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Targeting abnormal DNA repair in therapy-resistant breast cancers

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Abstract

Although hereditary breast cancers have defects in the DNA damage response that result in genomic instability, DNA repair abnormalities in sporadic breast cancers have not been extensively characterized. Recently we showed that, relative to non-tumorigenic breast epithelial MCF10A cells, estrogen receptor- and progesterone receptor-positive (ER/PR+) MCF7 breast cancer cells have reduced steady state levels of DNA ligase IV, a component of the major DNA-PK dependent non-homologous end-joining (NHEJ) pathway, whereas the steady state level of DNA ligase III α , a component of the highly error-prone alternative NHEJ (ALT NHEJ) pathway, is increased. Here we show that tamoxifen- and aromatase-resistant derivatives of MCF7 cells and ER/PR- cells have even higher steady state levels of DNA ligase III α and increased levels of poly (ADP-ribose) polymerase (PARP1), another ALT NHEJ component. This results in increased dependence upon microhomology-mediated ALT NHEJ to repair DNA double strand breaks (DSB)s and the accumulation of chromosomal deletions. Notably, therapy-resistant derivatives of MCF7 cells and ER/PR- cells exhibited significantly increased sensitivity to a combination of PARP and DNA ligase III inhibitors that increased the number of DSBs. Biopsies from ER/PR-tumors had elevated levels of ALT NHEJ and reduced levels of DNA-PK-dependent NHEJ factors. Thus, our results show that ALT NHEJ is a novel therapeutic target in breast cancers that are resistant to frontline therapies and suggest that changes in NHEJ protein levels may serve as biomarkers to identify tumors that are candidates for this therapeutic approach.

Keywords

breast cancer; therapy resistant breast cancers; DNA damage; DNA repair; Non homologous end-joining

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Conflict of interest

AET is a co-inventor on a patent application that covers the use of DNA ligase inhibitors as anti-cancer agents.

Introduction

The majority of breast cancers occur in postmenopausal women with 75% of these tumors being estrogen-dependent as defined by estrogen receptor (ER α) positivity (1). Tamoxifen, an anti-estrogen, has been the mainstay of treatment for hormone-dependent breast cancers. However, recent clinical trials have shown that inhibitors of aromatase, which catalyzes the rate-limiting step of estrogen biosynthesis, may be more effective than tamoxifen in treating hormone-dependent breast cancers in post menopausal women (2). Unfortunately, resistance to both these endocrine therapies is inevitable in metastatic breast cancer (3). Thus, there is a compelling need to develop more effective therapies for breast cancer patients with acquired anti-estrogen resistance in addition to those with intrinsic resistance to anti-estrogen and anti-HER2 therapies.

Genomic instability, including chromosomal abnormalities, is a characteristic of both hereditary and sporadic breast cancers (4). The tumors in inherited breast cancer that result from loss of either BRCA1 or BRCA2 function have a defect in the recombinational repair of replication-associated double strand break (DSB)s (5). This specific DNA repair defect in BRCA tumors not only underlies the numerous chromosomal rearrangements in these cells but also results in their hypersensitivity to inhibitors of poly (ADP-ribose) polymerase (PARP) that generate replication-associated DSBs by inhibiting the repair of DNA single-strand breaks (4, 6). Based on these pre-clinical studies, potent and specific inhibitors of PARP were developed and are currently being evaluated in clinical trials as therapeutic agents for inherited forms of breast and ovarian cancer (7, 8).

The promising initial results with PARP inhibitors have stimulated interest in DNA repair proteins as therapeutics targets and the characterization of DNA repair defects in sporadic cancers. Recent studies have begun to characterize an alternative (ALT) non-homologous end-joining (NHEJ) pathway that repairs DSBs and is much more error-prone than the major DNA-PK-dependent NHEJ pathway (9). Although ALT NHEJ is a relatively minor pathway in normal cells, there is emerging evidence that this pathway is upregulated whereas the DNA-PK-dependent pathway is downregulated in a significant fraction of cancers (10–12). These studies suggest that, while genomic instability generated by ALT NHEJ may drive disease progression, ALT NHEJ may be a cancer cell-specific therapeutic target.

In this study, we have examined the ALT NHEJ pathway in breast cancer. Notably, the steady state levels of PARP1 and DNA ligase III α , both of which participate in ALT NHEJ (11, 13–15), are significantly increased in breast cancer cell lines with acquired resistance to anti-estrogen therapies and in ER/PR- breast cancer cell lines. These cell lines also have reduced steady state levels of DNA ligase IV, a component of the DNA-PK-dependent pathway. Notably, cells with this DNA repair abnormality were hypersensitive to a PARP inhibitor in combination with an inhibitor of DNA ligase III, providing evidence that the ALT NHEJ pathway is a novel therapeutic target in breast cancers that have either failed or are intrinsically resistant to anti-estrogen therapies. Furthermore, since similar changes in expression of these DSB repair proteins were also observed in biopsies from ER/PR-tumors, our studies suggest that altered expression levels of NHEJ proteins may serve as biomarkers to identify breast cancer patients whose disease is likely to respond to DNA repair inhibitors that target ALT NHEJ.

Materials and Methods

Cell Culture

MCF10A, a non-tumorigenic epithelial cell line, was cultured in mammary epithelial cell growth medium (MEBM; Lonza) supplemented with the MEGM SingleQuot Kit (BPE, hEGF, insulin, and hydrocortisone; Lonza), and cholera toxin (100 ng/ml; Sigma). The ER/PR+ breast cancer cell line MCF7 and tamoxifen-resistant derivatives TAM1, TAM2, and TAM3 (kindly provided by Dr. Ben Park, JHU) were cultured in phenol red-free DMEM/F12 (1:1) with L-glutamine (Gibco), 5% fetal bovine serum (FBS; Sigma), and 1% penicillin-streptomycin (Invitrogen). TAM1, TAM2, and TAM3 were grown in the presence of tamoxifen (1 μ mol/L; Sigma). LTLT, an aromatase inhibitor resistant derivative of MCF7 isolated from long-term letrozole-treated xenograft tumors (16), was cultured in phenol-red free IMEM (Gibco) supplemented with 5% dextran-coated charcoal-treated serum (Thermo Scientific), 1% penicillin-streptomycin (Invitrogen), 750 μ g/mL G418 (American Bioanalytical) and 1 μ mol/L letrozole (kindly provided by Dr. Dean Evans, Novartis Pharma, Basel Switzerland.). The metastatic ER/PR/HER2- breast cancer cell line, MDA-MB-231, was cultured in high-glucose DMEM (Gibco) with 1% penicillin-streptomycin (Invitrogen) whereas the ER/PR- and HER2+ breast cancer cell line, SK-BR-3, was grown in McCoy's 5A modified medium (Stem Cell Technologies) with 1% penicillin-streptomycin (Invitrogen). Cells were incubated at 37°C in 5% CO₂.

Validation of cell lines

Cell line identification and validation of their clonality was performed using Promega's Power-Plex 16 Short Tandem Repeat assay system. Samples were amplified using the recommended protocol, and run on an Applied Biosystems 3100-Avant Genetic Analyzer using Power-Plex 16 Allelic Ladder mix and internal lane standards, ILS-600. Results were analyzed using GeneMapper ID3.2.

Chemical Synthesis of DNA ligase I/III inhibitor L67

The DNA ligase inhibitor L67 (10) was synthesized by incubation of ethyl chloroacetate with 3,5-dibromo-4-methylaniline in the presence of lithium carbonate in N-methyl-2-pyrrolidinone as the solvent at 100 °C for 16 hours to produce N-(3,5-dibromo-4-methylphenyl) glycine ethyl ester in 33% yield after recrystallization from ethanol. This compound was reacted with hydrazine hydrate in refluxing 1-propanol for 64 hours. The N-(3,5-dibromo-4-methylphenyl)glycine hydrazide produced (48% yield) was loaded onto a silica gel column and eluted with 10% ethanol in ethyl acetate. Condensation of the hydrazide with 5-nitrosalicylaldehyde in ethanol produced L67 in 76% yield. The chemical structure of L67 was confirmed by ¹H NMR.

Immunofluorescence Staining

Cells were grown on glass chamber slides (Lab-Tek II, Rochester, NY) and treated with the DNA ligase I/III inhibitor L67 (0.5 μ M) and/or the PARP inhibitor ABT888 (0.125 μ M; Abbott) for 24 hours. After treatment, cells were washed with PBS and fixed in 1% formaldehyde (P-6148; Sigma) for 10 minutes at room temperature. The cells were permeabilized by incubation in 70% EtOH for 10 minutes at room temperature and then incubated in a humidified chamber at 37°C for 1 hour in a solution of 10% FBS in TBS-Tween 20 (0.2%). After washing, slides were incubated for 1 hour with anti-phospho-histone H2AX antibody (1:100; Upstate) at 37°C in a humidified chamber and then with DyLight 594 anti-mouse (1:200; KPL, Gaithersburg, MD) secondary antibody for 1 hour. Slides were washed and dried prior to counterstaining with DAPI (Vector Laboratories, Burlingame, CA).

Western Blotting

Protein extractions were performed without the use of a detergent using the CellLytic NuClear Extraction Kit (Sigma) protocol. The proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using either 4% to 7% or 4% to 10% polyacrylamide gels and then transferred to a PVDF membrane. After blocking, membranes were sequentially incubated with a primary antibody and secondary antibodies as indicated below. Antigen-antibody complexes were detected using enhanced chemiluminescence (ECL; 100 mM Tris-HCl pH 8.5, luminal, coumaric acid, and hydrogen peroxide).

The following antibodies were used in the study: polyclonal anti-DNA ligase III (Sigma) produced in rabbit, 1:1000; monoclonal anti-PARP1 (eBioscience) produced in mouse, 1:1000; monoclonal anti-DNA ligase IV (Santa Cruz) produced in rabbit, 1:1000; monoclonal anti-Ku70 (Santa Cruz) produced in mouse, 1:1000; polyclonal anti-ER α (Santa Cruz) produced in rabbit, 1:1000; monoclonal anti-beta Actin (Abcam) produced in mouse, 1:5000; HRP goat anti-rabbit (Santa Cruz), 1:2000; and HRP goat anti-mouse (Santa Cruz), 1:5000.

In vivo NHEJ Repair Assay

*Eco*R1-linearized pUC18 plasmids (Fermentas) were transfected into cells using Amaxa Nucleofector Kit V. Plasmid DNA was extracted (Qiagen Plasmid Mini Kit) and used to transform *E. coli* DH5 α cells (Invitrogen), which were plated onto agar plates containing X-gal and IPTG. Colonies were analyzed by counting the total number of white (misrepaired) and blue (correctly repaired) colonies. Plasmids from white colonies were characterized by polymerase chain reaction (PCR) amplification of the break point region using primers 5'-CGGCATCAGAGCAGATTGTA-3' and 5'-TGGATAACCGTATTACCGCC-3'. Microhomologies are defined by two or more identical nucleotides at the breakpoint junctions. For each experiment, plasmids from 10 white colonies were analyzed. Results are representative of three independent experiments $\pm$ SEM.

CGH Array

Genomic DNA was isolated from frozen cell pellets using DNeasy tissue mini kit (Qiagen) following the manufacturer's protocol. Sample labeling was performed following Agilent's recommendation for 1M array CGH. Agilent Human High-Resolution Discovery 1 $\times$ 1M CGH microarrays containing probes representing 963,000+ human genomic sequences were used. Hybridization mixtures were first denatured at 95°C for 3 min and then immediately transferred to 37°C for 30 min. After hybridization to microarrays for 40 hours at 65°C in a rotating oven, the microarrays were washed and dried according to the manufacturer's protocols, and then imaged using an Agilent G2565BA microarray scanner. Data were extracted using Feature Extraction Software v9.5.3.1 (Agilent Technologies) and analyzed using Agilent's Genomic Workbench v 5.0. Noise was estimated for each sample array by calculating the spread of the log ratio differences between consecutive probes (DLRs_d) along all chromosomes, and dividing by sqrt (1) to counteract the effect of noise averaging. Aberrant regions (gains or losses) were then identified based on hidden Markov model (HMM) algorithm provided in the software (17).

MTT Cell Proliferation Assays

Cells were grown in 96-well plates with DNA ligase I/III inhibitor L67 (up to 60 μ M) and/or the PARP inhibitor ABT888 (up to 80 μ M) for 72 hours. Approximately 20 hours prior to evaluation, MTT labeling reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide in PBS; Roche) was added to each well. After incubating for 4 hours with the MTT labeling reagent, solubilization solution (10% SDS in 0.01 M HCl; Roche) was added to

solubilize the formazan salt crystals. The results were spectrophotometrically quantified using a VersaMax Microplate Reader at a wavelength of 550 nm and a reference wavelength of 650 nm. The effect of combining the DNA repair inhibitors was analyzed by determining the combination index (CI) described by Chou and Talalay (18, 19) using CalcuSyn software (version 2.0, Biosoft). This calculation takes into account of both potency (median dose Dm or IC₅₀) and the shape of the dose-effect curve (the *m* value) to calculate the CI. L67 and ABT888 were combined at fixed ratios of doses that corresponded to 0.0001, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 10 times the individual IC₅₀ values. Synergy, additivity, and antagonism are defined as CI < 1, CI = 1, and CI > 1, respectively.

Colony Survival Assays

Cells were seeded at a density of 1000 cells/well in methylcellulose-based medium in the presence of DNA Ligase I/III inhibitor, L67 (0.5μM) (10); PARP inhibitor, ABT888 (0.125μM); or L67 (0.5μM) and ABT888 (0.125μM) for approximately 10 days. Colonies were stained with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (1mg/mL) for 16 to 24 hours before evaluation. Vital colonies were counted using an automated image analysis system (Omincon FAS IV, BIOSYS GmbH, Karben, Germany). Experiments were performed at least three times and results are representative of the mean of three independent experiment ± SEM.

siRNA

Target plus SMART pool siRNA oligonucleotides for G22P1, DNA ligase III and Ku70 mRNAs were purchased from Dharmacon RNA Technologies (Chicago, IL). The oligonucleotides were transiently transfected into cells using Amaxa Nucleofector Kit V (VCA-1003) in Nucleofector II Amaxa biosystems. For transfection, 2 μL of 20 μM siRNA was used per 2 × 10⁶ cells. Colony survival assays were setup in combination with ABT888 (0.125 μM) following siRNA ligase III or Ku70 and L67 (0.5 μM) and/or ABT888 (0.125 μM). Cells were harvested for Western blotting 72 hours after transfection. siRNA non-targeting pool (Dharmacon RNA Technologies) was used as a control.

Patient samples and immunohistochemistry

All patient breast cancer and normal reduction mamoplasty samples were collected under the Umbrella Protocol at the University of Maryland Greenebaum Cancer Center. Immunohistochemical staining was performed on 5 μm-thick, formalin-fixed, paraffin-embedded tissue sections. Tissue slides were deparaffinized three times in xylene and rehydrated in graded series of ethanol. Sections were pretreated with heat-induced epitope retrieval using a pressure cooker and Target Retrieval Solution pH 6.1 (Dako TRS, S1699/1700), followed by endogenous peroxidase blocking for 5 minutes with 0.3% hydrogen peroxide. All sections were incubated with the primary antibody, PARP1 (1:200; C2-10, eBioscience, San Diego, CA) or Ku70 (1:100; C-19, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C in a hydration chamber. Primary antibody was visualized using the EnVision Dual Link system (Dako, Carpinteria, CA) for 30 min at room temperature. Slides were developed for 5 minutes using diaminobenzidine as the chromagen (Dako, Carpinteria, CA) and were counterstained with hematoxylin. Staining was performed on a DAKO automatic stainer.

Results

Altered expression of DNA repair proteins in breast cancer cell lines with acquired resistance to anti-estrogen therapeutics

While DSB repair defects in inherited breast cancers caused by mutations in either *BRCA1* or *BRCA2* have been extensively studied, DNA repair abnormalities in sporadic breast cancers are less well defined (4, 5). It was shown previously that the ER/PR+ MCF7 cell line established from a sporadic breast cancer had a lower steady state level of DNA ligase IV, a component of the major DNA-PK-dependent NHEJ pathway, and a higher steady state level of DNA ligase III α , a component of ALT NHEJ, compared with the non-tumorigenic MCF10A cell line established from normal breast epithelium (10). Here we find that MCF7 also has increased levels of PARP1, another component of ALT NHEJ, compared to MCF10A (Figure 1A). Tamoxifen (TAM1, TAM2, and TAM3) and letrozole (LTLT) resistant derivatives of MCF7 also exhibit significantly increased steady-state levels of the ALT NHEJ proteins PARP1 and DNA ligase III α and significantly decreased levels of DNA ligase IV compared to MCF10A (Figure 1A).

Breast cancer cell lines with acquired resistance to anti-estrogen therapeutics are hypersensitive to a combination of DNA ligase and PARP inhibitors

Since chronic myeloid leukemia cells with increased DNA ligase III α -dependent ALT NHEJ activity have abnormal DSB repair (11), we determined whether the breast cancer cell lines exhibited increased sensitivity to inhibitors of the overexpressed ALT NHEJ proteins. In the absence of a DNA ligase III-specific inhibitor, we initially examined the effects of the cytotoxic DNA ligase inhibitor L67 that specifically inhibits both DNA ligase I and III α (10). As single agents, both L67 and the PARP inhibitor ABT888 reduced the growth and viability of all the breast cancer cell lines with IC50s of about 6 μ M and 8 μ M, respectively while L67 and ABT888 reduced growth of MCF10A with IC50s of about 6 μ M and 10 μ M, respectively (Figure S1A–E). At lower concentrations of L67 (0.5 μ M) and ABT888 (0.125 μ M), both inhibitors alone caused small reductions in the colony survival of the tumorigenic cell lines compared with the MCF10A cell line (Figure 1B). More strikingly, the combination of the inhibitors significantly reduced the survival of the therapy-resistant derivatives of MCF7, compared with both parental MCF7 cells and non-tumorigenic MCF10A cells (Figure 1B). To determine whether the effects of the two repair inhibitors are additive or synergistic, we used the dose effect (ED) analyzer program CalcuSyn that uses the median effect method to quantify the effects of drug combinations. The combination index of L67 and ABT888 for all fractional effect levels in TAM1, and LTLT was <1 (Table 1) indicating that the DNA repair inhibitors are acting synergistically (Figure S1B–D).

In contrast to PARP inhibitors (20), the DNA ligase inhibitor L67, which inhibits DNA ligases I and III α , has been less extensively characterized. To determine whether the activity of the DNA ligase inhibitor L67 can be specifically attributed to its effects on DNA ligase III α , colony survival assays were performed following siRNA knockdown of DNA ligase III α alone and in combination with ABT888. Similar to L67, siRNA knockdown alone (Figure S1F) had little effect on colony survival of MCF7 and its tamoxifen-resistant derivatives, but in combination with ABT888 significantly decreased colony survival of the tamoxifen-resistant MCF7 derivatives (Figure 1C). Since L67 or siRNA knockdown of DNA ligase III α co-treatment with ABT888 produced the same decrease in colony survival, it appears likely that the activity of L67 is due to its inhibition of DNA ligase III α although we cannot definitively exclude off-target effects of L67.

Hormone therapy-resistant breast cancer cell lines are more dependent on the ALT NHEJ pathway for the repair of DSBs

Since both PARP1 and DNA ligase III α participate in the repair of DNA single strand breaks (SSBs; 11, 15, 21), we predicted that the combination of repair inhibitors would lead to an accumulation of DSBs, most likely as a consequence of inhibition of SSB repair as described by others (6, 8). Interestingly, prior to treatment, the therapy-resistant derivatives of MCF7 cells had significantly higher percentage of cells with spontaneous H2AX foci, an established marker for DSBs (22), than parental MCF7 cells and non-tumorigenic MCF10A cells (Figure 1D) but there were no differences between the cell lines in the levels of DNA SSBs, as measured by XRCC1 foci (data not shown; 23). To determine whether elevated levels of ROS may contribute to the elevated levels of DSB, we measured the levels of ROS (24) but found no differences between the cell lines (data not shown). Treatment with the DNA repair inhibitor combination resulted in a significant increase in the number of DSBs in the tumorigenic MCF7 cell line and its derivatives but not in the non-tumorigenic MCF10A cells (Figure 1D). Under the same conditions, we did not observe any increase in SSBs in the cancer cell lines compared with the non-tumorigenic MCF10A cells. Our results suggest that the repair of DSBs is abnormal in tumorigenic cells, in particular the therapy-resistant cell lines. It appears that these cell lines are more dependent upon the ALT NHEJ pathway than the non-tumorigenic cells and that the increased sensitivity of the therapy-resistant tumorigenic cell lines to the repair inhibitor combination is due, at least in part, to inhibition of ALT NHEJ.

Since the repair of DSBs by ALT NHEJ is error-prone, resulting in large deletions and chromosomal translocations, we predicted that there should be increased genomic instability in the anti-estrogen therapy-resistant derivatives of MCF7 compared with parental MCF7 cells. Therefore, we examined the frequency of genomic deletions and insertions in MCF7 cells and the therapy-resistant TAM3 and LTLT derivatives using High-Resolution Discovery 1M comparative genomic hybridization (CGH) microarrays (Agilent) containing probes representing 963,000 human genomic sequences. As expected, the therapy-resistant derivatives showed an increased incidence of genomic deletions and insertions, compared with the parental MCF7 cells (Figure 2A).

To measure the repair of DSBs by NHEJ, we used a plasmid-based assay in which a linearized plasmid is transfected into the tumor cell lines and then recovered for analysis after transformation into *E coli* (25). The NHEJ repair efficiency was higher in the tumorigenic cell lines, in particular the therapy-resistant derivatives, than in the non-tumorigenic MCF10A cells (Figure 2B). Treatment with the DNA repair inhibitor combination reduced the repair efficiency of the tumorigenic but not the non-tumorigenic cells. To provide evidence that the increased NHEJ in the plasmid-based assay is due to the ALT NHEJ pathway, we measured the size of deletions and the occurrence of DNA sequence microhomologies at the break sites in the repaired plasmids because deletions of more than a few nucleotides and microhomology-mediated joins distinguish ALT NHEJ from DNA-PK dependent NHEJ (11). The size of DNA deletions and frequency of microhomologies at DSB repair junctions was higher in plasmids recovered from the MCF7 cells compared with the non-tumorigenic MCF10A cells and these differences were even greater in the therapy-resistant derivatives of MCF7 (Figure 2C and D, S2B, S3). As expected, treatment with the DNA repair inhibitor combination significantly reduced the average size of deletions (Figure 2C, S3) and frequency of microhomologies (Figure 2D, Figure S2B) so that repair was similar to that of non-tumorigenic MCF10A cells. Based on these results, we conclude that ALT NHEJ makes a greater contribution to DSB repair in the tumorigenic cells, in particular in the therapy-resistant derivatives and is specifically inhibited by the combination of PARP and DNA ligase inhibitors.

ER/PR- Breast cancer cell lines have increased expression of DNA ligase III α and PARP1 and are hyper-sensitive to inhibition of ALT NHEJ

Since breast cancer cell lines with acquired resistance to anti-estrogen therapeutics have abnormalities in the repair of DSBs by NHEJ, we examined the steady state levels of NHEJ proteins in the intrinsically resistant, ER/PR- breast cancer cell lines, MDA-MB-231 and SK-BR-3. As was observed in the breast cancer cell lines with acquired resistance to anti-estrogen therapeutics, the steady levels of the ALT NHEJ proteins DNA ligase III α and PARP1 were significantly increased whereas the steady state levels of the DNA-PK-dependent NHEJ proteins, Ku70 and DNA ligase IV, were significantly decreased in both the ER/PR- cell lines, relative to non-tumorigenic MCF10A cells (Figure 3A). Similar to the breast cancer cell lines with acquired resistance to anti-estrogen therapies, the ER/PR- cell lines have elevated levels of endogenous DSBs (Figure S2A) and increased error-prone repair of DSBs by ALT NHEJ (Figure 3B–D, S2B). Treatment of the ER/PR- cell lines with a combination of PARP and DNA ligase inhibitors significantly reduced both the repair of DSBs by ALT NHEJ (Figure 3B–D, S2B) and cell survival (Figure 3E). The effect of the repair inhibitors was determined to be synergistic using the dose effect (ED) analyzer program CalcuSyn (Table 1, Figure S1E).

Expression of PARP1 and Ku70 in ER-/PR- breast tumor biopsies

Together our results show that increased dependence upon ALT NHEJ to repair DSBs is a common feature of breast cancer cell lines with either intrinsic or acquired resistance to anti-estrogen therapeutics. Since biopsies are not usually taken from patients with breast cancer that has acquired resistance to anti-estrogen therapeutics, we determined whether similar changes in the levels of NHEJ proteins can be detected in ER-/PR- breast cancer biopsies (3 triple negative ER-/PR-/HER-- and 2 ER/PR-/HER2+ samples) by immunochemistry. In accord with our data in breast cancer cell lines, the majority of the breast cancer specimens had increased PARP1 and reduced Ku70 expression, compared with normal breast tissue (Figure 4A–D, Table 2).

Down regulation of Ku70 results in increased expression of ALT NHEJ proteins and increased sensitivity to the combination of DNA ligase and PARP inhibitors

An intriguing feature that is particularly evident in ER/PR- breast cancer lines and primary biopsies is the reciprocal changes in the steady state levels of key factors involved in DNA PK-dependent and ALT NHEJ. To determine whether expression levels of ALT NHEJ and DNA PK-dependent NHEJ factors are related, we used siRNA knockdown to modulate the levels of Ku70. A 50% reduction of Ku70 levels in ER/PR+ MCF7 cells resulted in increased steady state levels of both PARP1 and DNA ligase III α (Figure 5A) with a concomitant increase in both the repair of DSBs by ALT NHEJ (Figure 5B–D). Interestingly, reducing Ku70 by siRNA also resulted in a significant decrease in the expression of both DNA ligase IV and ER α (Figure 5A) with the decreased ER α expression resulting in a decreased sensitivity to both tamoxifen and letrozole (Figure 5E). However, the increased dependence upon ALT NHEJ sensitizes the MCF7 cells to the combination of L67 and ABT888, resulting in a significant decrease in colony survival (Figure 5F) and NHEJ repair efficiency (Figure 5G).

Discussion

Although the majority of breast cancers are estrogen-dependent and effective anti-estrogen therapies have been developed, there is an urgent need to develop new and improved therapeutic strategies for patients whose disease is refractory to anti-estrogen therapies. Alterations in the network of pathways that respond to DNA damage and maintain genome stability are presumed to underlie the genomic instability and increased sensitivity of cancer

cells to cytotoxic DNA damaging agents used in cancer treatment (26). These abnormalities are potential targets for the development of therapeutics that either alone or in combination with cytotoxic DNA damaging agents, will specifically enhance killing of cancer cells. In this study, we show that an ALT NHEJ pathway is upregulated in breast cancer cell lines that are either intrinsically resistant or have acquired resistance to anti-estrogen therapies and that these cell lines are hypersensitive to DNA repair inhibitors that target the ALT NHEJ pathway.

In contrast to the major DNA PK-dependent NHEJ repair pathway, the ALT NHEJ pathway frequently generates large chromosomal rearrangements, including large deletions and translocations, during the repair of DSBs (9). Notably, the breast cancer cell lines with acquired resistance to anti-estrogen therapies have more endogenous DSBs compared with MCF10A and MCF7. This is consistent with a published study showing that MCF7 cells have decreased levels of antioxidants and increased levels of oxidative DNA damage (27). Furthermore, the therapy-resistant derivatives demonstrated higher incidence of large deletions compared with their parental MCF7 cells. This suggests that a combination of higher endogenous DNA damage, in particular complex clustered oxidative lesions (25) and increased activity of the error-prone ALT NHEJ pathway contribute to the increased genomic instability in these cell lines. While it is likely that the genomic instability drives disease progression and acquired resistance to cancer therapeutics, the DNA repair abnormality provides an opportunity to design a novel therapeutic strategy that selectively targets the therapy-resistant breast cancer cells.

Here, we have shown that therapy-resistant derivatives of estrogen-dependent MCF7 cells with increased ALT NHEJ activity are preferentially killed by a combination of DNA repair inhibitors that target two ALT NHEJ components, PARP1 and DNA ligase III α . The synergistic effect of combining the inhibitors is not consistent with them partially and/or completely inhibiting two components of the same pathway, suggesting that inhibition of other repair pathways contributes to cell killing. In addition to ALT NHEJ, both PARP1 and DNA ligase III α participate in the same SSB repair pathway (28, 29). Thus, although inhibition of the PARP1-dependent SSB repair results in replication-associated DSBs (7, 8), it cannot account for the observed synergy. In fact under conditions where the inhibitors caused an increase in DSBs, we did not observe an increase in SSBs (data not shown). It is possible that DNA ligase III α participates in the repair of replication-associated DSBs by homologous recombination. Alternatively, the inhibition of DNA ligase III α -dependent base excision repair of spontaneous DNA damage may add to the number of unrepaired SSBs that are then converted into DSBs as a consequence of DNA replication. We envision that ALT NHEJ acts as a back-up pathway for recombinational repair of replication-associated DSBs and so increasing the number of replication-associated DSBs combined with inhibition of ALT NHEJ and/or homologous recombination will result in the accumulation of DSBs and cell death. Alternatively, the efficacy of the repair inhibitor combination may be due to the targeting of other cellular pathways in addition to ALT NHEJ and SSB repair. For example, the PARP inhibitor may target cellular function involving other members of the PARP family (30) in addition to PARP1 whereas base excision repair and mitochondrial DNA will also be impacted by inhibition of DNA ligase III α (31, 32).

The higher steady state levels of PARP1 and DNA ligase III α correlate with the increased activity of the ALT NHEJ pathway and increased sensitivity to the combination of PARP and DNA ligase inhibitors. In addition, reduced levels of the canonical NHEJ components, Ku and DNA ligase IV, were also frequently observed in cells with up-regulated ALT NHEJ, suggesting that these DSB repair pathways are linked. In fact, recent studies have shown that Ku-dependent NHEJ suppresses the repair of DSBs by ALT NHEJ, providing an explanation as to why ALT NHEJ is more evident in cells that are deficient in the Ku-

dependent NHEJ pathway (33–36). Here we have shown that knockdown of Ku70 results in a significant increase in levels of PARP1 and DNA ligase III α . It is possible that increased levels of DSBs resulting from reduced Ku levels may increase expression of ALT NHEJ factors via DNA damage response signaling pathways (37). Alternatively, Ku may transcriptionally regulate genes encoding ALT NHEJ proteins (38–40).

Our results in cell lines suggest that dysregulation of NHEJ, resulting from abnormal expression of factors involved in DNA-PK dependent NHEJ proteins and ALT NHEJ, is a common characteristic of breast cancers with intrinsic or acquired resistance to hormone therapy. Notably, we observed similar changes in expression of Ku70 and PARP1 in biopsies from ER-/PR- breast tumors, indicating that the repair abnormality likely occurs in primary tumors, and that these expression patterns can be used to identify tumors with this DNA repair abnormality. A detailed characterization of breast cancer subtypes may lead to the delineation of additional groups of patients who may be candidates for therapy with this combination of DNA repair inhibitors when conventional frontline therapies have failed (41).

In summary, we have shown that breast cancers that are resistant to anti-estrogen therapies have a DNA repair abnormality, increased dependence upon an ALT NHEJ, which can be selectively targeted by the combined action of PARP and DNA ligase inhibitors. Tumors that are likely to respond to the repair inhibitor combination can be identified based on the expression levels of the ALT NHEJ factors and the DNA PK-dependent NHEJ factors. Although further work is needed to delineate the relationship between DNA PK-dependent and ALT NHEJ, the mechanisms underlying the synergistic activity of PARP and DNA ligase inhibitors, and the efficacy of the DNA repair inhibitor in mouse models of human breast cancer, our studies identify ALT NHEJ as a promising therapeutic target in forms of breast cancer for which there is a compelling need for more effective treatment options.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A, i, representative western blot and **ii**, relative steady state levels of Ku70, DNA ligase IV, PARP1, and DNA ligase III α proteins in cell extracts from non-tumorigenic breast epithelial MCF10A cells, MCF7 breast cancer cells and, tamoxifen (TAM1, 2, 3) and letrozole (LTLT)-resistant derivatives of MCF7. **B**, Colony survival of MCF10A, MCF7, TAM1, 2, 3, and LTLT cells after a 10-day growth in the presence of L67 (0.5 μ M) and/or ABT888 (0.125 μ M). **C**, Colony survival of MCF7, TAM1, 2, and 3 following siRNA knockdown of DNA ligase III α alone and in combination with ABT888 (0.125 μ M). **D. i**, representative nuclei from MCF10A, MCF7, TAM1, 2, 3, and LTLT cells immunostained for H2AX foci (red) in the presence of L67 and/or ABT888. Nuclei counterstained with DAPI (blue). **ii**, the percentage of cells with endogenous foci. Results are representative of the mean of three independent experiment $\pm$ SEM, * p <0.05 by Ttest.

Figure 2.

A, Genome view of DNA copy number variation on Agilent 1M array CGH. **i**, The upper panel shows log₂ ratio of TAM3(Cy5) versus MCF7(Cy3) and **ii**, the lower panel shows log₂ ratio of LTLT(Cy5) versus MCF7(Cy3). The numbers labeled on the horizontal axis is for different chromosomes. Log₂ ratios of signal intensities are plotted with horizontal central line equal to zero. Alterations above the line indicate amplifications and below the line indicate deletions. The copy number variations are shown as trend lines with 2MB base-pair moving average. The log₂ ratio on the vertical axis ranges from +4 (top) to -4 (bottom).

B–D, NHEJ repair assays measuring the repair of a single DSB within the LacZ gene of pUC18 plasmids in MCF10A, MCF7, tamoxifen resistant TAM1, 2, 3, and letrozole resistant LTLT cells following a 24 hour treatment with L67 (0.5 μ M) and/or ABT888 (0.125 μ M). **B**, The efficiency of repair as measured by the total number of colonies. **C**, Bar graph showing size of DNA deletions within the DSB region of repaired plasmids. **D**, DNA sequence microhomologies (≥ 2 bp) within repaired plasmids. Bar graph showing proportion of repaired DSBs with sequence microhomologies vs no microhomologies at the repair junctions. Results are representative of the mean of three independent experiment $\pm$ SEM, *p<0.05 by Ttest.

Figure 3.

A, i, representative western blot and **ii**, relative steady state levels of Ku70, DNA ligase IV, PARP1, and DNA ligase III α proteins in extracts from MCF10A normal breast epithelial cells and ER-/PR-breast cancer cells MDA-MB-231 and SK-BR-3. **B–D**, NHEJ repair assays measuring the repair of a single DSB within the LacZ gene of pUC18 plasmids in MCF10A, MDA-MB-231 and SK-BR-3 cells following a 24-hour treatment with L67 (0.5 μ M) and/or ABT888 (0.125 μ M). **B**, The efficiency of repair, as measured by the total number of colonies. **C**, Bar graph showing size of DNA deletions within the DSB region of repaired plasmids. **D**, DNA sequence microhomologies (≥ 2 bp) within repaired plasmids. Bar graph showing proportion of repaired DSBs with sequence microhomologies vs no microhomologies at the repair junctions. **E**, Colony survival of MCF10A, MDA-MB-231 and SK-BR-3 after a 10-day growth in the presence of L67 (0.5 μ M) and/or ABT888 (0.125 μ M). Results are representative of the mean of three independent experiment $\pm$ SEM, *p<0.05 by Ttest.

Figure 4.

Immunostaining for PARP1 and Ku70 in breast biopsies. **A**, Strong Ku70 expression in benign breast lobules, reduction mammoplasty specimen. **B**, Weak PARP expression in benign lobules (reduction mammoplasty specimen). **C**, Lack of Ku70 expression in invasive ductal carcinoma. Note positive immunostaining in a benign lobule adjacent to the tumor (arrow). **D**, Strong PARP expression in invasive ductal carcinoma. Note weak expression in entrapped benign breast epithelial profile (arrow).

Figure 5.

A, i, representative western blot for Ku70 in MCF7 cell extracts following siRNA knockdown of Ku70, compared with siRNA control. Actin was used as a loading control. **ii**, relative protein levels of Ku70, DNA ligase IV, PARP1, DNA ligase III α , and ER α in Ku70 knocked down MCF7 cells and controls. **B–D**, NHEJ repair assays measuring the repair of a single DSB within the LacZ gene of pUC18 plasmids following siRNA knockdown of Ku70 and a 24-hour treatment with L67 (0.5 μ M) and ABT888 (0.125 μ M) in MCF7. **B**, The relative efficiency of NHEJ repair. **C**, Scatter diagram showing size of DNA deletions within the DSB region of repaired plasmids. Individual repaired DSBs are represented by diamond symbols and the mean size of deletions is indicated by horizontal bars. **D**, DNA

sequence microhomologies (≥ 2 bp) within repaired plasmids. Bar graph showing proportion of repaired DSBs with sequence microhomologies vs no microhomologies at the repair junctions. **E–F**, Colony survival of MCF7 cells following siRNA knockdown of Ku70 and a 10-day growth in the presence of (**E**) tamoxifen (1 μ M) or letrozole (1 μ M) and (**F**) L67 (0.5 μ M) and/or ABT888 (0.125 μ M). **G**, NHEJ repair efficiency following a 24-hour treatment with L67 (0.5 μ M) and/or ABT888 (0.125 μ M). Results are representative of the mean of three independent experiment $\pm$ SEM, * $p < 0.05$ by Ttest.

Table 1

Summary of combination studies involving concurrent administration of L67 and ABT888

Cell lines	ED50	ED75	ED90
TAM1	0.04463	0.09622	0.20768
LTLT	0.01526	0.00871	0.00497
MDA-MB-231	0.10205	0.07717	0.05850

ED =dose effect

Table 2

Immunohistochemical Staining for Ku70 and PARP1 in Breast Biopsies

Patient#	Tissue Type	Tumor Grade	ER	PR	HER2	Ki67	Ku70	PARP1
1	IDC	3	0%	0%	3.4/P	36%	+1	+3
2	IDC	3	0%	0%	1.6/N	25%	+2	+1
3	IDC	3	0%	0%	0.4/N	47%	+1	+3
4	IDC/Brain metastasis	3	0%	0%	4.9/P	NA	0	+3
5	IDC	3	0%	0%	0/N	49%	+1	+3
6	Normal	n/a	n/a	n/a	n/a	n/a	+3	1-2
7	Normal	n/a	n/a	n/a	n/a	n/a	+3	1-2
8	Normal	n/a	n/a	n/a	n/a	n/a	+3	1-2

IDC-Invasive ductal carcinoma; P-positive; N-negative; NA-not available; n/a-not applicable

Normal-normal breast tissue from reduction mammoplasty