Enhancement of Synthetic Lethality via Combinations of ABT-888, a PARP Inhibitor, and Carboplatin In Vitro and In Vivo Using BRCA1 and BRCA2 Isogenic Models

Caroline C. Clark¹,², Jeffrey N. Weitzel³, and Timothy R. O'Connor²

Abstract

Individuals with an inherited BRCA1 or BRCA2 mutation have an elevated risk of developing breast cancer. The resulting tumors typically lack homologous recombination repair as do a subset of sporadic tumors with acquired BRCA deficiency. Clinical responses to monotherapy with platinum drugs or poly PARP inhibitors (PARPi) have been shown for BRCA-associated cancers. However, there are limited data on combination therapy with PARPi and platinum drugs, the mechanism of action of this combination, and the role of BRCA1 or BRCA2 in chemosensitivity. We compared the efficacy of ABT-888 (a PARPi) with that of cisplatin or carboplatin (platinum drugs) alone or in combinations by examining the survival of treated Brca-proficient and -deficient mouse embryonic stem cells. In addition, drug-induced growth inhibition of a BRCA1 and a BRCA2 null cell line were compared with their isogenic BRCA-complemented lines. Although each monotherapy killed or inhibited proliferation of Brca/BRCA-deficient cells, an enhanced effect was observed after treatment with ABT-888 in combination with carboplatin. Moreover, the ABT-888/carboplatin combination delayed tumor growth in Brca2 xenografts. The drugs caused DNA damage and apoptosis. Along with greater PARP activity in Brca/BRCA-deficient cells, these effects correlated with increased chemosensitivity. Our data suggest that ABT-888 and carboplatin combination treatment will be more successful than monotherapy in addressing many BRCA-associated cancers. A randomized phase II trial has recently been initiated to test this hypothesis to assist in the discovery of more effective therapies for patients with BRCA.

Preclinical Development

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Individuals with an inherited BRCA1 or BRCA2 mutation have an elevated risk of developing breast cancer. The resulting tumors typically lack homologous recombination repair as do a subset of sporadic tumors with acquired BRCA deficiency. Clinical responses to monotherapy with platinum drugs or poly PARP inhibitors (PARPi) have been shown for BRCA-associated cancers. However, there are limited data on combination therapy with PARPi and platinum drugs, the mechanism of action of this combination, and the role of BRCA1 or BRCA2 in chemosensitivity. We compared the efficacy of ABT-888 (a PARPi) with that of cisplatin or carboplatin (platinum drugs) alone or in combinations by examining the survival of treated Brca-proficient and -deficient mouse embryonic stem cells. In addition, drug-induced growth inhibition of a BRCA1 and a BRCA2 null cell line were compared with their isogenic BRCA-complemented lines. Although each monotherapy killed or inhibited proliferation of Brca/BRCA-deficient cells, an enhanced effect was observed after treatment with ABT-888 in combination with carboplatin. Moreover, the ABT-888/carboplatin combination delayed tumor growth in Brca2 xenografts. The drugs caused DNA damage and apoptosis. Along with greater PARP activity in Brca/BRCA-deficient cells, these effects correlated with increased chemosensitivity. Our data suggest that ABT-888 and carboplatin combination treatment will be more successful than monotherapy in addressing many BRCA-associated cancers. A randomized phase II trial has recently been initiated to test this hypothesis to assist in the discovery of more effective therapies for patients with BRCA. Mol Cancer Ther; 11(9); 1–11. ©2012 AACR.

Introduction

BRCA proteins are components of the homologous recombination repair (HRR) pathway, an error-free DNA double-strand break (DSB) repair pathway. Cells with 2 wild-type (WT) or 1 heterozygous BRCA alleles have intact HRR, and those cells can use HRR to repair DSBs in an error-free manner. BRCA-deficient (BRCA) cells/tumors lack HRR and are more susceptible to lethal DSBs because they must use a more error-prone DNA repair pathway (i.e., nonhomologous end joining; ref. 1) that can lead to increased genomic instability or cell death. BRCA1 has multiple functions, including roles in DNA repair, transcription, and the cell cycle, whereas BRCA2 has a major role in DNA repair (2).

PARP, a protein involved in single-strand break repair during base excision repair (BER), is a drug target for treatment of BRCA-associated tumors (3, 4). Parp1−/− mice are viable and healthy, but sensitive to DNA alkylating damage, suggesting that Parp1 is nonessential (5). PARP inhibitors (PARPi) block BER, which can lead to DSBs and replication fork collapse. Those DSBs are repaired by HRR in proficient cells but not in BRCA cells. Therefore, PARPi can specifically target BRCA tumors (6, 7). For this study, we used ABT-888, an efficient oral PARPi that targets PARP1 and PARP2, and is currently undergoing breast cancer clinical trial evaluation (8).

Platinum drugs, such as cisplatin and its analogue carboplatin, are not the standard-of-care for the treatment of BRCA-associated breast cancer. However, in vitro and in vivo BRCA cells are more sensitive to platinum drugs than are BRCA-proficient cells (9, 10). Nucleotide excision repair (NER) eliminates platinum drug–induced adducts/cross-links, but if that damage persists, they can stall replication forks and lead to DSBs. Cisplatin and carboplatin usage indicates that they have differing efficacies and side effects (11). Cisplatin side effects include nephrotoxicity, ototoxicity, and neurotoxicity, whereas carboplatin has myelosuppression side effects.

Presumably, targeting PARP in combination with a DNA-damaging agent in BRCA models could be more efficient than using the drugs singly. Drug combinations are often used to decrease the likelihood of drug resistance and improve the therapeutic index. However, assessing the efficacy of drug pairs requires empirical results with

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multiple drug treatments/schedule and a complex mathematical model (12). Combinations of PARPi and platinum drugs have shown enhanced tumor growth delay and survival in Brca-deficient mice (13–16), along with promising clinical results (17). Recent experiments using a PARPi combined with cisplatin elicited a synergistic effect on some triple-negative breast cancer (TNBC) lines (tumors with defective HER2, progesterone, and estrogen receptors; ref. 18). However, the mechanism underlying the action of the drug combination in BRCA cells remains unclear, and studies using PARP siRNA knockdown do not necessarily mirror cellular response to PARPi (19). Moreover, the effect also depends on the use of a particular PARPi or platinum drugs, which are scheduled in combination and models. Because carboplatin and cisplatin have different efficacy and toxicity profiles, it is important to examine whether ABT-888/carboplatin or ABT-888/cisplatin will lead to similar killing of BRCA-deficient cells without affecting normal cells. ABT-888/carboplatin combinations are currently in clinical trials to treat BRCA-associated breast cancer, but, until now, there has been no detailed preclinical report using that drug combination in isogenic BRCA models or on the cellular mechanism(s) underlying this combination. These additional preclinical data are needed to inform the clinical use of these combinations.

Our study differs from previous work in that, (i) we have investigated chemosensitization, cell survival, and drug interactions, for both single and dual drug treatments (ABT-888/cisplatin and ABT-888/cisplatin), as a function of BRCA status in vitro by comparing Brca/BRCA cell lines with their isogenic Brca/BRCA-proficient counterparts; (ii) we also examined ABT-888- and/or carboplatin-induced tumor growth delay in vivo in Brca2-deficient and isogenic-complemented xenografts; and (iii) we evaluated the mechanism of action of the ABT-888/carboplatin combination versus single agents by comparing ABT-888 and/or platinum-induced DNA damage, HRR, and cell death, as well as endogenous ERCC1 levels and PARP activity in Brca/BRCA isogenic cell lines.

Materials and Methods

Cell lines

Three mESC lines were used: AB2.2 WT cells (Brca-proficient), Brca1-deficient (Brca1, exon 11 deleted by gene targeting from E14T62a; obtained from Dr. Jeremy Stark, Department of Radiation Biology, City of Hope, Duarte, CA; ref. 20), and Brca2-deficient (Brca2, exon 26 partially and exon 27 completely deleted by gene targeting; obtained from Dr. Paul Hasty, Department of Molecular Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, TX; ref. 21). Cells were maintained as described (22). Human BRCA1 or BRCA2 deficient cell lines and their complementers were obtained from Dr. Simon Powell, Molecular Biology Program, Radiation Oncology, Sloan-Kettering Cancer Center, New York, NY. HCC1937 is a BRCA1-mutant cell line from a patient with breast cancer (23), and HCC1937+BRCA1 is the complemented cell line (24). EUFA423F is a BRCA2 mutant cell line from a patient with Fanconi anemia and EUFA423F+BRCA2 is the complemented cell line (24–28). VC8 and VC8+Brca2 Chinese hamster ovary (CHO) cells were previously established (obtained from Malgorzata Zdzienicka, Department of Molecular Cell Genetics, Nicolaus-Copernicus University in Torun, Bydgoszcz, Poland; refs. 29, 30). All cell lines were tested for the presence or absence of BRCA proteins using Western blot analysis and functionally for the capacity to form RAD51 foci that requires both BRCA1/Brca1 and BRCA2/Brca2 to occur. Cells lines were verified using these mean values within 6 months of conducting the analyses described.

Drug resuspension, cell survival, and proliferation assays

Cisplatin and carboplatin (Sigma-Aldrich or Hospira) were resuspended in saline and ABT-888 (Enzo Life Sciences or Abbott Laboratories/Cancer Therapy Evaluation Program) in dimethyl sulfoxide (in vitro) or saline (in vivo). Controls were treated with equivalent volumes of vehicle. For in vitro combination studies, drugs were added concomitantly and mESC survival was assessed by colony formation. Mitochondrial respiration of human cell lines was assessed by MTT assay. Drug interactions were determined using CalcuSyn software (Biosoft; ref. 12).

Immunostaining

Treated cells were fixed in paraformaldehyde and then immunostained with anti-γH2AX (Millipore), anti-RAD51 (Santa Cruz), and Alexa Fluor 488 goat-anti mouse IgG or 568 goat-anti-rabbit IgG (Invitrogen; ref. 31). Images were taken with an AX-70 microscope (Olympus) and analyzed using Image-Pro software (Media Cybernetics). More than 50 nuclei were counted per treatment; each experiment was carried out in triplicate.

Cell death analysis

Determination of cell death was conducted in duplicate or triplicate by flow cytometry with the Annexin V–FITC/Propidium Iodide (PI) Staining Kit (BD Pharmingen).

Western blot analysis

Cells were lysed and proteins separated using SDS-PAGE, wet transferred, incubated with primary antibodies, followed by incubation with near-infrared–conjugated secondary antibodies, and then imaged.

PARP activity

PARP activities in cell extracts were determined using the Trevigen Universal Chemiluminescent PARP Assay Kit (32).

Intracellular NADPH levels

NADPH levels were determined with a tetrazolium salt-based detection kit (Dojindo Molecular Technology; ref. 33).
Xenografts
VC8 and VC8+Brca2 CHO cells were injected subcutaneously into 4- to 6-week-old female NOD.Cg-PrkdcsldIle2rgtm1Wjl/SzJ (NSG) mice (NOD-SCID mice, Jackson Laboratories #005557). Mice with tumors grown for approximately 2 weeks were randomized into 4 groups of 5 mice for each CHO cell xenograft before beginning drug treatment. ABT-888 (25 mg/kg) was administrated daily by oral gavage and carboplatin (25 mg/kg) intraperitoneally every 5 days. Tumor volumes and mouse mass were measured twice per week. Mice were euthanized when the tumor volume in one of the mice reached 1,500 mm³.

Statistics
Paired, 2-tailed Student t tests were used to assess statistical significance.

Results
In Brca mESC, ABT-888/carboplatin combination treatments are mainly additive or synergistic, whereas ABT-888/cisplatin treatments are mainly antagonistic
We compared the clonogenic survival of WT, Brca1, and Brca2 cells (targeted deletions in Brca) treated with ABT-888, carboplatin, or cisplatin alone. As expected, Brca cell survival was lower than that of WT cells after single drug treatments with ABT-888 or either platinum drug (Fig. 1C and D). However, Brca1 cells were significantly more sensitive (IC₅₀: 0.87 μmol/L) to ABT-888 treatment than were Brca2 cells (IC₅₀: 3.6 μmol/L). Brca2 cells were only slightly more sensitive to carboplatin (IC₅₀: 1.9 μmol/L) than Brca1 cells (IC₅₀: 3.4 μmol/L; Supplementary Table S1). In contrast, Brca1 and Brca2 cells were almost equally susceptible to cisplatin (IC₅₀: ~0.2–0.3 μmol/L; Supplementary Fig. S1A and S1B).

We then examined clonogenic survival of mESC exposed to various combinations of ABT-888/platinum drugs (Fig. 1E and Supplementary Fig. S1C). For most of the ABT-888 concentrations used with fixed carboplatin concentrations, a synergistic or additive effect of ABT-888–carboplatin was observed for WT and Brca cells (Table 1 and Supplementary Fig. S2). However, the IC₅₀ for ABT-888–carboplatin treatment of Brca2 cells was 6.7 μmol/L as compared with 0.23 μmol/L for cisplatin–only treatment (Supplementary Table S1). Combination Index (CD) value calculations confirmed the antagonistic nature of ABT-888/cisplatin treatment. Even for a wide range of drug ratios, only a few combinations produced synergistic CD values with cisplatin, as compared with carboplatin (Table 1 and Supplementary Fig. S2). Therefore, although Brca cells were sensitive to treatment with cisplatin alone, ABT-888/cisplatin treatments either did not substantially lower the drug concentrations used or were antagonistic. In contrast, ABT-888/carboplatin combinations were either additive or slightly synergistic in killing Brca cells.

In human BRCA cancer cells, ABT-888/carboplatin combination treatment is mainly additive or synergistic, whereas ABT-888/cisplatin treatments is mainly antagonistic
To determine whether human cancer models produced similar results to those obtained for mESC lines, we used the HCC1937 and EUFA423F BRCA human tumor cell lines, as well as their respective BRCA-complemented cell lines. Because these cells grew slowly in colony forming assays, we monitored their survival by MTT assay. As expected, after single-agent treatment with ABT-888 or either platinum drug, both human BRCA cell lines showed lower mitochondrial respiration than the BRCA-complemented cell lines (Fig. 1F–J; Supplementary Fig. S1D–S1F). However, the BRCA1 line required almost 10-fold more ABT-888 (HCC1937, IC₅₀ = 122 μmol/L) to achieve an IC₅₀ as compared with the BRCA2 line (EUFA423F, IC₅₀ = 13.7 μmol/L; Supplementary Table S1). Although not as large a difference, the IC₅₀ values for mitochondrial respiration in response to carboplatin and cisplatin were also greater for the BRCA1-deficient line (46.2 and 3.3 μmol/L, respectively) as compared with the BRCA2 line (12.5 and 1.1 μmol/L, respectively; Supplementary Table S1).

We then treated the BRCA tumor lines with ABT-888/platinum drug combinations. BRCA2 cells showed either additive or synergistic response to ABT-888/carboplatin exposure, but showed an antagonistic response to ABT-888/cisplatin treatment (Fig. 1K, Table 1; Supplementary Fig. S2, Supplementary Table S1). BRCA1 cells also showed additivity or synergism with ABT-888/carboplatin, albeit only at high ABT-888 concentrations (200 μmol/L; Fig. 1H, Table 1, Supplementary Table S1). At lower ABT-888 concentrations (50 and 100 μmol/L), ABT-888/carboplatin was mostly antagonistic in BRCA1 cells, but at comparable ABT-888 concentrations, ABT-888/cisplatin was antagonistic or had higher IC₅₀ or CI values than single agents. Overall, ABT-888/carboplatin treatment had an additive to strong synergistic effect on respiration of human BRCA tumor cells, whereas cisplatin was antagonistic. Because ABT-888/cisplatin combination treatments were antagonistic, we focused on ABT-888/carboplatin combinations for further studies.

ABT-888/carboplatin combination treatment is synergistic in Brca2-deficient CHO cells in vitro and reduces tumor mass in vivo
We compared the responses of mice bearing VC8 (Brca2-deficient) or VC8+Brca2 (Brca2 complemented) xenografts tumors to treatment with ABT-888 and carboplatin, alone or in combination. Initially, we examined the in vitro clonal survival of VC8 and VC8+Brca2 cells and showed the combination treatment had a synergistic effect on VC8 cells (Supplementary Fig. S3). For the VC8+Brca2 xenografts, ABT-888 or carboplatin exposure delayed
tumor growth compared with the vehicle group, and the combination treatment was the most effective at slowing tumor growth, but tumors still grew compared with the size at the start of treatment (Fig. 2).

For the VC8 xenografts, ABT-888 and/or carboplatin exposure delayed tumor growth and reduced tumor mass compared with the vehicle group. Final tumor volume was reduced compared with the initial tumor volume after a 14-day treatment with either carboplatin or ABT-888/carboplatin (42% or 65% reduction, respectively; Supplementary Table S2). With vehicle, mitotic figures and necrosis were common, whereas with the combination treatments (Fig. 3A–D). In addition, the percentage of cells positive for phosphorylated H2AX (>0.05) following either single or combination treatment. Percentage inhibition for HCC1937 (BRCA1 or HCC1937 +BRCA1 (BRCA1 Comp.) cells after treatment with ABT-888 (F), carboplatin (G), and ABT-888/carboplatin combination (H). Percentage inhibition for EUSA423F (BRCA2) and EUSA423F +BRCA2 (BRCA2 Comp.) after treatment with ABT-888 (J), carboplatin (I), ABT-888/ carboplatin combination (K). Data are the average of triplicate measurements; error bars are ± SEM.

DSB formation is independent of BRCA status

Because differences in cell survival/proliferation could be because of differing damage levels, we quantified the number of human cells positive for phosphorylated Ser139 in histone H2AX (γH2AX) foci, a DSB marker. Similar levels of DNA damage were observed between BRCA cell lines and their respective BRCA-complemented cell lines (P > 0.05) following either single or combination treatments (Fig. 3A–D). In addition, the percentage of cells positive for γH2AX foci was greater in treated cells versus untreated control (P < 0.03) but was similar for all single and combination treatments. These data indicate that, at the concentrations of ABT-888 and/or carboplatin was not antagonistic and suggests this combination could prove useful in a therapeutic setting.
Table 1. Synergistic, additive, or antagonistic effects of combination treatments of cell lines using ABT-888 with either carboplatin or cisplatin

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Human cancer cell lines

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NOTE: Wild-type, Brca1, or Brca2 mESCs, and BRCA1 or BRCA2 human cancer cell lines and their complemented lines. ++++, strong synergism; +++, synergism; ++, moderate synergism; +, slight synergism; +/−, nearly additive; −, slight antagonism; −−, moderate antagonism; --, antagonism; ----, strong antagonism; -----, very strong antagonism.
tested, there was little difference in DNA damage, despite different BRCA status. Thus, cell proliferation changes are not directly linked to variations in DNA damage levels.

**RAD51 DNA damage response is dependent on BRCA status**

After DSBs form, RAD51 with the assistance of BRCA1 and BRCA2 coats single-stranded DNA at repair sites and indicates HRR. Therefore, we quantified the number of BRCA-defective and complemented cells that were RAD51-positive after similar treatments as those used for γH2AX foci formation (Fig. 3E and F, Supplementary Fig. S5). On the basis of the RAD51 foci ratio (treated/untreated), there were approximately 2-fold more RAD51 foci in treated HCC1937\*BRCA1 samples versus untreated. However, for HCC1937, none of the treatments increased RAD51 foci formation. This indicates that RAD51 foci are efficiently recruited to DSB sites in BRCA1-complemented cells, but not in BRCA1-deficient cells (p < 0.02). Nearly identical results were obtained using the EUFA423F and EUFA423F\+BRCA2 cells (p < 0.02; Fig. 3F). Therefore, both BRCA-complemented human cell lines show a normal response to DSBs by triggering HRR.

**BRCA1 cells manifest an apoptotic response to the ABT-888/carboplatin combination**

To monitor the apoptotic response following treatment of human cells with ABT-888 or carboplatin either singly or in combination, we used both Annexin V staining (Annexin V binds to cell surface phosphatidylserines during apoptosis) and caspase-3 expression (apoptosis leads to the cleavage of pro-caspase-3 into 19- and 17-kDa proteins). Compared with vehicle-treated cells, more apoptosis was seen in either BRCA cell line after single or combination agent treatment (Fig. 4). For HCC1937 cells, caspase-3 cleavage was not observed after either ABT-888 or carboplatin single treatment, but the ABT-888/carboplatin (200/25 μmol/L, respectively) combination had a pro-caspase-3 cleavage effect. Consistent with these results, in Brca1 mESCs, Parp1 cleavage (another apoptotic marker) was observed after ABT-888/carboplatin treatment (Supplementary Fig. S6). However, pro-caspase-3 cleavage was not detected in HCC1937\*BRCA1 cells. Thus, the ABT-888/carboplatin combination has an apoptotic effect in BRCA1 cells that is consistent with the synergistic effect observed for proliferation.

EUFA423F and EUFA423F\+BRCA2 cells were also treated with the ABT-888/carboplatin combination (12.5/12.5 μmol/L, respectively) to examine apoptosis. In EUFA423F, much less Annexin V staining was seen after treatment with ABT-888 than with carboplatin or ABT-888/carboplatin. We did not observe caspase-3 cleavage in EUFA423F and EUFA423F\+BRCA2 cells at drug concentrations that synergistically inhibited growth [ABT-888 (12.5 μmol/L)/carboplatin (12.5 μmol/L)].
ABT-888 and Platinum Drug Combinations

Low NADPH levels in BRCA-deficient cells reflect sensitivity to PARP inhibitors

Endogenous PARP activities in mESC and human cell lines were determined to assess PARP activity as a predictor of chemoresponse in Brca/BRCA cells. Increased PARP activity was greater in both Brca mESC lines and human BRCA cells as compared with the corresponding brca/BRCA proficient cells (Fig. 5A). However, although the difference in PARP activities between the human BRCA1 line and its complement were statistically valid, the difference in their activities was not large, suggesting the PARP activity assays are low estimates, possibly because of the presence of differing amounts of glycocyndrolase in the cell extracts. Nonetheless, the difference in PARP activity is noteworthy for the BRCA lines and is possibly linked to drug treatment response.

Formation of poly (ADP-ribose); (PAR) on target proteins depletes cellular NADPH and serves as an indirect measurement of endogenous PARP activity. Therefore, we determined the NADPH levels in all cell lines investigated. The lowest NADPH levels were found in BRCA cells, which was consistent with higher PARP activity (Fig. 5B). Thus, endogenous PARP activity was elevated in BRCA cells and correlated with low NADPH levels.

ERCC1 is a biomarker for carboplatin sensitivity in BRCA cells

NER is a major pathway used by mammalian cells for removal of DNA damage caused by platinum drugs (34). ERCC1 is involved in NER by cleaving DNA on the 5’ side of DNA damage sites. ERCC1 served as a biomarker for response to platinum drugs (35). Because there are differences in cellular responses to the platinum agents, we examined the levels of ERCC1 in human cell lines to determine if ERCC1 levels varied in our cell lines. BRCA2 cells had much lower ERCC1 levels than BRCA1 cells (Fig. 5C), and this difference reflected, in part, the approximately 3- or 4-fold greater sensitivity of the BRCA2 cells as compared with single drug treatments increase the survival of BRCA-deficient xenograft models (16, 36).

Discussion

PARPi/cisplatin or PARPi/carboplatin combinations as compared with single drug treatments increase the survival of BRCA-deficient xenograft models (16, 36). However, it is unclear whether a PARPi combined with cisplatin or carboplatin kills the tumor more efficiently, and only a limited number of studies have used isogenic models and examined the drug mechanisms. Here, we report that ABT-888/carboplatin, rather than ABT-888/cisplatin, is generally associated with a more favorable outcome: additive or synergistic killing or growth inhibition of Brca/BRCA cells.

However, single-agent carboplatin (25 μmol/L) and ABT-888 25μmol/L/carboplatin 25 μmol/L showed caspase-3 cleavage. Therefore, although ABT-888/cisplatin inhibits EUNA23F growth in comparison with complemented EUNA23F+BRCA2 cells, an effect similar to that in BRCA1-deficient cells is not seen in the BRCA2 cells. Moreover, the major apoptotic effect on BRCA2 cells treated with ABT-888/carboplatin is through carboplatin damage.

Figure 3. DNA damage response in BRCA and BRCA-complemented cell lines after ABT-888, carboplatin, or ABT-888/carboplatin combination treatment. All cells were analyzed after 24 hours of treatment and counterstained with 4’6-diamidino-2-phenylindole. A, immunostained γH2AX foci in HCC1937 or HCC1937+BRCA1 cells treated with vehicle or 200 μmol/L ABT-888/25 μmol/L carboplatin combination. B, immunostained γH2AX foci in EUNA23f or EUFA423F+BRCA2 cells treated with vehicle or 12.5 μmol/L ABT-888/12.5 μmol/L carboplatin combination. C, quantification of γH2AX foci formation in HCC1937 or HCC1937+BRCA1 cells treated with vehicle, 200 μmol/L ABT-888 (ABT), 25 μmol/L carboplatin (Pt), or 200 μmol/L ABT-888/25 μmol/L carboplatin combination. D, quantification of γH2AX foci formation in EUFA423F or EUFA423F+BRCA2 cells treated with vehicle, 12.5 μmol/L ABT-888 (ABT), 12.5 μmol/L carboplatin (Pt), or 12.5 μmol/L ABT-888/12.5 μmol/L carboplatin combination. E, quantification of RAD51 foci in HCC1937 and HCC1937+BRCA1 cells. F, quantification of RAD51 foci in EUFA423F and EUFA423F+BRCA2 cells. RAD51 foci were analyzed after the same treatment conditions as for the γH2AX foci. The RAD51 foci vehicle control was used as the denominator (foci untreated) to determine the fold increase with the RAD51 foci treated. Raw data examples for RAD51 foci are found in Supplementary Fig. S5.
Our examination of combination treatments in human and mouse cells dependent on Brca/BRCA status showed that ABT-888/carboplatin was overall more effective than ABT-888/cisplatin in enhancing cell killing/growth inhibition of Brca/BRCA cells. In Brca/BRCA cells, ABT-888/cisplatin combination treatment did not enhance lethality or reduce proliferation compared with ABT-888 or cisplatin treatments alone. In contrast, either synergistic or additive responses were observed in Brca/BRCA cells following ABT-888/carboplatin treatment. Several studies have already established that cisplatin has better activity than carboplatin in breast cancer (independent of BRCA status; refs. 37, 38). Therefore, it is likely that cisplatin potency was too high in our models to be enhanced in the combination studies. In addition, the 2 platinum drugs have differential kinetics for adduct formation (carboplatin is ~100-fold slower than cisplatin; ref. 39). It would be interesting to determine whether ABT-888, followed by cisplatin treatment (sequential treatment), would mimic the results obtained with simultaneous ABT-888/carboplatin treatment. The differences in cell sensitivity/synergism observed between cell lines are influenced by types and position of BRCA mutation, underlying genetic factors other than BRCA status, as well as their TNBC status (i.e., HCC1937 line). Currently, no specific targeted therapy exists for TNBC, but PARP targeting may be possible, even when tumors are not BRCA-linked (18).

Because ABT-888/carboplatin improved Brca/BRCA-deficient cell killing versus the drugs used singly in vitro, we determined whether tumor inhibition/response was also observed in vivo. So far, xenograft studies using the ABT-888/carboplatin combination have been carried out using only Brca/BRCA-deficient cells without any comparison to an isogenic BRCA-complemented line. We showed that the ABT-888/carboplatin combination not only delayed tumor growth in Brca2-deficient xenografts, but also in Brca2-complemented xenografts. However, an actual response to carboplatin and ABT-888/carboplatin treatment was observed only in the Brca2-deficient xenografts. Moreover, no toxicity was observed in either model. These in vitro and in vivo studies strongly suggest that although PARPi and carboplatin are not part of the first line of treatment of BRCA-associated breast cancer therapy, their use in combination should be considered. Hence, our interest is in evaluating the mechanism of ABT-888 and carboplatin combined versus single-agent treatment to understand the factors contributing to their efficacy.

ABT-888, carboplatin, or their combination generated DNA damage in all cell lines examined, but only the BRCA-complemented cells used HRR. To assess DNA damage, we evaluated γH2AX foci (40–43) in cells exposed to the drugs. A complexity in studying γH2AX response to ABT-888/carboplatin is that ABT-888 rapidly inhibits PARP activity, whereas carboplatin continues to form DNA adducts for up to 2 days in vitro. As expected, the number of cells positive for γH2AX foci following PARPi single-agent treatment was similar between HRR proficient and BRCA cells (3, 4, 36). However, we have shown that γH2AX foci produced after PARPi, carboplatin, or PARPi/carboplatin treatments in the tested cell lines are similar. RAD51 focus formation is dependent on both BRCA1 and BRCA2 and occurs following γH2AX focus formation (44). Both BRCA proteins are required for RAD51 foci formation, and only cells that are specifically
in S–G2-phase after drug treatment will show increased numbers of RAD51 foci (45). Upon treatment, increased formation of RAD51 foci occurred only in BRCA-complemented cells, attesting to the normal repair response of those cells upon complementation.

Although single drug treatments with carboplatin and cisplatin are known to trigger apoptosis, BRCA cell response to combinations of these agents with PARPi has not been extensively studied. All BRCA lines exhibited apoptosis post-treatment, indicating that this pathway is not disabled. However, an apoptotic response consistent with synergistic drug action was observed only for HCC1937 cells exposed to high ABT-888 concentrations (200 μmol/L) and was most apparent from caspase-3 cleavage. A similar apoptotic response was not detected in EUFA423F cells at the concentrations and times tested, but those cells are an Fanconi anemia line. Cell death mechanisms in Fanconi anemia cells are less well studied, and some cells constitutively produce caspase-3 and show PARP cleavage (46). Although we show evidence that the apoptotic response remains, this response might be different for BRCA lines that are not Fanconi anemia.

PARP activity correlated with chemoresponse to PARPi, in nonisogenic human BRCA2 cell lines (BXPC3 and CAPAN1) and isogenic VCS CHO cell lines (32). Here, we show that all the isogenic human and mESC BRCA1 and BRCA2 lines had higher PARP activity than their respective BRCA/Brca-proficient cell lines indicating that the PARP activity in cells with either BRCA deficiency could serve as a biomarker. PAR formation depletes NADPH. Similarly, increased PARP activity in BRCA cell extracts was associated with a decrease in NADPH. PARP activity could be greater in BRCA cells for several reasons, including changes in the HRR-deficient cells to a more open chromatin structure or increased reliance on BER in an HRR-deficient background. The lines most resistant to ABT-888 alone or in combination with carboplatin (Brca2 and BRCA1) had PARP activity more similar to their respective BRCA-proficient cell lines. Upregulation of PARP1 transcripts has recently been noted in TNBC (47), supporting the use of PARP1 RNA levels as a possible biomarker to guide treatment. Thus, PARP activity or NADPH levels could indicate patient response to PARPi.

Platinum drug lesions are generally repaired by NER and HRR (48). ERCC1, an NER protein, has been used to track the response of cells to platinum drugs. The high ERCC1 levels in BRCA1-complemented cells were associated with an approximately 3- or 4-fold higher carboplatin IC50 value as compared with the BRCA2-complemented cells. In addition, the BRCA1- or BRCA2-deficient lines had approximately 2-fold lower carboplatin IC50 values than their respective complemented cells. Thus, ERCC1 expression, which most likely correlates with cellular ability to remove adducts/cross-links, is a good indication of BRCA cell response to platinum drugs.

In conclusion, these data support the use of ABT-888/carboplatin combinations to treat BRCA tumors. Moreover, ABT-888/carboplatin seems to be a more effective combination than ABT-888/cisplatin, because after various doses and exposure times to BRCA cells, ABT-888/carboplatin was associated with a more additive or synergistic response than was ABT-888/cisplatin. Endogenous PARP activity and ERCC1 expression could serve as a guide for predicting treatment outcomes. However, further clinical and preclinical studies are needed to explore whether the efficiency of ABT-888/carboplatin is significantly enhanced in patient tumor cells that have high PARP activity and low ERCC1 levels. Overall, our data suggest that the ABT-888/carboplatin combination will likely be more successful than monotherapy in treating BRCA-associated cancers, and a phase II clinical trial (NCI#8264) is underway to test this hypothesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 5. PARP activity and NADPH levels in Brca/BRCA and HRR-proficient cells and ERCC1 levels in BRCA and BRCA-complemented cell lines. A, PARP activity in the cellular extracts of 3 mESC (WT, Brca1, and Brca2) and 4 human cell lines (HCC1937, HCC1937–BRCA1, EUFA423F, and EUFA423F–BRCA2). B, as an indirect measure of PARP activity, NADPH levels were determined using reduction of WST-8. C, Western blot analysis of ERCC1 levels in cell extracts.
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Molecular Cancer Therapeutics

Enhancement of Synthetic Lethality via Combinations of ABT-888, a PARP Inhibitor, and Carboplatin *In Vitro* and *In Vivo* Using BRCA1 and BRCA2 Isogenic Models

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