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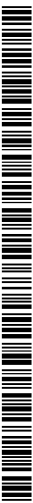


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(54) Title: USE OF RNAI INHIBITING PARP ACTIVITY FOR THE MANUFACTURE OF A MEDICAMENT FOR THE TREATMENT OF CANCER

(57) Abstract: The present invention relates to the use of an agent that inhibits the activity of an enzyme that mediates repair of a DNA strand break in the manufacture of a medicament for the treatment of diseases caused by a defect in a gene that mediates homologous recombination.

USE OF RNAI INHIBITING PARP ACTIVITY FOR THE MANUFACTURE OF A
MEDICAMENT FOR THE TREATMENT OF CANCER

This invention relates to the use of an agent that inhibits the activity of an
5 enzyme which mediates repair of DNA strand breaks in the treatment of certain forms of
cancer, in particular breast cancer.

Any discussion of the prior art throughout the specification should in no way be
considered as an admission that such prior art is widely known or forms part of common
general knowledge in the field.

10 Homologous recombination (HR) has been shown to play an important role in
repair of damage occurring at DNA replication forks in mammalian cells (2). Thus, cells
deficient in HR show retarded growth and exhibit higher level of genetic instability. It is
believed that genetic instability, due to loss of HR repair in human cancers, significantly
contributes to the development of cancer in these cells (1).

15 Post transcriptional modification of nuclear proteins by poly(ADP-ribosyl)ation
(PARP) in response to DNA strand breaks plays an important role in DNA repair,
regulation of apoptosis, and maintenance of genomic stability.

Poly(ADP-ribose) Polymerase-1 (PARP-1) is an abundant nuclear protein in
mammalian cells that catalyses the formation of poly(ADP-ribose) (PAR) polymers
20 using NAD⁺ as substrate. Upon DNA damage, PARP-1 binds rapidly to a DNA strand
break (single strand or double strand) and catalyses the addition of negatively charged
PAR chains to itself (automodification) and other proteins (see (3, 4) for reviews). The
binding of PARP-1 to DNA strand breaks is believed to protect DNA lesions from
further processing until PARP-1 is dissociated from the break by the accumulated
25 negative charge resulting from PAR polymers (5,6).

Although PARP-1 has been implicated in several nuclear processes, such as
modulation of chromatin structure, DNA replication, DNA repair and transcription,
PARP-1 knockout mice develop normally (7). Cells isolated from these mice exhibit a
hyper recombination phenotype and genetic instability in the form of increased levels of
30 SCE, micronuclei and tetraploidy (8-10). Genetic instability may also occur in these
PARP-1 knockout mice through telomere shortening, increased frequency of
chromosome fusion and aneuploidy (11), although all of these results could not be

repeated in another set of PARP-1 knock-out mice (12). In the former mice knockout, PARP-1 null mutation rescue impaired V(D)J recombination in SCID mice (13).

These results support the view suggested by Lindahl and co-workers that PARP-1 has a protective role against recombination (5). They proposed that binding of PARP-1 to DNA strand breaks prevents the recombination machinery from recognizing and processing DNA lesions or, alternatively, that the negative charges accumulated following poly ADP-ribosylation repel adjacent recombinogenic DNA sequences. Only the latter model is consistent with inhibition of PARP-1 itself and expression of a dominant negative mutant PARP-1, inducing SCE, gene amplification and homologous recombination (HR [14-18]).

Studies based on treating cells with inhibitors of PARP or cells derived from PARP-1 or PARP-2 knockout mice indicate that the suppression of PARP-1 activity increases cell susceptibility to DNA damaging agents and inhibits strand break rejoining (3, 4, 8-11, 19, 20, 47).

Inhibitors of PARP-1 activity have been used in combination with traditional anti-cancer agents such as radiotherapy and chemotherapy (21). The inhibitors were used in combination with methylating agents, topoisomerase poisons and ionising radiations and were found to enhance the effectiveness of these forms of treatment. Such treatments, however, are known to cause damage and death to non-cancerous or "healthy" cells and are associated with unpleasant side effects.

There is therefore a need for a treatment for cancer that is both effective and selective in the killing of cancer cells and which does not need to be administered in combination with radio or chemotherapy treatments.

The present inventors have surprisingly found that cells deficient in homologous recombination (HR) are hypersensitive to inhibitors of PARP as compared to wild type cells. This is surprising since PARP-1 knockout mice live normally, thereby indicating that PARP-1 is not essential for life. Thus, it could not be expected that cells would be sensitive to PARP inhibition.

According to a first aspect of the invention there is provided the use of an inhibitor of poly (ADP-ribose) polymerase (PARP) in the manufacture of a medicament for the treatment of homologous recombination (HR) defective cancer.

In one or more preferred embodiments, the present invention provides a method of treatment of a disease or condition in a mammal, including human, which is caused

by a genetic defect in a gene which mediates homologous recombination, which method comprises administering to the mammal a therapeutically effective amount of an agent which inhibits the activity of an enzyme which mediates repair of DNA strand breaks or other lesions present at replication forks.

5 In another embodiment said enzyme is PARP. In a further preferred aspect said agent is an inhibitor of PARP or an RNAi molecule specific to PARP gene.

In a further embodiment, the use is in the treatment of cancer.

Preferably the medicament is a pharmaceutical composition consisting of the inhibitor of PARP in combination with a pharmaceutically acceptable carrier or diluent.

10 The specific sensitivity of HR defective tumours to PARP-I inhibition means that normally dividing cells in the patient will be unaffected by the treatment. Treatment of HR defective cancer cells using an inhibitor of PARP also has the advantage that it does not need to be administered as a combination therapy along with conventional radio or chemotherapy treatments thereby avoiding the side effects associated with these
15 conventional forms of treatment.

A genetic defect in a gene, which mediates homologous recombination, may be due to a mutation in, the absence of, or defective expression of, a gene encoding a protein involved in HR.

In a second aspect, the present invention provides the use of an inhibitor of poly
20 (ADP-ribose) polymerase (PARP) in the manufacture of a medicament for inducing apoptosis in HR defective cells.

In one or more embodiments, the present invention provides a method of inducing apoptosis in HR defective cells in a mammal which method comprises administering to the mammal a therapeutically effective amount of an inhibitor of
25 PARP.

By causing apoptosis in HR defective cells it should be possible to reduce or halt the growth of a tumour in the mammal.

Preferably, the HR defective cells are cancer cells.

Cancer cells defective in HR may partially or totally deficient in HR. Preferably
30 the cancer cells are totally deficient in HR.

The term "cancer" or "tumour" includes lung, colon, pancreatic, gastric, ovarian, cervical, breast or prostate cancer. The cancer may also include skin, renal, liver,

bladder or cerebral cancer. In a preferred aspect, the cancer is in a mammal, preferably human.

The cancer to be treated may be an inherited form of cancer wherein the patient to be treated has a familial predisposition to the cancer. Preferably, the cancer to be
5 treated is gene-linked hereditary cancer. In a preferred embodiment of the invention the cancer is gene-linked hereditary breast cancer.

In one or more embodiments, the inhibitor of PARP is useful in the treatment of cancer cells defective in the expression of a gene involved in HR. Genes with suggested function in HR include XRCC1, ADPRT (PARP-1), ADPRTL2 (PARP-2), CTPS, RPA,
10 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, FANCG, RAD1, RAD9, FEN-1, Mus81, Emel, DDS1, BARD (see (2, 3, 5, 22-28) for reviews).

15 A gene involved in HR may be a tumour suppressor gene. The invention thus provides for the treatment of cancer cells defective in the expression of a tumour suppressor gene. Preferably, the tumour suppressor gene is BRCA1 or BRCA2.

Breast cancer is the most common cancer disease among women in the Western world today. Certain families have strong predisposition for breast cancer, which is often
20 owing to an inherited mutation in one allele of either BRCA1 or BRCA2. However, these patients still maintain one functional allele. Thus, these patient develop normally and have no phenotypic consequence from this mutation. However, in one cell, the functional allele might be lost, making this cell cancerous and at the same time deficient in homologous recombination (HR). This step is critical for the onset of a tumour [1].

25 The present inventors have surprisingly found that BRCA2 deficient cells are 100 times more sensitive to the cytotoxicity of the inhibitor of PARP, NU1025, than wild type cells.

Thus in an embodiment, the invention provides the use of an inhibitor of PARP in the manufacture of a medicament for the treatment of cancer cells defective in HR, e.g
30 due to the loss of BRCA1 and/or BRCA2 expression.

The cancer cells to be treated may be partially or totally deficient in BRCA1 or BRCA2 expression. BRCA1 and BRCA2 mutations can be identified using multiplex

PCR techniques, array techniques (29, 30) or using other screens known to the skilled person.

Inhibitors of PARP useful in the present invention may be selected from inhibitors of PARP-1, PARP-2, PARP-3, PARP-4, tankyrase 1 or tankyrase 2 (see 31 for a review). In a preferred embodiment, the inhibitor of PARP useful in the present invention is an inhibitor of PARP-1 activity.

Inhibitors of PARP useful in the present invention include benzimidazole-carboxamides, quinazolin-4-[3H]-ones and isoquinoline derivatives (e.g. 2-(4-hydroxyphenyl)benzimidazole-4-carboxamide (NU1085), 8-hydroxy-2-methylquinazolin-4-[3H]one (NU1025); 6(5H)phenanthridinone; 3 aminobenzamide; benzimidazole-4-carboxamides (BZ1-6) and tricyclic lactam indoles (T11-5) [32]. Further inhibitors of PARP may be identified either by design [33] or the novel FlashPlate assay [34].

The inhibitor of PARP formulated as a pharmaceutical composition may be administered in any effective, convenient manner effective for targeting cancer cells including, for instance, administration by oral, intravenous, intramuscular, intradermal, intranasal, topical routes among others. Carriers or diluents useful in the pharmaceutical composition may include, but are not limited to saline, buffered saline, dextrose, water, glycerol, ethanol and combinations thereof.

In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion. The inhibitor may be administered directly to a tumour or may be targeted to the tumour via systemic administration.

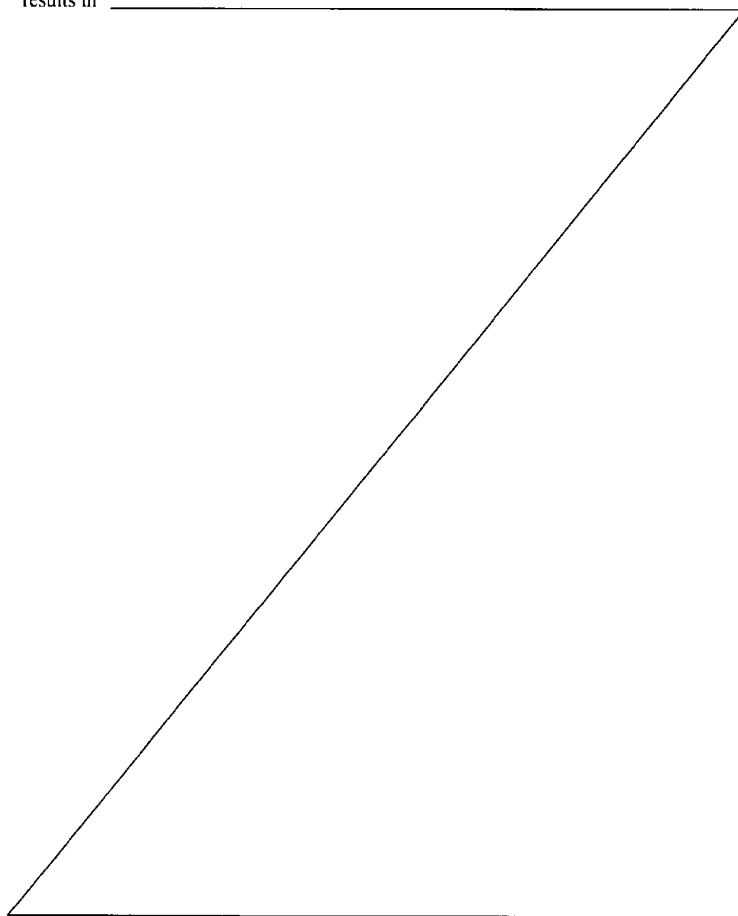
A therapeutically effective amount of the inhibitor is typically one which is sufficient to achieve the desired effect and may vary according to the nature and severity of the disease condition, and the potency of the inhibitor. It will be appreciated that different concentrations may be employed for prophylaxis than for treatment of an active disease.

For administration to mammals, and particularly humans, it is expected that the daily dosage level of the active agent will be up to 100mg/kg, for example from 0.01mg/kg to 50 mg/kg body weight, typically up to 0.1, 0.5, 1.0, 2.0 5.0, 10, 15, 20 or 30mg/kg body weight. Ultimately, however, the amount of inhibitor administered and the frequency of administration will be at the discretion of a physician.

A therapeutic advantage of using an inhibitor of PARP to treat cancer cells is that only very low doses are needed to have a therapeutic effect in treating cancer thereby reducing systemic build up of the inhibitors and any associated toxic effects.

In one or more embodiments the present invention provides an agent which is an
5 inhibitory RNA (RNAi) molecule.

A technique to specifically ablate gene function is through the introduction of double stranded RNA, also referred to as inhibitory RNA (RNAi), into a cell which results in



the destruction of mRNA complementary to the sequence included in the RNAi molecule. The RNAi molecule comprises two complementary strands of RNA (a sense strand and an antisense strand) annealed to each other to form a double stranded RNA molecule. The RNAi molecule is typically derived from exonic or coding
 5 sequence of the gene which is to be ablated.

Preferably said RNAi molecule is derived from the nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:

- a) a nucleic acid sequence as represented by the sequence in Figure 9, 10, 11,
 10 12, 13 or 14 or fragment thereof;
- b) a nucleic acid sequence which hybridises to the nucleic acid sequences of Figure 9, 10, 11, 12, 13 or 14 and encodes a gene for PARP;
- c) a nucleic acid sequence which comprise sequences which are degenerate
 15 as a result of the genetic code to the nucleic acid sequences defined in (a) and (b).

Recent studies suggest that RNAi molecules ranging from 100-1000bp derived from coding sequence are effective inhibitors of gene expression. Surprisingly, only a few molecules of RNAi are required to block gene expression which implies the
 20 mechanism is catalytic. The site of action appears to be nuclear as little if any RNAi is detectable in the cytoplasm of cells indicating that RNAi exerts its effect during mRNA synthesis or processing.

More preferably said RNAi molecule according has a length of between 10 nucleotide
 25 bases (nb) –1000nb. Even more preferably said RNAi molecule has a length of 10nb; 20nb; 30nb; 40nb; 50nb; 60nb; 70nb; 80nb; 90nb; or 100bp. Even more preferably still said RNAi molecule is 21nb in length.

Even more preferably still the RNAi molecule comprises the nucleic acid sequence
 30 aaa agc cau ggu gga gua uga (PARP-1)

Even more preferably still the RNAi molecule consists of the nucleic acid sequence
 aag acc aau cuc ucc agu uca ac (PARP-2)

Even more preferably still the RNAi molecule consists of the nucleic acid sequence aag acc aac auc gag aac aac (PARP-3)

The RNAi molecule may comprise modified nucleotide bases.

According to a third aspect, the present invention provides a method of treatment
5 of a disease or condition in a mammal which is caused by a genetic defect in a gene that mediates homologous recombination, which method comprises administering to the mammal a therapeutically effective amount of an inhibitor of poly (ADP-ribose) polymerase (PARP).

According to a fourth aspect, the present invention provides a method of
10 inducing apoptosis in HR defective cells in a mammal which method comprises administering to the mammal a therapeutically effective amount of an inhibitor of poly (ADP-ribose) polymerase (PARP).

According to a fifth aspect, the present invention provides a method of treating homologous recombination (HR) defective cancer comprising administering to a subject
15 in need of treatment a therapeutic amount of an inhibitor of poly (ADP-ribose) polymerase (PARP).

According to a sixth aspect, the present invention provides use of an inhibitor of poly (ADP-ribose) polymerase (PARP) in the manufacture of a medicament for the treatment of a disease or condition in a mammal which is caused by a genetic defect in a
20 gene that mediates homologous recombination

Unless the context clearly requires otherwise, throughout the description and the claims, the words "comprise", "comprising", and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

25 Preferred features of each embodiment of the invention are as for each of the other embodiments *mutatis mutandis*.

The present invention will now be described by way of example only with reference to the accompanying figures, wherein:

Figure 1 is a graph demonstrating that HR deficient cells are hypersensitive to
30 the toxic effect caused by inhibition of PARP-1. Colony outgrowth of the Chinese hamster cell lines AA8 (wild-type), irs1SF (deficient in HR[4]), CXR3 (irs1SF complemented with XRCC3 [2]), V79 (wild-type), irs1 (deficient in HR[5]) or irs1X2.2

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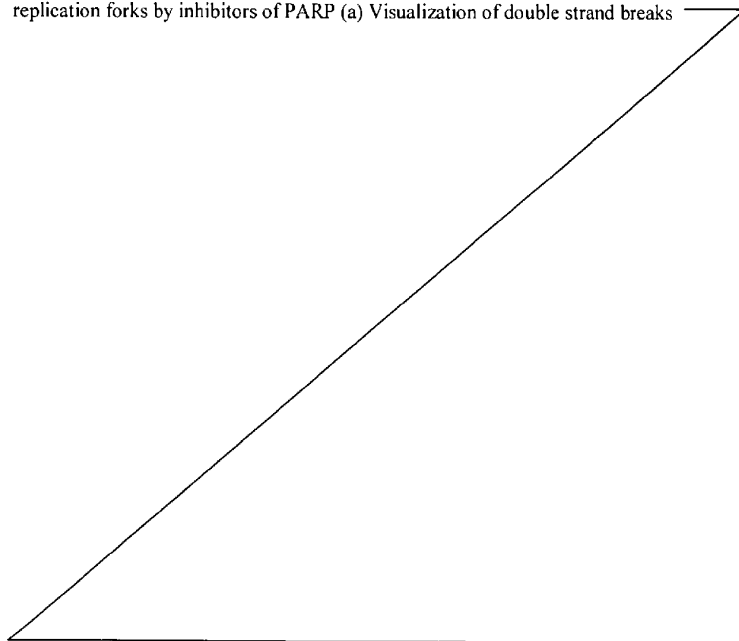
(irsl complimented with XRCC2 [1]) upon exposure to 3-AB (A), ISQ (B) or NU1025 (C). The means (symbols) and standard deviation (bars) of at least three experiments are shown. Colony outgrowth assay was used;

Figure 2 is a graph showing cell survival in the presence of an inhibitor of PARP
5 NU1025 in wt V79 cells, BRCA2 deficient VC-8 cells and VC-8 cells complimented with functional BRCA2 gene (VC-8#13, VC-8+B2). Colony outgrowth assay was used;

Figure 3 is a histogram showing the percentage of the cells in apoptosis following a 72 hour incubation with NU1025;

Figure 4 (a) Western blot analysis of protein lysates isolated from MCF-7 (p53^{wt})
10 or MDA-MB-231 (p53^{mut}) breast cancer cells following 48 hours transfection with siRNA (b) Colony outgrowth of siRNA-treated MCF-7 cells or (c) MDA-MB-231 cells following exposure to the inhibitor of PARP NU1025. The means (symbols) and standard deviation (bars) of at least three experiments are shown.

Figure 5 BRCA2 deficient cells fail to repair a recombination lesion formed at
15 replication forks by inhibitors of PARP (a) Visualization of double strand breaks



(DSBs) in BRCA2 proficient or deficient cells following a 24-hour treatment with NU1025 (0.1 mM) by pulse-field gel electrophoresis. Hydroxyurea 2 mM was used as a positive control. (b) Visualisation of γ H2Ax foci in untreated V-C8+B2 and V-C8 cells. Number of cells containing γ H2Ax foci (c) or RAD51 foci (d) visualised in V-C8+B2 and V-C8 cells following a 24-hour treatment with NU1025 (10 μ M). The means (symbols) and standard errors (bars) of three to nine experiments are shown. (e) A suggested model for cell death induced in BRCA2 deficient cells.

Figure 6. PARP-1 and not PARP-2 is important in preventing formation of a recombinogenic lesion, causing death in absence of BRCA2. (a) RT-PCR on RNA isolated from SW480SN.3 cells treated with BRCA2, PARP-1 and PARP-2 siRNA in combinations as shown for 48 hours. (b) Clonogenic survival following 48-hours depletion of BRCA2, PARP-1 and PARP-2. The means (symbols) and standard deviation (bars) of at least three experiments are shown. Two and three stars designate statistical significance in *t*-test $p < 0.01$ and $p < 0.001$, respectively. (c) Western blot for PARP-1 in SW480SN.3 cells treated with different siRNA.

Figure 7. (a) Visualisation of PAR polymers in untreated and (b) thymidine treated V79 cells (5 mM for 24 hours). (c) Percentage cells containing >10 sites of PARP activity following treatment with hydroxyurea (0.2 mM) and thymidine (5 mM). At least 300 nuclei were counted for each treatment and experiment. (d) Survival of V-C8+B2 cells following co-treatment with hydroxyurea or (e) thymidine and NU1025 (10 μ M). (f) The activity of PARP was measured by the level of free NAD(P)H¹¹, following treatment with MMS, hydroxyurea (0.5 mM) or thymidine (10 mM). The means (symbol) and standard deviation (error bars) from at least three experiments are depicted.

Figure 8. (a) Visualisation of PAR polymers in untreated V-C8 and (b) V-C8+B2 cells. (c) Quantification of percentage cells containing >10 sites of PARP activity in untreated V-C8 and V-C8+B2 cells. (d) Level of NAD(P)H measured in untreated V-C8 and V-C8+B2 cells. Three stars designate $p < 0.001$ in *t*-test. (e) Visualization of RAD51 and sites of PARP activity in V79 cells following a 24-hour thymidine

treatment (5 mM). (f) A model for the role of PARP and HR at stalled replication forks.

Figure 9 is the human cDNA sequence of PARP-1;

5

Figure 10 is the human cDNA sequence of PARP-2;

Figure 11 is the human cDNA sequence of PARP-3;

10

Figure 12 is the human gDNA sequence of Tankyrase 1;

Figure 13 is the human mRNA sequence of Tankyrase 2;

Figure 14 is the human mRNA sequence of VPARP.

15

Materials and Methods

Cytotoxicity of PARP inhibitors to HR-defective cells: XRCC2, XRCC3 or BRCA2

20

Cell culture

The irs1, irs1X2.1 and V79-4 cell lines were a donation from John Thacker [40] and the AA8, irs1SF and CXR3 cell lines were provided by Larry Thompson [41].

25

The VC-8, VC-8+B2, VC-8#13 were a gift from Malgorzata Zdzienicka [42]. All cell lines in this study were grown in Dulbecco's modified Eagle's Medium (DMEM) with 10% Foetal bovine serum and penicillin (100 U/ml) and streptomycin sulphate (100 µg/mL) at 37°C under an atmosphere containing 5% CO₂.

Toxicity assay - colony outgrowth assay

30

500 cells suspended in medium were plated onto a Petri dish 4 hours prior to the addition of 3-AB, ISQ or NU1025. ISQ and NU1025 were dissolved in DMSO to a final concentration of 0.2% in treatment medium. 7 - 12 days later, 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.

Apoptosis experiments

- 5 0.25x10⁶ cells were plated onto Petri dishes and grown for 4 hours before treatment with NU1025. After 72 hours, cells were trypsinized and resuspended with medium containing any floating cells from that sample. The cells were pelleted by centrifugation and resuspended for apoptosis analysis with FITC-conjugated annexin-V and propidium iodine (PI) (ApoTarget, Biosource International) according to
10 manufacturer's protocol. Samples were analysed by flow cytometry (Becton-Dickenson FACSsort, 488 nm laser), and percentage of apoptotic cells was determined by the fraction of live cells (PI-negative) bound with FITC-conjugated annexin-V.

15 Immunofluorescence

- Cells were plated onto coverslips 4 h prior to 24-h treatments as indicated. Following treatments the medium was removed and coverslips rinsed once in PBS at 37°C and fixed as described elsewhere [2]. The primary antibodies and dilutions used in this study were; rabbit polyclonal anti PAR (Trevigen; 1:500), goat polyclonal anti Rad51
20 (C-20, Santa Cruz; 1:200) and rabbit polyclonal anti Rad51 (H-92, Santa Cruz; 1:1000). The secondary antibodies were Cy-3-conjugated goat anti-rabbit IgG antibody (Zymed; 1:500), Alexa 555 goat anti-rabbit F(ab')₂ IgG antibody (Molecular Probes; 1:500), Alexa 546 donkey anti-goat IgG antibody (Molecular Probes; 1:500) and Alexa 488 donkey anti-rabbit IgG antibody (Molecular Probes; 1:500).
25 Antibodies were diluted in PBS containing 3% bovine serum albumin. DNA was stained with 1 µg/ml To Pro (Molecular Probes). Images were obtained with a Zeiss LSM 510 inverted confocal microscope using planapochromat 63X/NA 1.4 oil immersion objective and excitation wavelengths 488, 546 and 630 nm. Through focus maximum projection images were acquired from optical sections 0.50 µm apart
30 and with a section thickness of 1.0 µm. Images were processed using Adobe PhotoShop (Abacus Inc). At least 300 nuclei were counted on each slide and those containing more than 10 RAD51 foci or sites of PARP activity were classified as positive.

PARP activity assays

A water-soluble tetrazolium salt (5mM WST-8) was used to monitor the amount of NAD(P)H through its reduction to a yellow coloured formazan dye[43]. 5000 cells were plated in at least triplicate into wells of a 96 well plate and cultured in 100µl normal growth media for 4 h at 37°C. CK8 buffer (Dojindo Molecular Technology, Gaithersburg, USA), containing WST-8, was then added either with or without treatment with DNA damaging agents at concentrations indicated. Reduction of WST-8 in the presence of NAD(P)H was determined by measuring visible absorbance (OD₄₅₀) every 30 min. A medium blank was also prepared containing just media and CK8 buffer. Changes in NAD(P)H levels were calculated by comparing the absorbance of wells containing cells treated with DNA damaging agents and those treated with DMSO alone. Alternately relative levels of NAD(P)H in different cells lines were calculated after 4 h incubation in CK8 buffer.

The ability of NU1025 to inhibit PARP-1 activity was also assayed in permeabilised cells using a modification of the method of Halldorsson *et al* [44], and described in detail elsewhere [45]. Briefly: 300 µl of NU1025-treated (15 min) permeabilised cells were incubated at 26°C with oligonucleotide (final conc. 2.5 µg/ml), 75 µM NAD + [³²P] NAD (Amersham Pharmacia, Amersham, UK) in a total volume of 400 µl. The reaction was terminated after 5 min by adding ice cold 10%TCA 10%Na Ppi for 60 min prior to filtering through a Whatman GF/C filter (LabSales, Maidstone, UK), rinsed 6x with 1% TCA 1% NaPpi, left to dry and incorporated radioactivity was measured to determine PARP-1 activity. Data are expressed as pmol NAD incorporated/10⁶ cells by reference to [³²P] NAD standards.

25 Pulse-field gel electrophoresis

1.5x10⁶ cells were plated onto 100 mm dishes and allowed 4 h for attachment. Exposure to drug was for 18 h after which cells were trypsinised and 10⁶ cells melted into each 1% agarose insert. These inserts were incubated as described elsewhere (8) and separated by pulse-field gel electrophoresis for 24 h (BioRad; 120° angle, 60 to 240 s switch time, 4 V/cm). The gel was subsequently stained with ethidium bromide for analysis.

Pre-designed BRCA2 SMARTpool and scrambled siRNAs were purchased (Dharmacon, Lafayette, CO). 10000 cells seeded onto 6 well plates and left over night before transfected with 100nM siRNA using Oligofectamine Reagent (Invitrogen) according to manufacturers instructions. Cells were then cultured in normal growth media for 48 h prior to trypsinisation and replating for toxicity assays. Suppression of BRCA2 was confirmed by Western blotting (as described previously [46]) of protein extracts treated with siRNA with an antibody against BRCA2 (Oncogene, Nottingham, UK).

EXAMPLES

Homologous recombination deficient cells are hypersensitive to PARP-1 inhibition

To investigate the involvement of HR in cellular responses to inhibition of PARP-1, the effects of PARP-1 inhibitors on the survival of HR repair deficient cell lines were studied. It was found that cells deficient in HR (i.e., *irs1SF* which is defective in *XRCC3* or *irs1* which is defective in *XRCC2* [see Table 1] were very sensitive to the toxic effect of 3-aminobenzamide (3-AB) and to two more potent inhibitors of PARP-1: 1,5-dihydroxyisoquinoline (ISQ; [37]) or 8-hydroxy-2-methylquinazolinone (NU1025 [38, 39]) (Figure 1). The sensitivity in *irs1SF* cells to 3-AB, ISQ or NU1025 was corrected by the introduction of a cosmid containing a functional *XRCC3* gene (CXR3). Similarly, the sensitivity in *irs1* cells to 3-AB, ISQ or NU1025 was corrected by the introduction of a cosmid containing a functional *XRCC2* gene (*irs1X2.2*).

BRCA2 deficient cells are hypersensitive to PARP-1 inhibition

The survival of BRCA2 deficient cells (VC8) and wild type cells (V79Z) in the presence of inhibitors of PARP-1 was investigated. It was found that VC8 cells are very sensitive to the toxic effect of NU1025 (Figure 2). The sensitivity in VC8 cells was corrected by the introduction of a functional BRCA2 gene either on chromosome 13 (VC8#13) or on an overexpression vector (VC8+B2). This result demonstrates that the sensitivity to PARP-1 inhibitors is a direct consequence of loss of the BRCA2 function.

To investigate if inhibition of PARP-1 triggers apoptosis in BRCA2 deficient cells, the level of apoptosis 72 hours following exposure to NU1025 was investigated. It was found that NU1025 triggered apoptosis only in VC8 cells, showing that loss of PARP-1 activity in BRCA2 deficient cells triggers this means of death (Figure 3).

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BRCA2 deficient breast cancer cells are hypersensitive to PARP-1 inhibition

It was examined whether the MCF7 (wild-type p53) and MDA-MB-231 (mutated p53) breast cancer cell lines displayed a similar sensitivity to NU1025 upon depletion of BRCA2. It was found that PARP inhibitors profoundly reduced the survival of MCF7 and MDA-MB-231 cells only when BRCA2 was depleted with a mixture of BRCA2 siRNA (Figure 4). This shows that BRCA2 depleted breast cancer cells are sensitive to PARP inhibitors regardless of p53 status.

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BRCA2 deficient cells die from PARP-1 inhibition in absence of DNA double-strand breaks (DSBs) but in presence of γ H2Ax

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HR is known to be involved in the repair of DSBs and other lesions that occur during DNA replication [2]. To determine whether the sensitivity of BRCA2 deficient cells is the result of an inability to repair DSBs following NU1025 treatment, the accumulation of DSBs in V79 and V-C8 cells was measured following treatments with highly toxic levels of NU1025. It was found that no DSBs were detectable by pulsed field gel electrophoretic analysis of DNA obtained from the treated cells (Figure 5A), suggesting that low levels of DSBs or other recombinogenic substrates accumulated following PARP inhibition in HR deficient cells, which trigger γ H2Ax (Figure 5B). The reason why BRCA2 deficient cells die following induction of these recombinogenic lesions is likely to be due to an inability to repair such lesions. To test this, the ability of BRCA2 deficient V-C8 cells and BRCA2 complimented cells to form RAD51 foci in response to NU1025 was determined. It was found that RAD51 foci were indeed induced in V-C8+B2 cells following treatment with NU1025 (statistically significant in *t-test* $p < 0.05$; Figure 5D). This indicates that the recombinogenic lesions trigger HR repair in these cells allowing them to survive. In contrast, the BRCA2 deficient V-C8 cells were unable to form RAD51 foci in response to NU1025 treatment (Figure 5D) indicating no HR, which would leave the recombinogenic lesions unrepaired and thus cause cell death.

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PARP-1 and not PARP-2 is important in preventing formation of a recombinogenic lesion

There are two major PARPs present in the nucleus in mammalian cells, PARP-1 and PARP-2 and all reported PARP inhibitors inhibit both. In order to distinguish which PARP was responsible for the effect, we tested if the absence of PARP-1 and/or PARP-2 results in accumulation of toxic lesions, by depleting these and BRCA2 with siRNA in human cells (Figure 6a). We found that the clonogenic survival was significantly reduced when both PARP-1 and BRCA2 proteins were co-depleted from human cells (Figure 6b). Depletion of PARP-2 with BRCA2 had no effect on the clonogenic survival and depletion of PARP-2 in PARP-1 and BRCA2 depleted cells did not result in additional toxicity. These results suggest that PARP-1 and not PARP-2 is responsible for reducing toxic recombinogenic lesions in human cells. The cloning efficiency was only reduced to 60% of control in PARP-1 and BRCA2 co-depleted cells, while no HR deficient cells survived treatments with PARP inhibitors. This is likely to do with incomplete depletion of the abundant PARP-1 protein by siRNA (Figure 6c), which might be sufficient to maintain PARP-1 function in some of the cells.

PARP-1 is activated by replication inhibitors

HR is also involved in repair of lesions occurring at stalled replication forks, which may not involve detectable DSBs [2]. To test if PARP has a role at replication forks, PARP activation in cells treated with agents (thymidine or hydroxyurea) that retard or arrest the progression of DNA replication forks was examined. Thymidine depletes cells of dCTP and slows replication forks without causing DSBs. Hydroxyurea depletes several dNTP and block the replication fork, which is associated with the formation of DSBs at replication forks [2]. Both of these agents potentially induce HR [2]. V79 hamster cells treated for 24 hours with thymidine or hydroxyurea were stained for PAR polymers. This revealed a substantial increase in the number of cells containing sites of PARP activity (Figure 7C). This result suggests a function for PARP at stalled replication forks. It was also shown that inhibition of PARP with NU1025 enhances the sensitivity to thymidine or hydroxyurea in V-C8+B2 cells (Figure 7D,E). This result suggests that PARP activity is important in

repair of stalled replication forks or alternatively that it prevents the induction of death in cells with stalled replication forks.

PARP is rapidly activated at DNA single-strand breaks (SSB) and attracts DNA repair enzymes [3-6]. Methylmethane sulphonate (MMS) causes alkylation of DNA, which is repaired by base excision repair. PARP is rapidly activated by the SSB-intermediate formed during this repair, which depletes the NAD(P)H levels (Figure 7F). We found that the activation of PARP and reduction of NAD(P)H levels is much slower following thymidine or hydroxyurea treatments. This slow PARP activation can be explained by the indirect action of thymidine and hydroxyurea and the time required to accumulate stalled replication forks as cells enter the S phase of the cell cycle.

PARP-1 and HR have separate roles at stalled replication forks

The number sites of PARP activity in untreated BRCA2 deficient V-C8 cells was determined. It was found that more V-C8 cells contain sites of PARP activity compared to V-C8+B2 cells (Figure 8A,B,C). Also, the V-C8 cells have lower free NAD(P)H levels than the corrected cells (Figure 8D), as a likely result of the increased PARP activity. Importantly these sites of PARP activity do not overlap with RAD51 foci (Figure 8E).

The results herein suggest that PARP and HR have separate roles in the protection or rescue of stalled replication forks (Figure 8F). A loss of PARP activity can be compensated by increased HR while a loss of HR can be compensated by increased PARP activity. However, loss of both these pathways leads to accumulation of stalled replication forks and to death, as in the case of PARP inhibited BRCA2 deficient cells.

As shown in the model outlined in Figure 8F PARP and HR have complementary roles at stalled replication forks. (i) Replication forks may stall when encountering a roadblock on the DNA template. In addition, they may also stall temporarily, due to lack of dNTPs or other replication co-factors. (ii) PARP binds stalled replication forks or other replication-associated damage, triggering PAR polymerization. Resulting negatively charged PAR polymers may protect stalled replication forks, by repelling

proteins that normally would process replication forks (e.g., resolvases), until the replication fork can be restored spontaneously when dNTPs or other co-factors become available. Alternatively, PAR polymers or PARP may attract proteins to resolve the replication block by other means. (iii) In absence of PARP activity, HR
 5 may be used as an alternative pathway to repair stalled replication forks. This compensatory model explains the increased level of HR and RAD51 foci found in PARP deficient cells³⁻⁵ and higher PARP activity found in HR deficient cells (i.e. V-C8). Spontaneous replication blocks/lesions are only lethal in the absence of both PARP and HR.

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Table 1. Genotype and origin of cell lines used in this study.

Cell line	Genotype	Defect	Origin	Reference
AA8	Wt	Wt	CHO	[41]
irs1SF	<i>XRCC3</i> ⁻	<i>XRCC3</i> ⁻ , deficient in HR	AA8	[41]
CXR3	<i>XRCC3</i> ⁻ + <i>hXRCC3</i>	Wt	irs1SF	[41]
V79-4	Wt	Wt	V79	[40]
irs1	<i>XRCC2</i> ⁻	<i>XRCC2</i> ⁻ , deficient in HR	V79-4	[40]
irs1X2.2	<i>XRCC2</i> ⁻ + <i>hXRCC2</i>	Wt	irs1	[40]
V79-Z	Wt	Wt	V79	[42]
VC8	<i>BRCA2</i> ⁻	<i>BRCA2</i> ⁻ , deficient in HR	V79-Z	[42]
VC8#13	<i>BRCA2</i> ⁻ + <i>hBRCA2</i>	Wt	VC8	[42]
VC8+B2	<i>BRCA2</i> ⁻ + <i>hBRCA2</i>	Wt	VC8	[42]

REFERENCES:

- [1] A.R. Venkitaraman Cancer susceptibility and the functions of BRCA1 and BRCA2, *Cell* 108 (2002) 171-182.
- 5 [2] C. Lundin, K. Erixon, C. Armaudeau, N. Schultz, D. Jenssen, M. Meuth and T. Helleday Different roles for nonhomologous end joining and homologous recombination following replication arrest in mammalian cells, *Mol Cell Biol* 22 (2002) 5869-5878.
- [3] D. D'Amours, S. Desnoyers, I. D'Silva and G.G. Poirier Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions, *Biochem J* 342
10 (1999) 249-268.
- [4] Z. Herceg and Z.Q. Wang Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death, *Mutat Res* 477 (2001) 97-110.
- 15 [5] T. Lindahl, M.S. Satoh, G.G. Poirier and A. Khungland Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks, *Trends Biochem Sci* 20 (1995) 405-411.
- [6] M.S. Satoh and T. Lindahl Role of poly(ADP-ribose) formation in DNA repair, *Nature* 356 (1992) 356-358.
- 20 [7] S. Shall and G. de Murcia Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model?, *Mutat Res* 460 (2000) 1-15.
- [8] Z.Q. Wang, L. Stingl, C. Morrison, M. Jantsch, M. Los, K. Schulze-Osthoff and E.F. Wagner PARP is important for genomic stability but dispensable in apoptosis, *Genes Dev* 11 (1997) 2347-2358.
- 25 [9] C.M. Simbulan-Rosenthal, B.R. Haddad, D.S. Rosenthal, Z. Weaver, A. Coleman, R. Luo, H.M. Young, Z.Q. Wang, T. Ried and M.E. Smulson Chromosomal aberrations in PARP(-/-) mice: genome stabilization in immortalized cells by reintroduction of poly(ADP-ribose) polymerase cDNA, *Proc Natl Acad Sci U S A* 96 (1999) 13191-13196.
- 30 [10] J.M. de Murcia, C. Niedergang, C. Trucco, M. Ricoul, B. Dutrillaux, M. Mark, F.J. Oliver, M. Masson, A. Dierich, M. LeMeur, C. Walztinger, P. Chambon and G. de Murcia Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells, *Proc Natl Acad Sci U S A* 94 (1997) 7303-7307.

- [11] F. d'Adda di Fagagna, M.P. Hande, W.M. Tong, P.M. Lansdorp, Z.Q. Wang and S.P. Jackson Functions of poly(ADP-ribose) polymerase in controlling telomere length and chromosomal stability, *Nat Genet* 23 (1999) 76-80.
- [12] E. Samper, F.A. Goytisolo, J. Menissier-de Murcia, E. Gonzalez-Suarez, J.C. Cigudosa, G. de Murcia and M.A. Blasco Normal telomere length and chromosomal end capping in poly(ADP-ribose) polymerase-deficient mice and primary cells despite increased chromosomal instability, *J Cell Biol* 154 (2001) 49-60.
- [13] C. Morrison, G.C. Smith, L. Stingl, S.P. Jackson, E.F. Wagner and Z.Q. Wang Genetic interaction between PARP and DNA-PK in V(D)J recombination and tumorigenesis, *Nat Genet* 17 (1997) 479-482.
- [14] V. Schreiber, D. Hunting, C. Trucco, B. Gowans, D. Grunwald, G. De Murcia and J.M. De Murcia A dominant-negative mutant of human poly(ADP-ribose) polymerase affects cell recovery, apoptosis, and sister chromatid exchange following DNA damage, *Proc Natl Acad Sci U S A* 92 (1995) 4753-4757.
- [15] J.H. Kupper, M. Muller and A. Burkle Trans-dominant inhibition of poly(ADP-ribosyl)ation potentiates carcinogen induced gene amplification in SV40-transformed Chinese hamster cells, *Cancer Res* 56 (1996) 2715-2717.
- [16] J. Magnusson and C. Ramel Inhibitor of poly(ADP-ribose)transferase potentiates the recombinogenic but not the mutagenic action of alkylating agents in somatic cells in vivo in *Drosophila melanogaster*, *Mutagenesis* 5 (1990) 511-514.
- [17] A.S. Waldman and B.C. Waldman Stimulation of intrachromosomal homologous recombination in mammalian cells by an inhibitor of poly(ADP-ribosylation), *Nucleic Acids Res* 19 (1991) 5943-5947.
- [18] A. Semionov, D. Cournoyer and T.Y. Chow Inhibition of poly(ADP-ribose)polymerase stimulates extrachromosomal homologous recombination in mouse Ltk-fibroblasts, *Nucleic Acids Res* 27 (1999) 4526-4531.
- [19] F. Dantzer, V. Schreiber, C. Niedergang, C. Trucco, E. Flatter, G. De La Rubia, J. Oliver, V. Rolli, J. Menissier-de Murcia and G. de Murcia Involvement of poly(ADP-ribose) polymerase in base excision repair, *Biochimie* 81 (1999) 69-75.

- [20] F. Dantzer, G. de La Rubia, J. Menissier-De Murcia, Z. Hostomsky, G. de Murcia and V. Schreiber Base excision repair is impaired in mammalian cells lacking Poly(ADP-ribose) polymerase-1, *Biochemistry* 39 (2000) 7559-7569.
- [21] L. Tentori, I. Portarena and G. Graziani Potential clinical applications of poly(ADP-ribose) polymerase (PARP) inhibitors, *Pharmacol Res* 45 (2002) 73-85.
- [22] T. Lindahl and R.D. Wood Quality control by DNA repair, *Science* 286 (1999) 1897-1905.
- [23] K.W. Caldecott DNA single-strand break repair and spinocerebellar ataxia, *Cell* 112 (2003) 7-10.
- [24] D. D'Amours and S.P. Jackson The Mre11 complex: at the crossroads of dna repair and checkpoint signalling, *Nat Rev Mol Cell Biol* 3 (2002) 317-327.
- [25] A.D. D'Andrea and M. Grompe The Fanconi anaemia/BRCA pathway, *Nat Rev Cancer* 3 (2003) 23-34.
- [26] S.P. Jackson Sensing and repairing DNA double-strand breaks, *Carcinogenesis* 23 (2002) 687-696.
- [27] R. Kanaar, J.H. Hoeijmakers and D.C. van Gent Molecular mechanisms of DNA double strand break repair, *Trends Cell Biol* 8 (1998) 483-489.
- [28] D.C. van Gent, J.H. Hoeijmakers and R. Kanaar Chromosomal stability and the DNA double-stranded break connection, *Nat Rev Genet* 2 (2001) 196-206.
- [29] S.L. Neuhausen and E.A. Ostrander Mutation testing of early-onset breast cancer genes BRCA1 and BRCA2, *Genet Test* 1 (1997) 75-83.
- [30] G. Kuperstein, W.D. Foulkes, P. Ghadirian, J. Hakimi and S.A. Narod A rapid fluorescent multiplexed-PCR analysis (FMPPA) for founder mutations in the BRCA1 and BRCA2 genes, *Clin Genet* 57 (2000) 213-220.
- [31] A. Chiarugi Poly(ADP-ribose) polymerase: killer or conspirator? The 'suicide hypothesis' revisited, *Trends Pharmacol Sci* 23 (2002) 122-129.
- [32] C.R. Calabrese, M.A. Batey, H.D. Thomas, B.W. Durkacz, L.Z. Wang, S. Kyle, D. Skaltzky, J. Li, C. Zhang, T. Boritzki, K. Maegley, A.H. Calvert, Z. Hostomsky, D.R. Newell and N.J. Curtin Identification of Potent Nontoxic Poly(ADP-Ribose) Polymerase-1 Inhibitors: Chemopotential and Pharmacological Studies, *Clin Cancer Res* 9 (2003) 2711-2718.
- [33] D. Ferraris, Y.S. Ko, T. Pahutski, R.P. Ficco, L. Serdyuk, C. Alemu, C. Bradford, T. Chiou, R. Hoover, S. Huang, S. Lautar, S. Liang, Q. Lin, M.X.

- Lu, M. Mooney, L. Morgan, Y. Qian, S. Tran, L.R. Williams, Q.Y. Wu, J. Zhang, Y. Zou and V. Kalish Design and synthesis of poly ADP-ribose polymerase-1 inhibitors. 2. Biological evaluation of aza-5[H]-phenanthridin-6-ones as potent, aqueous-soluble compounds for the treatment of ischemic injuries, *J Med Chem* 46 (2003) 3138-3151.
- [34] K.J. Dillon, G.C. Smith and N.M. Martin A FlashPlate assay for the identification of PARP-1 inhibitors, *J Biomol Screen* 8 (2003) 347-352.
- [35] A.J. Pierce, R.D. Johnson, L.H. Thompson and M. Jasin XRCC3 promotes homology-directed repair of DNA damage in mammalian cells, *Genes Dev* 13 (1999) 2633-2638.
- [36] R.D. Johnson, N. Liu and M. Jasin Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination, *Nature* 401 (1999) 397-399.
- [37] G.M. Shah, D. Poirier, S. Desnoyers, S. Saint-Martin, J.C. Hoflack, P. Rong, M. ApSimon, J.B. Kirkland and G.G. Poirier Complete inhibition of poly(ADP-ribose) polymerase activity prevents the recovery of C3H10T1/2 cells from oxidative stress, *Biochim Biophys Acta* 1312 (1996) 1-7.
- [38] R.J. Griffin, S. Srinivasan, K. Bowman, A.H. Calvert, N.J. Curtin, D.R. Newell, L.C. Pemberton and B.T. Golding Resistance-modifying agents. 5. Synthesis and biological properties of quinazolinone inhibitors of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP), *J Med Chem* 41 (1998) 5247-5256.
- [39] S. Boulton, L.C. Pemberton, J.K. Porteous, N.J. Curtin, R.J. Griffin, B.T. Golding and B.W. Durkacz Potentiation of temozolomide-induced cytotoxicity: a comparative study of the biological effects of poly(ADP-ribose) polymerase inhibitors, *Br J Cancer* 72 (1995) 849-856.
- [40] C.S. Griffin, P.J. Simpson, C.R. Wilson and J. Thacker Mammalian recombination-repair genes XRCC2 and XRCC3 promote correct chromosome segregation, *Nat Cell Biol* 2 (2000) 757-761.
- [41] R.S. Tebbs, Y. Zhao, J.D. Tucker, J.B. Scheerer, M.J. Siciliano, M. Hwang, N. Liu, R.J. Legerski and L.H. Thompson Correction of chromosomal instability and sensitivity to diverse mutagens by a cloned cDNA of the XRCC3 DNA repair gene, *Proc Natl Acad Sci U S A* 92 (1995) 6354-6358.

- [42] M. Kraakman-van der Zwet, W.J. Overkamp, R.E. van Lange, J. Essers, A. van Duijn-Goedhart, I. Wiggers, S. Swaminathan, P.P. van Buul, A. Errami, R.T. Tan, N.G. Jaspers, S.K. Sharan, R. Kanaar and M.Z. Zdzienicka Brca2 (XRCC11) deficiency results in radioresistant DNA synthesis and a higher frequency of spontaneous deletions, Mol Cell Biol 22 (2002) 669-679.
- [43] J. Nakamura, S. Asakura, S.D. Hester, G. de Murcia, K.W. Caldecott and J.A. Swenberg Quantitation of intracellular NAD(P)H can monitor an imbalance of DNA single strand break repair in base excision repair deficient cells in real time, Nucleic Acids Res 31 (2003) e104.
- [44] H. Halldorsson, D.A. Gray and S. Shall Poly (ADP-ribose) polymerase activity in nucleotide permeable cells, FEBS Lett 85 (1978) 349-352.
- [45] K. Grube, J.H. Kupper and A. Burkle Direct stimulation of poly(ADP ribose) polymerase in permeabilized cells by double-stranded DNA oligomers, Anal Biochem 193 (1991) 236-239.
- [46] C. Lundin, N. Schultz, C. Arnaudeau, A. Mohindra, L.T. Hansen and T. Helleday RAD51 is Involved in Repair of Damage Associated with DNA Replication in Mammalian Cells, J Mol Biol 328 (2003) 521-535.
- [47] Schreider et al., Journal of Biological Chemistry 277: 23028-23036 (2002).

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. Use of an inhibitor of poly (ADP-ribose) polymerase (PARP) in the manufacture of a medicament for the treatment of homologous recombination (HR) defective cancer.
5
2. Use according to claim 1 wherein the inhibitor of PARP is an inhibitor of PARP-1.
3. Use according to claim 1 or claim 2 wherein the inhibitor of PARP is selected
10 from the group consisting of benzimidazole-carboxamides, quinazolin-4-[3H]-ones and isoquinolone derivatives.
4. Use according to any one of the preceding claims wherein the inhibitor of PARP is selected from the group consisting of 2-(4-hydroxyphenyl)benzimidazole-4-
15 carboxamide, 8-hydroxy-2-methylquinazolin-4-[3H]one, 6(5H)phenanthridinone, 3-amnobenzamide, benzimidazole-4-carboxamides and tricyclic lactam indoles.
5. Use according to any one of the preceding claims wherein the inhibitor of PARP is an RNAi molecule specific to a PARP gene.
20
6. Use according to claim 5 wherein the RNAi molecule is derived from a nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:
 - a) a nucleic acid sequence as represented by the sequence in Figure 9, 10, 11,12, 13 or 14, or a fragment thereof;
 - 25 b) a nucleic acid sequence which hybridises to the nucleic acid sequences of Figure 9, 10, 11,12, 13 or 14, and encodes a gene for PARP; or
 - c) a nucleic acid sequence which comprises sequences which are degenerate as a result of the genetic code to the nucleic acid sequences defined in (a) and (b).
30
7. Use according to claim 5 or claim 6 wherein the RNAi molecule comprises the nucleic acid sequence aaa agc cau ggu gga gua uga.

8. Use according to claim 5 or claim 6 wherein the RNAi molecule consists of the nucleic acid sequence aag acc aau cuc ucc agu uca ac.
9. Use according to claim 5 or claim 6 wherein the RNAi molecule consists of the
5 nucleic acid sequence aag acc aac auc gag aac aac.
10. Use according to any one of the preceding claims wherein the defect in the HR defective cancer is a mutation in a gene encoding a protein involved in HR.
- 10 11. Use according to any one of claims 1 to 9 wherein the defect in the HR defective cancer is the absence of a gene encoding a protein involved in HR.
12. Use according to any one of claims 1 to 9 wherein the defect in the HR defective cancer is in the expression of a gene encoding a protein involved in HR.
- 15 13. Use according to any one claims 10 to 12 wherein the gene encoding a protein involved in HR is selected from the group consisting of XRCC1, ADPRT (PARP-1), ADPRTL2 (PARP-2), CTPS, RPA, RPA1, RPA2, RPA3, XPD, ERCC1, XPF, MMS19, RAD51, RAD51B, RAD51C, RAD51D, DMC1, XRCC2, XRCC3, BRCA1, BRCA2,
20 RAD52, RAD54, RAD50, MRE11, NBS1, WRN, BLM, Ku70, Ku80, ATM, ATR, chk1, chk2, FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, RAD1, RAD9, FEN-1, Mus81, Emel, DDS1 and BARD.
14. Use according to any one of the preceding claims wherein the cancer is selected
25 from the group consisting of lung, colon, pancreatic, gastric, ovarian, cervical, breast and prostate cancer.
15. Use according to any one of the preceding claims wherein the cancer is in a human.
- 30 16. Use according to any one of the preceding claims wherein the cancer is gene-linked hereditary cancer.

17. Use according to any one of the preceding claims wherein the cancer is breast cancer.
18. Use according to any one of claims 14 to 17 wherein the cancer cells to be
5 treated are defective in BRCA1 expression.
19. Use according to any one of claims 14 to 17 wherein the cancer cells to be treated are defective in BRCA2 expression.
- 10 20. Use according to claim 18 or 19 wherein the cancer cells are partially deficient in BRCA1 and/or BRCA2 expression.
21. Use according to claim 18 or 19 wherein the cancer cells are totally deficient in BRCA1 and/or BRCA2 expression.
- 15 22. Use according to any one of claims 10 to 13 wherein the gene encoding a protein involved in HR is a tumour suppressor gene.
23. Use according to claim 22 wherein the tumour suppressor gene is BRCA1.
- 20 24. Use according to claim 22 wherein the tumour suppressor gene is BRCA2
25. Use of an inhibitor of poly (ADP-ribose) polymerase (PARP) in the manufacture of a medicament for inducing apoptosis in HR defective cells.
- 25 26. Use according to claim 25 wherein the HR defective cells are cancer cells.
27. Use according to claim 26 wherein the cancer cells defective in HR are partially deficient in HR.
- 30 28. Use according to claim 26 wherein the cancer cells defective in HR are totally deficient in HR.

29. A method of treatment of a disease or condition in a mammal which is caused by a genetic defect in a gene that mediates homologous recombination, which method comprises administering to the mammal a therapeutically effective amount of an inhibitor of poly (ADP-ribose) polymerase (PARP).

5

30. A method of inducing apoptosis in HR defective cells in a mammal which method comprises administering to the mammal a therapeutically effective amount of an inhibitor of poly (ADP-ribose) polymerase (PARP).

10 31. A method of treating homologous recombination (HR) defective cancer comprising administering to a subject in need of treatment a therapeutic amount of an inhibitor of poly (ADP-ribose) polymerase (PARP).

15 32. Use of an inhibitor of poly (ADP-ribose) polymerase (PARP) in the manufacture of a medicament for the treatment of a disease or condition in a mammal which is caused by a genetic defect in a gene that mediates homologous recombination.

20 33. Use of an inhibitor of poly (ADP-ribose) polymerase (PARP) in the manufacture of a medicament; a method of treatment of a disease or condition in a mammal which is caused by a genetic defect in a gene that mediates homologous recombination; a method for inducing apoptosis in HR defective cells; or a method of treating homologous recombination (HR) defective cancer in a mammal, substantially as herein described with reference to any one or more of the examples but excluding comparative examples.

5 Figure 1.

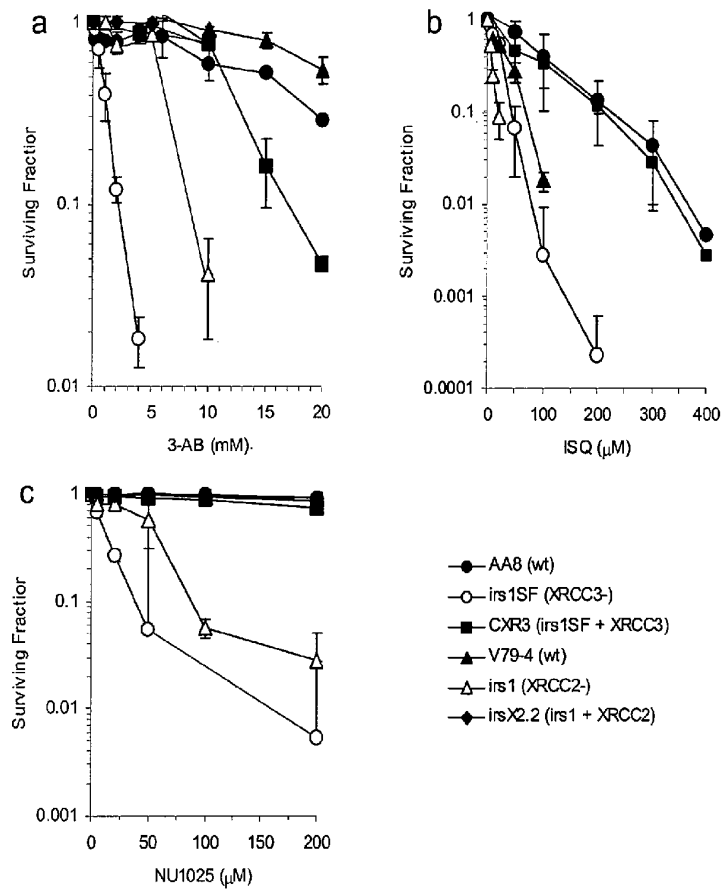


Figure 2.

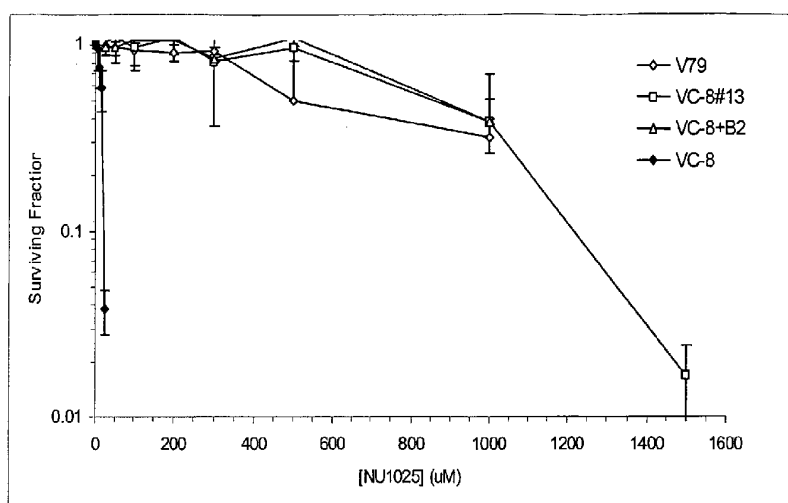
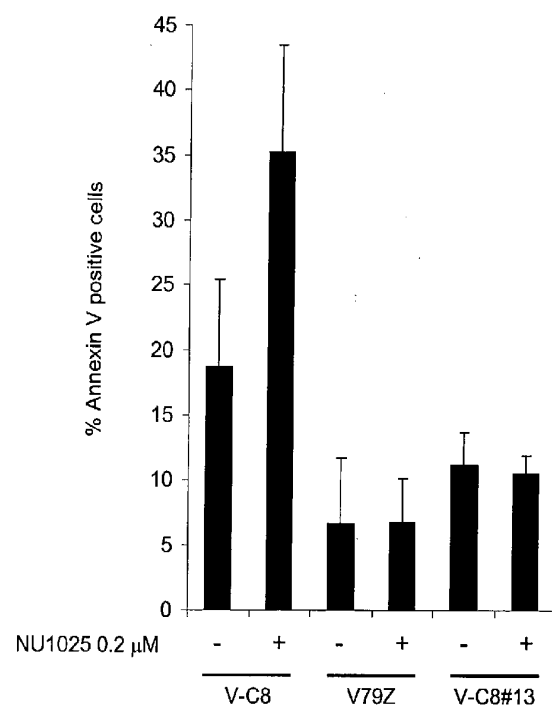


Figure 3



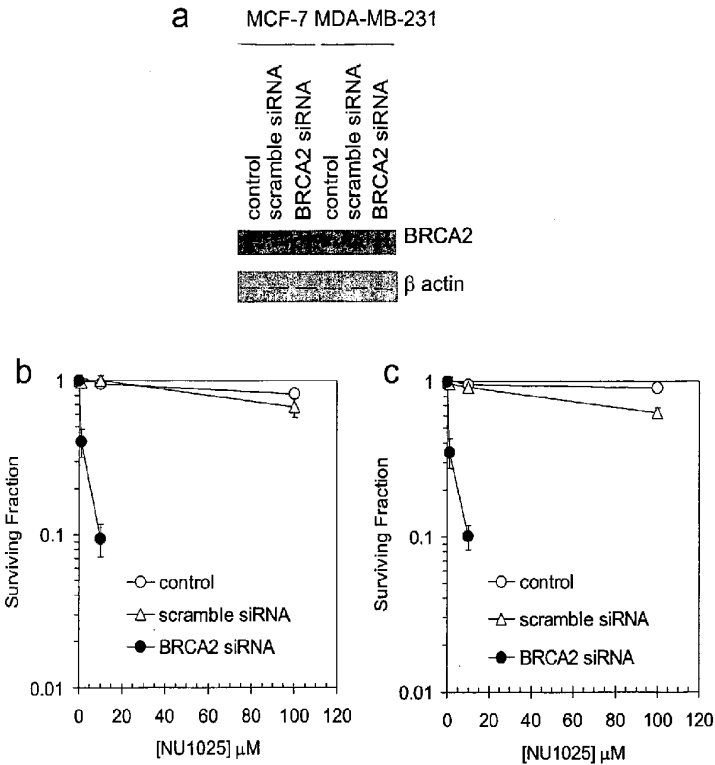
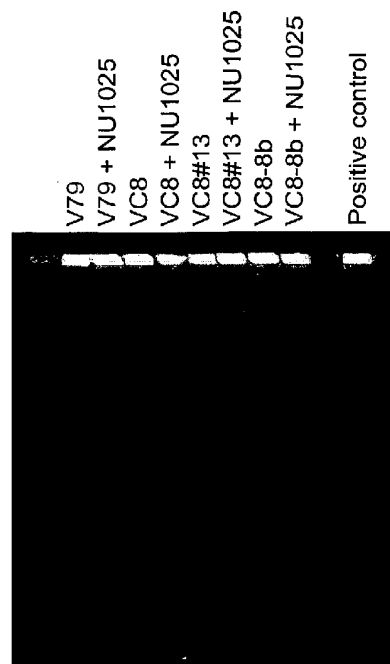
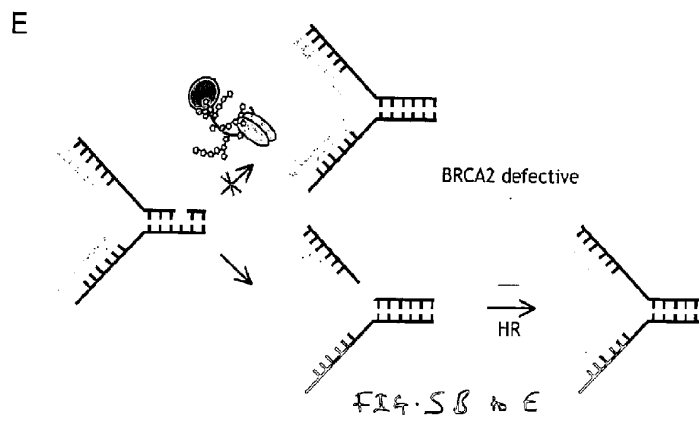
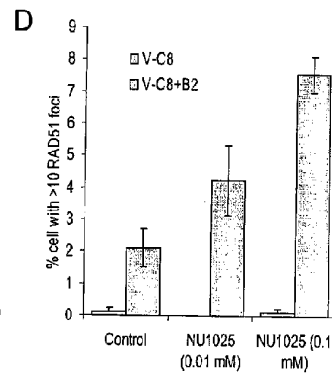
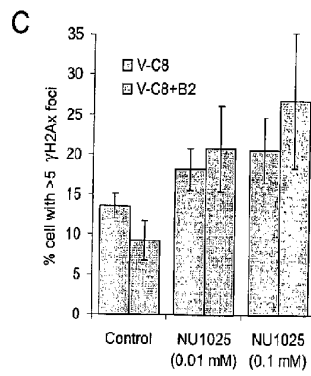
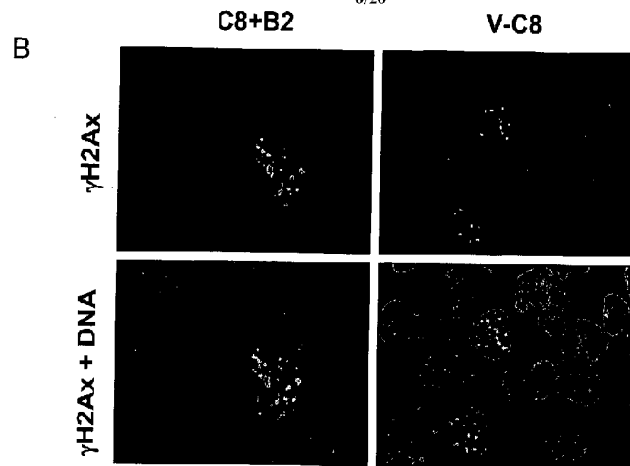


Figure 5A





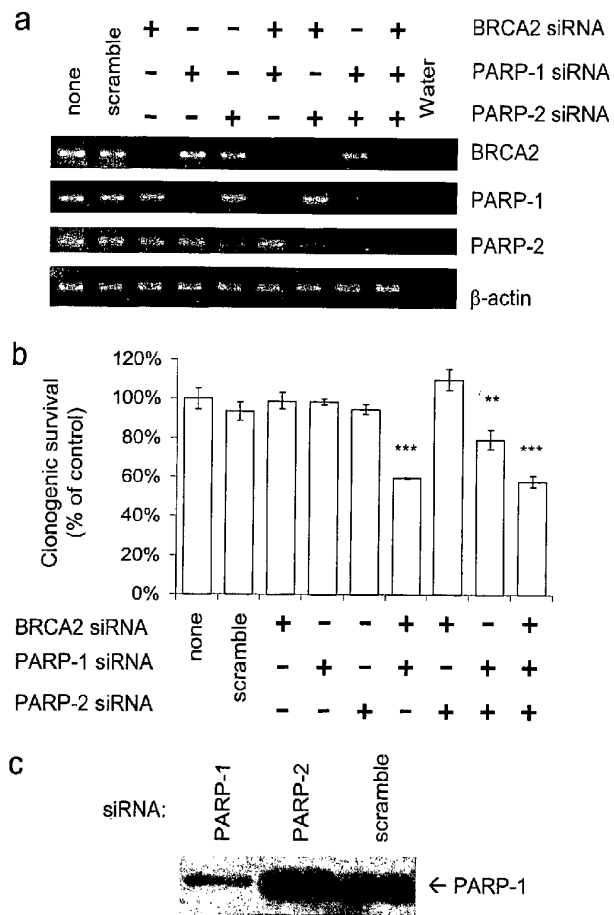


Fig. 6

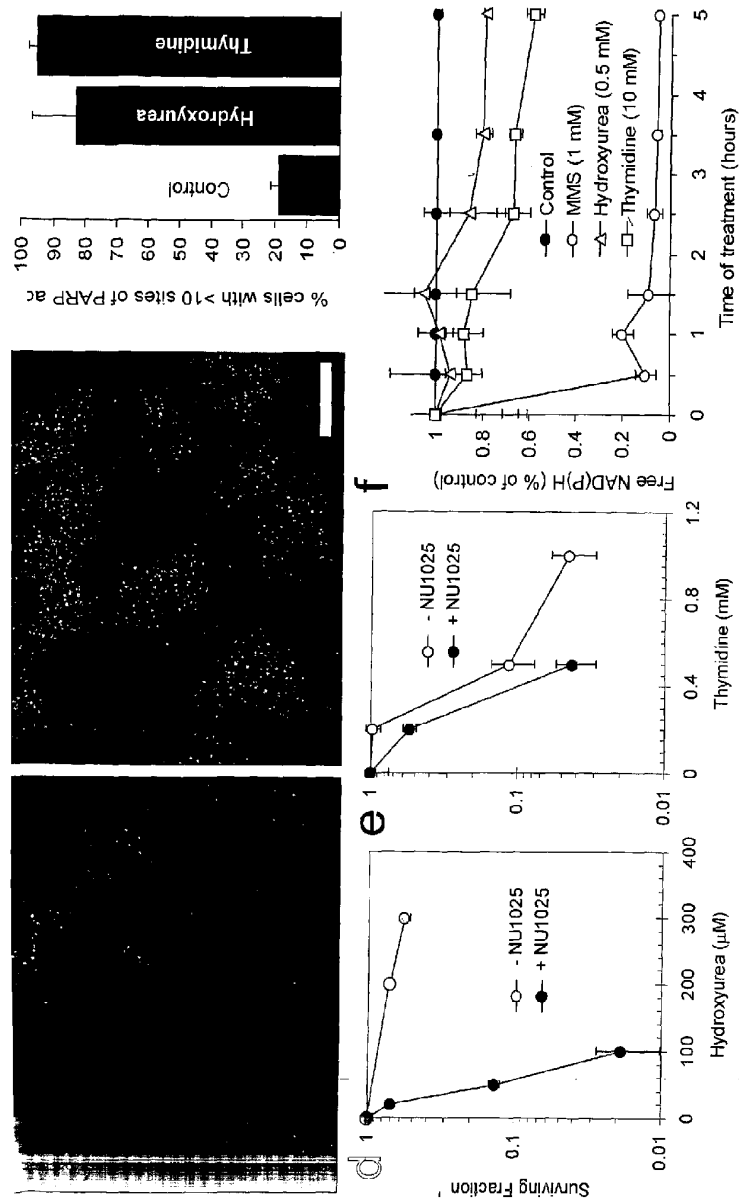


Fig. 7

a

b

c

d

e

f

Fig. 8

Figure 8 consists of several panels. Panels a and b are fluorescence microscopy images showing PARP1 (green) and PAR (red) in cells with stalled replication forks. Panel c is a bar graph showing PARP activity in cells with >10 sites of stalled forks. Panel d is a bar graph showing NAD(P)H levels in cells with >10 sites of stalled forks. Panel e shows fluorescence microscopy images of RAD51 (green), PAR (red), and DNA (blue) in cells with stalled replication forks. Panel f is a schematic diagram of the repair pathway, showing a stalled fork, PARP-1 recruitment, and the subsequent steps of HR and RAD51-mediated fork repair.

PARP activity

Condition	% cells with >10 sites of stalled forks
V-C8	~75
V-C8+B2	~25

NAD(P)H (% of control)

Condition	NAD(P)H (% of control)
V-C8	~60
V-C8+B2	~100

PAR

RAD51

RAD51 + PAR

RAD51 + PAR + DNA

(i) stalled fork

PARP

HR

(ii) PAR-protected fork

(iii) RAD51-mediated fork repair

restored fork

FIGURE 9

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Figure 10

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Figure 11

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Figure 12

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Figure 13

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Figure 14

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