Differences in expression of key DNA damage repair genes after epigenetic-induced BRCAness dictate synthetic lethality with PARP1 inhibition.

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Abbreviations: TNBC Triple Negative Breast Cancer; PARP poly-ADP-ribose-polymerase; HR Homologous Recombination; HDACi Histone Deacetylase Inhibitor; SAHA suberoylanilide hydroxamic acid; VPA valproic acid; ROMI romidepsin; NHEJ Non-homologous End Joining; GFP Green Fluorescent Protein; TLR Traffic Light Reporter; DSB Double Strand Break; DDR DNA Damage Response.

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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 poly-ADP-ribose-polymerase (PARP) inhibition in breast cancer cells which harbour silencing of the homologous recombination (HR) pathway. Histone deacetylase inhibitors (HDACis) are a class of drugs, which 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 the two of HDACis tested SAHA and ROMI but not VPA indeed inhibit HR repair and that RAD51, BARD1 and FANCD2 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 to mutant BRCA1 that results in enhanced polyploid 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 frontline clinical TNBC therapies are limited to cytotoxic chemotherapy with or without the anti-angiogenic agent (bevacizumab) (1). However, preclinical success has been gained by synthetic lethality, targeting genome instability with poly-ADP-ribose-polymerase (PARP) inhibition in breast cancer cells which harbour 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, which mediate epigenetic control of gene expression profiles. This occurs via HDACi-mediated genome-wide hyperacylation 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 tumours, reviewed in Slingerland et al. (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 (suberylinamide 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 sensitize TNBC to PARPi. Specific inhibition of HDAC1/2 has been shown to repress genes involved in non-homologous end joining (NHEJ) (6), while inhibition of HDAC 9/10 represses expression of homologous recombination (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 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 BRCAAness state, thus creating synthetic lethality with PARP inhibition.
Materials and Methods

Cell lines
MDA-MB-231, MDA-MB-436 were sourced from the American Type Culture Collection (ATCC) cell bank and were STI profiled and verified in our lab (Jan 2015), while the matched HCC1937-BRCA1\textsuperscript{MUT} and HCC1937-BRCA1\textsuperscript{RECON} have been authenticated and reported previously (12, 13) and subsequently verified for BRCA1 expression (Dec 2014).

**HDACi and PARPi individual drug dose curves**
A total of 5x10^4 cells per well of each cell line were incubated in the presence of escalating doses of either HDACi or PARPi (ABT-888 0-20μM) 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 cell lines 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 pCVL Traffic Light Reporter 1.1 Ef1a Puro, and selected for with puromycin. Positive colonies were screened for integration using Taqman probe ‘5'-FAM/TTCTAACAT/ZEN/GCGGTGACGTGGAGG/IABkFQ-3’. Cells were transduced with the Isce-1 enzyme (IFP tagged) and GFP complementary donor (BFP tagged) and repair measured after 72 hours end point 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 3x10^4 cells per well were incubated in presence of escalating doses up to ~IC20 of HDACi for 24 hours. The wells were then rinsed with fresh media and the cells further incubated in the presence of 2.5μM of ABT-888 or DMSO for 48 hours. The cells were harvested and fixed with 70% ethanol and analysed for cell cycle profile using propidium iodide and FACS. Plots were graphed using graph-pad prism and represent the mean +/- SEM of 4 independent experiments.

**Long-term colony assay**
After incubation with appropriate drug combinations, 1x10^3 cells were plated per well in a 6 well plate and incubated with DMEM supplemented with 10% FBS and antibiotics. After 14 days incubation the colonies were, washed with PBS and fixed with 70% ethanol stained with 0.5% crystal violet in PBS for 5min. Excess stain was washed away with running water and plates scanned and quantitated using Image J software. Plots represent the mean and SEM of 4 independent experiments.

**Expression analysis**

RNA was isolated from MDA-MB-231, HCC1937-BRCA1^{MUT} or HCC1937-BRCA1^{RECON} using QIAGEN RNAEasy Mini Kit (Cat 74104). cDNA was synthesized using RT² 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 (2ΔΔCt)+/- SEM.

**FACS analysis**

Cultures were trypsanized to single cells suspension, washed with PBS/10%FBS, staining with Hoechst 33342 and gating on the polyploid population. Cells were back gated and sorted for a purified polyploidy 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 Biotech) for 1hr, washed 3 times with 0.03% saponin in PBS and incubated with anti-rabbit 488 conjugated antibody for 1hr 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 (150mM NaCl, 10mM Tris-Cl pH 7.4, 5mM EDTA, 1% Triton X-100) supplemented with protease inhibitors (Leupeptin, Pepstatin and PMSF, Sigma Aldridge). Immunoblots were probed with anti-RAD51 (Santa Cruz Biotech), anti-PARP (Millipore), anti- PAR (BD Biosciences), DNA-PKc,P2609 (Cell Signaling) and anti-53BP1 (Bethyl), and with anti-Tubulin (Sigma) and anti-ß-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 Image J software (NIH).

**Immunofluorescence**

Approximately 5x10^4 cells were seeded onto 18-mm glass coverslips. At 48 h later, cells were gamma irradiated at 6 Gy. The coverslips were washed in phosphate-buffered saline (PBS), the cells were fixed in cold 70% ethanol and permeabilized in 0.5% Triton X-100 solution for 15 min at room temperature. Cells were blocked with 10% FBS in PBS and incubated with primary antibody (or alexa phalloidin) for 1 h and with secondary antibody for 30 min 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, γH2Ax 1:1000 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 BRCAAness) 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 (BRCA1A5396T) and HCC1937-BRCA1MUT (BRCA15382insC) and the reconstituted HCC1937-BRCA1RECON (BRCA1WT) over 48 hours, IC50s (Table S1). Each of the BRCA1 competent lines (Figure 1A, C) displayed reduced sensitivity to all HDACis compared to the BRCA1 mutant cell lines (Figure 1B, D). VPA was the least effective HDACi across all cell lines. Interestingly VPA was 1.8X more effective against MDA-MB-436 (IC50 19.1μM) than to MDA-MB-231 (IC50 34μM) (Figure 1A vs B, Table S1), but displayed little difference in IC50 when comparing HCC1937-BRCA1RECON and HCC1937-BRCA1MUT, 23.6μM vs 21.7μM respectively (Figure 1C vs D, Table S1). MDA-MB-436 also displayed exquisite sensitivity to ROMI 0.9μM (Figure 1B), most likely due to an additional RB1 homozygous mutation (c.607-608ins227) in the cell line which renders tumours sensitive to changes in acetylation and is an effective treatment for retinoblastoma (15). We also analysed 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 to the BRCA1 competent cell lines (Figure 1E). We did not observe the 1000 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 (Figure 1E) (16).

HDAC inhibitors suppress homologous recombination 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 in situ 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 analysed from the IFP+ population while HR was analysed from the IFP/BFP double positive
cells (Figure 1F). Positive Cherry cells representing NHEJ activity could be isolated form IFP+ population and GFP+ cells representing HR could be isolated from IFP/BFP double positive cells (Figure 1G). We next compared the response of all 4 cell lines to the 3 HDACi and utilized the DNA-PK inhibitor (NU7441) and 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 RAD51i induced NHEJ in all cell lines with little or no HR. In MDA-MB-231 cells, enhanced GFP signal detected in response to all HDACis (Figure 1H). In the reciprocal mCherry readout, a compensation shift toward NHEJ activity compared to HR was observed in the presence of SAHA and ROMI (Figure 1H). Due to the BRCA1 mutation, MDA-MB-436 cells display almost no HR activity when treated with HDACis and thus were effective at inducing a NHEJ response (Figure 1I). Analysis of HCC1937 matched lines revealed, that the BRCA1RECOn displayed increased DNA repair at 24 hours compared to 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 (Figure 1J). VPA also induced NHEJ but to a lesser extent (Figure 1J). Analysis of BRCA1MUT revealed a similar response as MDA-MB-231 to all HDACi with activation of NHEJ (Figure 1K). This suggests a continued reliance on NHEJ, which was not altered by reconstitution with exogenous BRCA1 (Figure 1J vs 1K).

We confirmed HDACi-mediated repression of HR in MDA-MB-231 using the DR-GFP construct and ISCE enzyme as described (18)(Figure S1A-C). DRGFP and IF assays correlated well with the traffic light reporter assay when analysing 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 analysed the ability of HDACi to sensitize TNBC to PARP inhibition through repression of HR. We performed dose curves of each HDACi (doses 0 to ~IC20) in combination with or without the PARP inhibitor, ABT-888 (2.5μM). 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 (Figure 2A). In MDA-MB-231 cells, the pan-HDACi, SAHA and HDAC1/2 specific HDACi, ROMI, induced ~80% cell death at the highest dose of each drug in
combination with PARPi as measured by sub-\(G_1\) fraction (Figure 2B, D). VPA did not induce any significant change in cell cycle profile even when used at 20 \(\mu\)M (Figure 2C). Of note ROMI alone induced an increase in super-\(G_2\) or polyploidy population in MDA-MB-231, suggesting induction of a BRCAness profile, seen normally in BRCA1-mutated cells (Figure 2D).

To quantitatively measure the dose-escalation relationship of the drugs in combination for synergy (combination index), an effective calculation is the use of median-drug effect analysis (Table S2). SAHA displayed synergy with ABT-888 at 2.5\(\mu\)M, however when the order of drug incubation was reversed (incubation with PARPi followed by HDACi) this synergy was not observed CI=1.251 (Figure 2H, Table S2). Increasing doses of ROMI in the absence of ABT-888 resulted in an increased fraction of multinucleated cells, confirmed by immunofluorescence (Figure S2A) and synergy with 2.5\(\mu\)M ABT-888 at all doses 0.156-2.5\(\mu\)M (Figure 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 (Figure 2I). In MDA-MB-436 cells the combination of high dose SAHA and ABT-888 resulted in 37% cell death (Figure 2E) while VPA response was similar to that observed with MDA-MB-231 with little change in cell cycle profile across all doses (Figure 2F). High dose ROMI induced a G1 arrest independent of ABT-888 (Figure 2G), which coincided with 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 (Figure 2J). Taken together these results suggest that a fully competent HR pathway in TNBCs enables synergy with PARP inhibition after HDACi-mediated epigenetic changes, specifically observed with SAHA and ROMI, while a non-functional BRCA1/PTEN background reduces PARPi synergism with HDACi.

**Combined depletion of RAD51, FANCD2 and BARD1 is required to sensitize VPA to PARP inhibition.**

To determine if differences in regulation of histone modification and DNA damage response (DDR) gene expression by VPA compared to other HDACi provides an explanation for lack of
synergy with ABT-888, we treated MDA-MB-231 cells with each of the HDACi’s for 24 hours and analysed expression of 82 different DDR genes and found 28 to show varying levels of expression change. These genes display 3 profiles; 1) genes which were repressed by VPA and ROMI but not the pan-HDACi SAHA (Figure S2B), 2) genes with increased expression when treated with VPA compared to SAHA and ROMI (Figure 3A) and 3) genes that were not repressed as effectively by ROMI compared to SAHA and VPA (Figure 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 8 genes, 3 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 5 genes (ABL1, BARD1, MAPK12, MPG 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 synergised 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 to other two HDACi and induction of p21 expression (Figure 3B). Similar to mRNA expression observed in the array data, BARD1, FANCD2 and RAD51 protein expression was not repressed by VPA in MDA-MB-231 cells (Figure 3B) and confirmed in MDA-MB-436 cells (Figure 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, FANCD2 or BARD1. Individual gene knockdown did not induce sensitivity (Figure S2F-H), only with depletion of all three genes did we achieve significant cell death 64.2% vs 6.9% (*p=0.0006) (Figure 3C vs 3D). Restoration of sensitivity translated to significant long-term inhibition of growth, inhibiting 83% colony formation (**p=0.003) (Figure 3E). Interestingly the RAD51/FANCD2 combination also displayed significant inhibition of colony formation, 37% inhibition compared to 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) (Figure 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 an ongoing and recently terminated Phase II clinical trials against breast cancer (NCT01010854, NCT00395655). Our results suggest that RAD51, FANCD2 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. HCC1937- BRCA1\textsuperscript{MUT} cell line displayed a slight enhanced G1 population fraction in response to SAHA and an increased polyploid population after the addition of PARPi (Figure 4A), which was also observed with ROMI and PARPi (Figure 4C). We confirmed enhanced multinucleation by immunofluorescence (Figure S3). This was consistent with the assumption that mutant BRCA1 and loss of functional HR drives aberrant repair resulting in multinucleation. In contrast to the other HDACi, VPA (10μM ~IC20) in combination with PARPi induced a slight G1 arrest rather than increase in polyploid fraction (Figure 4B). This lack of enhanced polyploid population was also observed in the BRCA1 reconstituted HCC1937 cells treated with each of the HDACi in combination with PARPi (Figure 4D-F). However, compared to BRCA1\textsuperscript{MUT}, BRCA1\textsuperscript{RECON} still displayed comparable levels of cell death in response to each HDACi in combination with PARPi (Figure 4D-F). Interpretation of the mechanisms at play suggest that the combination treatment in the presence of faulty HR displays aberrant DNA repair functions resulting in an increased polyploid population, whereas in the presence of competent HR epigenetic regulation of DNA repair gene expression forces a potential G1 arrest.

To determine whether the partial G1 arrest observed in combination with VPA and PARPi in HCC1937-BRCA1\textsuperscript{MUT} is the cause of ineffective therapeutic response and could be overcome we utilized the CHK1 inhibitor (AZD7762). Treatment with CHK1i alone induced a loss of S-phase population and enhanced G2 population, likely due to faster progression into G2/M phase while PARPi alone induced a slight increase in polyploidy population (Figure 4G and 4B). The combination of CHKi and PARPi induced a G2 arrest, which was also observed with CHK1i and VPA (Figure 4G). Most impressively the combination of CHKi, PARPi and VPA resulted in an enhanced polyploid population, similar to that observed with each of SAHA and ROMI with PARPi (Figure 4A and 4C). We suggest that inhibition of the G1-checkpoint and the consequent down regulation of DNA repair via VPA/ABT-888 combination on a BRCA1 functionally deficient background results in an unstable polyploidy population. In BRCA\textsuperscript{MUT} cells HDACi, SAHA and ROMI in combination with ABT-888 resulted in 20.5% and 11.8%
colony survival, respectively after 14 days (Figure 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 (Figure 4I). Taken together these results suggest that epigenetic BRCAness induced 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 HDAC/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 reconstitution of BRCA1 renders the HDACi or the combination of HDACi and PARPi less effective in HCC1937 cells, we analysed the gene expression changes in response to ROMI comparing BRCA1\textsuperscript{RECON} to BRCA1\textsuperscript{MUT}. Reconstitution of BRCA1 changes the cellular response from an increase in polyploidy population to an increased G1 population (Figure 4C vs 4F). The cytotoxic activity of PARP 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 lack of PARP activity, while loss of PTEN is thought to inhibit response (21). The observed G1 arrest in response to ROMI in cells with reconstituted BRCA1 (Figure 4F) is readily accounted for by the changes in gene expression including \textit{BARD1} a ubiquitin ligase that binds \textit{BRCA1} and regulates cell cycle and DNA repair, \textit{CHEK1} a cell cycle kinase that regulates checkpoint arrest and \textit{FANCD2} that controls chromosome stability (Figure 4J). Of note \textit{BRCA1} expression was increased validating the cell line genetics (Fig 4J). Interestingly a similar arrest was observed in MDA-MB-436 in response to ROMI, suggesting a greater role for the aforementioned \textit{BARD1}, \textit{CHEK1} and \textit{FANCD2} genes (Figure 2G). In contrast, the absence of functional \textit{BRCA1} in BRCA1\textsuperscript{MUT} cells meant 18% progressed through mitosis to a polyploidy state in the presence of ROMI (Figure 4C) and were unable to form colonies (Figure 4H). In this case we observed downregulation of DNA repair gene expression including HR regulators \textit{ATRIP}, \textit{RAD50}, \textit{RAD51}, \textit{RBBP8} and \textit{XRCC3}, NHEJ regulator \textit{PNKP}, the helicase \textit{BLM} and \textit{CIB1}, which is a negative regulator of p38 MAPK survival signaling (Figure 4K). Overall comparison of the
gene expression response to ROMI in HCC1937 demonstrates a BARD1-CHEKI-mediated G1-arrest in the presence of BRCA1 reducing effectiveness of PARPi-activity and a shift in the DNA repair profile towards mutagenic-NHEJ via down regulation of HR specific genes. This provides the genetic basis of our observations for the NHEJ shift observed with the HDACis in Figure 1F-M.

**Multinucleation is associated with high RAD51 expression and resistance to PARP inhibition.**

Treatment of HCC1937-BRCA1\(^{MUT}\) with ROMI and ABT-888 resulted in an enhanced polyploidy/multinucleation population (Figure 4C). This phenomenon was not observed in HCC1937-BRCA1\(^{RECON}\), most likely related to reduced HR activity (Figure 1K) and was similar to that seen in MDA-MB-231 (Fig 1I). To examine whether this polyploid population contributed to the small resistant population observed in colony assays (Figure 4G), we sorted the polyploid population post treatment (Figure 5A), confirmed multinucleated cells (Figure S3) and compared growth rate to unsorted cells. The polyploidy purified population only achieved 5% growth over 5 days compared to over 20% for unsorted cells (Figure 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). Protein expression analysis revealed no corresponding increase in FANCD2, BARD1 or PARP1 but enhanced RAD51 expression in the polyploid population (Figure 5C), which was confirmed by intracellular FACS staining in all cell lines (Figure 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 (Figure 5E). Increased expression of RAD51 independent of PARP1 expression increased IC50 of ABT-888 3.9 fold from 15.2μM vs 59.1μM (Figure 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 MDAM-MB-231 xenografts (19). There are several current 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 benefitted 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 in from HR to mutagenic-NHEJ activity (30). This creates the conditions required for PARP inhibition-mediated synthetic lethality and a rationale for use of HDACi to create an epigenetic-mediated BRCAness via downregulation of key HR gene expression. We observed the requirement for down regulation of RAD51, BARD1 and FANCD2 expression for effective HDAC inhibitor activity and a role for RAD51 in mechanisms of resistance to PARP 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 tumours compensating for the loss of HR, allowing carcinogenic progression and metastasis (31). Inhibition of RAD51 has been found to synergize with inhibition of PARP in both RNA knockdown screens and small molecule screens (32-34). Our study supports these observations with RAD51 overexpression enhancing resistance to PARPi. Further 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 pathological recombination events such as chromosomal amplifications, deletions and translocations resulting in loss of heterozygosity and aneuploidy (35). 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 co-operative 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 infer resistance to PARP inhibition.

In the breast cancer setting increased expression of FANCD2 is associated with high proliferation rates and replication stress (36) and associates with BRCA1 and RAD51 at sites of DNA damage to guarantee the integrity of genome during replication (37, 38). Interestingly in a panel of 1240 paraffin-embedded breast tumors, overexpression of FANCD2 was shown to be prognostic for poor patient survival whereas RAD51 was not (39). However this observation was in hormone receptor positive breast cancer, whereas RAD51 has been associated with poorer prognosis in TNBC (29). 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 auto-transcriptional 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 co-factor 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 (40) and recently complementation of BRCA1 deficient cell lines with FANCD2 was shown to restore normal HR function (41). We suggest that RAD51 is the main contributor to PARPi resistance as the depletion of RAD51 has been shown to be more potent than that of FANCD2 in sensitizing HeLa cells to PARPi (34).

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 (42), which directs HR-mediated repair via the BRCA1-BRCA2 linker protein PALB2 (43). Of importance is the observation that HDACi-mediated stabilization of mutant BRCA1 protein via hyperacetylation of Hsp90 (19), can enhance resistance to PARPi via stabilization of BRCA1/BARD1-PALB2-BRCA2/RAD51 complexes (44). This could account for our observed reduced efficacy of the HDACi/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 non-functional PTEN. The co-incidental 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 (45).

There is also a direct interplay between 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 to 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 (46). Of note BRCA1-independent functions of BARD1 have also been reported (47). 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.

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References


Table 1. Polyploidy and RAD51 expression infer resistance to PARP inhibition

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC50 Romidepsin</th>
<th>IC50 ABT-888</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Treated</td>
<td>10.62 μM</td>
<td>18.95 μM</td>
</tr>
<tr>
<td>Treated non sorted</td>
<td>8.75 μM</td>
<td>19.54 μM</td>
</tr>
<tr>
<td>Polyploid sorted</td>
<td>8.15 μM</td>
<td>27.20 μM</td>
</tr>
<tr>
<td>BRCA1 reconstituted</td>
<td>6.25 μM</td>
<td>52.13 μM</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. HDAC inhibitors change the profile of DDR from HR to NHEJ. MTS metabolism dose response curves (0-20μM) for three different HDACi (SAHA, VPA and ROMI) were determined for the TNBC cell lines (A) MDA-MB-231, (B) MDA-MB-436, (C) HCC1937-BRCA1REC and (D) HCC1937-BRCA1MUT. (E) The aforementioned cell lines were analysed for response to PARPi (ABT-888, 0-20μM). All experiments were performed in triplicate and plots represent mean ±SEM. A “traffic light” dual reporter system was utilized to assess simultaneous HR and NHEj readout. (F) Untreated cells were analysed as control, (G) IFP tagged ISCE construct and BFP GFP donor constructs were incorporated into target cell lines. Analysis of HR and NHEJ in response to various drugs was performed in (H) MDA-MB-231, (I) MDA-MB-436, (J) HCC1937-BRCA1REC, (K) HCC1937-BRCA1MUT. All cell lines were treated with individual HDACi at IC20 for 24 hours, DNA-PK inhibitor (0.1μM-NU7441) served as a control for inhibition of NHEJ, RAD51 inhibitor (10μM-B02) served as control for inhibition of HR, while –ISCE served as a negative control and +ISCE as a negative drug treatment control. Plots represent 1x10⁴ gated events.

Figure 2. HDAC inhibition and PARP inhibition synergize to kill TNBC. (A) The treatment schedule of pre-incubation with one of three HDACi for 24 hours followed by replacement of the media with PARP inhibitor for a further 48 hours or DMSO in controls. Cell cycle profile of MDA-MB-231 cells were analysed in response to (B) SAHA, (C) VPA and (D) ROMI. MDA-MB-436 cells were analysed in response to (E) SAHA, (F) VPA and (G) ROMI. All dose curves were formulated using concentrations diluted 2 fold from ∼IC20. (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 analysed after 72 hours. Surviving fractions of cells were analysed 14 days post-treatment in B-G highest doses for (I) MDA-MB-231 and (J) MDA-MB-436. Colony counts were expressed as a fraction of DMSO treated controls and represent mean ±SEM of triplicate experiments.

Figure 3. Effective killing of MDA-MB-231 cells requires loss of RAD51, FANCD2 and BARD1. (A) RNA expression analysis of DNA damage response (DDR) genes after treatment of MDA-MB-231 cells with HDACis. Fold change was normalized to DMSO treated controls and actin housekeeping gene. (B) Protein expression analysis of key DDR genes in response to ∼IC20 of HDACi in MDA-MB-231 cells. Cell cycle profile of VPA and ABT-888 treated
MDA-MB-231 cells with (C) a control non-targeting siRNA and (D) siRNA targeting RAD51, FANCD2 and BARD1. (E) Surviving fractions of cells were analysed after 14 days post-treatment VPA and siRNA. Colony counts were expressed as a fraction of DMSO treated controls and represent mean ±SEM of triplicate experiments *p=0.026,**p=0.003. (F) We analysed grade 3, TNBC probability of patient disease free survival based on gene expression of RAD51 (p=0.049), FANCD2 and BARD1.

Figure 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 (A) SAHA, (B) VPA and (C) ROMI. HCC1937-BRCA1RECON cells were analysed in response to (D) SAHA, (E) VPA and (F) ROMI. All dose curves were formulated using concentrations diluted 2 fold from ∼IC20. (G) Cell cycle profile of HCC1937-BRCA1MUT cells treated with as per (B) with the addition of CHK1i (AZD7762). Treatment of Surviving fractions of cells incubated with the highest dose drug combinations were analysed after 14 days post-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 DNA damage response (DDR) genes comparing reconstitution of BRCA1 in HCC1937-BRCA1RECON cells standardized to baseline expression in HCC1937-BRCA1MUT cells with (J) enhanced expression with reconstitution of BRCA1 and (K) reduced expression after reconstitution of BRCA1.

Figure 5. The polyploid population does not contribute to long-term cell survival but enhances resistance to PARP inhibition. (A) The resulting polyploidy population from HCC1937-BRCA1MUT treated with romidpesin/ABT-888 combination was isolated and compared to unsorted and untreated controls. (B) In situ analysis of realtime growth rates of polyploid population compared to an unsorted population over 4.5 days using Incucyte imaging. (C) Protein expression of key DDR proteins required for HDACi efficacy were analysed 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 (Grey 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.
Figure 1.

A. MDA-MB-231

B. MDA-MB-436

C. HCC1937-BRCA1\text{RECON}

D. HCC1937-BRCA1\text{MUT}

E. MDA-MB-231

F. MDA-MB-436

G. HCC1937-BRCA1\text{RECON}

H. MDA-MB-231

I. MDA-MB-436

J. HCC1937-BRCA1\text{RECON}

K. HCC1937-BRCA1\text{MUT}
Figure 2.

A. HDACi

- PARPi or DMSO

0  12  24  36  48  72 (Hrs)

B. Vorinostat (SAHA) +DMSO +2.5μM ABT-888

MDA-MB-231

% Cell number

0.3125 0.625 1.25 2.5 5.0 10.0 20.0 μM

C. Valproic Acid (VPA) +DMSO +2.5μM ABT-888

MDA-MB-231

% Cell number

0.3125 0.625 1.25 2.5 5.0 10.0 20.0 μM

D. Romidepsin (ROMI) +DMSO +2.5μM ABT-888

MDA-MB-231

% Cell number

0.3125 0.625 1.25 2.5 5.0 10.0 20.0 μM

E. Vorinostat (SAHA) +DMSO +2.5μM ABT-888

MDA-MB-436

% Cell number

0.3125 0.625 1.25 2.5 5.0 10.0 20.0 μM

F. Valproic Acid (VPA) +DMSO +2.5μM ABT-888

MDA-MB-436

% Cell number

0.3125 0.625 1.25 2.5 5.0 10.0 20.0 μM

G. Romidepsin (ROMI) +DMSO +2.5μM ABT-888

MDA-MB-436

% Cell number

0.3125 0.625 1.25 2.5 5.0 10.0 20.0 μM

H. ABT-888 +DMSO +2.5μM SAHA

MDA-MB-231

% Cell number

0.15625 0.3125 0.625 1.25 2.5 5.0 10.0 20.0 μM

I. Untreated -PARPi +PARPi

Survival Fraction

DMSO SAHA VPA ROMI

J. Untreated -PARPi +PARPi

Survival Fraction

DMSO SAHA VPA ROMI

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Figure 4.

A. Vorinostat (SAHA) +DMSO +2.5μM ABT-888

B. Valproic Acid (VPA) +DMSO +2.5μM ABT-888

C. Romidepsin (ROMI) +DMSO +2.5μM ABT-888

D. Vorinostat (SAHA) +DMSO +2.5μM ABT-888

E. Valproic Acid (VPA) +DMSO +2.5μM ABT-888

F. Romidepsin (ROMI) +DMSO +2.5μM ABT-888

G. Vorinostat (SAHA) +DMSO +10μM VPA

H. Survival Fraction

I. Survival Fraction

J. Relative Gene Expression fold change

K. Relative Gene Expression fold change

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Molecular Cancer Therapeutics

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