

The PARP-1 Inhibitor Olaparib Causes Retention of γ -H2AX Foci in *BRCA1* Heterozygote Cells Following Exposure to Gamma Radiation

Emma C. Bourton¹, Piers N. Plowman², Amanda J. Harvey¹, Sheba Adam Zahir¹,
Christopher N. Parris¹

¹Brunel Institute of Cancer Genetics and Pharmacogenomics, Division of Biosciences, School of Health Sciences and Social Care, Brunel University, Uxbridge, UK; ²Department of Radiotherapy, St Bartholomew's Hospital, West Smithfield, London, UK.
Email: christopher.parris@brunel.ac.uk

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ABSTRACT

A novel treatment for cancer patients with homozygous deletions of *BRCA1* and *BRCA2* is to use drugs that inhibit the enzyme poly(ADP-ribose) polymerase (PARP). Specific inhibition of PARP-1 can induce synthetic lethality in irradiated cancer cells while theoretically leaving normal tissue unaffected. We recently demonstrated in a cell survival assay that lymphoblastoid cells with mono-allelic mutations of *BRCA1* were hypersensitive to gamma radiation in the presence of the PARP-1 inhibitor Olaparib compared to normal cells and mono-allelic *BRCA2* cells. To determine if the enhanced radiation sensitivity was due to a persistence of DNA strand breaks, we performed γ -H2AX foci analysis in cells derived from two normal individuals, three heterozygous *BRCA1* and three heterozygous *BRCA2* cell lines. Cells were exposed to 2 Gy gamma radiation in the presence or absence of 5 μ M Olaparib. Using immunofluorescence and imaging flow cytometry, foci were measured in untreated cells and at 0.5, 3, 5 and 24 hours post-irradiation. In all lymphoblastoid cells treated with 2 Gy gamma radiation, there was a predictable induction of DNA strand breaks, with a modest but significant retention of foci over 24 hours in irradiated cells treated with Olaparib (ANOVA $P < 0.05$). However, in mono-allelic *BRCA1* cells, there was a failure to fully repair DNA double-strand breaks (DSB) in the presence of Olaparib, evidenced by a significant retention of foci at 24 hours' post irradiation (t -Test $P < 0.05$). These data show that the cellular hypersensitivity of mono-allelic *BRCA1* lymphoblastoid cells to gamma radiation in the presence of the Olaparib is due to the retention of DNA DSB. These data may indicate that patients with inherited mutations in the *BRCA1* gene treated with radiotherapy and PARP-1 inhibitors may experience elevated radiation-associated normal tissue toxicity.

Keywords: *BRCA1*; *BRCA2*; Heterozygote; Radiosensitivity; PARP Inhibitor; Gamma-H2AX; Imaging Flow Cytometry

1. Introduction

BRCA1 (Breast-Cancer susceptibility gene 1) and *BRCA2* are tumour suppressor genes, the protein products of which contribute to DNA repair and transcriptional regulation. The *BRCA* proteins are activated in response to DNA damage such as double-strand breaks (DSB), formed by exposure to ionising radiation [1]. DSB repair is mediated by two principal mechanisms: non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ occurs principally in non-cycling and G1 stage

cells, where the DNA DSB are ligated together in an error-prone manner. Here the *BRCA1* protein is associated with the MRN complex, which senses DSB and is responsible for DSB resolution. HR repairs DSB during the S and G2 phases of the cell cycle, when an intact sister chromatid can serve as a template for repair, a mechanism that is virtually error-free. Both *BRCA1* and *BRCA2* proteins are active in HR. *BRCA1* is a signal mediator, whereas *BRCA2* affects the initiation of repair by recruiting Rad51 to DSB. Hence *BRCA1* is a versatile protein with multiple functional domains that interacts with many

other proteins in both the NHEJ and HR pathways. *BRCA2*, however, is restricted to HR [2]. Both proteins are crucial components in the repair of ionising radiation-induced DSB.

A mutation in either of the *BRCA* genes predisposes individuals to a number of cancers, especially breast cancer. Somatic mutations are responsible for the majority of cases, however germline mutations in these genes are observed in approximately 5% - 10% of breast cancers [3]; such cases are also characterised by early onset [4]. In addition, mutations of these genes are implicated in cancers of the ovary, prostate, pancreas and male breast [3]. Such cancers are routinely treated with a regime of radiotherapy, chemotherapy or surgery, or a combination of these methods, which have proved over many decades to be highly effective in extending patient survival and eradicating certain types of tumours [5]. Radiotherapy is usually administered in fractionated doses, which enables the patient to better tolerate treatment by giving normal tissue time to recover in between fractions [6]. However, patients receiving identical radiotherapy treatments may experience widely-differing level of normal tissue toxicity (NTT), ranging from undetectable to unacceptably severe [7]. Such side-effects have been classified according to tissue type and symptoms exhibited; classification systems include that produced by The Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer (RTOG/EORTC), and the Late Effects Normal Tissue Task Force subjective, objective, management, and analytic (LENT/SOMA) [8]. Although the majority of NTT reactions can be predicted with reasonable accuracy, a small minority of patients experience severe responses to radiotherapy that fall outside the published NTT classifications and such individuals are referred to as "over-reactors". Mainly these individuals harbour inherited genetic defects in the repair of DNA DSB, including those affecting the ATM gene (Ataxia Telangiectasia) [9], the DNA-PKcs gene [10], and the Artemis gene, which results in Radiosensitive Severe Combined Immunodeficiency Disorder (RS-SCID) [11]. These cases exhibit clinical and cellular radiosensitivity so extreme as to be potentially fatal to the patient.

The crucial role of the *BRCA1* and *BRCA2* proteins in DNA DSB repair suggests that individuals carrying mutations in these genes may be susceptible to elevated radiotherapy-induced NTT, however published data is far from clear on this issue. For example, an *in vitro* study of lymphocytes and fibroblasts derived from patients heterozygous for either *BRCA1* or *BRCA2* found that the fibroblasts were radiation hypersensitive in a clonogenic assay and the lymphocytes displayed elevated chromosomal aberrations after radiation exposure [12]. These ob-

servations suggest that such individuals may experience increased NTT during radiotherapy. However, other studies have demonstrated that in those patients who do suffer elevated NTT, *BRCA1* and *BRCA2* heterozygosity is rarely or not observed [13]. This suggests that genes other than *BRCA1* and *BRCA2* probably account for most cases of clinical radiation hypersensitivity leading to NTT.

Additional to DSB, ionising radiation will also induce DNA single-strand breaks (SSB). Such damage will initiate the short-patch and long-patch base-excision repair (BER) pathways. Short-patch BER involves the removal of an incorrect or damaged base; members of the poly (ADP-ribose) polymerase (PARP) enzyme family are recruited to the damage site to replace the base and religate the DNA [14]. Nicotinamide, a catalytic by-product of these reactions, was identified as a weak PARP inhibitor, revealing a platform to develop synthetic PARP inhibitors, such as the benzamides [15]. These were superseded by more specific and potent compounds, including AZD-2281 (Olaparib). Studies on cells derived from head and neck tumours, and from lymphomas, showed that Olaparib produced a synergistic effect when used in concert with ionising radiation [16].

Our lab previously examined the response to ionising radiation of lymphoblastoid cells mono-allelic for either *BRCA1* or *BRCA2* mutations [17]. In this work, two lymphoblastoid cell lines derived from normal individuals and three from individuals with heterozygous mutations in the *BRCA1* or *BRCA2* genes were used. The cells were exposed to increasing doses of gamma radiation either alone or in concert with 5 μ M Olaparib, with the MTT assay used to measure cell survival. As expected, exposure to increasing doses of gamma radiation caused an increase in cell death of all cell types. Simultaneous exposure to gamma radiation and 5 μ M Olaparib did not enhance cell death in normal or *BRCA2* heterozygote cells, but significantly enhanced cell death in the mono-allelic mutated *BRCA1* cells. In the light of this finding, we cautioned that the treating cancer patients carrying *BRCA1* gene mutations with radiotherapy combined with Olaparib administration may enhance radiation-induced normal tissue toxicity (NTT).

The MTT assay is an established method to determine cell survival. However, we were interested in confirming our hypothesis that cell death was caused by the persistence of DNA single- and double-strand breaks due to the combined radiation exposure and PARP-1 inhibition by Olaparib. Theoretically, the number of DSB in the lymphoblastoid cells mono-allelic for *BRCA1* mutations would be greater in comparison to those in the normal cell lines and those mono-allelic for *BRCA2* mutations.

The phosphorylation of the histone protein H2AX is an important event in the signalling and subsequent re-

pair of DNA DSB. The protein product of this reaction (γ -H2AX) accumulates at the sites of DSB, forming nuclear foci where the number of foci is indicative of DNA DSB. These foci can be visualised with immunocytochemistry methods [18,19].

In cells normal for DNA DSB repair, these foci appear rapidly post-irradiation, then disappear within 1 - 3 hours, as the associated DSB are repaired and the γ -H2AX molecules become de-phosphorylated. In cells with defective DSB repair, the persistence of DNA strand breaks correlates with the persistence of γ -H2AX foci. Hence it is possible to visualise and quantitate in a time-dependent manner the repair of DSB in many cell lines.

In this study we demonstrate that the levels of cell death in normal, *BRCA1*-mutated and *BRCA2*-mutated cells exposed to the PARP inhibitor Olaparib and gamma radiation as observed in our previous work [17] correlated with the frequency and persistence of γ -H2AX foci in these same cells.

2. Materials and Methods

2.1. Cell Lines

Human B lymphocytes which had been immortalised using the Epstein-Barr virus were purchased from Coriell Cell Repositories (Camden, New Jersey, USA). Details of the cell lines are shown in **Table 1**. Cell lines “*BRCA1*” and “*BRCA2*” were heterozygotes for mutations in the *BRCA1* or *BRCA2* genes, respectively.

Table 1 provides a description of the B-lymphoblastoid cell lines used in the study together with the information on the mono-allelic mutation in the *BRCA1* and *BRCA2* heterozygous cell lines.

2.2. Cell Culture

The cell lines were routinely cultured in T75 cell culture flasks (PAA Laboratories Limited, Yeovil, Somerset, UK) in RPMI 1640 culture medium (Sigma-Aldrich, Poole, Dorset, UK) supplemented with 10% foetal bovine serum,

2.0 mM L-Glutamine and 100 Uml⁻¹ Penicillin and Streptomycin (PAA). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Cell concentration and viability values were determined using a “Countess™” automated cell counter based upon the method of trypan blue exclusion (Invitrogen, Paisley, Renfrewshire, UK). Cell cultures were used over a restricted range of ten passages, during which cell viability was not less than 80%.

2.3. Exposure to the PARP Inhibitor Olaparib

The highest non-cytotoxic concentration of Olaparib (LC Laboratories Inc, Woburn, Massachusetts, USA) was determined previously [17]. However, in brief, cells were exposed to increasing concentrations of the drug in the range of 1.0 μ M to 1000.0 μ M (log₁₀ scale). The cells were incubated in the drug for 3, 5 and 7 days and survival was determined using the MTT assay. The appropriate concentration of Olaparib was found to be 5.0 μ M following a 7 day exposure; this was the maximum concentration of Olaparib that did not cause significant cell death.

2.4. Exposure to Radiation

The cell suspension of each cell line was divided in half and the cells concentrated into pellets by centrifugation at 1500 rpm for 5 minutes using a table top centrifuge. Each cell pellet was re-suspended with either complete medium only or a 5.0 μ M solution of PARP inhibitor in complete medium to create two separate cell suspensions, which were incubated for 1 hour (incubation conditions detailed previously). One 5.0 ml aliquot from each cell suspension was designated as an un-irradiated control; the remaining suspensions were irradiated with 2 Gy gamma radiation from a ⁶⁰Cobalt source (Puridex Technologies, Oxfordshire, UK) sited at a distance of 25 cm with a dose rate of 0.9 Gy per minute.

The cell suspensions were returned to the incubator

Table 1. Description of B-lymphoblastoid cell lines used in study.

Cell Line	Type	Details of <i>BRCA</i> Gene Mutation
GM00893	Normal	None
GM05423	Normal	None
GM13705	<i>BRCA1</i>	4-base pair deletion in exon 11 = truncated protein.
GM14090	<i>BRCA1</i>	2-base pair deletion in exon 3 = truncated protein.
GM16105	<i>BRCA1</i>	Base substitution in intron 8.
GM14170	<i>BRCA2</i>	1-base pair deletion in exon 11 = frameshift mutation.
GM14622	<i>BRCA2</i>	2-base pair deletion in exon 11 = frameshift mutation = truncated protein.
GM14805	<i>BRCA2</i>	Base substitution in exon 7 = nonsense mutation.

(incubation conditions detailed previously), and 5.0 ml aliquots removed at 30 minutes, 3 hours, 5 hours and 24 hours after irradiation. Cells in each aliquot were washed once with ice-cold PBS (Severn Biotech Ltd, Kidderminster, Worcestershire, UK) and fixed in ice-cold methanol:acetone (50:50 v/v). Two compensation samples for imaging flow cytometry were prepared in the same way at the 30 minute time-point. Samples were stored at -20°C until the immunocytochemistry stage. The immunocytochemistry stage commenced within 5 days of the 24 hour time-point sample being fixed.

2.5. Immunocytochemistry

Cells were washed once in ice-cold PBS (Severn Biotech Ltd) then incubated with gentle agitation for 5 minutes at room temperature in permeabilisation buffer consisting of 0.5% TritonTM X-100 (Sigma-Aldrich) in PBS. Cells were then incubated with gentle agitation for 1 hour at room temperature in blocking buffer consisting of 5.0% rabbit serum (PAA) with 0.1% TritonTM X-100 in PBS. The blocking buffer was removed and the cells incubated with gentle agitation overnight at 4°C in primary antibody solution. The primary antibody solution consisted of an anti-phospho-histone H2AX (serine 139), mouse monoclonal IgG₁ antibody, clone JBW301 (Millipore, Watford, Hertfordshire, UK) diluted 1 in 10,000 in blocking buffer. Excess primary antibody was removed by washing twice with wash buffer, consisting of 0.1% TritonTM X-100 in PBS. The secondary antibody solution consisted of an Alexa Fluor[®]488 rabbit anti-mouse IgG antibody (Invitrogen) diluted 1 in 1000 in blocking buffer. This was added to each sample, except the DRAQ5TM compensation samples, and incubated with gentle agitation for 1 hour at room temperature. Excess secondary antibody was removed by washing twice with wash buffer. The cells were re-suspended in 100 μl AccumaxTM solution (PAA) and left overnight at 4°C (no agitation). 1.0 μl of 5 mM DRAQ5TM solution (Cell Signaling Technology, Hitchin, Hertfordshire, UK) was added to each sample, except the AlexaFluor[®]488 compensation samples. The samples were submitted for analysis by imaging flow cytometry.

2.6. Imaging Flow Cytometry

Imaging flow cytometry was conducted using the Imagestream^X system (Amnis Inc., Seattle, Washington, USA). This permits image capture of each cell in flow using a maximum of six optical channels. Using the InspireTM data acquisition software, images of 10,000 cells were captured on channel 1 for brightfield (BF); on channel 2 for Alexa Fluor[®]488 (AF), representing the green staining of γ -H2AX foci; and on channel 5 for the blue

DRAQ5TM (D5) staining of the nuclear region of each cell. Cell classifiers were applied to the BF channel to capture objects that ranged between 50 and 300 units on an arbitrary scale. These values were determined from previous analyses whereby this classifier range was observed to capture primarily single cell images. Following excitation with a 488 nm laser at a power setting of 40 mW, all images were captured using a 40 \times objective. Images of cells were acquired at a rate of 150 - 200 cell images per second.

2.7. Image Compensation

Image compensation was performed on populations of cells that had been fixed 30 minutes post-irradiation in which γ -H2AX staining intensity was likely to be highest. Cells that were stained with antibody only or DRAQ5TM only were used for generating the compensation matrix. These images were collected without brightfield or dark-field illumination since it was important to capture fluorescence intensity with the 488 nm laser as the single source of illumination. The IdeasTM software compensation wizard generates a table of coefficients whereby detected light that is displayed by each image is placed into the proper channel (channel 2 for antibody staining and channel 5 for DRAQ5TM) on a pixel-by-pixel basis. The coefficients were normalized to 1 and each coefficient represents the leakage of fluorescent signal into juxtaposed channels. Calculated compensation values were applied to all subsequent analyses as appropriate.

2.8. Analysis of Cell Images and Calculation of Foci Number

γ -H2AX foci were quantified in approximately 10,000 images of cells captured using the InspireTM imaging flow cytometry software. Foci were quantified in a similar manner as previously described [19], however, the spot counting wizard provided in IdeasTM was used to simplify foci quantitation. In brief, a series of simple building blocks are used to first identify and gate single cells, then a region is drawn to identify those single cells that are in the correct focal plane during imaging flow. Next, two truth populations were identified by the operator which includes images of cells that have few foci (less than 5) which equates to the first truth population. Finally a second truth population is created which identifies cells with a large number of foci (greater than 8 - 10). These truth populations are then saved and used by the IdeasTM software to create a feature which enumerates all of the foci in the 10,000 cells for each time point. A representative example of cells with increasing foci number is shown in **Figure 1**.

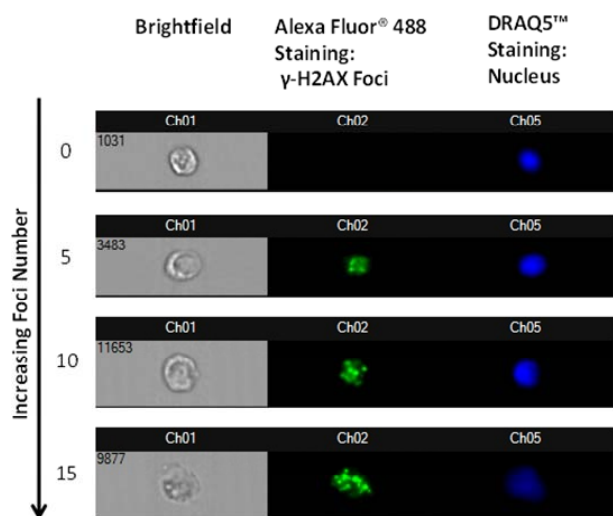


Figure 1. Figure 1 shows representative images of increasing number of γ -H2AX foci in the lymphoblastoid cells captured with imaging flow cytometry. The brightfield channel demonstrates the appearance of lymphoblastoid cells under light microscopy. The second channel represents the appearance of increasing numbers of γ -H2AX foci. The final column depicts the visualisation of the nuclear region of the cells by DRAQ5™ staining. Images of cells were captured at a magnification of 40 \times .

2.9. Statistical Analysis

Using the data analysis programme in Microsoft Excel, two-way analysis of variance was used to compare the distribution of foci in radiation-exposed cells in the presence or absence of Olaparib, across the complete time course of the experiment. A Student's unpaired *t*-Test was utilised to determine if there were differences in foci retention at specific time points following radiation exposure in the presence or absence of Olaparib. Data were regarded as significantly different in the P value was less than 0.05.

3. Results

3.1. Gamma H2AX Foci Analysis in Normal Lymphoblastoid Cells

Gamma H2AX foci analysis was performed in two normal lymphoblastoid cell lines (GM00893 and GM05423). Cells were exposed to 2 Gy gamma radiation in the presence or absence of the PARP-1 inhibitor Olaparib and gamma H2AX foci were measured in un-irradiated cells and at 30 minutes, 3, 5 and 24 hours post-irradiation. The profiles of foci induction combined for GM00893 and GM05423 are shown in **Figure 2**.

For normal cells with and without Olaparib exposure there is a predictable induction of foci at 30 minutes post-irradiation. In the cells not exposed to Olaparib there is a mean of 7.58 foci per cell, and in the Olaparib-

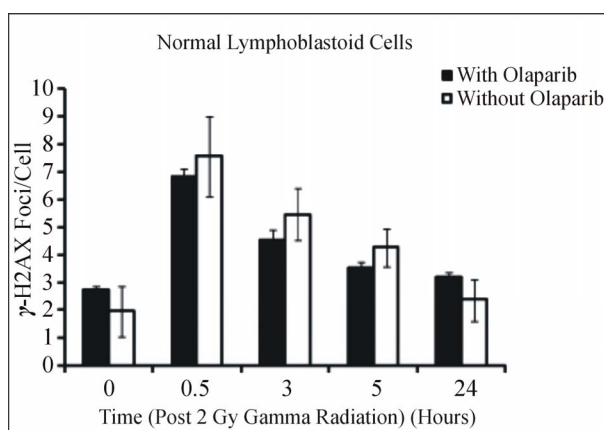


Figure 2. Figure 2 shows the induction of γ -H2AX foci in the normal lymphoblastoid cells GM00893 and GM05423 plotted as an average graph. Data are derived from enumeration of foci in the images of approximately 10,000 cells captured during imaging flow cytometry. Cells were treated with 2 Gy gamma radiation in the presence (black columns) or absence (white columns) of 5 μ m Olaparib. Foci were measured in untreated cells and those irradiated with 2 Gy gamma radiation at 0.5, 3, 5 and 24 hours post-irradiation. The data demonstrate a slight but significant retention of foci in Olaparib-treated cells over a 24 hour period (ANOVA $P > 0.05$). However by comparing the 24 hours post-irradiation time point alone, there was no significant difference in foci retention after irradiation with or without Olaparib (Student's unpaired *t*-Test $P < 0.05$).

treated cells this is slightly reduced at 6.83 foci per cell. Subsequently over a 24 hour period post-irradiation there is a decline in foci number in the normal cells, both Olaparib-treated and untreated. At 24 hours this is reduced to near un-irradiated levels in the non-Olaparib-treated cells, at 2.38 foci per cell, and in the Olaparib-treated cells this figure is slightly higher at 3.24 foci per cell.

Using analysis of variance to compare the time course distribution of post-2 Gy gamma radiation foci induction in the Olaparib-treated and untreated cells there is a significant difference in the distribution of foci ($p = 0.012$). However, using a Student's *t*-Test to compare foci retention at 24 hours in Olaparib-treated and untreated cells, there is no significant difference in foci numbers between treated and untreated cells ($p = 0.315$). This data indicates that Olaparib does not alter the repair of DNA DSB measured by gamma H2AX foci in lymphoblastoid cells derived from normal individuals.

3.2. Gamma H2AX Foci Analysis in *BRCA1* Heterozygous Lymphoblastoid Cells

Gamma H2AX foci analysis was performed following 2 Gy gamma radiation in three lymphoblastoid cell lines (GM13705, GM14090 and GM16105) (details of experiment and analysis as above). The profiles of foci induc-

tion combined for GM13705, GM14090 and GM16105 are shown in **Figure 3**.

For *BRCA1* heterozygous cells with and without Olaparib exposure there is a predictable induction of foci at 30 minutes post-irradiation. In the cells not exposed to Olaparib there is a mean of 5.23 foci per cell, and in the Olaparib-treated cells this is approximately the same at 5.00 foci per cell. Subsequently over a 24 hour period post-irradiation there is a decline in foci number in the cells not treated with Olaparib; at 24 hours there is a mean of 2.00 foci per cell. However, in the Olaparib-treated cells there is retention of foci over the 24 hour period, so that the mean at 24 hours is 4.27 foci per cell, over twice the number of foci than in the untreated cells.

Using analysis of variance to compare the time course distribution of post-2 Gy gamma radiation foci induction in the Olaparib-treated and untreated cells, there is a significant difference in the distribution of foci ($p = 0.004$). This significant difference is further verified with a Student's *t*-Test. At 24 hours there is a significant retention of foci in the Olaparib-treated cells ($p = 0.006$).

3.3. Gamma H2AX Foci Analysis in *BRCA1* Heterozygous Lymphoblastoid Cells

Gamma H2AX foci analysis was performed following 2 Gy gamma radiation in three lymphoblastoid cell lines (GM14170, GM14622 and GM14805) (details of experiment and analysis as above). The profiles of foci induction combined for GM14170, GM14622 and GM14805 are shown in **Figure 4**.

For *BRCA2* heterozygous cells with and without Olaparib exposure there is a predictable induction of foci at 30 minutes post-irradiation. In the cells not exposed to Olaparib there is a mean of 5.16 foci per cell, and in the Olaparib-treated cells this is approximately the same at 5.90 foci per cell. Subsequently over a 24-hour period post-irradiation there is a decline in foci number in the *BRCA2* heterozygous cells, both Olaparib-treated and untreated. At 24 hours this is reduced to slightly below un-irradiated levels in the non-Olaparib-treated cells, at 1.29 foci per cell, and in the Olaparib-treated cells this figure is slightly higher at 2.02 foci per cell.

Using analysis of variance to compare the time course distribution of post-2 Gy gamma radiation foci induction in the Olaparib-treated and untreated cells there is a significant difference in the distribution of foci ($p = 0.004$). However, using a Student's *t*-Test to compare foci retention at 24 hours in Olaparib-treated and untreated cells, there is no significant difference in foci numbers ($p = 0.181$). This data indicates that Olaparib does not alter the repair of DNA DSB measured by gamma H2AX foci in lymphoblastoid cells derived from *BRCA2* heterozygous individuals.

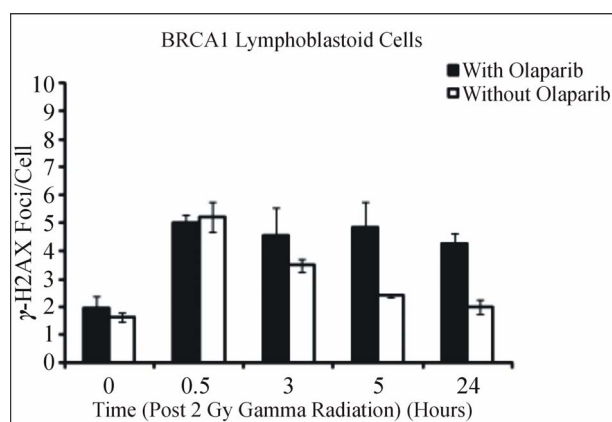


Figure 3. Figure 3 shows the induction of γ -H2AX foci in three *BRCA1* mono-allelic lymphoblastoid cell lines GM13705, GM14090 and GM16105 plotted as an average graph. Data are derived from enumerating the foci in the images of approximately 10,000 cells captured during imaging flow cytometry. Cells were treated with 2 Gy gamma radiation in the presence (black columns) or absence (white columns) of 5 μ m Olaparib. Foci were measured in untreated cells and those irradiated with 2 Gy gamma radiation at 0.5, 3, 5 and 24 hours post-irradiation. The data demonstrate a slight but significant retention of foci in Olaparib treated cells over a 24 hour period (ANOVA $P > 0.05$). However by comparing foci retention at 24 hours post-irradiation alone there is a significant retention of foci in the presence of Olaparib (Student's *t*-Test $P > 0.05$).

4. Discussion

Radiotherapy is routinely employed in the clinical management of cancer, frequently in conjunction with chemotherapy and/or surgery. The ionising radiation used in treatment creates single- and double-strand breaks in the DNA of both normal and tumour cells which if unrepaired can induce cell death. PARP inhibitors are chemotherapeutic agents prescribed to patients to accentuate the ionising radiation-induced DNA damage in tumour cells by inhibiting short-patch base-excision repair of DNA single-strand breaks. At replication forks where a SSB is encountered, the inhibition of PARP-1 by a drug such as Olaparib results in a DNA DSB [2], thus exacerbating radiation-induced DNA damage. These drugs are particularly effective in tumour cells homozygous for a mutation in the tumour suppressor genes *BRCA1* and *BRCA2* [16].

BRCA1 and *BRCA2* proteins are involved in NHEJ and HR. *BRCA1* is a versatile protein, participating in both the NHEJ and HR pathways, whereas *BRCA2* is restricted to HR. Hence cells where mutations in the *BRCA* genes have produced proteins with very little or no active function are likely to have reduced DSB repair capacity.

NTT is the manifestation of damage to normal, non-cancerous tissue as a side-effect of anti-cancer therapy. There is no evidence to suggest that PARP inhibitors

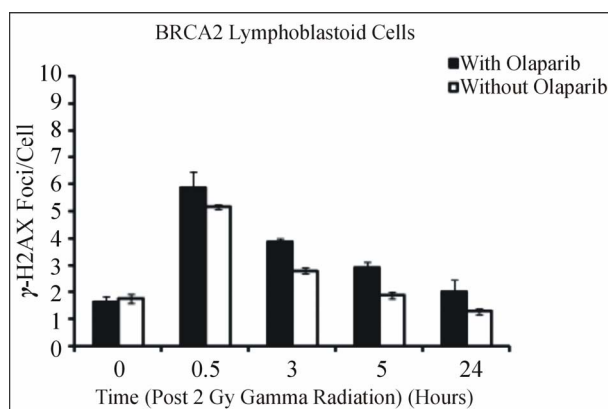


Figure 4. Figure 4 shows the induction of γ -H2AX foci in three *BRCA2* mono-allelic lymphoblastoid cell lines GM14170, GM14622 and GM14805 plotted as an average graph. Data are derived from enumerating the foci in the images of approximately 10,000 cells captured during imaging flow cytometry. Cells were treated with 2 Gy gamma radiation in the presence (black columns) or absence (white columns) of 5 μ m Olaparib. Foci were measured in untreated cells and those irradiated with 2 Gy gamma radiation at 0.5, 3, 5 and 24 hours post-irradiation. The data demonstrate a slight but significant retention of foci in Olaparib treated cells over a 24 hour period (ANOVA $P > 0.05$). However by comparing 24 hours post-irradiation time point alone, there was no significant difference in foci retention after irradiation with or without Olaparib (Student's unpaired t -Test $P < 0.05$).

used as a single modality of treatment affect the survival of cells heterozygous for mutations in *BRCA1* and *BRCA2* *i.e.* non-cancer cells [20]. However, PARP inhibitors used in conjunction with other forms of cytotoxic therapy (such as radiotherapy) may cause significant toxicity in these heterozygous/non-cancer tissue cells [17]. This has important implications for the treatment of those cancer patients carrying a *BRCA1* or *BRCA2* mutation; in a previous paper we cautioned that such patients may be susceptible to elevated levels of NTT [17].

The previous study examined the response to ionising radiation of lymphoblastoid cells mono-allelic for either *BRCA1* or *BRCA2* mutations, when the cells had been exposed to the PARP inhibitor Olaparib. Cell survival was measured using the MTT assay. The combined cytotoxic treatment did not affect cell survival in normal or *BRCA2* heterozygote cells, but significantly enhanced cell death in the *BRCA1* heterozygote cells.

We hypothesised that the enhanced cellular radiation sensitivity of *BRCA1* heterozygous cells exposed to Olaparib was due to DSB retention.

The number of foci per cell in each of the cell lines was measured over a time course of 24 hours post-irradiation, and the results combined depending on cell type (normal, *BRCA1*-mutated and *BRCA2*-mutated). The number of foci per cell in all cell types, both treated with

Olaparib and untreated, increased considerably 30 minutes post-irradiation, then declined over the time course as the DSB were repaired and the γ -H2AX proteins were de-phosphorylated. We did observe a slight retention of foci in all cell types at the 24 hour time point in those cells treated with Olaparib. The exception to this trend was observed in the *BRCA1*-mutated cells treated with Olaparib. They retained almost the same number of foci per cell over the 24 hours, whereas over the same time period the number of foci per cell in untreated *BRCA1*-mutated cells decreased almost to pre-irradiated levels. This confirms our hypothesis that the enhanced cell death detected by the MTT assay in heterozygous *BRCA1* cells was a result of unrepaired strand breaks in the DNA of these cells.

The reasons as to why this enhanced cell death is observed in heterozygous *BRCA1* cells treated with Olaparib, when compared with untreated *BRCA1* cells, is likely due to the specific functions of the PARP-1 inhibitor Olaparib and the *BRCA1* protein. The DNA single strand breaks created by exposure to ionising radiation are repaired by the BER pathway which requires the action of the PARP-1 enzyme. Inhibition of this enzyme by a drug such as Olaparib means SSB remain unrepaired. Furthermore, SSB are converted to DSB at replication fork sites, thus triggering the DNA DSB repair pathway (which includes the phosphorylation of the histone protein H2AX to γ -H2AX), and the formation of γ -H2AX foci [21].

The *BRCA1* protein has a variety of roles within DNA DSB repair, both in NHEJ and HR, and also interacts with tumour suppressor genes (e.g. p53) and other cell cycle regulators. The ubiquitous nature of the *BRCA1* protein in DNA repair means that a mutated/non-functioning protein may severely inhibit effective repair in both cycling and non-cycling cells.

Another noticeable difference in the results arises from comparing the number of foci per cell detected in the normal cell lines with that reported in the *BRCA*-mutated cell lines. It can be seen from **Figures 1-3** that normal cell lines report more foci per cell (and therefore more DNA DSB) at 30 minutes post-irradiation than either of the *BRCA*-mutated cell lines. There may be no single reason for this initially high level of reported DSB in normal cells, but a possible cause for this phenomenon includes the role of the *BRCA1* protein in signal mediation immediately post-damage. Hence mutated *BRCA1* proteins may inhibit the reporting of DNA damage in irradiated cells, although that does not explain the relatively low level of foci per cell in *BRCA2*-mutated cells 30 minutes post-irradiation. There may well be other, unidentified, reasons as to why this phenomenon occurs.

5. Conclusion

In summary, this fundamental study of DSB repair kinetics in a collection of lymphoblastoid cells mono-allelic for *BRCA1* and *BRCA2* indicates that the enhanced radiation sensitivity of *BRCA1* heterozygous cells to radiation in the presence of Olaparib is caused by a persistence of DNA DSB. We reiterate that cancer patients with *BRCA1* mutations may experience unexpectedly severe NTT when treated with radiotherapy and PARP inhibitors.

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Abbreviations

ATM: Ataxia Telangiectasia Mutated Gene

BER: Base Excision Repair

BRCA1: Breast Cancer Susceptibility Gene 1

BRCA2: Breast Cancer Susceptibility Gene 2

DNA: Deoxyribose Nucleic Acid

DRAQ 5: 1,5-Bis{[2-(di-methylamino) ethyl]amino}-4,8-dihydroxyanthracene-9, 10-dione

DSB: Double Strand Break

Gy: Gray

HR: Homologous Recombination

MRN: Mre11-Rad50-Nbs1 Complex

NHEJ: Non Homologous End Joining

NTT: Normal Tissue Toxicity

PARP: Poly(ADP-ribose)polymerase

PBS: Phosphate Buffered Saline