Tumor control and normal tissue toxicity: The two faces of radiotherapy

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Citation for published version (APA):
CHAPTER 3

Targeting DNA Double Strand Break Repair to Enhance the Effect of Radiation Treatment

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under review
ABSTRACT
Radiotherapy is based on the induction of lethal DNA damage, primarily DNA double-strand breaks (DSBs). However, effectiveness of therapy is reduced by efficient DSBs repair via Non-Homologous End Joining or Homologous Recombination. This study aims to enhance the radiation effect by suppressing DNA-DSB repair with hyperthermia (HT) and DNA-PKcs inhibitor NU7441 (DNA-PKcsi).

The sensitizing effect of HT for 1 hour at 42°C and DNA-PKcsi [1μM] to radiation treatment was investigated in cervical- and breast cancer cells, primary breast cancer stem cells (BCSCs) and in an in vivo human tumor model. A significant radio-enhancement effect of DNA-PKcsi and HT was observed for all cell types, and combination of both enhanced radiosensitivity to an even greater extent. Strikingly, combined treatment resulted in significant lower survival rates, 2 to 2.5 fold increase of apoptosis, more residual DNA-DSBs 6h post treatment and G2-phase arrest. In addition, tumor growth analysis in vivo showed significant reduction in tumor growth and elevated CASP3 activity after radiation combined with HT and DNA-PKcsi compared to radiation alone. Importantly, no toxic side effects of HT or DNA-PKcsi were found.

In conclusion, hampering DNA-DSB repair using HT and DNA-PKcsi before radiotherapy leads to enhanced cytotoxicity in cancer cells. This effect was even noticed in the more radio-resistant CSCs, which are clearly sensitized by combined treatment. Therefore, the addition of HT and DNA-PKcsi to conventional radiotherapy is promising and might contribute to a more efficient tumor control and patient outcome.
INTRODUCTION
The working mechanism of many anti-cancer treatments, including ionizing radiation, is the induction of lethal DNA double strand breaks (DSBs) [1, 2]. The more rapidly dividing tumor cells are thought to be more sensitive to ionizing radiation than healthy cells, and their subsequent DNA damage response less efficient[2]. However, tumor cells can still repair the induced DSBs thereby undermining the effectiveness of therapy. Furthermore, some tumor cells are thought to be less sensitive to radiation treatment[3], i.e. the cancer stem cells (CSC), which might resist therapy or repair DNA breaks more efficiently[4]. Therefore, a suggested mechanism to sensitize tumor cells and cancer stem cells to radiation is the inhibition of DNA-DSB repair proteins [5, 6]. In mammalian cells, DSBs are repaired predominantly by non-homologous end joining (NHEJ) or homologous recombination (HR) [7, 8]. A complex cascade of reactions is initiated after a DSB has been induced. ATM kinase and the Mre11/Rad50/NBS1 (MRN) complex are triggered and subsequently the histone protein H2AX is phosphorylated at the DSB sites to γ-H2AX, presenting one of the earliest markers of DSBs [9-11]. Other DSB repair proteins, including MDC1, 53BP1 and RAD51 are then attracted to the break ends and, accompanied by γ-H2AX, form ionizing radiation induced foci (IRIF) [12, 13]. After initial recognition, repair of DSBs can be executed. Failure of repair proteins to form IRIF has been linked to damage response deficiencies [14]. Interestingly, several studies correlate the induction and disappearance of γ-H2AX IRIF in vitro with treatment response in tumors and normal tissue [15-20]. The higher the number of induced γ-H2AX foci or slower disappearances rate, the more sensitive tumor cells are to radiation treatment. Furthermore, persisting γ-H2AX IRIF in normal cells 24h after radiation are associated with the development of late severe side effects.

HR requires a homologous DNA sequence to repair the broken strand and therefore is mainly active during the S and G2 phases of the cell cycle when a DNA template is available in the form of a sister chromatid [21]. The major HR factors include Rad51, Rad54, BRCA2 and RPA [22]. In contrast, NHEJ is active during all phases of the cell cycle as it ligates DNA break ends without requiring a homologous sequence. Therefore NHEJ is thought to be the less accurate form of DSBs repair [23]. One of the key proteins in the NHEJ process is DNA-PK. After induction of a DSB, the KU heterodimer, consisting of the KU70 and KU80 proteins, binds DNA break ends and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) leading to formation of the DNA-PK holo-enzyme [21]. DNA-PK then forms a functional complex with Artemis, which provides nucleolytic processing activity required to prepare DNA ends for ligation [24].

Hyperthermia (HT) is currently being used in the clinic and has proven to be a potent sensitizer of radiotherapy and/or chemotherapy [25, 26]. Krawczyck et al. [27] showed that hyperthermia transiently degrades the BRCA2 protein and subsequently abolishes the RAD51 protein to accumulate at the break ends. The inactivation of RAD51 and BRCA2 leads to a temporarily inhibition of the HR repair. However, blocking HR repair could lead to a
compensated NHEJ DSB repair [6, 28]. Therefore, we want to investigate the inhibition of both HR and NHEJ repair pathway. Here, NHEJ repair was inhibited by the specific DNA-PKcs inhibitor NU7441 (DNA-PKcsi) [29, 30]. Results show that a combination of both repair inhibitory modalities clearly enhanced radiosensitivity more than the single treatments, both in experimental cell lines and BCSCs as well as in human tumor mouse models.

MATERIALS AND METHODS

Cell cultures. Human cervical cancer cells: HeLa and SiHa and human breast cancer cells: MCF7 and T47D were obtained from the American Type Culture Collection (ATCC). Primary human BCSCs were obtained by mechanical and enzymatically digestion of breast cancer tissues, collected at the Department of Surgical, Oncological and Stomatological Sciences, in accordance with the ethical standards of the University of Palermo institutional committee, as previously described [31]. HeLa and SiHa cells were routinely cultured in Eagle’s Minimum Essential medium (EMEM, Gibco-brl technologies), MCF7 cells in Dulbecco’s modified Eagles medium (DMEM), and T47D cells in Iscove’s Modified Dulbecco’s medium (IMDM), all supplemented with 8% fetal bovine serum and antibiotic penicillin, streptomycin and glutamine (PSG) at 37°C in a 5% CO₂ humidified chamber. Primary breast cancer cells were cultured as spheroids in serum-free DMEM/F12 medium supplemented with 2% B27 (50X, Gibco), basic fibroblast growth factor (bFGF; 10ng/mL) and EGF (20 ng/mL) in ultra-low attachment flask (Corning).

Irradiation, hyperthermia and DNAPKcsi treatment. Radiation treatment was performed with γ-irradiation using a 137Cs source at a dose rate of about 0.5Gy/min. Levels of apoptosis and cell cycle distribution were measured after 4Gy irradiation, numbers of IRIF were detected after 1Gy, and for clonogenic assay survival analysis cells were irradiated with 0, 2, 4, 6, and 8Gy. Hyperthermia treatment was performed by incubating cells at 42°C for 1h in a thermostatically controlled water bath with additional CO₂. DNA-PKcs was inhibited using specific inhibitor NU7441, also known as KU-57788 (Selleckchem). NU7441 was dissolved in DMSO as 10 mM stock, further diluted in PBS to 1mM and added to culture medium at a final concentration of 1 μM. DNA-PK activity was measured in whole cell lysates from SiHa cells using the promega SignaTECT® DNA dependent protein kinase assay system, according to manufacturer’s protocol (Supplementary Figure S3 C).

In vivo tumor model and xenografts. Human cervical cancer SiHa cells were injected into the right hind leg of Athymic mice. In approximately 4 weeks, tumor volumes of 100 mm3 were reached and mice were divided randomly in 8 groups (n=6), existing of all different treatment combinations and controls. DNA-PKcs inhibitor NU7441 was dissolved in 40% PEG400/Saline and
injected i.p. (10mg/kg) [29] for 4 days before start of HT and RT treatment. For hyperthermia, a water bath system was used were only the right hind leg was treated for 1h at 42°C. HT was applied only on the first day of treatment. Mice were cooled to prevent an increase of the body core temperature, and anesthetized with a mixture of 2.5% isoflurane in oxygen. Radiation treatment was executed for 4 days with a daily dose of 3Gy using a X-ray RS320 Research cabinet (X-Strahl, 210kV, 15mA and 0.5mmCu filter). For tumor growth delay analyses, tumor volumes were measured twice per week and mice were sacrificed when tumor volumes above 1000mm³ were reached. Levels of CASP3 and γ-H2AX were detected in xenografts of mice sacrificed 6h, 24h or 48h after treatment. Per treatment condition, 3 xenografts were analyzed by immunohistochemistry. Animal experiments were approved by the animal welfare committee of the Academic Medical Center (AMC) as required by Dutch law LEX143.

**Clonogenic assay.** Adhering cells were plated in appropriate cell numbers in 6-well macroplates prior to treatment. After attachment, DNA-PKcsi NU7441 or DMSO only (mock treatment) was added and cells were treated with hyperthermia for 1h. Immediately after hyperthermia, cells were irradiated with doses up to 8Gy. Cells were incubated for 10 days to form colonies. After this period surviving colonies were fixated and stained with glutaraldehyde-crystal violet solution and counted manually. Spheroid BCSC cultures were dissociated and FACS deposited using FACSaria (BD Biosciences) in a limiting dilution manner at 5, 10, 25, 50, 100 and 200 cells per well in ultra-low 96-well plates (Corning). After sorting, DNA-PKcsi was added to medium and plates were subjected to HT and RT. Clonal frequency was evaluated with the Extreme Limiting Dilution Analysis ‘limdil’ function as described[32].

**Immunohistochemistry in vitro and in vivo.** Detection and scoring of immunofluorescence γ-H2AX and Rad51 in cell lines was performed as previously described [15, 27]. Xenografts were fixated in 3.6% paraformaldehyde (Aurion) and embedded in paraffin. Sections of 4μm were prepared for detection of both cleaved-Caspase3 and γ-H2AX and heat-induced antigen retrieval was performed at pH 6. CASP3 sections continued with peroxidase blocking for 20 min and serum blocking using Ultra-V (Immunologic) for 5 min. Primary antibody Cleaved-Caspase3 (anti rabbit, Cell Signaling) was applied 1:200 overnight at 4°C. Afterwards, sections were incubated with Powervision Poly-HRP-GAM/R/R IgG (Immunologic) for 30 min and PowerDAB (Immunologic) for 1-2 min, counterstained with haematoxylin (Fluka) and mounted with pertex. For γ-H2AX, sections were blocked after antigen retrieval in PBTB: PBS containing 0.1% Tween20 and 2% Bovine Serum Albumin (BSA) and incubated with primary antibody mouse monoclonal anti- γ-H2AX (Millipore) for 90 min (1:100 in PBTB) at room temperature. Secondary antibody goat anti-mouse Cy3 (Jackson Immunoresearch) was applied for 60min, and DAPI (Sigma-Aldrich) was used as counterstain. After washing, sections were embedded in Vectashield and analyzed.
**Cell Cycle and Apoptosis analyses.** Cell cycle analysis was carried out by flow cytometry using Bromodeoxyuridine (BrdU) and propidium iodide (PI) staining. BrdU (10μM) was administered to cell cultures at 16h after treatment. After 1h at 37°C, cells were harvested and fixed overnight in 70% ethanol in PBS. Fixed cells were centrifuged (1min, 2200 RPM), resuspended in 1ml pepsin-HCL (0.4 mg/ml 0.1N HCL), and incubated for 30 min. PBT (PBS with 0.05% Tween20) was added while vortexing, samples were centrifuged and incubated for 30 min in 1ml 2N HCL at 37°C. After washing with PBTb (PBT with 20 mg/ml BSA), the pellet was resuspended in 100μl rat anti-BrdU (Harlan Seralab) diluted 1:100 in PBTb for 60 min at 37°C. For secondary antibody step, cells were washed with PBTg (PBT with 1% v/v normal goat serum (DAKO)) followed by 60 min incubation at 37°C with 0.1 ml fluorescein conjugated goat anti-rat IgG (Jackson Immunoresearch) diluted 1:100 in PBTg. PI was added to a final concentration of 20μg/ml in PBS and samples were stored at 4°C before flowcytometric (FACS Canto, BD Biosciences) analysis.

**Apoptosis analysis.** The Nicoletti assay [33] was used to study apoptosis in adhering cell lines after different treatments. Cells were harvested 48h post treatment and resuspended in nicoletti buffer (0.1% w/v Sodium Citrate, 0.1% v/v Triton-X in ddH₂O, pH 7.4) and analyzed with flow cytometry (FACS Canto).

**Statistical Analysis.** All experiments were performed at least 3 times, independently, and results are shown as mean ± SD. Survival curves were analyzed using SPSS (Chicago) statistical software by means of fit of data by weighted linear regression, according to the linear-quadratic formula: $S(D)/S(0)=\exp(-\alpha D-\beta D^2)$ [34, 35]. For γ-H2AX and Rad51 foci detection, at least 100 cells per condition per experiment were scored and the data is presented as the mean ± standard error (SEM). GraphPad Prism 6 was used to perform ANOVA analysis, followed by unpaired Student t-test (two tails) for comparison of independent treatments. Significant P values are given, * indicates P<0.05, ** indicate P<0.01 and *** indicate P<0.001. ns indicates non statistically significant.

**RESULTS**

**DNA-PKcsi and hyperthermia sensitize cancer cells and BCSCs to radiation treatment**

Clonogenic survival assays were performed to study whether inhibition of HR in combination with prevention of NHEJ can lead to a more effective therapy. Results demonstrated a clear radio-enhancement when the cells are treated with either DNA-PKcsi or hyperthermia prior to irradiation. Overall lower survival fractions are found with increasing radiation dose in all assessed cell lines (Fig. 1 A-B, Supplementary Fig. S1 A-B).
Figure 1: Cancer cells and CSCs are clearly sensitized to ionizing radiation (RT) by DNA-PKcs inhibition and hyperthermia (HT). A and B: Clonogenic assay with increasing radiation dose after combined treatment in SiHa (A) and MCF7 (B) cells. Survival curves were established using the linear quadratic regression model, corresponding α and β values can be found in Supplementary Table S1. C: Levels of apoptosis 48 h after different combinations of treatment in SiHA cells. D: Levels of apoptosis of MCF7 cells harbouring CASP3 mutation, treated as in (C). E: Limiting dilution analysis for BCSCs treated as in (C). F: Flow cytometer plots presenting results of nicoletti assay in SiHa cells. All experiment were performed at least three times, independently and error bars represent SD.
Moreover, the radiosensitizing effect is observed in an even greater extent when the combination of both treatments is used. In Supplementary Table S1 values of the parameters of the Linear Quadratic model are presented. Hyperthermia, DNA-PKcs\textsuperscript{i} and the combination resulted in a higher induction of unrepairable DNA damage in cells compared to irradiation alone, indicated by the higher $\alpha$-values after combined treatment strategies. Furthermore, the reduced clonogenic survival is confirmed by increasing levels of apoptosis after combination treatments (Fig. 1 C and F, Supplementary Fig. S1 C-D). Importantly radiation alone does not induce apoptosis, but in combination with DNA-PKcs\textsuperscript{i} and HT a strong apoptotic response is detected. As MCF7 cells are deficient of caspase 3, no levels of apoptosis could be measured with Nicoletti assay in this cell line (Fig. 1 D)[36]. Limiting dilution analysis with spheroid BCSCs (Fig. 1 E) showed a significant decrease in clonogenic growth after radiation, and this decrease is further enhanced by HT or DNA-PKcs\textsuperscript{i}. Strikingly, combination of radiation with HT and DNA-PKcs\textsuperscript{i} resulted in a 3-fold reduction in clonogenic capacity compared to radiation alone ($p=0.001$).

**Delayed disappearance of DNA-DSB IRIF after HT and DNA-PKcs\textsuperscript{i}**

To examine whether the radiosensitizing effect of HT and DNA-PKcs\textsuperscript{i} are caused by hampered DNA-DSB repair, numbers of $\gamma$H2AX IRIF were scored at several time points post treatment (Fig. 2 A-C, Supplementary Fig S2 and Supplementary Table S2). The addition of HT and DNA-PKcs\textsuperscript{i} didn’t influence the initial induction of DNA-DSBs after radiation. Similar numbers of $\gamma$H2AX foci per cell are detected at 30min after different treatments for all cell lines, indicating that the amount of radiation-induced damage is the same in all conditions. However, 6h post treatment, significantly higher numbers of foci were found after RT combined with DNA-PKcs\textsuperscript{i} and/or HT compared to RT alone. The average numbers of DNA-DSBs per cell also highlighted the distinct effect of the triple treatment strategy compared to the double (RT with either HT or DNA-PKcs\textsuperscript{i}). Nevertheless, persisting DNA-DSBs seemed to be repaired later on, as no differences in numbers of IRIF are detected at 24h post treatment. Only for HeLa cells, DNA-DSB repair seemed hindered for a longer period as even after 24h significant higher numbers of foci were detected in the triple treatment compared to RT alone (Supplementary Fig S2 A). Mechanistically, a temporarily decrease of Rad51 accumulation at the site of $\gamma$H2AX IRIF (Supplementary Fig. S3) after hyperthermia treatment was indeed observed, and fully restored after 6h.

**Radiosensitization after HT and DNA-PKcs\textsuperscript{i} is accompanied with a G2-phase arrest**

The effect of combined treatment modalities on cell cycle distribution was measured 16h post treatment. In general, ionizing radiation induced an arrest of cell cycle progression in either G1 (SiHa, MCF7) or G2 (HeLa, T47D) phase depending on cell type. Interestingly, the combination of DNA-PKcs\textsuperscript{i} and HT with radiation resulted for all cell types in a marked accumulation of cells in G2 phase (Fig. 3 A-C and Supplementary Fig. S4).
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Figure 2: Persisting ionizing radiation induced foci (IRIF) in SiHa and MCF7 cells after radiation treatment combined with DNA-PKcs/i and HT. A: Visualization of γ-H2AX IRIF 30 min, 6h, and 24h after radiation treatment (1 Gy), HT 42°C and DNA-PKcs/i [1μm]. B-C: quantification of γ-H2AX IRIF in SiHa (B) and MCF7 (C) cells. For each radiation condition at least 100 cells per experiment (n=3) are counted, error bars are ±SEM. Bar is 5μm.

Tumor growth delay in vivo and higher levels of apoptosis in xenografts

To examine whether in vivo tumors can be radiosensitized to this extent as well, the different treatment modalities were investigated in tumors consisting of SiHa cells in athymic mice. DNA DSBs and apoptotic markers were analyzed shortly after treatment, while tumor growth was followed for approximately 30 days. Induction of DNA-DSBs was measured by scoring γ-H2AX IRIFs in xenografts 6h and 24h post treatment. Similar numbers of γ-H2AX foci were detected for all irradiated xenografts 6h post treatment, and after 24h numbers were only slightly reduced. No correlations were found with in vitro IRIF analysis or hindered DNA-DSB repair after DNA-PKcs/i or HT treatment (Fig 4 A-B). In contrast, levels of apoptosis in vivo were induced to a similar extent as measured in the cell lines by the combined treatment. Apoptosis in vivo was measured by the detection of cleaved caspase-3 48 h after treatment. Significant higher levels of cleaved caspase-3 were detected in xenografts that received radiation combined with HT and/or DNA-PKcs/i (See Figure 4 C-D). Interestingly, only in the xenografts of the triple treatment modality,
necrotic regions were observed. Furthermore, the triple treatment, ionizing radiation combined with HT and DNA-PKcsi, resulted in a significant tumor growth delay compared to ionizing radiation alone (p=0.004) and compared to ionizing radiation combined with only HT (p=0.03). In figure 4 E and Supplementary Figure S5, normalized tumor growth curves are presented of all treatment groups.

Figure 3: Radiosensitization of DNA-PKcsi and HT is accompanied with an induced G2/M arrest 16h after radiation. A-B: Cell cycle analysis of SiHa (A) and MCF7 (B) cells treated with radiation, hyperthermia and/or DNA-PKcsi. C: Flow cytometry plots of cell cycle distributions measured by the incorporation of bromodeoxyuridine (BrdU) in SiHa cells. As can be depicted, HT alone also affected SiHa cells in their cell cycle. For MCF7 this effect was not detected. Experiments are performed at least three times, error bars are SD.
Figure 4: Higher levels of apoptosis and clear tumor growth delay in vivo after combination treatments. A-B: Induction of γ-H2AX IRIF in xenografts collected 6h (A) or 24h (B) post treatment. C: Induced levels of apoptosis in xenografts (n=3) treated with radiation in combination with DNA-PKcsi and HT compared to radiation alone. Necrotic areas were observed in xenograft of triple treatment. D: Quantification of Caspase 3 staining in xenografts, as in (A). E: Tumor growth delay analysis after triple treatment compared to radiation alone. Tumor sizes were biweekly measured and normalized to initial size, every treatment group consisted of 6 mice.
DISCUSSION

This study investigated the additional effect of DNA-DSB repair inhibitors added to conventional radiotherapy. Our results show that both HT and DNA-PKcsi enhance the effect of radiation treatment significantly, especially when both modalities are combined. Lower surviving fractions, more residual DNA damage and a G2-phase arrest were detected after combined treatment in all examined cervical – and breast cancer cell lines. Interestingly, the assumed radio-resistant BCSCs were also affected to a higher degree after combined treatment strategies compared to radiation alone. Furthermore, in vivo results verify the importance of adding both HT and DNA-PKcsi to conventional radiation treatment. The highest level of apoptosis was detected and tumor growth was most delayed after the triple treatment strategy.

Results of the clonogenic survival assay showed that inhibiting both DNA DSB repair mechanism has a large radiosensitizing effect. The value of the linear parameter, α, increased with the different treatments being applied (HT, DNA-PKcsi and combined treatment respectively), corresponding with more cell reproductive dead in lower dose regions. Subsequently, the value of the quadratic parameter, β, dropped with each treatment solely and almost no functional DNA DSB repair was observed when both repair pathways are inhibited as the β-value reached zero.

In all cell lines, more persisting DNA-DSBs were detected at 6h after combined treatment modalities compared to radiation treatment alone. At 24h after treatment, numbers of γ-H2AX foci were reduced to numbers found in untreated, control samples, indicating that cells did eventually repair their DNA-DSB breaks. Thus the addition of HT and DNA-PKcs to radiation resulted in a slower loss of foci, e.g. slower repair rate, rather than no repair in the cancer cells who survived treatment. This can be explained by the fact that the effect of hyperthermia and NU7441 are only temporarly. Foci results obtained in this study, showed that Rad51 was detected at the sites of DSBs again 6h after HT treatment (Supplementary Fig S3 D), indicating active HR repair. This is congruent with other studies, which shown that hyperthermia degrades BRCA2 only for a few hours [26, 27]. In addition, Zhao et al [29] showed that the required concentration of NU7441 was only maintained in vitro up to 4 hours, meaning active NHEJ could occur again after 4 hours.

Furthermore, cell cycle analysis revealed an induced G2/M phase arrest after radiation treatment combined with HT and DNA-PKcsi. This is in line with the results of other studies [29, 30, 37], and might be explained by activation of other DNA damage pathways in the absence of HR and NHEJ. For example, activated ATR/Chk1 pathway is related to a profound accumulation in G2 phase in response to RT [38]. In addition, back-up End Joining (B-EJ) processes are also thought to benefit from a G2 arrest [39], allowing more time to repair DSBs.

Several studies examined the sensitizing effect of DNA-PKcsi [29, 30, 37] and HT [26, 27] separately, but the combination of both repair inhibitors has not been tested. As mentioned, blocking one repair pathway is thought to lead to the compensation of other repair pathway
Therefore, inhibiting both at the same time leads to a more complete, and pronounced radiosensitization. *In vitro* and *in vivo* results obtained in this study showed the largest sensitizing effect when both repair mechanisms are inhibited. Especially tumor growth delay and xenografts analysis elucidated the distinct effect of radiation combined with HT and DNA-PKcsi. Furthermore, the possibility of HT and DNA-PKcsi to sensitize BCSCs to radiation treatment is interesting and should be further investigated. CSCs are correlated with radioresistance and poor survival [41], and are thought to have an highly active DNA damage response [42]. Therefore, the inhibition of DSB repair mechanisms by HT and DNA-PKcsi might have an augmented effect in the CSC population of the tumor, leading to a better treatment response in poorly controlled tumors. In conclusion, the results obtained in this study elucidate the use of HT and DNA-PKcsi inhibition together with radiation treatment. The combination treatment enlarges the effect of conventional radiotherapy and is promising for clinical use.

**SUPPLEMENTARY INFORMATION**

Figure S1. Clonogenic survival analysis and apoptosis levels in HeLa and T47D cells.

Figure S2. DNA-DSBs after different treatment combinations in HeLa, T47D and BCSC cells.

Figure S3. Specificity of HT treatment and NU7441.

Figure S4. Cell cycle distributions in untreated and treated HeLa and T47D cells.

Figure S5. Tumor growth delay *in vivo* in untreated and non-irradiated control conditions.

**ACKNOWLEDGEMENTS**

The authors thank Arlene L. Oei for experimental help and input. We are grateful to the Maurits and Anna de Kock and the Nijbakker Morra foundations for sponsoring the fluorescence microscopes with software to study the γ-H2AX foci. The Dutch Cancer Foundation (nos. UVA 2008-4019) and the Stichting Vanderes are acknowledged for financing personnel support. This work was also supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) to Matilde Todaro (AIRC IG 14415). Simone Di Franco is an AIRC Fellowship for Italy recipient.

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Supplementary Figure S1: Clonogenic survival analysis and apoptosis levels after different treatment combinations: clear radiosensitization after triple treatment. A-B: Clonogenic survival analysis for HeLa (A) and T47D (B) cells. C-D: Nicoletti assay performed on HeLa (C) and T47D (D) cells. All experiment were performed at least three times, independently and error bars are SD.
Supplementary Figure S2: More residual DNA-DSBs after radiation treatment combined with HT and DNA-PKcsi indicate for less efficient DNA repair. A-C: Results of γ-H2AX IRIF analysis for HeLa (A), T47D (B) and BCSCs (C) at 30 min, 6h or 24h post treatment.
**Supplementary Figure S3**: Specificity of HT treatment and NU7441. A-B: Significant decrease of colocalisation of Rad51 and γ-H2AX foci directly after treatment with hyperthermia. At least 100 cells per condition were scored, experiment was done in triplicate, bar is 5 μm. C: DNA-PKcs activity is blocked in presence of DNA-PKcs inhibitor NU7441. Hyperthermia doesn’t affect DNA-PKcs activity. D: WB analysis of BRCA2 and DNA-PKcs elucidating the effect of HT treatment on HR pathway regulation.
Supplementary Figure S4: Cell cycle distributions in untreated and treated HeLa (A) and T47D (B) cells. Radiosensitization by HT and DNA-PKcsi. Experiments are performed at least three times, error bars represent SD.

Supplementary Figure S5: Tumor growth delay in vivo in untreated and non-irradiated control conditions. No growth delay between the different conditions could be observed.