

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
30 October 2008 (30.10.2008)

PCT

(10) International Publication Number  
**WO 2008/129239 A2**

(51) International Patent Classification:

A61K 31/00 (2006.01) C12N 15/11 (2006.01)

(21) International Application Number:

PCT/GB2008/001303

(22) International Filing Date: 15 April 2008 (15.04.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

0707556.7 19 April 2007 (19.04.2007) GB

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

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

Published:

- without international search report and to be republished upon receipt of that report
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

(54) Title: TREATMENT FOR CANCER

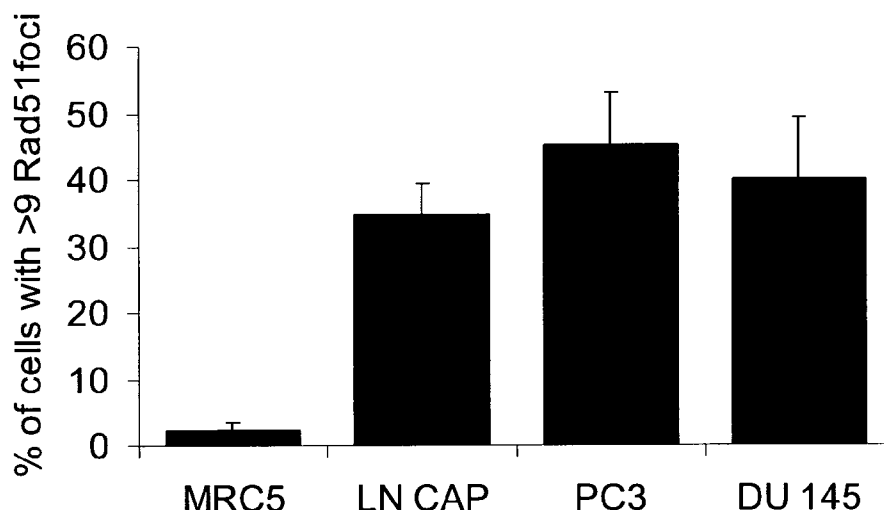


Figure 1A

(57) Abstract: The use of an agent that inhibits homologous recombination in the treatment of cancer or diseases with a hyper homologous recombination phenotype and a method of treatment of cells in a mammal, including a human, with a hyper homologous recombination phenotype comprising administering to the mammal a therapeutically effective amount of an agent that inhibits homologous recombination.

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## TREATMENT FOR CANCER

The present invention relates to the use of an agent that inhibits homologous recombination in the treatment of cancer or diseases with a  
5 hyper homologous recombination phenotype.

DNA replication is critical for the survival of both normal and cancer cells, however cancer cells often demonstrate some impairment of the repair mechanisms which naturally operate to ensure faithful DNA  
10 replication. In cancer cells this impairment results in sufficient errors to cause genetic instability in the cells, but not sufficient to cause cell death. It is this genetic instability that may lead to the development of cancer.

Mammalian cells have several pathways which are involved in the repair  
15 of replication lesions which arise when DNA is replicated (Eppink et al (2006) Exp Cell Res 312, 2660-2665). These different pathways collaborate to repair replication lesions, and a defect in one of the repair pathways may be compensated by other pathways (Bryant et al (2005) Nature 434, 913-917; Eppink et al (2006) Exp Cell Res 312, 2660-2665;  
20 Johansson et al (2006) DNA Repair 5, 1449-1458). However, damage to a repair pathway may result in non-accurate repair at replication forks and thus genetic instability, that in turn may drive the development of cancer (Lengauer et al (1998) Nature 396, 643-649).

25 A deficiency in homologous recombination has been linked to breast cancer. For example, in families with a strong predisposition for breast cancer, which is often due to an inherited mutation in one allele of either BRCA1 or BRCA2, breast cancer has been shown to develop when cells have a subsequent mutation in the functional BRCA1 or BRCA2 allele and  
30 have a reduction in the ability to use homologous recombination to repair damaged DNA (Venkitaraman A. R. (2002) Cell 108, 171-182). This

loss in ability to repair the DNA results in genetic instability, which is a likely cause of the cells becoming cancerous.

Many known anti-cancer drugs are designed to induce DNA damage, which in turn impairs DNA replication and kills all replicating cell. The problem of such treatments is that they are often tumour non-specific and have unpleasant and toxic side effects.

The present invention, relates to a novel treatment for cells which have elevated levels of homologous recombination. This is the opposite to BRCA1 or BRCA2 defective cancers where a reduction or loss of homologous recombination is seen. In particular, this treatment can be used to treat prostate cancer in which cells surprisingly display elevated levels of homologous recombination. Prostate cancer is the most common cancer disease among men in the Western world today.

According to a first aspect of the invention there is provided the use of an agent that inhibits homologous recombination in the manufacture of a medicament for the treatment of a disease characterised by a hyper homologous recombination phenotype.

According to another aspect of the invention there is provided an agent that inhibits homologous recombination for use in the treatment of a disease characterised by a hyper homologous recombination phenotype.

According to a further aspect, the invention provides a method of treatment of cells in a mammal, including a human, with a hyper homologous recombination phenotype comprising administering to the mammal a therapeutically effective amount of an agent that inhibits homologous recombination.

A disease with a hyper homologous recombination phenotype is a disease in which affected cells display elevated levels of homologous recombination compared to normal cells. Examples of diseases characterised by a hyper homologous recombination phenotype include  
5 some cancers, for example, prostate cancer and Bloom's syndrome.

Preferably cells with a hyper homologous recombination phenotype are defined as diseased cells which display at least about 40%, more preferably at least about 50%, more homologous recombination than a  
10 cell of the same type which is not diseased. Preferably cells with a hyper homologous recombination phenotype display at least about 60%, 70%, 80%, 90% or more homologous recombination than a cell of the same type which is not diseased.

15 Elevated levels of homologous recombination may occur due to defects in other DNA repair pathways, for example, cells with elevated levels of homologous recombination may have defects in the expression of proteins involved in other DNA repair pathways.

20 According to a further aspect the invention provides the use of an agent that inhibits homologous recombination in the manufacture of a medicament for the treatment of cancer.

According to another aspect of the invention there is provided an agent  
25 that inhibits homologous recombination for use in the treatment of cancer.

According to a further aspect, the invention provides a method of treatment of cancer in a mammal, including a human, comprising administering to the mammal a therapeutically effective amount of an  
30 agent that inhibits homologous recombination.

The invention provides a cancer treatment which takes advantage of cancer-specific defects in DNA replication repair. The benefit provided is that the treatments are toxic only to cancer cells and do not damage DNA or other cells.

5

Preferably the cancer to be treated by the agent of the invention is a cancer with a hyper homologous recombination phenotype, such as prostate cancer.

- 10 Preferably the cancer cells or diseased cells to be treated have a deficiency in another DNA repair pathway. For example, the cells may have a defect in the single break repair pathway. Also, a defect in response and repair pathways of oxidative damage would lead to a requirement for homologous recombination repair. Thus, this therapy
- 15 may be used to kill cells deficient in repairing oxidative damage. The cancer or diseased cells may have mutations in one or more genes involved in a DNA repair pathway other than homologous recombination repair pathway. The cancer or diseased cells may be totally deficient in one or more other, non-homologous recombination, repair pathways.

20

As the agent is preferably targeted to affect homologous recombination only, normal cells with other repair pathways operating will be able to repair DNA damage, and will not be killed by the agent of the invention.

- 25 The agent may inhibit homologous recombination by reducing the level of or completely eliminating homologous recombination. The agent may be targeted to all cells or just to a particular cell or cell type.

- Preferably the agent that inhibits homologous recombination does so by
- 30 reducing the expression and/or activity of a target molecule associated with homologous recombination. Preferably, the homologous

recombination inhibited by the agent is homologous recombination intended to correct errors introduced in DNA replication.

Preferably by inhibiting homologous recombination in cells with a hyper  
5 homologous recombination phenotype, or in cells with a defect in other DNA repair pathways, apoptosis, senescence or cell death can be induced in these cells.

The target molecule may be a protein, nucleic acid, or another  
10 metabolite. The target molecule may mediate homologous recombination in the cell. The target molecule may be essential to the mechanism of homologous recombination in a cell.

The agent may reduce the expression and/or activity of two or more  
15 target molecules.

The target molecule may be a one or more genes, and/or one or more proteins encoded by one or more genes, and/or one or more transcriptional or translational products of one or more genes, selected  
20 from the group comprising XRCC1, CTPS, RPA, RPA1, RPA2, RPA3, XPD, ERCC1, XPF, MMS19, RAD51, RAD51B, RAD51C, RAD51D, DMC1, XRCC2, XRCC3, BRCA1, BRCA2, RAD52, RAD54, RAD50, MRE11, NBS1, WRN, BLM, Ku70, Ku80, ATM, ATR, chk1, chk2, FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF,  
25 FANCG, FANCM, Hef, RECQ4, RECQ5B, RAD6, RAD18, PCNA, CLK-2, CHL1, RAD1, RAD9, FEN-1, Mus81, Ubc13. Eme1, DDS1, and BARD, or combinations thereof.

The target molecule may be a metabolite that is capable of interaction  
30 with and/or is essential to the normal function of a protein encoded by a gene selected from the group comprising XRCC1, CTPS, RPA, RPA1,

RPA2, RPA3, XPD, ERCC1, XPF, MMS19, RAD51, RAD51B, RAD51C, RAD51D, DMC1, XRCC2, XRCC3, BRCA1, BRCA2, RAD52, RAD54, RAD50, MRE11, NBS1, WRN, BLM, Ku70, Ku80, ATM, ATR, chk1, chk2, FANCA, FANCB, FANCC, FANCD1,  
5 FANCD2, FANCE, FANCF, FANCG, FANCM, Hef, RECQ4, RECQ5B, RAD6, RAD18, PCNA, CLK-2, CHL1, RAD1, RAD9, FEN-1, Mus81, Ubc13. Eme1, DDS1, and BARD, or combinations thereof.

The agent may be a protein, a nucleic acid, a small molecule, or any  
10 other suitable chemical.

Preferably the agent inhibits the activity of an enzyme that mediates homologous recombination, thus inhibiting the repair of replication lesions.

15

Two or more agents may be used separately or in combination.

Preferably the agent can be administered without the need for radiotherapy or chemotherapy, or other DNA damaging treatment.  
20 Preferably this will allow only cells with the specific phenotype to be specifically targeted, and preferably killed.

The agent may be an interfering or inhibitory RNA (RNAi) molecule. The RNAi molecule may be specific for one or more target molecules.  
25 The RNAi molecule may be specific for one or more genes or the transcriptional product of one or more genes involved in homologous recombination.

RNA interference is a technique used to specifically ablate gene function  
30 through the introduction of double stranded RNA, also referred to as RNAi, into a cell which results in the destruction of mRNA

complementary to one of the sequences of the RNAi molecule which forms the double stranded RNA. The RNAi molecule may be comprised of two separate strands, or it may be a single strand which forms a loop structure when the double stranded molecule is formed.

5

The RNAi molecule may comprise ribonucleic acid residues, or a mixture of deoxyribonucleic acid residues and ribonucleic acid residues. The RNAi molecule may comprise modified nucleotide bases.

10 The RNAi agent may be selected from the group comprising short interfering nucleic acid (siNA), microRNA (miRNA), small/short temporal RNA (stRNA), short hairpin RNA (shRNA) and double stranded RNA (dsRNA) or combinations thereof.

15 The RNAi molecule may on introduction to a cell cause the destruction or inactivation of mRNA complementary to the sequence of the RNAi molecule. Preferably the RNAi molecule is complementary to an exonic or coding sequence of a gene the expression of which is to be inhibited.

20 Preferably the RNAi molecule comprises a sequence complementary to the nucleic acid sequence of BRCA1 or BRCA2, [Ensembl Gene ID; BRCA1 ENSG00000012048, BRCA2 ENS000000139618] or is sufficiently complementary to the DNA or mRNA of BRCA1 or BRCA2 to inhibit their expression.

25

Preferably the RNAi molecule has a length between about 10 and about 1000 nucleotides, more preferably between about 10 and about 500, or between about 10 and about 100 nucleotides. Preferably the RNAi molecule has a length of between about 10 and about 50 nucleotides, more  
30 preferably between about 10 and about 30 nucleotides.



Preferably the RNAi molecule comprises the sequence AAC AAC AAU UAC GAA CCA AAC UU (SEQ ID NO. 1), or a sequence having one or more insertions, deletions or substitutions with at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% identity to AAC AAC AAU UAC GAA CCA AAC UU (SEQ ID NO. 1).

Percentage sequence identity is defined as the percentage of nucleotide residues in a sequence that are identical with the nucleotides in the provided sequence after aligning the sequences and introducing gaps if necessary to achieve the maximum percent sequence identity. Alignment for purpose of determining percent sequence identity can be achieved in many ways that are well known to the man skilled in the art, and include, for example, using BLAST and ALIGN algorithms.

According to a further aspect, the present invention provides a pharmaceutical composition comprising an agent for inhibiting homologous recombination and one or more pharmaceutically acceptable carriers, diluents and/or excipients.

The composition may be administered in any effective manner, for example, the composition may be administered orally, intravenously, intramuscularly, intradermally, intranasally, topically or via any other suitable route.

The composition may be for therapeutic or prophylactic use or both.

The composition may be for administration to a human or other mammal.

The composition may be administered directly to diseased cells, or may be targeted to diseased cells via systemic administration.

For administration to mammals, and particularly humans, it is expected that the daily dosage level of an active agent will be up to 100mg/kg, for example 0.01 to 50mg/kg body weight. A physician will be readily able to determine the appropriate amount of the agent for administration.

5

According to a further aspect the invention provides a method of treating prostate cancer comprising administering a therapeutically effective amount of a BRCA1 and/or a BRCA2 inhibitor to a subject with prostate cancer. The subject may be a human or other mammal.

10

According to another aspect the invention provides the use of a BRCA1 and/or a BRCA2 inhibitor in the manufacture of a medicament for the treatment of prostate cancer.

15 It will be appreciated that all the optional and/or preferred features of the invention can be applied to all aspects of the invention.

Preferred embodiments/aspects of the invention will now be described by way of example only with reference to the accompanying figures, in which:

20

**Figure 1a** – illustrates the percentage of cells exhibiting high levels (> 9) of RAD51 foci in prostate cancer cells in a histogram, this observation is consistent with a hyper homologous recombination phenotype in prostate cancer cells;

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**Figure 1b** – is a representative image illustrating that all the prostate cancer cell lines tested have an abnormally high level of RAD51 foci, and that this is maintained even in the absence of treatment with hydroxyurea;

30

**Figure 2a** – illustrates the levels of  $\gamma$ H2AX foci in the prostate cancer cell lines, these foci represent the sites of double-stranded breaks;

5 **Figure 2b** – is a histogram comparing the levels of  $\gamma$ H2AX foci in prostate cancer cells treated or not treated with aphidicolin (replication inhibitor) to the  $\gamma$ H2AX foci in osteosarcoma U2OS cells (which do not show hyper recombination phenotype, control), inhibiting replication further increased the foci in U2OS cells but not in the prostate cancer cells suggesting that the spontaneous double-  
10 stranded breaks in the prostate cancer cells are replication-associated.

**Figure 3a** – illustrates the high levels of single-stranded breaks observed in the prostate cancer cell lines LN CAP, DU 145 and PC3 compared with the osteosarcoma U2OS cells.

15 **Figure 3b** - is an immunoblot illustrating the proteins present in prostate cancer cell lines following depletion of BRCA2. BRCA2 has been removed using a shRNA vector;

**Figure 3c** - illustrates that prostate cancer cells show a significant decrease in their ability to form colonies following a 3 or 7 day  
20 depletion of BRCA2;

**Figure 3d** – illustrates that T47D breast cancer cells, which do not show elevated levels of homologous recombination, show only a slight decrease in their ability to form colonies following a 3 or 7  
25 day depletion of BRCA2;

**Figure 4a** – illustrates that prostate cancer cells show a decrease in survival after treatment with increasing doses of 17-AAG for 7 days. 17-AAG inhibits recombination by degrading BRCA2;

30 **Figure 4b** – illustrates that prostate cancer cells show a significant decrease in survival after treatment with 17-AAG compared to the osteosarcoma U2OS cells (control);

**Figure 5a** – is a histogram illustrating the levels of RAD51 foci in DU 145 and PC3 cells after treatment with inhibitors of different proteins involved in signalling and repair pathways that are important for the repair of replication lesions. Treatment with Parp inhibitor caused the most reduction in the levels of RAD51 foci; and **Figure 5b** – illustrates the decrease in survival in the prostate cancer cells compared to the normal fibroblasts (MRC5) after treatment with increasing doses of Parp inhibitor. The decrease in survival correlates with the decrease in RAD51 foci formation (and therefore levels of homologous recombination) after treatment with the Parp inhibitor.

## Methods

### Toxicity assay - colony outgrowth assay

6 well plates were plated with 200 cells/well, and grown in the presence of doxycyclin (dox; Sigma) for 3 and 7 days or in the absence of doxycyclin. Doxycyclin is a small steroid, which can be used, in the presence of a doxycyclin repressor expressed from a separate plasmid pUHD172-1 (Clontech, USA), to regulate transcription from a tetracycline sensitive promoter. In this way, BRCA2 shRNA expression is regulated such that it is expressed only in the presence of doxycyclin (see below for details). After 2 weeks, when colonies could be observed, these colonies were fixed and stained with methylene blue in methanol (4 g/l). Colonies consisting of more than 50 cells were subsequently counted.

### Immunofluorescence

Cell were either grown on coverslips or in 96 well plates and treated for 24h (or untreated) with various drugs. The medium was then removed, and the cover slips or wells were rinsed once in PBS (at room temperature) and fixed in 3% paraformaldehyde in PBS-T (PBS containing 0.1% Triton X-100) for 15 min. The coverslips or wells were rinsed 2x15 min in PBS-T and 1x10 min in PBS-T (0.3% Triton X-100) and blocked in PBS containing 3% bovine serum albumin prior to incubation with primary antibody overnight at 4°C. The primary antibodies used were: mouse monoclonal anti- $\gamma$ H2AX (Ser139, clone JBW30, peptide corresponding to amino acids 134-142 of human histone H2AX, Upstate) at a dilution of 1:1000 and rabbit polyclonal anti-RAD51 (H-92, epitope corresponding to amino acids 1-92 of RAD51 of human origin, Santa Cruz) at a dilution of 1:1000. Antibodies were diluted in PBS containing 3% bovine serum albumin. The coverslips or wells were rinsed 3x15 min in PBS-T and 1x10 min in PBS-T (0.3% T X100) followed by 1 hour incubation at room temperature with the appropriate secondary antibody and then rinsed 4x15 min in PBS-T. The secondary antibodies used were Alexa 488 goat anti-rabbit IgG antibody (Molecular Probes) at a concentration of 1:500, Alexa 488 goat anti-mouse IgG antibody (Molecular Probes) at a concentration of 1:500. Antibodies were diluted in PBS containing 3% bovine serum albumin. DNA was stained with 1 mg/ml To-Pro (Molecular Probes) or Dapi (Molecular Probes) at a concentration of 1:1000. Coverslips were mounted with SlowFade Antifade Kit (Molecular Probes) and images from cover slips were obtained with a Zeiss LSM 510 inverted confocal microscope. Images from 96 well plates were acquired using In Cell Analyzer 1000 (GE Healthcare) and analysed using the In Cell developer tool box software (GE Healthcare).

### 30 Immunoblotting

Cells were grown in the presence or absence of dox for 5 days, harvested, and lysed in buffer containing 100mM TRIS pH 7.4, 150mM NaCl, 1%NP40, protease inhibitors (cocktail) and phosphates inhibitors for 30min on ice, centrifuged for 15min 14000 RPM at +4°C and the supernatant was collected. Protein concentration was measured using Bradford assay and 150 µg of total protein was run on 4-12% PAAG (Invitrogen) and blotted onto nitrocellulose membrane for 2 hours at 100V and +4°C. Membrane was blocked in PBS-T (0.1% Tween20) containing 20% milk, 10% FBS, 3% BSA for 1 hour at room temperature prior to incubation with primary antibody overnight at 4°C. The primary antibody was an anti rabbit BRCA2 antibody (Santa Cr. H299) diluted 1:500 in blocking solution. The membrane was then rinsed 4x15 min with PBS-T followed by a 1 hour incubation at room temperature with the anti-rabbit HRP secondary antibody.

15

### Depletion of BRCA2

The aim was to produce stable and regulatable expression of BRCA2 shRNA. To achieve this BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit from Invitrogen was used according to the manufacturers protocol (Invitrogen, Sweden) to facilitate tetracycline-regulated expression of a short hairpin RNA of interest from an H1/TO RNAi cassette for use in RNA interference analysis in mammalian cells. The kit provides a Gateway® adapted entry vector designed to allow efficient transient or stable, regulated expression of shRNA in dividing mammalian cells or easy transfer of the H1/TO RNAi cassette into other suitable Gateway® destination vectors for other RNAi applications. The target sequence introduced to this system for BRCA2 was AAC AAC AAU UAC GAA CCA AAC UU (SEQ ID NO. 1) (Bruun et al., (2003) DNA Repair 2, 1007-1013.). The tet-regulator vector was pUHD172-1puro, made from the pUHD172-1 vector (Clontech, USA). Prostate cancer cells LN CAP,

30

PC3 and DU 145 (for details and source of cell lines see [www.atcc.org](http://www.atcc.org)) were transfected with both vectors at the same time, clones were picked up and analysed by immunoblotting.

## 5 Rezasurin Assay for Cellular Survival

4000 cells per well were seeded into 96 well plates in duplicates over night prior to treatment. The cells were then treated with various drugs at specified concentrations. After 7 days, the cells were washed twice with PBS and phenol red free media containing Rezasurin at a concentration of 10  $\mu\text{g/ml}$  was added to the wells. The cells were then incubated at 37°C for 1 hour. Fluroscence was measured at 544 nm excitation and 590 nm emission using a FLUOstar Omega plate reader (BMG LabTech). The relative fluroscence unit in untreated cells was taken as 1 and survival fractions were calculated. In the histogram, the relative fluroscence unit in untreated control cells was taken as 100% survival and the data are shown as percent of control.

## Alkaline DNA unwinding –Single strand break repair assay

The level of strand breaks was measured in cells previously inoculated on 24-well plates,  $1 \times 10^5$  cells per well, and labeled overnight with  $^3\text{H}$ -TdR. After labeling, the unwinding reaction was started by rinsing two times with 0.15M NaCl and adding 0.5 ml ice-cold unwinding solution containing 0.15 M NaCl and 0.03 M NaOH. After 30 min on ice in darkness, the unwinding was terminated by a forceful injection of 1 ml 0.02 M  $\text{NaH}_2\text{PO}_4$ . The molecular weight of the DNA was further reduced to about 3 kb by ultrasonic treatment for 15 sec (Branson sonifier B-12, equipped with a micro tip). Sodium dodecyl sulphate (SDS) was added to 0.25%. The 24-well plates were stored over night in a freezer (-20 °C). Separation of single and double-stranded DNA was performed on hydroxyapatite packed columns mounted in a thermostatic regulated (60

<sup>90</sup>C) aluminium block. Samples from the 24-well plates were thawed at room temperature and diluted to double volume with distilled water. Prior to use, all columns were washed with 2.5 ml of 0.5 M potassium phosphate and 0.01 M sodium phosphate to eluate, at a flow rate of 0.3 ml/min, any remaining DNA from previous samples followed by. 1.5 ml of cell lysate was loaded on each column followed by another wash with 4.5 ml 0.01 M sodium phosphate. Single and double-stranded DNA was eluted with 4.5 ml of 0.13 M and 0.25 M potassium phosphate, respectively, and collected in 2 separate vials for counting in a liquid scintillation spectrophotometer. All buffers used had pH 6.8-6.9. The ratio of ssDNA and double stranded DNA (dsDNA) were used to calculate the number of single strand breaks (SSB) per cell using a constant from a standard curve obtained after  $\gamma$ -irradiation [Erixon & Ahnström, 1979; Ahnström & Erixon, 1981]. Thus  $SSBs/cell = k_s \cdot (-\log) (Fds)$ , where  $Fds = \text{fraction of DS} = DS/(SS + DS)$ .

#### **Example 1 - Prostate cancer cell lines exhibit a hyper homologous recombination phenotype**

To investigate the role of homologous recombination in the repair of DNA in prostate cancer, the number of RAD51 foci in prostate cancer cell lines was determined. RAD51 foci represent active sites of homologous recombination repair and are normally formed at stalled or collapsed replication forks (Haaf et al (1995) Proc Natl Acad Sci U S A 92, 2298-2302; Lundin et al (2003) J Mol Biol 328, 521-535; Tashiro et al., (2000) J Cell Biol 150, 283-291). Normally the number of cells that carry >9 RAD51 foci is a very small percent of the total cell population in human cancer cell lines or in immortalised mammalian cells (Schultz et



al., (2003) Nucleic Acids Res, 31, 4959-4964; Sorensen et al., (2005) Nat Cell Biol, 7, 195-201). Figure 1a demonstrates that the spontaneous levels of RAD51 foci were highly elevated in the three prostate cancer cell lines tested, i.e., LN CAP, PC3 and DU 145 compared to the normal human fibroblasts MRC5. The prostate cancer cells were then treated with 2 mM of hydroxyurea, an agent that stalls replication forks (Bianchi et al (1986) J Biol Chem 261 16037-42), in order to study the induced levels of foci. Treatment with hydroxyurea normally results in an increase in the RAD51 foci (Sorensen et al., (2005) Nat Cell Biol, 7, 195-201; Tanaka et al (2007) Cytometry Part A, 71A, 648-661). However, as seen in Figure 1b, prostate cancer cells exhibit abnormal levels of the RAD51 foci even in the absence of Hydroxyurea, which is only slightly effected after treatment with hydroxyurea. This is a strong indication that homologous recombination is actively involved in DNA repair in prostate cancer cell lines, and suggests a hyper recombination phenotype in prostate cancer.

**Example 2 - The lesions that are repaired by the elevated homologous recombination in Prostate cancer are replication-associated double-stranded breaks.**

Homologous recombination is important in the repair of double-stranded breaks (DSBs) in the S-phase of the cell cycle and is a vital repair process of stalled or collapsed replication forks (Lundin et al (2002) Mol Cell Biol 22, 5869-5878). As shown in Figure 1, prostate cancer cells have highly elevated levels of recombination. In order to understand the lesions that are repaired by such high levels of recombination,  $\gamma$ H2AX foci, (phosphorylated histone H2AX, a marker for DSBs) were analysed in these cells. Intermediate DSBs formed during repair of DNA damage as well as DSBs that are formed during DNA replication trigger H2AX

phosphorylation (Chadwick et al (2005) *Chromosome*, 114, 432-439; Marti et al (2006) *Proc Natl Acad Sci USA*, 103, 9891-9896).

All the three prostate cancer cell lines tested (LN CAP, DU 145 and PC3) exhibited increased levels of  $\gamma$ H2AX foci compared to the normal fibroblasts MRC5 (Figure 2a). In order to understand the origin of these DSBs, the prostate cancer cell lines and U2OS (Osteosarcoma cell line that does not exhibit hyper recombination phenotype, control) were treated with replication inhibitor aphidicolin (10  $\mu$ M) for 24 hours and the  $\gamma$ H2AX foci were analysed (Figure 2b). Aphidicolin inhibits  $\alpha$ - like DNA polymerases and was reported to cause DNA damage-related H2AX phosphorylation induced by replication stress (Tanaka et al (2007) *Cytometry Part A*, 71A, 648-661). Hence, inhibiting replication using aphidicolin increases the  $\gamma$ H2AX foci, due to an increase in the DSBs caused by replication lesions. This is what was observed in the control (U2OS) cells (Figure 2b), in which the  $\gamma$ H2AX foci increased 2-fold up on treatment with aphidicolin. However, there was no further increase in the already high levels of  $\gamma$ H2AX foci in the prostate cancer cells, LN CAP and DU 145. In addition, there was a decrease in the  $\gamma$ H2AX foci in the PC3 cells after treatment with aphidicolin. This suggests that the DSBs observed in untreated prostate cancer cells are replication-associated and therefore treatment with a replication inhibitor caused either a decrease or no change in the DSBs. This further suggests the importance of homologous recombination in prostate cancer cells.

25

### **Example 3 Prostate cancer cells rely on homologous recombination for cellular survival**

Homologous recombination represents a final repair pathway for difficult replication lesions and it is used for lesions that are not possible to repair

30

by other repair pathways (Eppink et al (2006) Exp Cell Res 312, 2660-2665). In addition to homologous recombination, there are several other pathways to prevent replication collapse, such as DNA single-strand break repair pathways (Bryant et al (2005) Nature 434, 913-917; Saleh-Gohari et al (2005) Mol Cell Biol 25, 7158-7169).

As shown in Examples 1 and 2, prostate cancer cells have highly elevated levels of homologous recombination due to replication-associated damage, thus it is possible that prostate cancer cells have a defect in other repair pathways that help to preserve DNA replication. We have previously shown a defect in DNA single-strand break repair result in a hyper recombination phenotype (Bryant et al (2005) Nature 434, 913-917; Saleh-Gohari et al (2005) Mol Cell Biol 25, 7158-7169). We investigated the levels of DNA single-strand breaks in prostate cancer and find an increased level of DNA single-strand break in prostate cancer cells, supporting that these cells have a defect in DNA single-strand break repair or alternatively an increased level of oxidative lesions (Figure 3a). With an increased level of DNA single-strand breaks, it is postulated that they likely rely on a functional homologous recombination pathway for survival (Bryant et al (2005) Nature 434, 913-917; Saleh-Gohari et al (2005) Mol Cell Biol 25, 7158-7169). To test this a major component of the homologous recombination pathway, the BRCA2 protein (Davies et al (2001) Mol Cell 7, 273-282; Moynahan et al (2001) Mol Cell 7, 263-272), was deleted using an inducible shRNA depletion method. Three different prostate cancer cell lines were stably transfected with the constructs described above under the heading "Depletion of BRCA2". An almost 100% efficiency in BRCA2 depletion was observed following 5 days expression of a BRCA2 RNA interfering shRNA (Figure 3b).

Surprisingly, it was found that all three prostate cancer cell lines tested failed to survive depletion of BRCA2, and thus inactivation of

homologous recombination (Figure 3c). This result shows that homologous recombination is required for survival of prostate cancer cells. Depletion of homologous recombination is therefore an efficient way of killing prostate cancer cells, and indeed other cells which display  
5 a hyper homologous recombination phenotype, or which rely only on homologous recombination to correct lesions in the DNA which occur during DNA replication.

By way of comparison, BRCA2 was depleted from other tumour cells that do not show elevated levels of homologous recombination. More  
10 specifically T47D breast cancer cells (available from ATCC, USA) were depleted of BRCA2 in the same way as prostate cancer cells were. In the T47D cells an initial slight decrease in clonogenic ability was observed (Figure 3d), however, this was not as profound as in the prostate cancer cells. Furthermore, a seven day depletion of BRCA2 did not cause any  
15 further reduction in cell survival, demonstrating that the T47D cells survive long term depletion of BRCA2 (Figure 3d). These results show that loss of BRCA2 is achievable and is not generally toxic to all cells.

**Example 4 – 17-allyl-aminogeldanamycin, a chemical inhibitor of Heat shock  
20 protein 90 that degrades BRCA2 is toxic to prostate cancer cells.**

As shown in Example 3, depletion of BRCA2 results in cytotoxicity in prostate cancer cells. In order to test if chemical inhibitors that degrade BRCA2 result in a similar cytotoxicity, survival of the prostate cancer  
25 cells was measured after treatment with 17-allyl-aminogeldanamycin (17-AAG). 17-AAG is an inhibitor of Heat shock protein 90 (HSP90), which is over expressed in numerous tumour cells and forms multi-chaperone complexes with client proteins (Whitesell et al (2005) Nature Rev Cancer, 5, 761-772). In tumour cells, 17-AAG specifically inhibits the chaperone

functions of HSP90 and induces degradation of its client proteins (Kamal et al (2003) Nature, 425, 407-410). 17-AAG has been shown to significantly disturb homologous recombination as a consequence of degradation of the BRCA2 protein (Noguchi et al (2006) Biochem Biophys Res Commun, 351, 658-663). Prostate cancer cells DU 145 and PC3 and the osteosarcoma cell line U2OS (control) were treated with either increasing doses of 17-AAG for 7 days (Figure 4a) or pre-treated with 100 nM of 17-AAG for 16 hours followed by continuous treatment with 50 nM of 17-AAG for 7 days (Figure 4b) and survival was measured using the Rezasurin assay.

As shown in Figure 4a, the prostate cancer cells are more sensitive to treatment with 17-AAG at higher doses compared to U2OS cells. This became even more apparent when the cells were pre-treated with 100 nM of 17-AAG followed by continuous treatment with 50 nM of 17-AAG for 7 days. Only about 5 % of the cells survived when compared to the untreated cells. These results further suggest that inhibiting homologous recombination is an effective way of killing the prostate cancer cells that exhibit hyper homologous recombination phenotype.

20

**Example 5: Inhibition of Parp-1 in prostate cancer cells results in decrease of homologous recombination and survival.**

In order to identify other inhibitors that effect homologous recombination in the Prostate cancer cells, they are treated with inhibitors that affect different proteins involved in signalling pathways or repair pathways that are important for replication lesions. Phosphatidylinositol 3-kinase (PI3K) kinases ATM, ATR, DNA-PKcs and a downstream target for ATR and ATM, Chk1 are known to be involved in DNA damage response in S phase (Abraham (2001) Genes Dev, 15, 2177-2196; Durocher and Jackson (2001) Curr Opin Cell Biol, 13, 225-231). They are also known to initiate HR in response to DSBs and

replication stress (Bolderson et al (2004) Hum Mol Genet, 13, 2937-2945; yajima et al (2006) Mol Cell Biol, 26, 7520-7528). AKT, which lies downstream of PI3K has a large number of downstream targets implicated in survival and cell-cycle regulation (Katso et al (2001) Annu Rev Cell Dev Biol, 17, 615-675). Poly(ADP-ribose) polymerase (PARP-1) is shown to regulate homologous recombination through its involvement in the repair of DNA single-strand breaks that persist in S phase (Helleday et al (2005) Cell Cycle, 9,1176-1178; Bryant et al, submitted). These proteins were specifically targeted using chemical inhibitors: ATM with 10  $\mu$ M of KU55933 (ATMi), PARP-1 with 100  $\mu$ M of 4 amino 1,8-naphthalamide (Parpi), DNA-PKcs with 10  $\mu$ M of KU51777 (DNA-PKi), Chk1 with 500 nM of CEP3891 (Chk1i). Non-specific inhibitors of PI3Ks, caffeine (10mM) and wortmannin (125 nM) were also used. 0.4  $\mu$ M of PI103 (AKTi) was used to target the AKT pathway.

Among all the inhibitors tested, Parp inhibitor caused the most reduction in RAD51 foci in both PC3 and DU 145 (Figure 5a). It has been shown previously that loss of Parp-1 results in an increase in spontaneous RAD51 foci (Schultz et al, (2003) Nucleic Acids Res, 17, 4959-64) but a reduction in RAD51 foci induced due to replication stress (Bryant et al, submitted). This is consistent with the data in example 2, which suggests that the hyper recombination phenotype of prostate cancer cells is due to replication-associated damage in these cells. It was then examined if the decrease in RAD51 foci formation (homologous recombination) results in cytotoxicity in the prostate cancer cell lines. To test this, prostate cancer cells were treated with increasing doses of Parp inhibitor for 7 days and survival was measured using Rezasurin assay. As seen in Figure 5c, all the prostate cancer cells exhibit a reduction in survival compared to MRC5 cells. Moreover, the reduction in the survival correlates with the reduction in RAD51 foci formation (homologous recombination) in these cells. DU 145 cell line that exhibited maximum reduction in RAD51 foci formation up on treatment with Parp inhibitor (Figure 5a) also exhibited

most sensitivity to Parp inhibitor compared to the other cell lines. This further suggests that prostate cancer cells require homologous recombination for their survival.

## CLAIMS

1. The use of an agent that inhibits homologous recombination in the preparation of a medicament for the treatment of a disease characterised  
5 by a hyper homologous recombination phenotype.

2. An agent that inhibits homologous recombination for use in the treatment of a disease characterised by a hyper homologous recombination  
10 phenotype.

3. A method of treatment of cells in a mammal, including a human, with a hyper homologous recombination phenotype comprising administering to the mammal a therapeutically effective amount of an agent that inhibits homologous recombination.  
15

4. The use according to claim 1, the agent according to claim 2 or the method according to claim 3 wherein the disease with a hyper homologous recombination phenotype is selected from the group comprising cancer and Bloom's syndrome.  
20

5. The use, agent or method of claim 4 wherein the cancer is prostate cancer.

6. The use, agent or method of any preceding claim wherein cells to  
25 be treated have a deficiency in a DNA repair pathway other than the homologous recombination pathway.

7. The use, agent or method of any preceding claim wherein the agent inhibits homologous recombination by reducing the expression and/or  
30 activity of a target molecule associated with homologous recombination.



8. The use, agent or method of claim 7 wherein the target molecule is selected from the group comprising:

- (i) one or more genes;
- (ii) one or more proteins encoded by one or more genes;
- 5 (iii) one or more transcriptional or translational products of one or more genes;
- (iv) a metabolite that is capable of interaction with and/or is essential to the normal function of a protein encoded by one or more genes;

10 wherein the one or more genes are selected from the group comprising XRCC1, CTPS, RPA, RPA1, RPA2, RPA3, XPD, ERCC1, XPF, MMS19, RAD51, RAD51B, RAD51C, RAD51D, DMC1, XRCC2, XRCC3, BRCA1, BRCA2, RAD52, RAD54, RAD50, MRE11, NBS1, WRN, BLM, Ku70, Ku80, ATM, ATR, chk1, chk2, FANCA, FANCB,  
15 FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCM, Hef, RECQ4, RECQ5B, RAD6, RAD18, PCNA, CLK-2, CHL1, RAD1, RAD9, FEN-1, Mus81, Ubc13, Eme1, DDS1, and BARD, or combinations thereof.

20 9. The use, agent or method of any preceding claim wherein the agent is an interfering or inhibitory RNA (RNAi) molecule.

10. The use, agent or method of claim 9 wherein the RNAi molecule is specific for one or more genes, or the transcriptional product of one or  
25 more genes, selected from the group comprising XRCC1, CTPS, RPA, RPA1, RPA2, RPA3, XPD, ERCC1, XPF, MMS19, RAD51, RAD51B, RAD51C, RAD51D, DMC1, XRCC2, XRCC3, BRCA1, BRCA2, RAD52, RAD54, RAD50, MRE11, NBS1, WRN, BLM, Ku70, Ku80, ATM, ATR, chk1, chk2, FANCA, FANCB, FANCC, FANCD1,  
30 FANCD2, FANCE, FANCF, FANCG, FANCM, Hef, RECQ4, RECQ5B,

RAD6, RAD18, PCNA, CLK-2, CHL1, RAD1, RAD9, FEN-1, Mus81, Ubc13, Eme1, DDS1, and BARD, or combinations thereof.

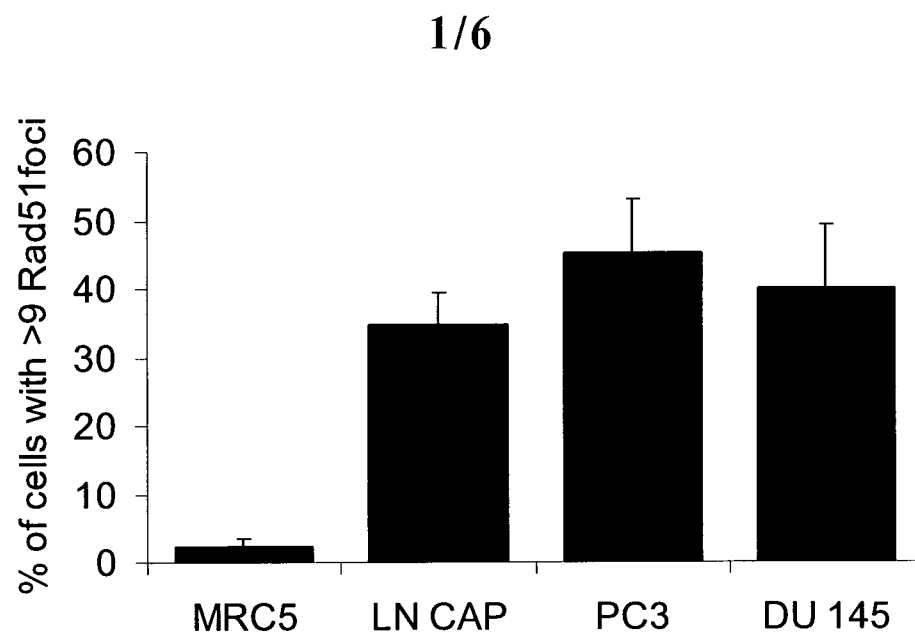
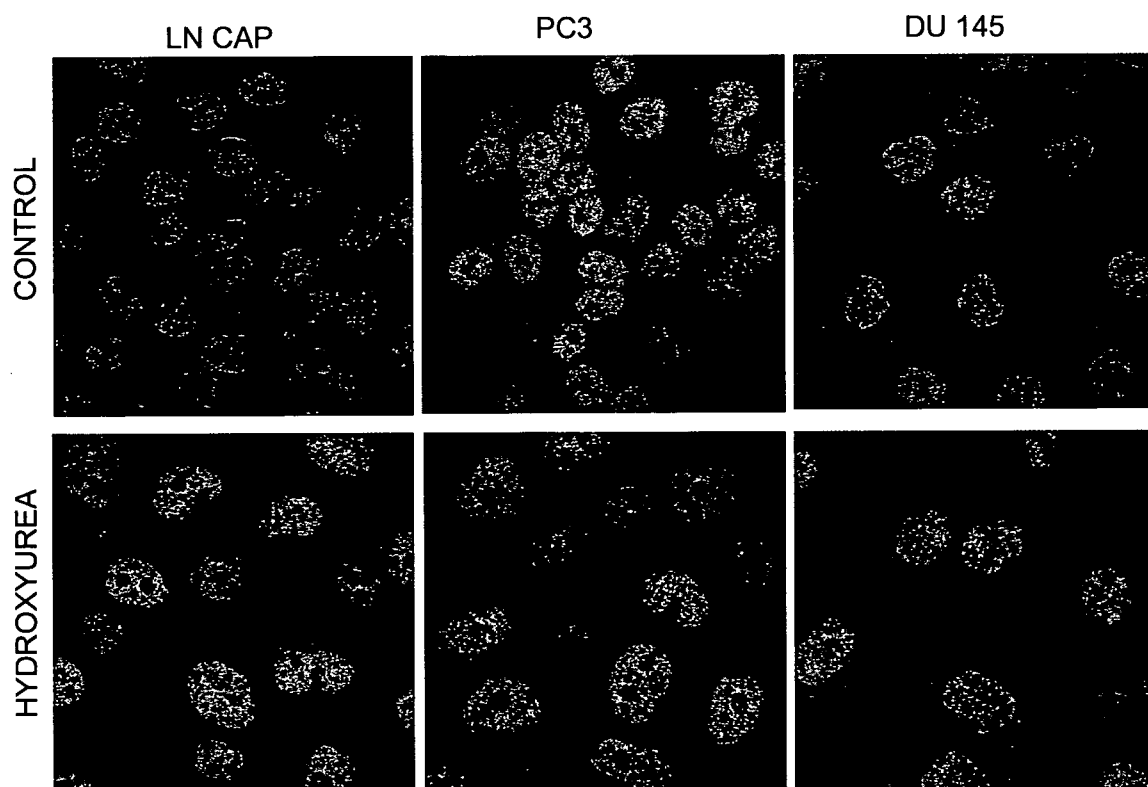
11. The use, agent or method of claim 10 wherein the RNAi molecule  
5 comprises a sequence sufficiently complementary to part of the nucleic acid sequence of BRCA1 or BRCA2 to inhibit their expression.

12. The use, agent or method of claim 10 wherein the RNAi molecule  
10 comprises the sequence AAC AAC AAU UAC GAA CCA AAC UU (SEQ ID NO. 1), or a sequence having one or more insertions, deletions or substitutions with at least 50% identity to AAC AAC AAU UAC GAA CCA AAC UU (SEQ ID NO. 1).

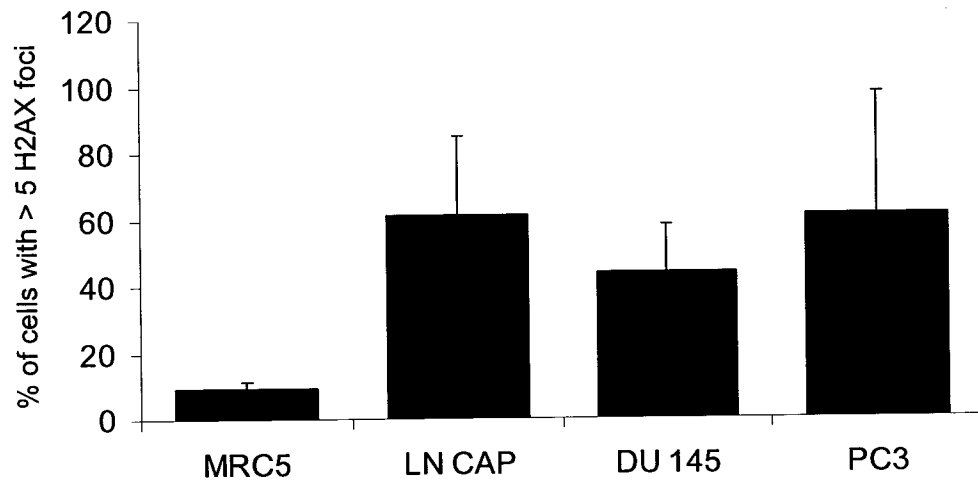
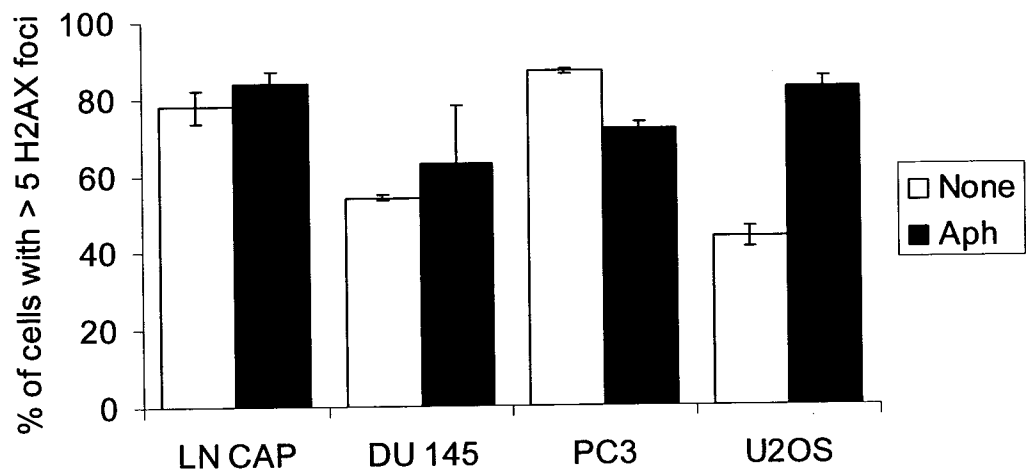
13. A pharmaceutical composition comprising an agent according to  
15 any preceding claim and one or more pharmaceutically acceptable carriers, diluents and/or excipients.

14. A method of treating prostate cancer comprising administering a  
20 therapeutically effective amount of a BRCA1 and/or a BRCA2 inhibitor to a subject with prostate cancer.

15. The use of a BRCA1 and/or a BRCA2 inhibitor in the manufacture of a medicament for the treatment of prostate cancer.

**Figure 1A****Figure 1B**

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**Figure 2a****Figure 2b**

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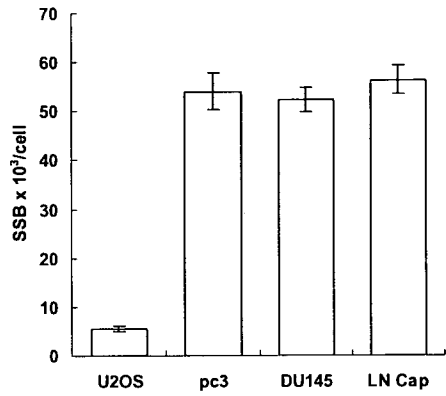


Figure 3a

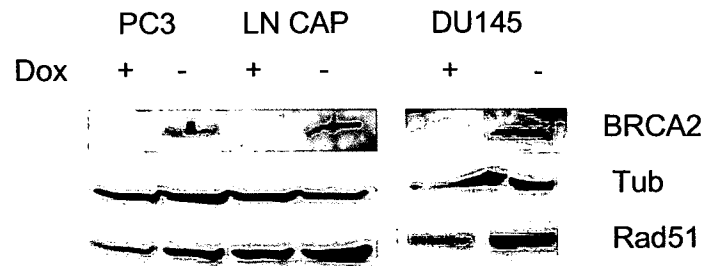


Figure 3b

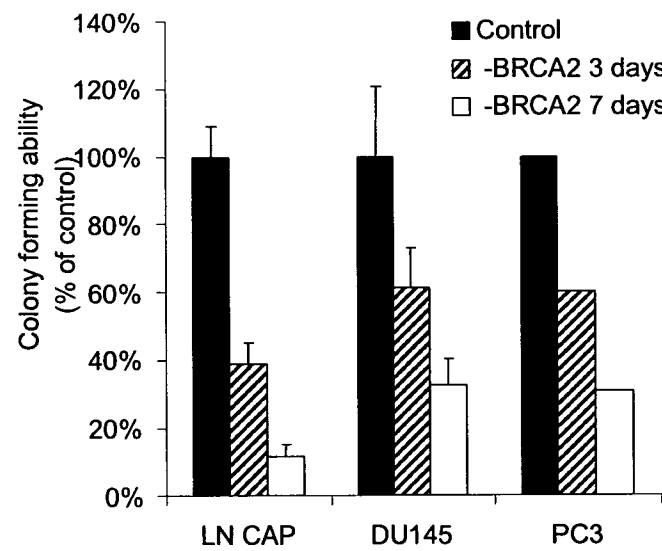
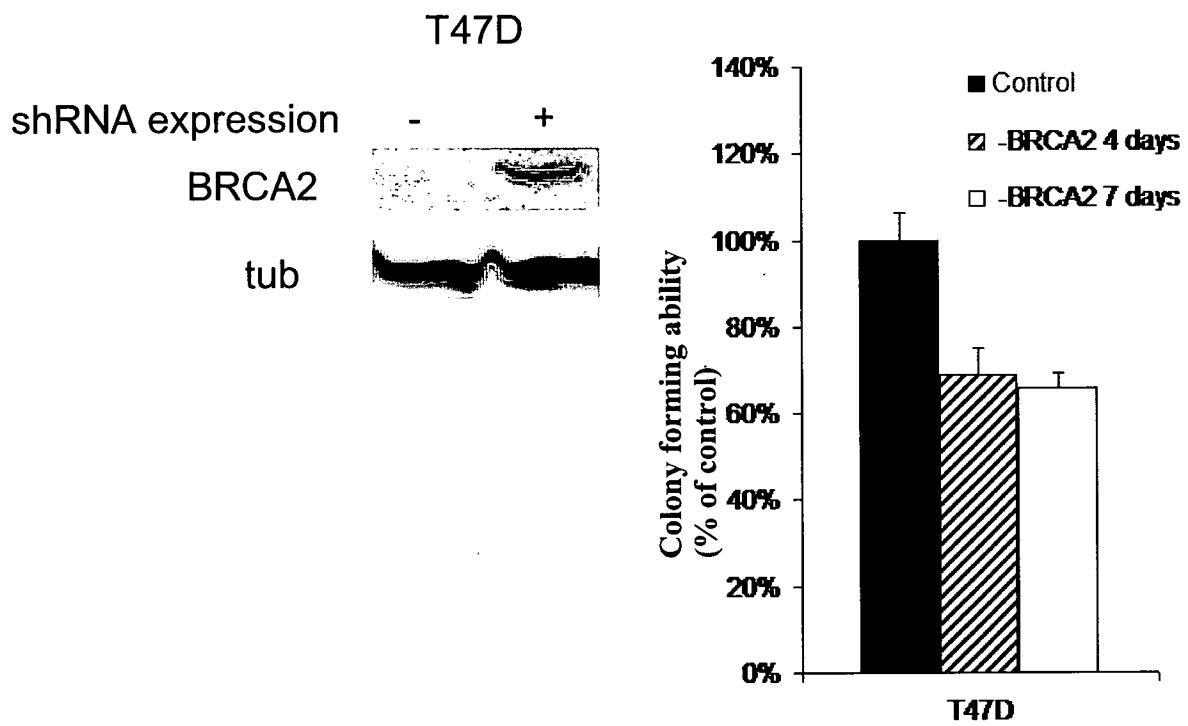


Figure 3c

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**Figure 3d**

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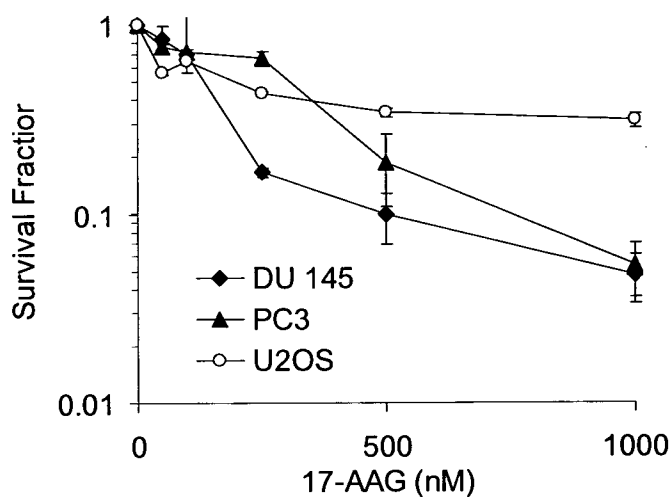


Figure 4a

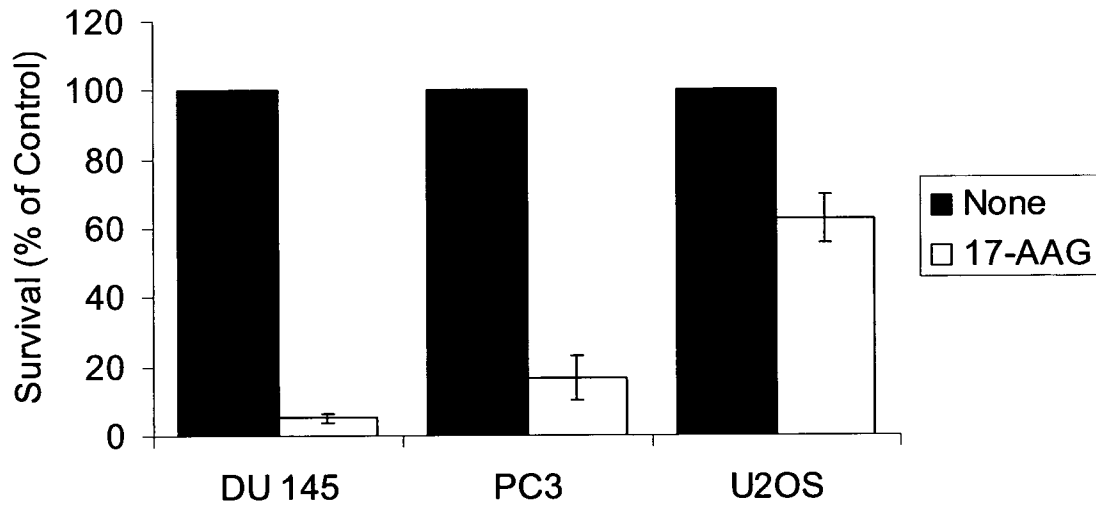


Figure 4b

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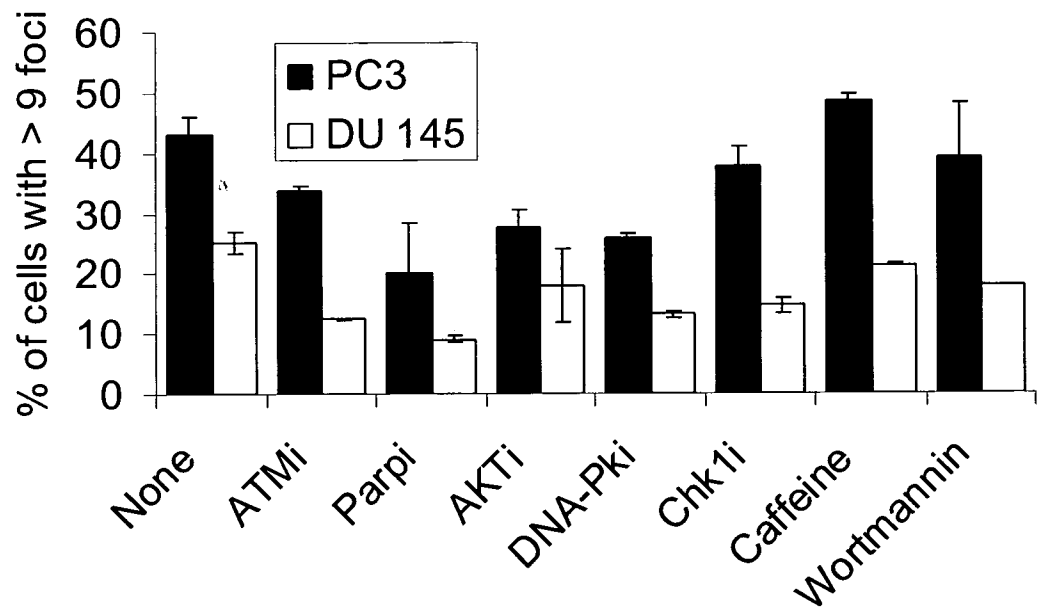


Figure 5a

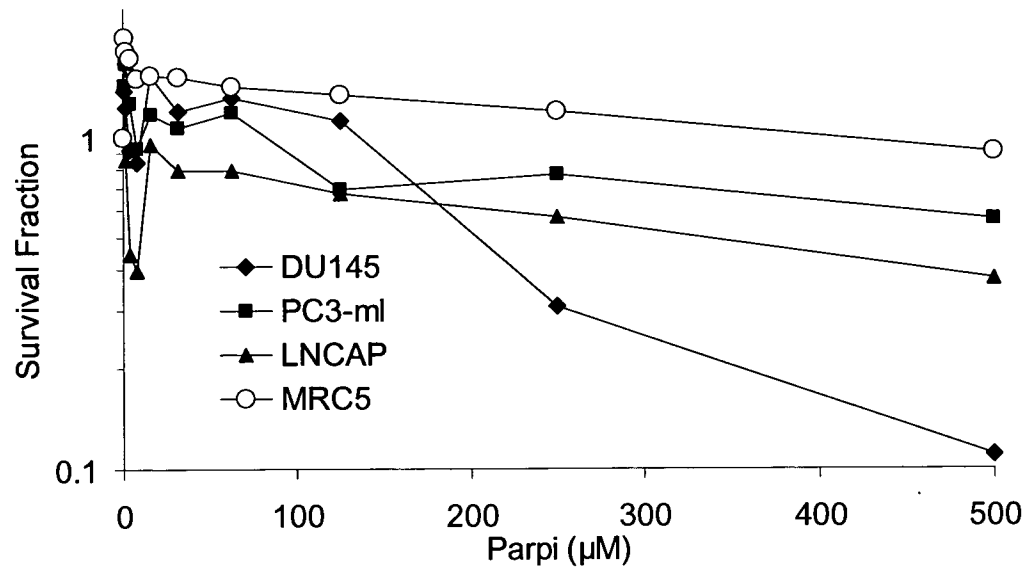


Figure 5b