Differences in Expression of Key DNA Damage Repair Genes after Epigenetic-Induced BRCAness Dictate Synthetic Lethality with PARPi Inhibition

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Abstract

The triple-negative breast cancer (TNBC) subtype represents a cancer that is highly aggressive with poor patient outcome. Current preclinical success has been gained through synthetic lethality, targeting genome instability with PARP inhibition in breast cancer cells that harbor silencing of the homologous recombination (HR) pathway. Histone deacetylase inhibitors (HDACi) are a class of drugs that mediate epigenetic changes in expression of HR pathway genes. Here, we compare the activity of the pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), the class I/IIa HDAC inhibitor valproic acid (VPA), and the HDAC1/2-specific inhibitor romidepsin (ROMI) for their capability to regulate DNA damage repair gene expression and in sensitizing TNBC to PARPi. We found that two of the HDACIs tested, SAHA and ROMI, but not VPA, indeed inhibit HR repair and that RAD51, BARD1, and FANCID2 represent key proteins whose inhibition is required for HDACi-mediated therapy with PARP inhibition in TNBC. We also observed that restoration of BRCA1 function stabilizes the genome compared with mutant BRCA1 that results in enhanced polyploidy population after combination treatment with HDACi and PARPi. Furthermore, we found that overexpression of the key HR protein RAD51 represents a mechanism for this resistance, promoting aberrant repair and the enhanced polyploidy observed. These findings highlight the key components of HR in guiding synthetic lethality with PARP inhibition and support the rationale for utilizing the novel combination of HDACi and PARPi against TNBC in the clinical setting.

Introduction

Triple-negative breast cancer (TNBC) represents an especially aggressive and hard-to-treat subtype. The current first-line clinical TNBC therapies are limited to cytotoxic chemotherapy with or without the antiangiogenic agent bevacizumab (1). However, preclinical success has been gained by synthetic lethality, targeting genome instability with PARP inhibition in breast cancer cells that harbor genetic or epigenetic silencing of the homologous recombination (HR) pathway routinely via the DNA damage repair mediator BRCA1 (2). Histone deacetylase inhibitors (HDACi) are a class of drugs that mediate epigenetic control of gene expression profiles. This occurs via HDACi-mediated genome-wide hyperacetylation of histones, resulting in a reordering of the chromatin structure, increasing accessibility to DNA for either induction or repression of transcriptional complexes (3, 4). Several studies have documented HDACi-mediated repression of DNA repair protein expression and associated HDACi-induced DNA damage in solid tumors, reviewed by Slingerland and colleagues (5).

Structurally different HDACi have been purified from natural sources or synthetically developed, which can be divided into six classes based on their chemical structure; these include short-chain fatty acids (valproic acid, VPA), hydroxamic acids (suberoylanilide hydroxamic acid, SAHA), benzamides, cyclic tetrapeptides (romidepsin, ROMI), electrophilic ketines, and a remaining class of inhibitors with miscellaneous structures. Here, we compare the activity of the pan-HDAC inhibitor (SAHA), the class I and IIa HDAC inhibitor (VPA), and the HDAC1 and 2-specific inhibitor (ROMI) for their capacity to regulate DNA damage repair gene expression and hence sensitise TNBC to PARPi. Specific inhibition of HDAC1/2 has been shown to repress genes involved in nonhomologous end joining (NHEJ; ref. 6), while inhibition of HDAC 9/10 represses expression of HR genes (7). Several HDAC inhibitors have been shown to repress expression of several HR proteins, including RAD51, via reduced recruitment of the transcription factor E2F1 (8, 9). There is a great deal of heterogeneity in the genome maintenance mechanisms in sporadic TNBC (10). In addition to the epigenetic silencing of the BRCA gene, other drivers of impaired HR have been identified in TNBC (11). We hypothesize that repression of specific members of the HR pathway are required to sensitize aggressive TNBC to HDAC and PARP inhibition. We suggest that different HDACi have different efficacies in modulating expression of HR genes and that HDACi-catalyzed epigenetic regulation of HR gene expression is an effective means to induce a BRCAness state, thus creating synthetic lethality with PARP inhibition.
Materials and Methods

Cell lines
The MDA-MB-231 and MDA-MB-436 cells were sourced from the ATCC cell bank and were STI profiled and verified in our laboratory (January 2015), while the matched HCC1937-BRCA1MUT and HCC1937-BRCA1RECON cell lines have been authenticated and reported previously (12, 13) and subsequently verified for BRCA1 expression (December 2014).

HDACi and PARPi individual drug dose curves
A total of 5 × 10^4 cells per well of each cell line were incubated in the presence of escalating doses of either HDACi or PARPi (ABT-888 0–20 μmol/L) for 48 hours. Cell viability was measured against DMSO-treated controls in an MTS metabolism assay. Each plot represents mean ± SEM of triplicate experiments.

Simultaneous HR and NHEJ "traffic light" assay
All cells were engineered to express the traffic light reporter system to analyze the activation status of both NHEJ and HR (14). In brief, cells were transfected with lentiviral packaging of pCCL Traffic Light Reporter 1.1 E1a Puro, and selected for puromycin. Positive colonies were screened for integration using TaqMan probe 5'-FAM-TCTTACAAT/ZEN/GCCGTGAGCTGAGG/CybKFPQ-3'. Cells were transduced with the Ise-c1 enzyme (IFP tagged) and GFP complementary donor (BFP tagged) and repair measured after 72 hours endpoint on FACS comparing GFP (in IFP/ BFP-positive cells) to Cherry signal (IFP-positive) ratio ± HDACi.

Combination drug-dose curves
In a 48-well plate, 36 wells of 3 × 10^4 cells per well were incubated in the presence of escalating doses up to ~IC50, for MDA-MB-231 (SAHA 2.5 μmol/L, VPA 20 μmol/L and ROMI 1.25 μmol/L) and for MDA-MB-436 (SAHA 0.3125 μmol/L, VPA 0.625 μmol/L, and ROMI 0.15625 μmol/L) of HDACi for 24 hours. The wells were then rinsed with fresh media, and the cells were further incubated in the presence of 2.5 μmol/L of ABT-888 or DMSO for 48 hours. The cells were harvested and fixed with 70% ethanol and analyzed for cell-cycle profile using propidium iodide and FACS. Plots were graphed using Graphpad prism and represent the mean ± SEM of four independent experiments.

Long-term colony assay
After incubation with appropriate drug combinations, 1 × 10^5 cells were plated per well in a 6-well plate and incubated with DMEM supplemented with 10% FBS and antibiotics. After 14 days of incubation, the colonies were washed with PBS and fixed with 70% ethanol stained with 0.5% crystal violet in PBS for 5 minutes. Excess stain was washed away with running water and plates scanned and quantitated using ImageJ software. Plots represent the mean and SEM of four independent experiments.

Expression analysis
RNA was isolated from MDA-MB-231, HCC1937-BRCA1MUT, or HCC1937-BRCA1RECON using a Qiagen RNAeasy Mini Kit (cat. 74104). cDNA was synthesized using an RT" First Strand Kit (Qiagen; cat. 330401). cDNA was loaded in duplicate on The Human DNA Damage Signaling Pathway RT2 Profiler PCR Array containing 82 genes (Qiagen; cat. PAHS-029Z) and analyzed on Roche Lightcycler 480. Fold expression change was calculated against actin and expressed as base-two exponential increase in RNA levels (2ΔACt) ± SEM.

FACS analysis
 Cultures were trypsinized to single-cell suspension, washed with PBS/10% FBS, stained with Hoechst 33342, and gated on the polyploid population. Cells were back gated and sorted for a purified polyploid population. Propidium iodide staining with RNase and cell-cycle analysis was performed to confirm the purity. For intracellular RAD51 analysis, cells were further incubated with 0.3% saponin and 1:250 of anti-RAD51 (Santa Cruz Biotechnology) for 1 hour, washed 3 times with 0.03% saponin in PBS, and incubated with anti-rabbit 488–conjugated antibody for 1 hour in 0.3% saponin. Cells were washed and resuspended in propidium iodide containing RNase. For general cell-cycle analysis, cells were fixed with 70% cold ethanol and stained with propidium iodide containing RNase. FACS analysis was performed on 10,000 gated events per sample.

Immunoblotting
Protein lysates were prepared via whole-cell lysis in ice-cold lysis buffer (150 mmol/L NaCl, 10 mmol/L Tris–Cl pH 7.4, 5 mmol/L EDTA, 1% Triton X-100) supplemented with protease inhibitors (Leupeptin, Pepstatin, and Puromycin; Sigma Aldrich). Immunoblots were probed with anti-RAD51 (Santa Cruz Biotechnology), anti-PARP (Millipore), anti-PARP (BD Biosciences), anti-DNA-PKcs (Cell Signaling Technology) and anti-53BP1 (Bethyl), and with anti-tubulin (Sigma), and anti-β-actin, (Sigma) as controls. Membranes were developed using fluorescent-labeled secondary antibodies and visualized using the Odyssey system. Protein expression levels were determined by optical density versus actin loading controls using ImageJ software (NIH, Bethesda, MD).

Immunofluorescence
Approximately 5 × 10^4 cells were seeded onto 18-mm glass coverslips. Forty-eight hours later, cells were gamma irradiated at 6 Gy. The coverslips were washed in PBS, the cells were fixed in 1% cold 70% ethanol and permeabilized in 0.5% Triton X-100 solution for 15 minutes at room temperature. Cells were blocked with 10% FBS in PBS and incubated with primary antibody (or Alexa phallloidin) for 1 hour and with secondary antibody for 30 minutes at room temperature. All antibodies were diluted in 5% FBS–PBS. Cells were then washed, counterstained with 4,6-diamidino-2-phenylindole (DAPI), and mounted. Primary antibody dilutions were as follows: Rad51, 1:100; H2AX 1:1,000 and 1:500, respectively. All secondary antibodies were used at 1:200.

Results

Functional BRCA1 loss sensitizes to PARP or HDAC inhibitors
TNBCs are characterized by chromosomal instability, some of which might be caused by defects in HR (described as BRCA1ness) and display synthetic lethality in response to PARP inhibition (1). We investigated the ability of three different HDACi, SAHA, VPA, and ROMi to impact on cell survival in four TNBC cell lines: MDA-MB-231 (BRCA1WT), MDA-MB-436 (BRCA1ASSHOT), and HCC1937-BRCA1MUT (BRCA1S326C) and the reconstituted HCC1937-BRCA1RECON (BRCA1WT) over 48 hours. IC50 (Supplementary Table S1). Each of the BRCA1-competent lines (Fig. A and C) displayed reduced sensitivity to all HDACis compared with the BRCA1-mutant cell lines (Fig. 1B and D). VPA was the least effective HDACi across all cell lines. Interestingly VPA was 1.8 times more effective against MDA-MB-436 (IC50 19.1 μmol/L) than MDA-MB-231 (IC50 34 μmol/L; Fig. 1A and B; Supplementary
but displayed little difference in IC50 when compared with HCC1937-BRCA1RECON and HCC1937-BRCA1MUT, respectively (Fig. 1C and D). MDA-MB-436 also displayed exquisite sensitivity to ROMI 0.9 μmol/L (Fig. 1B), most likely due to an additional RB1 homozygous mutation (c.607–608ins227) in the cell line that renders tumors sensitive to changes in acetylation and is an effective treatment for retinoblastoma (15). We also analyzed the response of the four cell lines to PARP inhibitor ABT-888 for 24 hours, and again found the BRCA1-mutant lines displayed higher sensitivity to PARPi compared with the BRCA1-competent cell lines (Fig. 1E). We did not observe the 1,000-fold enhanced sensitivity comparing MDA-MB-231 and MDA-MB-436 achieved by the van’t Veer group using olapirib; however, our 9-fold sensitivity was at 48 hours not 15 days (Fig. 1E; ref. 16).

HDAC inhibitors suppress HR activity in breast cancer cells independent of BRCA1 status

To determine the effect of epigenetic changes mediated by HDACi on DNA repair pathways, we utilized the "traffic light" dual reporter (TLR) assay. This assay provides simultaneous readouts of HR and NHEJ, via I-SCE (IFP-tagged) induced double-strand break (DSB) in the eGFP gene and restoration of green-fluorescence signal when repaired by gene conversion of a BFP-tagged exogenous template. Repair of the DSB by error-prone NHEJ results in a +2 shift in mCherry reading frame, resulting in red fluorescence (14). NHEJ was analyzed from the IFP population, while HR was analyzed from the IFP/BFP double–positive cells (Fig. 1F). Positive Cherry cells representing NHEJ activity could be isolated from the IFP population, and GFP cells representing HR could be isolated from IFP/BFP double–positive cells (Fig. 1G). We next compared the response of all four cell lines to the three HDACi and utilized the DNA-PK inhibitor NU7441 and the Rad51 inhibitor B02 (17) as controls for inhibition of NHEJ and HR, respectively. DNA-PKi induced HR in all cell lines with minor levels of NHEJ, while RAD51 inhibitor (10 μmol/L-B02) served as control for inhibition of HR, while –ISCE served as a negative control and +ISCE served as a negative drug treatment control. Plots, 1 × 10^4 gated events.

Table S1), but displayed little difference in IC50 when compared with HCC1937-BRCA1RECON and HCC1937-BRCA1MUT, 23.6 versus 21.7 μmol/L, respectively (Fig. 1C and D). Supplementary Table S1). MDA-MB-436 also displayed exquisite sensitivity to ROMI 0.9 μmol/L (Fig. 1B), most likely due to an additional RB1 homozygous mutation (c.607–608ins227) in the cell line that renders tumors sensitive to changes in acetylation and is an effective treatment for retinoblastoma (15). We also analyzed the response of the four cell lines to PARPi inhibitor ABT-888 for 24 hours, and again found the BRCA1-mutant lines displayed higher sensitivity to PARPi compared with the BRCA1-competent cell lines (Fig. 1E). We did not observe the 1,000-fold enhanced sensitivity comparing MDA-MB-231 and MDA-MB-436 achieved by the van’t Veer group using olapirib; however, our 9-fold sensitivity was at 48 hours not 15 days (Fig. 1E; ref. 16).

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Analysis of HCC1937 matched lines revealed that the BRCA1RECON displayed increased DNA repair at 24 hours compared with the other cell lines. In response to SAHA and ROMI, the cells displayed similar induction of NHEJ and HR to that observed in MDA-MB-231 response to SAHA (Fig. 1J). VPA also induced NHEJ but to a lesser extent (Fig. 1J). Analysis of BRCA1MUT revealed a similar response as MDA-MB-231 to all HDACi with activation of NHEJ (Fig. 1K). This suggests a continued reliance on NHEJ, which was not altered by reconstitution with exogenous BRCA1 (Fig. 1J vs. K).

We confirmed HDACi-mediated repression of HR in MDA-MB-231 using the DR-GFP construct and ISCE enzyme as described (ref. 18; Supplementary Fig. S1A–S1C). DRGFP and IF assays correlated well with the traffic light reporter assay when analyzing HR function in response to HDACi. Our results correlated with several published studies in that HDACi is able to repress HR activity in breast cancer cells (19, 20). In addition, we observe a corresponding shift to activation of mutagenic NHEJ. Interestingly, we find that HDACi are able to further influence DNA repair in BRCA1-mutant cell lines despite having reduced cellular HR activity.

Cell growth response of BRCA1 wild-type and mutated TNBC lines to the combination of HDAC and PARP inhibition

We analyzed the ability of HDACi to sensitize TNBC to PARP inhibition through repression of HR. We performed dose curves of each HDACi (doses 0 to \(\sim \text{IC}_{50}\)) for MDA-MB-231 (SAHA 2.5 \(\mu\text{mol/L}\), VPA 20 \(\mu\text{mol/L}\), and ROMI 1.25 \(\mu\text{mol/L}\)) and for MDA-MB-436 (SAHA 0.3125 \(\mu\text{mol/L}\), VPA 0.625 \(\mu\text{mol/L}\), and ROMI 0.15625 \(\mu\text{mol/L}\)) in combination with or without the PARP inhibitor ABT-888 (2.5 \(\mu\text{mol/L}\)). Drug scheduling allowed HDACi-mediated epigenetic change over the first 24 hours followed by inhibition of DNA repair with PARPi for a further 48 hours (Fig. 2A). In MDA-MB-231 cells, the pan-HDACi, SAHA, and HDAC1/2-specific HDACi, ROMi, induced approximately 80% cell death at the highest dose of each drug in combination with PARPi as measured by the sub-G1 fraction (Fig. 2B and D). VPA did not induce any significant change in the cell-cycle profile even when used at 20 \(\mu\text{mol/L}\) (Fig. 2C). Of note, ROMi alone induced an increase in super-G2 or polyploidy population in MDA-MB-231, suggesting induction of a BRCA1ness profile, seen normally in BRCA1-mutated cells (Fig. 2D).

To quantitatively measure the dose-escalation relationship of the drugs in combination for synergy (combination index), an

![Figure 2](https://example.com/fig2.png)

HDAC inhibition and PARP inhibition synergize to kill TNBC. A, the treatment schedule of preincubation with one of three HDACi for 24 hours followed by replacement of the media with PARP inhibitor for a further 48 hours of DMSO in controls. Cell-cycle profile of MDA-MB-231 cells were analyzed in response to SAHA (B), VPA (C), and ROMI (D). MDA-MB-436 cells were analyzed in response to SAHA (E), VPA (F), and ROMI (G). All dose curves were formulated using concentrations diluted 2-fold from \(\sim \text{IC}_{50}\). H, the treatment schedule from A was reversed with cell incubated in the presence of PARPi/DMSO for 24 hours then incubation with SAHA. Cell cycle was analyzed after 72 hours. Surviving fractions of cells were analyzed 14 days after treatment in B-G highest doses for MDA-MB-231 (I) and MDA-MB-436 (J). Colony counts were expressed as a fraction of DMSO-treated controls and represent mean \(\pm\) SEM of triplicate experiments.
effective calculation is the use of median-drug effect analysis (Supplementary Table S2). SAHA displayed synergy with ABT-888 at 2.5 μmol/L; however, when the order of drug incubation was reversed (incubation with PARPi followed by HDACi), this synergy was not observed (CI, 1.25; Fig. 2H; Supplementary Table S2). Increasing doses of ROMI in the absence of ABT-888 resulted in an increased fraction of multinucleated cells, confirmed by immunofluorescence (Supplementary Fig. S2A), and synergy with 2.5 μmol/L ABT-888 at all doses 0.156–2.5 μmol/L (Fig. 2G). The observed synergism translated to long-term inhibition with SAHA or ROMI significantly retarding colony formation to 12% and 6% to that of controls, while MDA-MB-231 cells retained 80% colony formation in the presence of VPA and ABT-888 (Fig. 2I). In MDA-MB-436 cells, the combination of high-dose SAHA and ABT-888 resulted in 37% cell death (Fig. 2E), while VPA response was similar to that observed with MDA-MB-231, with little change in the cell-cycle profile across all doses (Fig. 2F). High-dose ROMI induced a G1 arrest independent of ABT-888 (Fig. 2G), which coincided with a reduced polyploid population, suggesting reduced aberrant progression through the cell cycle. This would suggest a possible mechanism for inhibition of ABT-888 activity, which requires active cycling cells for induced DNA damage (2). A recent study also attributed PTEN expression as a determinant for sensitivity to the combination of SAHA and PARPi (21). MDA-MB-231 has functional PTEN while MDA-MB-436 does not express the protein (22). This might suggest why the combinations were less effective in long-term assays in MDA-MB-436 (Fig. 2I). Taken together, these results suggest that a fully competent HR pathway in TNBCs enables synergy with PARPi inhibition after HDACi-mediated epigenetic changes, specifically observed with SAHA and ROMI while a nonfunctional BRCA1/PTEN background reduces PARPi synergism with HDACi.

Combined depletion of RAD51, FANCD2, and BARD1 is required to sensitize VPA to PARPi inhibition

To determine whether differences in the regulation of histone modification and DNA damage response (DDR) gene expression by VPA compared with other HDACi provides an explanation for the lack of synergy with ABT-888, we treated MDA-MB-231 cells with each of the HDACi’s for 24 hours and analyzed expression of 82 different DDR genes and found 28 to show varying levels of expression change. These genes display three profiles: (i) genes that were repressed by VPA and ROMI but not the pan-HDACi SAHA (Supplementary Fig. S2B); (ii) genes with increased expression when treated with VPA compared with SAHA and ROMI (Fig. 3A); and (iii) genes that were not repressed as effectively by ROMI compared with SAHA and VPA (Supplementary Fig. S2C). The intriguing gene set is the second as it represents the genes possibly responsible for the lack of VPA synergy with ABT-888 observed in MDA-MB-231 cells. Of the eight genes, three were induced by VPA, namely FANCD2, XRCC1, and XRCC6. The latter two were also induced by ROMI and thus not specific to VPA activity. Of the remaining five genes (ABL1, BARD1, MAPK12, MNG, and RAD51), BARD1 and RAD51 directly function as part of HR-mediated repair, while ABL1-mediated phosphorylation of RAD51 can regulate RAD51 function (23, 24). It is interesting to note that RAD51 was identified in a set of 13 genes whose loss of expression synergized with PARPi, validating our experiment (25–27). We confirmed that all the HDACi imparted epigenetic changes on MDA-MB-231 cells via changes in histone H3 and H4 acetylation although VPA was less effective compared with other two HDACi and induction of p21 expression (Fig. 3B). Similar to mRNA expression observed in the array data, BARD1, FANCED2, and RAD51 protein expression was not repressed by VPA in MDA-MB-231 cells (Fig. 3B) and confirmed in MDA-MB-436 cells (Supplementary Fig. S2D).

To provide direct evidence that these genes are critical for the cooperative anti-breast cancer activity of HDACi and PARPi, we next evaluated whether we could restore VPA activity in combination with ABT-888 by knockdown of RAD51, FANCED2, or BARD1. Individual gene knockdown did not induce sensitivity (Supplementary Fig. S2F–S2H), only with depletion of all three genes did we achieve significant cell death, 64.2% versus 6.9% (\(^*\), \(P = 0.0006\); Fig. 3C and D). Restoration of sensitivity translated to significant long-term inhibition of growth, inhibiting 83% colony formation (\(^*\), \(P = 0.003\); Fig. 3E). Interestingly, the RAD51/FANCD2 combination also displayed significant inhibition of colony formation, 37% inhibition compared with the drug combination alone (\(^*\), \(P = 0.008\)). Of the three genes, only RAD51 overexpression is significantly correlated with worse disease-free survival in TNBC patients (\(P = 0.049\); Fig. 3F). VPA has been utilized in a phase II clinical trial against castration-resistant prostate cancers, which demonstrated some encouraging results (28) and is currently in ongoing and recently terminated phase II clinical trials against breast cancer (NCT01010854 and NCT00395655). Our results suggest that RAD51, FANCED2, and BARD1 are key mediators of HDACi-induced sensitization to PARPi therapy.

Checkpoint activation is a key mechanism in resistance to HDACi and PARPi combination

We observed that the MDA-MB-436 cells had reduced HR-mediated repair capacity and were generally less responsive to the combination therapy, which could be attributed to functional loss of BRCA1 (21). We wanted to validate these results using the HCC1937 breast cancer cell line. The HCC1937–BRCA1MUT cell line displayed a slight enhanced G1 population in response to SAHA and an increased polyploid population after the addition of PARPi (Fig. 4A), which was also observed with ROMI and PARPi (Fig. 4C). We confirmed enhanced multinucleation by immunofluorescence (Supplementary Fig. S3). This was consistent with the assumption that mutant BRCA1 and loss of functional HR drives aberrant repair, resulting in multinucleation. In contrast with the other HDACi, VPA (10 μmol/L) significantly correlated with worse disease-free survival in TNBC patients (\(P = 0.049\); Fig. 3F). VPA has been utilized in a phase II clinical trial against castration-resistant prostate cancers, which demonstrated some encouraging results (28) and is currently in ongoing and recently terminated phase II clinical trials against breast cancer (NCT01010854 and NCT00395655). Our results suggest that RAD51, FANCED2, and BARD1 are key mediators of HDACi-induced sensitization to PARPi therapy.

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while PARPi alone induced a slight increase in the polyploidy population (Fig. 4G and B). The combination of CHKi and PARPi induced a G2 arrest, which was also observed with CHK1i and VPA (Fig. 4G). Most impressively, the combination of CHKi, PARPi, and VPA resulted in an enhanced polyploidy population, similar to that observed with each of SAHA and ROMI with PARPi (Fig. 4A and C). We suggest that inhibition of the G1 checkpoint and the consequent downregulation of DNA repair via VPA/ABT-888 combination on a BRCA1 functionally deficient background results in an unstable polyploidy population. In BRCA1 cells,
HDACi, SAHA, and ROMI in combination with ABT-888 resulted in 20.5% and 11.8% colony survival, respectively, after 14 days (Fig. 4H), suggesting indeed that the polyploid population may be unstable. Similarly, the cells reconstituted for BRCA1 function displayed a similar trend, 43.4% and 22.3% colony survival with the combination of SAHA or ROMI with ABT-888, respectively (Fig. 4). Reconstitution of BRCA1 stabilizes the genome in response to HDACi/PARPi combination and changes DDR gene expression profile. Cell-cycle profile of DMSO or ABT-888 treated HCC1937-BRCA1MUT cells in response to SAHA (A), VPA (B), and ROMI (C). HCC1937-BRCA1RECON cells were analyzed in response to SAHA (D), VPA (E), and ROMI (F). All dose curves were formulated using concentrations diluted 2-fold from -IC50. G, cell-cycle profile of HCC1937-BRCA1MUT cells treated with the combination as per B with the addition of CHK1i (AZD7762). Treatment of surviving fractions of cells incubated with the highest dose drug combinations were analyzed after 14 days after treatment from H (A–C) and I (D–F). Colony counts were expressed as a fraction of DMSO-treated controls and represent mean ± SEM of triplicate experiments. RNA expression analysis of DDR genes comparing reconstitution of BRCA1 in HCC1937-BRCA1RECON cells standardized to baseline expression in HCC1937-BRCA1MUT cells with enhanced expression with reconstitution of BRCA1 (J) and reduced expression after reconstitution of BRCA1 (K).
(Fig. 4I). Taken together, these results suggest that epigenetic BRCA1 loss in BRCA1 reconstitution is less sensitive than genetic mutant BRCA1 due to aberrant DNA repair, resulting in polyploidy and cell death. However, these observations are cell line specific, as MDA-MB-231 or MDA-MB-436 did not display any enhanced polyploidy in response to the combination of HDACi/PARPi. This could be attributed to higher RAD51 expression in HCC1937 cells (29) and RAD51-guided aberrant DNA repair that we suggest is not related to PTEN status.

**BRCA1 expression changes DDR expression profile and cellular response to HDAC inhibition**

To examine why the reconstitution of BRCA1 renders the HDACi or the combination of HDACi and PARPi less effective in HCC1937 cells, we analyzed the gene expression changes in response to ROMI comparing BRCA1-depleted with BRCA1-reconstituted. The reconstitution of BRCA1 changes the cellular response from an increase in the polyploidy population to an increased G1 population (Fig. 4C vs. F). The cytotoxic activity of PARPi inhibition relies upon unresolved ssDNA damage to create dsDNA breaks upon replication fork collapse and activation of apoptosis (2). Cell-cycle arrest stifles this mechanism and can, in part, provide an explanation for the lack of PARPi activity, while loss of PTEN is thought to inhibit response (21). The observed G1 arrest in response to ROMI in cells with reconstituted BRCA1 (Fig. 4F) is readily accounted for by the changes in gene expression, including BARD1, a ubiquitin ligase that binds BRCA1 and regulates cell cycle and DNA repair, CHEK1, a cell-cycle kinase that regulates checkpoint activation, and FANCD2 that regulates chromosome stability (Fig. 4I). Of note, BRCA1 expression was increased, validating the cell line genetics (Fig. 4I). Interestingly, a similar arrest was observed in MDA-MB-436 in response to ROMI, suggesting a greater role for the aforementioned BARD1, CHEK1, and FANCD2 genes (Fig. 2G). In contrast, the absence of functional BRCA1 in BRCA1-depleted cells meant 18% progressed through mitosis to a multinucleation population only achieved 5% growth over 5 days compared with over 20% for unsorted cells (Fig. 5B). Comparison of the purified polyploid population to untreated and unsorted populations for sensitivity to ROMI and ABT-888 revealed that the sorted population retained similar sensitivity as unsorted cells to ROMI but displayed increased resistance to ABT-888, but not to the level afforded by BRCA1 reconstitution (Table 1). Bold is related to enhanced resistance in this population. Protein expression analysis revealed no corresponding increase in FANCD2, BARD1, or PARPi but enhanced RAD51 expression in the polyploid population (Fig. 5C), which was confirmed by intracellular FACS staining in all cell lines (Fig. 5D). This suggests that RAD51 contributes to survival of the polyploid population and resistance to PARPi. To confirm that RAD51 contributes to PARPi resistance, we overexpressed RAD51 in the low RAD51–expressing breast cancer cell line Hs578t (Fig. 5E). Increased expression of RAD51, independent of PARPi expression, increased IC50 of ABT-888 3.9-fold from 15.2 versus 59.1 μmol/L (Fig. 5F). Taken together, the results suggest that after treatment with the combination of HDACi and PARPi, BRCA1-deficient cells initially survive by upregulating RAD51 expression and bypassing cell-cycle arrest, resulting in polyploidy. However, these cells display no sustained survival, which could be due to lack of BARD1 and/or FANCD2 expression.

**Discussion**

The observation that HDACis suppress multiple DNA damage response pathways, including HR, creates the opportunity for synthetic lethality in solid cancers. In vivo studies have demonstrated that the combination of ABT-888 and SAHA is able to retard growth of established MDA-MB-231 xenografts (19). There are several ongoing clinical trials utilizing the HDACis we studied, including a pilot study for SAHA against TNBC (NCT01695057) and phase I trial with a ROMI and cisplatin combination against breast cancer (NCT02393794) and phase II trial with VPA against breast cancer. There is also a recently terminated phase II trial with VPA against breast cancer (NCT00395655). This trial could have benefited from our potential biomarkers of resistance to VPA, namely BARD1, FANCD2, and RAD51. In addition, another key clinical marker for HDAC activity is the observed shift from HR to mutagenic-NHEJ activity (30). This creates the conditions required for PARPi inhibition–mediated synthetic lethality and a rationale for the use of HDACi to create an epigenetic-mediated BRCA1 loss via downregulation of key HR gene expression. We observed the requirement for downregulation of RAD51, BARD1, and FANCD2 expression for effective HDACi inhibitor activity and a role for RAD51 in mechanisms of resistance to PARPi inhibition–induced polyploidy. Therefore, we suggest that RAD51 expression is a key regulator of synthetic lethality.

RAD51 is a key protein in the final steps of HR and functions to bind the resected ssDNA and search for the complementary sequence on the sister chromatid, resulting in high fidelity repair. The expression of RAD51 is known to be upregulated in BRCA1-deficient tumors compensating for the loss of HR, allowing carcinogenic progression and metastasis (31). Inhibition of RAD51 has been found to synergize with inhibition of PARPi in both RNA knockdown screens and small molecule screens (25–27). Our study supports these observations with RAD51 overexpression enhancing resistance to PARPi. Furthermore, we showed that the PARPi-resistant polyploid population displayed...
increased expression of RAD51. This suggests that RAD51 is a driver of aberrant recombination, suggesting a mechanism for cell survival. Indeed, overexpression of RAD51 in normal cells drives pathologic recombination events such as chromosomal amplifications, deletions, and translocations resulting in loss of heterozygosity and aneuploidy (32). Lack of suppression of RAD51 expression in the presence of VPA might explain the observed resistance to PARPi; however, depletion of RAD51 alone was not enough to restore activity and required cooperative loss of FANCD2 for significance. These findings suggest that inherently unstable genomes are stabilized by RAD51 expression as a mechanism for cell survival, which can confer resistance to PARP inhibition.

In the breast cancer setting, increased expression of FANCD2 is associated with high proliferation rates and replication stress (33) and associates with BRCA1 and RAD51 at sites of DNA damage to guarantee the integrity of genome during replication (34, 35). Interestingly, in a panel of 1,240 paraffin-embedded breast tumors, overexpression of FANCD2 was shown to be prognostic for poor patient survival, whereas RAD51 was not (36). However, this observation was in hormone receptor–positive breast cancer, whereas RAD51 has been associated with poorer prognosis in TNBC (29). The BRCA1/BARD1 complex is known to regulate FANCD2 via monoubiquitination; however, we observe a transcriptional upregulation of FANCD2 after BRCA1 reconstitution in HCC1937 cells. BRCA1 has autotranscriptional activity, but can also be regulated by E2F1, a transcription factor shared with FANCD2, hinting at a role for E2F1 in resistance to DNA damage–induced cell death. The transcriptional mechanism by which FANCD2 is regulated is yet to be fully delineated. We speculate that BRCA1 could act as a transcriptional cofactor to induce FANCD2 expression, which could contribute to resistance to PARPi. Of note, FANCD2 upregulation has indeed been associated with HDAC and PARP inhibition–resistant hepatocellular carcinoma cells (37) and, recently, complementation of BRCA1-deficient cell lines with FANCD2 was shown to restore normal HR function (38). We suggest that RAD51 is the main contributor to PARPi resistance as the depletion of RAD51 has been shown to be

### Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>$IC_{50}$ romidepsin</th>
<th>$IC_{50}$ ABT-888</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated</td>
<td>10.62 μmol/L</td>
<td>18.95 μmol/L</td>
</tr>
<tr>
<td>Treated nonsorted</td>
<td>8.75 μmol/L</td>
<td>19.54 μmol/L</td>
</tr>
<tr>
<td>Polyploid sorted</td>
<td>8.15 μmol/L</td>
<td>27.20 μmol/L</td>
</tr>
<tr>
<td>BRCA1 reconstituted</td>
<td>6.25 μmol/L</td>
<td>52.13 μmol/L</td>
</tr>
</tbody>
</table>

NOTE: Bold text indicates enhanced resistance in this population compared to nonsorted.

Figure 5.
The polyploid population does not contribute to long-term cell survival but enhances resistance to PARP inhibition. A, the resulting polyploid population from HCC1937-BRCA1MUT treated with romidepsin/ABT-888 combination was isolated and compared with untreated and untreated controls. B, in situ analysis of real-time growth rates of polyploid population compared with an unsorted population over 4.5 days using Incucyte imaging. C, protein expression of key DDR proteins required for HDACi efficacy were analyzed in the three aforementioned cell populations. D, FACS analysis of RAD51 expression in various cell-cycle phases was compared between BRCA1-competent lines MDA-MB-231 and HCC1937-BRCA1RECON, and BRCA1-mutant lines MDA-MB-436 and HCC1937-BRCA1MUT (gray shaded histogram IgG control). E, parental, control empty vector, and RAD51-overexpressing Hs578t cell lines were compared for RAD51 and PARP1 protein expression. F, comparison of RAD51-overexpressing line and control to increasing doses of ABT-888 was performed in triplicate and expressed as mean ± SEM.
more potent than that of FANCD2 in sensitizing HeLa cells to PARPi (27).

Along with RAD51 and FANCD2, loss of BARD1 expression was required to sensitize BRCA1-competent cells to combination of VPA and PARPi. BARD1 facilitates BRCA1 nuclear translocation for DNA repair and enhances DNA binding. Upon DNA damage, BARD1/BRCA1 heterodimers form nuclear foci in complexes that can include BRCA2 and RAD51 (39), which directs HR-mediated repair via the BRCA1–BRCA2 linker protein PALB2 (40). Of importance is the observation that HDAC-mediated stabilization of mutant BRCA1 protein via hyperacetylation of Hsp90 (19) can enhance resistance to PARPi via stabilization of the BRCA1/ BARD1–PALB2–BRCA2/RAD51 complexes (41). This could account for our observed reduced efficacy of the HDAC/PARPi combination in BRCA1-mutant cells in short-term assays. However, alternate studies demonstrate that loss of PTEN is the main mechanism for resistance to SAHA and PARPi in TNBC (21). This could be true for MDA-MB-436, which harbors a PTEN mutation; however, we were able to induce cell death in HCC1937 that also harbor a nonfunctional PTEN. The coincidental loss of functional BRCA1 in HCC1937 also sensitized the cells to the HDAC/PARPi combination and suggests that the dual mutations cancel each other out functionally (42).

There is also a direct interplay among BRCA1, BARD1, and RAD51 resulting in the ability of wild-type BRCA1 to regulate the cellular response to each of the HDACi tested and metastatic potential. Compared with mutant cells, those with reconstituted BRCA1 displayed increased BARD1 expression. BARD1 expression has been observed to facilitate RAD51 overexpression in a TNBC metastatic model to the brain (43). Of note, BRCA1-independent functions of BARD1 have also been reported (44).

In general, the presence of functional BRCA1 and BARD1 can direct HR-mediated repair, supporting RAD51 expression and inducing resistance to PARP inhibition. Thus, SAHA and ROMI-induced suppression of BARD1/RAD51 suggest good anti-metastatic activities. On the other hand, in the absence of functional BRCA1, we also observed a BARD1-independent increase in RAD51 expression in the polyploid population, which also drives resistance to PARP inhibition. Taken together, we suggest that BARD1 may play a role in the interplay between RAD51 and BRCA1 and the DNA repair mechanisms regulating PARP inhibitor resistance. More definitively, we state that the cellular response to PARPi is directed by levels of HR proteins most efficiently depleted by SAHA and that BRCA1 functional status can determine the cellular response of cells to epigenetic expression changes induced by SAHA, VPA, and ROMI.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A.P. Wiegmans, K.K. Khanna
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.P. Wiegmans, P.-Y. Yap, A. Ward
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.P. Wiegmans, Y.C. Lim
Writing, review, and/or revision of the manuscript: A.P. Wiegmans, Y.C. Lim, K.K. Khanna
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.K. Khanna
Study supervision: K.K. Khanna

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