Synergistic Chemosensitivity of Triple-Negative Breast Cancer Cell Lines to Poly(ADP-Ribose) Polymerase Inhibition, Gemcitabine, and Cisplatin

Kedar Hastak, Elizabeth Alli, and James M. Ford

Abstract

The basal-like subtype of breast cancer is characterized by a triple-negative (TN) phenotype (estrogen receptor, progesterone receptor, and human epidermal growth factor receptor-2/neu negative). TN breast cancers share similar gene expression profiles and DNA repair deficiencies with BRCA1-associated breast cancers. BRCA1-mutant cells exhibit sensitivity to gemcitabine, cisplatin, and poly(ADP-ribose) polymerase (PARP) inhibition; therefore, we hypothesized that TN cancer cells may also exhibit sensitivity to these drugs. In this study, we report that TN breast cancer cells are more sensitive to these drugs compared with non-TN breast cancer cells. Moreover, combination treatments indicated that PARP inhibition by the small-molecule inhibitor PJ34 or siRNA knockdown synergized with gemcitabine and cisplatin in TN cells but not in luminal cancer cells. TN cells exhibited reduced repair of UV-induced cyclobutane pyrimidine dimers after PARP inhibition, suggesting that the synergistic effect of PJ34 and gemcitabine or cisplatin reflected inefficient nucleotide excision repair. Mechanistic investigations revealed that in TN cells, PJ34 reduced the levels of ΔNp63α with a concurrent increase in p73 and its downstream target p21. Thus, the sensitivity to combination treatment seemed to be mediated by sustained DNA damage and inefficient DNA repair triggering p63/p73–mediated apoptosis. Our results suggest a novel therapeutic strategy to treat women with TN breast cancer, an aggressive disease that presently lacks effective treatment options. Cancer Res; 70(20); 7970-80. ©2010 American Association for Cancer Research.

Introduction

Breast cancer is the most common cause of malignancy and the second most common cause of cancer death in women (1). This heterogeneous disease is composed of five major biological subtypes, which are based on microarray gene classifications: luminal A, luminal B, normal breast-like, human epidermal growth factor receptor 2 (HER2), and basal-like breast cancers (1, 2). Whereas many of these subtypes can be treated with much success, the basal-like carcinomas are associated with high rates of relapse following chemotherapy (3, 4). Basal-like breast tumors are largely estrogen receptor (ER), progesterone receptor (PR), and HER2/neu negative [triple negative (TN)] and express genes characteristic of basal epithelial and normal breast myoepithelial cells (5–7). However, the genes responsible for the etiology and aggressive phenotype of basal-like breast cancers remain unknown.

As many cancer chemotherapeutic drugs and radiation therapy cause DNA damage, tumor cells defective in DNA repair pathways are predicted to be sensitive to their effects. Indeed, cell lines deficient in BRCA1 (and BRCA2) have been shown to be sensitive to the DNA cross-linking agents cisplatin and mitomycin C (8, 9), the topoisomerase inhibitor etoposide (10), and oxidative DNA damage (11). Recently, we have shown that BRCA1-deficient cells are sensitive to gemcitabine (2′,2′-difluoro-2′-deoxyctydine; ref. 12), an analogue of cytosine arabinoside that exhibits anticancer properties due to potent inhibition of DNA synthesis (13). Gemcitabine is often used either alone or in combination with other drugs such as taxanes, vinorelbine, carboplatin, or trastuzumab in metastatic breast cancer. However, there are no reports of the efficacy of gemcitabine in the basal-like subtype of breast cancers.

The BRCA1 breast cancer susceptibility gene is known to be involved in a number of DNA repair pathways, including DNA double-strand break repair through homologous recombination (HR; ref. 14), nucleotide excision repair (NER; ref. 15), and base excision repair (BER) of oxidative DNA damage (11). Recently, BRCA1- and BRCA2-deficient cells have also been shown to be sensitive to inhibitors of poly(ADP-ribose) polymerase (PARP; refs. 16, 17), an enzyme involved in BER, which, when inhibited, leads to DNA strand breaks and cell death. In this scenario, BRCA-mutant tumor cells with primary defects in DNA repair are particularly sensitive to small-molecule inhibitors of BER, such as PARP inhibitors.
Recent studies indicate that sporadic basal-like or TN tumors bear a striking resemblance to breast tumors that arise in hereditary BRCA1 mutation carriers. These similarities strongly suggest that sporadic basal-like tumors might bear defects in BRCA1-mediated DNA repair pathways (11) and exhibit similar sensitivities to DNA-damaging agents and PARP inhibitors.

Therefore, using a panel of breast cancer cell lines, we examined the cytotoxic effects of gemcitabine, cisplatin, and a PARP inhibitor alone and in combination. We show that, like BRCA1-mutant cells, basal-like TN breast cancer (TNBC) cells are sensitive to the PARP inhibitor PJ34, gemcitabine, and cisplatin. We further show that PJ34 acts synergistically with both gemcitabine and cisplatin in TNBC cells, but not in luminal breast cancer cell lines. Moreover, we show that PJ34 and gemcitabine disrupt NER, suggesting a novel mechanism of sensitivity to these drugs in TNBC cells.

Materials and Methods

Cell lines and reagents
All cell lines were used within 6 months of purchase. MDAMB468, hss578, MCF7, BT549, and BT474 cell lines were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM with 10% fetal bovine serum (FBS). T47D and HCC1806 cells (ATCC) were maintained in RPMI 1640 with 10% FBS. ATCC provides molecular authenticity in support of their collection through their genomics, immunology, and proteomic cores, as described, using DNA barcoding and species identification, quantitative gene expression, and transcriptome analyses (18). SUM149PT cells were obtained from Asterand Plc, where they were authenticated through gene expression data through the use of Affymetrix GeneChips and biomolecular markers, such as ER and HER2 status. They were maintained in Ham's F-12 medium with 10% FBS. HCC1806 cells were transfected with the shRNAmir retroviral vector in pSM2 against nonspecific silencing control, PARP1 or PARP2 (Thermo Fisher Scientific). Stable clones were maintained with 1 μg/ml puromycin and confirmed by Western blotting. The PARP inhibitor PJ34 was purchased from EMD Biosciences. Methylene blue, MTT, and cisplatin were purchased from Sigma Chemicals. Gemcitabine was a gift from Eli Lilly Pharmaceuticals.

Cell viability and colony formation assay
Cell viability was measured by an MTT assay. For the MTT assay, cells were seeded in 96-well plates and treated with PJ34, gemcitabine, and cisplatin as indicated. MTT reagent was added to the cells, which was reduced to purple formazan crystals by the mitochondria of living cells. The reduced crystals were solubilized with DMSO, and the absorbance was measured at 570 nm by spectrophotometry. All experiments were done in triplicate and also repeated three independent times, and data were plotted as mean ± SD. Data from a representative experiment are shown in the figures. For colony formation assay, HCC1806 and MCF7 were plated at equal density and treated with 10 μmol/L PJ34, 0.6 nmol/L gemcitabine, or 4 μmol/L cisplatin for 72 hours. After treatment, cells were counted and either 500 or 1,000 cells were plated. After 15 days, the cells were stained with methylene blue and individual colonies were counted.

Drug combination studies
For combination studies, BT474, MCF7, HCC1806, and MDAMB468 cells were seeded in triplicate in 96-well plates and treated with PJ34, gemcitabine, or cisplatin alone or with the combination of PJ34 and gemcitabine or PJ34 and cisplatin at the indicated doses. MTT assays were performed after 72 hours of treatment. The data were plotted using CalcuSyn-Biosoft software (19, 20). Combination index (CI) and isobolograms were plotted using the CI equation of Chou-Talalay (21). A nonconstant ratio drug combination design was used and normalized isobolograms were constructed using CalcuSyn-Biosoft software. The experimental protocol and the isobologram plotted along with the equation for calculating CI are shown in Supplementary Fig. S1. CI < 1 was synergistic, CI = 1 was additive, and CI > 1 was antagonistic. Study was repeated three independent times and representative data are shown.

γH2AX and Rad51 staining
BY474, MDAMB468, HCC1806, HCC1806shP1, and HCC1806shP2 cells were plated overnight in chambered slides (1,500 cells per chamber). Cells were treated with 10 μmol/L PJ34 or 5 nmol/L gemcitabine for either 1 to 4 hours for Rad51 staining or 24 hours for γH2AX. Controls included primary alone, isotype control, and secondary alone. After treatment, cells were fixed in 4% paraformaldehyde and stained overnight with primary antibody for Rad51 (H-92; 1:200 dilution; Santa Cruz Biotechnology) or γH2AX-Ser139 (1:500 dilution; Cell Signaling). Cells were washed with TBS/bovine serum albumin and incubated for 1 hour at room temperature with either Alexa 488 or Alexa 594 (Invitrogen) secondary antibody for Rad51 or γH2AX, respectively. Cells were fixed in Prolong gold antifade with 4′,6-diamidino-2-phenylindole (Invitrogen) and cured at room temperature for 24 hours before visualizing. For quantification of Rad51 foci and γH2AX foci, at least 100 cells from each group were visually scored. Cells showing more than three foci were counted as positive for γH2AX or Rad51. The ratio of Rad51 foci in the control versus the treated groups was represented as fold change. For γH2AX, the fold change was the ratio of γH2AX-positive cells in HCC1806 control cells versus shPARP1 and shPARP2. Images from random fields were taken using a Nikon Eclipse E 800 microscope with an attached camera and using Spot Software from Diagnostic Instruments (Diagnostic Instruments). Images were taken with a 40× lens (40×/1.0 DIC H Plan APO oil immersion lens).

Apoptosis detection
Apoptosis was detected by staining HCC1806 cells for Annexin V and cleaved caspase-3. Cells were plated overnight in chambered slides followed by treatment with 10 μmol/L PJ34, 0.6 nmol/L gemcitabine, or 4 μmol/L cisplatin for 24 hours. For Annexin V-FITC, the standard manufacturer's

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protocol was followed (Sigma). For caspase-3, cells were stained with a 1:250 dilution of cleaved caspase-3 antibody (Asp175, Cell Signaling) and visualized as mentioned above.

**Nucleotide excision repair assay**

MDAMB468, HCC1806, HCC1806 control, HCC1806shP1, and HCC1806shP2 cells were grown overnight in six-well plates (in triplicate). Cells were rinsed with PBS and then irradiated with 20 J/m² UVC. MDAMB468 and HCC1806 cells were treated with 10 μmol/L PJ34 or 5 nmol/L gemcitabine, whereas medium without drugs was added to PARP1- and PARP2-knockdown cells. Genomic DNA was extracted (QIAamp DNA mini kit, Qiagen) at 0 to 24 hours. Repair of cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PPs) was measured using an ELISA. Briefly, genomic DNA was distributed in triplicate onto microtiter plates precoated with 0.003% protamine sulfate. DNA lesions were detected with either 1:5,000 TDM-2 (for CPDs) or 1:5,000 64M-2 (for 6-4PPs; a gift from Dr. Toshio Mori, Radioisotope Research Center, Nara Medical University School of Medicine, Nara, Japan; ref. 22). The signals were amplified and subsequently developed with 3,3′,5′-tetramethylbenzidine (Sigma Chemicals). Absorbance was measured at 450 nm. Each experiment was repeated three independent times, and representative data are shown.

**Western blot analyses**

Total cellular protein was isolated by lysing the cells in modified radioimmunoprecipitation assay buffer. Proteins were resolved on SDS-PAGE and transferred to nitrocellulose. Primary antibodies were used at the following dilutions: cleaved caspase-3 (1:1,000, Cell Signaling), PARP1 and PARP2 (1:1,000, Cell Signaling), and PARP-1 (1:1,000, Cell Signaling). After incubation with the respective secondary antibodies, blots were developed with chemiluminescence reagent (Amersham). Blots were imaged using a ChemiDoc XRS+ system (Bio-Rad).
(25–50 μg/lane) were separated by electrophoresis (10% SDS-PAGE) and electroblotted onto polyvinylidene difluoride membranes (GE Healthcare). The membranes were probed with antibodies against PARP, p63, p73, p21, actin (Santa Cruz Biotechnology), or MCM (BD Biosciences). Protein levels from the blots were evaluated using the gel analysis software ImageJ (NIH), and the ratio of protein levels in the control versus the treated groups was represented as fold change.

Results

Sensitivity of basal-like breast cancer cells to PJ34, gemcitabine, and cisplatin

To test our hypothesis that PARP inhibitors that target BRCA1-pathway dysfunction might also be efficacious in the treatment of TNBCs, a panel of sporadic TNBC cells (BT549, HCC1806, and MDAMB468) along with a BRCA1-mutant TN cell line (SUM149PT) and luminal breast cancer cell lines (BT474, MCF7, and T47D) were tested for their sensitivity to PJ34 (0–62.5 μmol/L), gemcitabine (0–4.8 nmol/L), and cisplatin (0–10 μmol/L). After 72 hours of treatment, cell viability was measured by an MTT assay. All TNBC cell lines were significantly more sensitive to PJ34, gemcitabine, and cisplatin treatment than the luminal breast cancer cell lines (Fig. 1A, Supplementary Table S1). We confirmed the sensitivity of TNBC cells to PJ34, gemcitabine, and cisplatin by colony formation assay, and as shown in Fig. 1C, HCC1806 cells were highly sensitive to all the three drugs compared with the luminal MCF7 cells. Furthermore, all the drugs induced apoptosis in TNBC cells as evidenced by caspase-3 cleavage and Annexin V staining (Fig. 1B). Therefore, we found that, similar to BRCA1-deficient cells, TNBC cells were selectively sensitive to PARP inhibition, gemcitabine, and cisplatin compared with the other breast subtypes. Moreover, the resistance of the luminal cell lines does not depend on HER2 status, as BT474 is HER2/neu positive whereas MCF7 is HER2/neu negative.

### Table 1. Synergistic effect of PJ34 with either gemcitabine or cisplatin in breast cancer cell lines

<table>
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<tr>
<th></th>
<th>A Gemcitabine (μmol/L)</th>
<th>PJ34 (μmol/L)</th>
<th>CI</th>
<th>Effect</th>
<th></th>
<th>B Cisplatin (μmol/L)</th>
<th>PJ34 (μmol/L)</th>
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<td>Synergism</td>
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NOTE: HCC1806, MDAMB468, MCF7, and BT474 cells were treated with PJ34, gemcitabine, or cisplatin alone or a combination of the drugs at the indicated concentrations for 72 h, and an MTT assay was performed. CI was calculated by CalcuSyn-Biosoft software. (A) CI values of PJ34 in combination with gemcitabine; (B) CI values of PJ34 in combination with cisplatin.


Synergism between PJ34 and gemcitabine or cisplatin

Our results show that TNBC cells are selectively sensitive to PJ34, gemcitabine, and cisplatin when used individually. However, to determine whether inhibition of PARP by PJ34 acts in a synergistic, additive, or antagonistic fashion with gemcitabine or cisplatin, we treated BT474, MCF7, HCC1806, and MDAMB468 cell lines with the agents alone or in combination for 72 hours. Cells were treated either with 1 μmol/L PJ34 and 0.0016 to 1 μmol/L gemcitabine or with 1.875 to 15 μmol/L PJ34 and 10 μmol/L cisplatin. CalcuSyn software was used to calculate the CI and plot normalized isobolograms (Supplementary Figs. S2 and S3; refs. 19, 21). CI < 1, CI = 1, and CI > 1 quantitatively indicate synergism, additivity, and antagonism, respectively. We also calculated the linear coefficient $r$ value to estimate the accuracy of measurement, and all our experiments had an $r$ value $>0.90$ for the median-effect plot.

As shown in Table 1A (i and ii), TNBC cell lines HCC1806 and MDAMB468 exhibited synergism for all the different combinations of PJ34 and gemcitabine doses, whereas the luminal BT474 and MCF7 cell lines (Table 1A, iii and iv) exhibited antagonistic effects for most dose combinations. Similarly, PJ34 and cisplatin had additive to synergistic effects in both HCC1806 and MDAMB468 cells (Table 1B, i and ii), whereas antagonism was observed in the luminal BT474 and MCF7 cell lines (Table 1B, iii and iv).

We also treated the cells with varied concentrations of PJ34 and kept the concentration of gemcitabine constant, and conversely, we kept the concentration of PJ34 constant while changing the concentration of cisplatin. Again, TNBC cell lines exhibited additive to synergistic effects, whereas antagonism was observed in BT474 and MCF7 cell lines (data not shown).

PARP knockdown sensitizes cells to gemcitabine and cisplatin

Many PARP inhibitors are known to inhibit PARP1 and PARP2, both of which are involved in DNA repair pathways. Therefore, to investigate the role of PARP inhibition in sensitizing TNBC cells to gemcitabine and cisplatin, we generated stable clones of PARP1- and PARP2-knockdown cells in the TN HCC1806 cell line. Decreased protein expression of PARP1 and PARP2 protein was confirmed by immunoblotting (Fig. 2A). We then treated HCC1806 nonsilencing control and HCC1806 shPARP1 and shPARP2 clones (two each) with either 0.15 to 4.8 nmol/L gemcitabine or 0.25 to 8 μmol/L cisplatin for 72 hours, measured cell viability by an MTT assay, and calculated IC_{50} values by Prism software. As shown in Fig. 2B and C, both PARP1 and PARP2 knockdown sensitized HCC1806 cells to gemcitabine and cisplatin. PARP2 knockdown significantly ($P < 0.05$) sensitized cells to gemcitabine compared with HCC1806 (Fig. 2B). Conversely, PARP1 knockdown significantly ($P < 0.05$) sensitized cells to cisplatin (Fig. 2C).

DNA damage in basal-like breast cancer cell lines after PJ34 and gemcitabine treatment

Because PARP and gemcitabine play a major role in DNA repair and inhibition of DNA synthesis, respectively, we investigated the effect of PJ34 and gemcitabine on DNA damage by staining for Rad51 foci and $\gamma$H2AX, which accumulate at sites of broken DNA.

To study Rad51 foci formation, the luminal BT474 and TN MDAMB468 cell lines were treated with either 10 μmol/L PJ34 or 5 nmol/L gemcitabine for 1 to 4 hours. As shown in Fig. 3A, 4 hours of PJ34 or gemcitabine treatment increased the Rad51 foci in MDAMB468 cells. BT474 cells had a higher
basal level of Rad51 foci, but PJ34 and gemcitabine treatment did not further increase the number of foci. We next counted the number of Rad51 foci and observed a 2-fold increase after PJ34 and gemcitabine treatment (Fig. 3C, top and middle). Similar to PJ34 treatment, knockdown of PARP1 and PARP2 also increased the number of Rad51 foci compared with HCC1806 control cells, as seen and quantified in Fig. 3B and C (bottom).

We next treated BT474, MDAMB468, and HCC1806 cells with 10 μmol/L PJ34 or 5 nmol/L gemcitabine for 24 hours and stained the cells for γH2AX. As shown in Fig. 4A, following treatment, there was no increase in the number of γH2AX-positive cells in BT474 cell line. On the other hand, HCC1806 and MDAMB468 cell lines showed a significant increase in the number of γH2AX-positive cells. PARP1- and PARP2-knockdown cells also exhibited 2-fold increases in the number of γH2AX-positive cells compared with HCC1806 control cells, as shown and quantified in Fig. 4B and C. These observations suggest that TNBC cells accumulate DNA damage due to inhibition of PARP activity or due to inhibition of DNA synthesis by gemcitabine. Moreover, cell cycle analysis (data not shown) illustrates that PJ34 treatment arrested cells in the G2-M phase and gemcitabine induced an S-phase block in TNBC cells, consistent with cell cycle arrest after DNA damage.

**Inefficient repair of UV-induced DNA damage after PJ34 and gemcitabine treatment**

Recent studies have shown that PARP is activated by UV irradiation (23, 24); however, the role of PARP after
UV-induced DNA damage is not clear. Previously, our laboratory showed that human and mouse BRCA1-mutant cells are defective in global genomic repair (GGR) compared with BRCA1 wild-type and luminal breast cancer cells (15). Because TNBC cells bear resemblance to BRCA-mutant cells, we investigated UV-induced DNA damage repair through the GGR pathway in TNBC cells treated with either PJ34 or gemcitabine and in PARP1- and PARP2-knockdown cells.

As shown in Fig. 5A and B, both MDA468 and HCC1806 cells treated with either PJ34 or gemcitabine were efficient in repairing 6-4PPs (top). However, treatment of cells with PJ34 or gemcitabine completely inhibited the repair of CPDs (bottom). Similarly, knockdown of both PARP1 and PARP2 rendered the cells inefficient in repairing CPDs with no change in the ability to repair 6-4PPs (Fig. 5C). Therefore, the synergistic effect observed in TNBC cells between PJ34 and cisplatin or gemcitabine may be, in part, due to inhibition of the GGR pathway along with defects in other DNA repair pathways.

**Role of p63 and p73 in sensitizing basal-like breast cancer cells to PJ34 and gemcitabine**

Studies have suggested that 0% to 30% of invasive ductal breast carcinomas express ΔNp63α protein (25–27). p73 can induce apoptosis by p53-independent mechanisms, making it particularly important for therapeutics in basal-like breast

![Figure 4](https://example.com/figure4.png)

**Figure 4.** DNA damage in cells after PARP inhibition or gemcitabine treatment. A, representative images of γH2AX in BT474, MDA468, and HCC1806 cells treated with 10 μmol/L PJ34 or 5 nmol/L gemcitabine for 24 h. Inset, magnified image showing distinct punctate staining. B, γH2AX-positive cells in HCC1806 control and HCC1806 shPARP1- and shPARP2-knockdown cells. C, quantification of γH2AX foci in HCC1806 control and PARP1- and PARP2-knockdown cells.
carcinomas, which are most often null or mutant for p53. We found that p63 is significantly overexpressed in TNBC cell lines by analyzing gene expression data from a study published by Neve and colleagues (28). Because p63 can act as an oncogene (25), the correlation between p63 expression and PJ34- and gemcitabine-mediated cell death becomes even more important.

As shown in Fig. 6A, PJ34-treated hs568t, MDAMB486, and HCC1806 TN cells exhibit a more than 3-fold decrease in the expression of ΔNp63α. ΔNp63α binds to p73 and prevents its proapoptotic activity. We therefore analyzed the levels of p73 protein and observed an increase in the expression of p73 in PJ34-treated cells with a concurrent increase in the expression of p21 (Fig. 6B). A recent study showed that p53 and p73, through p21, can repress minichromosome maintenance (MCM) proteins (29). Because analysis of the published microarray data (28) revealed that MCM proteins are overexpressed in TNBC cells, we examined the expression of MCM proteins after PJ34 treatment. As shown in Fig. 6C, PJ34 treatment decreased the levels of MCM4 and MCM7 in TNBC cells. We next treated PARP1- and PARP2-knockdown cells with gemcitabine for 24 or 48 hours, and as shown in Fig. 5D, gemcitabine treatment downregulated the protein level of ΔNp63α with a concurrent increase in p73 and p21. Thus, the results suggest that DNA damage may trigger the p63/p73 pathway to induce cell cycle arrest and apoptosis.

Discussion

The discovery of molecular subclasses of breast cancer suggests that treatments may be targeted more selectively with improved outcomes. Currently, a major challenge is to identify such targets and more effective therapeutic regimens for TNBCs that are not responsive to endocrine therapy or trastuzumab. BRCA1 mutation carriers commonly develop basal-like breast tumors with defects in DNA repair and have been shown to have altered sensitivity to certain cytotoxic DNA-damaging agents. In the current study, we explored whether agents known to selectively target DNA-repair–deficient BRCA1-mutant cells would also be effective in TNBC cells.

Previously, we have established that BRCA1-mutant cell lines are more sensitive to cisplatin and gemcitabine than matched BRCA1 wild-type cells (12). Mechanisms to explain this observation include defects in DNA repair pathways involved in HR, NER, and resolution of the intra- and interstrand DNA cross-links induced by cisplatin. In our current...
study, we found that TNBC cell lines are sensitive to cisplatin compared with luminal cells. We also report for the first time that TNBC cell lines exhibit profound sensitivity to gemcitabine, compared with the luminal types, which are not sensitive to gemcitabine. These novel findings suggest a targeted chemotherapeutic approach to TN and BRCA1-deficient breast cancer.

PARP1 and PARP2 are involved in various DNA repair mechanisms. They bind to the DNA damage sites and activate themselves by automodification. Recent studies have suggested that decreasing PARP expression by RNA interference or by chemical inhibitors sensitizes BRCA1- and BRCA2-deficient cells to cell death through synthetic lethality with their DNA repair defects (16, 17). Our finding that TNBC cells share DNA repair defects with BRCA1-mutant cells (11) suggests that PARP inhibitors may be effective in treating these tumors, as well. In fact, our results show that TNBC cells are more susceptible to PARP inhibition than the luminal type of breast cancer cells. Moreover, their sensitivity to PARP inhibitors is similar to that of BRCA1-mutant cells.

Because PARP plays a major role in the response to DNA damage, we also wished to examine whether inhibitor of PARP acts synergistically with DNA-damaging cytotoxic agents. Therefore, to explore the effects of drug combinations in breast cancer subtypes, we performed isobologram analyses and found that the combination of PJ34 with gemcitabine or cisplatin had a synergistic effect in TNBC cells. Remarkably, however, the combination proved antagonistic in repair-proficient luminal breast cancer cell lines.

A recent study (30) showed that PARP1 and PARP2 are required to reactivate replication at stalled DNA forks. Thus, the synergism observed between gemcitabine and PARP inhibition in TNBC cells may be attributed to stalled replication forks caused by incorporation of gemcitabine into replicating DNA and failure to reactivate replication at the stalled fork due to inhibition of PARP activity. These results have clinical significance and suggest that a regimen combining a platinum agent with gemcitabine and a PARP inhibitor may have unique efficacy in TNBC but may not prove effective in other subtypes of breast cancers. Interestingly, knocking down PARP2 further sensitized TN cells to gemcitabine, whereas PARP1-knockdown cells were sensitive to cisplatin. It is therefore possible that PARP1 and PARP2 responded preferentially to DNA damage caused by different DNA-damaging agents, and this observation is being further investigated.

Our work shows that H2AX is phosphorylated and forms distinct nuclear foci in response to gemcitabine in TNBC cell lines, similar to the effect of other deoxycytidine nucleoside analogues, such as 1-h-d-arabinofuranosylcytosine and troxacitabine (31). Additionally, consistent with previous studies (32, 33), we found that gemcitabine treatment caused an accumulation of Rad51 nuclear foci. It is not clear if these foci result from gemcitabine-induced stalled replication forks and/or gemcitabine-induced accumulation of cells in S phase.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Modulation of p63, p73, p21, and MCMs in TNBC cells. Western blot analysis of ΔNp63α in MDAMB468 and hs578t cells treated with PJ34 (A); p73 and p21 in hs578t, MDAMB468, and HCC1806 cells treated with PJ34 (B); and MCM4 and MCM7 in MDAMB468 and HCC1806 cells treated with PJ34 (C). D, expression of PARP, ΔNp63α, p73, and p21 in HCC1806 and HCC1806 shPARP1 and shPARP2 cells treated with 0.6 nmol/L gemcitabine.
However, it is possible that stalled replication forks caused by gemcitabine trigger HR repair of chemotherapy-induced DNA damage.

Similarly, inhibition of PARP either by drugs or by RNAi also led to an increase in γH2AX foci and Rad51 foci, consistent with the idea that loss of PARP might increase the formation of DNA strand breaks that are repaired by HR (34, 35). Recently, we (11) have shown that TNBC cells are defective in BER; moreover, these cells may also be defective in HR. Thus, inhibition of PARP along with defective DNA repair mechanisms may lead to synthetic lethality.

Although UV-induced activation of PARP has been reported, the possible role of PARP in NER has not received much attention. Studies have shown that repair of 8-oxoG is stimulated by XPC (36) and CSB (37, 38), and because our laboratory has shown that BRCA1-mutant cells are defective in the GGR pathway of NER, but not in transcription-coupled repair (15) and BER (11), we investigated the role of PARP in GGR. We showed that PARP inhibition chemically or by RNAi decreased the capacity of TNBC cells to remove UVC-induced CPD lesions, a result consistent with previous reports of PARP playing a role in GGR (39, 40). NER is known to be involved in platinum-DNA adduct repair. Therefore, the synergism observed with PARP inhibition and cisplatin may be partly due to inhibition of NER. However, whether the repair is facilitated by the transcription-coupled repair pathway of NER is not established by this study, and further investigation into the mechanism is under way.

Understanding the molecular mechanism behind drug treatment is critical to predict the clinical efficacy of treatment. Meta-analyses of published microarray data (28) for gene expression changes common to the basal-like and BRCA1-mutated cell lines identified p63 and MCM family members to be significantly overexpressed ($P < 0.05$) in both groups. Our study showed that treatment of TNBC cells with PJ34 or gemcitabine resulted in a decreased expression of ΔNP63α with a concurrent increase in the expression of p73 and the cyclin-dependent kinase inhibitor p21, which, in turn, repressed the MCM proteins (41). MCMs are required for licensing of origins, providing a signal for initiating replication in S phase, and are frequently overexpressed in cancer cells (42–44).

Our data showed that MCM4/MCM7 are downregulated in TNBC cells treated with PJ34. Based on these lines of evidence, we hypothesize that PJ34 treatment leads to downregulation of ΔNP63α with a concurrent increase in the expression of p73 and p21, which, in turn, decreases the expression of MCM4/MCM7, leading to cell cycle arrest and cell death. Overall, we found that human TNBC cells, similar to BRCA1-deficient cell lines, were more sensitive to PJ34, gemcitabine, and cisplatin and exhibited synergistic responses to combinations of these agents. This sensitivity seems to be dependent on inefficient DNA repair mechanisms, causing sustained DNA damage that may trigger the p63/p73-mediated apoptotic signaling cascade. These data suggest novel options for targeted treatment of TNBCs.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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