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

AUTHORS and AFFILIATIONS

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

Author Affiliations: ¹Graduate School of Biological Sciences, ²Department of Cancer Biology, ³Departments of Population Sciences and Medical Oncology, Beckman Research Institute of the City of Hope, Duarte, CA

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ABT-888 and Platinum Drug Combinations

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Corresponding Author: Timothy R. O’Connor, Department of Cancer Biology, Beckman Research Institute of City of Hope, 1500 East Duarte Road, Duarte, CA 91010. Phone: 626-301-8220; Email: toconnor@coh.org

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Abstract

Individuals with an inherited BRCA1 or BRCA2 mutation have an elevated risk of developing breast cancer (BC). 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(ADP)ribose polymerase (PARP) inhibitors (PARPi) have been demonstrated for BRCA-associated cancers. However, there is 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 survival of treated Brca-proficient and -deficient mouse embryonic stem cells (mESC). In addition, drug-induced growth inhibition of a BRCA1 and a BRCA2 null cell line were compared to their isogenic BRCA-complemented lines. Whereas 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, apoptosis and greater PARP activity in Brca/BRCA-deficient cells, and 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 BRCA patients.
Introduction

BRCA proteins are components of the homologous recombination repair (HRR) pathway, an error-free DNA double strand break (DSB) repair pathway. Cells with two (WT) or one (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.*, non-homologous end joining) 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.

Poly(ADP-ribose) polymerase (PARP), a protein involved in single-strand break (SSB) 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 alkylation damage, suggesting that Parp1 is non-essential (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 BC 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 BC. 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/crosslinks, but if that damage persists, they can stall replication forks and lead to DSBs. Cisplatin and carboplatin use indicates they have differing efficacies and side effects (11). Cisplatin side effects include nephro-, oto-, 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 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 BC (TNBC) lines (tumors with defective HER2, progesterone, and estrogen receptors) (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, 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 BC, 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 clinical use of these combinations.

Our study differs from previous work in that: (a) we have investigated chemosensitization, cell survival, and drug interactions for both single and dual drug treatments (ABT-888/carboplatin and ABT-888/cisplatin) as a function of BRCA status in vitro by comparing Brca/BRCa cell lines to their isogenic Brca/BRCa-proficient counterparts. (b) We also examined ABT-888- and/or carboplatin-induced tumor growth delay in vivo in Brca2-deficient and isogenic complemented xenografts. (c) 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) (20) and Brca2-deficient (Brca2, exon 26 partially and exon 27 completely deleted by gene targeting) (obtained from Dr. Paul Hasty) (21). Cells were maintained as described (22). Human BRCA1 or BRCA2 deficient cell lines and their complements were obtained from Dr. Simon Powell. HCC1937 is a BRCA1 mutant cell line from a breast cancer patient (23), and HCC1937+BRCA1 is the complemented cell line (24). EUFA423F is a BRCA2 mutant cell line from a Fanconi anemia (FA) patient and EUFA423F+BRCA2 is the complemented cell line (24-28). VC8 and VC8+Brca2 CHO cells were previously established (obtained from Małgorzata Zdzienicka) (29, 30). All cell lines were tested for the presence or absence of BRCA proteins using Western blots 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 means within 6 months of performing 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 DMSO (in vitro) or saline (in vivo). Controls were treated with equivalent volumes of vehicle. For in vitro combination studies, drugs were added concomitantly. 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, UK) (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) (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 performed in triplicate.
Cell death analysis

Determination of cell death was performed in duplicate or triplicate by flow cytometry with the Annexin V-FITC/Propidium Iodide (PI) staining kit (BD Pharmingen).

Western blots

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) (33).

Xenografts

VC8 and VC8+Brca2 CHO cells were injected subcutaneously into 4–6-week-old female NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice (NOD-SCID mice, Jackson Laboratories #005557). Mice with tumors grown for ~2 weeks were randomized into 4 groups of 5 mice for each CHO cell xenograft prior to 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 1500 mm<sup>3</sup>.

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-D). However, Brca1 cells were significantly more sensitive (IC\textsubscript{50}: 0.87 \(\mu\)M) to ABT-888 treatment than were Brca2 cells (IC\textsubscript{50}: 3.6 \(\mu\)M). Brca2 cells were only slightly more sensitive to carboplatin (IC\textsubscript{50}: 1.9 \(\mu\)M) than were Brca1 cells (IC\textsubscript{50}: 3.4 \(\mu\)M) (Supplementary Table S1). In contrast, Brca1 and Brca2 cells were almost equally susceptible to cisplatin (IC\textsubscript{50}: \(\sim\)0.2–0.3 \(\mu\)M) (Supplementary Fig. S1A-B).

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). In contrast, for varying ABT-888/cisplatin ratios at a fixed cisplatin concentration (0.25 \(\mu\)M), the IC\textsubscript{50} value for Brca1 cells was almost the same as that for cisplatin-only treatment. The IC\textsubscript{50} for ABT-888/cisplatin treatment of Brca2 cells was 6.7 \(\mu\)M as compared to 0.23 \(\mu\)M for cisplatin-only treatment (Supplementary Table S1). CI 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 CI values with cisplatin, as compared to 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-G, 1I-J, Supplementary Fig. S1D-F). However, the BRCA1 line required almost 10-fold more ABT-888 (HCC1937, IC\textsubscript{50}=122 $\mu$M) to achieve an IC\textsubscript{50} as compared to the BRCA2 line (EUFA423F, IC\textsubscript{50}=13.7 $\mu$M) (Supplementary Table S1). Although not as large a difference, the IC\textsubscript{50} values for mitochondrial respiration in response to carboplatin and cisplatin were also greater for the BRCA1-deficient line (46.2 $\mu$M and 3.3 $\mu$M, respectively) as compared to the BRCA2 line (12.5 $\mu$M and 1.1 $\mu$M, 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 $\mu$M) (Fig. 1H, Table 1, Supplementary Table S1). At lower ABT-888 concentrations (50 and 100 $\mu$M), ABT-888/carboplatin was mostly antagonistic in BRCA1 cells, but at comparable ABT-888 concentrations, ABT-888/cisplatin was antagonistic or had higher IC\textsubscript{50} or CI values than as 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 \textit{in vitro} and reduces tumor mass \textit{in vivo}.
We compared the responses of mice bearing VC8 (Brca2-deficient) or VC8+Brca2 (Brca2-complemented) xenograft 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 to the vehicle group, and the combination treatment was the most effective at slowing tumor growth, but tumors still grew compared to the size at the start of treatment (Fig.2).

For the VC8 xenografts, ABT-888 and/or carboplatin exposure delayed tumor growth and reduce tumor mass compared to the vehicle group. Final tumor volume was reduced compared to 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, while with the combination, there were fewer xenograft cells and mitotic figures (Supplementary Fig. 4). The final body masses for drug or vehicle treated mice were similar, indicating no overt sign of toxicity. Thus, treatment with ABT-888/carboplatin was not antagonistic and suggests this combination could prove useful in a therapeutic setting.

**DSB formation is independent of BRCA status**

Because differences in cell survival/proliferation could be due to 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 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 double-strand breaks 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-F, Supplementary Fig. S5). Based on the RAD51 foci ratio (treated/untreated), there were ~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 to 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 μM/25 μM, 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 μM/12.5 μM, respectively) to examine apoptosis. In EUFA423F and 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 μM/carboplatin 12.5 μM). However, single agent carboplatin (25 μM) and ABT-888 25μM/carboplatin 25μM showed caspase 3 cleavage. Therefore, although ABT-888/carboplatin inhibits EUFA423F growth in comparison to complemented EUFA423F+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.

**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 to 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 due to the presence of differing amounts of glycohydrolase 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 ~3 or 4-fold greater sensitivity of the BRCA2 cells as assessed by cell proliferation following carboplatin and cisplatin treatment, respectively (Supplementary Table S1). Therefore, these data suggest the levels of ERCC1 could also likely indicate response to platinum treatment in these BRCA cells.

Discussion

PARPi/cisplatin or PARPi/carboplatin combinations as compared to 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.

Our examination of combination treatments in human and mouse cells dependent on Brca/BRCA status demonstrated that ABT-888/carboplatin was more effective overall 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 to 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 BC (independent of BRCA status) (37, 38). Therefore, it is likely that cisplatin potency was too high in our in models to be enhanced in the combination studies. In addition, the two platinum drugs have differential kinetics
for adduct formation (carboplatin is ~100 fold slower than cisplatin) (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 if 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 for BRCA-associated BC therapy, their use in combination should be considered. Hence, our interest in evaluating the mechanism of ABT-888 and carboplatin combined versus single agent treatment in order 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 demonstrated 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 μM) 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 FA line. Cell death mechanisms in FA 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 FA.

PARP activity correlated with chemoresponse to PARPi, in non-isogenic human BRCA2 cell lines (BXPC3 and CAPAN1) and isogenic VC8 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/Berca-proficient cell lines, indicating that 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 a ~3 or 4-fold higher carboplatin IC$_{50}$ value as compared to the BRCA2-complemented cells. Additionally, the BRCA1- or BRCA2-deficient lines had ~2-fold lower carboplatin IC$_{50}$ values than their respective complemented cells. Thus, ERCC1 expression, which most likely correlates with cellular ability to remove adducts/crosslinks, 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 appears 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.

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Table 1. Synergistic, additive or antagonistic effects of combination treatments of cell lines using ABT-888 with either carboplatin or cisplatin. Wild Type, Brca1, or Brca2 mESCs and BRCA1 or BRCA2 human cancer cell lines and their complemented lines.

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<th>Carboplatin (μM)</th>
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+++ Strong synergism, +++ Synergism, ++ Moderate synergism, + Slight synergism, +/- Nearly additive, - Slight antagonism, -- Moderate antagonism, --- Antagonism, ---- Strong antagonism, ----- Very Strong Antagonism
Figure Legends

**FIGURE 1:** Clonogenic cell survival and MTT assays of mouse and human BRCA-deficient or -proficient cell lines treated with ABT-888 or carboplatin as single agents or in combination. (A) ABT-888 structure. (B) Carboplatin structure. (C-E) WT (AB2.2, Brca1\(^{+/+}\)), Brca1 (Brca1\(^{-/-}\)), or Brca2 (Brca2\(^{-/-}\)) mESCs were treated for 48 h with the indicated drugs and concentrations. (C) ABT-888, (D) carboplatin, (E) ABT-888/carboplatin. (F-K) Human BRCA-deficient and BRCA-complemented cell growth was assessed by MTT assay after 72 h of continuous drug treatment. Percent inhibition for HCC1937 (BRCA1) or HCC1937+BRCA1 (BRCA1 Comp.) cells after treatment with (F) ABT-888 (G) carboplatin and (H) ABT-888/carboplatin combination. Percent inhibition for EUFA423F (BRCA2) and EUFA423F+BRCA2 (BRCA2 Comp.) after treatment with (I) ABT-888, (J) carboplatin, (K) ABT-888/carboplatin combination. Data are the average of triplicate measurements; error bars are ± SEM.

**FIGURE 2:** *In vivo* efficacy of ABT-888 in combination with carboplatin in Brca2 isogenic CHO xenograft models. (A) Relative tumor volume (RTV) growth in mice (5 per group) bearing VC8 or VC8+Brca2 xenografts after vehicle, ABT-888, carboplatin or ABT-888/carboplatin treatment (relative to the volume on day 1 of treatment). (B) RTV after the 14 day treatment course. Horizontal lines are averages. (C) Tumor mass (mg) after the 14 day treatment course. Horizontal lines are averages. (D) Representative image of one tumor sample for each treatment group endpoint.

**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 24h 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 μM ABT-888/25 μM carboplatin combination. (B) Immunostained γH2AX foci in EUFA423F or EUFA423F+BRCA2 cells treated with vehicle or 12.5 μM ABT-
888/12.5 μM carboplatin combination. (C) Quantification of γH2AX foci formation in HCC1937 or HCC1937+BRCA1 cells treated with vehicle, 200 μM ABT-888 (ABT), 25 μM carboplatin (Pt), or 200 μM ABT-888/25 μM carboplatin combination. (D) Quantification of γH2AX foci formation in EUFA423F or EUFA423F+BRCA2 cells treated with vehicle, 12.5 μM ABT-888 (ABT), 12.5 μM carboplatin (Pt), or 12.5 μM ABT-888/12.5 μM 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.

**FIGURE 4:** Cell death of BRCA and BRCA-complemented cell lines after treatment with ABT-888, carboplatin, or ABT-888/carboplatin combination. All cells were analyzed 72 hours after initial drug treatment. (A) Percentage of apoptotic cells determined from Annexin V staining of HCC1937 and HCC1937+BRCA1 cells incubated in medium containing vehicle or ABT-888 (200 μM), carboplatin (12.5 μM) or ABT-888/carboplatin (12.5 μM/200 μM). (B) Western blots of full and cleaved caspase 3 in HCC1937 and HCC1937+BRCA1 cells. (C) Percentage of apoptotic cells determined from Annexin V staining of EUFA423F or EUFA423F+BRCA1 cells incubated in medium containing vehicle or ABT-888 (12.5 μM), carboplatin (12.5 μM) or ABT-888/carboplatin (12.5 μM/12.5 μM). (D) Western blots of full and cleaved caspase 3 in EUFA423F and EUFA423F+BRCA2 cells.

**FIGURE 5:** PARP activity and NADPH levels in Brca/BRCA and HRR-proficient cells and ERCC1 levels in BRCA and -complemented cell lines. (A) PARP activity in the cellular extracts of three mESC (WT, Brca1, and Brca2) and four 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 of ERCC1 levels in cell extracts.
Figure 1

(A) ABT-888

(B) Carboplatin

(C) % Survival

(D) % Survival

(E) % Survival

(F) % Inhibition

(G) % Inhibition

(H) % Inhibition

(I) % Inhibition

(J) % Inhibition

(K) % Inhibition

[ABT-888] μM

[Carboplatin] μM

[Carboplatin] 2.5 μM

[ABT-888] 200 μM

[ABT-888] 12.5 μM

WT

Brca2

Brca1

BRCA1

BRCA1 Comp.

BRCA2

BRCA2 Comp.
Figure 2
(A) PARP Activity (units/µl)

(B) NADPH (% Control)

Cell Line

(C) ERCC1 Actin

Figure 5
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

Caroline C. Clark, Jeffrey N. Weitzel and Timothy R O'Connor

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