Hyperthermia Inhibits Recombination Repair of Gemcitabine-Stalled Replication Forks

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Background

Gemcitabine is a potent nucleoside analogue against solid tumors, but development of drug resistance is a substantial problem. Removal of gemcitabine incorporated into DNA by repair mechanisms may contribute to resistance in chemo-refractory solid tumors. Human hepatocellular carcinoma (HCC) is usually very chemoresistant to gemcitabine.

Methods

We treated HCC in vitro and in vivo (orthotopic murine model with human Hep3B or HepG2 xenografts, 7–10 CB17SCID mice per group) with gemcitabine. The role of homologous recombination repair proteins in repairing stalled replication forks was evaluated with hyperthermia exposure and cell-cycle analysis. The Student t-test was used for two-sample comparisons. Multiple group data were analyzed using one-way analysis of variance. All statistical tests were two-sided.

Results

We demonstrated that Mre11-mediated homologous recombination repair of gemcitabine-stalled replication forks is crucial to survival of HCC cells. Furthermore, we demonstrated inhibition of Mre11 by an exonuclease inhibitor or concomitant hyperthermia. In orthotopic murine models of chemoresistant HCC, the Hep3B tumor mass with radiofrequency plus gemcitabine treatment (mean ± SD, 180 ± 91 mg) was statistically significantly smaller compared with gemcitabine alone (661 ± 419 mg, P = .0063).

Conclusions

This study provides mechanistic understanding of homologous recombination inhibiting-strategies, such as non-invasive radiofrequency field-induced hyperthermia, to overcome resistance to gemcitabine in refractory human solid tumors.


Primary hepatocellular carcinoma (HCC) is an aggressive disease. Globally, about one million new cases of HCC are diagnosed each year with an identical cause-specific mortality rate (1). Most patients are not eligible for curative intent local-regional therapies (1). Conventional cytotoxic therapy has been shown to be of minimal benefit. This has largely been attributed to over-expression of multidrug resistance-associated efflux protein (2–4). The clinical response of HCC to sorafenib, a multi-kinase inhibitor, is limited and transient (5). We purposely focused our investigation on gemcitabine, which is not used for the treatment of HCC because of substantial chemoresistance. We believe it is a priority to understand the mechanisms of gemcitabine chemoresistance and to develop methods to overcome this resistance.

Gemcitabine (2’, 2’ - difluoro 2’-deoxyctydine, dFdC) is a nucleoside analogue and a prodrug that is incorporated into the DNA of replicating cancer cells after activation. Despite resistance of HCC to gemcitabine, the mechanisms of resistance are largely elusive. Study of gemcitabine resistance in other cancers has focused on pathways involving its transport and metabolism, or those of altered apoptosis and survival. It is unclear if mechanisms engaged in repairing gemcitabine-stalled replication forks are important in resistance. Aberrant mismatched nucleotides are removed from the DNA by 3’-5’ exonuclease activity of DNA polymerase ε (6). It was demonstrated that dFdCMP residues are difficult to excise from the DNA, in part because of masked-chain termination in comparison with dCMP residues (7). Recent data suggests that restart requires regression of the stalled fork into a chicken-foot structure (8). This replication fork intermediate is sensed by poly (ADP-ribose) polymerase 1 (PARP1). Poly-ADP ribose residues associate with the chromatin recruit Mre11, a 3’-5’ exonuclease for DNA end processing (9). The DNA end processing is essential for loading of Rad51 recombinase on the DNA that forms a RAD51 nucleoprotein filament assisted by BRCA-2 (10). This complex subsequently catalyzes sister chromatid homology search and strand invasion to complete homologous recombination repair (HRR) (8). The HRR pathway attempts to restore error-free replication; however, once malignant transformation has occurred, cancer cells may rely on DNA repair pathways such as HRR to propagate the mutated genome. Importantly, it has been clearly demonstrated that cells treated with replication inhibitors exhibit...
pronounced activation of HRR and that this pathway is essential for survival during recovery from stalled replication forks (10). At least one report suggests localization of HRR pathway proteins to sites of gemcitabine-stalled replication forks (11).

Recent studies have demonstrated that hyperthermia has pronounced inhibitory effects on HRR pathways, mainly mediated through inhibition of PARP-1, MRN-complex, or BRCA-2 in the context of DNA double strand break repair (12–18). It is not known, however, if HRR of stalled replication forks is an important mechanism that contributes to chemosensitivity of gemcitabine. We hypothesize that hyperthermia can inhibit homologous recombination after gemcitabine-stalled replication forks through its effects on key components of the HRR pathway, hence contributing to chemoresistance in hepatocellular cancer, a malignancy not usually treated with gemcitabine because of drug resistance.

Methods

Cell Lines, Reagents, and Transfection
All cell lines (Hep3B, HepG2, and SNU449) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and used according to the supplier’s protocol within six months of acquisition. For transected cell lines, short tandem repeat fingerprint was confirmed by the Cell Line Characterization Core Service (M. D. Anderson Cancer Center, Houston, TX). Media, ie, RPMI-1640 (for SNU449) or MEM (for HepG2 or Hep3B) was supplemented with 10% (v/v) fetal bovine serum. For fluorescence microscopy, the following primary antibodies were used: rabbit anti-PAR (Trevengen, Gaithersburg, MD), rat anti-RPA32 (4E4, Cell Signaling, Danvers, MA), rabbit anti-Mre11 (GenTex, San Antonio, TX), rabbit anti-rad51 (H-92, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-γH2AX (Upstate-Millipore, Billerica, MA), and rat anti-BrdU (BU1/75[ICR1], Abcam, Cambridge, MA). Primary antibodies were detected using the following secondary antibodies; Alex Fluor 488 conjugated donkey anti-rat, Alex Fluor 546 conjugated donkey anti-mouse, and Alex Fluor 647 conjugated donkey anti-rabbit antibodies (Invitrogen, Grand Island, NY).

For western blot analysis, the following antibodies were used: mouse anti-PARP (Trevengen, Gaithersburg, MD), rabbit anti-NBS1 (Cell Signaling, Danvers, MA), rabbit anti-Rad50 (Cell Signaling, Danvers, MA), rabbit anti-mre11 (Gentex, San Antonio, TX), rabbit anti-rad51 (H-92, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-p53 (DO-1, Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-BRCA2 (Calbiochem, Billerica, MA). Primary antibodies were detected using HRP-linked goat anti-rabbit or goat anti-mouse antibodies (Jackson Immunoresearch, West Grove, PA). GFP-supplemented Renilla luciferase containing plasmid pRL-TK (Promega, Madison, WI) was introduced in Hep3B and HepG2 cells using lentiviral infection. For generating an Mre11-knockdown cell line, we used GP1Z lentiviral shRNA (Open Biosystems, Lafayette, CO) according to the supplied protocol. Further details are available in the Supplementary Methods (available online).

Immunocytochemistry
Approximately 50,000 cells were seeded in each well of a 12-well plate and grown on circular #1.5 cover slips (Electron Microscopy Sciences, Hatfield, PA). After 24 hours, cells were exposed to various treatment conditions as described “Results”. At the end of treatment, cells were fixed, permeabilized, blocked, labeled with primary antibody, and then with secondary antibody in consecutive steps. For confocal imaging, Fluoview - FV1000 Olympus Confocal Microscope (Center Valley, PA) was used. Acquisition parameters and processing are summarized in the Supplementary Methods (available online). Immunohistochemistry was performed similarly with some modifications, described in the Supplementary Methods (available online).

Clonogenic Assays
Clonogenic assays estimate single-cell reproductive viability by measuring the ability of a single cell to form a colony of 50 cells or more. Clonogenic assays in this study were performed as described previously (19) and in the Supplementary Methods (available online).

Cell Cycle Analysis
For cell cycle analysis a BrdU-labeling protocol from BrdU Flow Kit (BD Pharmingen, San Diego, CA) was used according to the supplied instructions. Laser and filter settings are described in Supplementary Methods (available online). Data was analyzed using FlowJo 7.63 (Tree Star, Inc., Ashland, OR).

Mouse Model of Hepatocellular Carcinoma
For in vivo studies, an implanted mouse model of human HCC was generated in CB17SCID mice (Taconic, Hudson, NY). Female mice between 4 and 5 weeks in age were purchased and acclimated in M.D. Anderson Animal facilities for up to one week. All animals were handled, housed and studied in accordance with the Institutional Animal Care and Use Committee. Tumors were generated after orthotopic implantation of approximately 1.6 million cells into the liver of each mouse (10 mice per group). Bioluminescence imaging indicated tumor development in the majority of mice three weeks after implantation. Further details are available in the Supplementary Methods (available online).

Radiofrequency Generator Setup
A Kanzius noninvasive external radiofrequency (RF) generator (ThermMed, LLC, Erie, PA) was used for animal hyperthermia exposures as described before (20) and in the Supplementary Methods and Supplementary Figure 1 (available online).

Statistical Analyses
The data were plotted and analyzed in GraphPad Prism (version 5, La Jolla, CA). For data with Gaussian distribution and when comparing two groups, the Student t test was used. Multiple group data were analyzed using one-way analysis of variance (ANOVA). Where necessary, ad-hoc post-tests were performed, and the type of test used is reported with the results. For all inferential statistics a P value less than .05 was considered significant. All statistical tests were unpaired two-sided unless noted otherwise.

Results

Effect of Hyperthermia on HRR-Pathway Proteins
To investigate the effects of hyperthermia on HRR-pathway proteins, we evaluated three different human HCC cells lines, ie,
Hep3B, HepG2, and SNU449. Cells were subjected to hyperthermia at 42.5°C for two hours in an incubator. Protein expression levels were monitored at varying time points. The data are shown in Figure 1, A and B, and Supplementary Figure 2 (available online).

With hyperthermia exposure, levels of Nbs1, Rad50 and Rad51 showed minimal change. The levels of Mre11 gradually declined after heat shock in all cell lines to less than half of the control levels at 24 hours, ie, normalized mean absorbance units ± SD at 24 hours for Hep3B cell line were 0.64 ± 0.09, P = .0024; for HepG2 cell line was 0.45 ± 0.07, P < .0001; and for SNU449 cell line was 0.61 ± 0.19, P = .024, compared with respective control groups. It has been previously reported that BRCA2 is an important target of heat radiosensitization (18). We found that the effect of hyperthermia on BRCA2 levels was cell-line–dependent. The HepG2 cells and SNU449 cells demonstrated negligible changes in BRCA2 levels after thermal exposure (statistically not significant); however, Hep3B cells demonstrated a slight decrease in BRCA2 levels, ie, normalized mean absorbance units ± SD at 24 hours for Hep3B cell line were 0.70 ± 0.13, P = .02, compared with the control group.

Localization of HRR-Pathway Proteins to Gemcitabine-Stalled Replication Forks

We first sought to investigate γH2AX-p as a marker of stalled replication forks. Hep3B cells were pulsed with BrdU for 30 minutes prior to addition of gemcitabine. This allowed us to label the DNA just downstream to the stalled replication fork site. When these cells were labeled with γ-H2AX antibody, we found that almost all γ-H2AX foci localize with BrdU foci, confirming their presence at stalled replication forks (Supplementary Figure 3, available online). Cells that were treated with hyperthermia alone also demonstrated γ-H2AX foci. However, the relative mean (±SD) numbers of γ-H2AX pixels per cell were statistically significantly fewer than those in cells treated with gemcitabine alone (0.28 ± 0.17 vs 1.0 ± 0.26, P = .017, Student’s t test, Figure 2D). We also note that γ-H2AX foci were only found in cells positive for BrdU, suggesting specificity for S-phase (Supplementary Figure 4, available online). Of note, in control experiments (ie, cells where BrdU label was added but no hyperthermia or gemcitabine treatment was given), γ-H2AX foci could not be found for further quantification (Supplementary Figure 4, available online). Hyperthermia immediately followed by gemcitabine treatment did not increase mean (±SD) γ-H2AX pixels per cell compared with those in cells treated with gemcitabine alone (1.0 ± 0.20 vs 1.0 ± 0.26, P = .8, Student’s t test, Figure 2D). This suggests a common etiology to the origin of γ-H2AX foci with hyperthermia, ie, stalled replication forks. Transient stalling of replication forks is known to occur with hyperthermia, based on prior reports (21). If this is correct, hyperthermia induced- γ-H2AX foci should colocalize with single-stranded DNA resulting from stalled replication forks. Using replication protein A (RPA) as a marker of single-stranded DNA, we found that γ-H2AX foci after hyperthermia exclusively colocalize with RPA foci. This confirms that γ-H2AX phosphorylation after hyperthermia corresponds to sites of single-stranded DNA (Supplementary Figure 5, available online).

After establishing γ-H2AX foci as a marker of stalled replication forks, we evaluated recruitment of downstream pathway proteins
to these sites. We exposed cells to mild hyperthermia at 42.5°C for 75 minutes followed immediately by a high concentration of gemcitabine (10 μg/mL). This concentration was chosen to induce maximal stalling of replication forks and hence to produce the maximum number of γ-H2AX foci in each cell. Stalling leads to stretches of single-stranded DNA. Replication protein A (RPA) has a high affinity for single stranded DNA and is important for recruitment of downstream HRR-pathway proteins to stalled replication forks. Pretreatment with hyperthermia does not inhibit localization of RPA to stalled replication forks (Supplementary Figure 5, available online). Downstream, RPA recruits proteins of the Mre11-Rad50-Nbs1 (MRN) complex. Within this complex, Mre11 is the key effector with known 3′ to 5′ exonuclease as well as 5′ to 3′ endonuclease activity. In particular, Mre11 nucleolytic activity allows loading of Rad51 recombinase by processing DNA ends at stalled replication forks. We note that pretreatment with mild hyperthermia decreases overall levels of Mre11 as well as impairs localization of Mre11 to sites with γ-H2AX foci; mean Pearson correlation (a.u.) and error bars represent the SD from three independent experiments with 10 replicates each (*P < .05, **P < .01). Gem = Gemcitabine; HT = Hyperthermia; N/A = not applicable, i.e., in hyperthermia treated cells BrdU staining is not detected as distinct foci and therefore correlation could not be quantified; ns = not significant, by the Student unpaired two-sided t test in comparison with gemcitabine alone group. Also, see Supplementary Figures 3–5 (available online).

**Figure 2.** Inhibition of Mre11 and Rad51 recruitment at gemcitabine-stalled replication forks by hyperthermia. A) Hep3B cells were exposed to hyperthermia and/or gemcitabine. B and C) Mre11 and Rad51 were recruited at gemcitabine-stalled replication forks. Cells treated with hyperthermia or hyperthermia with gemcitabine demonstrated decreased colocalization of Mre11 and Rad51 with γ-H2AX foci. Scale bar = 10 µm. D) Quantification of data from colocalization experiments. Data are mean Pearson correlation (a.u.) and error bars represent the SD from three independent experiments with 10 replicates each (*P < .05, **P < .01). Gem = Gemcitabine; HT = Hyperthermia; N/A = not applicable, i.e., in hyperthermia treated cells BrdU staining is not detected as distinct foci and therefore correlation could not be quantified; ns = not significant, by the Student unpaired two-sided t test in comparison with gemcitabine alone group. Also, see Supplementary Figures 3–5 (available online).

**Cell Cycle Alterations**

To test the hypothesis that hyperthermia would inhibit the repair of gemcitabine-stalled replication forks and thus delay progression through the cell cycle, we exposed Hep3B cells to gemcitabine for 24 hours (approximate doubling time of Hep3B cells) at a concentration...
of 1 μM. This concentration is comparable to the peak intracellular concentration achieved with clinically used fixed-dose rate regimens (22). Since incorporation of gemcitabine only occurs during S-phase, exposing Hep3B cells for 24 hours ensures incorporation of gemcitabine in all cells. For the last two hours of incubation, cells were or were not exposed to hyperthermia at 42.5 °C. Cell cycle progression was analyzed over time (Figures 3 and 4).

A 24-hour incubation with 1 μM gemcitabine completely halts cell cycle progression by activating a G1/S checkpoint (Figure 3). Once gemcitabine is removed, cells resume synchronized DNA synthesis in 24 hours, suggesting that gemcitabine-induced cell cycle arrest is reversible at clinically relevant concentrations. The delay caused by hyperthermia in progression through early and mid S-phase was negligible. We did note that gemcitabine followed by hyperthermia causes a much slower progression through late-S and G2/M phases compared with treatment with gemcitabine alone. The effect of hyperthermia on repair of stalled replication forks is transient (lasting a few hours), as most cells progress to the G0/G1 phase eventually.

Cells that fail to resolve stalled replication forks should demonstrate persistent staining for γ-H2AX phosphorylation sites. We treated Hep3B cells with gemcitabine (100 nM) for 24 hours. For the last two hours, cells were treated with or without hyperthermia at 42.5 °C. The medium was then replaced and cells were allowed to recover. We noted that when cells were treated with a combination of gemcitabine and hyperthermia, γ-H2AX positive cells persisted for a longer duration compared with treatment with hyperthermia or gemcitabine alone (Figure 3). The resolution of γ-H2AX positivity coincided with the progression of cell cycle observed previously.

**Figure 3.** Hyperthermia inhibition of postreplication recombination repair at gemcitabine-stalled replication forks. A) Experimental scheme. B) Cell cycle progression was monitored using flow cytometry in Hep3B cells after release from gemcitabine-induced G1/S arrest. C) Median DNA content of Hep3B cells was quantified using flow cytometry after treating them according to the design in (A). (Data points: means of median DNA content; error bars represent SD from three independent experiments with 10,000 counted events per experiment. Gem vs Gem + HT, *P < .05 by the Student unpaired two-sided t test at the specified time point). D) Hep3B cells were treated with gemcitabine +/- hyperthermia and cells positive for γ-H2AX foci were quantified. (Data points: mean percent of γ-H2AX positive cells; error bars represent SD from three independent experiments with at least 10 replicates each. Gem vs Gem + HT, *P < .05 by the Student unpaired two-sided t test at the specified time point). Gem = Gemcitabine; HT = Hyperthermia; t = Time.
Clonogenic Survival and Viability
Clonogenic assays were performed on Hep3B cells and SNU449 cells after one of two combinations of hyperthermia and gemcitabine compared with hyperthermia or gemcitabine alone. Figure 4 shows a dose-dependent enhancement of gemcitabine toxicity by hyperthermia in both SNU449 and Hep3B cells irrespective of the dose schedule used. Next, we evaluated clonogenic viability at a gemcitabine concentration of 5 ng/mL and varied the duration
of hyperthermia (30 minutes to 4 hours). The data demonstrate a thermal dose-dependent enhancement in toxicity for gemcitabine and hyperthermia.

Since we demonstrated that Mre11 is a thermolabile target of hyperthermia, we asked if inhibition of Mre11 exonuclease activity by a specific inhibitor, mirin, would result in similar enhancement of gemcitabine toxicity. Experiments were repeated with and without a sub-cytotoxic dose of mirin (25 μM). We found that inhibition of Mre11 exonuclease activity by mirin substantially enhanced gemcitabine-induced clonogenic cell death (Figure 5). Addition of hyperthermia did not enhance this toxicity, suggesting that thermal enhancement of gemcitabine toxicity is mediated through an Mre11-dependent pathway.

To rule out the possibility of off-target effects of mirin, we developed a partial Mre11 knockdown (shMre11) Hep3B cell line. Western blot densitometry confirmed depletion of Mre11 by 72 ± 11% (mean, SD) in the shMre11 cell line compared with the shControl cell line. We found that the shMre11 cell line was more sensitive to gemcitabine in comparison with the shControl cell line. Thermal enhancement of gemcitabine toxicity was noted for shControl but not shMre11 cells.

Animal Model Studies

Development of mouse models and details of RF exposure and thermography are discussed in Supplementary Figures 6 and 7 (available online). Mice bearing Hep3B orthotopic tumors were randomized to one of five groups: untreated, RF exposure alone, gemcitabine alone, gemcitabine followed 24 hours later by RF exposure, or RF exposure immediately followed by gemcitabine. The treatments were administered twice a week for three weeks for a total of six treatments. The gemcitabine dose administered was 70 mg/kg/dose or 150 mg/kg/week. This is approximately half the dose used in humans (1000 mg/m²/week dose in a 1.7m human = 300 mg/kg/week dose in a mouse). Twenty-four hours after the last treatment, mice were killed and tumors were harvested, weighed, and fixed in formalin for later analysis (Figure 6A). We noted that tumors in all treatment groups were statistically significantly smaller and had a lower tumor mass than untreated controls (P < .05). Combination therapy was more effective than gemcitabine alone based on tumor mass, irrespective of the sequence of combination therapy (mean tumor mass ±SD) for gemcitabine vs RF then gemcitabine, 661 ± 419 mg vs 180 ± 91 mg, P = .0063; gemcitabine vs gemcitabine then RF, 661 ± 419 mg vs 291 ± 126 mg, P = .022). This experiment was repeated in the slow-growing HepG2 xenograft model with weekly treatments for three weeks with similar results (Figure 6B) (mean tumor mass ±SD) for gemcitabine vs RF then gemcitabine, 170 ± 51 mg vs 107 ± 64 mg, P = .032; gemcitabine vs gemcitabine then RF, 170 ± 51 mg vs 74 ± 45 mg, P = .0005). Preliminary in vivo experiments to assess the thermomimetic effect of mirin demonstrated that the mice treated with mirin and gemcitabine had the lowest median tumor mass compared with other groups (Supplementary Figure 8, available online). Consistent with in vitro data, we observed decreased localization of Mre11 and Rad51 to sites of stalled replication forks following RF-induced hyperthermia. There was a relatively higher proportion of γ-H2AX-positive cells in tumors treated with combination therapy in comparison with other groups (mean γ-H2AX positive pixels/ HPF ±SD) for gemcitabine vs RF then gemcitabine, 27258 ± 8022 vs 45430 ± 18980, P = .042; gemcitabine vs gemcitabine then RF, 27258 ± 8022 vs 46968 ± 10284 P = .0007, Supplementary Figures 9–11, available online).

Discussion

Several pathways have been implicated in chemoresistance of solid tumors to gemcitabine. Most involve reduced conversion of the prodrug (gemcitabine) to active drug (gemcitabine triphosphate), leading to decreased incorporation into DNA. Our data demonstrate that a clinically achievable intracellular concentration (1 μM) was sufficient to arrest the cell cycle in hepatocellular carcinoma cells, suggesting adequate incorporation of gemcitabine into the DNA. Pathways to repair gemcitabine-stalled replication forks exist and may contribute to drug resistance.
We evaluated proteins of the HRR-pathway believed to be important in the restart of hydroxyurea-stalled replication forks. We find that RPA, Mre11 and Rad51 readily accumulate at sites of stalled replication forks. These findings are consistent with prior reports \(^9\),\(^{15}\),\(^{23}\). When evaluating HRR protein levels after hyperthermia, we noted that partial degradation of Mre11 was consistently observed in all cell lines. Thermolability of Mre11 has been recently reported in a study where only 10 min at 42.5 °C reduced Mre11 exonuclease function to 10% of untreated control \(^{22}\). In addition, we found that Mre11 and, as a result, downstream Rad51 failed to localize to gemcitabine-stalled replication forks in cells pretreated with mild hyperthermia. There was a prolonged passage of cancer cells treated with gemcitabine and hyperthermia through late S and G2 phase, which is characteristic for cells deficient in post-replication recombination repair \(^{24}\). When evaluating synergy of gemcitabine and hyperthermia, Vertees et al. noted a similar enhancement of cells arrested in G2/M phase with combination therapy \(^{25}\). As a consequence, inhibition of the Mre11-dependent HRR pathway after exposure to gemcitabine by hyperthermia, mirin, or Mre11 knockdown is responsible for decreased clonogenic survival and cell death. A recent study demonstrated that nonhomologous end joining (NHEJ) represents a salvage pathway for cancer cells in which HRR of double-strand breaks is inhibited by hyperthermia \(^{26}\). While this is a possibility, the role of NHEJ as an alternative fork restart pathway remains inconclusive \(^{27}\). In addition, the effect of hyperthermia on Mre11 levels was not evaluated in that study. Although we have limited our discussion to the role of Mre11 in HRR, it is possible that inhibition of Mre11 by hyperthermia also inhibits the salvage NHEJ pathway. This hypothesis is supported by the findings of Zhuang et al. but remains to be investigated in the context of hyperthermia in future studies \(^{28}\).

It is important to discuss thermal degradation of BRCA2 and its effects on HRR of stalled replication forks because BRCA2 plays two essential roles at the stalled replication forks. BRCA2 prevents excessive nucleolytic degradation of stalled forks by Mre11, an effect associated with genomic instability \(^{29}\). Therefore, degradation of BRCA2 is expected to increase excision of gemcitabine by Mre11 and contribute to chemoresistance. Conversely, BRCA2 participates in Rad51 loading in the HRR pathway \(^{30}\). In this case, degradation of BRCA2 is expected to inhibit the repair of gemcitabine-stalled replication forks and have the opposite effect. Because of this paradoxical effect at stalled replication forks, it is unlikely that BRCA2 is responsible for thermal enhancement of gemcitabine toxicity as observed here. These findings also imply that synergistic interaction between hyperthermia and gemcitabine will ultimately depend on the relative effect of heat on BRCA2 compared with that on Mre11. For instance, thermal degradation of BRCA2 without Mre11 inhibition may not only contribute to gemcitabine resistance but also genomic instability as detailed in a report by Schlacher et al. \(^{29}\). Conversely, tumors already deficient in BRCA2 may be more susceptible to thermal sensitization of gemcitabine therapy. This speculation is supported by findings of Ying et al., who showed BRCA2 deficient cells to be more susceptible to Mre11 inhibition \(^{23}\). These speculations, however, need to be tested further in future studies.

![Figure 6. Efficacy of gemcitabine and RF combination therapy in mice bearing orthotopic hepatocellular carcinoma xenografts. Tumor weight and percent growth inhibition are shown for Hep3B (A) and HepG2 (B) xenografts. Data points represent mean tumor mass (mg) and error bars represent SD, n = 7–10, *P < .05, **P < .001 vs gemcitabine by the Student unpaired two-sided t test. RF = Radiofrequency. Also, see Supplementary Figures 6–11 (available online).](jnci.oxfordjournals.org)
This study also had some limitations. We used human hepatocellular cancer xenografts in an immunocompromised murine model. The livers in these animals, unlike the livers in the majority of human hepatocellular carcinoma patients, are not chronically inflamed or cirrhotic. This is also not a spontaneous or induced model of development of hepatocellular carcinoma. These studies will be repeated in our laboratory in a porcine model of carcinogen-induced chronic liver injury leading to cirrhosis and hepatocellular carcinogenesis in order to study the effects of RF-induced hyperthermia and gemcitabine treatment in animals with chronically injured livers and hepatocellular cancer. This will be critical to understanding the efficacy and potential toxicity profile differences in a more clinically relevant model. This initial study has demonstrated a potentially important interaction between noninvasive RF field-induced hyperthermia and cytotoxic chemotherapy, including overcoming a molecular mechanism involved in chemotherapy resistance. We must now study RF field dosimetry, including electric field strength, treatment duration, treatment frequency, and changes in the RF frequency used in order to optimize this treatment benefit.

We conclude that the HRR pathway is important in the repair of gemcitabine-stalled replication forks. Inhibition of the HRR-pathway protein Mre11 enhances the toxicity of gemcitabine in cancer cells in vitro and in vivo. Thermal enhancement of the anti-tumor effect of gemcitabine is mediated through inhibition of Mre11-dependent HRR pathways by denaturation and degradation of Mre11. Noninvasive RF field-induced hyperthermia in combination with gemcitabine is superior to either modality alone in orthotopic murine cells in vitro and in vivo. Thermal enhancement of the anti-tumor effect of gemcitabine is mediated through inhibition of Mre11-dependent HRR pathways by denaturation and degradation of Mre11. Noninvasive RF field-induced hyperthermia in combination with gemcitabine is superior to either modality alone in orthotopic mouse models of hepatocellular carcinoma. Future studies already underway will build on these findings to develop a noninvasive combined modality treatment for patients with hepatocellular carcinoma.

References

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