Efficacy of Carboplatin Alone and in Combination with ABT888 in Intracranial Murine Models of BRCA-Mutated and BRCA-Wild-Type Triple-Negative Breast Cancer

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Abstract

Patients with breast cancer brain metastases have extremely limited survival and no approved systemic therapeutics. Triple-negative breast cancer (TNBC) commonly metastasizes to the brain and predicts poor prognosis. TNBC frequently harbors BRCA mutations, translating to platinum sensitivity potentially augmented by additional suppression of DNA repair mechanisms through PARP inhibition. We evaluated brain penetrance and efficacy of carboplatin (± PARP inhibitor ABT888), and investigated gene-expression changes in murine intracranial TNBC models stratified by BRCA and molecular subtype status. Athymic mice were inoculated intracerebrally with BRCA-mutant: SUM149 (basal), MDA-MB-436 (claudin-low); or BRCA–wild-type (wt): MDA-MB-468 (basal), MDA-MB-231BR (claudin-low). TNBC cells were treated with PBS control (intraperitoneal [IP], weekly), carboplatin (50 mg/kg/wk, IP), ABT888 (25 mg/kg/d, oral gavage), or their combination. DNA damage (γ-H2AX), apoptosis (cleaved caspase-3, cC3), and gene expression were measured in intracranial tumors. Carboplatin ± ABT888 significantly improved survival in BRCA-mutant intracranial models compared with control, but did not improve survival in BRCA-wt intracranial models. Carboplatin + ABT888 revealed a modest survival advantage versus carboplatin in BRCA-mutant models. ABT888 yielded a marginal survival benefit in the MDA-MB-436, but not in the SUM149 model. BRCA-mutant SUM149 expression of γ-H2AX and cC3 proteins was elevated in all treatment groups compared with control, whereas BRCA-wt MDA-MB-468 cC3 expression did not increase with treatment. Carboplatin treatment induced common gene-expression changes in BRCA-mutant models. Carboplatin ± ABT888 penetrates the brain and improves survival in BRCA-mutant intracranial TNBC models with corresponding DNA damage and gene-expression changes. Combination therapy represents a potential promising treatment strategy for patients with TNBC brain metastases warranting further clinical investigation.

Introduction

Brain metastases are a particularly devastating complication for patients with triple-negative breast cancer (TNBC) with a limited survival and few therapeutic options (1). TNBC lacks expression of the estrogen receptor (ER), the progesterone receptor (PR), and amplification of the HER-2 gene (2). When compared with other breast cancer subtypes, TNBC has an increased potential to spread to the central nervous system (CNS; refs. 3–5); approximately 50% of patients with advanced TNBC will recur in the brain (4). Despite local cranial therapies (i.e., radiation and/or neurosurgery), overall survival (OS) from detection of CNS metastases for patients with TNBC is less than 6 months (1). Currently, there are...
no FDA-approved systemic or targeted therapies for patients with TNBC brain metastases. The treatment of CNS metastases arising from TNBC is limited by both the lack of defined biologic targets and the inability of the majority of anticancer agents to penetrate the blood–brain barrier (BBB). Thus, there is an urgent need to develop safe and effective treatments for this aggressive disease.

Although TNBC is a distinct clinical subtype, it is a molecularly heterogeneous disease. Given the lack of defined biologic targets in TNBC, it is important to study possible molecular drivers to develop successful therapies. Gene-expression studies have shown that greater than 75% of TNBC are basal-like or claudin-low subtype (6, 7). With approximately 20% of TNBC patients harboring a mutation in either the BRCA1 or BRCA2 genes (8), exploitation of this molecular pathway with brain-penetrant cytotoxic agents could be used as a therapeutic strategy to treat TNBC brain metastases.

The BRCA family is responsible for repairing DNA double-stranded breaks (DSB) via homologous recombination (HR; ref. 9). Thus, tumors harboring BRCA mutations are sensitized to DNA-damaging cytotoxic agents such as platinum derivatives (i.e., cisplatin or carboplatin) that bind to DNA and form DNA cross-links, leading to DNA DSBs (10). Following platinum treatment of BRCA-mutated breast cancer cells, at least two possibilities may occur: (i) the cells are unable to repair widespread DSBs and undergo apoptosis, or (ii) the cells rely on the base excision machinery to rescue DSBs via the enzyme PARP (11, 12). One promising strategy to enhance the cytotoxic effect of platinum therapies in BRCA-mutated TNBC is concomitant PARP inhibition. This strategy may be particularly advantageous for TNBC BRCA–mutated brain metastases as several clinically available PARP inhibitors cross the BBB (13).

Historically, platinums in combination with PARP inhibitors have shown activity in in vitro and in vivo models of BRCA-associated TNBC (14–16), and are brain penetrant. The combination of platinum/PARP inhibitor therapy has never been examined in breast cancer brain metastases. The novelty of this preclinical study is the exploration and efficacy of brain-permeable, clinically available compounds in animal models of TNBC brain metastases, with the goal to translate these findings into the clinical setting. We present a novel study using preclinical murine models of intracranial TNBC, comparing both BRCA-mutant and wild-type (wt) models, as well as basal-like and claudin-low subtypes in response to systemic platinum ± PARP inhibitor combination therapy. This study uniquely demonstrates brain penetration of both systemically administered carboplatin and ABT888, improved survival in BRCA-mutant intracranial TNBC models, and molecular mechanisms of DNA damage and increased apoptosis in response to therapy. Our results suggest that carboplatin ± ABT888 may be a viable therapeutic option for patients with TNBC brain metastases.

Materials and Methods

Cell lines and culture conditions

SUM149 (BRCA1-mutant and basal-like), MDA-MB-436 (BRCA1-mutant and claudin-low), MDA-MB-468 (BRCA1-wt, basal-like), and MDA-MB-231BR (BRCA1-wt, claudin-low) were selected for the study and obtained from the ATCC unless otherwise specified. The identity of the cell lines was confirmed by gene expression (September 2010). All media and additives were purchased from Invitrogen. SUM149 (17) was cultured in HuMEC with supplements and 5% FBS. MDA-MB-468 was cultured in RPMI-1640 plus 10% FBS in a flask with plug seal cap (Corning). MDA-MB-231BR and MDA-MB-436 were maintained in DMEM (high glucose) plus 10% FBS. All cell lines except for MDA-MB-436 were transduced with pTK1261 vector carrying firefly luciferase as previously described (18). Cell lines were maintained at 37°C and 5% carbon dioxide in the presence of antibiotic–antimycotic solution (Invitrogen). Cells were harvested immediately before intracranial implantation.

Intracranial tumor implantation and animal handling

Ten-week-old female Fomlin/nu mice (UNC Animal Studies Core) weighing at least 20 g before intracranial tumor implantation were used for all studies. Intracranial tumor implantation was performed as previously described (18). All animals were handled and monitored for health conditions according to the Institutional Animal Care and Use Committee (IACUC) approved protocols and entered the study only if recovered following surgery. Bioluminescence imaging was performed weekly to monitor intracranial tumor growth as described previously (18).

Efficacy study design

For intracranial tumor models expressing Luciferase (SUM149, MDA-MB-468, and MDA-MB-231BR), bioluminescence signal was used to evenly distribute tumor size among the treatment groups within each model. The average bioluminescence signal between treatment groups within each model was not statistically different before treatment (data not shown). MDA-MB-436–injected animals were assigned to treatment groups randomly.

At 14 days following tumor implantation for all models except MDA-MB-231BR (7 days due to aggressive tumor phenotype), treatment started. Health and weight of the animals were monitored at least three times weekly until sacrifice due to clinical symptoms representing poor health condition (i.e., decreased response to stimuli, neurologic dysfunction, weight loss of 20%, and/or a body composition score of 2 or less) or 12 weeks after intracranial implantation as dictated by prespecified IACUC guidelines for humane reasons. Treatment doses were selected on the basis of previous literature (16, 19, 20) and dose-escalation guidelines for humane reasons. Treatment doses were selected on the basis of previous literature (16, 19, 20) and dose-escalation studies (data not shown), and were: 100 μL PBS control (IP, weekly), 50 mg/kg/wk carboplatin (IP; 10 mg/mL sterile aqueous solution, University of North Carolina Hospital Pharmacy, Chapel Hill, NC). 25 mg/kg/d ABT888 (oral gavage, OG; Chemistry Center for Integrative Chemical Biology and Drug Discovery, UNC) dissolved in PBS (Life Technologies) immediately before OG, or combination 50 mg/kg/wk carboplatin + 25 mg/kg/d ABT888 (IP and OG, respectively). For the SUM149 model, survival data from three independent experiments were combined on the basis of similar median survival for all control groups (data not shown, P = 0.35). No toxicities were found throughout the survival studies in any model or treatment group.

Magnetic resonance imaging and analysis of BBB permeability

Four mice from each intracranial model were used. Gadolinium enhanced brain MRI on a Biospec 9.4 T small animal imaging system (Bruker Corporation) under isoflurane anesthesia began 1 week following MDA-MB-231- BR injection and 2 weeks following SUM149, MDA-MB-436, and MDA-MB-468 injection. Precontrast coronal T1 and T2 images were obtained for localization. Precontrast variable flip angle gradient echo T1 sequences
(TE = 1.5 msec, TR 15 msec, flip angles 2, 5, 10, and 15 degrees) were performed followed by a dynamic scan using 15 degree flip angle was repeated for 130 scans (8.6 seconds/repeat) for dynamic contrast enhancement. After the fifth scan, gadopentetate dimeglumine (Magnevist; Bayer Healthcare) was administered at 0.1 mmol/kg followed by saline flush via tail vein catheter. Coronal post-contrast T1 images were obtained. Standard physiologic monitoring occurred during imaging procedure. Animals were recovered and returned to standard housing. Mice were reimaged each week until time of sacrifice due to 20% weight loss or signs of tumor burden.

Using the T1 MR 20th and third scan of the dynamic sequence, a difference map was generated and relative enhancement (RE) was calculated as follows:

\[
\text{RE} = \frac{S_{t0} - S_{t}}{S_{t0}} = \frac{\Delta S_{t0}}{S_{t0}}
\]

where \(\Delta S_{t0}\) and \(\Delta S_{t}\) represent contrast changes in tissue and plasma during time zero and t, respectively, \(S_{t0}\) is signal intensity in selected tissue (tumor, contralateral normal appearing brain tissue, or left lateral ventricular choroid plexus) at time point t, \(S_{t}\) is signal intensity in plasma at time point t, and \(S_{t0}\) and \(S_{t}\) are precontrast signal intensity in selected tissue or plasma, respectively.

Multiple regions of interest (ROI) sized 10 to 15 pixel (0.1 mm²) were manually drawn by one blinded reader on T2 images, guided by the lesion shape, avoiding macroscopic vessels, necrosis, and areas of hemorrhage. Region with greatest signal change was selected as the tumor ROI. ROIs were propagated to the subtracted T1 DCE MR images, acquired in anatomic registration. The same- size ROIs were drawn on contralateral amygdala, choroid plexus of the contralateral lateral ventricle, and contralateral posterior facial vein lumen.

**Pharmacodynamic study design**

**In vivo PAR pharmacodynamic study.** An in vivo PAR assay was performed using intracranial tissue from the TNBC SUM149 intracranial murine model. Animals were divided into treatment groups within each model and treated for 14 days after tumor injection with 3 doses of carboplatin, 15 doses of ABT888, or carboplatin + ABT888 in combination. Brain tissues from 3 animals per group were collected in formalin the day following the last dose of carboplatin and/or immediately after the last dose of ABT888. Whole mouse brains fixed in formalin were cut parasagittally approximately 2 mm of midline, incubated in 70% Ethanol, embedded in paraffin, and sectioned immediately before staining.

**Immunohistochemistry study design and tissue preparation**

For IHC, we collected tissue from BRCA-mutant SUM149 and BRCA-wt MDA-MB-468 TNBC intracranial murine models. Animals were divided into treatment groups within each model and treated for 14 days after tumor injection with 3 doses of carboplatin, 15 doses of ABT888, or carboplatin + ABT888 in combination. Brain tissues from 3 animals per group were collected in formalin the day following the last dose of carboplatin and/or immediately after the last dose of ABT888. Whole mouse brains fixed in formalin were cut parasagittally approximately 2 mm of midline, incubated in 70% Ethanol, embedded in paraffin, and sectioned immediately before staining.

**Immunohistochemical staining for γ-H2AX and cleaved caspase-3**

The following antibodies were used to stain 4-μm sections placed on coated glass slides: Rabbit monoclonal anti–phospho-ser139-H2AX [γ-H2AX] (1:2,000, 2 hours; Cell Signaling Technology), rabbit polyclonal cleaved caspase-3 (cC3; 1:50, 1 hour; Biocare Medical). Staining was performed as previously described (21, 22). IHC was carried out on the Bond Autostainer, and all solutions were from Leica Microsystems. Briefly, slides were deparaffinized in Bond dewax solution (AR9222) and hydrated in Bond wash solution (AR9590). Antigen retrieval of γ-H2AX was performed for 30 minutes at 100°C in Bond-epitope retrieval solution 1 pH 6.0 (AR9961) and in solution 2 pH 9.0 (AR9640) for cC3. After incubation with the appropriate antibody, detection was performed with the Bond Polymer Refine Detection System (DS9800). Stained slides were dehydrated and coverslipped. Positive and negative (no primary antibody) controls were included for each antibody. Sections from two to three biologic replicas and two to three technical replicas (total 6 sections) per treatment group were stained.

**Digital imaging and image analysis of immunohistochemical staining**

Hematoxylin and eosin (H&E)– and IHC-stained sections were digitally imaged (×20 objective) using Aperio ScanScope XT (Aperio Technologies) and analyzed within the Aperio Spectrum Database. Folded tissues, nontumor areas, and artifacts were excluded from the analysis using a negative pen. Tumor area identification was guided by H&E staining of an adjacent section. The expression of γ-H2AX and cC3 was measured using the Aperio Nuclear V9 (cell quantification) algorithm. Positive and negative (no primary antibody) controls were included for each antibody. The intensity score and the percentage of γ-H2AX–positive nuclei in the tumor area obtained with the modified nuclear v9 algorithm at each intensity level were used to calculate the H-Score using the formula: 

\[
\text{H-Score} = (\% \text{ at } \text{1}+) \times 1 + (\% \text{ at } \text{2}+) \times 2 + (\% \text{ at } \text{3}+) \times 3.
\]

The H-Score was
normalized to a γ-H2AX–positive SK-MEL 181 cell line control included in each staining. Expression of cC3 protein was calculated using the percentage of cC3-positive nuclei.

Microarrays and RNA purification
RNA from BRCA-mutant intracranial SUM149 and MDA-MB-436 tumors was purified using the RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol, labeled as previously described (23), amplified incorporating cyanine-5 (Cy5) dye for the tumor samples and cyanine-3 (Cy3) dye for the reference (24) using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies), and hybridized to 4 × 44 K Customized Human Oligomicroarrays (Agilent Technologies). Arrays were scanned with either the Agilent Scanner (Agilent Technologies) or the GenePix Scanner (Molecular Devices) as previously described (25). All microarray data have been deposited in the Gene Expression Omnibus under the accession number GSE55399.

SUM149 arrays scanned with the GenePix scanner were normalized to be comparable with the Agilent scanner, were adjusted according to Supplementary Materials and Methods (Supplementary Fig. S1). Principle component analysis demonstrates effective removal of scanner bias (Supplementary Fig. S2). Treatment-specific gene sets were identified using R v3.0.2 and the two class unpaired significance analysis of microarray (v3.11; ref. 26) and as further described in Supplementary Materials and Methods (Supplementary Fig. S3). The following comparisons were conducted within each model: Control versus carboplatin, Control versus ABT888, Control versus carboplatin + ABT888, and carboplatin versus carboplatin + ABT888 (Supplementary Table S1B–S1I). All genes with an FDR = 0 in control versus carboplatin intracranial SUM149 or MDA-MB-436 tumors were analyzed with DAVID (27, 28). Pathways are reported with a Bonferroni corrected P value of <0.05 (Supplementary Table S1J–S1K). For comparative visualization, SUM149 arrays were scaled to the same dynamic range as the MDA-MB-436 arrays (Supplementary Fig. S4). Unsupervised and supervised hierarchical clustering were clustered using centroid linkage in Gene Cluster 3.0 v1.52 (29) and viewed with Java Treeview v1.1.6r4 (30).

Statistical analysis
Results for BBB permeability are reported as mean RE ± SEMs. PAR assay results were normalized to the respective control group’s median and are presented as mean of the normalized values ± SEM. IHC to evaluate DNA damage and apoptosis is reported as mean values ± SEM. One-way ANOVA tests were used to compare mean values between treatment groups using GraphPad Prism 6.04. Unpaired t tests were performed for all pair-wise comparisons, with Bonferroni correction for multiple comparisons when applicable.

The Kaplan–Meier method and log-rank tests were used to compare OS among treatment groups. Mice were censored at prespecified study endpoints if they had not been previously sacrificed. Overall and pair-wise tests were completed, and unadjusted P values are reported. For bioluminescence imaging, fold changes were calculated relative to the start date of treatment, and if present, negative imaging values (due to correction for background) were recorded and set to zero. The common logarithm of these values was used for this analysis. For every time point where at least two animals were alive in the treatment group, the median level and interquartile range (IQR: 25th–75th percentile) for bioluminescence imaging were calculated. For gene-expression analysis, significance analysis of microarray was used through R v. 2.15.0 (26).

Results
Our preclinical study evaluates efficacy of carboplatin therapy as a single agent and in combination with the PARP inhibitor, ABT888, in intracranial orthotopic murine models of BRCA-mutant and BRCA-wt TNBC.

BBB permeability across models
To assess the inherent differences in therapy accessibility due to the vasculature across all intracranial murine models, we performed MRI with Gadolinium contrast. Because contrast can only enter brain tissue in which the BBB is disrupted, RE estimates the BBB permeability. Tumor mean RE for each intracranial model was not significantly different across models (SUM149, 1.081 ± 0.1084; MDA-MB-436, 0.8711 ± 0.1788; MDA-MB-468, 0.7683 ± 0.2210; MDA-MB-231BR, 0.6835 ± 0.2052; P = 0.48). In addition, RE was significantly higher in all tumor groups compared with matched normal brain tissue across all models (P < 0.001; Fig. 1A), indicating a similarly compromised, leaky BBB within all intracranial tumor models. There was no significant difference across models within the normal (P = 0.14), tumor (P = 0.18), and choroid plexus (P = 0.923).
ABT888 inhibits the function of PARP in SUM149 intracranial tumors

PARP catalyzes the formation of poly(ADP-ribose) (PAR) chains on a variety of proteins, including PARP itself. To assess penetration of the PARP inhibitor ABT888 across the BBB and monitor inhibitory activity, we measured net PAR levels (±SEM) in SUM149 intracranial tumors following 14 days of ABT888 treatment. PAR levels in ABT888-treated SUM149 intracranial tumors (18.76 ± 7.566) were significantly decreased as compared with control tumors (119.8 ± 30.37; P = 0.0091), indicating 84.3% inhibition and intracranial tumor exposure with ABT888 treatment (Fig. 2A).

To assess differences in the ability to form PAR chains in BRCA-mutant as compared with BRCA-wt models, in vitro PAR assays of BRCA-mutant SUM149 and BRCA-wt MDA-MB-231BR models were performed following 2 hours of treatment with 60 μmol/L of ABT888. PAR levels in SUM149 cells were significantly decreased with ABT888 treatment (5.820 ± 2.044) as compared with control (72.89 ± 14.25; P = 0.0002). In parallel, PAR levels in MDA-MB-231BR were significantly decreased in ABT888 treated cells (5.518 ± 2.763) as compared with control cells (114.1 ± 10.01; P < 0.0001). Similar percentage inhibition was observed between the BRCA-mutant and BRCA-wt cell lines (SUM149, 92.0% inhibition; MDA-MB-231BR, 95.2%; Fig. 2B). Taken together, these data demonstrate effective ABT888 penetration into intracranial tumor and similar relative inhibition in vitro between a BRCA-mutant and BRCA-wt model.

Treatment with carboplatin alone and in combination with ABT888 improves survival of BRCA-mutant, but not BRCA-wt, TNBC intracranial models

Recognizing the BBB permeability of carboplatin and ABT888, the efficacy of platinum agents and PARP inhibitors in advanced extracranial BRCA-mutant TNBC, and the potential rational therapeutic combination to treat breast cancer brain metastases, we evaluated the efficacy of carboplatin and ABT888 as single agents and in combination in both BRCA-mutant and BRCA-wt TNBC intracranial murine models. Median survivals are reported for each model by the treatment group (Table 1).

In the basal-like BRCA1-mut SUM149 intracranial model, the median survival of animals treated with carboplatin ± ABT888 was both significantly longer than control [carboplatin, 58 days (95% confidence interval (CI), 47–67 days]; carboplatin + ABT888: 64 days (95% CI, 59–75 days); Control: 36 days (95% CI, 34–40 days); both treatment groups P < 0.0001 relative to control; Table 1 and Fig. 3A). The difference between median survival of control and single-agent ABT888 treated animals [39 days; 95% CI, 30–46 days] was not statistically significant (P = 0.2). In addition, ABT888 alone was inferior compared with therapies with carboplatin as a single agent (P = 0.007) or carboplatin + ABT888 combination (P = 0.0012). Despite theoretical promise, the carboplatin + ABT888 combination therapy exhibited modest improvement of survival in comparison with single-agent carboplatin, and was not statistically significant (P = 0.4; Table 1 and Fig. 3A). Of note, 2 of 16 and 2 of 17 animals from the carboplatin and carboplatin + ABT888 groups, respectively, remained alive at the prespecified study completion date (12 weeks after intracranial implantation of tumor cells).

Recognizing the heterogeneity of TNBC, we investigated response to carboplatin and carboplatin + ABT888 in a second BRCA-mutant intracranial TNBC model, MDA-MB-436. Median survival following treatment with carboplatin alone (86 days; 95% CI, 61 to undefined) and with combination carboplatin + ABT888 showed...
Carboplatin ± ABT888 in BRCA and Non-BRCA Intracranial TNBC

Table 1. Median survival of intracranial TNBC murine models treated with carboplatin with or without ABT888 in comparison with control

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>PBS, control</th>
<th>ABT888</th>
<th>Carboplatin</th>
<th>Carboplatin + ABT888</th>
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<tr>
<td>SUM149 (BRCA-mut, pTEN- and basal)</td>
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<tr>
<td>Mice per group (n)</td>
<td>17</td>
<td>10</td>
<td>16</td>
<td>17</td>
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<tr>
<td>Median survival (days)</td>
<td>36 (34–40)</td>
<td>39 (30–46)</td>
<td>58 (47–67)</td>
<td>56 (59–75)</td>
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<tr>
<td>95% CI (days)</td>
<td>N/A</td>
<td>p = 0.211</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
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<tr>
<td>MDA-MB-436 (BRCA1-mut, pTEN+, and claudin-low)</td>
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<tr>
<td>Mice per group (n)</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Median survival (days)</td>
<td>25 (12–30)</td>
<td>37 (33–54)</td>
<td>37 (33–54)</td>
<td>37 (33–54)</td>
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<tr>
<td>95% CI (days)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Abbreviations: CI, confidence interval; N/A, not applicable.</td>
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ABT888 (not reached, ≥65 days) was both significantly longer (both P = 0.001) as compared with control (44 days (95% CI, 35–47 days); Table 1, Fig. 3B). Single-agent ABT888 resulted in a modest survival advantage compared with control (57 days; 95% CI, 44–74 days; P = 0.03). The addition of ABT888 to carboplatin did not yield a significant improvement in survival in the MDA-MB-436 model compared with carboplatin alone (P = 0.2); however, 4 of 5 animals in the combination group versus 2 of 5 animals in the carboplatin alone group remained alive 12 weeks after cell injection, suggesting benefit of the addition of ABT888 to carboplatin.

To examine the efficacy of carboplatin and carboplatin + ABT888 in BRCA-wt intracranial TNBC models, we applied the same experimental design to BRCA-wt intracranial TNBC models, basal-like MDA-MB-468 and claudin-low MDA-MB-231BR. In contrast with the BRCA-mutant TNBC intracranial models, neither single-agent carboplatin, ABT888, nor combination carboplatin + ABT888 resulted in an improved median survival compared with control in either BRCA-wt TNBC intracranial model (Supplementary Fig. S5A and S5B; MDA-MB-468, P = 0.8; MDA-MB-231BR, P = 0.1).

Treatment with carboplatin alone or in combination with ABT888 impairs tumor growth in the BRCA-mutant basal-like TNBC intracranial murine model

In addition to the survival analysis, we examined the effect of treatment with carboplatin alone or in combination with ABT888 on intracranial tumor growth via bioluminescence in the BRCA-mutant SUM149 model. Consistent with OS results, dynamic changes in the intracranial tumor growth as measured by median log-fold change of the bioluminescence signal intensity (photons/second) from the start of the treatment were lowest following

Figure 3.

Figure 3. Treatment with carboplatin ± ABT888 improves survival in BRCA-mutant intracranial TNBC. Animals in both models were treated with PBS (control), carboplatin 50 mg/kg/wk (IP), ABT888 25 mg/kg/d (OG), or combination carboplatin + ABT888 (doses as in single agent therapy). Treatment began 14 days after intracranial implantation. A total of 4 animals in the SUM149 model and a total of 6 animals in the MDA-MB-436 model treated with carboplatin and carboplatin + ABT888 were sacrificed 12 weeks after intracranial implantation, as defined by IACUC protocol guidelines, and were censored for the purpose of the analysis. A, median survival of the SUM149 intracranial model. B, median survival of the MDA-MB-436 intracranial model. C, dynamic changes in intracranial tumor growth measured by median fold change of the bioluminescence signal intensity from the start of the treatment in the SUM149 TNBC murine model. The median log-fold changes for each treatment group were calculated weekly. Data for each time point were plotted if at least 2 animals per treatment group remained alive. The vertical bars represent the IQRs (25th–75th percentiles).
treatment with carboplatin + ABT888 (Fig. 3C). The median log-fold change in intracranial tumors treated with either carboplatin or carboplatin + ABT888 was similar at 8 weeks of treatment (median 3.3; IQR, 2.85–4.14 vs. median 3.32; IQR, 2.56–3.97, respectively). Although based on small numbers of animals remaining, at 10 weeks after treatment, the median log-fold change for the carboplatin + ABT888 group remained low until the end of the study, whereas median fold change for the carboplatin single-agent group continued to increase over time (Fig. 3C).

Treatment with carboplatin and carboplatin + ABT888 induces DNA damage and apoptosis in BRCA-mutant intracranial tumor, but not BRCA-wt intracranial tumors

To investigate whether processes of DNA damage and apoptosis are activated in intracranial tumors in response to treatment, we evaluated expression of γ-H2AX protein, a known biomarker of DNA damage, and cC3, the central protein in the terminal phase of apoptosis (15, 32).

Expression of γ-H2AX was elevated in all treatment groups compared with control following 14 days of treatment in BRCA-mutant SUM149 intracranial tumors (Fig. 4A, P = 0.0106). DNA damage was highest in the carboplatin group (91.0 ± 3.7, P = 0.001) followed by combination carboplatin + ABT888 (76.6 ± 8.8, P = 0.12), which were both higher than control (58.5 ± 6.0). ABT888 treatment resulted in a γ-H2AX H-score (75.4 ± 4.3) that was higher than control (P = 0.045).

With similar BBB permeability observed between the BRCA-wt and BRCA-mutant models, significant differences in DNA damage were also observed in the BRCA-wt MDA-MB-468 intracranial model for both carboplatin and carboplatin + ABT888 treated groups as compared with control (P = 0.0015). Expression of γ-H2AX, however, was notably weaker in the MDA-MB-468 intracranial tumors as compared with the SUM149 intracranial model (P < 0.0001 MDA-MB-468 Control vs. SUM149 Control, Fig. 4A, C, and D). Attenuated DNA damage after DNA adduct formation rather than differences in exposure to carboplatin may provide an explanation to why carboplatin treatment ± ABT888 was effective in the BRCA-mutant and not in the BRCA-wt intracranial models.

In addition to γ-H2AX expression, the same tumor samples from BRCA-mutant SUM149 and BRCA-wt MDA-MB-468 intracranial models were analyzed to determine expression levels of cC3 protein. In BRCA-mutant SUM149 intracranial tumors, mean percentages of cC3-positive cells for all treatments were significantly higher in comparison with Control (7.7 ± 0.4, P = 0.0297, Fig. 4B, E, and F); however, mean percentages of cC3-positive cells (±SEM) by the treatment group were not significantly different from each other (ABT888 15.5 ± 2.6, carboplatin 12.8 ± 1.5, and carboplatin + ABT888 12.2 ± 1.5; P = 0.46).

In the BRCA-wt MDA-MB-468 intracranial model, mean values of the percentage of cC3-positive cells for ABT888 (14.5 ± 2.7), carboplatin (7.0 ± 2.0), and carboplatin/ABT888 (13.0 ± 2.0) exhibited overall borderline significance when compared with control (8.2 ± 1.3; P = 0.051; Fig. 4B, Supplementary Fig. S6).

Taken together, these data support previous findings that BRCA status has a significant impact on sensitivity to carboplatin treatment through increased DNA damage and apoptosis levels, and provides a possible explanation to why BRCA-wt intracranial tumors are less responsive to carboplatin treatment ± ABT888.

Figure 4.
Immunohistochemical staining to evaluate DNA damage and apoptosis via γ-H2AX and cC3 protein expression in BRCA-mutant and BRCA-wt intracranial TNBC. Gamma-H2AX (γ-H2AX) (A) and cC3 (B) protein expression in BRCA-mutant SUM149 and BRCA-wt MDA-MB-468 intracranial tumors evaluated after 14 days of treatment with PBS, carboplatin, and carboplatin + ABT888 groups by IHC staining. Data, H-score mean values (±SEM) for γ-H2AX protein expression, and as the mean percentage (±SEM) of cC3-positive cells to reflect cC3 protein expression. P values of all treatment groups compared with control are presented for each model. Representative sections stained with γ-H2AX after treatment with carboplatin are displayed for SUM149 model (C) and MDA-MB468 models (D), magnification ×10. Representative sections stained with cC3 after treatment with PBS (control, E) or carboplatin (F) are displayed for the SUM149 model (magnification, ×20; zoom, 10×).
Intracranial TNBC gene-expression changes in response to carboplatin with and without ABT888

To examine the dynamic transcriptional changes that occur in intracranial tumors in response to treatment, we performed gene-expression analysis of BRCA-mutant intracranial tumors from mice treated with control, ABT888, carboplatin, and carboplatin + ABT888 in the BRCA-mutant basal-like SUM149 and the BRCA-mutant claudin-low MDA-MB-436 intracranial models.

Unsupervised hierarchical clustering of all samples identified cell line–specific delineations as well as distinct clusters defined by carboplatin treatment ± ABT888 (Supplementary Fig. S7). Independent analyses within each model further demonstrated little to no contribution with the addition of ABT888 to control or carboplatin treatments (Fig. 5A and B).

To quantify gene-expression changes that occurred with treatment, each treatment group was compared with control within SUM149 and separately within MDA-MB-436 tumors (Supplementary Table S1B–S1I). Treatment with ABT888 as compared with control (Supplementary Table S1B and S1C) resulted in 19 differentially expressed genes for the SUM149 model, and 153 differentially expressed genes for the MDA-MB436 model. In contrast, comparison of carboplatin treatment with control (Supplementary Table S1D and S1E) demonstrated a substantially higher number of genes differentially expressed within each model (carboplatin vs. control: SUM149 = 176; MDA-MB-436 = 5125). Supervised clustering of these genes reveals consistent gene-expression patterns throughout each cluster of samples (Supplementary Fig. S8A and S8B). Similar results were seen in comparing carboplatin + ABT888 with Control, with 53 genes differentially regulated in SUM149 tumors, and 4183 genes altered in MDA-MB-436 tumors (Supplementary Table S1F and S1G). When carboplatin was compared with carboplatin + ABT888 treatment, less than 10 differentially expressed transcripts were identified, and none were shared between the two models (SUM149: n = 9; MDA-MB-436 n = 2; Supplementary Table S1H and S1I). Thus, although not inhibiting the ability of carboplatin to induce dynamic transcriptional changes, ABT888 minimally changes gene expression when combined with carboplatin treatment.

To annotate the transcriptional changes occurring with carboplatin treatment, pathway analysis of genes altered with carboplatin treatment within each model was analyzed with DAVID (27, 28). Upregulated genes in both SUM149 and MDA-MB-436 were enriched for "signal peptide," "glycopeptide," and "SH3 domain" (Supplementary Table S1J). Interestingly, in MDA-MB-436, neuronal development, neuronal differentiation, and neuronal morphogenesis were significantly upregulated (Supplementary Table S1J). Downregulated pathways in DAVID were only significant in the MDA-MB-436 model, with 16 of the top 30 genes in common with the SUM149 model.
Interestingly, the addition of a PARP inhibitor, which impairs base excision repair, increased levels of cC3 following treatment with carboplatin. BRCA hypothesized to be a failure to effectively repair DNA damage by assessing expression in purified tumor cells (39), passaging metastases cells in vitro (41), or by using species-specific microarrays (43). Exploration of potentially targetable upregulated genes in combination with carboplatin treatment that are low or absent in normal brain tissue will be critical to future rational combination therapies to avoid neurologic side-effects.

Our results demonstrate efficacy of well-tolerated therapeutics in model systems for a patient population with few treatment options. Currently in the field of TNBC brain metastases, a lack of therapeutic targets leaves clinicians with few beneficial choices aside from neurosurgical resection or cranial radiation. In addition, TNBC brain metastases are commonly (>80% of the time) accompanied by extracranial metastases, warranting effective systemic therapies capable of controlling both CNS and non-CNS disease (4). Other studies have demonstrated that irinotecan and iniparib yield an approximately 30% clinical benefit rate in patients with progressive TNBC brain metastases (44). In addition, a recent phase II study showed an approximately 65% intracranial response rate as measured by volumetric imaging following treatment with carboplatin and Bevacizumab in patients with progressive HER2-negative brain metastases (45). Despite these promising results, there remains no FDA-approved systemic therapy for TNBC brain metastases. Our data continue to support the role of carboplatin for the treatment of breast cancer brain metastases, specifically in TNBC with BRCA mutations, and the rationale of using carboplatin ± ABT888 combination in the clinical setting to determine whether ABT888 provides an additional benefit in patients with brain metastases.

Although our study adds to the literature surrounding TNBC brain metastases treatment, there are several limitations to our study. Direct intracranial implantation, as opposed to hematogenous spread of tumor cells to the CNS, may not recapitulate human spread of metastases; however, the purpose of this study was to evaluate established brain metastases, not prevention, in response to drug delivery and its impact on the intracranial tumor. In addition, although ABT888 reduced PAR levels in intracranial tumors, suggesting effective BBB penetration, limited efficacy and few gene-expression changes were observed. Future studies may aim to investigate other PARP inhibitors with varying degrees of catalytic activity, and thus may be more effective and synergistic with carboplatin treatment in brain metastases (46).

In conclusion, to our knowledge this study is the first to demonstrate a significant survival advantage in response to systemic carboplatin ± ABT888 treatment in BRCA-mutant TNBC intracranial murine models, as well as a mechanistic explanation of these results. Moreover, we have shown that PARP inhibition as a single agent, although effectively penetrating the BBB, does not yield significant improvement in survival or have a dynamic impact on gene expression. These results provide strong rationale to translate our findings into the design of early-phase clinical trials for patients with BRCA-mutant TNBC brain metastases testing carboplatin ± ABT888, with the ultimate goal of improving the survival of patients with an incurable disease who, at present, have limited systemic therapeutic options.

**Disclosure of Potential Conflicts of Interest**

Y.Z. Lee reports receiving a commercial research grant from Carestream, Inc., and is a consultant/advisory board member for Merrimack Pharmaceuticals. C.K. Anders is a consultant/advisory board member for Novartis, Sanoﬁ/BiPAR, to-BBR, GERON, Angiochem, Merrimack, and Lilly. No potential conflicts of interest were disclosed by the other authors.

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Efficacy of Carboplatin Alone and in Combination with ABT888 in Intracranial Murine Models of BRCA-Mutated and BRCA–Wild-Type Triple-Negative Breast Cancer

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