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Nitric oxide-donor/PARP-inhibitor combination: A new approach for sensitization to ionizing radiation

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ABSTRACT

Recently, clinical development of PARP inhibitors (PARPi) expanded from using them as a single agent to combining them with DNA-damaging therapy to derive additional therapeutic benefit from stimulated DNA damage. Furthermore, inhibiting PARP in cancers with *BRCA1/2* mutations has been shown to be an effective synthetic lethality approach either as a single agent or in combination with the different DNA damaging agents: chemotherapy or ionizing radiation (IR). However, inherited *BRCA1/2* mutations account only for 5–10% of breast cancers, 10–15% of ovarian cancers, and lesser for the other cancers. Hence, for most of the cancer patients with *BRCA1/2*-proficient tumors, sensitization to DNA-damaging agents with PARPi is significantly less effective. We recently demonstrated that moderate, non-toxic concentrations of NO-donors inhibited *BRCA1* expression, with subsequent inhibition of error-free HRR and increase of error-prone non-homologous end joining (NHEJ). We also demonstrated that the effect of NO-dependent block of *BRCA1* expression can only be achieved in the presence of oxidative stress, a condition that characterizes the tumor microenvironment and is also a potential effect of IR. Hence, NO-donors in combination with PARPi, with effects limited by tumor microenvironment and irradiated area, suggest a precise tumor-targeted approach for radio-sensitization of BRCA1/2-proficient tumors. The combination with NO-donors allows PARPi to be successfully applied to a wider variety of tumors. The present work demonstrates a new drug combination (NO-donors and PARP-inhibitors) which demonstrated a high potency in sensitization of wide variety of tumors to ionizing radiation treatment.

1. Introduction

Current efforts to develop poly (ADP-ribose) polymerase inhibitors (PARPi) as anticancer drugs represent the culmination of over 40 years of research. Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme, essential for the repair of DNA single-strand breaks (SSB) through the base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR) [1–4]. When PARP1 or PARP2 are inhibited, these lesions are unresolved and subsequently converted to DNA double-strand breaks (DSBs) by the cellular replication and/or transcription machinery [5]. Breast cancer type susceptibility proteins (BRCA1/2) are essential for homologous recombination repair (HRR) of DNA DSB. Loss of BRCA1/2 activities, as in BRCA-mutant tumors, leads to genomic instability and chromosomal rearrangements. The genetic interaction between PARP and BRCA can be described as the effect of synthetic lethality. It occurs between two genes where individual loss of either gene's activity is compatible with cell survival, but a simultaneous loss of both genes' activities results in cell death [6]. This suggests that a synthetic lethality mechanism can be used in the treatment of cancer [7] and the PARP-BRCA interaction provides a successful

Abbreviations: BER, base excision repair; BRCA, breast cancer type susceptibility proteins; DSB, double-strand break; FITC, fluorescein isothiocyanate; HRR, homologous recombination repair; IR, ionizing radiation; MMR, mismatch repair; MTM, mean tail moment; NER, nucleotide excision repair; NHEJ, non-homologous end joining; PARP, poly (ADP-ribose) polymerase; PARPi, poly (ADP-ribose) polymerase inhibitor; PI, Propidium Iodide; RBL2, Retinoblastoma-like protein 2; SD, standard deviation; SSB, single-strand break

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synthetic lethal approach for clinical trials [8,9].

Recently, the clinical development of PARPi expanded from using them as a single agent to a combination of PARPi with DNA-damaging therapy to derive additional therapeutic benefit from stimulated DNA damage. The approach of pairing PARPi with DNA-damaging agents to achieve sensitization is based on extensive preclinical studies showing that PARPi enhance the action of methylating agents, topoisomerase poisons, platinum drugs, and ionizing radiation in tumor cell lines *in vitro* and in human tumor xenografts *in vivo* [10–14]. Furthermore, inhibiting PARP in cancers with *BRCA1/2* mutations has been shown to be an effective synthetic lethality approach either as a single agent or in combination with the different chemotherapeutic agents or ionizing radiation (IR). However, inherited *BRCA1/2* mutations account only for 5–10% of breast cancers, 10–15% of ovarian cancers, and lesser for the other cancers. Hence, for most cancer patients with *BRCA1/2*-proficient tumors, sensitization to DNA-damaging agents with PARPi is significantly less effective.

To stimulate sensitization of tumors with the intact DNA HRR, there is a need to establish a new combination of PARPi and the DNA HRR blocking agent. Adding of this agent should not increase the normal tissue toxicity and blocking of DNA HRR by this agent should be limited by the tumor or the irradiated area. One potential class of these agents includes nitric oxide releasing compounds, or NO-donors. These compounds contain different subclasses and are being actively investigated in preclinical and clinical studies in cardiovascular, inflammatory, bacterial, fungal, viral, parasitic, and ocular diseases as well as cancer [15–19]. We recently demonstrated that moderate, non-toxic concentrations of NO-donors inhibited *BRCA1* expression, with subsequent inhibition of error-free HRR and increase of error-prone non-homologous end joining (NHEJ) [20]. NO-donors actively suppress DNA HRR and, in combination with PARPi, potentiate synthetic lethality in *BRCA1/2*-normal cancer cells. In the present work, we demonstrate that NO-donors in combination with PARPi is an effective approach for radio-sensitization of *BRCA1/2*-proficient tumors.

2. Materials and methods

2.1. Cell culture and γ-H2AX immunofluorescence (foci formation assay)

Human lung adenocarcinoma epithelial cells A549 and H-1299 were obtained from American Type Culture Collection and grown as recommended. All cell lines were used within 6 months after resuscitation. For γ-H2AX immunofluorescence, H-1299 or A-549 cells were seeded on coverslips in 6-well dishes at a density of 1×10^5 cells/well and allowed to attach for 24 h. These were then treated with the ABT-888, DETA or combined for 4 h and then exposed to a single dose of IR (MDS Nordion Gammacell 40 research irradiator, ON, Canada). Then cells were incubated for 4 h and 24 h post-irradiation. After washing in PBS three times, cells were fixed in 4% formaldehyde for 20 min and permeabilized with 0.2% TritonX-100 in 5% BSA/PBS for 30 min. The coverslips were then incubated in primary antibodies placed on parafilm strips and incubated at RT for 1 h. The coverslips were washed in PBS three times for 5 min each. They were then incubated with secondary antibodies at RT for 1 h. The coverslips were washed with PBS three times for 5 min each, air-dried and then mounted in Fluoroshield mounting medium with DAPI (Abcam) and viewed using a fluorescence microscope.

2.2. Clonogenic assay and apoptosis assay

For the clonogenic assay, cells were seeded into 60-mm culture dishes. After an incubation period of 2 weeks, the colonies were fixed with methanol and stained with crystal violet. Cell apoptosis was determined through APC Annexin V/Propidium Iodide (PI) assay staining by using the APC-Annexin V Apoptosis Detection Kit (BioLegend) according to the manufacturer's protocol. At specific time-points, cells were stained and analyzed by flow cytometry (FACS Canto II flow cytometer, BD Biosciences).

2.3. Western blotting

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were exposed to antibodies at specific dilutions. Specific protein bands were detected using infrared-emitting conjugated secondary antibodies: anti-mouse 680 Alexa Fluor (Molecular Probes) or anti-rabbit IR Dye 800 (Rockland Immunochemicals), using the Odyssey Infrared Imaging System and the Application software version 2.0 from Li-Cor Biosciences as was previously described [20]. Blots were quantified by densitometry using GelEval 1.37 software.

2.4. Antibodies, reagents, and siRNAs

Primary antibodies used for immunostaining and Western blotting: anti-phospho-Histone S139 (γ-H2AX) and anti-β-tubulin (Cell Signaling), anti-BRCA1 (Calbiochem), anti-RBL2 (BD Transduction Laboratories). Secondary antibodies for immunostaining: goat anti-rabbit CFL488 (Santa Cruz) and goat anti-rabbit Alexa Fluor 488 (Life Technologies). NO donors SNAP and DETA NONOate (DETA) were purchased from Cayman Chemical. FlexiTube siRNA mixtures (Qiagen) for siRNA transfection included Hs_BRCA1_13 (SI02654575), Hs_RBL2_6 (SI02664473), and AllStars non-coding negative control (SI03650318). Transfections with 50 nMol/L siRNAs were conducted according to the manufacturer's recommendations.

2.5. Neutral Comet Assay

Single cell gel electrophoresis or comet assay using neutral conditions was carried out using the manufacturer's instructions (Trevigen). Briefly, cells were seeded at a density of 0.5×10^5-0.8×10^5 cells per well in a 6-well tissue culture dish and allowed to attach for 24 h. The cells were then treated with the drugs, ABT-888, DETA or combination of both for 4 h and then exposed to a single dose of IR. After 24 h cells were trypsinized, washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4) and resuspended in 1 mL of cold PBS. Ten μL of the cell suspension was mixed with 100 μL of low melting agarose maintained at 37 °C and 60 μL of this mixture was spread onto the comet slides and allowed to solidify in the dark at 4 °C. These slides were treated with ice-cold lysis buffer provided in the Trevigen comet assay kit for 1.5 h at 4 °C in the dark. Following lysis, the slides were immersed in ice-cold 1XTBE for 15 min. Electrophoresis was performed using 1XTBE buffer (as per manufacturer's instructions) at 21 V for 40 min under cold conditions. The slides were rinsed in water for 5 min, immersed in 70% ethanol for 5 min, and then dried at 37 °C for 15 min. The slides were stained with SYBR green for 30 min at RT in the dark and then visualized using a fluorescence microscope. At least 50–60 comets were scored for each sample and analyzed using the ImageJ software with the OpenComet plugin.

3. Results

3.1. Selection of optimal doses of SNAP, DETA NONOate (DETA), and ABT-888 for combination treatment

Previously we demonstrated that NO-donors actively block BRCA1 expression and DNA HRR in the different BRCA1/2-proficient cell lines in concentrations of 50 μM and higher [20,21]. To demonstrate this with A-549 and H-1299 lung cancer cell lines, cells were incubated for 12 h with different doses of NO-donors: SNAP and DETA NONOate (DETA). In both cell lines treatment with SNAP or DETA demonstrated dose-dependent downregulation of BRCA1 protein level in concentrations ≥50 μM (Fig. 1). Next, we tested the effect of NO-donor (SNAP

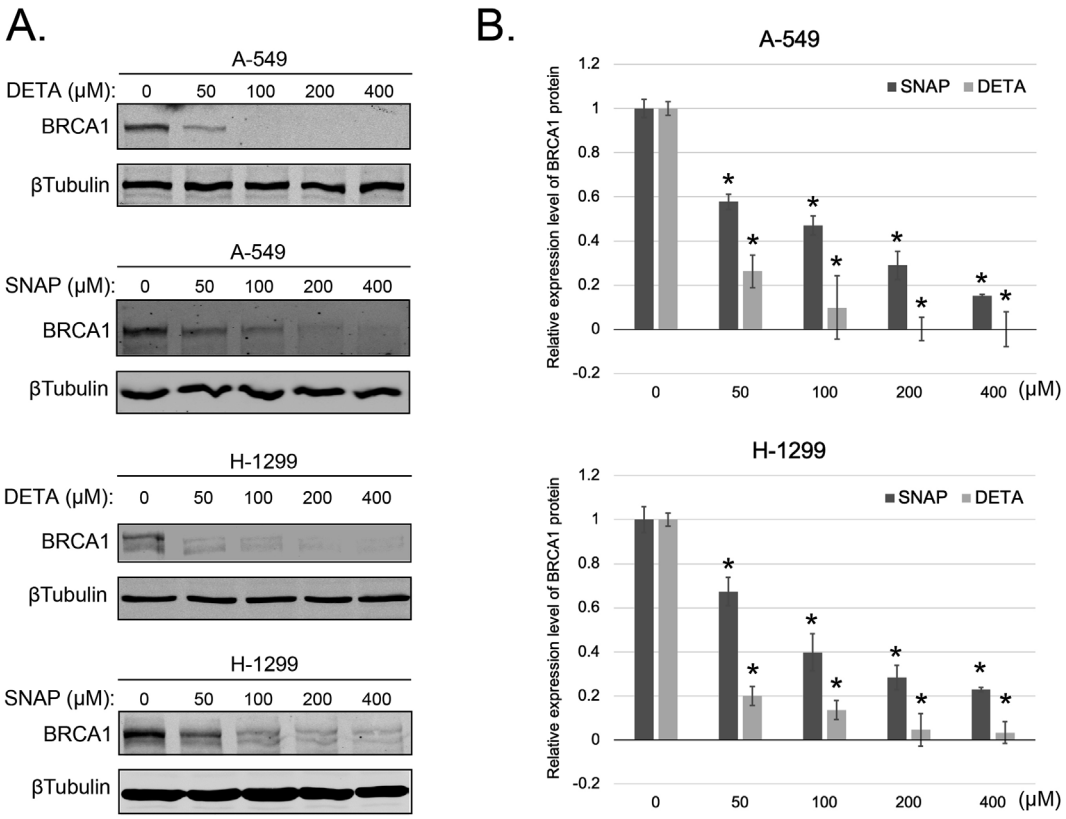


Fig. 1. Dose-dependent downregulation of BRCA1 protein by NO-donors: DETA and SNAP. (A) A-549 and H-1299 cells were incubated with different concentrations of DETA and SNAP. After 12 h of incubation, cells were lysed and total cell lysates were probed for antibodies against BRCA1 and β-Tubulin (as a loading control). **(B)** Analysis of WB results of three independent experiments. Results were expressed as fold changes of control. Experimental data are presented as the mean ± SD. The *P*-value was calculated with the Student t-test and shown as: *– $p < 0.01$.

and DETA) and PARPi (ABT-888) treatments on cell survival with or without IR (Fig. 2). A-549 and H-1299 were seeded into 60-mm culture dishes and treated with different concentrations of SNAP, DETA, or ABT-888, and then half of the dishes were irradiated with 2Gy. For non-irradiated cell lines, neither SNAP nor DETA treatment demonstrated a significant decrease in cell survival in the concentration range 50–200 μM (Fig. 2A). Unlike non-irradiated control, A-549 and H-1299 irradiated with 2Gy demonstrated the dose-dependent decrease of surviving fractions with a statistically significant difference: for SNAP ≥100 μM (in A-549 cell line) and ≥300 μM (in H-1299 cell line); for DETA ≥100 μM (in A-549 and H-1299 cell lines); for ABT-888 ≥ 10 μM (in A-549 and H-1299 cell lines) (Fig. 2). The higher activity of DETA in comparison with SNAP can be explained by its longer half-life in a cell media (~20 h vs ~6 h respectively). The PARP inhibitor ABT-888 by itself had no effect on cell survival in a dose range 10–40 μM but demonstrated a significant dose-dependent sensitization to IR in the same concentration range (Fig. 2B). To study the sensitization effect of NO-donors, PARPi, and their combination to IR, it is logical from a therapeutic perspective to use them at concentrations which can affect the irradiated cells but have no significant effect on non-irradiated ones. Therefore, for the subsequent experiments, the following concentrations were selected: 100 μM for DETA, 200 μM for SNAP, and 10 μM for ABT-888.

3.2. NO-donors significantly stimulated radiosensitization effect of ABT-888

A-549 and H-1299 cell lines were subjected to clonogenic assay with radiation doses: 0, 2, 4, 6, 8Gy. SNAP, DETA, or ABT-888 were applied 4 h before IR. Culture media were replaced 24 h post-IR with the fresh media without drugs or vehicle added. Survival fraction was estimated for 6 treatment groups: 1) Vehicle control (with DMSO); 2) 10 μM ABT-888; 3) 100 μM DETA; 4) 200 μM SNAP; 5) 100 μM DETA + 10 μM ABT-888; 6) 200 μM SNAP + 10 μM ABT-888 (Fig. 3). Both, NO-donors SNAP and DETA and PARPi ABT-888 as a single agent demonstrated the effect of radiosensitization; however, the combination of ABT-888 with SNAP or DETA significantly enhanced the effect of radiosensitization (Fig. 3A). Normalization to Vehicle control showed that the sensitization effect of NO-donors SNAP and DETA was not associated with IR: sensitization power was not dependent on the dose of IR and stayed at the same level for all doses of IR (Fig. 3B). ABT-888 demonstrated an increase of sensitization according to the increase of IR dose. Normalization to Vehicle control revealed that SNAP/ABT-888 and DETA/ABT-888 combinations enhanced the effect of radiosensitization in a synergistic manner (Fig. 3B).

3.3. Combination of ABT-888 with DETA stimulates synthetic lethality

First, we tested how the ABT-888/DETA combination affects IR-dependent formation of DNA DSB. Two different approaches for estimation of DNA DSB formation were used: γ-H2AX foci formation assay [22] and Neutral Comet assay [23,24]. A-549 and H-1299 cell lines were treated with DETA, ABT-888 or a combination of drugs and then 4 h later exposed to a single IR dose. Formation of γ-H2AX was analyzed by using the immunofluorescence assay for A-549 and H-1299 cells at non-irradiated controls, 4 h, and 24 h after a single IR dose: 10Gy for A-549 and 6Gy for H-1299 cell line. A lower dose of IR for H-1299 was used due to a relatively high initial level of γ-H2AX foci in this cell line. Both cell lines showed similar reaction to the same type of treatment (Fig. 4A). Non-irradiated control treated with Vehicle (DMSO) demonstrated no or a few γ-H2AX foci with a significant increase of γ-H2AX foci 4 h after IR. Recovery of γ-H2AX foci close to non-irradiated

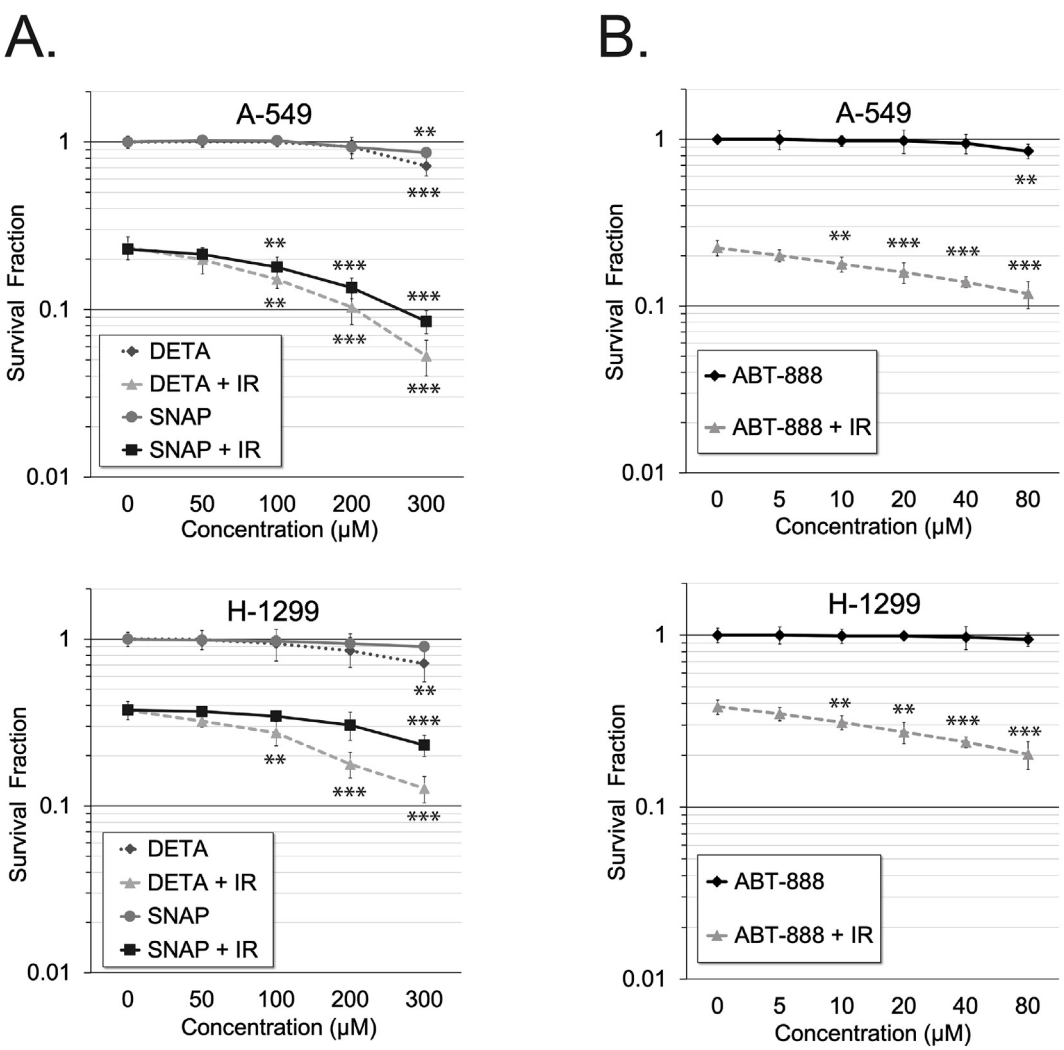


Fig. 2. Combined treatment of NO-donors (SNAP, DETA) and PARP inhibitor ABT-888 with IR. (A) Clonogenic analysis for A-549 and H-1299 cells treated with NO-donors (SNAP and DETA) with or without IR (4Gy). **(B)** Clonogenic analysis for A-549 and H-1299 cells treated with the combination of ABT-888 and IR (4Gy). Experimental data are presented as the mean ± SD for quadruplicate samples. The *P*-value was calculated with the Student t-test and shown as: **– $p < 0.05$ and ***– $p < 0.001$.

controls was observed in vehicle controls 24 h post-IR demonstrating that for the most part complete repair of IR-initiated DNA DSBs. Pre-treatment with DETA did not change the level of γ-H2AX foci in non-irradiated cells and on 4 h after IR comparing with Vehicle control. At the same time, on 24 h after IR cells pretreated with DETA demonstrated an insignificantly higher γ-H2AX foci number comparing with vehicle control. Pretreatment with ABT-888 significantly increased the amount of γ-H2AX foci in non-irradiated cells as well as 4 h after IR compared with vehicle control (Fig. 4A). There was no decrease in the number of γ-H2AX foci 24 h after IR plus pre-treatment with ABT-888, demonstrating a significant delay in DNA DSB repair. Importantly, the combination ABT-888 and DETA was able to simulate a significant increase of γ-H2AX foci number in non-irradiated cells. As well as for cells treated with ABT-888, cells pretreated with ABT-888/DETA combination demonstrated no decrease in γ-H2AX foci number 24 h after IR.

The neutral Comet assay was used as an additional measure of DNA DSB level in non-irradiated cells and 24 h after a single IR dose. Cells were treated with DETA, ABT-888 or their combination followed 4 h later by exposure to 10Gy for A-549 and 6Gy for H-1299 cells (Fig. 4B). Similar to the results obtained with the γ-H2AX assay, both cell lines showed no significant difference in Mean Tail Moment (MTM) before and 24 h after IR for vehicle control (DMSO). (MTM level for A-549 cells: 10.21 ± 1.39 before and 11.87 ± 2.15 after IR; $p = 0.38$. MTM level for H-1299 cells: 14.61 ± 1.97 before and 18.21 ± 1.77 after IR; $p = 0.079$). Pretreatment with DETA alone also did not affect the MTM level before and 24 h after IR in both cell lines (MTM level for A-549 cells: 10.59 ± 2.19 before and 13.53 ± 0.99 after IR; $p = 0.179$. MTM level for H-1299 cells: 14.92 ± 1.58 before and 18.19 ± 3.09 after IR; $p = 0.189$). Pretreatment with ABT-888, however, significantly increased MTM in both cell lines (MTM level for A-549 cells: 18.18 ± 0.98, $p < 0.005$; MTM level for H-1299 cells: 21.09 ± 2.12, $p = 0.006$). The MTM in H-1299 cells pretreated with ABT-888 was significantly higher 24 h post-IR compared with non-irradiated control (MTM level for H-1299 cells: 21.09 ± 2.12 before and 18.19 ± 3.09 after IR; $p = 0.007$), whereas A-549 pretreated with ABT-888 showed no difference before and 24 h after IR (MTM level for A-549 cells: 18.18 ± 0.98 before and 19.91 ± 1.17 after IR; $p = 0.122$.) (Fig. 4B). Non-irradiated cells treated with ABT-888/DETA combination demonstrated significantly higher MTM comparing with non-irradiated incubated with ABT-888 only (For A-549 cell line: MTM level for ABT-888 treatment −18.18 ± 0.98, MTM level for ABT-888/DETA combination −25.89 ± 2.62 after IR; $p = 0.007$. For H-1299 cell line: MTM level for ABT-888 treatment - 21.09 ± 2.12, MTM level for ABT-888/DETA combination - 25.95 ± 1.07 after IR; $p = 0.024$). Finally, irradiated cell lines pretreated with ABT-888/DETA combination demonstrated a significant synergistic increase of MTM

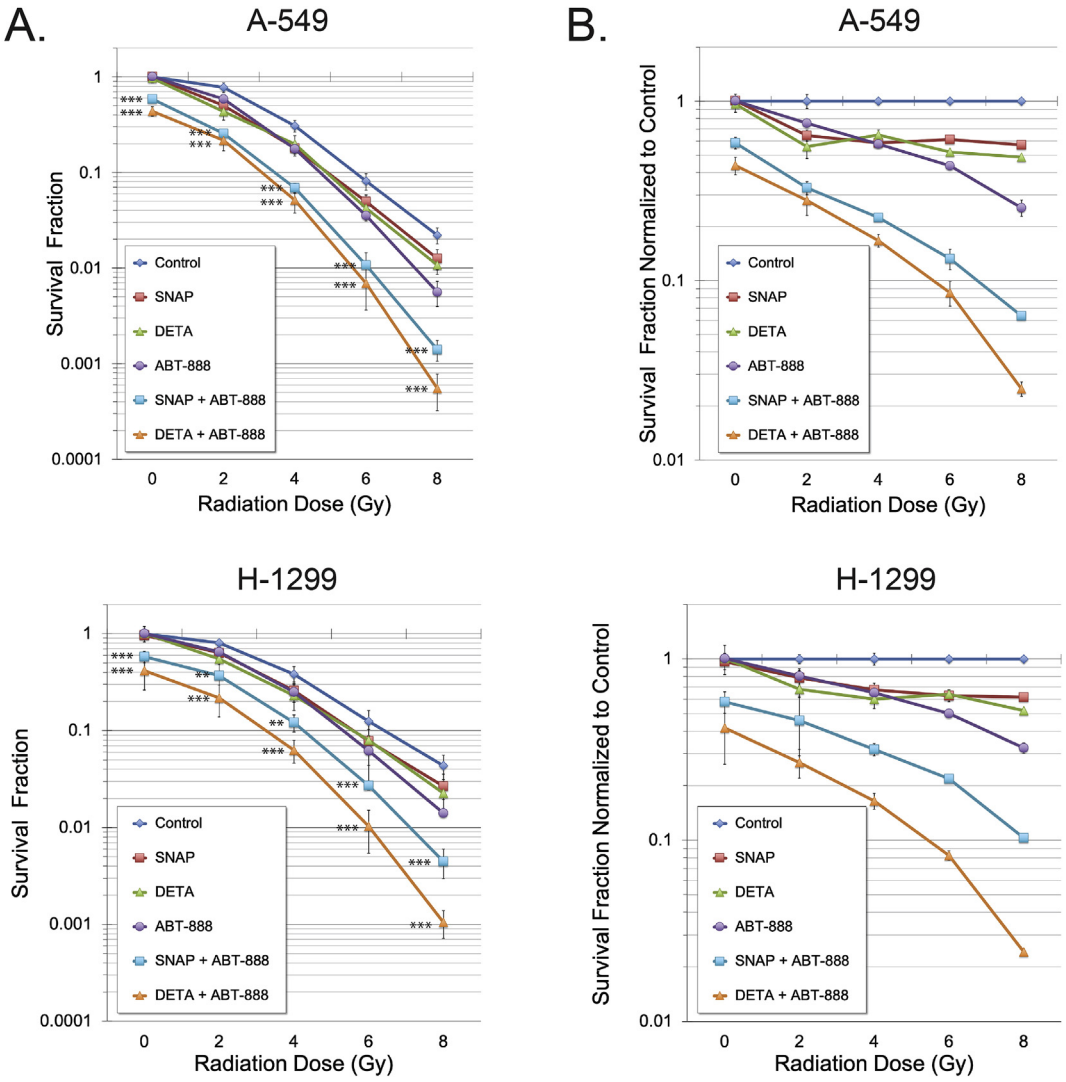


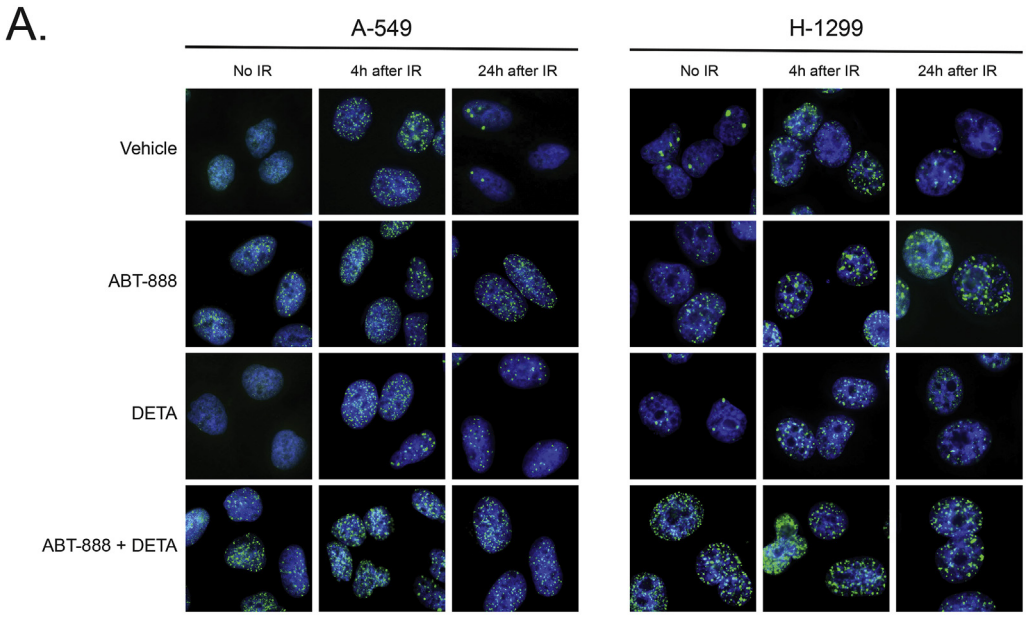
Fig. 3. Combining treatment of NO-donors (SNAP or DETA) with PARP-inhibitor ABT-888 induces sensitization to IR. A-549 and H-1299 cells were pretreated 4 h before IR with 200 μM of SNAP, 100 μM of DETA, 10 μM of ABT-888 or the combination of NO donor and ABT-888. Controls were pretreated with vehicle (DMSO). (A) Experimental data are presented as the mean ± SD for quadruplicate samples. (B) Experimental data was normalized to Vehicle control. Sensitization by ABT-888/SNAP and ABT-888/DETA combinations was compared with sensitization activity of ABT-888 for the different radiation doses. The *P*-value was calculated with the Student t-test and shown as: **– $p < 0.05$ and ***– $p < 0.001$.

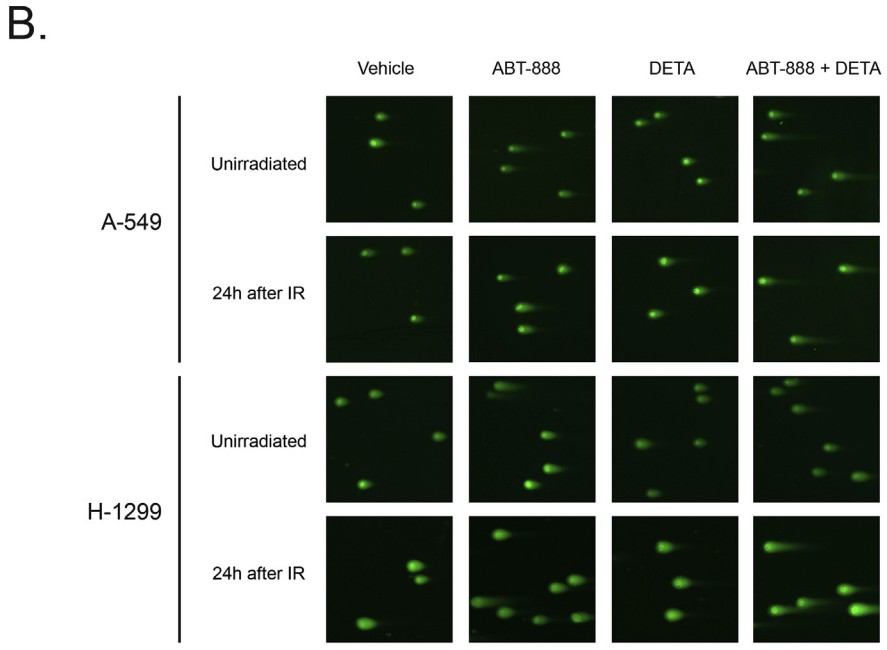
compared with irradiated cells pretreated with ABT-888 only (For A-549 cell line: MTM level for ABT-888 treatment – 19.91 ± 1.17, MTM level for ABT-888/DETA combination – 37.74 ± 3.72 after IR; $p < 0.005$. The synergy was estimated after normalization to vehicle control. For H-1299 cell line: MTM level for ABT-888 treatment - 30.1 ± 2.08, MTM level for ABT-888/DETA combination - 44.38 ± 5.42 after IR; $p < 0.005$) (Fig. 4B).

Second, to study the effect of ABT-888, DETA, and their combination on stimulation of cell apoptosis with and without IR, we used the APC Annexin V/PI assay. Cells were irradiated with a single 4Gy dose and the level of apoptosis (including early and late apoptosis) was estimated 24, 48, and 72 h after IR (Fig. 5A). As with the other assays, cells were treated with ABT-888, DETA, or their combination 4 h before IR and incubation of drugs continued 24 h after IR. For non-irradiated controls, cells were incubated with ABT-888, DETA, or their combination for 28 h and apoptosis was estimated 72 h after beginning incubation with reagents. Incubation with vehicle (DMSO) was used as a non-treated control. Incubation of A-549 cell line with a single agent ABT-888 or DETA resulted in moderate stimulation of apoptosis compared with vehicle control 72 h after IR (Fig. 5B). However, this stimulation was not statistically significant. At the same time, pretreatment of H-1299 cells with DETA revealed statistically significant induction of apoptosis at 48 h and 72 h after IR (compared with vehicle control at the same time-points) (Apoptotic cells (%) 48 h after IR: Vehicle control – 3.9 ± 1.77, DETA – 6.7 ± 1.16; p = 0.043. Apoptotic cells (%) 72 h after IR: Vehicle control – 5.2 ± 1.47, DETA – 8.7 ± 0.41; p = 0.021). Pretreatment of H-1299 with ABT-888 also significantly stimulated apoptosis compared with vehicle control 72 h after IR (p = 0.028) (Fig. 5B). Both cell lines showed significant synergistic stimulation of apoptosis after pretreatment with ABT-888/DETA combination: A-549 cells at 48 h and 72 h; and H-1299 cells at 24 h, 48 h, and 72 h. Interestingly, treatment with ABT-888/DETA combination also significantly increased the levels of apoptosis even without radiation (p = 0.027) (Fig. 5B).

3.4. Synergistic effect of ABT-888/DETA combination depends on RBL2/BRCA1 pathway

Our previous studies demonstrated that incubation of cells with NO-donors reduces BRCA1 expression by stimulating the PP2A-RBL2-E2F4 pathway and that blocking RBL2 protein expression attenuates NO-dependent downregulation of BRCA1(20). In the present work





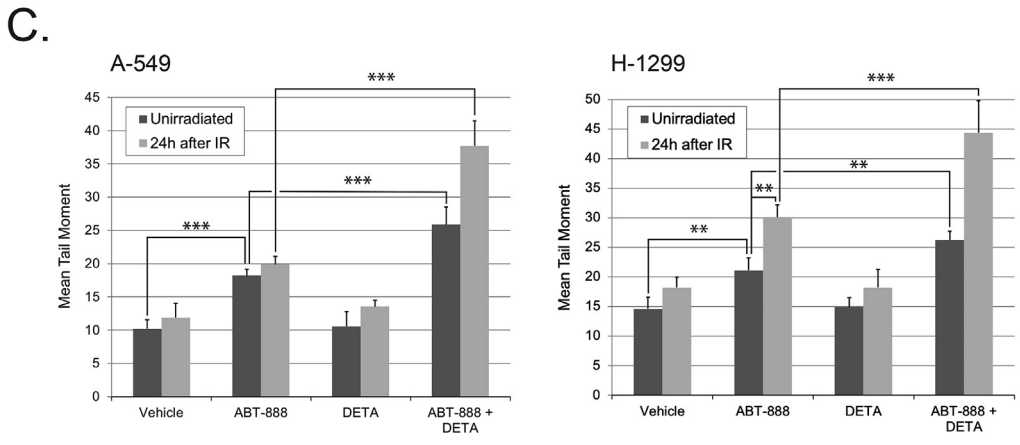


Fig. 4. Effects of pre-treatment with ABT-888, DETA and their combination on IR-induced γ-H2AX foci formation and DNA double-strand breaks (DSBs). A-549 and H-1299 cells were treated with 100 μM of DETA, 10 μM of ABT-888 or combination of drugs followed 4 h later by a single IR exposure. **(A)** γ-H2AX foci formation was analyzed using the immunofluorescence assay for A-549 and H-1299 cells at non-irradiated controls, 4 h, and 24 h after a single IR exposure. **(B)** Level of DNA DSBs was measured by Neutral Comet Assay for A-549 and H-1299 cells at non-irradiated controls and 24 h after a single IR exposure. **(C)** Experimental data of three independent Neutral Comet Assays are represented as the mean ± SD. At least 50–60 comets were scored for each sample and analyzed using the ImageJ software with the OpenComet plugin. The *P*-value was calculated with the Student t-test and shown as: **– $p < 0.05$ and ***– $p < 0.001$.

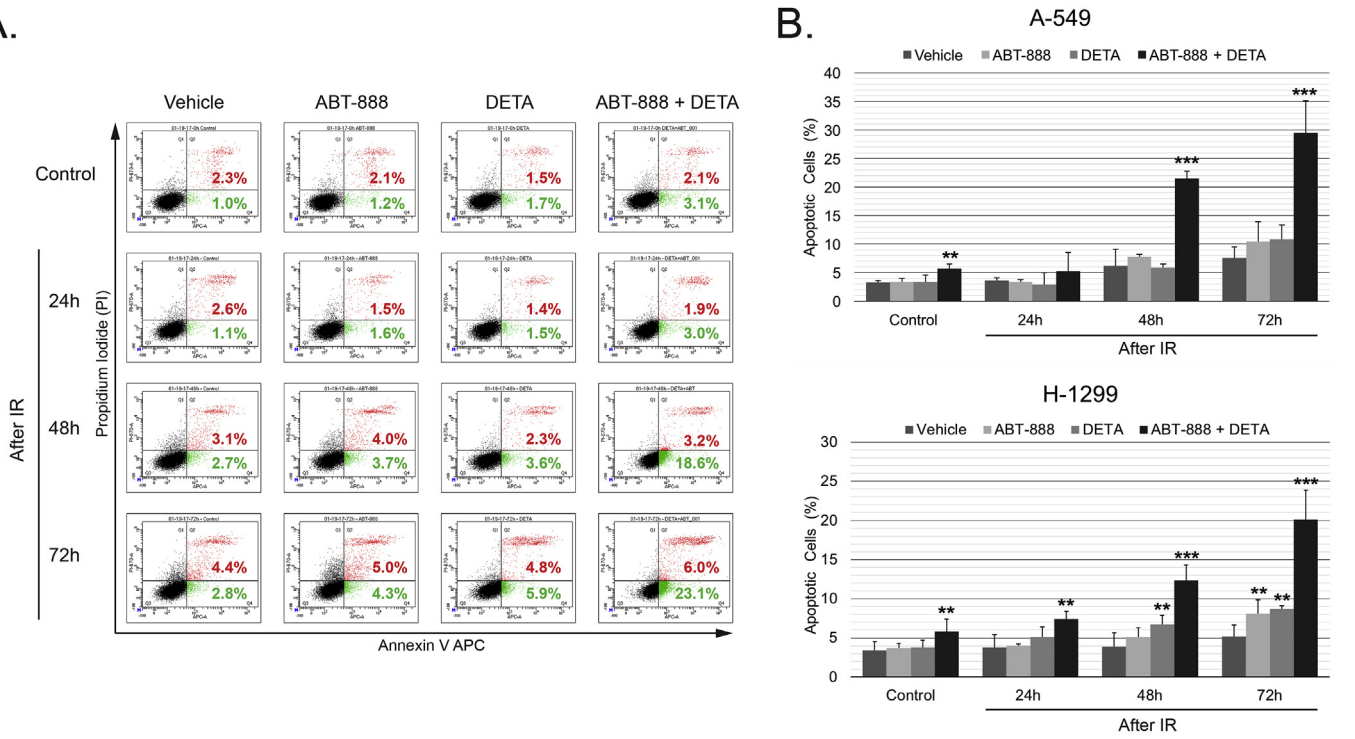


Fig. 5. Combination of DETA with ABT-888 enhances IR-stimulated apoptotic cell death. (A) Flow cytometric analysis of apoptosis in A-549 cell lines at different time-points after 4Gy IR using Annexin V-fluorescein isothiocyanate (FITC)/Propidium iodide (PI) assay. Quadrants: Q3 (normal cells), Q4 (early apoptotic cells), Q2 (late apoptotic/necrotic cells). Numbers represent a percentage of cells in the relevant quadrants. **(B)** Summary of flow cytometry analysis for A-549 and H-1299 cell lines. Columns represent the means ± SD values for apoptotic cells (Q2 + Q4) obtained from three individual experiments. The *P*-value was calculated with the Student t-test and shown as: **– $p < 0.05$ and ***– $p < 0.001$.

inhibiting RBL2 expression with siRNA dramatically reduced the IR-sensitization effect of the ABT-888/DETA. Compared with cells transfected with scrambled siRNA control, cells transfected with RBL2 siRNA also demonstrated significantly reduced levels of apoptosis and enhanced clonogenic survival after pre-treatment with ABT-888/DETA combination and IR (Fig. 6A and C). In contrast, inhibiting BRCA1 expression by siRNA stimulated apoptosis ($p < 0.005$) and decreased clonogenic cell survival in cells treated with ABT-888 alone plus IR. Inhibiting *BRCA1* expression had no effects on apoptosis and clonogenic survival in cells treated with the ABT-888/DETA combination and IR (Fig. 6B and C).

4. Discussion

PARP inhibition as a therapeutic strategy in oncology is generating increasing enthusiasm from researchers in academia and the pharmaceutical industry because of its potential to widen the therapeutic index of chemotherapy and radiotherapy, its low side effect profile, and the possibility that target populations with higher sensitivity can be identified. However, PARPi have mostly demonstrated significant effectiveness in sensitization to the different types of DNA-damaging therapy with BRCA1/2-deficient tumors [25,26]. The present work represents one approach to overcome this limitation of PARPi-based sensitization for the BRCA1/2-proficient tumors to IR.

Our previous investigations revealed that moderate, non-toxic concentrations of NO-donors inhibited *BRCA1* expression in different *BRCA1/2*-proficient cancer cell lines [20,21]. We also demonstrated that the effect of NO-dependent block of *BRCA1* expression can only be achieved in the presence of oxidative stress [21]. Oxidative stress is a condition that characterizes the tumor microenvironment and is also a potential effect of IR [27–29]. Hence, NO-donors, with effects limited by tumor microenvironment and irradiated volume, in combination with PARPi, suggest a precise tumor-targeted approach for radiosensitization of BRCA1/2-proficient tumors. However, combination of NO-donors with PARPi can be potentially toxic for non-irradiated normal tissues and additional approaches for targeting NO-donors are needed. As an example, a recently described light-activated nitrosyl ruthenium-antibody complexes can be used for more precise NO delivery to the tumor [30]. A research team of da Silva demonstrated targeting of light-activated complexes to mitochondrial VDAC in liver cancer cells, but other designs could be envisioned to other target sites as needed to downregulate BRCA1 expression.

In the present project, we demonstrated that although PARPi ABT-888 or NO-donors (SNAP and DETA) stimulated a minor sensitization to IR, their combination displayed a strong synergistic effect for IR-sensitization (Fig. 3). Based on both γ-H2AX and Neutral Comet assays, there is a significant increase of DNA DSBs early after IR (4 h) followed by a subsequent decrease to levels at 24 h approximating initial DNA DSBs before IR (Fig. 4). The amount of DNA DSBs induced by IR was enhanced with ABT-888 and this was maintained at high levels for at least 24 h post IR (Fig. 4A). This result was expected because PARP inhibition by ABT-888 increases the incidence of collapsed replication forks, converting DNA SSB into persistent DNA DSBs [31,32]. It is obvious that this effect depends on the level of cell replication. Hence, different levels of DNA DSBs in A-549 and H-1299 cell lines after IR and ABT-888 treatment can be explained by the initial difference in non-treated/non-irradiated control, but also by the difference in cell proliferation rate. Treatment with DETA did not alter the level of DNA DSBs in non-irradiated controls, 4 h, and 24 h after IR in both cell lines (Fig. 4). Unlike ABT-888, DETA also did not produce new DNA DSBs, but blocked expression of *BRCA1*, with subsequent reduction of the ability to repair DNA DSBs through the high-fidelity HRR compensated by a switch to the error-prone NHEJ [20,33]. This shift from error-free HRR to error-prone NHEJ promotes genomic instability with the subsequent accumulation of the DNA errors and stimulation of apoptosis and cell death. It also explains a moderate sensitization to IR

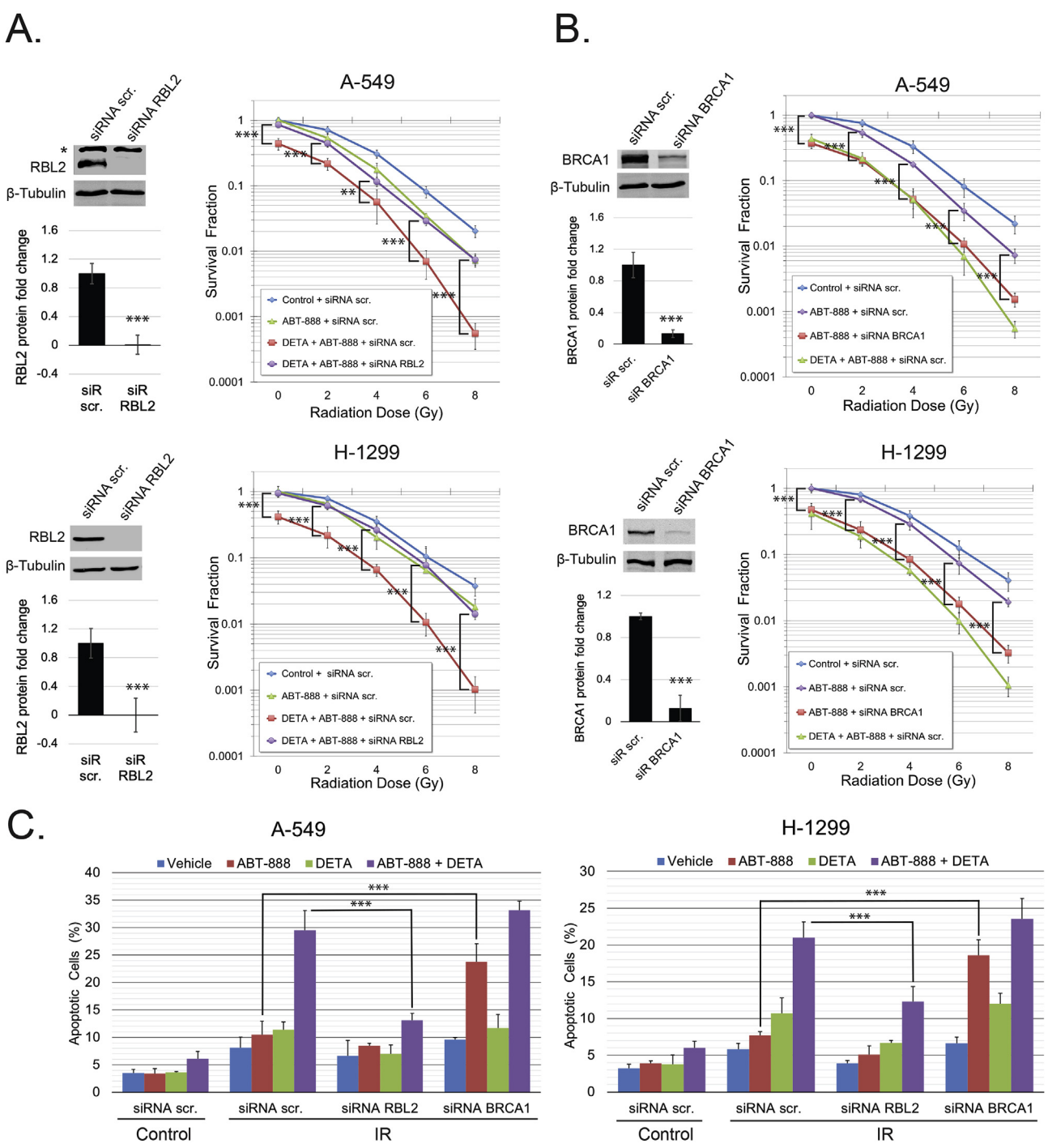


Fig. 6. Sensitization to IR by NO-donor/PARP-inhibitor combination is BRCA1-dependent. A-549 and H-1299 cells were transfected with corresponding siRNA and 24 h after transfection cells were trypsinized and one part of the cells were subjected to clonogenic assay (as described in Fig. 3), the second part was subjected for Annexin V-fluorescein isothiocyanate (FITC)/PI assay (as described in Fig. 5), and the last part was lysed and total cell lysates were probed for antibodies against BRCA1, RBL2, and β-Tubulin (as a loading control). Transfection with AllStars siRNA was used as a negative control; **(A)** Block of RBL2 expression significantly reduced effect of sensitization to IR by DETA/ABT-888 combination. Inserted Western blots and graphs demonstrates RBL2 protein expression downregulation by siRNA transfection. Graphs represent WB results of three independent experiments. Results were expressed as fold changes of control. Experimental data are presented as the mean ± SD; **(B)** Block of BRCA1 expression significantly stimulated effect of sensitization to IR by ABT-888 pretreatment. Inserted Western blots and graphs demonstrate effect of BRCA1 protein downregulation by siRNA transfection. Graphs represent WB results of three independent experiments. Results were expressed as fold changes of control. Experimental data are presented as the mean ± SD **(C)** Summary of flow cytometric analysis of apoptosis for A-549 and H-1299 cell lines: non-irradiated controls and 72 h after 4Gy of IR (as shown in Fig. 5). Columns represent the means ± SD values for apoptotic cells obtained from three individual experiments. The *P*-value was calculated with the Student t-test and shown as: **– $p < 0.05$ and ***– $p < 0.001$.

demonstrated by NO-donors SNAP and DETA (Fig. 3). However, the combination of ABT-888 and DETA demonstrated synergistic sensitization to IR through the mechanism of synthetic lethality: while ABT-888 forces conversion of DNA SSB into DSB, treatment with DETA directs repair of accumulated DSB through the error-prone NHEJ. This mechanism is illustrated in Fig. 7. Stimulation of synthetic lethality promotes growth of genomic instability, accumulation of critical DNA errors, and cellular death activation. That ABT-888/DETA combination

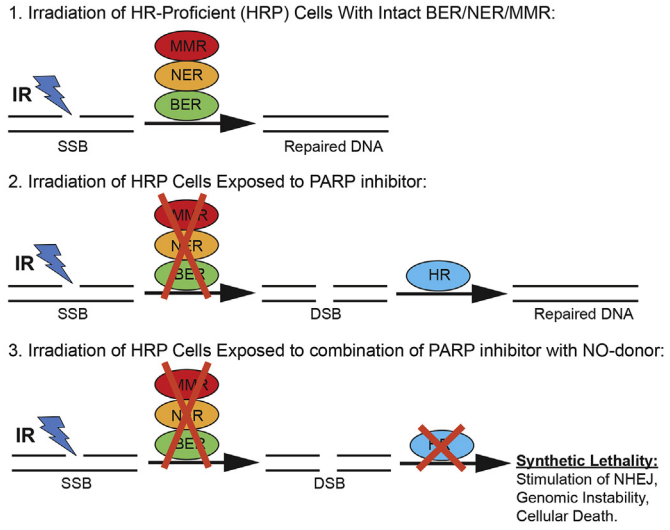


Fig. 7. A proposed stimulation of synthetic lethality by the combination of NO-donor and PARP-inhibitor.

stimulates effect of synthetic lethality is confirmed in the synergistic stimulation of apoptosis (Fig. 5). This combination of ABT-888/DETA also stimulated a significant increase of apoptosis even in non-irradiated control (Fig. 5B).

In summary, a combination of PARPi/NO-donor demonstrated a very high potency in sensitization of *BRCA1/2*-proficient cancer cell lines to IR through the stimulation of synthetic lethality. The combination with NO-donors allows PARPi to be successfully applied to a wider variety of tumors. The next step would be to conduct preclinical small animal studies to find an optimal regime of treatment for the NO-donor/PARPi combination, estimate its effectiveness *in vivo* for sensitization to IR, and evaluate toxicity.

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Disclosure of potential conflicts of interest

The authors disclose no potential conflicts of interest.

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