Published OnlineFirst March 24, 2016; DOI: 10.1158/0008-5472.CAN-15-2908

Priority Report

Cancer Research

Hyperthermia Synergizes with Chemotherapy by Inhibiting PARP1-Dependent DNA Replication Arrest

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Abstract

Although hyperthermia offers clinical appeal to sensitize cells to chemotherapy, this approach has been limited in terms of long-term outcome as well as economic and technical burden. Thus, a more detailed knowledge about how hyperthermia exerts its effects on chemotherapy may illuminate ways to improve the approach. Here, we asked whether hyperthermia alters the response to chemotherapy-induced DNA damage and whether this mechanism is involved in its sensitizing effect in BRCA-competent models of ovarian and colon cancer. Notably, we found that hyperthermia delayed the repair of DNA damage caused by cisplatin or doxorubicin, acting upstream of different repair pathways to block histone polyADP-ribosylation (PARylation), a known effect of chemotherapy. Furthermore, hyperthermia blocked this histone modification as efficiently as pharmacologic inhibitors of PARP (PARPi), producing comparable delay in DNA repair, induction of double-strand breaks (DSB), and cell cytotoxicity after chemotherapy. Mechanistic investigations indicated that inhibiting PARylation by either hyperthermia or PARPi induced lethal DSB upon chemotherapy treatment not only by reducing DNA repair but also by preventing replication fork slowing. Overall, our work reveals how PARP blockade, either by hyperthermia or small-molecule inhibition, can increase chemotherapy-induced damage in BRCA-competent cells. *Cancer Res; 76(10); 2868–75. ©2016 AACR.*

Introduction

It has been demonstrated that hyperthermia augments the efficacy of DNA-damaging agents both in preclinical studies (1, 2) as well as in clinical trials of hyperthermic intraperitoneal chemotherapy (HIPEC) in peritoneal carcinosis patients (2, 3), indicating that one mechanism of the sensitizing effect of elevated temperature is induction of DNA repair dysfunctionality. Indeed, direct interactions between hyperthermia and repair of γ irradiation–induced DNA damage have been reported (4). Importantly, enhanced temperatures sensitize to different cytotoxic drugs, such as platinum compounds (1), doxorubicin (2), and mitomycin-C (5), inducing different types of DNA damage and consequently activating different repair pathways. This indicates that hyperthermia might affect common enzymatic reactions shared by different DNA repair pathways. One prerequisite for DNA repair in heterochromatic DNA is chromatin relaxation rendering DNA lesions accessible to repair proteins. This is predominantly facilitated through PARP1-mediated polyADP-ribosylation (PARylation) making PARP1 an essential player in different repair pathways, such as base excision repair (BER; ref. 6), nucleotide excision repair (7), homologous recombination (HR; ref. 8), and nonhomologous end joining (9). We, here, report that hyperthermia inhibits chemotherapy-induced PARylation as efficiently as pharmacologic PARP inhibitors (PARPi). Importantly, both hyperthermia and PARPi potentiate chemotherapy by reducing DNA repair and preventing replication fork slowing, leading to elevated double-strand break (DSB) formation and eventually reduced long-term survival.

Material and Methods

Cell lines and patient tumors

OVCAR8 and OVCAR3 were purchased from the NCI (Rockville, MD). HCT116 and LOVO are from ATCC. All four cell lines were authenticated by STR profile analysis (PowerPlex 21HS, Promega), with frozen aliquots from same passages used for experiments. Tumor tissues were obtained from 15 peritoneal carcinosis patients (9 from ovary and 6 from colon) directly after cytoreductive surgery (CRS). All patients underwent CRS and HIPEC in our hospital. The investigation was approved by the local ethics committee (159/2011BO2), and informed consent was obtained from the patients. Culture was performed as described previously (Supplementary Material; ref. 10).

Immunofluorescent staining

Staining procedure and foci scoring was performed according to standard protocols (described in Supplementary Material) with

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-15-2908

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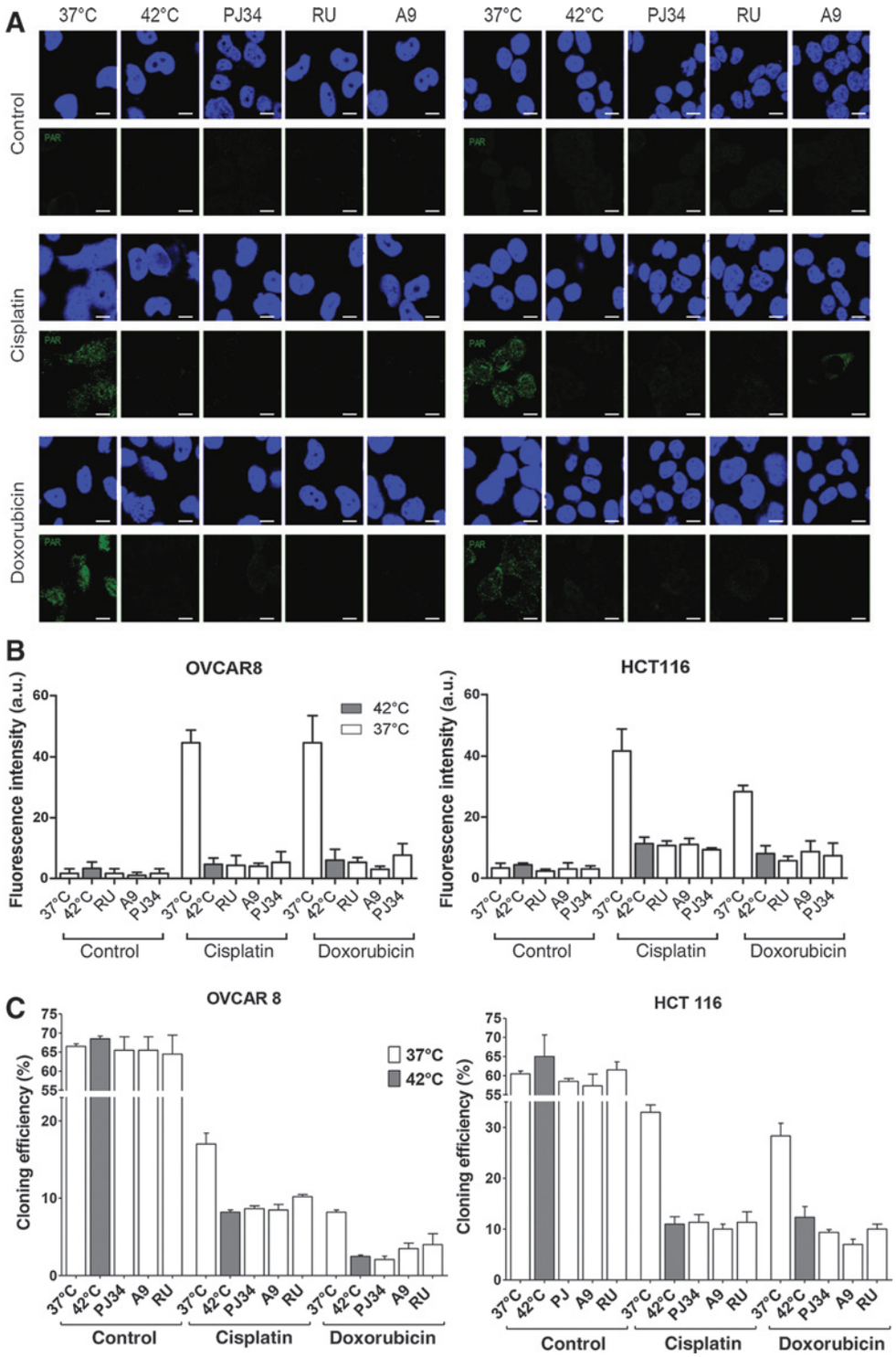

Figure 1.

Hyperthermia potentiates chemotherapy by blocking PARylation. OVCAR8 and HCT116 were cultivated at 37°C and 3% oxygen. Treatment was performed at either 37°C or 42°C under atmospheric oxygen. Cisplatin was used at 40 μmol/L at both temperatures, as we found no differences in quantity of PtDNA adducts at 37°C or 42°C (Supplementary Fig. S1). Doxorubicin dosages had to be adapted to reach similar intracellular concentrations at both temperatures: 15 μmol/L at 37°C and 6.5 μmol/L or 7.0 μmol/L at 42°C for HCT116 or OVCAR8, respectively (Supplementary Fig. S1). PARP inhibitors were added 2 hours before chemotherapy/hyperthermia treatment. Cells were then immediately harvested and fixed for immunofluorescence staining or seeded for colony assays. A, representative PAR staining of cells incubated with/without hyperthermia, with/without cisplatin or doxorubicin, and with/without rucaparib (RU), PJ34, or A9 (scale bars, 10 μm). B, quantitative analysis of PAR staining using Definiens Tissue Studio. Values reflect means ± SD from three independent experiments. C, for colony assays, cells were seeded at 500 and 1,000 cells for controls and treated cells, respectively. Following 10 days of culture at 37°C and 3% oxygen, colonies with more than 50 cells were counted. The values reflect means ± SD from three independent experiments. a.u., arbitrary units.

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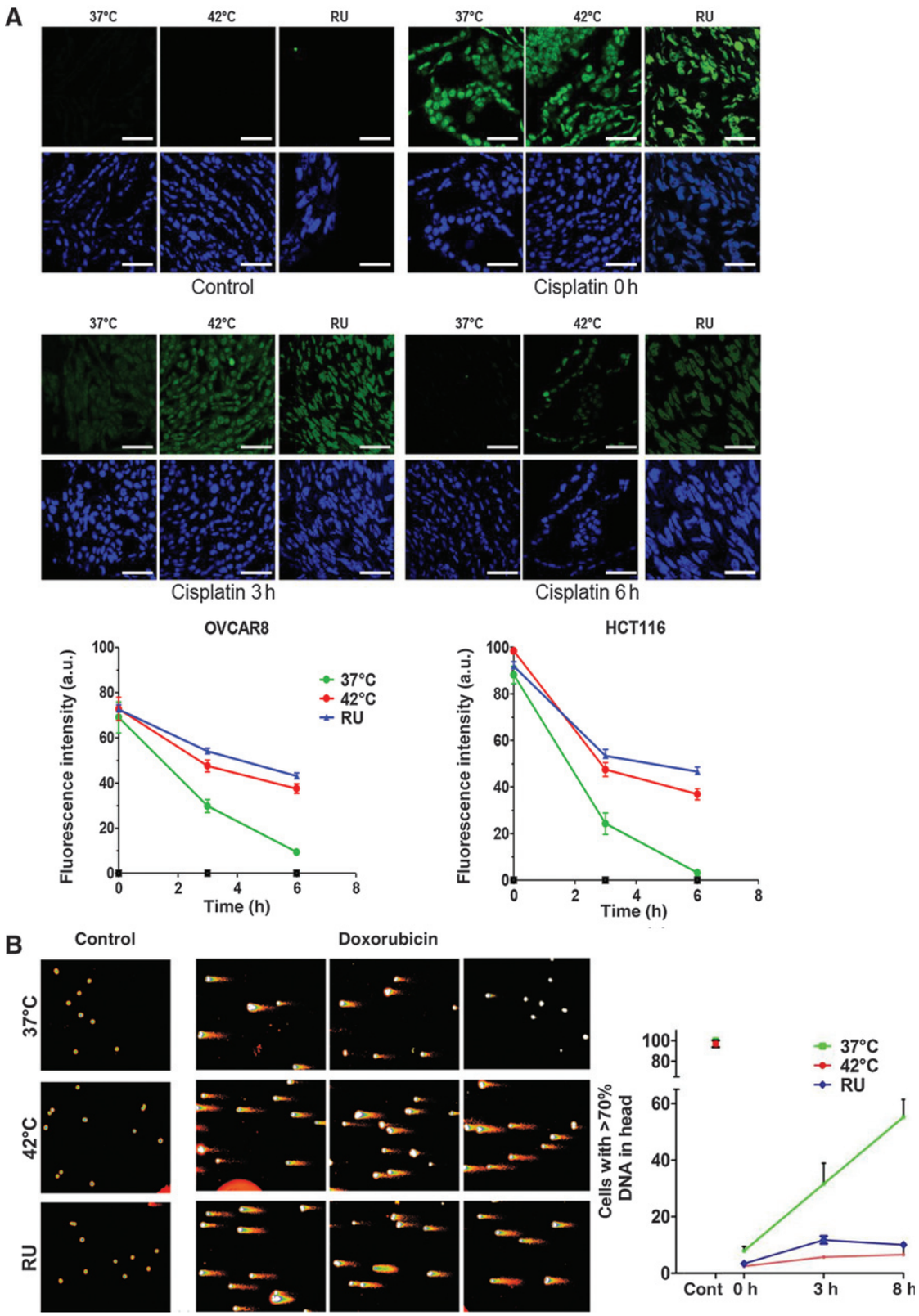

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the following primary antibodies: anti-geminin (#10802-1-AP; Proteintech), anti-γH2AX (#ab26350-9F3; Abcam), anti-phospho-53BP1 (#2675; Cell Signaling Technology), anti-Rad51 (#GTX70230; GeneTex), and anti-PARP (#4335-MC-100; Trevigen).

Apoptosis

Cells were stained for FACS using Annexin V-FITC (BD Pharmingen) and propidium iodide (Sigma-Aldrich) as described previously (11).

RNA expression

RNA extraction and PCR were performed as described previously (11). TaqMan assays were from Applied Biosystems (Supplementary Table S1).

Protein expression

Cells were lysed according to standard protocols. Western blot analysis was performed using SDS-PAGE. The following primary antibodies were used: PARP (#NB100-113; Novus Biologicals), PARG (#ab16060, Abcam), BCRA1 (#ab16781; Abcam), and BRCA2 (#ab9143; Abcam).

Colony formation assay

Cells for control (500) or treated (1,000) samples were seeded into 25-cm^2 flasks and plated in triplicates. After 10 days of incubation, colonies were fixed with methanol and stained with hematoxylin. Colonies with more than 50 cells were counted using Definiens Tissue Studio.

Comet assay

For quantification of DNA strand breaks, we used a standard alkaline COMET assay (described in the Supplementary Material).

DNA fiber assay

Cells were pulse labeled for 45 minutes with 25 μmol/L 5-iodo-2′-deoxyuridine (IdUrd), followed by 250 μmol/L 5-Cloro-2′-deoxyuridine (CldU; Sigma-Aldrich). During IdUrd labeling, cells were incubated with/without cisplatin or doxorubicin. Labeled cells were harvested, and fiber spreads were prepared and stained as described previously (Supplementary Material; ref. 12).

Statistical analysis

Data analysis was performed using Genedata Analyst (Genedata) and Prism 5 (GraphPad). Data were expressed as SD of the means. Changes in paired samples were analyzed using two-sided t test. $P < 0.05$ was considered significant.

Results and Discussion

Hyperthermia blocks chemotherapy-induced PARylation as efficiently as pharmacologic PARPi

Previously, it has been demonstrated that hyperthermia inhibits HR by BRCA2 degradation, leading to an enhanced sensitivity to PARPi (4). We here addressed the question if hyperthermia may also act upstream of DNA repair processes by direct alteration of chemotherapy-induced PARP activity. For this, we incubated BRCA-competent, HR-proficient cells [demonstrated by their expression of BRCA proteins and their ability to form Rad51 foci (Supplementary Fig. S1; ref. 13)] derived from colon and ovarian cancer with or without adapted cisplatin or doxorubicin concentrations at 37°C or 42°C (Supplementary Fig. S2) and subsequently analyzed global PARylation. Treatment was performed for one hour to mimic the clinical HIPEC setting. As shown in Fig. 1, hyperthermia completely blocked cisplatin- and doxorubicin-mediated PARylation. The efficacy of this blockade was comparable with that observed after pharmacologic inhibition of PARP with three different PARPi (Fig. 1A and B). Besides the pan-PARPi PJ34, we selected a specific PARP1/2 inhibitor (A966492) and rucaparib currently used in phase II trials of patients with peritoneal carcinosis (14). Importantly, the synergistic effect of hyperthermia and cisplatin or doxorubicin on long-term survival was comparable with that observed after concomitant use of PARPi and cisplatin or doxorubicin (Fig. 1C). These data indicate that inhibition of PARP enzymatic activity is critical for the potentiating effect. This is supported by the finding that hyperthermia or PARPi failed to increase cytotoxic effects of compounds not inducing acute PARylation, such as paclitaxel or 5FU (Supplementary Fig. S3A). It is unlikely that hyperthermia leads to the degradation of PARP1 or induction of PARG, as protein levels were unchanged in OVCAR8 and only minimally regulated in HCT116 (Supplementary Fig. S3B). Interestingly, Petesch and Lis recently showed an even enhanced PARylation upon hyperthermia at a specific site of the *Hsp70* promoter (15). Our experiments reveal an almost complete hyperthermia-mediated inhibition of global PARylation upon DNA damage. Together, these data indicate a differential impact of elevated temperature on PARP, namely an enhanced activation as cotranscription factor and a concomitant reduced function as repair protein.

Hyperthermia or PARPi significantly delays repair of cisplatin and doxorubicin-induced DNA damages

We next asked whether hyperthermia may influence the efficacy of Pt-DNA adduct removal upon cisplatin as well as DNA strand break repair upon doxorubicin treatment. We therefore performed time course experiments following short-term incubation of cells with or without chemotherapy

Figure 2.

Hyperthermia leads to a delayed repair of cisplatin and doxorubicin-induced DNA damages. Cells were treated according to the procedure described in Fig. 1 legend with cisplatin (A) or doxorubicin (B) at 37°C or 42°C and in presence or absence of PARPi. Cells were then further incubated in the absence of drugs at 37°C and 3% oxygen and harvested at indicated time points. A, immunofluorescence analyses of Pt-DNA adducts. Top, representative images of OVCAR8 cells (scale bars, 50 μm); bottom, fluorescence intensity quantified using Definiens Tissue Studio. Data reflect means ± SD from three independent experiments performed with OVCAR8 cells (left) and HCT116 (right). B, following incubation with/without doxorubicin and with/without rucaparib (RU) at either 37°C or 42°C, cells were incubated at 37°C in the absence of drugs for indicated times and then harvested for alkaline COMET assay. Left, representative images; right, percentages of cells with more than 70% of DNA in the COMET head. For each experiment, at least 100 cells were quantified using CometScore. Values reflect means ± SD from three independent analyses. a.u., arbitrary units; Cont, control.

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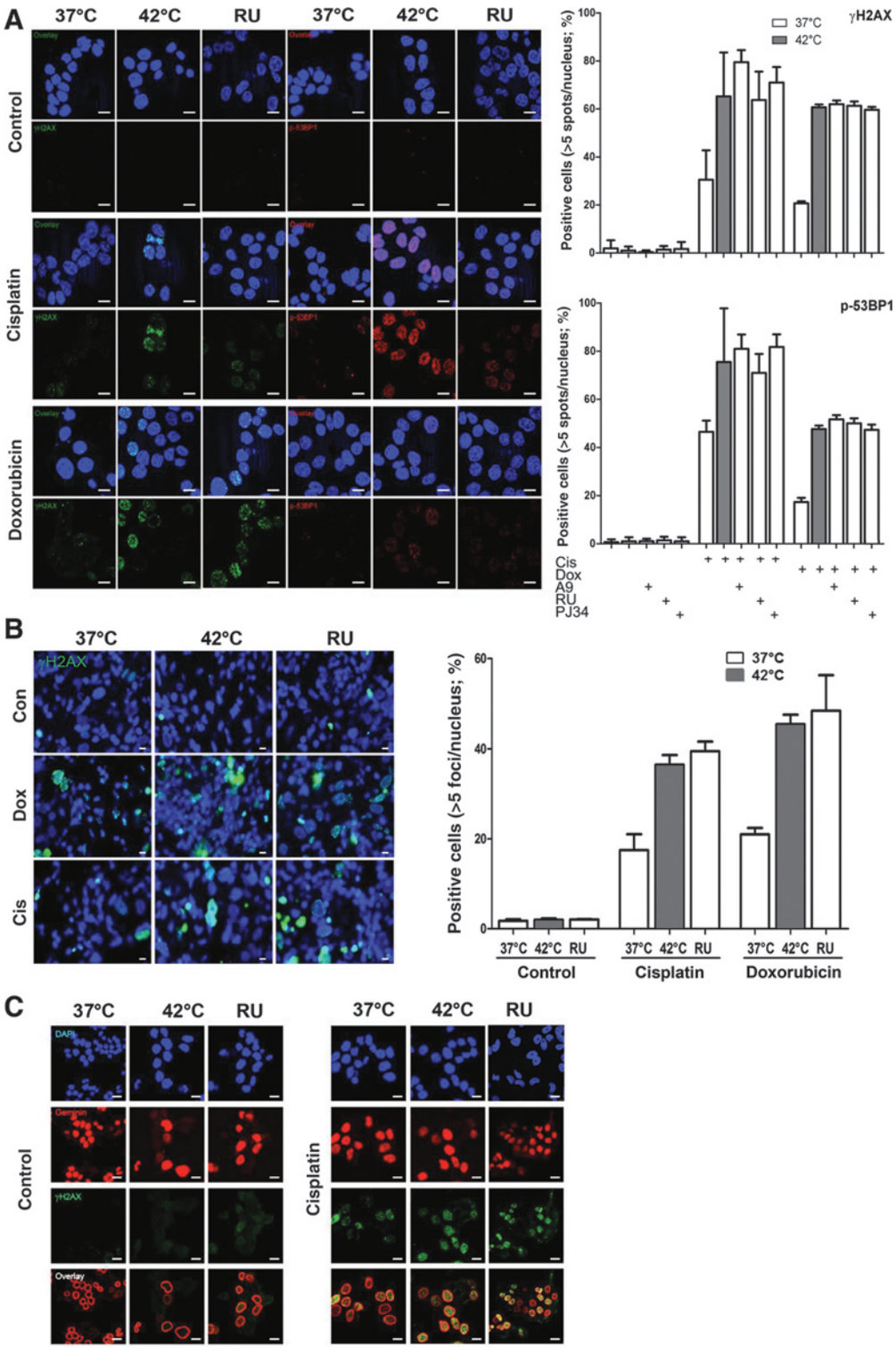

Figure 3.

Hyperthermia enhances cisplatin and doxorubicin-induced DSB formation in S–G_2-phase cells. OVCAR8 (A) or precision-cut tissue slices from patient tumors (B) were treated according to the procedure described above with cisplatin (Cis) or doxorubicin (Dox) at 37°C or 42°C and in the presence or absence of rucaparib (RU). Cells were then incubated for 3 hours in the absence of drugs at 37°C and 3% oxygen. DSBs were detected via immunofluorescence with antibodies recognizing γH2AX and P-53BP1. Left, representative images; right, percentages of cells with more than 5 foci per nucleus. Values reflect means ± SD from three independently cultivated tissue slices derived from one ovarian cancer patient tissue. C, to evaluate whether DSB formation is dependent on proliferation status of the cells, we performed costaining experiments using geminin to detect S–G_2-phase cells (scale bars, 10 μm). Con, control.

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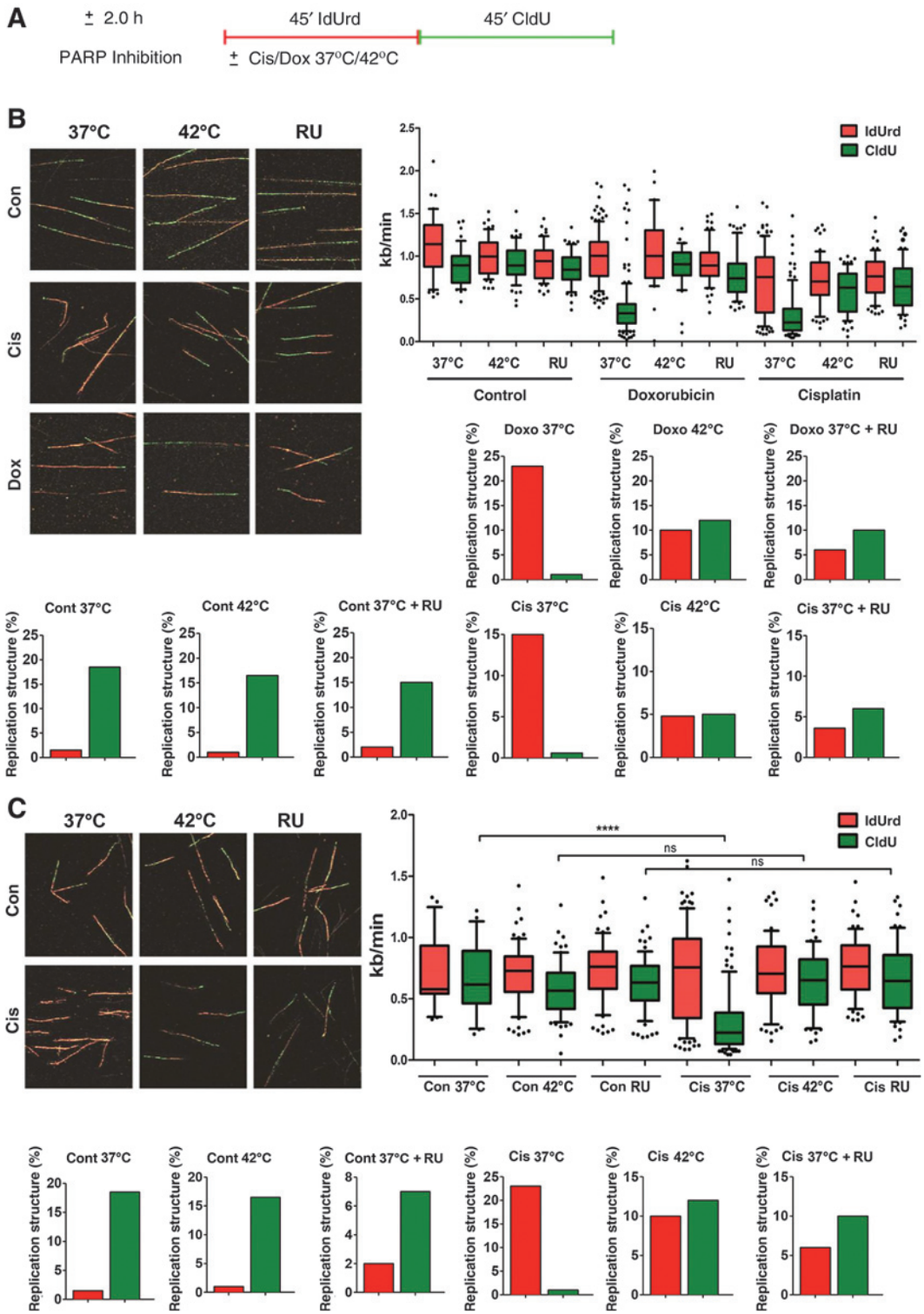

Figure 4.

Both hyperthermia and PARPi prevent DNA damage–induced replication fork slowing upon cisplatin or doxorubicin. A, experimental setup for DNA fiber analysis. Cells were preincubated with/without rucaparib (RU) for 2 hours and then labeled with IdUrd for 45 minutes in the presence or absence of cisplatin (Cis) or doxorubicin (Dox) at either 37°C or 42°C. After washing, CldU labeling was performed for another 45 minutes (′) in the absence of drugs. B and C, representative fiber tracks in OVCAR8 cells (B) and isolated cancer cells from peritoneal carcinosis tissues derived from one ovarian cancer patient tumor (C) are shown (left); right, IdUrd track length measurements. At least 150 replication tracks were quantified in each dataset. Whiskers show the 10% and 90% percentiles; outliers are indicated with dots. Statistical analysis was performed according to Mann–Whitney (****, $P < 0.0001$). Bottom, analysis of IdUrd- and CldU-only tracks. ns, not significant.

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at different temperatures. Both cisplatin adducts and doxorubicin-induced strand breaks were efficiently repaired within the first 6 hours at 37°C. In contrast, at 42°C, we observed a significant slowdown of Pt-DNA adduct removal (Fig. 2A) and an almost complete blockade of strand break repair upon doxorubicin treatment (Fig. 2B and Supplementary Fig. S4). Importantly, similar effects were observed after pharmacologic inhibition of PARP1/2 (Fig. 2). These findings are corroborated by previous data from lung cancer (16), indicating that PARP is not only implicated in BER and DNA stand break repair but also in Pt-DNA adduct removal.

Hyperthermia or PARPi enhances cisplatin and doxorubicin-induced DSB formation in replicating cells

Deficient repair of DNA alterations leads to secondary DNA DSB (17). Our data demonstrate that the combination of cisplatin or doxorubicin with hyperthermia induced significantly higher numbers of γH2AX/P-53BP1 foci per cell as compared with cisplatin or doxorubicin alone. Again, the same degree of foci formation was seen after combined treatment of cisplatin or doxorubicin with different PARPi (Fig. 3A). To prove whether the same mechanisms pertain in the complexity of a primary tumor, precision-cut tumor tissue slices from patients with peritoneal carcinosis were treated and stained for γH2AX/P-53BP1. As shown in Fig. 3B, again, significantly increased DSBs were detected upon combination with hyperthermia or PARP inhibition. Of note, all samples were positive for BRCA1 and BRCA2, and we detected geminin-positive cancer cells with Rad51 foci, indicative for HR competence (13) in 14 of 15 investigated tumors (Supplementary Fig. S5).

Whereas γ irradiation led to foci formation in 100% of the cells (Supplementary Fig. S6), chemotherapy-induced DSBs were restricted to a subset of cells both with and without hyperthermia or PARPi. We hypothesized that this effect may depend on cell-cycle status and performed costaining with geminin, detecting S–G_2-phase cells. As shown in Fig. 3C, γH2AX foci formation upon treatment with cisplatin and hyperthermia or PARPi was restricted to geminin-positive cells, indicating that PARP inhibition via hyperthermia or pharmacologic intervention increases DSB only during S–G_2-phase.

Hyperthermia or PARPi prevents DNA damage–induced replication fork slowing upon cisplatin or doxorubicin

Our finding that synergistic effects of hyperthermia or PARPi with doxorubicin and cisplatin on DSB formation are only observed in S–G_2-phase prompted us to investigate possible influences of this combinatory treatment on replication. Therefore, we performed DNA fiber spreading analyses (Fig. 4). Cisplatin and doxorubicin led to an immediate slowdown of replication fork progression, as demonstrated by reduced CldU tract length distribution (Fig. 4B). Similar effects were observed in primary tumor cells isolated from peritoneal carcinosis (Fig. 4C). The effect was accompanied by an almost complete stop of new origin firing as indicated by an accumulation of IdUrd-only paralleled by a loss of CldU-only fibers in cell lines and primary cancer cells (Fig. 4B and C). Hyperthermia or PARPi alone had no effect on replication fork progression (Fig. 4). Importantly, however, hyperthermia as well as pharmacologic inhibition of PARP1/2 completely abolished chemotherapy-induced fork slowing in cell lines and primary cancer cells. The inability of replication fork arrest upon chemotherapy in cells with blocked PARP1/2 activity is confirmed by new origin firing and reduced replication stops (Fig. 4). Of note, the degree of fork slowing upon cisplatin or doxorubicin alone and its blockade after coincubation with hyperthermia or PARPi observed in our study is similar to the data published by Berti and colleagues, who investigated effects of camptothecin together with PARPi (12). They demonstrated a central role of PARylation in replication fork regression upon camptothecin through inhibition of the helicase RECQ1. Inhibition of PARP1/2 restored RECQ1 activity, leading to a deregulated untimely restart of reversed forks and eventually to DSB formation (12). Importantly, replication fork reversal seems not to be restricted to torsional DNA stress upon TopI inhibition but is also present in cells with unrepaired interstrand cross-links produced, for instance, by platinum compounds (18). Interestingly, anthracyclines, like doxorubicin, also produce interstrand adducts (19), which are claimed to be predominantly involved in cell death upon doxorubicin (20).

Taken together, we demonstrate that hyperthermia acts via abolishing DNA damage–induced PARylation, leading to the inhibition of DNA repair and prevention of replication fork slowing. Probably together with the previously described effect on HR proficiency (4), this results in elevated DSB formation and eventually reduced long-term survival. The findings that elevated temperature neither influenced the induction of acute apoptosis (Supplementary Fig. S7A) nor regulation of bona fide apoptosis genes (Supplementary Fig. S7B and S7C; Supplementary Table) and that simultaneous treatment of cells with hyperthermia and PARPi only marginally enhanced the potentiating effect of hyperthermia or PARPi alone (Supplementary Fig. S8) indicate that the synergistic effect produced by hyperthermia acts predominantly through the inhibition of global PARylation and subsequent deregulation of DNA repair and replication fork progression upon chemotherapy. The finding that comparable effects were observed upon pharmacologic PARP inhibition at normothermic conditions implies that the combination of PARPi with replication stress–inducing drugs provides an attractive alternative to HIPEC.

Our work not only unravels a major molecular mechanism for hyperthermia-related chemotherapy-induced DNA damage response but might have important future clinical implications: (i) agents to be combined with hyperthermia can be selected more specifically, that is, particularly drugs with a high potential for inducing PARylation; and (ii) repeated normothermic chemotherapy perfusion simultaneously with the application of a systemic PARPi might overcome severe adverse side-effects of hyperthermia by HIPEC procedures. In addition, sufficient killing of proliferating cells due to the timely limited nature of hyperthermia will be counteracted.

Another important novel aspect of our work implicates that PARPi might have significant effects not only in BRCA-deficient but also in BRCA-competent cells when combined with replication stress–inducing chemotherapeutics. This might open an avenue of effective pharmacologic treatment of patients with peritoneal carcinosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Published OnlineFirst March 24, 2016; DOI: 10.1158/0008-5472.CAN-15-2908

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Development of methodology: L. Schaaf, C. Ulmer, T.E. Mürdter

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Schaaf, C. Ulmer, S. Heine, J.O. Schmid

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Schaaf, C. Ulmer, T.E. Mürdter, G. Sauer, W.E. Aulitzky, H. van der Kuip

Writing, review, and/or revision of the manuscript: L. Schaaf, M. Schwab, C. Ulmer, T.E. Mürdter, G. Sauer, W.E. Aulitzky, H. van der Kuip

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Ulmer, T.E. Mürdter, G. Sauer

Study supervision: C. Ulmer, G. Sauer, W.E. Aulitzky, H. van der Kuip

Acknowledgments

The authors thank Kerstin Willecke for technical assistance.

Grant Support

This research has received support from the Robert Bosch Foundation (project 13-11).

Received October 22, 2015; revised February 19, 2016; accepted March 7, 2016; published OnlineFirst March 24, 2016.

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Published OnlineFirst March 24, 2016; DOI: 10.1158/0008-5472.CAN-15-2908

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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