HHS Public Access

Author manuscript

Pharmacogenomics J. Author manuscript; available in PMC 2015 June 01.

Published in final edited form as:

Pharmacogenomics J. 2013 February ; 13(1): 94–104. doi:10.1038/tpj.2011.48.

A genomic approach to predict synergistic combinations for breast cancer treatment

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Abstract

We leverage genomic and biochemical data to identify synergistic drug regimens for breast cancer. In order to study the mechanism of the histone deacetylase (HDAC) inhibitors valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA) in breast cancer, we generated and validated genomic profiles of drug response using a series of breast cancer cell lines sensitive to each drug. These genomic profiles were then used to model drug response in human breast tumors and show significant correlation between VPA and SAHA response profiles in multiple breast tumor datasets, highlighting their similar mechanism of action. The genes deregulated by VPA and SAHA converge on the cell cycle pathway (Bayes Factor 5.21, and 5.94, respectively, p-value $10^{-8.6}$ and 10^{-9}, respectively). In particular, VPA and SAHA upregulate key cyclin-dependent kinase (CDK) inhibitors. In two independent datasets, cancer cells treated with CDK inhibitors have similar gene expression profile changes to the cellular response to HDAC inhibitors. Together, these results led us to hypothesize that VPA and SAHA may interact synergistically with CDK inhibitors such as PD-033299. Experiments show that HDAC and CDK inhibitors have statistically significant synergy in both breast cancer cell lines and primary 3-dimensional cultures of cells from pleural effusions of patients. Therefore, synergistic relationships between HDAC and CDK inhibitors may provide an effective combinatorial regimen for breast cancer. Importantly, these studies provide an example of how genomic analysis of drug response profiles can be used to design rational drug combinations for cancer treatment.

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Conflict of Interest: The authors have no financial conflicts of interest to declare.

Author Contributions: AHB and RS designed the study. AHB, ALC, and RS wrote the manuscript. RS and LC performed the in vitro experiments. ALC and YS performed the microarray analysis, profile generation, and profile validation. ALC and AHB performed the gene ontology analysis. ALC, PM, and AHB performed the data analysis, statistical analysis, and synergy calculations.

Keywords

Pharmacogenomics; histone deacetylase inhibitors; cyclin-dependent kinase inhibitors; drug synergy; breast cancer

Introduction

Most clinical trials apply single-agent and combinatorial regimens to unselected patients in a random manner, diluting the ability to find successful treatment approaches. This indiscriminate approach has failed to identify curative regimens for many breast cancer patients. In fact, approximately 17% of women with regional breast cancer and 74% of women with metastatic breast cancer will die from their disease within 5 years [1]. Advances using therapies targeted at deregulated pathways have had some successes, but the ability to systematically assess the sensitivity of individual cancers to effective drugs remains to be refined. As with chemotherapy, it is highly likely that combinations of targeted therapies will be critical for effective treatment of breast cancer.[2] Furthermore, as more and more potent single-agent inhibitors are developed, the question becomes how to find useful combinations without resorting to large mechanism-blind clinical trials.

One class of drugs that we do not know appropriate combination regimens for is the histone deacetylase (HDAC) inhibitors. Epigenetic modifications affect a wide range of biological processes and play key roles in development and tumorigenesis [3, 4]. Among the key chromatin modifying enzymes that affect epigenetic states and gene transcription are the histone deacetylases (HDACs). HDACs have been shown to impact tumor development and progression [5–8]. Overexpression of HDACs have been found in several cancers, including breast, colon, and prostate cancer [9–12]. Drugs that target HDACs have been used in clinical trials for multiple types of solid tumors with some success [13, 14]. We used gene expression profiling to explore the mechanism of action of HDAC inhibitors in order to rationally combine appropriate therapies.

The effects of HDAC inhibitors include induction of differentiation, arrest in cell cycle in G1 and/or G2, and induction of apoptosis [15, 16]. Cell cycle arrest at G1/S boundary can be associated with the induction of members of the CIP/KIP family of CDKs inhibitors, such as CDKN1A (p21, WAF/CIP1) and CDKN1C (p57, KIP2). Induction of CDK inhibitors results in p53-independent hypophosphorylation of the tumor suppressor retinoblastoma gene product, the phosphorylation of which is required for the progression from G1 to S phase in the cell cycle [17, 18]. In vitro experiments with cell lines have shown that treatment with HDAC inhibitors can increase CDK inhibitor expression, including CDKN1C[18–21]. In breast cancer, tumors do not typically express CDKN1C due to promoter hypermethylation and histone deacetylation [22–25]. Importantly, low expression of CDKN1C is associated with poor clinical outcome, and the reintroduction of CDKN1C expression in vitro results in suppression of cell transformation, suggesting that CDKN1C may act as a tumor suppressor in breast cancer[26, 27].

Our overarching goal is to use genomics to rationally identify optimal combination regimens for cancer. In principle, two drugs that produce similar effects can be synergistic when used

concurrently[28]. We generate gene expression profiles of drug response to VPA and SAHA, two HDAC inhibitors. In order to capture the diversity of breast cancer, we developed profiles using panels of breast cancer cell lines of various phenotypes that are sensitive to each specific drug profiled. Examination of gene expression changes in response to HDAC inhibitors highlight the critical role of cell cycle regulated genes. These results led to the hypothesis that CDK inhibitors would synergize with HDAC inhibitors in the treatment of breast cancer. We demonstrate synergy between HDAC inhibitors and CDK inhibitors in breast cancer cell lines and primary patient tumors grown in 3-dimensional culture. Together, these results highlight a novel therapeutic combination rationally designed by genomic analysis.

Methods

Cell cultures and drugs

Breast cancer cell lines HCC1143, HCC38, HCC1806, BT483, BT549, MDA-MB-435, MDA-MB-453, SKBR3, and T47D were maintained in culture in RPMI (SIGMA) containing 2 mM L-Glutamine (Invitrogen), 10 mM HEPES (Invitrogen), 1 mM Sodium Pyruvate (Invitrogen), 4.5 g/L glucose (Invitrogen), and 10 % fetal bovine serum (Sigma). BT474, MDA-MB-361, CAMAI, MCF7, and Hs578t breast cancer cell lines were maintained in culture in DMEM (SIGMA) containing 2 mM L-Glutamine (Invitrogen), 1 mM NEAA (Invitrogen), 1 mM Sodium Pyruvate (Invitrogen), 4.5 g/L glucose (Invitrogen), and 10 % fetal bovine serum (Sigma). Valproic Acid (VPA) (SIGMA) was dissolved in water at stock concentration of 500 mM; suberoylanilide hydroxamic acid (SAHA) (Cayman Chemical) was resuspended in DMSO at stock concentration of 20 mM; PD-0332991 (Selleck) was dissolved in DMSO at stock concentration of 10 mM.

Generation of drug response profiles for HDAC inhibitors

Breast cancer cell lines sensitive to HDAC inhibitors as determined by their EC50 (HCC38, BT483, BT549, BT474, MDA-MB-361, MDA-MB-435s, MDA-MB-453, SKBR3, ZR75, CAMAI, MCF7, Hs578t, T47D) representing the major breast cancer phenotypes (ER−/PR −/ErbB2−; ER+/ PR+/ ErbB2 +; ER−/PR−/ ErbB2+) were seeded in 100 mm plates (NUNC) in media containing 5% fetal bovine serum, at 75% density. After 24 h, VPA or SAHA were added at the final concentration of 5 mM VPA and 1.5 μM SAHA. After 6 h the cells were harvested and subjected to RNA purification by RNeasy Kit (Qiagen), and 1 μg of each sample was later hybridized to Affymetrix U133 microarrays according to the manufacturer's protocol. To verify VPA and SAHA efficacy on HDACs activity, a portion of each sample was subjected to histone purification (Active Motif) and resolved by 15% SDS-PAGE. Western blots were hybridized with anti-acetyl-histone H4 (Millipore) antibody to evaluate the histone acetylation status, and the proteins visualized with the ECL Plus Western Blot Detection system (GE Healthcare) (data not shown).

Microarray data were analyzed as described in the supplemental methods. In brief, microarray data were MAS 5.0 normalized, scaled by log base 2, and then quantile normalized. Distance weighted discrimination was used to eliminate differences between the training cell lines and datasets. A Bayesian analysis binary regression model was then used

to derive the profiles.. To enable complete reproduction of our results, the input files, output files and the binary regression program used in this study are available at: http://io.genetics.utah.edu/files/Pharmacogenomics_synergy

Western blot analysis of CDKN1C expression and activation following HDAC inhibitor treatment

Breast cancer cells were seeded in 100 mm plates in media containing 5% fetal bovine serum, at 75% density. After 24h, 5 mM VPA or 1.5 uM SAHA were added to the media. After 24 and 48h the cells were harvested and lysed in lysis buffer (50 mM TRIS (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) containing 0.1 mM sodium orthovanadate, 2 mM phenylmethanesulfonyl fluoride(PMSF), and 100μM protease inhibitors cocktail (Sigma). After centrifugation at 14000 rpm for 20 minutes at 4°C, the protein yield was quantified by Bradford assay (Pierce), and equivalent amounts of protein were resolved on a 10% SDS-PAGE gels and transferred to nitrocellulose. Total CDKN1C and CDKN1A expression was detected by primary antibody to CDKN1C (Cell Signaling), and mouse p21(Amersham).. GAPDH (AbCam) was used as loading control. Western blots were developed with the ECL Plus Western Blot Detection system.

Statistical analysis

Gene Ontology calculations were done using http://gather.genome.duke.edu and werw limited to genes significantly deregulated following drug treatment (p value<=0.001 by paired t-test). Bayes factors (the ratio of the posterior probability of associating a particular Gene Ontology category and the given gene list to the probability in a random gene list) and p-values were calculated. In all figures, error bars represent 95% confidence intervals unless otherwise noted. Bliss Interaction Index for dose d1 of drug A and dose d2of drug B was calculated as:

$$I=\frac{f_{d1,A+d2,B}}{f_{d1,A}+f_{d2,B}-f_{d1,A}\times f_{d2,B}}$$

where $f_{d1,A}$ is the percent inhibition in cell number when treated with drug A at dose d1 and $f_{d2,B}$ is the percent inhibition in cell number when treated with drug B at dose d2. Two drugs are synergistic if the Bliss Interaction Index is greater than 1 and antagonistic if it is less than 1. Each combination was tested in triplicate for 2-D cultures and duplicate for 3-D cultures, and variability of Bliss Interaction Index was confirmed by 2-way ANOVA. For those cell lines with 2-way ANOVA p-values < 0.05, Bonferroni post-testing was performed to compare each dose combination with a triplicate of 1 by a two-sided t-test with alpha=0.05. The p-value for the dose combination with the Bliss Interaction Index furthest from 1 is reported. Graphs and calculations were generated in MATLAB 2009 and Graphpad Prism v4.02.

Analysis of CDK inhibition

Gene expression microarray CEL files were downloaded from GEO GSE18552 [29] and GSE15396 [30]. The GSE18552 dataset contains gene expression data from an ovarian cancer

cell line (A2780) and a breast cancer cell line (MCF-7) before and after treatment with a variety of CDK inhibitors (CDK-125, CDK-887, and CDK 509). The GSE15396 dataset contains gene expression data from peripheral blood mononuclear cells, a prostate cancer cell line (DU145), and a colon cancer cell line (HCT116) before and after treatment with the CDK inhibitor R547 or DMSO. The CEL files were mas5 normalized, scaled by log base 2, and quantile normalized.

Analysis of synergistic effect of HDAC/CDK inhibitors

Breast cancer cell lines were seeded in 384 well plates (NUNC) in media (DMEM or RPMI) containing 5% fetal bovine serum, at a density to yield 80% confluence in control treated wells at t=96 hours post-treatment (as determined by growth curves) in either triplicate or quadruplicate. After 24 h, VPA, SAHA, and PD-0332991 were added singularly or in combination. The indicated doses were chosen within the average linear range of drug effect: VPA (64 mM to 0.25 mM), SAHA (3 μM to 0.05 μM), and PD-0332991 (100 μM to 0.05 μM). A BIOMEK 3000 (Beckamn Coulter) robot was used to seed the cells and dispense the drugs. After 96 h the cells were harvested and CellTiter-Blue Reagent (Promega) was added to test cell viability. After 2 h of incubation at 37°C, the fluorescence was recorded (560(20)Ex/590(10)Em) using a Victor3V 1420 Multilabel Counter (PerkinElmer) plate reader. The Bliss formula was used to calculate synergy (detailed below).

Breast cancer pleural effusion collection, growth in 3D cultures, and synergy assays

After obtaining informed consent, pleural effusion samples were collected by thoracentesis from breast cancer patients at the University of Utah. Three samples from breast reduction were also collected at the University of Utah as cancer-free controls. Samples were grown in a 24 Well Ultra Low Attachment tissue culture plate (Corning inc.) for 24 hours at 37°C, in 5% CO2, with MEGM in order to form organoid structures. Cells density was determined with a hemacytometer (Bright-line). Cells were diluted in media and added to BD MatrigelR Matrix, Growth Factor Reduced (BD Biosciences) for total volume of 80% matrix to media. 30μL of the cocktail was seeded into a 96 well half area assay plate (Corning inc.) at a density of 8 $x10^3$ organoids/well. After 24h, the drugs were added at a range of dosages determined by our drug response assay: valproic acid (4 mM and 1 mM), SAHA (0.3 uM and 1 uM), and PD-0332991 (1.5 uM and 5 uM). After 96 hours CellTiter-BlueR (Promega) was added to test viability. After 4h of incubation at 37°C, the fluorescence was recorded (560(20)Ex/590(10)Em) using a Victor3V 1420 Multilabel Counter (PerkinElmer) plate reader. Synergy was calculated by Bliss formula. The assay was performed in triplicate. At least two wells per experiment were treated with LIVE/DEAD Cell viability assay (Invitrogen) and the images were taken using an Olympus IX81-ZDC DSU with ORCAER camera and Slidebook 5.0 software.

Results

Generation and validation of drug response profiles

To investigate patterns of response to drug treatment in breast cancer samples placed onto microarray, drug response profiles for VPA and SAHA were created using a series of breast

cancer cell lines that were sensitive to each drug. Importantly, our series of breast cancer cell lines represents a diversity of breast cancer phenotypes, including estrogen receptor positive and negative cells and HER2 positive cell lines. While other studies have profiled gene expression changes following treatment with these inhibitors, only a couple of cell lines were used, which may not represent the gene expression changes across different tumor subtypes[31]. Sensitivity for each cell line was determined using dose-response analysis on a panel of breast cancer cell lines listed in the Methods section. Cell lines most sensitive to the drug (Figure 1A, dose-response curves highlighted in red) were then included in the generation of the profile. In order to generate the drug response profiles, we treated sensitive cell lines with an HDAC inhibitor (5 mM VPA or 1.5 μM SAHA) and collected RNA 6 hours later. Microarray analysis was performed, and the top 100 genes correlating to drug response across all cell lines were identified. Heat maps and leave-one-out cross validation curves for each profile are shown in figure 1B. As shown, there are distinct sets of genes that reflect response to HDAC inhibitors in common to the sensitive breast cancer cell lines. Further, leave-one-out cross validation shows the robustness and internal consistency of the drug response profiles. Next, profiles were validated by projecting them into samples from the Connectivity map treated with VPA, a public dataset of prostate cancer cells treated with SAHA (GSE9000), and a public dataset of normal ovarian theca cells treated with VPA (GSE 1615)[31–33]. Results show that the VPA and SAHA response profiles generated above can accurately identify drug treatment in external datasets in which cells are treated with the inhibitors (Figure 1C).

We then projected the profiles into two large independent gene expression datasets of primary breast tumors (GSE6532 and GSE5460, 539 tumors total). Across the 539 breast tumors analyzed for drug response, there is significant correlation between VPA and SAHA (Figure 2A&B). These results highlight the similar mechanisms of action of the two drugs, and their similar response profiles in breast tumors. When breast tumors were sorted by different clinical phenotypes (ER−/+, HER+/−, tumor size, and tumor grade), no significant interaction between HDAC inhibitor response and subtype was found, suggesting that response to HDAC inhibitors is independent of these factors (Supplemental Table 1).

CDK inhibitors are upregulated in breast cancer cells following treatment with HDAC inhibitors

As genes involved in the cell cycle are significantly enriched following HDAC inhibitor treatment, we investigated individual cell cycle specific genes deregulated following response to VPA and SAHA that could be targets for potential synergistic therapeutic regimens. For this analysis, we calculated the average change after VPA or SAHA treatment in the eight breast cancer cell lines used in the HDAC inhibitor profiles for all cyclin-dependent kinases (CDK) or CDK inhibitor genes. Those genes with highest significant change are shown in Figure 3A. In particular, CDKN1C (p57KIP2) was highly upregulated by both drugs. CDKN1C is a member of the CDKN1 family of CDK inhibitors, which, together with CDKN1A (p21CIP1) orchestrates the G0/G1-M phase transition by regulating cyclin/CDK interactions. The HDAC inhibitor-induced over-expression of CDKN1C is particularly interesting since this CDK inhibitor is not normally expressed in human tumors due to promoter hypermethylation and histone deacetylation [22–25]. Other CDK inhibitors

were also upregulated following VPA and SAHA treatment, suggesting a key role for CDK inhibitors in drug response.

To assess whether the upregulation of CDK inhibitor expression seen on microarray corresponded to increased protein levels and activation, we next examined the protein level of CDKN1C and CDKN1A. As shown in Figure 3B, HDAC inhibitor treatment causes increased expression of CDK inhibitor protein levels, with large levels of CDKN1C upregulation. These data suggest that CDK inhibitors may play a crucial role in the HDAC inhibitor-dependent decrease in proliferation observed in breast cancer cells

Correlation of HDAC inhibitor response and CDK inhibition

As our data suggests that VPA and SAHA function, at least in part, by deregulation of CDK-related pathways, we next tested if HDAC inhibitors and drugs targeting CDKs have similar genomic profiles. To assess the relationship between HDAC inhibition and CDK inhibition, we projected the HDAC inhibitor drug response profiles into two datasets of cells treated with various CDK inhibitors. If there are similar mechanisms of action, we expect the HDAC inhibitor signatures to correlate with drug response in CDK inhibitor treated cells. In particular, cancer cells treated with multiple CDK inhibitors (CDK-509, CDK-887, and CDK-125) have gene expression patterns significantly more similar to both VPA and SAHA than untreated controls (Figure 4A). Similarly, in an independent gene expression dataset, cells treated with the CDK inhibitor R547 have gene expression patterns significantly more similar to both VPA and SAHA when the dose is high (IC90 or greater)(Figure 4B). Interestingly, a similar effect was not seen for normal peripheral blood mononuclear cells (data not shown), suggesting that there may be a cancer-specific effect of HDAC inhibitors on CDK inhibition. Together, these results indicate some overlap in the mechanism of action for VPA, SAHA and CDK inhibitors, and suggest these compounds may be synergistic.

To confirm the relationship between HDAC inhibition and CDK inhibition, we examined the genes significantly (p value<0.001) up or down regulated on average after treatment with HDAC inhibitors VPA and SAHA. Genes that were significantly deregulated following VPA or SAHA treatment were then examined for enrichment of pathways using GATHER (Gene Annotation Tool to Help Explain Relationships, www.gather.duke.edu). The top five gene ontologies significantly enriched in the cells treated with VPA/SAHA are given in supplemental table 2. KEGG pathway analysis revealed a significant deregulation of cell cycle related genes for VPA and SAHA (Bayes factor 5.21 and 5.94, respectively, and p value $10^{-8.6}$ and 10^{-9}, respectively). Often, synergy between two drugs occurs when they target the same pathway. Therefore, we hypothesized that drugs targeting CDKs would be ideal candidates for combination with VPA/SAHA.

HDAC inhibitors synergize with a CDK inhibitor in breast cancer cell lines

Our global gene expression analysis strongly suggests that cell cycle regulation and CDK inhibition are important components to cellular response to HDAC inhibitors. From these results, we hypothesized that inhibition of multiple targets in the CDK pathway would synergistically decrease breast cancer cell proliferation. To verify whether the combination of HDAC inhibition and CDK inhibition leads to a synergistic response in breast cancer, we

tested the potency of combination therapy with VPA or SAHA and the CDK inhibitor PD-0332991. PD-0332991 targets the CDK4 and CDK6 containing complexes[34, 35]. For these studies, we chose to use HER2-positive cells (BT474 and MDA-MB-361), ER-positive cells (MCF7 and T47D), and negative for hormone receptors and HER2, i.e., triple-negative, (HCC1806, BT549, HCC1143, HCC38, and MDA-MB-453).[36] All tested cell lines are shown. These cell lines were treated with a range of doses with each drug alone or with a combination CDK inhibitor-HDAC inhibitor regimen.

Additivity occurs when the Bliss Interaction Index equals one, and synergy occurs when the Bliss Interaction Index is greater than one. Using the replicates for each combination of doses and each cell line, we calculated the average Bliss Interaction Index. In order to control for multiple comparisons, we first used 2-way ANOVA to determine if the distribution of Bliss Interaction Index across all doses for each cell line differed significantly additivity, i.e., from a distribution where all the dose combinations had Bliss Interaction Index equal to all ones OneHER2-positive cell line showed signifigant synergy for both SAHA and VPA. Figure 5). Both of the ER-positive cell lines showed significant synergy for VPA but not SAHA. All of the triple-negative cell lines showed significant synergy with VPA, but some did not show significant synergy for SAHA. (Figure 5). Indeed, most cell lines had some drug combination with Bliss Interaction Index greater than 2. The highest synergy was often seen when the VPA dose was ~0.25mM or the SAHA dose was ~0.3mM. These are doses well below the EC50 of these drugs, which is not surprising because showing synergy at doses where most cells are killed is difficult. Importantly, these are clinically achievable serum concentrations of these drugs with few side effects. These results highlight the importance of identifying tumors sensitive to specific drugs and the ability to rationally identify effective synergistic regimens using drug response profiles.

HDAC inhibitors synergize with a CDK inhibitor in patient tumors grown in 3D culture

To further validate the synergistic effect of the two classes of drugs in actual patient tumors, we used short-term, 3-dimensional (3-D) cultures of patient breast tumor samples from pleural effusions. The 3-D culture microenvironment more closely resembles the in vivo cellular microenvironment and provides a more biologically relevant growth setting than standard 2-D microenvironments [37, 38]. Further, as breast cancer cell lines commonly gain additional genetic variation not found in the primary tumors due to long-term growth in culture, testing drug sensitivity in fresh patient samples that have not been grown in culture may be closer to the clinic.

Dose response assays in 3-D growth conditions were performed for normal breast tissue from breast reductions and for tumors representing various subtypes of breast cancer shown. Samples were treated at doses expected to be synergistic based on the 2-D culture assays. All samples tested are shown in Figure 6. Importantly, no synergy was observed in the normal breast samples, showing that this drug regimen specifically targets tumor cells. The average Bliss Interaction Index in the primary tumor samples was 2.2 (CI 1.3–3.2). Synergy was seen in Her2+ samples, triple-negative samples, and the ER-positive sample. Bliss Interaction Index and confidence intervals for the individual samples are shown in Figure 6A. The effect of drug treatment on the tumor organoids was also assessed visually by light

microscopy with fluorescent dyes to identify live (green) and dead cells (red) (Figure 6B). Tumor organoids treated with VPA/PD0332991 or SAHA/PD0332991 exhibited reduced structural integrity and increased cellular scatter with increasing concentrations of the drugs. A marked increase in ethidium bromide uptake was observed concomitantly with the loss of organoid integrity, demonstrating a cytotoxic effect of the drugs on the primary tumor organoids. Again, minimal cytotoxicity was seen in the normal breast samples. Together, these results strongly support the synergistic effect of an HDAC inhibitor-CDK inhibitor regimen on aggressive breast tumors and the ability of using gene expression profiles to identify efficacious regimens.

Discussion

Breast cancer is the most common female cancer, but despite advances in early detection and systemic treatment approximately 40,000 women in the U.S. per year die of breast cancer [39]. Numerous therapeutic approaches have been used with some success in breast cancer to target hormone responsive breast cancers with estrogen antagonists or aromatase inhibitors [40] and to target tumors that overexpress the human epidermal growth factor 2 (HER2) with trastuzumab or lapatinib. Unfortunately, metastatic breast cancer is still an incurable disease, highlighting the need for more effective regimens that selectively interfere with the key cancer cell targets to ensure a more positive clinical outcome.

The recent findings that HDAC inhibitors have anticancer properties in various cancer types have opened new options in the malignancies treatment [41–43]. In clinical trials, VPA has promising activity against breast cancer in combination with anthracyclines or demethylating agents [44–46]. The optimal combinations containing HDAC inhibitors are not known, however. One of the mechanisms behind the anticancer properties of HDAC inhibitors may involve overexpression of cyclin-dependent kinase inhibitors, a large family of proteins that regulates cell cycle progression, proliferation, and differentiation.

Because of the success of gene expression profiles in elucidating other aspects of breast cancer biology, including molecular subtypes [47], we examined gene expression profiles of breast cancer cell lines treated with valproate or SAHA to explore the cellular response to HDAC inhibition. Our analysis shows a marked upregulation of numerous CDK inhibitors in breast tumor cells of diverse phenotypes. The inhibitory effect of the CIP/KIP family affects the kinase activity of pre-activated G1 protein complexes such as cyclin E-CDK2 and cyclinD-CDK4/6. Thus, we hypothesized that dual inhibition of cyclin dependent kinases by epigenetic induction of CDK inhibitor proteins combined with a pharmacologic CDK inhibitor would synergistically affect tumor cell growth. Synergistic combinations of CDK inhibitors with an HDAC inhibitor may facilitate use of these drugs in broader settings, as currently CDK inhibitors have limited use in cancer patients due to relatively high toxicity.

The prospect of HDAC inhibitor/CDK inhibitor combination treatment is extremely attractive if HDAC inhibition can increase cell cycle inhibition and decrease apoptotic threshold, thereby reducing the necessary CDK inhibitor dosage to a more tolerable level. In this context, we analyzed combination therapy of HDAC inhibitors with PD-0332991, a small molecule competitive inhibitor of the ATP-binding site of CDK2, currently in clinical

trials as single agents and combined with other targeted agents. Our findings show that in breast cancer cells HDAC inhibitors and CDK inhibitors synergistically affect cell proliferation. Combinations of HDAC inhibitors and CDK inhibitors produced a striking response observed in primary cells from breast cancer patients grown in 3-D system, suggesting an acceptable therapeutic index. It is possible that the synergistic inhibition of cyclin-CDK complex formation due to overexpression of CDKN1C is sufficient to induce a cytotoxic effect in tumor tissue originally lacking of the enzyme. We do not see cytotoxicity in normal breast tissue treated with HDAC/CDK inhibitors; therefore, it is possible that in healthy tissue, where CDKN1C is normally expressed, the additional expression is not sufficient to affect the regulation of the cell cycle progression.

In conclusion, the data presented in our study support the use of gene expression analysis of drug response to uncover effective combination therapeutic regimens in a rational manner. This approach will be particularly important for drugs such as VPA and SAHA, in which the mechanism of action is not well defined. By investigating the genes central to drug response using global gene expression profiling, we can identify key targets for therapeutic selection of combination therapies that may be synergistic. As an example, we generate profiles of drug response for VPA and SAHA, and identify pathways central to drug response. Specifically, the assessment of genes and pathways affected by VPA and SAHA treatment resulting from the microarray analysis suggests a deregulation of CDK inhibitors, leading to the hypothesis that combinational therapeutic treatment HDAC inhibitors with inhibitors of specific CDK may represent a new therapeutic approach to the treatment of breast cancer. We validate the synergistic effects of this combination therapy regimen in both breast cancer cell lines and primary patient tumors grown in 3-dimensional culture. We conclude that additional clinical studies of HDAC inhibitor/CDK inhibitors are warranted. Importantly, we show that analysis of gene expression profiles can identify synergistic drug combinations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support provided by the National Institute of Health (R01GM085601, AHB); the Pharmaceutical Research and Manufacturers of America (AHB); a Multidisciplinary Cancer Research Training Program award (T32 CA93247, RS and AC), and an award from the MIDT cancer center support grant. The Breast Interdisciplinary Group of the University of Utah is acknowledged for their assistance in breast tumor collection.

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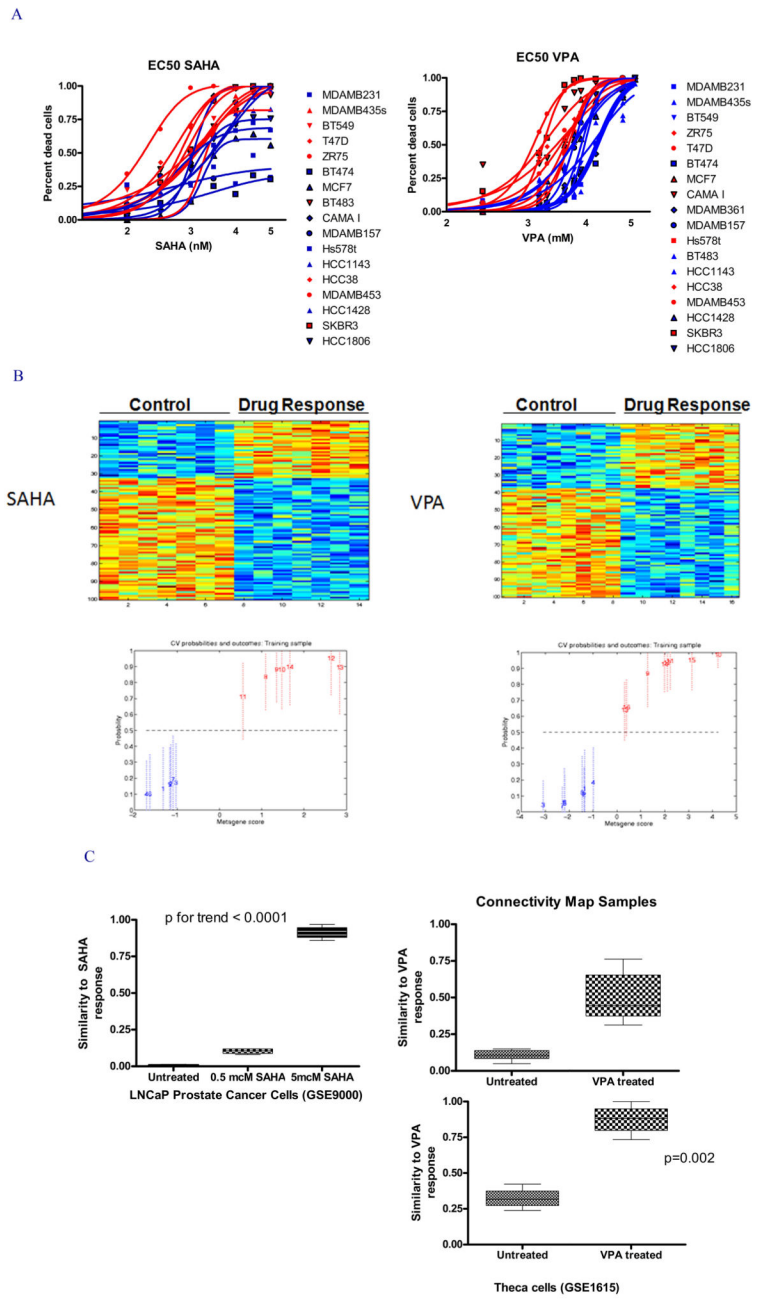

Fig 1. Generation and validation of genomic signatures for VPA and SAHA

(A) Eighteen breast cancer cell lines were tested for sensitivity to HDAC inhibitors VPA and SAHA. Following treatment of cells with a range of doses, the effective concentration at which 50% of cells respond (EC50) is calculated. The cell lines with dose response curves marked in red showed a higher sensitivity to the drugs compared to cell lines insensitive to drugs (marked in blue), and thus were subsequently used to evaluate the gene expression changes resulting upon drug treatment. (B) Heat maps and leave-one-out cross validation curves for each drug response profile are shown. Genomic signatures are comprised of 100 genes significantly deregulated upon response to drug in sensitive cell lines identified above.

Leave-one-out cross validation shows the robustness and internal consistency of the drug response profiles. (C) Validation of predictive accuracy of genomic signatures. Profiles were tested by predicting drug response in samples from three external and independent datasets, including the Connectivity map treated with VPA, a public dataset of prostate cancer cells treated with SAHA (GSE9000), and a public dataset of ovarian theca cells treated with VPA (GSE1615).

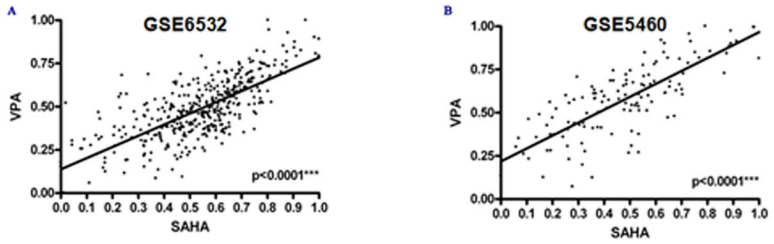

Fig 2. Predicted response to HDAC inhibitors VPA and SAHA show significant correlation in breast tumor datasets

Genomic signatures for VPA and SAHA were used to predict correlation with drug response in breast tumor gene expression datasets. Similarities with drug response are significantly correlated in two large independent gene expression datasets of breast tumors.

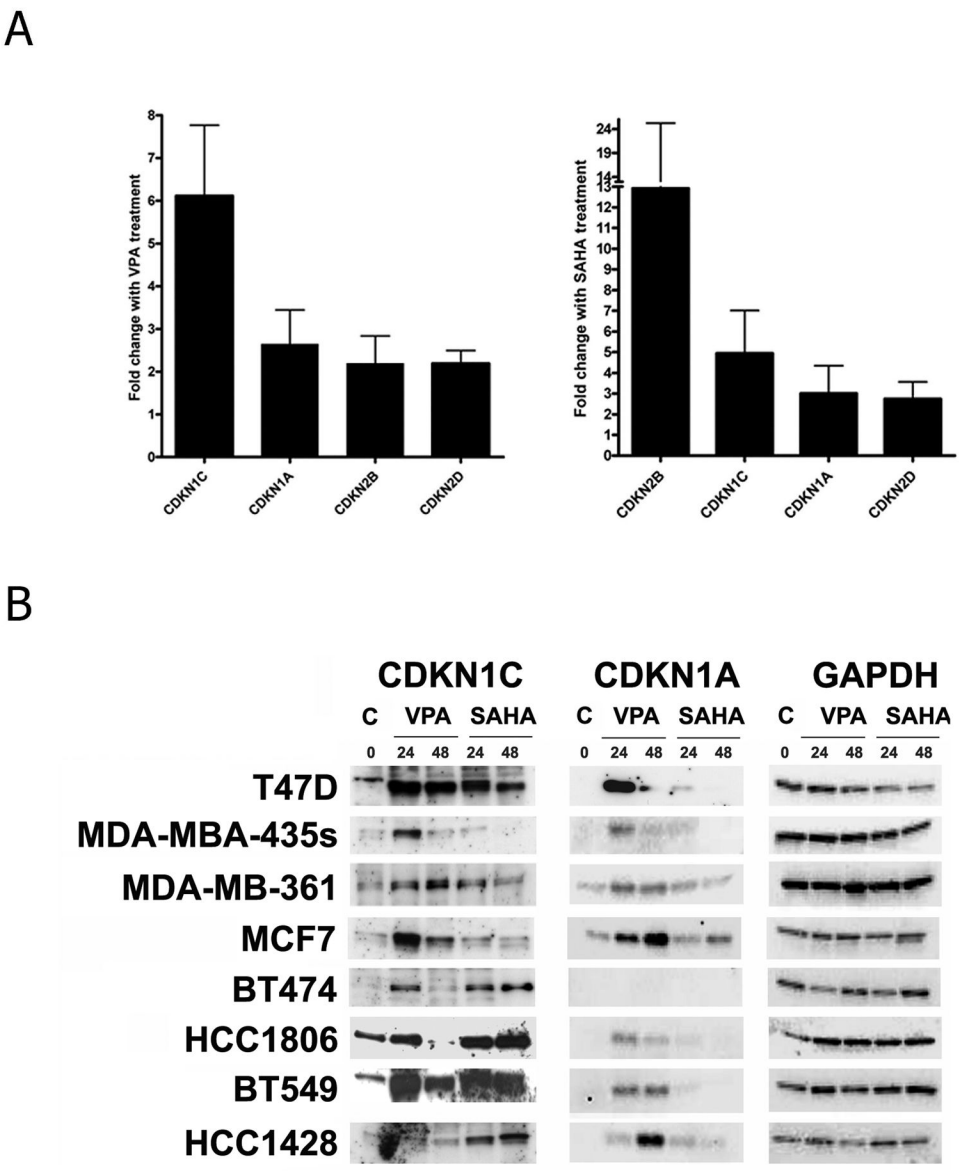

Fig 3. CDK inhibitors are upregulated in breast cancer cells following treatment with HDAC inhibitors

(A) CDK inhibitor genes CDKN1C, CDKN1A, CDKN2B, and CDKN2D were significantly (p value<0.05) deregulated after treatment with HDAC inhibitors VPA and SAHA in the eight breast cancer cell lines used to develop the drug signature. (B) Western blot analysis shows that CDKN1C (p57KIP2) is upregulated by both VPA and SAHA in breast cancer cell lines.

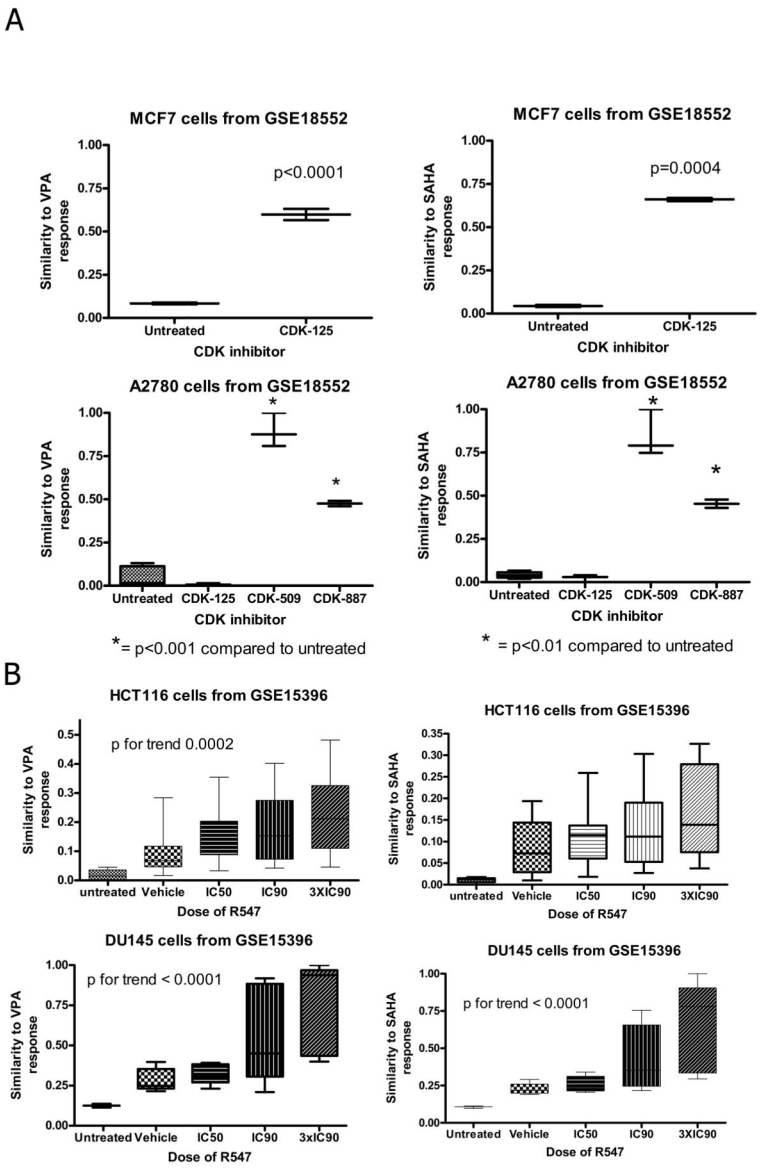

Fig 4. Response to HDAC inhibitors correlates with CDK inhibition

(A) The HDAC inhibitor signatures were projected into a dataset of cancer cell lines treated with CDK-125, also known as PHA-848125, CDK-509, also known as PHA-690509, and CDK-887, also known as PHA-793887. Each point is an individual sample, and the horizontal line indicates the median. (B) The HDAC inhibitor signatures were projected into a dataset of normal cells and cancer cell lines treated with different concentrations of the CDK inhibitor R547.

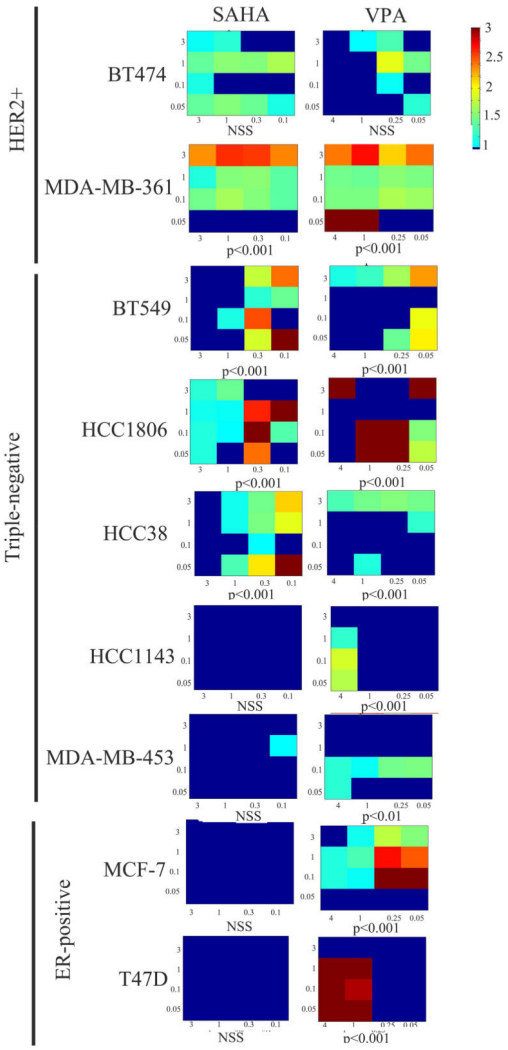

Fig 5. Analysis of synergistic effect of HDAC/CDK inhibitors on breast cancer cell lines

Breast cancer cell lines were tested for synergistic responses to an HDAC inhibitor/CDK inhibitor combination treatment regimen. Four doses of both HDAC inhibitors and the CDK inhibitor PD-0332991 were tested (doses are in mM for VPA and μM for SAHA and PD-0332991) in each cell line, with at least three replicates for each experiment. The average Bliss Interaction Index at all combinations of drugs was calculated and plotted as a heatmap where red represents high synergy and blue represents low or no synergy. The p-value listed are for the dose combination with the most significant t-test compared to additivity. NSS indicates that no significant synergy was found at any dose combination. Complete list of Bliss Interaction Indices and p-values is given in Supplemental Table 3.

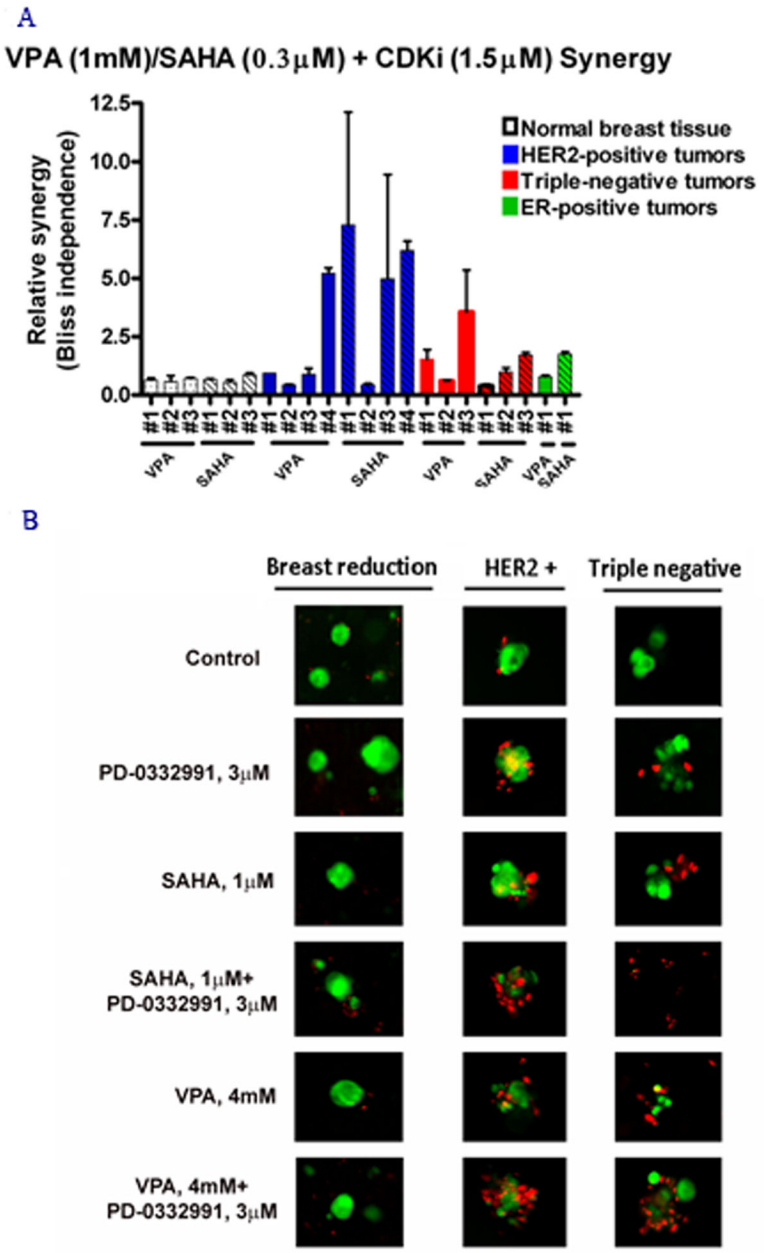

Fig 6. Patient tumor cell sensitivity to combinatorial treatment with HDAC/CDK inhibitors in 3D culture

(A) Control normal breast epithelial cells or cancer cells from the pleural effusion of breast cancer patients were embedded in Matrigel and treated with VPA, SAHA, and PD0332991 alone or in combination. Proliferation was measured, and synergy calculated using the Bliss independence algorithm. Dose response analysis shows higher sensitivity to combination HDAC inhibitor/CDK inhibitor treatment for breast cancer cells versus normal breast reduction cells. Samples for each cell/tumor subtype are labeled sequentially. (B) The effect of each drug or drug combination was assessed by fluorescent dye to identify live (green)

and dead cells (red). Cells from breast reduction patients were used as non-cancer control in the assay. Experiment was performed in triplicate, one representative example is shown.