Tumor cells can’t stand the heat

Boosting the effectiveness of hyperthermia in cervical carcinoma

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Chapter 3

Hyperthermia affects both BRCA2-proficient and BRCA2-deficient cell lines

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Under Revision
Abstract

Poly(ADP-ribose)polymerase1 (PARP1) is an important enzyme in regulating DNA replication. Inhibitors of PARP1 can lead to collapsed DNA forks which cause genomic instability. This makes the DNA more susceptible to the development of fatal DNA double strand breaks. PARP1-induced DNA damage is generally repaired by homologous recombination (HR), for which BRCA2 proteins are essential. Therefore, BRCA2-deficient tumor cells are more susceptible to treatment with PARP1-inhibitors (PARP1-i). Recently it has been shown that BRCA2 is temporarily downregulated by hyperthermia (HT), thereby inactivating HR for several hours.

In the present study we investigated whether HT exclusively interferes with HR by testing the hyperthermic radiosensitivity on BRCA2-proficient and deficient cells. Moreover, the effectiveness of HT in BRCA2-proficient and deficient cells was compared. After elucidating the equitoxicity of PARP1-i on BRCA2-proficient and deficient cells, cell survival, apoptosis, DNA damages (γ-H2AX foci and comet assay) and cell cycle distribution after different treatments were investigated. The study confirmed that sensitivity to PARP1-i strongly depends on the BRCA2 status. Both BRCA2-proficient and deficient cells show radiosensitization by HT, indicating that HT does not exclusively act by inhibition of HR. In all cell lines, the addition of HT to radiotherapy and PARP1-i resulted in the lowest cell survival, the highest levels of DNA damages and apoptotic levels compared to the duo-modality treatments. In conclusion, HT not only inhibits HR, HT is also capable of radiosensitizing BRCA2-deficient cells.

Novelty and Impact

PARP1-i are currently under investigation in clinical trials for BRCA2-mutation (HR-deficient) carriers to induce synthetic lethality. Hyperthermia interferes with HR repair, but remarkably, BRCA2-deficient cell lines still show radiosensitization by hyperthermia. Thus, combining hyperthermia with PARP1-i may boost the effectiveness of treatments for BRCA2-mutation carriers and combination therapy could give the opportunity to treat all patient with PARP1-i regardless of their BRCA status.
Introduction

DNA is replicated during the S-phase of the cell cycle. Replication is initiated by a helicase that unzips DNA strands, creating a replication fork. At the Y-shaped site of the replication fork, close to the unwounded position, multiple proteins restore DNA damages to prevent genome instability, before DNA replication may proceed (Branzei & Foiani, 2010). If DNA replication is compromised, DNA damages, such as single strand breaks (SSBs) may occur. One of the proteins that is recruited at sites of stalled forks is Poly(ADP-ribose) polymerase1 (PARP1), which consequently binds to the DNA and together with other repair proteins, it can repair DNA damages and restart replication at the stalled forks (Satoh & Lindahl, 1992). If PARP1 cannot cope with these SSBs during this semi-conservative repair process, these SSBs can be converted to DNA double strand breaks (DSBs) or maybe even better referred to as a double strand end (DSE) (Shrivastav et al, 2008), which will primarily activate homologous recombination (HR) to repair DNA damages in the S- and G2-phases of the cell cycle (Essers et al, 2000; Rothstein et al, 2000). Thus, as PARP1 is required for regulating replication integrity, interference by PARP1-inhibitors (PARP1-i) gives rise to DSEs caused by failures during DNA replication.

Subsequently, if DNA damages caused by inactive PARP1 cannot be restored by the generally used backup mechanism HR, synthetic lethality is induced. For this reason, PARP1-i are under investigation for treatment of HR-deficient tumors or more specifically BRCA2-mutated tumors, as the BRCA2 proteins are essential in HR (Bryant et al, 2005; Eppink et al, 2012; Farmer et al, 2005).

Hyperthermia (HT) - heating the tumor within a range of 40-42.5°C for approximately 1 hour - has multiple anti-cancer effects (Falk & Issels, 2001; Horsman & Overgaard, 2007; Oei et al, 2015a; Oei et al, 2015b; Oleson & Dewhirst, 1983), like stimulating the heat shock proteins, triggering the immune system and creating a window for a few hours, during which the activity of BRCA2 is suppressed by downregulation of the protein (Krawczyk et al, 2011), resulting in an increased number of unrepaired radiation-induced DSBs (El-Awady et al, 2001). HT is an established clinically therapy that in combination with radiotherapy or chemotherapy has proven to improve local tumor control and survival for a selected number of tumors (Datta et al, 2016a; Datta et al, 2016b; Issels, 2008; Snider et al, 2016; van der Zee et al, 2000). In the present study, radiosensitization by hyperthermia was tested in both BRCA2-proficient and deficient tumor cells. Since BRCA2 is a pivotal protein in HR, it can be hypothesized that HT specifically interferes with DNA repair by HR (Krawczyk et al, 2011), although it is not clear whether HT may also affects other DNA repair pathways, i.e. non-homologous end joining (NHEJ) (Oei et al, 2015b). Moreover, discussion is ongoing whether HT induces DNA breaks (Kampinga & Laszlo, 2005) or not (Velichko et al, 2012).
A very common technique to quantify the amount and the presence of DNA double strand breaks is the γ-H2AX foci staining. Immediately when a double strand break is formed, histone proteins H2AX is phosphorylated by a kinase ATM (ataxia telangiectasia mutated) at the site of this break. This phosphorylated histone H2AX (called γ-H2AX) signals DNA repair proteins to restore the DNA breaks. Remarkably, after HT, a slight induction of γ-H2AX foci can already be found. It is still a question whether these are actual double strand breaks, or that HT induces phosphorylation of histone H2AX proteins, and thus leads to false positive signals.

In this study, the impact of HT and PARP1-i on DNA repair mechanisms is investigated by subjecting BRCA2-proficient and deficient cells to different treatment combinations with RT, PARP1-i and HT. In particular, we studied whether HT only inhibits HR, by exploring the combinational treatment of HT and PARP1-i in both BRCA2-proficient and deficient cells, and by testing whether HT induces DSBs.

Results

BRCA2-deficient cells lack homologous recombination which makes them sensitive to PARP1-i
In both BRCA2-proficient and deficient cells, a few spontaneous DSBs were found in untreated cells (Figure 1A). Ionizing radiation (2 Gy) increased the number of γ-H2AX. In BRCA2-deficient cells, no co-localization with RAD51 was found, whereas co-localization of γ-H2AX and RAD51 was observed in the BRCA2-proficient cells. After combination of HT with ionizing radiation, no co-localization of γ-H2AX and RAD51 is observed.

In order to determine the sensitivity of BRCA2-proficient and deficient cells, first, cells were treated with different doses of PARP1-i and cell survival was studied using clonogenic assay (Figure 1B). Consistent results were obtained in both BRCA2-deficient cells (KB2p1.21 and KB2p3.4) and both BRCA2-proficient cell lines (KB2p1.21R and KB2p3.4R3), demonstrating an approximately 60% cell survival after a single treatment with 25 μM PARP1-i in BRCA2-deficient cells and after 50 μM PARP1-i in BRCA2-proficient cells. These equitoxic concentrations were used in further experiments.

BRCA2-deficient cells are sensitive to HT
To study whether BRCA2-deficient cells can be radiosensitized by HT, cells were exposed to combinational therapy of ionizing radiation and HT. Cell survival was studied after ionizing radiation and ionizing radiation with HT. Figure 2 demonstrates that both BRCA2-proficient and deficient cells have lower cell survival after combination with HT. The changes in values of the LQ-parameters,
α and β, induced by HT treatment are of the same order of magnitude for both BRCA2-proficient and deficient cells, indicating that radiosensitization by HT is also significant in BRCA2-deficient cells wherein HR is disabled (Table 1).

Figure 1. Sensitivity of cells to PARP1-i. (A) In BRCA2-deficient cells, no accumulation of RAD51 is observed at sites of a double strand breaks after ionizing radiation. In BRCA2-proficient cells, there is colocalization of γ-H2AX (red) and RAD51 (green). Scale bar indicates 10 μm (B) To find an equitoxicity for PARP1-i, cells are exposed to different doses of PARP1-i. From left to right: KB2p1.21 and KB2p3.4 (BRCA2-deficient cells), KB2p1.21R and KB2p3.4R3 (BRCA2-proficient cells). An equitoxicity of ~60% cell survival was found after treatment with 25 μM PARP1-i in BRCA2-deficient cells and 50 μM PARP1-i in BRCA2-proficient cells. Experiments were performed in duplicates.
A 90-min incubation with PARP1-i prior to ionizing radiation resulted in a lower cell survival in the BRCA2-proficient and deficient cell lines, with a larger effect in BRCA2-deficient cells, although the differences are not pronounced. The triple treatment with PARP1-i prior to ionizing radiation and HT caused the most cell kill in all cell lines, especially in BRCA2-proficient cell lines.

DNA damage in BRCA2-deficient cells is enhanced by HT

The comet assay was used to study whether BRCA2-deficient cells have increased DNA damages after treatments with PARP1-i, HT and ionizing radiation and to test if HT directly induced DNA damages or not (Figure 3A). By measuring the induction of DNA damages in all cells, the effectiveness in

![Graphs showing cell survival fraction against dose for different treatments.]

Figure 2. BRCA2-deficient cells demonstrate hyperthermic radiosensitivity. In BRCA2-deficient cells (top panel) a lower cell survival is observed after addition of HT (42°C for 1 hour) to ionizing radiation. Addition of PARP1-i (25 μM for BRCA2-deficient cells and 50 μM for BRCA2-proficient cells) to ionizing radiation demonstrated a slightly lower cell kill compared to the combinational treatment of HT and ionizing radiation. The BRCA2-proficient cell lines (bottom panel) showed some additional effect of PARP1-i to ionizing radiation, while the effects of HT are more pronounced. The triple combination resulted in the highest cell kill. Means of at least three independent experiment with the standard error of the mean.
Table 1. Linear-quadratic (LQ) parameters for cell survival after various treatments

<table>
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* α (Gy⁻¹) and β (Gy⁻²)
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A

B

C

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Figure 3. Higher induction of DNA damages in BRCA2-deficient cells after treatment with PARP1-i. (A) Different treatments have different effects on DNA damages. Representative cells of treated KB2P3.4R are shown to demonstrate the effects of the comet assay. Scale bar indicates 10 μm. (B) Quantification of at least 100 cells are measured, which are presented by the mean with the standard error of the mean. Ionizing radiation (RT) has a strong effect on induction DNA breaks, HT hardly induces any DNA breaks, and PARP1-i is more effective in BRCA2-deficient cells than in BRCA2-proficient cells. Combinational treatments all have the ability to create DNA breaks, but the longest tails are observed after treatment including RT. Treatment with HT and PARP1-i has the most effects on BRCA2-deficient cells. (C) DNA DSBs can also be monitored with a γ-H2AX foci staining. Representative cells of treated KB2P1.21(R) illustrate the effects of various treatments. (D) The bar graphs demonstrate the number of γ-H2AX foci is counted for three independently performed experiments and per experiment a minimum of 100 cells were counted, which is presented by mean with the standard error of the mean. Scale bar indicates 5 μm.
sensitivity to PARP1-i and HT was tested. In Figure 3A, representative comets of treated KB2p3.4R cells are shown. Irradiation to 4 Gy highly increased the tail length, while HT alone did not induce any tail. Quantification of at least 100 cells per treatment are performed and illustrated in Figure 3B. Cells were treated with various combinations of HT (42°C for 1 hour), PARP1-i at the doses obtained from Figure 1 and a single dose of 4 Gy (RT). In the top lane irradiated treated BRCA2-deficient cells show a 15-20 times longer tail length than the controls without RT. Whereas HT does not increase the tail length, a 72-hour incubation with PARP1-i increases the tail length almost to comparable levels as RT. Any combinational treatment of these cells with either RT or PARP1-i shows almost similar lengths of tails as RT or PARP1-i alone. In BRCA2-proficient cells, shown in the bottom lane, a single treatment with 4 Gy also demonstrate a tail, while HT alone does not. PARP1-i as a monotherapy induced shorter tails and the triple treatment induced the longest nuclear tails.

Figure 4. G₂-arrest after addition of PARP1-i to HT and ionizing radiation. Cell cycle was determined after BrdU incorporation using FACS analysis. HT and HT+RT and showed a clear increase in cells in G₂-phase. Addition of PARP1-i to HT+RT increased the level of cells in G₂-phase even more.
The remaining number of DNA DSBs, 24 hours after treatment, was also tested by γ-H2AX foci staining (Figure 3C). Nuclei are stained in blue and each red γ-H2AX foci represents one double strand break. The results show for BRCA2-deficient cells (top lane) a rather low background number of foci spots (~8 per cell) and a clear increase after 2 Gy irradiation (Figure 3D). After HT only a minor increase in γ-H2AX foci is found, whereas treatment with PARP1-i demonstrates a quite high induction of γ-H2AX foci. The dual modality treatments with ionizing radiation (RT and HT or RT and PARP1-i) demonstrated an even higher number of γ-H2AX foci, while HT and PARP1-i showed less DSBs. The triple modality had the highest number of remaining γ-H2AX foci 24 hours after treatment.

Figure 5. Induction of apoptosis after HT was most pronounced in BRCA2-proficient cells. Apoptotic levels were measured by the Nicoletti assay. Both RT and HT are able to induce increased apoptotic levels in BRCA2-deficient cells. However, PARP1-i hardly increased apoptosis in the BRCA2-deficient cells. The dual and triple modality all increased apoptotic levels. In BRCA2-proficient cells, considering the monotherapies, only HT had the ability to induce apoptosis. The dual modalities were all able to induce apoptosis, while the triple modality had the most pronounced increase in BRCA2-proficient cells.
The BRCA2-proficient cells lines (bottom lane) show a similar trend in remaining γ-H2AX foci.

**Addition of PARP1-i increased G2-arrest after to radiotherapy and hyperthermia, regardless of the BRCA2 status**
At 16 hours post-treatment, cell cycle distribution was measured. In the untreated samples, a high percentage of cells are in the S-phase of the cell cycle (Figure 4). Of all monotherapies, HT had the largest effect in inducing a G2-arrest. Even larger effects were observed for treatment with HT plus ionizing radiation. The most obvious effect was seen after the triple modality in all cell lines.

**Hyperthermia induces apoptosis in BRCA2-deficient cells**
At 48 hours post treatment, apoptotic levels were measured by flow cytometry. Figure 5, top lane: BRCA2-deficient cells treated with 4 Gy, show a high increase of apoptosis compared to controls. Treatment with HT resulted in even higher apoptotic levels, whereas monotherapy with PARP1-i only resulted in a minor increase. Dual and triple modalities increased apoptotic levels in BRCA2-deficient cells. However, apoptotic levels were not higher than after HT alone. Bottom lane: BRCA2-proficient cells treated with RT hardly showed higher levels of apoptosis, while HT was effective to induce apoptosis. Treatment of HT and ionizing radiation in these cells, the triple modality resulted clearly in the highest levels of apoptosis.

![Western blots demonstrating the effects of HR and NHEJ after hyperthermia. Hyperthermia downregulates ligase IV (NHEJ) but not BRCA2 (HR) in BRCA2-deficient cells, and vice versa downregulates BRCA2 (HR) but not ligase IV (NHEJ) in BRCA2-proficient cells.](image-url)
**HT impairs both HR and NHEJ**

Previous experiments had demonstrated that BRCA2-deficient cells can also be sensitised by HT. Now we investigated whether HT also affects NHEJ, the second most important DNA repair pathway. In the BRCA2-deficient cells lines, BRCA2 does not change after HT, while the ligase IV (major protein of the NHEJ mechanism) levels are downregulated after HT (Figure 6). In BRCA2-proficient cells, BRCA2 is degraded, whereas ligase IV is upregulated.

**Discussion and conclusions**

When PARP1, an important protein in regulating DNA replication, is inhibited, the integrity of DNA is salvaged by HR. However, when HR is blocked, for instance by HT, DNA damage will accumulate, resulting in higher cell kill. With the experiments performed in this study we aimed to treat cells with a dose of PARP1-i that caused equitoxicity in both BRCA2-proficient and deficient cell lines. As expected, the equitoxicity dose yielded similar cell survival fractions and an almost similar amount of radiosensitization when PARP1-i was added to ionizing radiation. However, since the doses of PARP1-i differed, BRCA2-deficient cells lines were clearly more sensitive to the combination of PARP1-i with ionizing radiation (Figure 2). Furthermore, the hyperthermic radiosensitization also had an abundant effect on BRCA2-deficient cell lines, indicating that these cells are sensitive to HT as well. This suggests, since HR is not active in BRCA2-deficient cell lines, this dismissed our hypothesis that hyperthermic radiosensitization is solely or not even mainly caused by inhibition of HR, and that other molecular mechanism may be involved. However, the triple modality did not result in a lower survival fraction on KB2p1.21 cells compared to the dual modalities, although in the KB2p3.4 cells there seems to be a slightly additional effect of PARP1 inhibition. In BRCA2-proficient cell lines, radiosensitization by PARP1-i and HT had almost equal effects, but triple modality definitely showed a lower cell survival. However, *in vivo* experiments are necessary to confirm our *in vitro* findings.

The induction of DNA damages, measured by the comet assay, was demonstrated by the long tails after ionizing radiation, indicating the presence of high levels of DNA DSBs (Figure 3A). A treatment with PARP1-i alone, resulted in more pronounced DNA damages in BRCA2-deficient cells than in BRCA2-proficient cells. These findings confirm previously published results (Evers *et al.*, 2010).

What did our present results add to the controversy whether HT induces DNA breaks (Kampinga & Laszlo, 2005; Takahashi *et al.*, 2016) or not (Hunt *et al.*, 2007; Oei *et al.*, 2015b)? We found that HT alone does not induce a
tail in the comet assay, or at most a very minor one, not even in the BRCA2-deficient cells. This suggests that HT does not, at least not directly, induce DNA double strand breaks. Only in the BRCA2-proficient cell lines, the triple modality led to a higher number of DNA double strand breaks. In the KB2p1.21 no significant difference was observed between dual modalities and the triple modality. Thus, addition of HT to PARP1-i and RT or supplementing PARP1-i to the combination of RT+HT did not increase the induction of DNA DSBs. However, in KB2p3.4 there is a slight increase in induction of DNA DSBs compared to dual modalities. To detect the remaining levels of DNA DSBs after DNA repair has taken place, at 24 hours after treatment, γ-H2AX foci were studied (Figure 3C). Likewise, these experiments provide more evidence that HT does not directly induce high levels of major DNA damage.

HT is the only monotherapy that has the ability to highly increase apoptotic levels (Figure 4) and a large G2-arrest (Figure 5). In combination with ionizing radiation, these effects were more pronounced and the largest effects were found after the triple combination. The G2-arrest is more increased in the KB2p3.4 cells compared to the KB2P1.21 cells.

Using γ-H2AX and RAD51 foci stainings, we confirmed that BRCA2-deficient cells do not have functional HR, as a single dose of 2 Gy did not cause co-localization of γ-H2AX and RAD51 in BRCA2-deficient cells, while this did occur in BRCA2-proficient cells. After HT, co-localization disappeared in BRCA2-proficient cells, as HT inhibits the HR, and as a consequence, RAD51 is not accumulated at sites of DNA DSBs (Bergs et al, 2013; Genet et al, 2013; Krawczyk et al, 2011). There seem to be contradictory results on the influences of HT on NHEJ. Whereas several publications demonstrate that NHEJ is upregulated when HR is inhibited by HT (Bergs et al, 2013; van Oorschot et al, 2016), others suggest that HT downregulates the NHEJ (Ihara et al, 2014; Kampinga et al, 2004) and in some cases no differences were found in NHEJ (Woudstra et al, 1999). Moreover, several groups claim that the hyperthermic effects on NHEJ do not play a crucial role in enhancement of hyperthermic radiosensitivity (Kampinga et al, 2004; Woudstra et al, 1999).

According to previous findings and our recent results, it seems that it is cell line and temperature dependent whether the effects on NHEJ are noticeably, up- or downregulated. Our results demonstrate that BRCA2-deficient cells can also be sensitised by HT and affect the NHEJ protein ligase IV in both the BRCA2-deficient and proficient cells. In the BRCA2-deficient cell lines, BRCA2 does not change after HT - as this protein is not functional, while the ligase IV levels are reduced after HT. Perhaps, because these cells rely completely on NHEJ, effects are noticed within this pathway. In BRCA2-proficient cells, on the other hand, BRCA2 is downregulated, while ligase IV is upregulated. Since the BRCA2-proficient cell lines have active HR and NHEJ in untreated samples, when HT downregulated HR, cells that need to restore DNA damages will chose the NHEJ, causing an increase in ligase IV.
Overall, as PARP1 levels are increased after ionizing radiation induced DNA damages (Schiewer et al., 2012; Zaremba et al., 2011), we have shown that PARP1-i is capable of increasing the amount of DNA damages, resulting in reduction of cell survival. Also in BRCA2-proficient cells lines, treatment with PARP1-i resulted in a lower surviving fraction.

Previous studies demonstrated the selective sensitivity of BRCA2-mutated tumors to PARP1-i (Bangham et al., 2016; Tinker & Gelmon, 2012). Here, we demonstrate that BRCA2-deficient cells are also sensitive to HT, resulting in higher levels of unreppaired ionizing radiation-induced DNA damages, resulting in higher cell kill. The BRCA2-proficient cells treated with RT, HT and PARP1-i show higher values of the linear parameter α when compared to BRCA2-deficient cells treated with RT and PARP1-i. These results suggest that PARP1-i may also be advantageous in cancer patients who are non-BRCA2 carriers, if this treatment would also include radiotherapy with HT. The next step towards clinical investigations require *in vivo* confirmation of effectivity and investigation of normal tissue toxicity.

**Competing interests**
Authors declare that they have no competing interests.

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Material and Methods

Cell culture. The mouse mammary cell lines KB2p1.21, KB2p3.4 (BRCA2-deficient) and KB2p1.21R, KB2p3.4R3 (BRCA2-proficient) were established in the Netherlands Cancer Institute (NKI) as described in (Evers et al., 2008; Evers et al., 2010). These cell lines were kindly provided by Jos Jonkers (Netherlands Cancer Institute). These mammary cell lines were grown in DMEM F-12 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 units/mL penicillin, 50 μg/ml streptomycin (Life Technologies), 5 μg/ml insulin (Sigma), 5 ng/mL epidermal growth factor (Life Technologies) and 5 ng/mL cholera toxin (Gentaur). The reconstituted BRCA2-deficient cells line, KB2p1.21R and KB2p3.4R3 were purified by selection with 400 μg/ml hygromycin (Invitrogen). γ-H2AX co-localization to test the BRCA2-proficient cells on their functional BRCA2 reconstitution has been tested before on these cell lines (Evers et al., 2008).

Reagents. First experiment was performed to find equitoxicity to PARP1-i (NU1025, Tocris Bioscience) in both BRCA2-proficient and BRCA2-deficient cell lines. To that end, PARP1-i was dissolved in DMSO to treat cell lines with different single doses (0, 5, 10, 25, 50 and 100 μM). Afterward BRCA2-proficient cells were treated with a single treatment of 50 μM and as BRCA2-deficient cells were more sensitive to PARP1-i they were treated with a dose of 25 μM. PARP1-i was added 30 min prior to HT and 90 min before RT for almost all experiments. Except for the comet assay, in those experiments, PARP1-i was added 72 hours before HT and RT, because the electrophoresis should be performed immediately after RT and cells should undergo cell divisions in order to obtain PARP1-i induced DNA damages.

Hyperthermia. Cells were treated with HT was performed by partially submerging culture dishes in a thermostatically controlled water bath (Lauda aqua line AL12, Beun de Ronde, Abcoude, The Netherlands) for 1 h at 42°C. Temperature check was performed in parallel dishes and the desired temperature (±0.1°C) was reached in approximately 5 min. Cells were heated in a 5% CO₂/95% air atmosphere with an air inflow of 2 L/min.

Radiation treatment. Ionizing radiation post PARP1-i and HT was combined with multiple radiation doses (0, 2, 4, 6 and 8 Gy) to study clonogenic cell survival using a gamma-rays from a 137Cs source at a dose rate of approximately 0.5 Gy/min. DNA damages monitored by the comet assay, apoptotic levels measured with the Nicoletti assay and cell cycle analyses were analyzed after combinational treatment of PARP1-i, HT and a single treatment of 4 Gy. γ-H2AX foci was studied after combinational treatments of cells with a single dose of 2 Gy.

Immunohistochemical detection of γ-H2AX. To study the effects of combinational treatments in the number of DSBs, γ-H2AX foci assay was conducted. Cells were seeded at a density of 3.0 x 10⁵ cells on top of a sterile cover slip (21 x 26 mm) in a 6 cm culture dish. Approximately 2 hours after cell seeding, medium was added to a total volume of 3 ml per dish. The next day, cells were treated with PARP1-i, HT and or irradiated and again incubated at 37°C. On the third day, approximately 24 hours after irradiation, cells were washed with PBS before a 15-min fixation with 2% paraformaldehyde (PFA). After removing PFA with three times washing step of PBS, cells were permeabilized for
30 min with TNBS (PB containing 0.1% Triton X-100 and 1% FCS) at room temperature. Afterwards 50 μl per sample of the primary mouse monoclonal antibody anti-γ-H2AX (Millipore, diluted 1:100 in TNBS) was added for 90 min under a parafilm, again at room temperature. Next, parafilm was removed and cells were washing once with PBS and twice with TNBS. Subsequently, a 30-min incubation with 50 μl per sample of the secondary goat-anti-mouse-Cy3 antibody (Joackson-Immunoresearch, diluted 1:100 in TNBS) covered with a parafilm, was performed. Again, at room temperature, but cells were kept in the dark. After removing the parafilm, cells were washed with TNBS, three times and finally to stain the nuclei, 50 μl of DAPI (2.5 μg/ml. Life technologies, USA) were added per sample for approximately 10 min. Eventually cells were washed once with PBS and coverslips were transferred upside down to microscope cover slides, before scoring foci under the fluorescence microscope (Leica). After washing, cells were incubated with TNBS for 30 min. Finally, vectashield with DAPI was dropped at the slide and the coverslip is turned upside down on the slide, for scoring under the fluorescence microscope.

Clonogenic assays. Cell survival was studied on BRCA2-proficient and BRCA2-deficient mammary cells lines after different treatment combinations; ionizing radiation (0, 2, 4, 6, and 8 Gy), HT (1 h at 42°C) and PARP1-i (50 μM for BRCA2-proficient cells and 25 μM in BRCA2-deficient cells). Clonogenic assays were performed as described by Franken et al (Franken et al, 2006). First, cells were seeded 4 hours prior to treatment into 6-well culture plates (Costar, USA). The 6-well plates were placed in an incubator with 5% CO2 at 37°C until sufficiently large clones were formed (approximately 12-16 days). Afterwards the medium was removed and cells were washed once with PBS. A 2-3 ml of a 6.0% glutaraldehyde and 0.5% crystal violet mixture was added for at least 30 min at room temperature. Next, dishes were washed with water and dried in normal air at room temperature. Colonies were counted under a light microscope (Bergs et al, 2006) and by dividing the plating efficiency of treated cells by that of control cells, surviving fractions were calculated (van Bree et al, 2001). Survival curves were analyzed using the linear-quadratic formula: S(D)/S(0)=exp-(αD+βD²) (Barendsen, 1997). S(D) is the survival at dose D and S(0) is the survival at dose 0.

Comet assay. The comet assay is a gel electrophoretic-based technique to explore DNA damage in individual cells. As DNA can get compromised by exposure to therapies, DNA breaks can occur. By permeabilizing cells with a lysis buffer broken DNA ends can migrate out of the nucleus, and the negatively charged DNA breaks will migrate toward the anode during a 20-min electrophoresis. More damages will create a longer tail. Intact DNA is too large to migrate and therefore no or very minor tails may be observed. The comet assay if performed as in described by Olive and colleagues (Olive & Banath, 2006). First, 1% low-gelling-temperature agarose was mixed in distilled water, heated in a ~100°C water bath until it is completely dissolved (see-through). Next, the glass beaker is transferred to a 40°C water bath to keep the agarose warm. Dust-free frosted-end microscope slides are pre-coated with this agarose mixture. 500 μl of agarose is pipetted on the slide, covered with a coverslip (21 x 60 mm) and kept overnight in the fridge. The next day, after samples are treated, a 0,5% agarose mixture is prepared in the same way as before. After the agarose mixture is transferred to the 40°C water bath, a cell pellet with approximately 50,000 cells is resuspended in 200 μl of agarose and pipetted on top of the pre-coated slide.
Again, this is immediately covered with a coverslip (21 x 60 mm) and kept in the fridge for approximately 15 min. Next, cover slips are removed and cells are lysed in an alkaline lysis buffer (1.2 M NaCl, 100 mM Na₂EDTA, 0.1% sodium lauryl sarcosinate, 0.26 NaOH, pH > 13) for 60 min in the fridge. Subsequently, microscope slides are transferred into an electrophoresis system and run for 20 min at 180 V. Finally, PI (2.5 μg/ml) was added on top of the slides before scoring cells under the fluorescence microscope (Leica).

**Cell cycle analysis.** Cell cycle distribution was studied by incorporation of the thymidine analogue 5-Bromo-2'-deoxy-uridine (BrdU, Sigma Aldrich, USA) in S-phase cells (60 min at 37°C). A 1-hour incubation at 37°C with BrdU was performed 16 h after treatment and 1 h prior to fixation in 2 ml PBS and 6 ml of 100% ethanol. Next, cells were centrifugated at 1200 rpm for 5 min. Pellets were resuspended in pepsin-HCl (0.4 mg/ml, 0.1 N HCl) and cells were stored 30 min at room temperature. Cells were washed with PBT (0.5% Tween-20, Sigma Aldrich USA in 0.5 l PBS) and centrifugated afterwards. Pellets were resuspended in HCl (2 N, Merck) and cells were incubated 30 min at 37°C. Subsequently, cells were washed twice with PBS and once with PBTb (1% bovine serum albumin, Sigma, in PBT). After the last centrifugation, 100 μl primary antibody rat-anti-BrdU (Abcam, UK) in PBTb was added to the pellets for 1 h at 37°C. Next, cells were washed twice with PBS and once with PBTg (1% normal goat serum, Dako, USA, in PBT). After the last centrifugation, 100 μl IgG goat-anti-rat FITC (Abcam, UK) in PBTg was added and left for 1 h at 37°C. Again, cells were washed twice with PBS and finally, cells were resuspended in 500 μl and PI (2.5 μg/ml Sigma-Aldrich, USA) was added, cell suspensions were vortexed and directly measured using flow cytometry (FACS Canto, BD Biosciences, USA).

**Apoptosis assay.** To study apoptotic levels in cells after several combinational treatments, the Nicoletti assay (Riccardi & Nicoletti, 2006) was conducted in all cell lines. At 48 h after treatment cells, medium was collected in one 15 ml tube per treatment. Cells were washed with PBS and this was also collected in the same 15 ml. After trypsinization, cells were neutralized with and resuspended in the medium of the tubes. Next, cells were centrifugated at 1200 rpm for 2 min. Eventually pellets were resuspended in Nicoletti buffer (0.1% w/v Sodium citrate, 0.1% v/v Triton X-100 in demi water, pH 7.4), before analyzing samples using flow cytometry (FACS Canto, BD Biosciences, USA).

**Western blotting.** The effects of hyperthermia on HR or NHEJ were studies using western blotting. To this end BRCA2, a protein from the HR and ligase IV, a member of the NHEJ were tested on their response to hyperthermia. Controls and treated cells were harvested 30 min after treatment. Pellets were lysed for 30 min on ice in ice-cold RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin) containing protein inhibitors (van Bree et al, 2004). Laemmli buffer with 2-mercaptoethanol (355 mM) was added to the supernatant (1:1), before heated in boiling water for 2-5 min. Finally, samples were sonificated (Sonic & Materials Inc). One μg of protein was resolved by 4-15% SDS-PAGE gradient precast gels (BioRad) and transferred to PVDF membranes. Equal protein loading was checked by Ponceau S staining.
Immunodetection was performed for anti-BRCA2 (antibodies-online), anti-ligase IV (abcam), anti-ERK2 (Bethyl Laboratories) and a secondary anti-rabbit (Invitrogen Life Technologies). All samples were enhanced using chemoluminescence (Amersham Pharmacia Biotech). Eventually, blots were analyzed using LAS4000 (GE, Healthcare life sciences).

**Statistics.** All represented *in vitro* data have been performed at least three times, represented with ± standard error of the mean. SPSS (Chicago, IL, USA) was used to analyze cell survival and apoptosis, using statistical software using a non-parametric Mann-Whitney test.
References


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