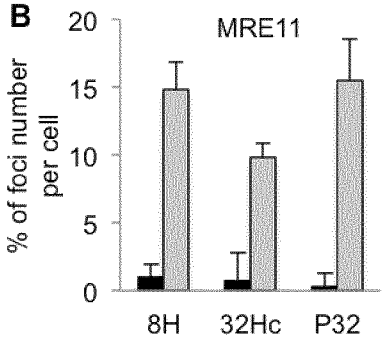


Fig 8C

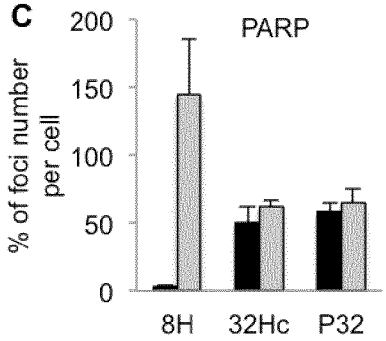


Fig 9A

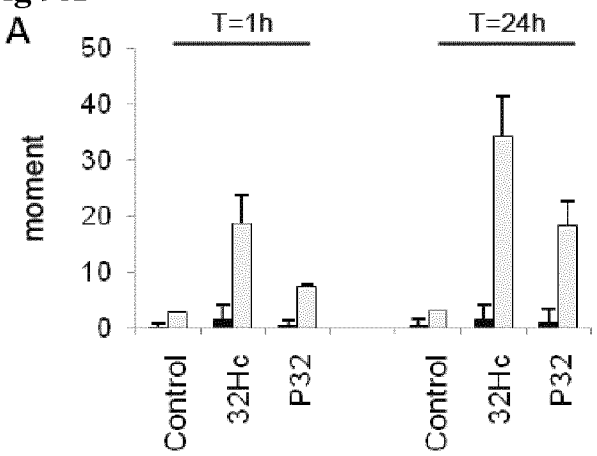


Fig 9B

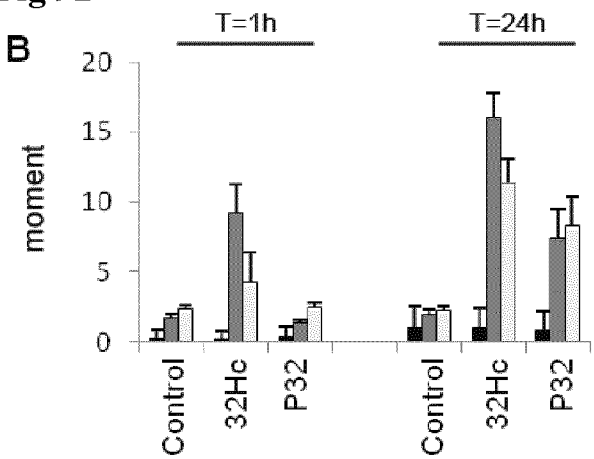


Fig 9C

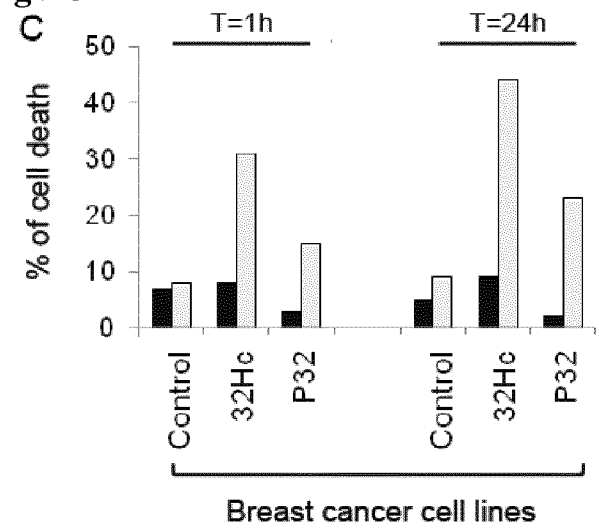


Fig 9D

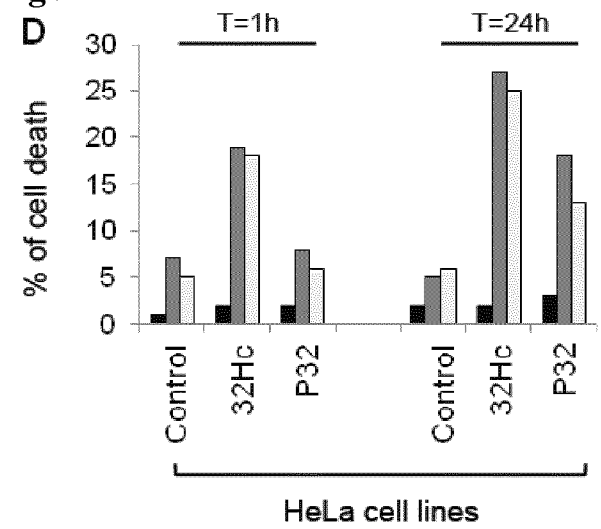


Fig 10A

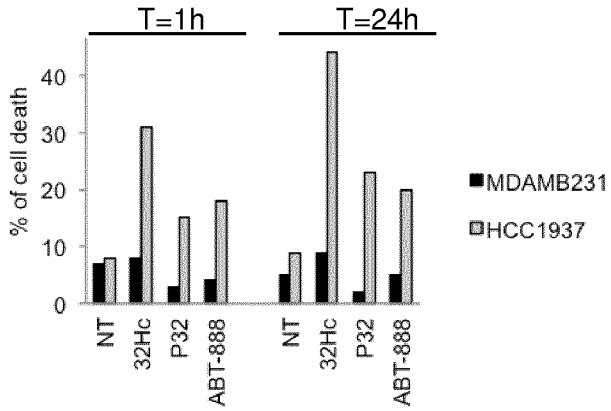
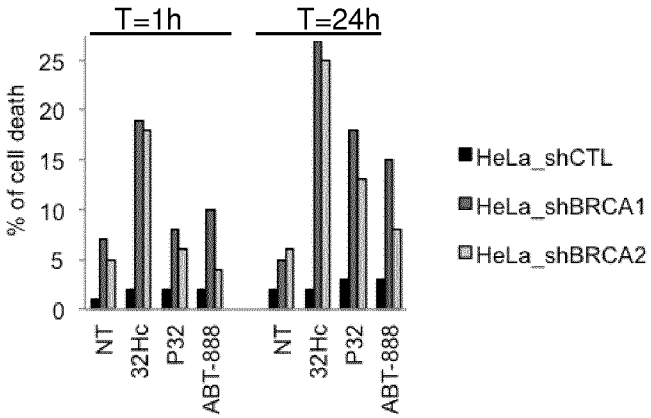


Fig 10B



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/057904

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/11 A61K31/712 A61P35/00
ADD.

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

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 practicable, search terms used)
EPO-Internal, BIOSIS, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Maria Quanz ET AL: "SiDNA and Other Tools for the Indirect Induction of DNA Damage Responses" In: "Selected Topics in DNA Repair", 26 October 2011 (2011-10-26), INTECH, XP055124790, ISBN: 978-9-53-307606-5 pages 333-368,	1-16
Y	paragraph [0004]; figures 1, 4	1-16
X	EP 1 669 450 A1 (ANGES MG INC [JP]) 14 June 2006 (2006-06-14) the whole document	1-9,11

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

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 June 2014	01/07/2014

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	Spindler, Mark-Peter

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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	R. PRASAD ET AL: "Pol associated complex and base excision repair factors in mouse fibroblasts", NUCLEIC ACIDS RESEARCH, vol. 40, no. 22, 5 October 2012 (2012-10-05), pages 11571-11582, XP055078497, ISSN: 0305-1048, DOI: 10.1093/nar/gks898 figure 3B	1-6
X	YAMAKAWA H ET AL: "Properties and anti-hiv activity of nicked dumbbell oligonucleotides", NUCLEOSIDES & NUCLEOTIDES, MARCEL DEKKER INC, US, vol. 15, no. 1-3, 1 January 1996 (1996-01-01), pages 519-529, XP002975792, ISSN: 0732-8311 the whole document	1-9
X	GAO H ET AL: "Double-stranded cyclic oligonucleotides with non-nucleotide bridges", BIOCONJUGATE CHEMISTRY, ACS, WASHINGTON, DC, US, vol. 5, no. 5, 1 September 1994 (1994-09-01), pages 445-453, XP002549926, ISSN: 1043-1802, DOI: 10.1021/BC00029A011 [retrieved on 2009-10-09]	1-6
Y	figure 1	6
X	HOSOYA T ET AL: "Sequence-specific inhibition of a transcription factor by circular dumbbell DNA oligonucleotides", FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 461, no. 3, 19 November 1999 (1999-11-19), pages 136-140, XP004260536, ISSN: 0014-5793, DOI: 10.1016/S0014-5793(99)01450-7 figure 1	1-6

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SEBASTIAN EUSTERMANN ET AL: "The DNA-Binding Domain of Human PARP-1 Interacts with DNA Single-Strand Breaks as a Monomer through Its Second Zinc Finger", JOURNAL OF MOLECULAR BIOLOGY, ACADEMIC PRESS, UNITED KINGDOM, vol. 407, no. 1, 14 January 2011 (2011-01-14), pages 149-170, XP028184061, ISSN: 0022-2836, DOI: 10.1016/J.JMB.2011.01.034 [retrieved on 2011-01-22] figure 1	1-6

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A	WO 2010/091140 A1 (BIPAR SCIENCES INC [US]; SHERMAN BARRY M [US]; BRADLEY CHARLES [US]; O) 12 August 2010 (2010-08-12) the whole document	1-16

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/057904

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