

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 reexpression of genes encoding important therapeutic targets, including the estrogen receptor (ER). We conducted a multicenter phase II study of 5-azacitidine and entinostat in women with advanced hormone-resistant or triple-negative breast cancer (TNBC).

Experimental Design: Patients received 5-azacitidine 40 mg/m² (days 1–5, 8–10) and entinostat 7 mg (days 3, 10) on a 28-day cycle. Continuation of epigenetic therapy was offered with the addition of endocrine therapy at the time of progression [optional continuation (OC) phase]. Primary endpoint was objective response rate (ORR) in each cohort. We hypothesized that ORR would be $\geq 20\%$ against null of 5% using Simon two-stage design. At least one response was required in 1 of 13

patients per cohort to continue accrual to 27 per cohort (type I error, 4%; power, 90%).

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 posttreatment (58% paired) with either up- or downregulation of ER observed in approximately 50% of posttreatment biopsies in the hormone-resistant, but not TNBC cohort.

Conclusions: 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. *Clin Cancer Res*; 23(11); 2691–701. ©2016 AACR.

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 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 hematologic 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 results in superior ER reexpression and greater restoration of tamoxifen responsiveness compared with HDAC inhibitor alone and prompted the development of

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Translational Relevance

We conducted a multicenter phase II clinical trial of the DNA methyltransferase inhibitor 5-azacitidine and the histone deacetylase inhibitor entinostat in women with advanced hormone-resistant or triple-negative breast cancer (TNBC). We observed one partial response with epigenetic therapy alone in the hormone-resistant cohort and another in a patient-offered continuation of epigenetic therapy with the addition of endocrine therapy at the time of progression. No responses were seen in the TNBC cohort. Mandatory tumor samples were obtained pre- and posttreatment (58% paired) with modulation of the estrogen receptor observed in approximately 50% of posttreatment 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.

this clinical trial (12). The combination of the HDAC inhibitor scriptaid and decitabine was more effective in inducing estrogen receptor (ER) in ER⁻ 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 and the HDAC inhibitor entinostat would yield objective disease responses in advanced HER2⁻ 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 5-azacitidine and entinostat in women with advanced breast cancer, including triple-negative [ER/progesterone receptor (PR)/HER2⁻, TNBC] and hormone-resistant cohorts, incorporating blood and tissue biomarker evaluation.

Materials and 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⁻ (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 Protocol Chair discretion, or be intolerant of endocrine therapy. Eastern Cooperative Oncology Group 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 1 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⁻ (TNBC or hormone-resistant) breast cancer received 5-azacitidine (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 5-azacitidine 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 reexpression with epigenetic agents, patients were offered continuation of 5-azacitidine 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 3 years postregistration 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 5-azacitidine in liver) activity, and change in candidate gene reexpression/DNA methylation in mandatory tumor samples pre- and posttherapy. 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 3 years postregistration 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 5-azacitidine for pharmacodynamic analyses. Mandatory study-specific tumor biopsies of an accessible tumor site were performed

at baseline and after 8 weeks of therapy. Optional biopsies were obtained at 6 months in consenting patients, including those who had stopped taking the study drugs.

Correlative analysis

Concentrations of 5-azacidine and entinostat were determined using a validated LC/MS-MS method (19, 20). 5-Azacidine pharmacokinetic parameters were determined as described previously (21). Entinostat trough concentrations (C_{min}) were considered reportable if they were collected pretreatment 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), respectively. RNA quality was determined using a 2100 Bioanalyzer and hybridized to Agilent 4 × 44 k 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; ref. 23). Sex chromosomes (X, Y) and probes within 10 bp of an SNP 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 analysis

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 5-azacidine 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 (CI) did not account for the sequential design. PFS and TTD were described using Kaplan–Meier method with 95% CIs. 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 described previously (25). Tumor purity for primary tumors was estimated from gene expression using the Estimate-Project method (26). Expression of genes was evaluated on the basis of the microarray data. PANTHER (27) and gene set enrichment analysis (28) were 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 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.

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 posttreatment to assess the association of the fold change of gene expression at 8 weeks posttreatment to pretreatment [$\log_2(\text{post/pre})$] 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 FDR of 0.05 (31). The analyses were carried out using SAS software (v9.3, SAS Institute) 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 2 (range, 0–9). Sixteen patients (40%) proceeded to the optional continuation phase (Supplementary Table S1).

Treatment and treatment safety

All patients received the predefined starting doses of 5-azacidine and entinostat. Hematologic and nonhematologic 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 nonhematologic adverse events were urinary tract infection (10%), hypophosphatemia (5%), and fatigue (5%).

Doses of 5-azacidine and entinostat were reduced in 7 patients due to grade 3–decreased white blood cell/absolute

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%)
Nonvisceral	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%)

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

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; Fig. 1A and B).

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; Fig. 1C and D).

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 S1). 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 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 S1). 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 three 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 the addition of tamoxifen for 9 months. She had received one prior line of endocrine therapy in the 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 the addition of letrozole for 8 months. She had received three 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 Fig. S1).

Pharmacokinetics and CDA activity

Pharmacokinetic data were obtained from 24 patients treated at 5-azacitidine dose level of 40 mg/m²/day. As previously reported (21), 5-azacitidine 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 AUC ($AUC_{0-\infty}$) for 5-azacitidine were 634 ± 286 ng/mL and $730 \pm 248 \times \text{hr/mL}$. 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 5-azacitidine 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 5-azacitidine 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 posttreatment, and one (3%) patient at 6 months posttreatment. Matched baseline and 2-month samples were obtained for 58% of patients. Posttreatment biopsy was performed 13 to 21 days after last dose

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
Blood and lymphatic system disorders								
Anemia (decreased hemoglobin)	33 (82.5)	32 (80)	—	1 (2.5)	13 (81)	13 (81)	—	—
General disorders and administration site conditions								
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)	—	—
Gastrointestinal disorders								
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)	—	—
Infection and infestations								
Urinary tract infection	4 (10)	—	4 (10)	—	0 (0)	—	—	—
Infection, Other	1 (2.5)	—	1 (2.5)	—	2 (12.5)	1 (6)	1 (6)	—
Investigations								
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)	—
Metabolism and nutrition disorders								
Anorexia	10 (25)	10 (25)	—	—	4 (25)	4 (25)	—	—
Hypophosphatemia	9 (22.5)	7 (17.5)	2 (5)	—	4 (25)	3 (19)	1 (6)	—
Musculoskeletal and connective tissue disorders								
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)	—	—
Nervous system disorders								
Dysgeusia	2 (5)	2 (5)	—	—	2 (12.5)	2 (12.5)	—	—
Headache	10 (25)	10 (25)	—	—	2 (12.5)	2 (12.5)	—	—
Psychiatric disorders								
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)	—	—	—
Skin and subcutaneous tissue disorders								
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.

Abbreviations: ≤G2, grade 1 and 2; G3, grade 3; G4, grade 4; NOS, not otherwise specified.

of therapy in 87.5% of cases. Tumor purity was >70% in the majority of samples, with small random variations observed between pre- and posttreatment biopsies (Supplementary Fig. S2). The median RNA integrity number for the hormone-resistant and TNBC RNA samples was 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 5-azacitidine and entinostat (Fig. 2A; Supplementary Fig. S3A). Fold change is calculated as $\log_2(\text{post/pre})$, 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 (Fig. 2A; Supplementary Fig. S3). There were no statistically significant changes in gene expression in the paired biopsies from patients with TNBC, possibly due to the small sample size (Fig. 2B).

To better understand the function of these genes, we queried them through the PANTHER database (pantherdb.org), using Gene Ontology terminology to assign the genes to biological

processes. Thirteen biological processes were identified (Supplementary Fig. S3B) as containing genes that were altered in the posttreatment biopsies as compared with the pretreatment biopsies. Additional studies will be required to determine the significance of these changes to breast tumor biology.

Characterization of the pre- and posttreatment biopsies using GeneFu and the PAM50-intrinsic gene set to classify samples agreed well with clinical subtypes assigned at study entry, with 16 of 19 (84%) paired biopsies receiving the same classification by both methods. Pre- and posttreatment subtypes were in disagreement for only 2 of 19 (11%) cases (Supplementary Table S2). 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 noncoding repetitive elements, was conducted in 15 paired biopsies from patients in the hormone-resistant cohort and

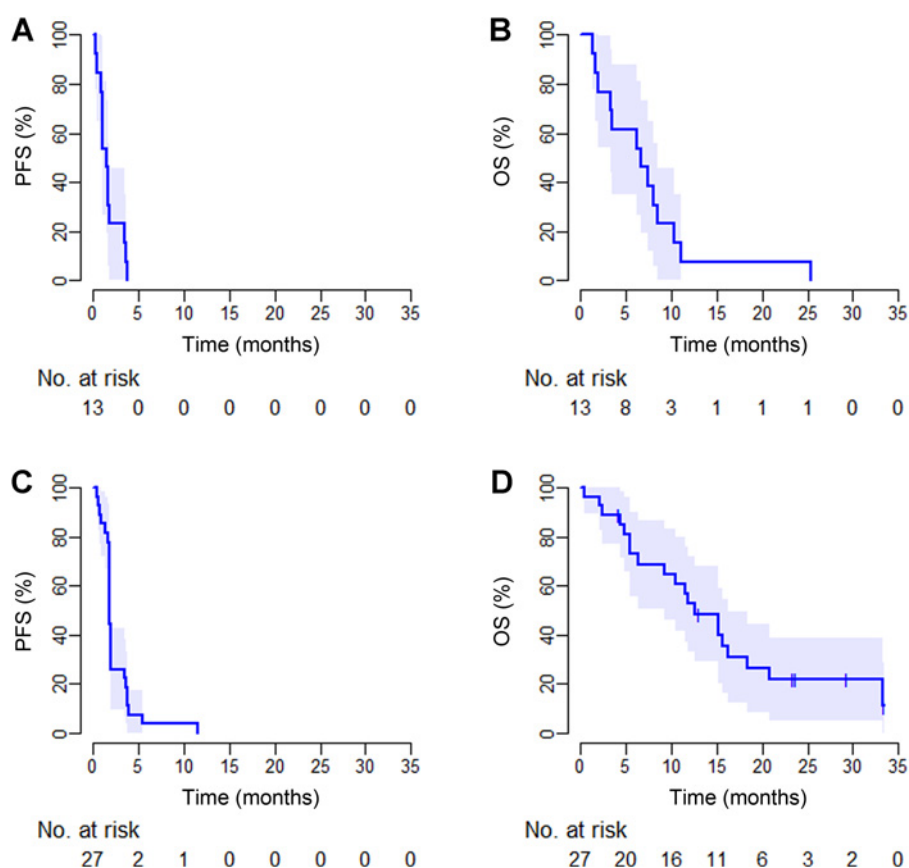


Figure 1. **A–D**, Kaplan-Meier curves for PFS (**A**) and OS (**B**) with the combination of 5-azacitidine and entinostat in women with advanced TNBC; PFS (**C**) and OS (**D**) with the combination of 5-azacitidine and entinostat in women with advanced hormone-resistant breast cancer. The 95% confidence limits are shaded; tick marks indicate censored data.

5 from patients in TNBC cohort. Because 5-azacitidine 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 $\beta_{pre} - \beta_{post} \geq 0.20$. Global percent demethylation was calculated as the percentage of CpG sites meeting this criterion. Widespread decreases in methylation were observed in some posttreatment biopsies at 8 weeks and 6 months posttreatment (Fig. 2C; Supplementary Fig. S4A and S4B) 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 ($n = 3$) with those who survived <10 months ($n = 10$; Fig. 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 S3 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 S3).

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 ($n = 12$; Fig. 1D). Paired biopsies were available for 3 of the 6 patients living >20 months

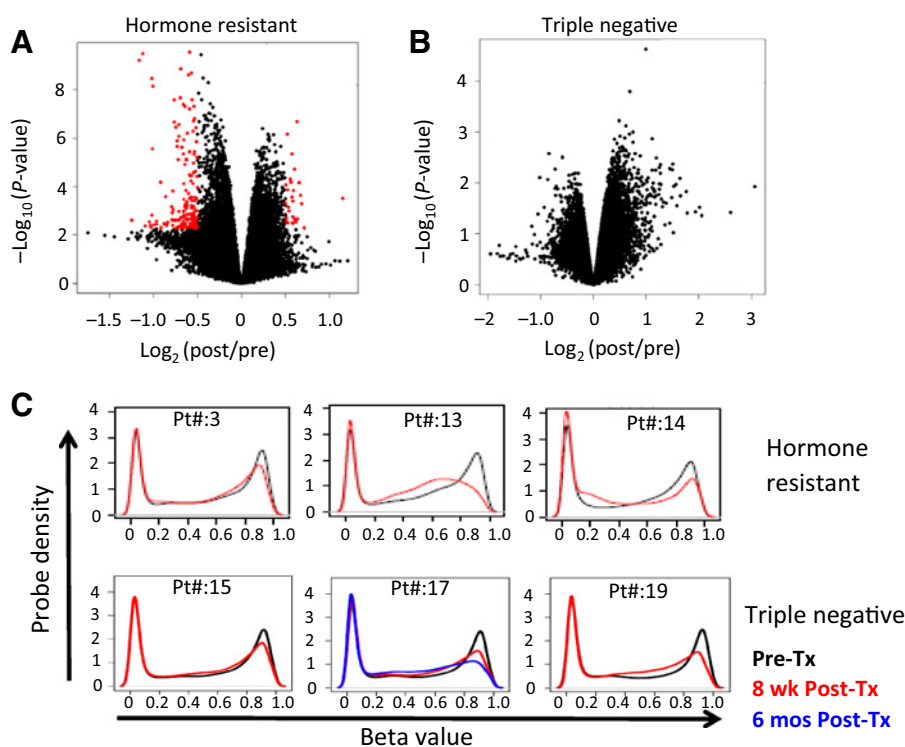
(Pt #3, 13, and 14) and showed median percent demethylation of 11.5% (range, 2.7%–22.2%). Paired biopsies were available for 12 of the 21 patients living <20 months and also showed lower median percent demethylation of 0.85% (range, 0%–10.2%).

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 (HR = 0.83; 95% CI, 0.71–0.98) when comparing samples with global percent demethylation above the median with 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, (HR = 0.92; 95% CI, 0.83–1.01) and also percent CpG island demethylation (HR = 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 (Fig. 3A). Change in ER gene expression was noted in the posttreatment biopsies from the hormone-resistant cohort (6/14 $\geq 0.5 \log_2$ fold change and 2/14 $\leq -0.5 \log_2$ fold change), but this was not observed for the TNBC cohort (Fig. 3B). Interestingly, paired biopsies for 2 patients (7.4%) in the hormone-resistant cohort who were on trial for more than 6 months demonstrated an increase in ER expression after epigenetic therapy, but no significant changes were observed in ER CpG island DNA methylation status (Supplementary Fig. S5, identified as ">6 months"). Increases in ER expression could also be due to changes in protein

Figure 2.

Changes in genomic expression and methylation analyses in hormone-resistant and triple-negative tumor biopsies. **A and B**, Differential gene expression analysis was conducted on paired (post-/pretreatment) biopsies using limma in R language. The fold change is calculated as $\log_2(\text{post-/pretreatment})$, and significantly altered genes (red dots) are determined using a threshold cutoff of ≥ 0.5 and an 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 upregulated (right side of the plot), and 157 were downregulated (left side of the 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 posttreatment 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.



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 reexpression in the TNBC tumors may be due to the fact that ER promoter was not methylated in the pretreated 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 5-azacitidine 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, 5-azacitidine and entinostat, was well tolerated in patients with advanced HER2⁺ 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 10 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 TTP 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 5-azacitidine 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 (Fig. 3). Either upregulation or downregulation of ER was noted in approximately half of posttreatment biopsies from the hormone-resistant cohort, which did not correlate with the variability in CDA expression, 5-azacitidine, or entinostat exposure. One could speculate how either ER upregulation followed by antiestrogen therapy, or ER downregulation in an estrogen driven tumor, may yield antitumorigenic, therapeutic results. Indeed, we observed that 2 patients in the hormone-resistant cohort who enrolled in the optional continuation phase and remained on therapy for 8 to 11 months had increases in ER expression in their 8-week posttreatment biopsy when compared with 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⁺ tumors than in basal-like tumors, which are usually TNBCs (9, 10).

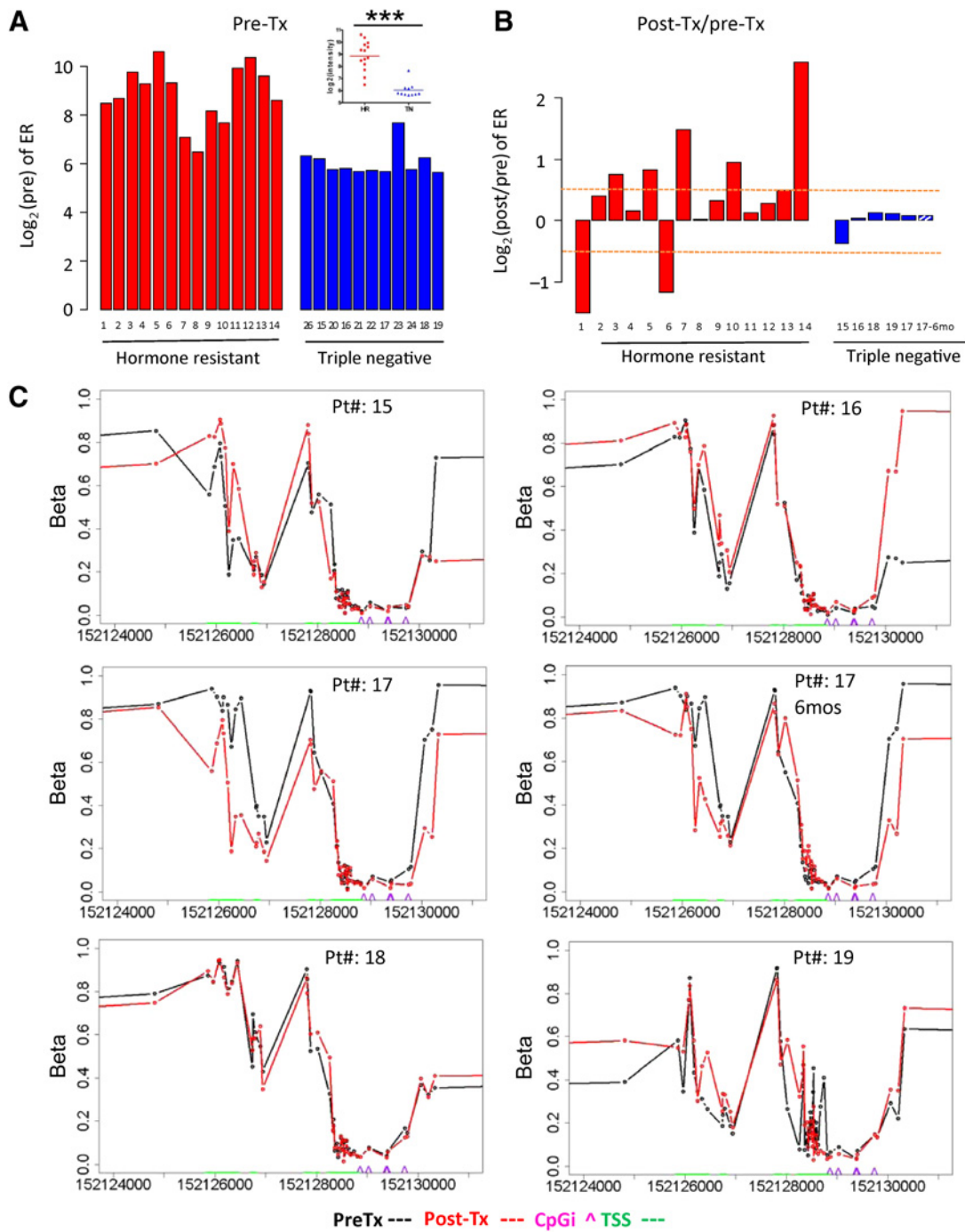


Figure 3.

ER gene expression and methylation changes in pre- and posttreatment biopsies. **A**, ER gene expression in pretreatment hormone-resistant biopsies (red bars) in comparison with ER expression in the pretreatment triple-negative biopsies (blue bars). Student *t* test confirmed the significant difference in basal ER gene expression between HR and triple-negative cohort ($P < 0.0001$, see inset graph). **B**, Significantly altered fold changes in ER expression were observed in 7 paired hormone-resistant biopsies [$\text{log}_2(\text{post/pre}) \geq 0.5$ 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.

Interestingly, more downregulation of gene expression than upregulation was observed in samples from the hormone-resistant cohort at 8 weeks posttherapy, 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 reexpressed by epigenetic modifiers (32, 33). Therefore, it may be difficult to observe significant and sustained reexpression of these genes at 8 weeks posttherapy. 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 5-azacitidine 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 correlate 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 (Fig. 2C; Supplementary Fig. S4A and S4B). 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 that 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 reexpress 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 reexpression 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 with 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 nonsteroidal 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, NCT02115282). Results from a study using the combination of 5-azacitidine 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., PD-L1/PD-1; refs. 16, 41). Indeed, we have demonstrated upregulation of a 5-AZA immune gene set after 5-azacitidine 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 postepigenetic 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, nonrandomized 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 whether 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 5-azacitidine (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 reintroduction 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).

Disclosure of Potential Conflicts of Interest

R.C. Jankowitz is a consultant/advisory board member for Adaxis and Biotheranostics. N. Ahuja has ownership interests (including patents) with Cepheid, is a consultant/advisory board member for Ethicon, and reports receiving commercial research grants from Astex. D.J. Weisenberger has ownership interests (including patents) with Epigenomics/USC and is a consultant/advisory board member for Zymo Research Corporation. G. Somlo reports

receiving speakers bureau honoraria from Millennium and is a consultant/advisory board member for AstraZeneca and Pfizer. P.A. Jones is a consultant/advisory board member for Zymo. C.A. Zahnow reports receiving commercial research grants from Janssen. V. Stearns reports receiving commercial research grants from Abbvie, Celgene, Medimmune, Merck, Novartis, Pfizer, and Puma. R.M. Connolly reports receiving commercial research grants from Novartis, Puma Biotechnology, Merrimack Pharmaceuticals, Clovis Oncology, and Genentech. No potential conflicts of interest were disclosed by the other authors.

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