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Combination Epigenetic Therapy in Advanced Breast Cancer with 5-Azacitidine and Entinostat: a Phase II National Cancer Institute/Stand Up to Cancer Study

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Abstract

Purpose—In breast cancer models, combination epigenetic therapy with a DNA methyltransferase inhibitor and a histone deacetylase inhibitor led to re-expression of genes encoding important therapeutic targets including the estrogen receptor (ER). We conducted a multicenter phase II study of 5-azacitidine (AZA) and entinostat in women with advanced hormone-resistant or triple-negative breast cancer (TNBC).

Patients and Methods—Patients received AZA 40 mg/m² (days 1–5, 8–10) and entinostat 7 mg (days 3,10) of 28 day cycle. Continuation of epigenetic therapy was offered with addition of endocrine therapy at time of progression (optional continuation, OC phase). Primary endpoint was objective response rate (ORR) in each cohort. We hypothesized that ORR would be 20% against null of 5% using Simon two-stage design. At least 1 response was required in 1st of 13 patients per cohort to continue accrual to 27 per cohort. Type I error 4%, power 90%.

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Results—There was one partial response among 27 women with hormone-resistant disease (ORR=4%, 95% CI=0–19%), and none in 13 women with TNBC. One additional partial response was observed in the OC phase in the hormone-resistant cohort (n=12). Mandatory tumor samples were obtained pre- and post-treatment (58% paired) with either up- or down-regulation of ER observed in approximately 50% of post-treatment biopsies in the hormone-resistant, but not TNBC cohort.

Conclusion—Combination epigenetic therapy was well tolerated but our primary endpoint was not met. OC phase results suggest that some women benefit from epigenetic therapy and/or reintroduction of endocrine therapy beyond progression but further study is needed.

Keywords

Advanced breast cancer; epigenetics; 5-azacitidine; entinostat

Introduction

Cancer initiation and progression may be due to inherited or somatic genetic mutations, or epigenetic alterations in the genome. In contrast to genetic mutations, epigenetic alterations are not due to modifications in the gene primary nucleotide sequence, but include abnormal cytosine deoxyribonucleic acid (DNA) methylation and histone hypoacetylation in the promoter region of important genes.(1, 2) This may result in an altered chromatin structure leading to a repressive chromatin state and transcriptional silencing that can contribute to tumor development, growth and drug resistance. Several drugs that target epigenetic alterations, including inhibitors of DNA methyltransferases (DNMT) and histone deacetylases (HDAC), are currently approved for treatment of hematological malignancies and are being investigated in solid tumors.(3–5)

Epigenetic alterations are prevalent in breast cancer, prompting interest in their clinical significance and potential to be targeted by epigenetic modifiers. Breast cancer-related genes, tumor suppressor genes, and those involved with growth regulation, such as the estrogen receptor (ER, ER α , *ESR1*) have been shown to be epigenetically silenced.(6–8) Mounting evidence suggests that hormone receptor-positive breast cancers harbor more extensive DNA hypermethylation than hormone receptor-negative subtypes.(9, 10) ER silencing has been associated with poor prognosis and resistance to endocrine therapy.(11) Preclinical studies in breast cancer models have shown that the combination of DNMT and HDAC inhibitors result in superior ER re-expression and greater restoration of tamoxifen responsiveness compared to HDAC inhibitor alone, and prompted the development of this clinical trial.(12) The combination of the HDAC inhibitor scriptaid and decitabine was more effective in inducing ER in ER-negative cell lines than either agent alone.(13) Clinical studies have also demonstrated that epigenetic modulation can result in clinical benefit in solid malignancies including breast cancer.(14–16)

We therefore hypothesized that clinically tolerable doses of the DNMT inhibitor 5-azacitidine (AZA) and the HDAC inhibitor entinostat would yield objective disease responses in advanced HER2-negative breast cancer, and result in modulation of DNA methylation and gene expression. To test these hypotheses, we performed a multicenter

phase II clinical trial combining AZA and entinostat in women with advanced breast cancer, including triple-negative (ER/progesterone receptor [PR]/HER2-negative, TNBC) and hormone-resistant cohorts, incorporating blood and tissue biomarker evaluation.

Methods

Patients

Women 18 years of age or older, with histologically-proven infiltrating carcinoma of the breast with locally advanced or metastatic measurable disease were eligible. Patients with HER2-negative (TNBC or hormone receptor-positive) tumors were included.(17) Women must have experienced disease progression after at least one prior chemotherapy in any setting. Patients with hormone receptor-positive disease were required to have progressed through two lines of endocrine therapy (adjuvant or metastatic), display hormone-resistance clinically based on rate of disease progression or short interval time on first line endocrine therapy before progression per investigator and Study Chair discretion, or be intolerant of endocrine therapy. Eastern Cooperative Oncology Group (ECOG) performance status 0–1, and adequate hematologic, renal and liver function were required. After 50% of patients were enrolled, the study was amended to allow only patients with < 30% liver involvement based on clinical observation that patients with TNBC who enrolled with significant burden of liver disease were developing rapid disease progression in cycle one of therapy in keeping with the disease biology of this breast cancer subtype, and because epigenetic modifiers take longer to work than standard chemotherapy. The study was registered at clinicaltrials.gov (NCT01349959), and participants signed a written informed consent approved by the Institutional Review Boards of participating institutions.

Clinical Trial Design

In this single arm, multicenter, phase II study, two cohorts of women with advanced HER2-negative (TNBC or hormone-resistant) breast cancer received AZA (40 mg/m² subcutaneously, days 1–5, 8–10) and entinostat (7 mg orally, days 3 and 10) every 28 days (cycle). Treatment continued until progressive disease or unacceptable toxicity. Up to two dose reductions were allowed for AZA and one dose reduction for entinostat, unless permission was given by the Protocol Chair. A 5-HT₃ receptor antagonist was administered as premedication to prevent nausea. Because of the potential for ER re-expression with epigenetic agents, patients were offered continuation of AZA and entinostat at progression with the addition of endocrine therapy per physician discretion (optional continuation phase). Tamoxifen was recommended in the premenopausal setting and letrozole in the postmenopausal setting. Those patients who did not enter the optional continuation phase per treating physician's discretion were followed to capture subsequent therapies and disease status until either three years post-registration or death, whichever was earlier (event monitoring).

The primary endpoint was objective response rate (ORR) per Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1). Secondary endpoints were safety and tolerability, progression-free survival (PFS), time to death since progression (TTD) and overall survival (OS). Exploratory endpoints included the safety, toxicity, feasibility and response rate for the

optional continuation phase, pharmacokinetics, cytidine deaminase (CDA, metabolizes AZA in liver) activity, and change in candidate gene re-expression/DNA methylation in mandatory tumor samples pre- and post-therapy. Common Terminology Criteria for Adverse Events (CTCAE, version 4.0) was used to grade treatment-related toxicity.

Assessments

Baseline evaluations included routine history and physical examination, complete blood counts, serum chemistries and radiologic evaluations. Clinical evaluations and laboratory tests were repeated on day 10 (labs only) and monthly thereafter. Responses of measurable lesions were evaluated using RECIST criteria after every two cycles.(18) Upon discontinuation of treatment, patients were followed for outcomes until either three years post-registration or death. Patients removed from study for unacceptable adverse events were monitored until resolution or stabilization of the adverse event. All patients were followed for toxicity assessment for 30 days after going off-study.

Research blood samples were drawn on days 1 and 10 of cycle 1 for pharmacokinetic analyses, as well as prior to treatment on day 10 in cycles 1 and 2, and on day 1 of cycle 3 prior to receiving AZA for pharmacodynamic analyses. Mandatory study-specific tumor biopsies of an accessible tumor site were performed at baseline and after eight weeks of therapy. Optional biopsies were obtained at six months in consenting patients, including those who had stopped taking the study drugs.

Correlative Analysis

Concentrations of AZA and entinostat were determined using a validated liquid chromatography-tandem mass spectrometry (LC/MS/MS) method.(19, 20) AZA pharmacokinetic parameters were determined as previously described.(21) Entinostat trough concentrations (C_{min}) were considered reportable if they were collected pre-treatment on day 10. CDA activity was assayed following a simplified spectrophotometric method based upon the release and detection of ammonium from cytidine.(22)

DNA and RNA were extracted from fresh frozen biopsies using DNeasy Blood & Tissue Kit and RNeasy Mini Kit (Qiagen, Valencia, California) respectively. RNA quality was determined using a 2100 Bioanalyzer and hybridized to Agilent 4×44k Human Gene Expression v2 arrays (Agilent Technologies) in the Sidney Kimmel Comprehensive Cancer Center Microarray Core.

DNA samples underwent bisulfite conversion and quality control using MethyLight-based, real-time PCR control assays (Campan 2009 DNA Methylation Methods Protocols 507,325) followed by hybridization to the Infinium HumanMethylation450 (HM450) BeadChip (Illumina, San Diego, California).(23) Sex chromosomes (X, Y) and probes within 10bp of a Single Nucleotide Polymorphism were removed before analysis. DNA methylation levels at each CpG site were reported as beta values(23), calculated as $\beta = M/(U+M)$ where U and M are the intensities of unmethylated and methylated probes, respectively. A CpG site was considered to be demethylated, after treatment, if the DNA methylation level decreased so that $\beta_b - \beta_p \geq 0.20$. Global percent demethylation was calculated as the percentage of CpG

sites meeting this criterion. Percent demethylation of promoter CpG islands was calculated similarly, but using only CpG island probes within the promoter region.

Statistical Considerations

The sample size and decision rules were determined according to a two-stage three-outcome design(24) with an interim analysis to assess the efficacy of AZA and entinostat in the two patient cohorts in parallel. This design incorporates a possibility to declare an inconclusive outcome (i.e., reject neither null nor alternative hypothesis), allowing for further assessments before reaching a conclusion whether or not the regimen is considered promising. A minimum of 13 and maximum of 27 evaluable patients were to be accrued to each cohort with the hypotheses that a 20% response rate would be of interest and 5% considered ineffective in this population. The design had a 4% chance of finding the regimen to be effective when truly not (i.e., Type I error) and 80% chance of declaring this regimen warranted further study (i.e. statistical power) when the true response rate was 20%. The probability of determining that the study was inconclusive was 20% when the true response rate was 10%. Interim safety and lack of efficacy analyses were planned after the first 13 patients enrolled in each cohort. ORR was estimated independently for each cohort by the number of complete or partial responses divided by the total number of evaluable patients. Computation of the associated 95% confidence intervals did not account for the sequential design. PFS and TTD were described using Kaplan-Meier method with 95% confidence intervals. Analyses of OS were descriptive in nature and may be contaminated because the choice of optional continuation or event monitoring was subject to selection bias.

Preprocessing of expression data, including loess normalization to correct for dye bias, as well as differential expression analysis were performed using the *limma* package as previously described.(25) Tumor purity for primary tumors was estimated from gene expression using the Estimate-Project method.(26) Expression of genes was evaluated based on the microarray data. PANTHER(27) and gene set enrichment analysis (GSEA)(28) was used to characterize the most differentially expressed biological pathways. Samples were assigned to PAM50 classes(29) using the GeneFu package from Bioconductor.(30)

Pharmacokinetic parameters were summarized using descriptive statistics. Spearman's rank correlation coefficients were used to assess correlations between pharmacokinetic parameters and CDA activity. Kruskal-Wallis tests were used to compare medians between the groups with respect to drug exposure, response, toxicity, and change in ER expression.

In order to explore the potential prognostic effect of each gene in women with hormone resistant breast cancer, we performed landmark analyses with *a priori* defined landmark time at 8 weeks post treatment to assess the association of the fold change of gene expression at 8 weeks post treatment to pre-treatment ($\log_2[\text{post/pre}]$) with OS via Cox proportional hazards models. All statistical tests were two-sided and considered statistically significant at $P < 0.05$ unless otherwise specified. The analyses that involved large number of comparisons with respect to gene expression data were considered statistically significant at a Benjamini-Hochberg false discovery rate (FDR) of 0.05.(31) The analyses were carried out using SAS software (v9.3, SAS Institute, Cary, NC) and the R statistical software suite and programming environment (www.r-project.org).

Results

Patient Characteristics

From August 2011 to September 2013, 40 evaluable women (13 TNBC, 27 hormone-resistant) enrolled in the study and their characteristics are summarized in Table 1. No patients were enrolled in the hormone-resistant cohort based on the eligibility criteria of “intolerance of endocrine therapy.” Median age was 55 years in the hormone-resistant, and 47 years in the TNBC cohorts. The population of patients enrolled was heavily pretreated with the median number of prior chemotherapy regimens for advanced disease equal to two (range 0–9). Sixteen patients (40%) proceeded to the optional continuation phase (Supplementary Table 1).

Treatment and Treatment Safety

All patients received the pre-defined starting doses of AZA and entinostat. Hematological and non-hematological toxicities are shown in Table 2. The preplanned blinded interim toxicity analysis did not meet the criteria for early termination. Grade 3 and 4 drug-related toxicities were infrequent, with the most common hematologic adverse events in the primary phase including neutropenia (17.5%) and leukopenia (17.5%). The most frequent non-hematologic adverse events were urinary tract infection (10%), hypophosphatemia (5%), and fatigue (5%).

Doses of AZA and entinostat were reduced in seven patients due to grade 3 decreased white blood cell/absolute neutrophil count (ANC) (n=5), grade 4 decreased white blood cell/ANC (n=1), and grade 3 hypophosphatemia (n=1). One patient had a second dose reduction for grade 3 decreased white blood cell/ANC. Entinostat was dose reduced alone in one patient due to grade 3 nausea/vomiting.

Treatment Efficacy

We did not observe any clinical response in the first stage of 13 patients with TNBC, and this cohort was closed to further accrual. At a median follow-up of 6.6 months (range 1.3–25.3), all patients in this cohort had progressed and died. Median PFS was 1.4 months (95% CI = 0.9 – 1.8) and median OS was 6.6 months (95% CI, 2.0 – 10.3) (Figure 1A, 1B).

We observed one partial response with epigenetic therapy alone among 13 evaluable patients in the hormone-resistant cohort in the first stage and the accrual continued to the second stage with 14 additional patients. No further responses were seen with epigenetic therapy alone and the study therefore did not meet its primary endpoint, resulting in an ORR of 4% (95% CI, 0–19%). At a median follow up of 10.4 months (range 0.5–28.2), 26 participants had progressed and 20 died. Median PFS was 1.8 months (95% CI, 1.7–1.9) and median OS was 12.6 months (95% CI, 6.3–16.3) (Figure 1C, 1D).

Optional Continuation Phase

Four women (31%) in the TNBC cohort continued epigenetic therapy with the addition of endocrine therapy as part of the optional continuation phase, and received a median of 1.5 additional cycles (range 1–6) with no tumor responses observed (Supplementary Table 1).

Interestingly, one patient who enrolled in this phase after earlier documentation of tumor progression following two cycles of epigenetic therapy alone remained on therapy for an additional 6 months. The median time to progression (TTP) for this cohort was 3.6 months (95% CI, 1.6–3.8) versus 1 month (95% CI, 0.3–1.6) for those who did not enter this phase (event monitoring). The median TTD after progression was 7.1 months (95% CI, 5.0–21.5) for patients who continued with endocrine therapy and 3.0 months (95% CI, 0.2–7.1) for those with event monitoring.

Among the 27 patients in the hormone-resistant cohort, 12 (44%) transitioned to the optional continuation phase and 15 (56%) continued event monitoring. Patients in the optional continuation phase received a median of 2.5 additional cycles (range 1–9) (Supplementary Table 1). We observed one partial response (liver) in this phase (ORR 8%, 95% CI, 0.2–38%) in a woman who progressed after two cycles of epigenetic therapy alone, and remained on study for an additional 8 months. This patient (no paired biopsies available thus no Pt #) had received one line of endocrine therapy (anastrozole) in the adjuvant setting and relapsed with advanced disease after 13 months of therapy. She was then treated with 3 lines of chemotherapy prior to referral for clinical trial. She was deemed clinically endocrine resistant, and had extensive bone and liver metastases. Two patients had prolonged disease stabilization on this phase; Pt #7 received 2 months of epigenetic therapy alone prior to disease progression, and then remained on the epigenetic therapy with addition of tamoxifen for 9 months. She had received one prior line of endocrine therapy in adjuvant setting (2 years of tamoxifen) and two lines of chemotherapy for advanced disease. The second (no paired biopsies available thus no Pt #) was on epigenetic therapy alone for 2 months and remained on the study with addition of letrozole for 8 months. She had received 3 prior lines of endocrine therapy including anastrozole but not letrozole. Median TTP was similar between patients who went on optional continuation phase (1.9 months, 95% CI, 1.7–3.7) and those with event monitoring who did not (1.8 months, 95% CI, 0.8–1.9). The median TTD after progression was 13.9 months (95% CI=3.7–29.3) for patients with optional continuation and 10 months (95% CI, 1.8–17.7) with event monitoring (Supplementary Figure 1).

Pharmacokinetics and Cytidine Deaminase Activity

Pharmacokinetic data were obtained from 24 patients treated at AZA dose level of 40 mg/m²/day. As previously reported,(21) AZA was rapidly absorbed and eliminated with the time to maximal concentration (T_{max}) occurring at 0.38 hours (median; range 0.18 – 0.67 hours) and half-life ($t_{1/2}$) of 0.90 ± 0.40 hours (average $\pm$ sd). Maximum concentration (C_{max}) and area under the curve ($AUC_{0-\infty}$) for AZA were 634 ± 286 ng/mL and 730 ± 248 ng*hr/mL. Entinostat trough concentrations measured on day 10 were 0.53 ± 0.42 ng/mL. CDA activity was variable at 1.91 ± 1.29 AU/mg (n=30). There was no statistically significant correlation between the AZA exposure and CDA activity (C_{max} $r=-0.047$, $P=0.84$; $AUC_{0-\infty}$ $r=-0.024$, $P=0.94$). There were also no statistically significant correlations between the worst grade of toxicity and AZA or entinostat exposure or CDA activity ($P>0.05$).

Gene Expression and DNA Methylation Alterations after Treatment

Overall, study specific core biopsies were obtained in 38 patients (95%) at baseline, 24 (60%) at 2 months post-treatment and one (3%) patient at 6 months post-treatment. Matched baseline and 2 month samples were obtained for 58% of patients. Post-treatment biopsy was performed 13–21 days post last dose of therapy in 87.5% of cases. Tumor purity was >70% in the majority of samples, with small random variations observed between pre- and post-treatment biopsies (Supplementary Figure 2). The median RNA Integrity Number (RIN) for the hormone-resistant and TNBC RNA samples were 7.55 and 5.5, respectively.

Differential gene expression analysis of 14 paired biopsies from patients in the hormone-resistant cohort showed significant changes in 186 genes (FDR <0.05) after treatment with AZA and entinostat (Figure 2A and Supplementary Figure 3A). Fold change is calculated as $\log_2$ post/pre, and significantly altered genes were required to have an absolute log fold change > 0.5 and an FDR <0.05. Twenty-nine genes were upregulated, and 157 downregulated after treatment (Figure 2A and Supplementary Figure 3). There were no statistically significant changes in gene expression in the paired biopsies from patients with TNBC, possibly due to the small sample size (Figure 2B).

To better understand the function of these genes, we queried them through the PANTHER database (pantherdb.org), using Gene Ontology (GO) terminology to assign the genes to biological processes. Thirteen biological processes were identified (Supplementary Figure 3B) as containing genes that were altered in the post-treatment biopsies as compared to the pre-treatment biopsies. Additional studies will be required to determine the significance of these changes to breast tumor biology.

Characterization of the pre- and post- treatment biopsies using GeneFu and the PAM50 intrinsic gene set to classify samples agreed well with clinical subtypes assigned at study entry, with 16/19 (84%) paired biopsies receiving the same classification by both methods. Pre- and post-treatment subtypes were in disagreement for only 2/19 (11%) of cases (Supplementary Table 2). In addition, none of the genes had shown statistically significant association of the fold change (post/pre) with OS using an FDR of <0.105 for the hormone-resistant cohort.

A Global DNA methylation analysis, which measures cytosine (CpG) methylation in the entire genome, including gene regions, and non-coding repetitive elements, was conducted in 15 paired biopsies from patients in the hormone-resistant cohort and 5 from patients in TNBC cohort. Because AZA is a demethylating agent, we looked for losses in methylation across the entire genome. Decreasing beta values correlate with decreases in DNA methylation (i.e increases in demethylation). A CpG site was considered to be demethylated, after treatment, if the beta value for the DNA methylation level decreased so that $\beta_{\text{pre}} - \beta_{\text{post}} \geq 0.20$.

Global percent demethylation was calculated as the percentage of CpG sites meeting this criterion. Widespread decreases in methylation were observed in some post-treatment biopsies at eight weeks and six months post treatment (Figure 2C, Supplementary Figures 4A–B) as evidenced by a shift to the left in the methylation distribution plots and a decrease in the beta values.

In a post-hoc analysis, we compared the percent demethylation from those patients in the TNBC cohort who survived > 10 months (n=3) to those who survived < 10 months (n=10) (Figure 1B). Paired biopsies were available for 2 of the 3 patients living > 10 months (Pt #17 and 19) and showed percent demethylation of 11.5% (Pt # 17 at 6 months) and 4% (Pt #19 at 8 weeks) respectively. Supplementary Table 3 notes an increase in percent demethylation in biopsies from Pt #17 from 2.2% at 8 weeks to 11.5% at 6 months. Paired biopsies were available for 3 of the 10 patients living < 10 months and showed lower percent demethylation; 0.1%, 1.8%, and 6.6% (Supplementary Table 3).

We compared the percent demethylation from those patients in the hormone-resistant cohort who survived > 20 months (n=6) to those who survived < 20 months (n=12) (Figure 1D). Paired biopsies were available for 3 of the 6 patients living > 20 months (Pt #3, 13 and 14) and showed median percent demethylation of 11.5% (range 2.7–22.2%). Paired biopsies were available for 12 of the 21 patients living < 20 months and also showed lower median percent demethylation of 0.85% (range 0–10.2%).

Cox proportional hazards regression analysis was also conducted for OS and PFS with the percent global demethylation and percent CpG island demethylation respectively. Greater global percent demethylation appears to be associated with longer survival (hazard ratio=0.83, 95% CI=0.71–0.98) when comparing samples with global percent demethylation above the median to those with lower global percent demethylation, although it will be necessary to formally test this observation in independent samples before statistical significance can be assessed. A similar trend was observed for the association of PFS with global percent demethylation, (hazard ratio=0.92, 95% CI, 0.83–1.01) and also percent CpG island demethylation (hazard ratio=0.33, 95% CI, 0.12–0.93).

As expected, higher baseline ER expression was noted in biopsies from the hormone-resistant cohort versus TNBC (Figure 3A). Change in ER gene expression was noted in post-treatment biopsies from the hormone-resistant cohort (6 of 14 0.5 log₂-fold-change and 2 of 14 –0.5 log₂-fold-change), but this was not observed for the TNBC cohort (Figure 3B). Interestingly, paired biopsies for two patients (7.4%) in the hormone-resistant cohort who were on trial for more than six months demonstrated an increase in ER expression after epigenetic therapy, but no significant changes were observed in ER CpG island DNA methylation status (Supplementary Figure 5, identified as “>6 months”). Increases in ER expression could also be due to changes in protein acetylation status and chromatin remodeling due to effects from the HDAC inhibitor entinostat. The acetylation status of the ER protein was not examined in this study. The lack of DNA demethylation and ER re-expression in the TNBC tumors may be due to the fact that ER promoter was not methylated in the pre-treated samples from patients with TNBC, or in other words that CpG island DNA hypermethylation was not present (Fig 3C, purple triangles). There were also no correlations between the change in ER gene expression and AZA or entinostat exposure or CDA activity ($P > 0.05$).

Discussion

In a multicenter phase II study, we have demonstrated that the combination of epigenetic modifiers, AZA and entinostat, was well tolerated in patients with advanced HER2-negative breast cancer with few grade 3/4 adverse events attributed to the epigenetic therapy. There was no clinical activity in 13 patients with advanced TNBC. We observed one partial response with epigenetic therapy alone in 27 women with hormone-resistant disease and thus the trial did not meet the primary endpoint. In addition, we observed one partial response in a patient in the hormone-resistant cohort in the optional continuation phase, after initial progression following two cycles of epigenetic therapy in the primary phase of the study. The partial response occurred after two additional cycles of epigenetic therapy in combination with letrozole, and persisted until ten cycles of therapy had been received. It must be noted that this patient had received only one line of prior endocrine therapy, however, she was deemed clinically hormone-resistant by her treating physician prior to study entry. We also observed that the median TTD was longer in the optional continuation phase than in those who did not enter this phase (event monitoring), with median time to progression nearly identical between these two patient groups. These results are likely due to small patient numbers and/or selection bias but it is possible that some women with hormone-resistant disease may benefit from continuation of epigenetic therapy and/or reintroduction of endocrine therapy beyond progression. Future studies could investigate more robustly whether the addition of endocrine therapy to AZA and entinostat (concurrent or at time of progressive disease) provides clinical benefit to patients with hormone-resistant breast cancer. Our efficacy results (ORR) are similar to the findings in clinical trials incorporating this combination of epigenetic agents with same dosing and schedule in patients with advanced non-small cell lung cancer. (16)

Intriguing data from the correlative studies support our clinical observations. There were no significant changes in gene expression in the available paired biopsies from patients with TNBC, which may be due to the lack of ER promoter DNA methylation (Figure 3). Either up regulation or down regulation of ER were noted in approximately half of post treatment biopsies from the hormone-resistant cohort, which did not correlate with the variability in CDA expression, AZA or entinostat exposure. One could speculate how either ER upregulation followed by anti-estrogen therapy, or ER down-regulation in an estrogen driven tumor, may yield anti-tumorigenic, therapeutic results. Indeed, we observed that two patients in the hormone-resistant cohort who enrolled in the optional continuation phase and remained on therapy for 8–11 months had increases in ER expression in their 8-week post treatment biopsy when compared to baseline. Although this is only hypothesis generating due to small numbers, it raises the question as to whether epigenetic therapy may sensitize some patients to endocrine therapy. These data agree with published reports that DNA methylation is more abundant in ER-positive tumors than in basal-like tumors, which are usually TNBCs.(9, 10)

Interestingly, more downregulation of gene expression than upregulation was observed in samples from the hormone-resistant cohort at eight weeks post therapy which was contrary to our original hypothesis. In any single tumor there may only be a few hundred genes that are demethylated and silenced, and even fewer will be re-expressed by epigenetic modifiers.

(32, 33) Therefore it may be difficult to observe significant and sustained re-expression of these genes at 8 weeks post-therapy. Moreover, many of these demethylated genes may encode transcription factors and DNA binding proteins that may be transcriptionally repressive. We speculate that the downregulation observed in gene expression could be due to the indirect, downstream signaling effects of demethylated genes analyzed several weeks after the administration of the last dose of AZA and entinostat. Another explanation is that in addition to promoter and enhancer demethylation, gene bodies may also be demethylated by epigenetic modifiers, leading to altered gene expression profiles. Interestingly, recent reports show that DNA methyltransferase 3B (DNMT3B) is involved in DNA methylation of gene body regions that correlates with expression of the gene.(34, 35)

Global demethylation was observed in patient biopsies, suggesting that the patient's tumor tissue received exposure to the epigenetic therapy (Figure 2C, Supplementary Figures 4A–B). Our data suggest that greater percent demethylation may be associated with longer survival. However, the small sample size and confounding factors may have influenced these hypothesis generating results. Further evaluation of this observation is ongoing in our laboratory and could be investigated in the clinical trial setting.

The optional continuation phase was a unique component of our clinical trial which offered patients in either cohort the opportunity to transition to the same epigenetic therapy at progression with the addition of endocrine therapy. The rationale for this phase was partially based on experience in the hematologic malignancy setting suggesting that epigenetic modifiers require more time than standard chemotherapies to elicit disease responses.(36) In addition, epigenetic modifiers appear to have the potential to re-express genes encoding important therapeutic targets, including ER, and to restore sensitivity to endocrine therapy. That ER is frequently silenced by promoter DNA hypermethylation in cell lines prompted us to include a TNBC cohort in this study.(11, 37, 38) Entinostat itself has been shown to induce re-expression of ER, and the aromatase enzyme (CYP19) both *in vitro* and in TNBC xenografts, as well as sensitize breast cancer cells to estrogen and letrozole.(39) The combination of entinostat with letrozole or exemestane also resulted in a significant and durable reduction in letrozole-resistant xenograft tumor volumes when compared to treatment with either agent alone.(40) These results prompted development of the phase II ENCORE301 clinical trial, in which patients who had progressed following treatment with a non-steroidal aromatase inhibitor were randomized to exemestane with entinostat or placebo, with the entinostat arm resulting in an improvement in both PFS and OS.(14) These findings support the hypothesis that epigenetic therapy may overcome treatment resistance and sensitize to endocrine therapy and has led to the launch of a phase III double-blind placebo-controlled registration study in this patient population (E2112, NCT 02115282). Results from a study using the combination of AZA and entinostat in non-small cell lung cancer also suggests that epigenetic therapy may sensitize tumors to subsequent therapy, including immune checkpoint blockade (e.g. PDL1/PD1).(16, 41) Indeed, we have demonstrated up-regulation of a 5-AZA Immune gene set (AIMs) after AZA and entinostat in samples from select patient biopsies from our breast cancer study, providing additional support for epigenetic modifiers in priming breast tumors for immune modulation.(25)

Additional strengths of this study include the multicenter prospective design and the inclusion of separate breast cancer cohorts (TNBC and hormone-resistant). We have been extremely successful in collecting mandatory serial tumor biopsies from the same tumor location both pre- (95%) and post-epigenetic therapy (58% matched). Tumor purity and RNA quality were high, indicating the feasibility of such an approach in studies attempting to further understand tumor biology and drug mechanism of action, or identify predictors of response to therapy. A wealth of relevant correlative analyses was embedded in this clinical trial including pharmacokinetic, CDA and pharmacodynamic analyses. The major limitation of this study is that this was a single arm, non-randomized trial without a comparator arm. The combination of DNMT and HDAC inhibitors was chosen based on preclinical data supporting this combination over single-agent therapy; however, it remains unclear if this combination is more efficacious than either agent alone in the clinical setting or what the optimal dose and schedule may be of the combination. A phase II clinical trial in hematologic malignancies showed no benefit of this combination over treatment with single agent AZA.(42) Our trial also indicates a lack of benefit of the treatment regimen in patients with TNBC; however, patients were generally heavily pretreated and were not selected by any biomarker other than hormone-receptor status.(43) One patient in the TNBC cohort remained on continuation therapy for a total of 10 months, with the best response being stable disease. It is still possible that there is a subgroup of patients with TNBC who may benefit from epigenetic therapy, perhaps at an earlier stage of the disease, or combined with immune checkpoint inhibitors.

In conclusion, our study indicates the feasibility of conducting multicenter studies that use novel agents, and incorporate serial tissue biopsies and blood samples for biomarker development in patients with advanced breast cancer. A strong collaboration between the laboratory and the clinic was present from study design through completion and will be critical for the effective design of future trials. Although our study did not meet the primary endpoint of overall response rate, a subgroup of women with hormone-resistant disease may derive clinical benefit from epigenetic therapy and/or re-introduction of endocrine therapy. This question would need to be investigated in future clinical trials and with additional correlative analysis. Moreover, combination epigenetic therapy with DNMT and HDAC inhibitors may lead to sensitization to other standard or novel therapies. Ongoing trials are investigating the addition of epigenetic therapy to endocrine therapy in patients with hormone-resistant disease (NCT02115282) and whether epigenetic therapy can sensitize to chemotherapy or immune checkpoint agents based on promising preclinical and clinical data (NCT01935947, NCT01928576, NCT02453620).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

We conducted a multicenter phase II clinical trial of the DNA methyltransferase (DNMT) inhibitor 5-azacitidine (AZA) and the histone deacetylase (HDAC) inhibitor entinostat in women with advanced hormone-resistant or triple-negative breast cancer (TNBC). We observed 1 partial response with epigenetic therapy alone in the hormone-resistant cohort and another in a patient offered continuation of epigenetic therapy with addition of endocrine therapy at time of progression. No responses were seen in TNBC cohort. Mandatory tumor samples were obtained pre- and post-treatment (58% paired) with modulation of the estrogen receptor observed in approximately 50% of post-treatment biopsies in the hormone-resistant, but not TNBC cohort. A subset of women with hormone-resistant breast cancer may thus benefit from epigenetic therapy and/or reintroduction of endocrine therapy with epigenetic therapy beyond progression. Ongoing and future studies testing epigenetic agents in combination with other therapeutics aim to identify potential predictive biomarkers of response to these agents.

Figure 1. Kaplan-Meier curves for PFS (A) and OS (B) with the combination of 5-azacitidine (AZA) and entinostat in women with advanced triple-negative breast cancer; PFS (C) and OS (D) with the combination of AZA and entinostat in women with advanced hormone-resistant breast cancer. The 95% confidence limits are shaded; Tick marks indicate censored data. PFS = progression-free survival, OS = overall survival.

Figure 2. Changes in genomic expression and methylation analyses in hormone-resistant and triple-negative tumor biopsies. **A–B.** Differential gene expression analysis was conducted on paired (post-/ pre-treatment [tx]) biopsies using *limma* in R language. The fold change is calculated as $\text{Log}_2(\text{post-/pre-tx})$ and significantly altered genes (red dots) are determined using a threshold cutoff of 0.5 and a false discovery rate (FDR) of <0.05 . **A.** Analysis of 14 paired hormone-resistant biopsies showed significant changes (red dots) in 186 genes. A total of 29 genes were up-regulated, (right side of plot) and 157 were down-regulated (left side of plot). **B.** Analysis of 5 paired triple-negative biopsies did not reveal any significant gene changes. **C.** Global DNA methylation analysis of DNA from selected patients showing the most prominent decreases in DNA methylation (observed as a decreased beta value or shift to the left) in post-tx biopsies (red and blue curves). Probe density is shown on the y-axis. Beta value reflects methylation percentage is shown on the x-axis. Tx: Therapy.

Figure 3.

Estrogen receptor (ER) gene expression and methylation changes in pre- and post-treatment biopsies. **A.** ER gene expression in pre-tx hormone-resistant biopsies (red bars) in comparison to ER expression in the pre-tx triple-negative biopsies (blue bars). Student t-test confirmed the significant difference in basal ER gene expression between HR and TN cohort ($p < 0.0001$, see insert graph). **B.** Significantly altered fold changes in ER expression were observed in 7 paired hormone-resistant biopsies ($\log_2(\text{post/pre}) \geq 0.5$ or ≤ -0.5) but no significant changes of ER expression were observed in triple-negative biopsies. **C.** ER

promoter DNA methylation analysis using the Illumina Infinium HM450 DNA methylation arrays. No CpG island DNA hypermethylation (purple) was observed in the CpG island region, but DNA methylation changes were observed for some patients in probes correlating with the transcription start site (green). CpGi: CpG island, TSS: transcription start site, Tx: therapy

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Table 1

Patient characteristics (Primary Phase)

Characteristics	Hormone-resistant (n=27)	Triple-negative (n=13)
Age, years		
Median	55	47
Range	(35–70)	(31–67)
Race		
White	21 (78%)	10 (77%)
Black	3 (11%)	3 (23%)
Other	3 (11%)	0 (0%)
ECOG Performance Status		
0	11 (41.5%)	7 (54%)
1	15 (55%)	6 (46%)
2	1 (0.5%)	0 (0%)
Disease status		
Locally advanced	1 (0.5%)	1 (0.7%)
Metastatic	26 (95.5%)	12 (93.3%)
Location of disease		
Visceral	24 (89%)	10 (77%)
Non-visceral	3 (11%)	3 (23%)
Median number of prior therapies (all settings) (range)		
Hormonal	3 (1–5)	0 (0–1)
Cytotoxic	3 (1–10)	3 (1–6)
Median no. of regimens for metastatic disease (range)		
Hormonal	2 (0–4)	0 (0–1)
Cytotoxic	2 (0–9)	2 (0–4)
Optional Continuation Phase	12 (44%)	4 (31%)
Event Monitoring	15 (56%)	9 (69%)

ECOG, Eastern Cooperative Oncology Group

Table 2

Treatment-related side effects occurring in > 5% of patients across primary and continuation treatment phases

Toxicity	Primary Treatment Phase (N= 40)				Optional Continuation Phase (N= 16)			
	Total Events n (%)	By Grade: n (%)			Total Events n (%)	By Grade: n (%)		
		G2	G3	G4		G2	G3	G4
<i>Blood and Lymphatic System Disorders</i>								
Anemia (decreased hemoglobin)	33 (82.5)	32 (80)	-	1 (2.5)	13 (81)	13 (81)	-	-
<i>General Disorders and Administration Site Conditions</i>								
Chills	4 (10)	4 (10)	-	-	1 (6)	1 (6)	-	-
Fatigue	30 (75)	28 (70)	2 (5)	-	8 (50)	8 (50)	-	-
Fever	4 (10)	4 (10)	-	-	0 (0)	-	-	-
Injection site reaction	28 (70)	28 (70)	-	-	12 (75)	12 (75)	-	-
<i>Gastrointestinal Disorders</i>								
Bloating	3 (7.5)	3 (7.5)	-	-	0 (0)	-	-	-
Constipation	15 (37.5)	15 (37.5)	-	-	6 (37.5)	6 (37.5)	-	-
Diarrhea	2 (5)	2 (5)	-	-	2 (12.5)	2 (12.5)	-	-
Dyspepsia/GERD	3 (7.5)	3 (7.5)	-	-	2 (12.5)	2 (12.5)	-	-
Nausea	25 (62.5)	24 (60)	1 (2.5)	-	6 (37.5)	6 (37.5)	-	-
Vomiting	12 (30)	11 (27.5)	1 (2.5)	-	1 (6)	1 (6)	-	-
<i>Infection and Infestations</i>								
Urinary tract infection	4 (10)	-	4 (10)	-	0 (0)	-	-	-
Infection, Other	1 (2.5)	-	1 (2.5)	-	2 (12.5)	1 (6)	1 (6)	-
<i>Investigations</i>								
Neutrophil count decreased	20 (50)	11 (27.5)	7 (17.5)	2 (5)	7 (44)	4 (25)	2 (12.5)	1 (6)
Platelet count decreased	11 (27.5)	11 (27.5)	-	-	2 (12.5)	2 (12.5)	-	-
Weight loss	2 (5)	2 (5)	-	-	1 (6)	1 (6)	-	-
White blood cell decreased	28 (70)	20 (50)	7 (17.5)	1 (2.5)	13 (81)	11 (69)	2 (12.5)	-
<i>Metabolism and Nutrition Disorders</i>								
Anorexia	10 (25)	10 (25)	-	-	4 (25)	4 (25)	-	-

Toxicity	Primary Treatment Phase (N= 40)			Optional Continuation Phase (N= 16)			
	Total Events n (%)	By Grade: n (%)		Total Events n (%)	By Grade: n (%)		
		G2	G3		G2	G3	G4
Hypophosphatemia	9 (22.5)	7 (17.5)	2 (5)	4 (25)	3 (19)	1 (6)	-
<i>Musculoskeletal and Connective Tissue Disorders</i>							
Back pain	1 (2.5)	1 (2.5)	-	2 (12.5)	2 (12.5)	-	-
Bone pain	2 (5)	2 (5)	-	1 (6)	1 (6)	-	-
Pain, NOS	2 (5)	2 (5)	-	1 (6)	1 (6)	-	-
Pain, Other	9 (22.5)	9 (22.5)	-	4 (25)	4 (25)	-	-
<i>Nervous System Disorders</i>							
Dysgeusia	2 (5)	2 (5)	-	2 (12.5)	2 (12.5)	-	-
Headache	10 (25)	10 (25)	-	2 (12.5)	2 (12.5)	-	-
<i>Psychiatric Disorders</i>							
Anxiety	2 (5)	2 (5)	-	1 (6)	1 (6)	-	-
Depression	2 (5)	1 (2.5)	1 (2.5)	2 (12.5)	2 (12.5)	-	-
Insomnia	3 (7.5)	3 (7.5)	-	0 (0)	-	-	-
<i>Skin and Subcutaneous Tissue Disorders</i>							
Pruritus	4 (10)	4 (10)	-	1 (6)	1 (6)	-	-

Note: Number of worst grade adverse events possibly, probably, or definitely attributed to study drug administration. Toxicities are graded per the NCI CTCAE Version 4 criteria. G2=Grade 1 and 2, G3=Grade 3, G4=Grade 4. NOS= not otherwise specified.