Published OnlineFirst May 29, 2013; DOI: 10.1158/1078-0432.CCR-13-0033

Cancer Therapy: Clinical

Clinical Cancer Research

Biomarker Modulation following Short-Term Vorinostat in Women with Newly Diagnosed Primary Breast Cancer

Vered Stearns[1], Lisa K. Jacobs[2], MaryJo Fackler[1], Theodore N. Tsangaris[2], Michelle A. Rudek[1], Michaela Higgins[1], Julie Lange[2], Zandra Cheng[5], Shannon A. Slater[1], Stacie C. Jeter[1], Penny Powers[1], Susanne Briest[1], Calvin Chao[7], Carl Yoshizawa[7], Elizabeth Sugar[1,4], Igor Espinoza-Delgado[6], Saraswati Sukumar[1], Edward Gabrielson[3], and Nancy E. Davidson[1]

Abstract

Purpose: Agents that target the epigenome show activity in breast cancer models. In preclinical studies, the histone deacetylase inhibitor vorinostat induces cell-cycle arrest, apoptosis, and differentiation. We evaluated biomarker modulation in breast cancer tissues obtained from women with newly diagnosed invasive disease who received vorinostat and those who did not.

Experimental Design: Tumor specimens were collected from 25 women who received up to 6 doses of oral vorinostat 300 mg twice daily and from 25 untreated controls in a nonrandomized study. Candidate gene expression was analyzed by reverse transcription PCR (RT-PCR) using the Onco*type* DX 21-gene assay, and by immunohistochemistry for Ki-67 and cleaved caspase-3. Matched samples from treated women were analyzed for gene methylation by quantitative multiplex methylation-specific PCR (QM-MSP). Wilcoxon nonparametric tests were used to compare changes in quantitative gene expression levels pre- and post-vorinostat with changes in expression in untreated controls, and changes in gene methylation between pre- and post-vorinostat samples.

Results: Vorinostat was well tolerated and there were no study-related delays in treatment. Compared with untreated controls, there were statistically significant decreases in the expression of proliferation-associated genes Ki-67 ($P = 0.003$), STK15 ($P = 0.005$), and Cyclin B1 ($P = 0.03$) following vorinostat, but not in other genes by the Onco*type* DX assay, or in expression of Ki-67 or cleaved caspase-3 by immunohistochemistry. Changes in methylation were not observed.

Conclusions: Short-term vorinostat administration is associated with a significant decrease in expression of proliferation-associated genes in untreated breast cancers. This demonstration of biologic activity supports investigation of vorinostat in combination with other agents for the management of breast cancer. *Clin Cancer Res; 19(14); 4008–16. ©2013 AACR.*

Introduction

Most women with early breast cancer survive the disease, but others suffer recurrence despite completion of recommended local and systemic therapy. Once metastatic, breast cancer is seldom curable, emphasizing the need for new therapeutic options. Carcinogenesis, including cancer initiation and progression, is a multistep process. Ample evidence shows that, in addition to the role that inherited or sporadic mutations play, epigenetic alterations can lead to abnormal gene expression and subsequent tumorigenesis (1). Epigenetic alterations can include histone modifications such as acetylation or methylation as well as abnormal

Authors' Affiliations: Departments of [1]Oncology, [2]Surgery, and [3]Pathology, Johns Hopkins University School of Medicine; [4]Departments of Epidemiology and Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore; [5]Anne Arundel Medical Center, Annapolis; [6]National Cancer Institute, NIH, Bethesda, Maryland; and [7]Genomic Health, Inc., Redwood City, California

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Current address for M. Higgins: Massachusetts General Hospital Cancer Center, Boston, MA 02114. Phone: 617-726-4920; Fax: 617-643-0589; E-mail: mjhiggins@partners.org; current address for T.N. Tsangaris: Yale School of Medicine, 20 York Street, North Pavilion, First Floor, Suite A, New Haven, CT 06510. Phone: 203-200-2328; Fax: 203-200-2075; E-mail: theodore.tsangaris@yale.edu; current address for S. Briest: Head of the Breast Cancer Center, University of Leipzig, Liebigstr. 20A, Leipzig 04103, Germany. Phone: 49-341-9723943; E-mail: Susanne.Briest@medizin.uni-leipzig.de; current address for Z. Cheng: Center for Breast Wellness, Griffin Hospital, 67 Maple Ave, Derby, CT 06418. Phone: 203-732-7233; E-mail: zcheng@griffinhealth.org; and current address for N.E. Davidson: University of Pittsburgh Cancer Institute and UPMC Cancer Center, 5150 Centre Ave, Suite 500, Pittsburgh, PA 15232. Phone: 412-623-3205; Fax: 412-623-3210; E-mail: davidsonne@upmc.edu

Corresponding Author: Vered Stearns, Bunting-Blaustein Cancer Research Building, 1650 Orleans Street, Room 144, Baltimore, MD 21231. Phone: 443-287-6489; Fax: 410-614-4073; E-mail: vstearn1@jhmi.edu

doi: 10.1158/1078-0432.CCR-13-0033

Published OnlineFirst May 29, 2013; DOI: 10.1158/1078-0432.CCR-13-0033

Translational Relevance

Epigenetic alterations are common in multiple genes in breast cancer and may predict inferior prognosis and response to standard therapies. Agents that modulate epigenetic alterations including demethylating agents and histone deacetylase (HDAC) inhibitors are currently available. Preclinical studies in breast cancer model systems suggest that administration of HDAC inhibitors alone or combined with hormonal agents, cytotoxics, or other biologics is associated with significant antitumor activity. In human breast cancer cell lines, the HDAC inhibitor vorinostat induces growth arrest, resulting in differentiation or apoptosis. Here, we show that short-term oral vorinostat administered to women with primary breast cancer is associated with significant reduction in expression of proliferation-related genes. Our results confirm preclinical and clinical data suggesting that vorinostat may have single-agent activity in breast cancer. Because these effects are modest and likely reversible upon drug discontinuation, agents that target epigenetic alterations should be studied in combination with other drugs.

methylation of DNA in the promoter region of important genes (2). Epigenetic alterations are observed in virtually all breast cancers and may be reversible; their modulation through histone deacetylase (HDAC) or DNA methyltransferase (DNMT) inhibitors, which have been shown to reverse such alterations, has become an attractive area of new drug investigation.

Several classes of HDAC inhibitors have been developed and are currently under investigation or in clinical use. Vorinostat [suberoylanilide hydroxamic acid (SAHA), NSC 701852] is a potent HDAC inhibitor that can be administered orally with excellent bioavailability. Oral vorinostat was available for clinical investigation through the National Cancer Institute's (NCI; Bethesda, MD) Cancer Therapy Evaluation Program (CTEP), and the maximum-tolerated doses identified for further study included 400 mg daily, 200 mg twice a day, an intermittent dose schedule of 300 mg twice a day for 3 days per week, or 250 mg 3 times daily for 14 days followed by 7 days of rest (3, 4). Vorinostat 400 mg daily was approved in 2006 by the United States Food and Drug Administration for the treatment of progressive, persistent, or recurrent cutaneous T-cell lymphoma following 2 systemic therapies.

Several preclinical studies supported a role for HDAC inhibitors and, in particular, vorinostat in breast cancer. In a N-nitrosomethylurea–induced rat mammary tumorigenesis model, vorinostat reduced tumor incidence by 40% (5). *In vitro* studies showed that vorinostat inhibited clonogenic growth of the breast cancer cell lines MCF-7, MDA-231, and MDA-435 by inducing G_1 and G_2–M cell-cycle arrest and subsequent apoptosis within 24 to 72 hours following drug administration (6). Vorinostat-induced apoptosis is reversed by the administration of a caspase inhibitor, suggesting that caspases are involved in vorinostat-induced apoptosis. Importantly, the effects on cell growth and death were not as pronounced in the normal breast epithelial MCF-10 cells or fibroblasts, suggesting a therapeutic window (7). Vorinostat also induced morphologic and other changes consistent with differentiation in tumor cells with different properties, including estrogen receptor (ER)-negative, HER-2/*neu*-amplified, and EGF receptor (EGFR)-amplified cell lines (8). Of note, the effects of vorinostat on the cells were reversible upon drug discontinuation, supporting the need for chronic administration.

Together, these preclinical studies suggested that HDAC inhibitors may have antitumor activity in breast cancer, but the effects of single-agent vorinostat on human breast tumors were unknown. We initiated a prospective clinical study to evaluate the safety, tolerability, and biomarker modulation associated with short-term administration of vorinostat to women with primary breast cancer before definitive breast surgery or other primary treatment. A nonrandomized control cohort was also recruited to understand the variation in biomarker marker expression in the same tumor over time in the absence of any intervention. The results can be used to design future studies of the combination of vorinostat and other standard or novel agents.

Materials and Methods

Patients

Women aged 18 years or older, awaiting definitive surgery or preoperative therapy for a histologically confirmed invasive breast cancer of clinical tumor size 1 cm or more were eligible for the study. Additional inclusion criteria included Eastern Cooperative Oncology Group performance status 0 to 2 and adequate blood counts and organ function, including leukocytes $\geq$3,000/mm^3, absolute neutrophil count $\geq$1,500/mm^3, platelets $\geq$100,000/mm^3, bilirubin within normal limits, aspartate aminotransferase/alanine aminotransferase within 2.5 times the upper limit of normal, and prothrombin time (PT) $\leq$14 seconds. Major exclusion criteria included hormone contraceptive and replacement therapy use within 30 days of diagnostic biopsy (vaginal preparations were allowed), prior or concomitant treatment for the current cancer, history of prior irradiation to the involved breast, uncontrolled intercurrent illness that could limit compliance, pregnancy, or known HIV-positive status due to the potential for drug interaction between vorinostat and antiretroviral therapy. A nonrandomized control cohort of women meeting the same inclusion criteria, who were unwilling to receive vorinostat but willing to donate tissue for the study analyses, was also assembled.

Study design

The study, including all amendments and revisions, was approved by CTEP and by the Johns Hopkins Institutional Review Board. An Investigational New Drug application was filed and held by CTEP. Women enrolled from 2 sites, Johns Hopkins Medical Institutes (Baltimore, MD) and Anne Arundel Medical Center (Annapolis, MD). Informed

Published OnlineFirst May 29, 2013; DOI: 10.1158/1078-0432.CCR-13-0033

consent was obtained from all participants in the vorinostat and control groups. Women in the vorinostat group were scheduled to receive 6 doses of oral vorinostat at 300 mg twice daily, with the last dose administered by study personnel approximately 2 hours before the scheduled breast surgery (or biopsy). No additional systemic therapy was allowed between pre- and posttreatment biopsies. The schedule was selected on the basis of the phase I study in solid malignancies recommending a maximum-tolerated dose of vorinostat 300 mg twice a day for 3 out of 7 days (4). Pre- and post-vorinostat samples were evaluated for candidate biomarkers that may predict response to vorinostat. Women who declined vorinostat, but agreed to donate tissues for biomarker assessment, signed a separate informed consent and were enrolled as controls.

Because vorinostat's dose-limiting toxicities include anorexia, dehydration, diarrhea, and fatigue, patients were instructed to maintain adequate fluid and food intake. Patients who suffered grade 2 or greater dehydration, diarrhea, or anorexia were evaluated by a member of the study staff and treated appropriately. To assess for toxicity, a study team member contacted each treated participant following the second or third anticipated vorinostat dose. Treatment-related adverse events were reported using NCI Common Terminology Criteria for Adverse Events version 3.0.

On the day of tissue collection, study personnel assessed vital signs and toxicities, and collected study blood samples. Laboratory tests collected following the last dose to ensure safety included a complete blood count, chemistry panel, and PT/partial thromboplastin time. The subjects were also seen during the postoperative clinic visit to assess toxicities, obtain vital signs, and recheck any previous abnormal values. Subjects were contacted approximately 30 days following the final dose of vorinostat to monitor for adverse events and to evaluate whether postoperative or other primary treatment complications may have resulted from the study drug.

Biomarker analysis

Paraffin-embedded tissue was obtained from the diagnostic biopsy and from the definitive surgical specimens, and subjected to biomarker analysis. Prespecified markers included expression, methylation, and histone acetylation of candidate genes.

We conducted immunohistochemistry (IHC) for Ki-67 and cleaved caspase-3 using commercially available monoclonal antibodies under the direction of the study pathologist (E. Gabrielson) who was blinded to patient data during analyses. We prepared slides using Target Retrieval Solution S1699 (DAKO) and incubated slides with antibodies to Ki-67 (DAKO; 1:100 dilution) or cleaved caspase 3 (Cell Signaling; 1:200 dilution), followed by incubation with secondary antibody and development using the DAKO LASB/HRP system according to manufacturer's instructions. Normal lymph node tissues were also stained and examined for expected staining patterns as controls (positive and negative) for both antibodies. At least 100 cells were counted from at least 2 separate fields of tumor (200 cells total) for samples to be evaluable. Percent positive cells were calculated by counting and dividing the numbers of apoptotic cells or cells labeling with Ki-67 by the total number of cells scored.

Formalin-fixed paraffin-embedded tumor blocks or slides containing at least 30 μm of tissue were sent to Genomic Health, Inc. for reverse transcription PCR (RT-PCR) analysis. Briefly, RNA was extracted (MasterPure kit, Epicentre-Technologies, Inc.), total RNA content was measured, and the absence of DNA contamination was verified by a quantitative TaqMan PCR assay for β-actin DNA, which includes both positive and negative controls. Gene expression profiling was conducted according to standardized operating procedures for quantitative RT-PCR for the Onco*type* DX® 21-gene assay (9). Reference-normalized expression measurements of 16 individual cancer-related genes are expressed on the $\log_2$ scale and typically range from 2 to 15 where each 1-unit increase reflects approximately a 2-fold increase in RNA (9).

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples (10–30 mL collected in heparinized tubes) and frozen (cell pellet) for future extraction. PBMCs were to be isolated by centrifugion and nuclei from mononuclear cells isolated in lysis buffer, with histones then isolated, as previously described (10). Quantitative multiplex methylation-specific PCR (QM-MSP) was used to evaluate candidate gene methylation. Details of this method and primer sequences have been reported previously (11, 12).

Pharmacokinetics

Blood samples (5 mL collected in serum tubes) for vorinostat concentrations were collected before dosing and on the day of tissue collection. Initially, samples were obtained as a trough (minimal concentration) and later switched to the presumed maximal concentration time (approximately 30 minutes after dose administration). Samples were allowed to clot at 4°C for 20 to 30 minutes, and then centrifuged at 2,000 × *g* for 15 minutes at 4°C. The resulting serum was transferred to polypropylene cryotubes and stored at −70°C until analyzed for vorinostat concentrations over the range of 3 to 1,000 ng/mL with a validated liquid chromatography/tandem mass spectrometry assay (13).

Statistical analysis

The primary study objectives were to evaluate the safety and tolerability of 3 days of oral vorinostat 300 mg twice a day in women with primary breast cancer before definitive breast surgery or other primary treatment, and to evaluate baseline and change in proliferation and apoptosis by IHC in pre- and posttreatment tumor specimens in women who received vorinostat compared with untreated controls. Exploratory objectives included baseline and change in gene methylation silencing and expression of candidate genes in vorinostat-treated women.

Wilcoxon signed rank tests were used to compare pre- and posttreatment pharmacodynamic values within each group, and Wilcoxon rank-sum tests were used to compare the differences (post–pre) between groups. Exact 2-sided *P* values were calculated. Because this analysis is primarily

Published OnlineFirst May 29, 2013; DOI: 10.1158/1078-0432.CCR-13-0033

exploratory, adjustments for multiple comparisons were not made. Pharmacokinetic parameters were summarized using descriptive statistics. Vorinostat concentrations were correlated with the percentage change in each biomarker using the Pearson correlation coefficient. A priori level of significance was $P < 0.05$.

For each methylated gene

$$\%M = \frac{\text{Methylated copies}}{\text{Unmethylated} + \text{methylated copies}}[100];$$

the cumulative methylation index (CMI) was reported as the sum of all %M for all genes. To compare CMI between pre- and posttreatment samples, Wilcoxon signed rank tests (2-tailed) were conducted using GraphPad Prism version 5.04 for Windows, GraphPad Software, www.graphpad.com.

Results

Patient characteristics

From March 2006 to October 2008, 25 women enrolled in the study and received at least one dose of vorinostat, and 29 additional women enrolled as untreated controls (Fig. 1). Of the women receiving vorinostat, 22 received all 6 doses, and 3 women received 1, 4, and 5 doses, respectively (Fig. 1). Twenty-four vorinostat-treated women underwent surgery or a biopsy as scheduled, and surgery was delayed for one patient for reasons unrelated to the study. Patient characteristics are summarized in Table 1. All 29 enrolled controls proceeded to surgery as planned; however, matched tissues from diagnostic biopsy and definitive surgical specimen were available for biomarker studies from 25 women who are included in the analysis. Four additional samples were not released for research purposes due to a final tumor pathologic size less than 1 cm.

Safety and tolerability

Oral vorinostat was well tolerated. Grade 1 nonhematologic toxicities included diarrhea (28%), fatigue (16%), taste changes (16%), anorexia (12%), nausea (16%), and headaches (4%). Grade 1 leukopenia was seen in 24% of the patients but there were no significant changes in hemoglobin, platelet count, electrolytes, renal and liver function laboratory tests, or coagulation factors. All surgical procedures were conducted without study-related delays, change in surgical plans, or complications. Likewise, 2 women were able to initiate recommended preoperative chemotherapy without delays.

Biomarker analysis

In the vorinostat-treated group, the last dose was administered by a study team member before tissue collection in 23 women. Of those, subsequent tissue collection time was not recorded in one case. The average time to tissue sample collection was 4.2 hours (1.2–7.9 hours). One additional woman had a non study-related delay in surgery where no tissue was collected, and another had discontinued the study drug following the first dose and the collection was delayed (time to surgery 62.2 hours).

Matched tumor tissue from baseline and surgery was successfully collected for biomarker analysis from 24 treated women and 25 controls and subjected to IHC and RT-PCR analysis. However, not all matched specimens were evaluable for prespecified marker analysis as described below. Only 2 women agreed to an optional pre-vorinostat breast tumor biopsy that was placed in RNAlater Solution. Most women declined the optional biopsy due to the required intensity of clinical and study procedures in the presurgical period, whereas others declined due to concerns of the associated discomfort for a nonclinically-indicated biopsy.

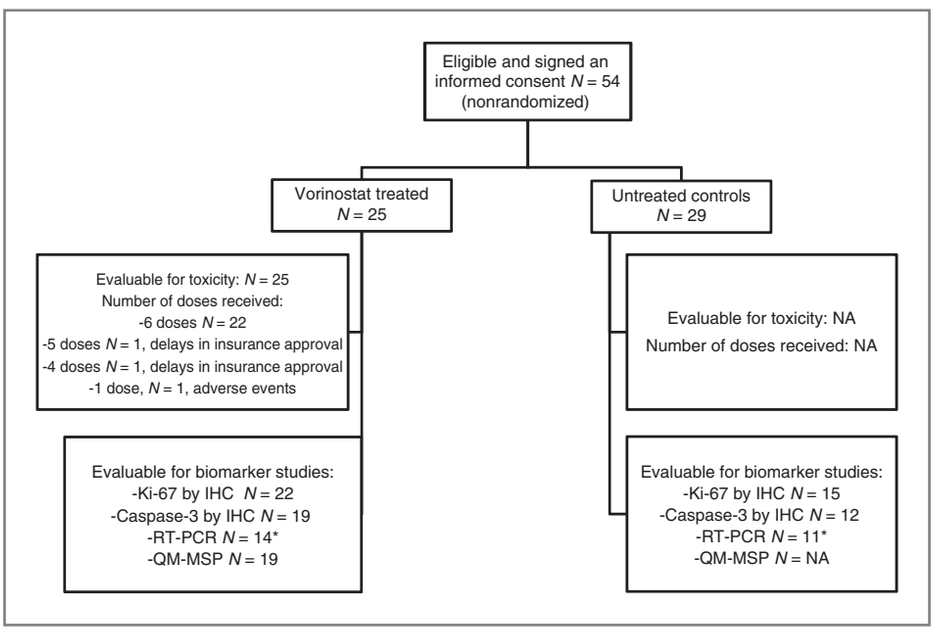

Figure 1. Study enrollment and samples evaluable for biomarker analysis. *, demographics comparable with the entire group. N, number; NA, not applicable.

Published OnlineFirst May 29, 2013; DOI: 10.1158/1078-0432.CCR-13-0033

Table 1. Patient characteristics

Characteristic	Vorinostat ($N = 25$)	Controls ($N = 25$)[a]
Median age (range)	55 (34–71)	52 (34–79)
Median tumor size (range)[b]	2 cm (1.1–5.3)	1.9 cm (1.0–4.8)
Tumor size[b]		
0–2	13 (52%)	13 (52%)
>2–5	11 (44%)	12 (48%)
>5	1 (4%)	0 (0)
Nodal status		
Node-negative	11 (44%)	14 (56%)
Node-positive	14 (56%)	11 (44%)
ER/PR Status		
ER^+/PR^+	14 (56%)	15 (60%)
ER^+/PR^-	6 (24%)	0 (0%)
ER^-/PR^-	5 (20%)	10 (40%)
HER2 Status		
$HER2^+$	3 (12%)	4 (16%)
$HER2^-$	21 (84%)	20 (80%)
Unknown	1 (4%)	1 (4%)
Triple negative		
Yes	4 (16%)	7 (28%)
No	20 (80%)	17 (68%)
Unknown	1 (4%)	1 (4%)
Type of specimen collected		
Lumpectomy	17 (68%)	13 (52%)
Mastectomy	5 (20%)	12 (48%)
Core biopsy	2 (8%)	0 (0)
None	1 (4%)	0 (0)

[a]Twenty-nine women enrolled but only 25 had evaluable matched samples.

[b]Pathologic tumor size was recorded in 23 women, and clinical measurements on 2 women undergoing neoadjuvant chemotherapy.

Matched samples for Ki-67 by IHC were available from 22 (92%) treated and from 15 (60%) controls. There was no change in Ki-67 by IHC compared with baseline in the treated or untreated patients, and there were no differences between groups in the changes from baseline ($P = 0.42$; Fig. 2A). Likewise, there were no differences in cleaved caspase-3 by IHC within or between groups ($P = 0.50$; Fig. 2B); however, matched samples from only 19 (71%) treated and 12 (48%) controls were evaluable for analysis of the marker. Fewer samples were evaluable for the caspase-3 analysis because of a low total number of cells, which did not allow for a reliable reading.

Candidate gene mRNA expression was assessed by the Onco*type* DX assay. Only 14 (58%) and 11 (44%) paired samples from treated and controls, respectively, were available for the Onco*type* DX assay. The samples evaluable for this assay were from participants with similar characteristics to the entire cohort (data not shown). The smaller proportion of evaluable matched samples for the assay was primarily due to the requirement for several consecutive slides, which were often not available from the initial core biopsy. Despite the small sample size, we observed statistically significant greater reductions in the mRNA expression of the proliferation-associated genes, Ki-67 (Fig. 3A; $P = 0.003$), STK15 (Fig. 3B; $P = 0.005$), and cyclin B1 (Fig. 3D; $P = 0.03$), in the samples from vorinostat-treated women as compared with samples from untreated women. Directionally consistent patterns were observed in the other 2 proliferation-associated genes included in the assay (Fig. 3C; MYBL2; $P = 0.20$; Fig. 3E; survivin; $P = 0.11$). Overall, a statistically significant larger reduction was observed in the proliferation axis, which includes the 5 proliferation-associated genes (Fig. 3F; $P = 0.01$), in tissues derived from vorinostat-treated women when compared with control women.

For 25 evaluable matched sample sets for both IHC and RT-PCR, 76% had ER-positive tumors by both assays (100% concordance). There was no statistically significant difference in ER expression between the treated and

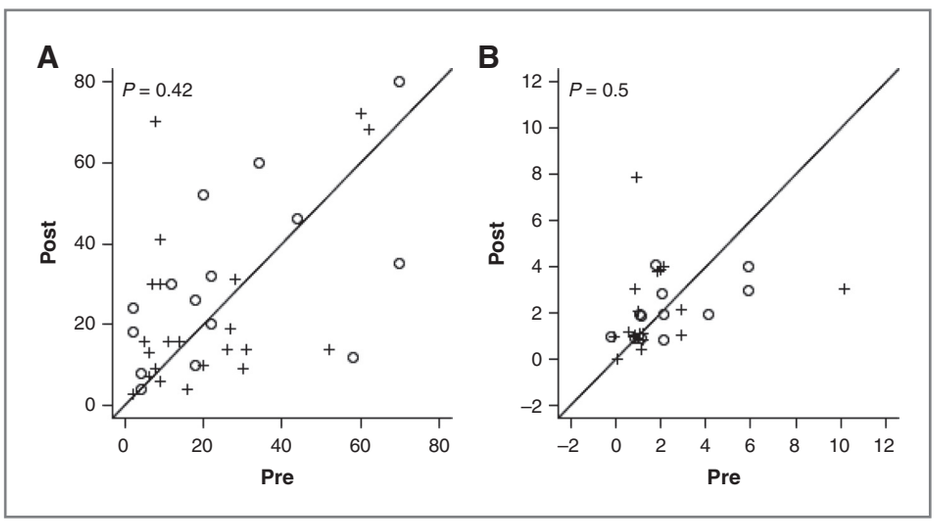

Figure 2. Pre- and post-vorinostat values of gene expression by IHC. Ki-67 (A) and cleaved caspase-3 (B) in vorinostat-treated women (+) versus control (○). Exact 2-sided *P* values are from the 2-sample Wilcoxon rank-sum test.

Published OnlineFirst May 29, 2013; DOI: 10.1158/1078-0432.CCR-13-0033

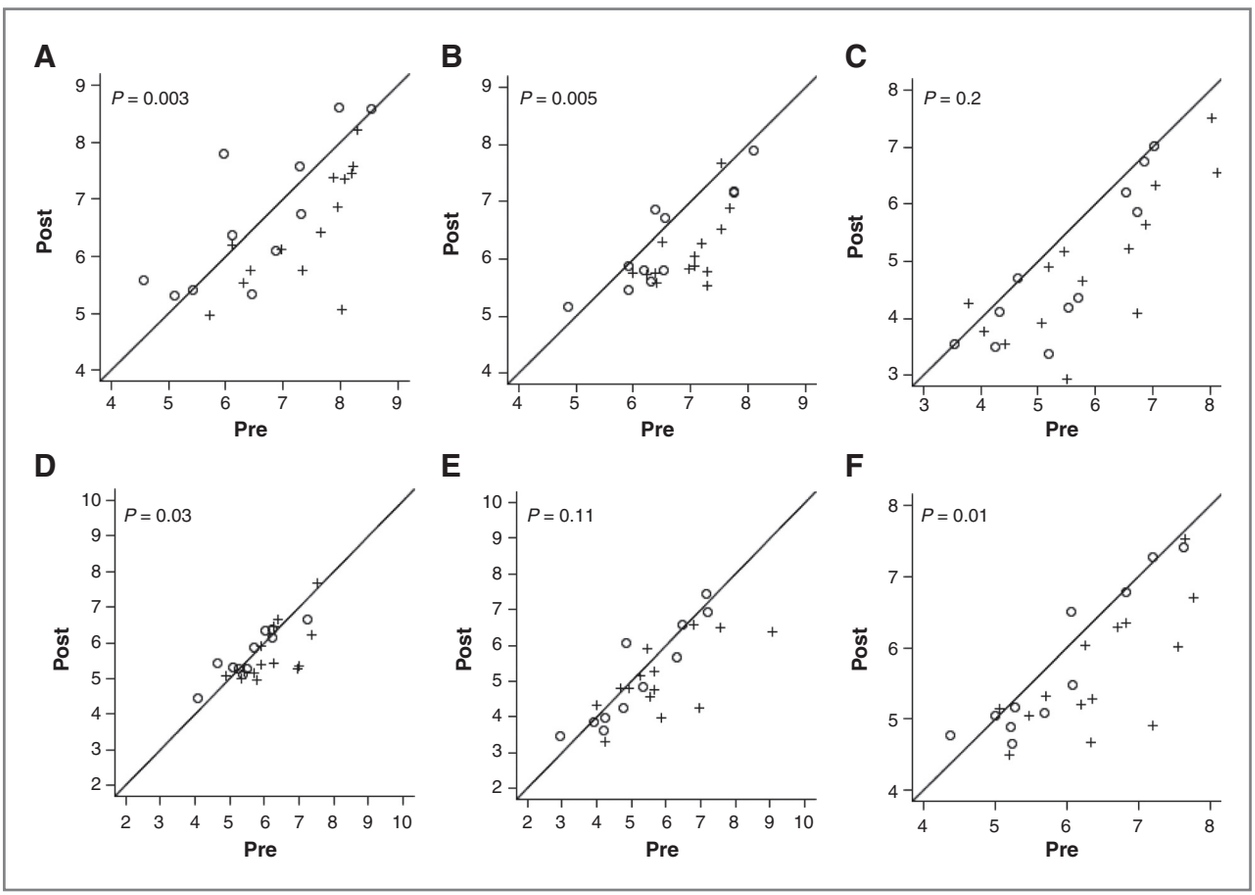

Figure 3. Pre- and post-vorinostat values of proliferation-associated gene expression using the Onco*type* DX 21-gene assay. Vorinostat-treated women (+, $N = 14$) versus control (○, $N = 11$) for Ki-67 (A), STK15 (B), MYBL2 (C), cyclin B1 (D), survivin (E), and the proliferation axis (F). Values are on a $\log_2$ scale. Exact 2-sided *P* values are based upon the 2-sample Wilcoxon rank-sum test.

untreated groups (Fig. 4A). The differences in expression of progesterone receptor (PR) that were observed (Fig. 4B) were due to an increased expression in the untreated group, which may be due to chance, and is also reflected in the change in ER-axis, which includes ER and PR (Fig. 4C). There were no consistent changes among the pairs for genes in the Invasion or HER-2 groups included in the Onco*type* DX assay (Supplementary Fig. S1).

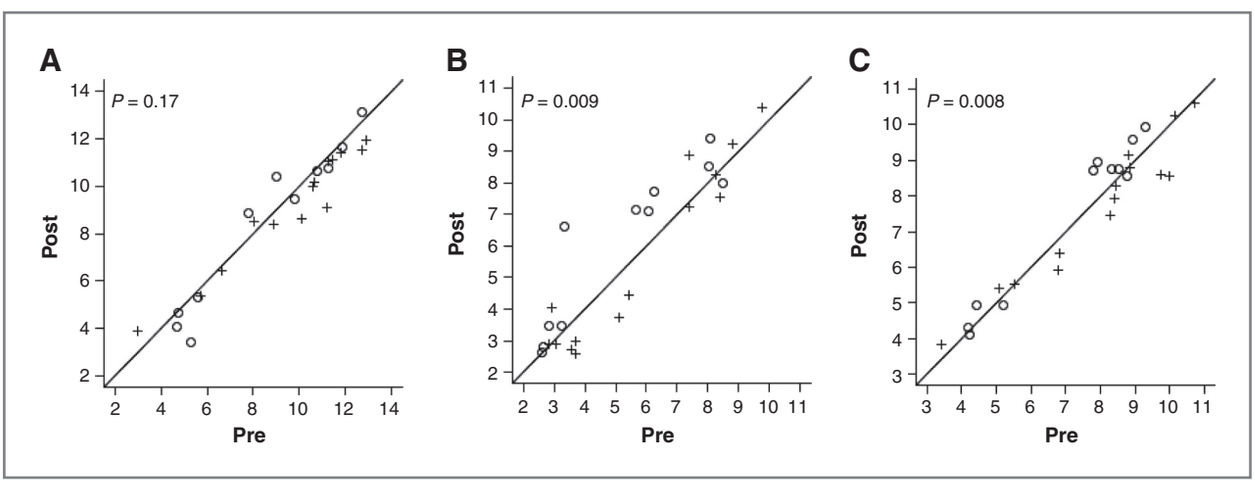

Figure 4. Pre- and post-vorinostat values of ER-related gene expression using the Onco*type* DX 21-gene assay. Vorinostat-treated women (+, $N = 14$) versus control (○, $N = 11$) for ER (A), PR (B), and the ER-axis (C). Values are on a $\log_2$ scale. Exact 2-sided *P* values are from the 2-sample Wilcoxon rank-sum test.

Published OnlineFirst May 29, 2013; DOI: 10.1158/1078-0432.CCR-13-0033

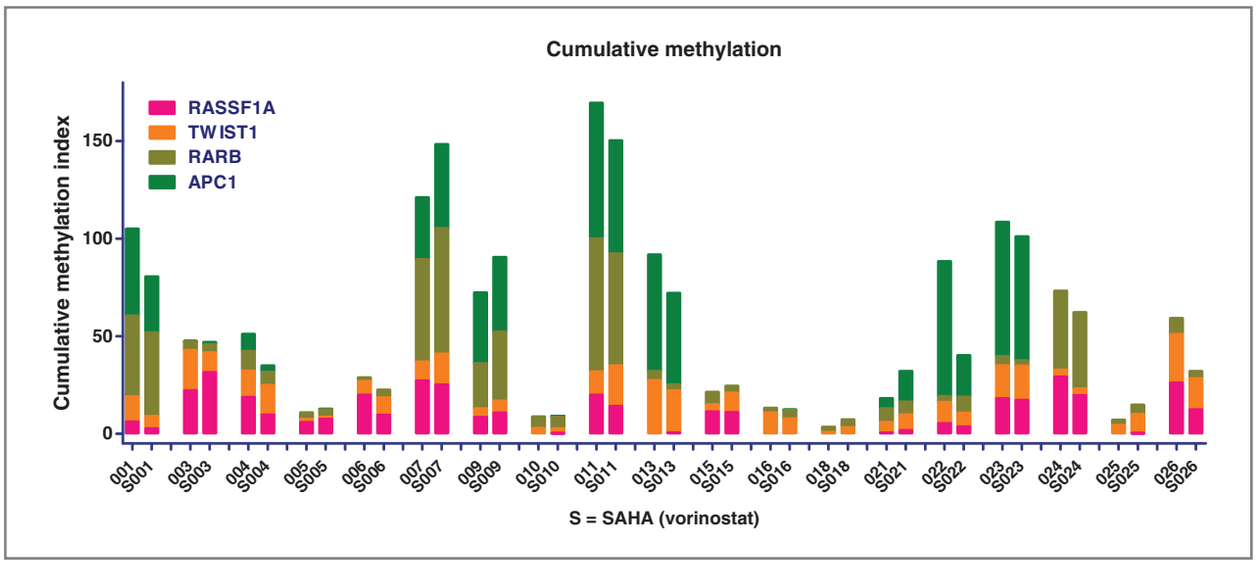

Figure 5. Baseline and change in CMI in vorinostat-treated women ($N = 19$, $P = 0.24$).

Changes in methylation of candidate genes as assessed by QM-MSP were compared in 19 evaluable matched pre- and post-vorinostat samples. Pre- and posttreatment samples were associated with very similar methylation profiles (Fig. 5). As hypothesized, no change in CMI was seen ($P = 0.24$; Wilcoxon signed rank test) after vorinostat treatment. No association was observed between vorinostat concentrations and biomarker expression ($P > 0.05$). Although PBMC were isolated from blood samples and cell pellets frozen, we were unfortunately not able to successfully dissolve the pellet in lysis buffer, and the samples were not subjected to histone acetylation analysis.

Pharmacokinetics

Samples for trough vorinostat concentration were drawn immediately before the last dose in the first 13 patients. Vorinostat trough concentrations were 71.7 ± 90.7 ng/mL in 7 of the 13 patients, and below the limits of quantification in the rest of the patients. Therefore, the sampling schema was altered to capture the maximal exposure (C_{max}) in the remaining 11 patients (i.e., samples drawn 30–60 minutes following the last dose of vorinostat). Vorinostat C_{max} was detectable in all patients (234.0 ± 180.1 ng/mL).

Discussion

Breast cancer is a consequence of the accumulation of multiple genetic and epigenetic alterations. We hypothesized that epigenetic modifications, which unlike genetic alterations, are potentially reversible and represent valid clinical targets that can be exploited in combination strategies. To set the foundation for such combination studies, we conducted a "window of opportunity" study of preoperative vorinostat to provide evidence for single-agent activity. In this study, short-term vorinostat was safe, adverse events were modest, and were not associated with delays in surgical plans or outcomes. Although only 44% to 92% of samples were available for biomarker analysis, we observed a significant reduction in expression of proliferation-associated genes using the RT-PCR assay, suggesting that vorinostat is biologically active.

The main strengths of our study include the enrollment of women who have not received prior treatment for their current cancer and the collection of all samples from treated individuals at a uniform time, 2 hours following administration of an oral agent with a short half-life, though the collection time may not have been optimal to capture the maximal pharmacodynamic effect or account for intraindividual differences in vorinostat pharmacokinetics. Another important strength of our trial is the inclusion of a parallel untreated control group to allow evaluation of biomarker expression from the same tumor at 2 different times.

The main limitation of the trial is the unexpectedly low proportion of matched evaluable samples available for the biomarkers studied. The goal of obtaining usable matched specimens from 80% or more of participants was not achieved for all prespecified biomarkers. Our results suggest that women with tumors 1.5 to 2 cm or more may be better candidates for window of opportunity studies, and our recent studies have been amended accordingly. As has been recommended by others, a randomized placebo-controlled trial should be considered when conducting window trials, although the design is often unappealing for patients (14). It is possible that we would have had a higher proportion of evaluable control samples should the study have been randomized. We also recommend a close collaboration between members of the multidisciplinary team including oncologists, surgeons, and pathologists to allow optimal presentation of the study objectives and plan.

In addition, the large number of parameters evaluated increases the potential for false discoveries. We selected biomarkers based on our preclinical studies and the breast

Published OnlineFirst May 29, 2013; DOI: 10.1158/1078-0432.CCR-13-0033

cancer clinical trial field to guide selection of endpoints (e.g., Ki-67). Nonetheless the biomarker analyses should be regarded as exploratory and will be used to design studies of combinations of vorinostat with other agents.

Compared with controls, samples from vorinostat-treated women showed a significant reduction in proliferation-related genes despite the short treatment duration. Thus we achieved our goal of showing evidence of biologic activity with an HDAC inhibitor in newly diagnosed untreated breast cancers. Although we have not observed a reduction in Ki-67 by IHC, previous studies have shown poor correlation between IHC and RT-PCR determination of the marker (15). There were no significant changes in expression of other candidate genes in the Onco*type* DX assay including ER or HER-2 although the number of patients with ER-negative or HER-2–positive tumors was low because many of these women with these tumor characteristics presented with stage II-III cancer and received preoperative chemotherapy. It is possible that novel agents may also be tested in this setting, but because effective systemic treatments are available and required, it is difficult to conduct a long-term study of a novel drug administered as a single agent.

Other potential confounding factors may be that the dose or schedule chosen was insufficient to achieve adequate exposure. The selected candidate markers may not have been optimal. For example, others use a 400 mg daily vorinostat dose for 2 or 3 consecutive weeks. *In vitro* studies have shown that HDAC inhibitors may lead to either up- or downregulation of ER, depending on the tumor subtype, and downregulation of EGFR or HER-2. Because the antitumor effects are likely reversible upon drug discontinuation, our goal was not to develop the drug for single-agent administration but to confirm and define its effects on breast tumors to help design future studies in which vorinostat is administered in combination with other drugs.

Our laboratory data suggest that HDAC inhibitors do not result in demethylation of methylated gene promoters of candidate genes, even genes like ER whose mRNA expression is reinduced in ER-negative human breast cancer cell lines (16). We therefore hypothesized that HDAC inhibition will not be associated with reversal of gene methylation in candidate genes in human tumors. Our results show concordance of candidate gene promoter methylation between the pre- and post-vorinostat samples, yet lack of reversal of methylation with vorinostat exposure, which may be due to either biologic reality or inadequate vorinostat exposure. Indeed, the vorinostat exposure observed in the current study ranged from trough concentrations that were undetectable to 72 ng/mL (~0.3 μmol/L) and C_{max} values of 234 ng/mL (~0.9 μmol/L). These concentrations were lower than the 1.25 to 3 μmol/L typically used preclinically to achieve gene reexpression (7). In the context of this limitation, we did not observe correlations between vorinostat concentrations and changes in gene expression or other biomarkers.

Substantial preclinical data show the activity of vorinostat or other HDAC inhibitors with endocrine interventions, cytotoxic agents, anti-HER2 agents, or novel agents including other epigenetic modifiers and have been reviewed recently (17). Importantly, both the efficacy of HDAC inhibitors and reexpression of candidate genes are especially enhanced when combined with other epigenetic modifiers such as DNMT inhibitors (5-azacytidine or decitabine; refs. 19, 20). Clinical trials are already underway based on these preclinical data from several labs including our own. In a traditional phase II trial in women with refractory metastatic breast cancer, administration of oral vorinostat 200 mg twice a day for 14 days of each 21 day cycle was not associated with complete or partial responses in the 14 patients who enrolled in the first stage of the study, leading to trial closure. However, 4 patients (29%) had stable disease with times to progression of 4, 8, 9, and 14 months (21). Munster and colleagues completed a phase II trial of vorinostat and tamoxifen in women with ER-positive metastatic breast cancer who have progressed on prior endocrine therapy. The investigators reported an objective response rate of 19% and a clinical benefit rate of 40%, including in patients who received prior tamoxifen (22). We used our preclinical and clinical results to initiate a study of daily vorinostat 400 mg and tamoxifen 20 mg for 14 days in the preoperative window setting. Two women with ER-positive breast cancer enrolled in the study and received the combination without significant side effects or surgical delays (data not shown) before difficulty in drug supply and poor accrual led to early trial closure. In an ongoing trial, we are evaluating the combination of entinostat and azacitidine in women with hormone-resistant or triple-negative breast cancer; women who develop progressive disease on this regimen may enroll in an optional continuation cohort where either tamoxifen or an aromatase inhibitor is added to the epigenetic therapy to further test the possibility of epigenetic sensitization to other treatments (23).

In summary, short-term administration of the oral HDAC inhibitor vorinostat to women with early-stage breast cancer is well tolerated and associated with reduction in mRNA expression of proliferation index genes in primary breast cancer tissue. This observation in human tissues recapitulates results from extensive preclinical studies. This successful translation of preclinical data from lab to clinic supports ongoing efforts to move promising combination therapies such as HDAC inhibitor plus a demethylating agent or HDAC inhibitor plus chemotherapy agents into clinical investigation in the hope that such approaches may enhance cell killing, improve response rates, and improve long-term outcomes.

Disclosure of Potential Conflicts of Interest

V. Stearns has commercial research grants from Merck, Novartis, Pfizer, Abraxis, and Abbott. S. Sukumar is a consultant/advisory board member of CBCRF. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: V. Stearns, C. Chao, E. Sugar, I. Espinoza-Delgado, S. Sukumar, N.E. Davidson

Development of methodology: V. Stearns, L.K. Jacobs, M.J. Fackler, S. Sukumar, N.E. Davidson

Published OnlineFirst May 29, 2013; DOI: 10.1158/1078-0432.CCR-13-0033

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Stearns, L.K. Jacobs, M.J. Fackler, T.N. Tsangaris, M.J. Higgins, J.R. Lange, Z. Cheng, S.C. Jeter, P. Powers, S. Briest, S. Sukumar, E. Gabrielson, N.E. Davidson
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Stearns, M.J. Fackler, M.A. Rudek, M.J. Higgins, C. Chao, C. Yoshizawa, E. Sugar, I. Espinoza-Delgado, S. Sukumar, E. Gabrielson, N.E. Davidson
Writing, review, and/or revision of the manuscript: V. Stearns, L.K. Jacobs, T.N. Tsangaris, M.A. Rudek, M.J. Higgins, J.R. Lange, Z. Cheng, S. C. Jeter, C. Chao, C. Yoshizawa, E. Sugar, I. Espinoza-Delgado, S. Sukumar, N.E. Davidson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Stearns, M.J. Fackler, S.A. Slater, S.C. Jeter, C. Chao
Study supervision: V. Stearns, S. Sukumar

Acknowledgments

The authors thank Drs. Pedram Argani, Ting Bao, Michael Carducci, Lorraine Tafra, Stanley Watkins, Ms. Nina Kouprina, Ms. Lillie Shockney, and the staff of the Avon Breast Center for help in study design and conduct, Dr. Steven Shak for collaboration and Roberto Bugarini for biostatistical support, Diana Cherbavaz for scientific support in Genomic Health's development laboratory, and the late Dr. Merrill Egorin for providing analytical support for vorinostat concentrations.

Grant Support

This work was supported by the CTEP, NIH U01 CA 70095 (to V. Stearns VS and M.A. Rudek) and SAIC (P6914 to V. Stearns), P30CA47904 (to M. Egorin) and U01-CA099168 (to M. Egorin), American Society of Clinical Oncology Advanced Clinical Research Award (to V. Stearns), Young Investigator Award (to M.J. Higgins), Specialized Program of Research Excellence in Breast Cancer (P50 CA88843 to S. Sukumar and N.E. Davidson), and by the Avon Foundation. Vorinostat for the vorinostat and tamoxifen study was supplied by Merck. The Onco*type* DX 21-gene assays were conducted by Genomic Health, Inc.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 4, 2013; revised April 12, 2013; accepted May 5, 2013; published OnlineFirst May 29, 2013.

References

1. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042–54.
2. Stearns V, Zhou Q, Davidson NE. Epigenetic regulation as a new target for breast cancer therapy. Cancer Invest 2007;25:659–65.
3. Kelly WK, Richon VM, O'Connor O, Curley T, MacGregor-Curtelli B, Tong W, et al. Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid administered intravenously. Clin Cancer Res 2003;9(10 Pt 1):3578–88.
4. Kelly WK, O'Connor OA, Krug LM, Chiao JH, Heaney M, Curley T, et al. Phase I study of an oral histone deacetylase inhibitor, suberoylanilide hydroxamic acid, in patients with advanced cancer. J Clin Oncol 2005;23:3923–31.
5. Cohen LA, Marks PA, Rifkind RA, Amin S, Desai D, Pittman B, et al. Suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, suppresses the growth of carcinogen-induced mammary tumors. Anticancer Res 2002;22:1497–504.
6. Huang L, Pardee AB. Suberoylanilide hydroxamic acid as a potential therapeutic agent for human breast cancer treatment. Mol Med 2000;6:849–66.
7. Munster PN, Troso-Sandoval T, Rosen N, Rifkind R, Marks PA, Richon VM. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. Cancer Res 2001;61:8492–7.
8. Zhou Q, Chaerkady R, Shaw PG, Kensler TW, Pandey A, Davidson NE. Screening for therapeutic targets of vorinostat by SILAC-based proteomic analysis in human breast cancer cells. Proteomics 2010;10: 1029–39.
9. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med 2004;351:2817–26.
10. Yoshida M, Kijima M, Akita M, Beppu T. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J Biol Chem 1990;265:17174–9.
11. Fackler MJ, McVeigh M, Mehrotra J, Blum MA, Lange J, Lapides A, et al. Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. Cancer Res 2004;64:4442–52.
12. Swift-Scanlan T, Blackford A, Argani P, Sukumar S, Fackler MJ. Two-color quantitative multiplex methylation-specific PCR. Biotechniques 2006;40:210–9.
13. Parise RA, Holleran JL, Beumer JH, Ramalingam S, Egorin MJ. A liquid chromatography-electrospray ionization tandem mass spectrometric assay for quantitation of the histone deacetylase inhibitor, vorinostat (suberoylanilide hydroxamicacid, SAHA), and its metabolites in human serum. J Chromatogr B Analyt Technol Biomed Life Sci 2006;840: 108–15.
14. Ratain MJ. Bar the windows but open the door to randomization. J Clin Oncol 2010;28:3104–6.
15. Cobleigh MA, Tabesh B, Bitterman P, Baker J, Cronin M, Liu ML, et al. Tumor gene expression and prognosis in breast cancer patients with 10 or more positive lymph nodes. Clin Cancer Res 2005;11(24 Pt 1): 8623–31.
16. Sharma D, Saxena NK, Davidson NE, Vertino PM. Restoration of tamoxifen sensitivity in estrogen receptor-negative breast cancer cells: tamoxifen-bound reactivated ER recruits distinctive corepressor complexes. Cancer Res 2006;66:6370–8.
17. Connolly R, Stearns V. Epigenetics as a therapeutic target in breast cancer. J Mammary Gland Biol Neoplasia 2012;17:191–204.
18. Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F. Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 2003;63:7291–300.
19. Zhu WG, Otterson GA. The interaction of histone deacetylase inhibitors and DNA methyltransferase inhibitors in the treatment of human cancer cells. Curr Med Chem Anticancer Agents 2003;3: 187–99.
20. Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG, Davidson NE. Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. Cancer Res 2001; 61:7025–9.
21. Luu TH, Morgan RJ, Leong L, Lim D, McNamara M, Portnow J, et al. A phase II trial of vorinostat (suberoylanilide hydroxamic acid) in metastatic breast cancer: a California Cancer Consortium study. Clin Cancer Res 2008;14:7138–42.
22. Munster PN, Thurn KT, Thomas S, Raha P, Lacevic M, Miller A, et al. A phase II study of the histone deacetylase inhibitor vorinostat combined with tamoxifen for the treatment of patients with hormone therapy-resistant breast cancer. Br J Cancer 2011;104: 1828–35.
23. Connolly RM, Jankowitz RC, Andreopoulou E, Allred JB, Jeter SC, Zorzi J, et al. A phase 2 study investigating the safety, efficacy and surrogate biomarkers of response of 5-azacitidine (5-AZA) and entinostat (MS-275) in patients with advanced breast cancer. Cancer Res 2011;7:Supplement 3, OT-01-6: A.

Published OnlineFirst May 29, 2013; DOI: 10.1158/1078-0432.CCR-13-0033

Clinical Cancer Research

Biomarker Modulation following Short-Term Vorinostat in Women with Newly Diagnosed Primary Breast Cancer

Vered Stearns, Lisa K. Jacobs, MaryJo Fackler, et al.

Clin Cancer Res 2013;19:4008-4016. Published OnlineFirst May 29, 2013.