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PARP Inhibitors Synergize with Gemcitabine by Potentiating DNA Damage in Non-Small Cell Lung Cancer

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Abstract

Poly (ADP-ribose) polymerase (PARP) inhibitors have demonstrated great promise in the treatment of patients with deficiencies in homologous recombination (HR) DNA repair, such as those with loss of *BRCA1* or *BRCA2* function. However, emerging studies suggest that PARP inhibition can also target HR-competent cancers, such as non-small cell lung cancer (NSCLC), and that the therapeutic effect of PARP inhibition may be improved by combination with chemotherapy agents. In this the present study, we found that PARP inhibitors talazoparib (BMN-673) and olaparib (AZD-2281) both had synergistic activity with the common first-line chemotherapeutic gemcitabine in a panel of lung cancer cell lines. Furthermore, the combination demonstrated significant *in vivo* antitumor activity in an H23 xenograft model of NSCLC compared to either agent as monotherapy. This synergism was occurred without loss of HR repair efficiency. Instead, the combination induced synergistic single-strand DNA breaks, leading to accumulation of toxic double-strand DNA lesions *in vitro* and *in vivo*. This study elucidates the underlying mechanisms of synergistic activity of PARP inhibitors and gemcitabine, providing a strong motivation to pursue this combination as an improved therapeutic regimen.

Keywords

PARP Inhibitors; Non-small cell lung cancer (NSCLC); DNA Damage; Gemcitabine

INTRODUCTION

Lung cancer is the most common tumor in the world. It almost invariably has a poor prognosis, and it is the most frequent cause of cancer deaths (1.1 million annually)¹. Non-

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

small-cell lung cancer (NSCLC) accounts for more than 80% of all lung cancer cases². About two thirds of all patients with newly diagnosed NSCLC have advanced disease (stage IIIB or IV) that can only be managed using palliative chemotherapy if they cannot benefit from targeted drugs. The standard primary treatment in these patients is a combination of platinum with an agent, such as gemcitabine, paclitaxel, vinorelbine, or docetaxel³.

Unfortunately, use of platinum-based chemotherapeutics is hampered by high toxicity and acquired resistance. Specifically, it is plagued by nephrotoxicity and myelosuppression⁴⁻⁶, and several studies of prolonged initial platinum-based combination chemotherapy demonstrated resistance to it with no evidence of additional benefit after two or three rapidly administered cycles rapidly. Four treatment cycles are recommended to elicit the maximum benefit of a platinum-based combination, with a reduced risk of toxicity caused by prolonged treatment during a relatively short overall survival period^{6, 7}. The development of new effective combination regimens for NSCLC therapy is a major unmet medical need. Poly (ADP-ribose) polymerase (PARP) inhibitors offer an attractive new NSCLC therapeutic avenue, as they are largely less toxic than platinum agents⁸⁻¹⁰.

A fundamental feature of cancer is genome instability¹¹. In tumor cells, genomic instability is likely the combined effect of DNA damage, tumor-specific DNA repair defects, and failure to stall or stop the cell cycle before damaged DNA is passed on to daughter cells¹². Although these processes lead to genomic instability and ultimately disease progression, they also provide therapeutic opportunities¹². Defective DNA damage response, such as dysregulation of DNA damage repair and checkpoint signaling^{13, 14}, is known to be associated with predisposition to cancer and affects tumor response to DNA-damaging anticancer therapy¹⁵. As DNA repair pathways have multiple redundancies to ensure genome stability, so while a tumor cell with deficient DNA damage response may be killed by exposure to agents that damage DNA or inhibit repair pathways, any negative effects will be largely minimized on normal tissue¹⁶. Therefore, exploitation of DNA damage response defects by synthetic lethality is a promising approach to cancer therapy¹⁵.

PARP is involved in DNA repair and transcriptional regulation and is recognized as a key regulator of cell survival and cell death as well as a master component of a number of transcription factors involved in tumor development and inflammation¹⁷. PARP1, which accounts for at least 85% cellular PARP activity¹⁷, binds to single-strand DNA, such as stalled replication forks and base-excision repair intermediates, to facilitate repair processes¹⁸. Once this function is disabled by treatment with a PARP inhibitor¹⁹, cells rely on homologous recombination (HR) for DNA damage repair. Dysfunction of HR (such as in *BRCA1* or *BRCA2*-deficient cells) results in synthetic lethality with PARP inhibition^{20, 21}. Researchers have used this synthetic lethal relationship in patients with HR-incompetent cancers, such as BRCA-mutated breast and ovarian cancer^{16, 21-24}. Recent studies demonstrated that PARP inhibitors act synergistically with both gemcitabine and cisplatin in triple-negative breast cancer cells with defects in HR-directed repair²⁵. However, the benefit in of PARP inhibitor monotherapy and combinations in BRCA-proficient solid tumors remain unclear.

In this study, we demonstrated that inhibition of PARP has single-agent activity in NSCLC, which is further potentiated by the current first-line chemotherapeutic gemcitabine *in vitro*. This synergistic activity was conserved with multiple cell lines and PARP inhibitors. Furthermore, this combined therapy for NSCLC was beneficial *in vivo* with the use of the highly potent PARP inhibitor talazoparib (BMN 673), which recently entered phase I testing in lung cancer patients²⁶. We found that this synergism occurred without loss of HR activity but depended on accumulation of single-strand DNA breaks. These results suggest that co-administration of gemcitabine in a plausible strategy for expanding the therapeutic use of PARP inhibitors to a broader spectrum of lung cancers.

MATERIALS AND METHODS

Cell culture and chemicals:

H23 and H522 lung adenocarcinoma cells, as well as SK-MES-1 squamous lung cancer cells were obtained from ATCC (Manassas, VA, USA). They were grown in RPMI 1640 supplemented with glutamine and 10% fetal bovine serum at 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% air. Cell lines were validated by STR profiling at the MD Anderson Cancer Center core facilities and routinely tested for mycoplasma. The PARP inhibitors olaparib (AZD2281) and talazoparib (BMN673) were purchased from Selleckchem (Houston, TX, USA). BMN673 and AZD2281 were dissolved in dimethyl sulfoxide (DMSO) and kept as 10 mmol/L stock solutions in small aliquots at -20°C. The following primary antibodies were used: phosphorylated RAD17 (p-RAD17), p-CHK2, cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA), RAD51, γH2AX antibody (Millipore), and p-RPA32 (Bethyl Laboratories, Montgomery, TX, USA). The transfection reagent Lipofectamine 3000 was purchased from Life Technologies (Carlsbad, CA, USA).

Cell viability assay:

Cancer cells were seeded in 96-well cell culture plates and treated on the second day with gemcitabine alone, BMN673 alone, gemcitabine combined with BMN673, or DMSO (vehicle control). At the end of 5 days of treatment, cell numbers were estimated using a sulforhodamine B assay. The relative cell viability was calculated using the equation $ODT/ODC \times 100\%$, in which ODT is the absorbance of the treatment group and ODC is the absorbance of the control group. The results represent the means from three independent experiments conducted in duplicate. Half-maximal inhibitory concentrations (IC₅₀) values were calculated using dose-response curves with the Prism software program (GraphPad Software, San Diego, CA, USA).

Drug combination analysis:

Drug combinations were analyzed according to the Chou-Talalay equation, which accounts for both the potency (median inhibitory concentration) and the shape of the dose-effect curve. The cytotoxicity of these combinations was compared with that of each drug alone using the combination index (CI), with CIs less than 0.9, from 0.9 to 1.1, and greater than 1.1 indicating synergistic, additive, and antagonistic effects, respectively. The combination index (CI) was determined using the CompuSyn software program^{27, 28}.

Immunofluorescence staining and microscopy:

To detect DNA damage-induced γ -H2AX, native bromodeoxyuridine (BrdU) and RAD51 foci formation, cells were treated with either gemcitabine (5 nM) or BMN 673 (50 nM) for 48 hours. Cells were extracted with cytoskeletal and stripping buffers, fixed with 4% paraformaldehyde, and subjected to permeabilization with 0.5% NP-40 and 1% Triton X-100. The cells were then incubated with a primary antibody (anti-human γ -H2AX or rabbit anti-RAD51, 1:400; Abcam, Cambridge, UK) for 2 hours at room temperature and incubated with a secondary antibody (Alexa Fluor 488-conjugated donkey anti-rabbit, 1:400; Life Technologies) for 1 hour at room temperature. Slides were mounted in medium containing DAPI (H-1200; Vector Laboratories, Burlingame, CA, USA) and analyzed under a fluorescence microscope (Eclipse TE2000E; Nikon Instruments, Melville, NY, USA). At least 8 images for biological replicate were captured and quantified using custom image analysis algorithms in MATLAB.

Annexin V/propidium iodide-based flow cytometric analysis:

Apoptosis was determined via flow cytometry using an Annexin V-FITC Apoptosis Kit from BD Biosciences (San Jose, CA, USA). H23 and SK-MES-1 cells were plated in six-well plates at a density of 5×10^5 cells per well overnight and then treated with gemcitabine, BMN 673, or a combination of the two for 48 hours. Labeling was performed according to the manufacturer's instructions, and flow cytometry was conducted using a FACS Aria flow cytometer (BD Biosciences). The percentage of early apoptotic cells was calculated by annexin V-positivity and propidium iodide (PI) negativity, whereas the percentage of late apoptotic cells was calculated according to annexin V and PI dual positivity.

HR repair analysis:

H23 and SK-MES-1 lung cancer cells were transiently co-transfected with the HR repair reporter substrate direct-repeat-GFP (DR-GFP) and pCBASceI as described previously²⁹ (gifts from Dr. Maria Jasin, Memorial Sloan-Kettering Cancer Center, New York, NY, USA). The GFP-expressing plasmid pEGFP-C1 was used as a transfection efficiency control. Gemcitabine was added 12 hours after transfection, and then cells were incubated for 48 hours before flow cytometric analysis of GFP intensity.

Western blotting:

Total proteins were collected in urea lysis buffer (1 M Tris-HCl, pH 7.5, 8 M urea, 150 mM β -mercaptoethanol, and fresh protease inhibitors). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay. Equal amounts of protein from each sample were separated on a SDS-PAGE. After electrophoresis, proteins were electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked at room temperature and then incubated at 4°C overnight with desired primary antibodies, washed three times in PBS, and then incubated with HRP-conjugated secondary antibodies. After secondary incubation, the membranes were washed three times with PBS, and was visualized using an enhanced chemiluminescence kit (GenDEPOT, Harris, TX).

Cell-cycle assays:

Cells were harvested by trypsinization, washed twice with cold PBS, fixed with ice-cold 70% ethanol, and fixed at -20°C at overnight. Cells were then washed with PBS and incubated with 25 $\mu\text{g/mL}$ propidium iodide (PI) containing 30 $\mu\text{g/mL}$ ribonuclease A for 30 min at room temperature. Cells were analyzed using a FACSCalibur flow cytometer with the CellQuest software program (BD Biosciences), and quantification performed using FlowJo.

***In vivo* tumorigenesis assay:**

All animal studies were conducted in compliance with animal protocols approved by The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee. Six-week-old male and female athymic nu/nu mice (average weight, 25 g) were used for all *in vivo* experiments. H23 cells (8×10^6) were inoculated subcutaneously into the right dorsal flanks of the nude mice^{30, 31}. When the resulting tumors reached an average volume of about 50–75 mm^3 , the mice were randomly placed in control and treatment groups ($n=8$ animals per group). Daily treatment with a vehicle control, gemcitabine (80 mg/kg)³², BMN 673 (0.333 mg/kg)²⁸, or a combination of gemcitabine and BMN 673 was given via oral gavage. The mice underwent treatment for 33 days. Their body weights were monitored, and the perpendicular diameter of each tumor was measured twice a week using a digital caliper. Tumor volumes were calculated using the following formula: $(\text{length} \times \text{width}^2)/2$. At the end of the 33-day period, the animals were sacrificed, and their tumors removed. Tumor tissues were formalin fixed and embedded in paraffin. In some instances, end-point blood was harvested in heparin-coated tubes, transferred to heparin-coated capillary tubes and centrifuged at 10,000 RPM for 10 minutes. Compact blood volume (hematocrit) was determined in ImageJ.

Immunohistochemistry:

Sections were deparaffinized in xylene and serial ethanol dilutions. Antigens retrieval was performed with citric acid, and the endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 10 min. The sections were then blocked with 10% normal sheep serum for 30 min. After overnight incubation with primary antibodies, including $\gamma\text{-H2AX}$ (dilution, 1:500) and cleaved caspase-3 (dilution, 1:400), the sections were probed with biotinylated secondary antibodies and then incubated with a streptavidin-biotin complex (Lab Vision, Fremont, CA, USA). The sections were then stained with DAPI (3,3'-diaminobenzidine tetrahydrochloride solution) (Lab Vision), counterstained with Mayer's hematoxylin (Thermo Fisher Scientific, Pittsburgh, PA, USA), dehydrated, and mounted.

Statistical analysis:

Unless otherwise noted, statistical significance of differences between multiple groups was performed using ANOVA with appropriate post-hoc tests. P values less than 0.05 were considered statistically significant.

RESULTS

PARP inhibitors synergize with gemcitabine in NSCLC cell lines

Gemcitabine is a front-line DNA-damaging chemotherapy used to treat NSCLC. Because PARP inhibition is reported to enhance the effects of other DNA-damaging anticancer drugs, we hypothesized that PARP inhibitors would enhance the cytotoxicity of gemcitabine in NSCLC cell lines. We tested this in a panel of three lung cancer cell lines with two different PARP inhibitors in clinical trials for lung cancer therapy: talazoparib (BMN673) and olaparib (AZD2281). In H23 cells, the combination of either BMN673 (Fig. 1a) or AZD2281 (Fig. 1b) with gemcitabine potently inhibited cell viability. We observed similar effects in H522 and SK-MES-1 cells (Fig. 1c–f). As shown in Figure 1g, all tested cell lines displayed observed significant synergy of PARP inhibitors with gemcitabine based on the calculated CI values²⁷. In addition, the combinations of PARP inhibitors and gemcitabine had equal or better CI values in lung cancer cells as the standard lung cancer chemotherapy regimen of cisplatin and gemcitabine (Fig. 1g, Supplementary Fig. 1).

Combination of PARP inhibitors and gemcitabine induces apoptosis

To monitor the apoptotic response following treatment of lung cancer cells with gemcitabine and BMN673 alone and combined, we evaluated cleavage of caspase-3 using Western blot as well as flow cytometric analysis of single cells following dual Annexin V/PI staining (Annexin V binds to cell surface phosphatidylserines during apoptosis). As shown in Fig. 2a, we observed caspase-3 cleavage in H23 cells after either gemcitabine- or BMN673-based monotherapy, but the combination of the two agents induced much more caspase-3 cleavage than either single agent. Furthermore, flow cytometric analysis using annexin V/propidium iodide staining demonstrated that after treatment with gemcitabine and BMN673 for 48 hours, the proportions of early and late apoptotic H23 and SK-MES-1 cells were markedly increased (Fig. 2c–e), with the largest changes occurring with the combined treatment, suggesting that the combination induced lung cancer cell apoptosis better than either drug did alone. Thus, the combination of gemcitabine and BMN673 has a synergistic apoptotic effect on NSCLC cells that is consistent with their observed effect on proliferation.

Combination treatment with PARP inhibitors and gemcitabine results in tumor regression in NSCLC xenograft models

We further assessed the effects of treatment with BMN673 and gemcitabine alone and combined on H23 tumor xenografts *in vivo*. Exposure to BMN673 or gemcitabine delayed tumor growth more so than that in the vehicle group. The combination of BMN673 and gemcitabine showed superior tumor growth inhibition compared to monotherapy with BMN673 ($p=0.019$) or gemcitabine ($p=0.033$) (Fig. 3a–b). Furthermore, we observed neither significant body weight loss in mice from any treatment arm (Fig. 3c), nor significant reduction in hematocrit levels indicative of anemia at the conclusion of treatment (Fig. 3d), indicating that the combination was well tolerated by the animals.

Analysis of H23 tumors at the termination of the study demonstrated that tumors treated with the combination of BMN673 and gemcitabine had increased apoptosis as indicated by immunohistochemical staining of cleaved caspase-3 (Fig. 3e) above either single agent. This

was consistent with the cleavage of caspase-3 observed *in vitro* (Fig. 2a–b), with the largest increase in observed in cells treated with the gemcitabine/BMN673 combination. Thus, combining gemcitabine with PARP inhibitors may improve therapeutic efficacy in NSCLC patients without increasing toxicity in normal cells.

Gemcitabine does not suppress homologous recombination repair

We found synergism of gemcitabine with PARP inhibitors, which has previously been reported to be induced by induction of homologous recombination (HR) defects³³. Thus, we hypothesized that gemcitabine likewise may suppress HR in NSCLC. To test this, we used a fluorescent reporter construct in which a functional GFP gene was reconstituted following an HR event, and analyzed the percentage of GFP+ (HR competent) cells by flow cytometry. Not only did gemcitabine not reduce HR repair capacity, we actually observed an increase in the percentage of GFP+ cells in both H23 and SK-MES-1 cells (Fig. 4a). This result suggests that gemcitabine does not inhibit HR repair, contrary to the hypothesis that the synergy is induced by induction of an HR defect^{18, 34–36}. To verify this, we further analyzed expression of RAD51, a marker of HR repair. Analysis of total protein levels by western blot demonstrated no alterations in total RAD51 protein expression (Fig. 4b). Furthermore, immunofluorescence staining for RAD51 foci also demonstrated no reduction in RAD51 foci formation after 48 hours of treatment (Fig. 4c), with slightly more RAD51 foci forming in H23 cells treated with both agents than in those treated with either agent alone or vehicle control (Fig. 4d, $p>0.05$). This result persisted after drug washout for at least 48 hours (Fig. 4e).

Combination treatment with a PARP inhibitor and gemcitabine alters cell-cycle dynamics:

To investigate alternative mechanisms by which the combination of gemcitabine and BMN673 may act synergistically, we profiled cell-cycle distributions following treatment with the two drugs alone and combined over the first three days of drug exposure. We stained the cells for their DNA content using propidium iodide and performed cell-cycle analysis using flow cytometry. As shown in Figure 5, within 24 hours BMN673 produced a mild G2/M arrest that persisted across all time points evaluated. In contrast, at 24 hours gemcitabine produced accumulation of cells at S-phase that dissipated over the course of treatment. When the drugs were combined the initial S-phase arrest of gemcitabine was reproduced, but a unique G2/M-phase arrest was observed after 72 hours of treatment. Quantification of cell cycle distribution indicated over 50% of combination treated cells were stalled in G2/M-phase, nearly double the amount observed with BMN673 alone (Fig. 5b). The G2/M checkpoint is activated in response to S-phase DNA damage associated with replication, so we next hypothesized that the combination was increasing replication-associated DNA damage.

Combination treatment with a PARP inhibitor and gemcitabine induces synergistic DNA damage mediated by increased accumulation of single-strand DNA breaks

Gemcitabine is known to stall replication forks upon incorporation into DNA as evidence by S-phase arrest following 24 h of treatment (Fig. 5), and PARP1 is known to be recruited to stalled replication forks. We hypothesized that this replication fork stalling, known as replication stress, contributes to the synergistic activity of gemcitabine and BMN673. To

gain further insight into this, we examined the changes in a panel of DNA damage response proteins. We found that the combination induced significant DNA damage, with increased levels of double-strand DNA break marker γ H2AX and even greater phosphorylation of single-strand break markers Rad17 and RPA indicative of increased replication stress (Fig. 6a).

To confirm this observation, we examined the formation of DNA damage foci by immunostaining for γ H2AX as a double-strand DNA break marker and native BrdU as a single-strand DNA break marker indicative of replication stress. Image quantification demonstrated that the intensity of γ H2AX staining was greater in cells subjected to combination treatment with gemcitabine and BMN673 than in those given monotherapy or vehicle control ($p<0.05$), which was consistent with the western blot results. The incidence of single-strand DNA breaks, as indicated by native BrdU staining, increased much more dramatically (combination versus gemcitabine alone, $p<0.01$; combination versus BMN 673 alone, $p<0.05$) (Fig. 6b–c). Moreover, while there was a strong correlation between staining intensity for single- and double-strand DNA breaks in all treatment conditions (Spearman correlation coefficient >0.5), this correlation was strongest in the combination treatment (Spearman correlation coefficient = 0.91) (Fig. 6d). Immunohistochemical analysis of H23 tumor samples at the termination of the study demonstrated this DNA damage was also observed *in vivo*, with large increases in γ -H2AX staining intensity in combination gemcitabine- and BMN673-treated tumors (Fig. 6e). Taken together, these results suggest that treatment with this combination increases accumulation of double- and single-strand DNA breaks in NSCLC cells, contributing to the synergism of gemcitabine and PARP inhibitors.

DISCUSSION

The use of DNA-damaging chemotherapy for lung cancer has been a mainstay of cancer treatment over the past 50 years³⁷. The anticancer activity of most chemotherapeutic drugs relies on induction of DNA damage in rapidly cycling tumor cells with inadequate DNA repair³⁸. DNA repair pathways can enable cancer cells to survive DNA damage induced by chemotherapy¹⁰. Therefore, heightened DNA repair capacity can cause resistance to DNA-damaging chemotherapy¹⁵. Conversely, deficient DNA repair capacity can induce unreparable and more cytotoxic DNA damage in cancer cells by selecting the appropriate chemotherapy. Therefore, DNA damage response inhibitors can expand the range of tumor types that can be treated with conventional drugs¹⁰. The best possible treatments may be combinations of DNA-damaging anticancer agents with DNA damage response inhibitors, which prevent therapy resistance and increase the efficacy of the cancer treatment by inhibiting DNA repair-mediated removal of toxic DNA lesions. The sensitivity of NSCLC to DNA-damaging chemotherapy suggests that targeted inhibition of the DNA repair pathways operant in NSCLC is a particularly attractive strategy and can substantially augment the efficacy of therapies in the current armamentarium.

In the present study, we tested the anticancer activity of PARP inhibitors in combination with the chemotherapeutic agent gemcitabine in NSCLC cell lines and animal models of NSCLC without defects in homologous DNA repair. We found that the combination of

gemcitabine and multiple PARP inhibitors had synergistic activity in three NSCLC cell lines. The combination index values for these combinations were similar to or better than that for the combination of gemcitabine and cisplatin, which has been the standard-of-care chemotherapy for this disease for more than 10 years. These findings provide rationale for further investigation into translation of combinations of PARP inhibitors with gemcitabine into clinical use as alternative forms of platinum-based combination chemotherapy, the side effects of and resistance to which have limited the efficacy of such combinations in most patients^{6, 39}. While monotherapy with either the PARP inhibitor BMN673 or gemcitabine showed *in vivo* activity, their combination showed maximal inhibition of tumor growth and induction of cleaved caspase 3 with minimal signs of toxicity (Fig. 3).

PARP inhibitors are largely thought to act through synthetic lethality with cells that show defective homologous recombination, though emerging evidence suggests they can likewise target HR-proficient NSCLC (8). We observed functional HR repair in the NSCLC both in presence and absence of gemcitabine using both the DR-GFP reporter assay and analysis of RAD51 foci formation (Fig. 4). However, administration of this compound to HR-deficient NSCLC may show an even greater therapeutic benefit. The maintenance of HR activity with gemcitabine diverges from most PARP inhibitor synergy literature reports where synergy is achieved through induction of HR defects^{28, 40}. Although we found no evidence of deficient HR repair, we did find that the combination of BMN673 and gemcitabine induced synergistic DNA damage in NSCLC cell lines. Compared with BMN673- and gemcitabine-based monotherapy, combination treatment increased phosphorylation the double-strand DNA break marker γ -H2AX, with even greater increases in the single-strand DNA break markers phosphorylated RAD17 and RPA, suggesting accumulation of unrepaired single-strand DNA breaks. We verified this result on a single cell level by staining for γ -H2AX to examine double-strand DNA breaks and for native BrdU foci to examine single-strand DNA breaks (Fig. 6). Our xenograft studies demonstrated that increased γ -H2AX was conserved *in vivo*, as well. These *in vitro* and *in vivo* findings strongly demonstrated that use of the combination of BMN673 and gemcitabine leads to not only an increase of single-strand DNA breaks but also accumulation of double-strand DNA breaks, which contributes to the sensitivity to combined PARP inhibition and treatment with gemcitabine.

Gemcitabine is a nucleoside analog that is incorporated into the DNA of replicating cancer cells⁴¹. It not only inhibits ribonucleotide reductase and DNA polymerases but also is incorporated into replicating DNA, resulting in partial chain termination, stalling of replication forks, and single-stranded DNA breaks^{42–44}. The replication fork stalling induces S-phase checkpoint activation, a subsequent decrease in initiation of replication origins⁴³, and S-phase arrest^{45, 46}. PARP enzymes have an active role in protecting stalled replication forks and mediating replication restart^{47, 48}. PARP is also implicated to have a role in stabilizing replication fork arrest upon topoisomerase I poisoning⁴⁹. Upon gemcitabine-based treatment, replication forks rapidly experience stalling, and may fail to restart due to PARP inhibition, leading to increases in toxic double-strand DNA breaks. This combination leads to incomplete replication and persistent fork stalling, causing double-strand DNA breaks via eventual fork collapse and/or processing. This offers insight into the molecular mechanisms of the synergistic activity of gemcitabine and PARP inhibitors.

In conclusion, we report herein on the *in vitro* and *in vivo* synergy of PARP inhibitors with gemcitabine in NSCLC cell lines. This synergy occurs with alterations in HR activity and may be explained by exposure to gemcitabine leading to stalled replication forks, requiring PARP activity for restart. Taken together, our findings may open new routes to treatment of NSCLC with PARP inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Impact Statements

In this work, the authors demonstrate that poly (ADP-ribose) polymerase (PARP) inhibitors and gemcitabine have synergistic activity in non-small cell lung cancer (NSCLC) both *in vitro* and *in vivo*, demonstrating promise as a front-line regimen in treatment of NSCLC. In contrast to most documented synergizing agents, this phenomenon was independent of alterations of homologous recombination, but instead depends on gemcitabine-induced stalled replication forks, which fail to recover in presence PARP inhibition.

Figure 1. PARP inhibitors synergize with gemcitabine in NSCLC cells.

(a-f) Cell proliferation assay results for H23, H522, and SK-MES-1 cells treated with gemcitabine (GEM) and the PARP inhibitors BMN673/talazoparib (a-c) and AZD2281/olaparib (d-f) at different concentrations for 5 days. Each value is relative to the value for cells treated with DMSO vehicle control. Results are shown as means $\pm$ standard deviation from three independent experiments. (g) Comparison of the cytotoxicity of the combinations of gemcitabine with PARP inhibitors and cisplatin using the combination index (CI). The CI reflects the signs and magnitude of drug-drug interactions; in this study, CIs less than 0.9, ranging from 0.9 to 1.1, and greater than 1.1 indicated synergistic, additive, and antagonistic effects, respectively.

Figure 2. Induction of apoptosis in NSCLC cell lines by treatment with gemcitabine and BMN673 alone or combined.

(a) Western blot showing changes in cleaved caspase-3 protein expression in H23 and SK-MES-1 cells after 48 hours of treatment with gemcitabine (GEM; 5 nM), BMN673 (50 nM), both agents, or DMSO vehicle control. (b) Quantification of changes in relative cleaved caspase-3 protein expression in H23 and SK-MES-1 cells after 48 hours of treatment with gemcitabine (5 nM), BMN673 (50 nM), both agents combined, or DMSO vehicle control. (c) Analysis of apoptosis in H23 and SK-MES-1 cells treated with BMN673 (50 nM), gemcitabine (5 nM), or both for 48 hours. The percentage of apoptotic cells was determined using Annexin V/propidium iodide staining. (d) Quantification of flow cytometry experiments from 2c.

Figure 3. PARP inhibitors sensitize NSCLC xenografts to chemotherapy with gemcitabine (GEM).

Male and female nude mice (n = 8 per group) orthotopically implanted with H23 tumor xenografts and given treatment with a vehicle control, gemcitabine (80 mg/kg), BMN 673 (0.333 mg/kg), or a combination of these two agents via oral gavage or intraperitoneal injection for 33 days. (a) Resulting tumor volumes measured on the indicated days of treatment. Results are shown as means $\pm$ standard error of the mean. Two-way analysis of variance was used to determine the statistical significance of differences between groups. *p<0.05; **p<0.01. (b) Representative photograph of orthotopically implanted tumors in each mouse group at the time of study termination (day 33) Scale bar = 1 cm. (c) Body-weight time curve for mice with H23 xenografts. Two-way analysis of variance was used to determine the statistical significance of differences between groups. n.s., Not significant. (d)

Hematocrit levels of end-point mice after 33 days of treatment. Dots represent individual mice, bar represents average value. (e) Photographs of H23 xenografts subjected to immunohistochemical analyses using anti-cleaved caspase-3 antibodies.

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Figure 4. Homologous recombination is not inhibited following treatment with gemcitabine. (a) Results of a DR–GFP reporter assay performed to measure HR-mediated double-strand DNA break repair in H23 and SK-MES-1 cells. The frequency of GFP+ cells according to flow cytometry after infection with I-Sce1 endonuclease and incubation for 48 h with and without treatment with 5 nM gemcitabine is shown. (b) Western blot of RAD51 protein expression in H23 cells after 48 hours of treatment with gemcitabine (GEM; 5nM), BMN673 (50 nM), a combination of these two agents, or DMSO. (c) Immunofluorescence stains of H23 cells for RAD51 after treatment with BMN673 (50 nM), gemcitabine (5 nM), combination of both, or DMSO vehicle control for 48 hours. Scale bars, 20 μ m. (d) Quantification of RAD51 foci formation in H23 cells from images in 4c. Error bars represent the standard error of the mean from three independent experiments. (e)

Quantification of RAD51 foci following drug washout for 3, 6, 24, and 48 hours after 48 hours of treatment with indicated drugs.

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Figure 5. Effect of treatment with gemcitabine (GEM) and BMN673 on cell-cycle progression in H23 cells.

(a) H23 cells were treated with gemcitabine (5 nM), BMN673 (50 nM) a combination of both, or DMSO for 24, 48, and 72 hours. The cells were then harvested, and their cell-cycle distributions were analyzed using flow cytometry. (b) Quantification of cell cycle distributions from 5a. Error bars represent the standard error of the mean from three independent experiments.

Figure 6. DNA damage in NSCLC cell lines after treatment with gemcitabine, BMN673, or both. (a) Western blots of protein expression changes about DNA damage response in H23 and SK-MES-1 cells after 48 hours of treatment with gemcitabine (GEM; 5 nM), BMN673 (50 nM), a combination of these two agents, or DMSO. (b) Immunofluorescent stains of γ -H2AX (double-strand DNA breaks) and native BrdU (single-strand DNA breaks) in H23 cells after treatment with BMN673 (50 nM) and gemcitabine (5 nM) for 48 hours. Scale bars, 20 μ m. (c) Quantification of γ H2AX and BrdU foci formation in H23 cells in Fig. 6b. DSB, double-strand DNA break; ssDNA, single-stranded DNA; Theor. Combo, theoretical value if DNA damage was purely additive. Error bars represent the standard error of the mean from three independent experiments. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$ (one-way ANOVA and post-hoc analysis) compared with DMSO control unless bars indicate.

otherwise. (d) Correlation of log-transformed intensity values for γ -H2AX (double-strand DNA breaks) and native BrdU (single-strand DNA breaks) from Fig. 6b. r, Spearman correlation coefficient. (e) Immunohistochemical analysis of tumor samples using anti- γ H2AX antibodies.

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