Synergistic Loss of Prostate Cancer Cell Viability by Coinhibition of HDAC and PARP

Olivia S. Chao1 and Oscar B. Goodman Jr1,2

Abstract

Tumors with BRCA germline mutations are defective in repairing DNA double-strand breaks (DSB) through homologous recombination (HR) pathways, making them sensitive to PARP inhibitors (PARPi). However, BRCA germline mutations are rare in prostate cancer limiting the ability to therapeutically target these pathways. This study investigates whether histone deacetylase (HDAC) inhibitors (HDACi), reported to modulate DSB repair pathways in sporadic cancers, can downregulate DSB repair pathways and sensitize prostate cancer cells to PARPi. Prostate cancer cells cotreated with the HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA) and the PARPi, olaparib, demonstrated a synergistic decrease in cell viability compared with single-agent treatment (combination index < 0.9), whereas normal prostatic cells did not. Similarly, clonogenicity was significantly decreased after cotreatment. Flow cytometric cell-cycle analysis and Annexin-V staining revealed significant apoptosis upon treatment with SAHA + olaparib. This coincided with increased DNA damage observed by immunofluorescence microscopy analysis of γH2AX foci, a marker of DSBs. In addition, immunoblot analysis showed a significant and persistent increase in nuclear γH2AX levels. Both SAHA and olaparib downregulated the expression of HR-related proteins, BRCA1 and RAD51, whereas SAHA + olaparib had an additive effect on RAD51. Silencing RAD51 sensitized prostate cancer cells to SAHA and olaparib alone. Collectively, cotreatment with HDACi and PARPi downregulated HR-related protein expression and concomitantly increased DNA damage, resulting in prostate cancer cell death.

Implications: These findings provide a strong rationale for supporting the use of combined HDAC and PARP inhibition in treating advanced prostate cancer. Mol Cancer Res; 12(12); 1755–66. ©2014 AACR.

Introduction

Prostate cancer is the most commonly diagnosed solid malignancy and second leading cause of cancer-specific mortality in American men (1). Although few initially present with distant disease, up to 40% of patients will eventually develop metastatic disease despite local therapy (2). Currently, the standard treatment for metastatic prostate cancer is androgen deprivation therapy; however, most patients will progress to develop incurable castration-resistant prostate cancer (CRPC). Although overall survival in prostate cancer has improved modestly, a recent study of 19,336 men with de novo metastatic prostate cancer found that the mortality rate from advanced prostate cancer remained mostly unchanged over the past 25 years (3), underscoring the need for novel therapeutic approaches.

Advanced prostate cancers often display increased genomic instability that can be attributed to defects in DNA damage response (4). Paradoxically, these cancers go on to develop dependencies on certain DNA repair pathways to prevent excessive DNA damage in the tumor cells. As these defects are specific to tumor cells, targeting them should offer an enhanced therapeutic window and avoid unwanted toxicities in normal tissue. DNA double-strand breaks (DSB), considered the most lethal form of DNA damage, are repaired by the either the error-prone non-homologous end joining (NHEJ), which involves KU70/80, DNA-PK, and DNA ligase IV, or the high fidelity homologous recombination (HR) mechanism mediated by BRCA1, BRCA2, and RAD51. Cancers with mutations causing deletion or functional impairment of critical DNA damage response proteins, including BRCA1/2, have defective DNA damage response making them highly sensitive to DNA perturbations.

PARP inhibitors (PARPi) have garnered much interest as a novel class of anticancer therapeutics due to their clinical activity in breast and ovarian cancers carrying BRCA germ-line mutations (5–7). Tumors with BRCA1 mutations are exquisitely sensitive to PARPi through the mechanism of

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synthetic lethality, whereby the perturbation of one gene (or DNA repair pathway) is nontoxic but the simultaneous perturbation of two genes or pathways results in cellular death (8). PARP catalyzes the polymerization of PAR on target proteins and activates DNA damage response to repair single-strand breaks via nucleotide excision repair or base excision repair pathways. Inhibition of PARP leads to accumulation of single-strand DNA breaks, resulting in collapsed replication forks and formation of potentially lethal DSBs. Tumors with BRCA deficiency are defective in repairing DSBs and thus sensitive to PARP inhibition. Olaparib is an oral PARPi and the first to reach human clinical trials. A phase I study of olaparib in patients with BRCA1/2-mutated tumors resulted in substantial clinical responses in several subjects, including a patient with metastatic CRPC with *BRCA2* mutation who experienced >50% PSA drop with radiographic resolution of bone metastases (6). In a follow-up phase II study, olaparib monotherapy yielded positive clinical response in 50% of the 8 patients with advanced prostate cancer with *BRCA1/2* mutations and halted disease progression for at least 8 weeks in 25% of the patients.

While germline *BRCA* mutations are rare in prostate cancer (0.44% for *BRCA1*; 1%–2% for *BRCA2*), castration-resistant prostate cancers often harbor genetic or epigenetic modifications that affect genes involved in HR DNA repair (9). Consequently, these tumors may display HR deficiency or "BRCAness," referring to the phenotypic traits that sporadic cancers share with *BRCA*-mutated tumors (10). While there are significant efforts to identify other predictive biomarkers of HR deficiency, others have developed strategies to pharmacologically manipulate HR repair for sensitization to PARPi. One emerging class of therapeutic agents is the histone deacetylase (HDAC) inhibitors. Suberoylanilide hydroxamic acid, SAHA (vorinostat) is a widely studied and well-tolerated pan-HDAC inhibitor that is clinically approved for treatment of cutaneous T-cell lymphoma (11). An important observation with HDAC inhibitor reported in preclinical studies is that they can induce loss of viability in transformed cells at concentrations which have little effect on normal cells (12, 13), making them ideal for cancer therapy. Unfortunately, HDAC inhibitors as monotherapy in solid tumors, including prostate cancers, (14) have been disappointing; however, they appear to be more effective when combined with other drugs. HDACs regulate gene expression by modifying the chromatin structure as well as regulating the function of nonhistone proteins (15). HDAC inhibition has been reported to downregulate the expression of proteins involved in HR leading to impaired repair of DSBs, i.e., increase "BRCAness" in various types of cancers (16–19). We hypothesize that HDAC inhibition by SAHA can modulate the expression of proteins involved in DSB repair and abrogate DNA damage response in prostate cancer cells, thereby sensitizing them to PARP inhibition.

In the present study, we demonstrate that cotreatment with SAHA and olaparib synergistically decrease cell viability and induce apoptosis in prostate cancer cells. Furthermore, both SAHA and olaparib as single agents downregulate HR-related protein expression and cotreatment lead to increased DNA damage. Our results provide a rationale for combining HDAC inhibitors and PARPi as a novel therapeutic approach in the treatment of sporadic prostate cancers.

**Materials and Methods**

**Cell lines, antibodies, and reagents**

DU145, PC-3, and RWPE-1 cell lines were obtained from ATCC and grown in 5% CO₂, 37°C incubator. DU145 and PC-3 cell lines were cultured in RPMI1640 medium supplemented with 10% FBS and penicillin/streptomycin antibiotics. RWPE-1 cells were cultured in keratinocyte serum-free medium supplemented with 0.05 mg/mL bovine pituitary extract and 5 ng/mL human recombinant EGF (Invitrogen). SAHA and olaparib (AZD2281) was purchased from ChemieTek.

**Cell viability assay**

Cell viability was assessed with the standard MTT colorimetric assay (20). To test the sensitivity of prostate cells to SAHA and olaparib, cells were treated with SAHA (0.1–2.5 μmol/L) or olaparib (0.1–50 μmol/L) and cell viability was assessed at day 0, 1, 3, and 5. To determine the potential synergy between SAHA and olaparib, prostate cells were treated with specified doses of the drugs, alone or in combination, for 3 days. Doses of each drug were added in a constant ratio, and combination index (CI) values were calculated. The assays were performed in triplicates at least three times.

**Clonogenic survival assay**

For clonogenic assays, cells were plated in 6-well plates overnight, SAHA and olaparib were then added at indicated doses as single agent or in combination for 3 days. Fresh growth media with olaparib or vehicle were added to the cells for another two days before replacing with growth media. After 5 days, cells were fixed and stained with 0.75% crystal violet solution in 50% ethanol and 1.75% formaldehyde. Colonies with >50 cells were scored under the microscope and survival fraction was calculated as the average number of colonies in drug-treated over vehicle-treated wells.

**Apoptosis assay**

Apoptosis was assessed by two methods: cell-cycle analysis of sub-G₁ population in propidium iodide (PI)-stained cells and analysis of FITC-Annexin-V/PI staining. For cell-cycle analyses, after drug treatment for 5 days, cells were collected and fixed with 70% ethanol overnight at −20°C. Cells were then stained with PI and DNA content/cell of 10,000 events was analyzed using a BD Accuri C6 flow cytometer (BD Biosciences). Percentage apoptosis was quantified as the percentage of population in sub-G₁ phase. Annexin-V/PI staining was performed using the FITC Annexin-V Apoptosis Detection Kit I (BD Pharmingen) as per manufacturer’s instruction. Briefly, cells were incubated in FITC Annexin-V solution and PI staining solution for 15 minutes at room temperature in the dark and then analyzed immediately using a BD Accuri C6 flow cytometer. The
percentage of apoptotic cells was defined as the population of cells that are FITC Annexin-V<sup>+</sup> and PI<sup>−</sup>.

**γH2AX immunofluorescence staining**

Cells were grown in poly-d-lysine-coated Nunc Lab-Tek II Chamber Slide (Thermo Scientific) and treated with SAHA and/or olaparib for 48 hours. After treatment, cells were fixed in ice-cold methanol, permeabilized with 0.5% Triton X-100 and then incubated with mouse anti-γH2AX (phosphoS139, Abcam) overnight. Cells were then washed and incubated with goat anti-mouse IgG TRITC (Abcam) secondary antibody and nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI).

**Confocal microscopy and foci analysis**

Image acquisition was performed with a Nikon A1R confocal imaging system (Nikon Instruments Inc.) using a galvano scanner and a Nikon CFI Apochromat TIRF 60×/1.49 oil objective lens. A minimum of 50 cells were imaged per sample. Image processing and foci analysis were performed using Fiji software (21). Details of image acquisition and foci analysis can be found in Supplementary Materials and Materials. Nucleus with ≥10 foci was considered positive for DNA damage.

**Small RNA interference**

Cells were transfected with siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen) as per manufacturer's instructions. After 16 hours, the culture media were replaced with normal growth media and incubated for 24 hours before analyzing for levels of gene silencing by Western blotting. For experiments with drug treatments, SAHA and olaparib were added to the cells 48 hours after transfection and treated for 3 days before subjecting cells to flow cytometric analysis for apoptosis. The siRNAs used in the experiments were: siGENOME Human RAD51 Smartpool, siGENOME Human BRCA1 smartpool, and ON-TARGETplus Non-Targeting pool (Dharmacon), each consisting of a mixture of 4 target siRNAs.

**Statistical analysis**

Each experiment was repeated at least three times independently. Results were analyzed using MS Excel and presented as mean ± SD or SEM as indicated. Statistical significance was calculated by Student t test using two-tailed analysis. P values of less than 0.05 were considered significant. CI was calculated using the CalcuSyn software (Biosoft) based on the Chou–Talalay method (22). A CI < 0.9 indicates synergism, CI = 0.9–1.1 indicates additivity, and CI > 1.1 indicates antagonism at the effective dose (ED) for 50%, 75%, 90%, and 95% of the fraction affected.

**Results**

Prostate cancer cells are more sensitive to SAHA than normal prostate epithelial cells

We first determined the effect of SAHA and olaparib individually on cell viability in two androgen-independent prostate cancer cell lines, DU145 and PC-3, and a normal prostatic epithelial cell line, RWPE-1. Cells were grown in increasing concentration of SAHA, ranging from 0.1 μmol/L to 10 μmol/L for up to 5 days (Supplementary Fig. S1A) and cell viability was determined using the standard MTT assay. After 72 hours of incubation with SAHA, DU145 showed higher sensitivity (IC<sub>50</sub> = 0.9 μmol/L) than PC-3 (IC<sub>50</sub> = 1.6 μmol/L), whereas RWPE-1 showed the least sensitivity (IC<sub>50</sub> = 8.5 μmol/L; Fig. 1A). This result is consistent with prior findings that showed normal prostate cells to be more resistant to SAHA-induced cell death than transformed cells (12). Conversely, all three prostate cell lines showed relative resistance to olaparib, with less than 50% loss of cell viability after 72 hours of incubation with olaparib (0.1–50 μmol/L; Fig. 1B). PC-3 demonstrated a higher sensitivity at the lower doses of olaparib tested. Prolonged incubation with high dose of olaparib (50 μmol/L) did decrease cell viability markedly in DU145 and RWPE-1 cells (Supplementary Fig. S1B). However, this dose was much higher than the highest concentration (20 μmol/L) used in the subsequent combination studies, and is beyond therapeutic levels achieved in the plasma in the clinical settings (23).

Cell viability in prostate cancer cells synergistically decreased with SAHA and olaparib treatment

Next, DU145, PC-3, and RWPE-1 cells were treated with SAHA and olaparib alone and the combination of SAHA and olaparib (SAHA + olaparib) at the indicated doses.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Sensitivity of prostate cancer and normal prostate epithelial cells to SAHA and olaparib. DU145, PC-3, and RWPE-1 were grown increasing dose of (A) SAHA (0.1 μmol/L to 10 μmol/L) and (B) olaparib (0.1 μmol/L–50 μmol/L) for 3 days. Cell viability was determined using MTT assay. Graph represents mean cell viability (%) ± SD of three experiments performed in octuplicates.
Figure 2. Cotreatment of SAHA and olaparib synergistically decrease cell viability in prostate cancer cells. Prostate cell lines were treated with SAHA and olaparib (OLA), alone or in combination, at indicated doses and cell viability was measured by MTT assay after 72 hours. SAHA and olaparib concentrations were kept at a constant ratio of 1:20 for DU145 (A) and 1:4 for PC-3 (C). (Continued on the following page.)
SAHA and olaparib concentrations were adjusted to maintain a constant ratio of SAHA:olaparib ratio at 1:20 for DU145 cells and 1:4 for PC-3 cells due to the relatively higher sensitivity of PC-3 to olaparib. After 72 hours of drug exposure, simultaneous treatment with SAHA and olaparib resulted in significantly decreased cell viability compared with each drug alone for DU145 and PC-3 cells (Fig. 2A and C). RWPE-1 cells treated with the same drug combination concentrations did not show significant decrease in cell viability compared with the single agents (Fig. 2B and D). Table 1 shows the CI for ED50, ED75, ED90, and ED95 for DU145, PC-3, and RWPE-1 cells treated with the combination of SAHA and olaparib. The combined treatment was synergistic in DU145 and PC-3 (CI < 0.9) compared with RWPE-1. The ability of SAHA to sensitize prostate cancer cells to olaparib was confirmed with clonogenic survival assays. Prostate cancer cells were incubated with SAHA and olaparib for 5 days before replacing with growth media for another 5 days. In both DU145 and PC-3 cells, the combination of SAHA + olaparib significantly reduced clonogenicity compared with either drug alone for all the doses tested (Fig. 2E and F). Similar to the MTT assay, CI values for ED50, ED75, and ED90 were all <0.9 (Supplementary Table S1) indicating a synergistic response to the drug combination. Notably, the differences in survival fractions of combined drug treatment versus the single agent were more dramatic in the clonogenic assay relative to the MTT assay. While this is partly reflective of the differing treatment schedules, clonogenic assays provide a better indication of long-term cell viability and are independent of cell death pathways. Assessment of histone H4 acetylation and PAR levels in the cell lysate of treated DU145 and PC-3 cells confirmed the specificity of SAHA and olaparib in targeting HDACs and PARP, respectively, at the doses used in viability assays (Fig. 2G and H).

**Table 1.** Prostate cells were treated with SAHA and olaparib in a constant ratio and CI values were calculated at the effective dose (ED) for 50%, 75%, 90%, and 95% of the fraction affected.

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>CI values</th>
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<tbody>
<tr>
<td></td>
<td>ED50</td>
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<tr>
<td>SAHA: Olaparib (1:20)</td>
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<tr>
<td>DU145</td>
<td>0.87</td>
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<tr>
<td>RWPE-1</td>
<td>1.30</td>
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<tr>
<td>PC-3 (1:4)</td>
<td>0.66</td>
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<tr>
<td>RWPE-1 (1:4)</td>
<td>1.48</td>
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NOTE: CI < 0.9 indicates synergism, CI = 0.9–1.1 indicates additivity, and CI > 1.1 indicates antagonism.

**Cotreatment with SAHA and olaparib induced apoptosis in prostate cancer cells**

To investigate whether synergistic loss of cell viability in prostate cancer cells was due to apoptotic cell death, prostate cancer cells were treated with SAHA (0.5–1 μmol/L) and olaparib (10–20 μmol/L) alone or in combination for 5 days and cell-cycle analysis was performed. Analysis of the sub-G1 apoptotic population showed that SAHA + olaparib induced 14% apoptosis compared with 4% in SAHA (0.5 μmol/L) or 5% in olaparib (10 μmol/L) alone. At higher concentration of SAHA (1 μmol/L) and olaparib (20 μmol/L), there was an 8% and 7% apoptotic population, respectively, and an increase to 41% apoptosis in the combination treatment (Fig. 3A and B). Interestingly, cell-cycle analysis demonstrated lack of sub-G1 population in PC-3 cells treated with doses of SAHA + olaparib that decreased cell viability, indicating apoptosis is not the mechanism of cell death (data not shown). To confirm the drug treatments were inducing apoptosis in DU145 cells, flow cytometric analysis of FITC Annexin-V staining was performed. Results showed that there was minimal apoptosis occurring after a 12-hour incubation for all treatment conditions (Fig. 3C). A significant extent of apoptosis was only detected after 24 hours, with significantly higher apoptosis in SAHA + olaparib-treated cells (40%) compared with those treated with SAHA (13%) and olaparib (15%) alone after 72 hours of incubation (Fig. 3C and D). These findings suggest that SAHA in combination with olaparib induced apoptosis in DU145 cells in a manner consistent with replication-dependent cell death as the early apoptotic events were only observed after 24 hours of drug incubation.

**Cotreatment with SAHA and olaparib result in increased DNA damage**

When DSBs occur, one of the earliest DNA damage responses is H2AX phosphorylation at Ser139 (γH2AX) to form distinct foci at the site of DSBs (24). As shown in Fig. 4A, in DU145 cells, combined treatment with SAHA and olaparib induced formation of γH2AX foci, observed as punctate staining in the nuclei of a majority of cells, indicating DNA damage. Comparatively, there were fewer cells demonstrating similar γH2AX staining when SAHA and olaparib were used alone. Analysis of foci number per cell demonstrated that more than 60% of the cells had formed distinct foci at the site of DSBs (24). As shown in Fig. 4A, in DU145 cells, combined treatment with SAHA and olaparib induced formation of γH2AX foci, observed as punctate staining in the nuclei of a majority of cells, indicating DNA damage. Comparatively, there were fewer cells demonstrating similar γH2AX staining when SAHA and olaparib were used alone. Analysis of foci number per cell demonstrated that more than 60% of the cells had formed distinct foci at the site of DSBs (24). As shown in Fig. 4A, in DU145 cells, combined treatment with SAHA and olaparib induced formation of γH2AX foci, observed as punctate staining in the nuclei of a majority of cells, indicating DNA damage. Comparatively, there were fewer cells demonstrating similar γH2AX staining when SAHA and olaparib were used alone. Analysis of foci number per cell demonstrated that more than 60% of the cells had formed distinct foci at the site of DSBs (24). As shown in Fig. 4A, in DU145 cells, combined treatment with SAHA and olaparib induced formation of γH2AX foci, observed as punctate staining in the nuclei of a majority of cells, indicating DNA damage. Comparatively, there were fewer cells demonstrating similar γH2AX staining when SAHA and olaparib were used alone. Analysis of foci number per cell demonstrated that more than 60% of the cells had formed distinct foci at the site of DSBs (24). As shown in Fig. 4A, in DU145 cells, combined treatment with SAHA and olaparib induced formation of γH2AX foci, observed as punctate staining in the nuclei of a majority of cells, indicating DNA damage. Comparatively, there were fewer cells demonstrating similar γH2AX staining when SAHA and olaparib were used alone. Analysis of foci number per cell demonstrated that more than 60% of the cells had formed distinct foci at the site of DSBs (24). As shown in Fig. 4A, in DU145 cells, combined treatment with SAHA and olaparib induced formation of γH2AX foci, observed as punctate staining in the nuclei of a majority of cells, indicating DNA damage. Comparatively, there were fewer cells demonstrating similar γH2AX staining when SAHA and olaparib were used alone. Analysis of foci number per cell demonstrated that more than 60% of the cells had formed distinct foci at the site of DSBs (24). As shown in Fig. 4A, in DU145 cells, combined treatment with SAHA and olaparib induced formation of γH2AX foci, observed as punctate staining in the nuclei of a majority of cells, indicating DNA damage. Comparatively, there were fewer cells demonstrating similar γH2AX staining when SAHA and olaparib were used alone. Analysis of foci number per cell demonstrated that more than 60% of the cells had formed distinct foci at the site of DSBs (24). As shown in Fig. 4A, in DU145 cells, combined treatment with SAHA and olaparib induced formation of γH2AX foci, observed as punctate staining in the nuclei of a majority of cells, indicating DNA damage. Comparatively, there were fewer cells demonstrating similar γH2AX staining when SAHA and olaparib were used alone. Analysis of foci number per cell demonstrated that more than 60% of the cells had formed distinct foci at the site of DSBs (24).
SAHA and olaparib decrease HR DNA repair proteins

HDAC inhibitors have been reported to downregulate HR-related proteins. We next investigated whether SAHA was sensitizing prostate cancer cells to olaparib and enhancing DNA damage by regulating the expression of HR-related proteins. DU145 cells were incubated with increasing concentrations of SAHA (0.25–2.0 μmol/L) and cell fractionation was performed at 24 and 48 hours to assess BRCA1 and RAD51 protein expression in the nucleus and cytoplasm. SAHA decreased RAD51 expression in both nuclear and cytoplasmic fraction maximally at 24 hours, with 1 μmol/L of SAHA reducing nuclear and cytoplasmic RAD51 to approximately 50% and 60% of vehicle control, respectively (Fig. 5A and Supplementary Fig. S3A and B). However, RAD51 repression by SAHA reverted after 48 hours. Concomitantly, BRCA1 protein expression in the nucleus was also downregulated with increasing concentrations of SAHA, demonstrating a decrease to approximately 50% of control after 48-hour incubation with 1 μmol/L of SAHA (Fig. 5B and Supplementary Fig. S3C). BRCA1 protein was undetectable in the cytoplasmic fraction even in vehicle-treated samples, indicating an accumulation of BRCA1 in the nucleus of DU145 cells. Interestingly, olaparib also downregulated RAD51 and BRCA1 expression after 48-hour treatment. As shown in Fig. 5C, olaparib (5–20 μmol/L) decreased both nuclear and cytoplasmic RAD51 by 40% to 50% (Fig. 5C and Supplementary Fig. S3D). In addition, nuclear BRCA1 expression was reduced to approximately 30% of control with low dose of olaparib (5 μmol/L), which did not reduce cell viability significantly, whereas higher doses of olaparib (10–20 μmol/L) reduced BRCA1 expression to <20% of control (Fig. 5D and Supplementary Fig. S3E). There was no significant change in RAD51 and BRCA1 protein expression at 24-hour treatment with olaparib (data not shown). As both SAHA and olaparib downregulated RAD51 and BRCA1, we examined whether the SAHA + olaparib combined treatment had an additive effect on protein expression. At 48 hours, SAHA + olaparib decreased nuclear RAD51 expression to approximately 40% of control, compared with approximately 70% for SAHA (1 μmol/L) or approximately 65% for olaparib (20 μmol/L) alone, indicating an additive effect on RAD51 downregulation (P < 0.01 vs. SAHA; P < 0.03 vs. olaparib; Fig. 5E and F). Downregulation of BRCA1 by olaparib (20 μmol/L) at 48 hours was greater than 80% and was not enhanced with addition of SAHA (Fig. 5E and G). Together, these results demonstrate that both HDAC and PARPi are able to independently downregulate HR-related proteins, albeit with variable kinetics and potency.

Knockdown of BRCA1 and RAD51 expression increases sensitivity to SAHA and olaparib

To confirm the role of BRCA1 and RAD51 in modulating the sensitivity of prostate cancer cells to HDAC and PARPi, we knocked down the expression of BRCA1 and RAD51 by RNA interference, treated the cells with SAHA or olaparib, and assessed for apoptosis. DU145 cells were transfected with siRNA targeting BRCA1 or RAD51, and immunoblot analysis was performed 48 hours after transfection. Immunoblot analysis performed 48 hours after transfection showed >90% reduction in nuclear BRCA1 protein levels, whereas cytoplasmic and nuclear RAD51 levels were not significantly affected in DU145 cells transfected with BRCA1 siRNA compared with nontargeting siRNA (NT; Fig. 6A, lane 1 vs. lane 2). Similarly, RAD51 siRNA reduced nuclear and cytoplasmic RAD51 expression by >80% and 90% of NT control, respectively (Fig. 6A, lane 1 vs. lane 3). SAHA (1 μmol/L) and olaparib (20 μmol/L) were added to the cells 48 hours after transfection and incubated for 3 days before assessing for apoptosis. Flow cytometry analysis of Annexin-V positively stained cells showed that knocking down BRCA1 and RAD51 protein expression alone induced increased apoptosis in vehicle-treated cells (nontarget, 3%; BRCA1, 13%; RAD51, 14%), underlining the role of BRCA1 and RAD51 proteins in protecting the cells from naturally occurring DNA damage (Fig. 6B). Taking into account the higher basal apoptotic levels in BRCA1 and RAD51 knockdowns (%apoptosis of vehicle-treated samples were deducted from the drug-treated samples for each respective siRNA), the results show that knocking down RAD51 sensitized prostate cancer to SAHA and olaparib as single agents (Fig. 6C). Moreover, knocking down RAD51 further enhanced apoptosis in SAHA + olaparib–treated cells, which may be attributed to a more complete repression of RAD51 protein, compared with the inhibitors alone, or is indicative of other targets of SAHA and olaparib. Knocking down BRCA1 expression increased apoptosis compared with NT control in olaparib–treated cells but not SAHA-treated cells. These results suggest that RAD51 protein level is critical in protecting prostate cancer cells from apoptosis caused by SAHA + olaparib, whereas there is some redundancy for BRCA1. Knocking down BRCA1 in SAHA + olaparib–treated cells had a protective effect (compare nontarget vs. BRCA1 siRNA in SAHA + olaparib). This seems to coincide with the pleiotropic roles of 1760 Mol Cancer Res; 12(12) December 2014 Molecular Cancer Research Published OnlineFirst August 15, 2014; DOI: 10.1158/1541-7786.MCR-14-0173

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BRCA1 protein in DNA damage repair, cell-cycle checkpoint regulation, and transcriptional regulation (25). Together with other proteins in the DNA damage response pathways, BRCA1 has been shown to have dual roles, both as part of the DNA repair machinery and as inducer of apoptosis when there is excessive DNA damage (26).

**Discussion**

The landmark finding of the synthetically lethal relationship between PARP inhibition and BRCA1/2 mutations has led to renewed interest in the therapeutic potential of PARPi (27, 28). Although clinical successes have been limited to cancers with germline BRCA1/2 mutations, there is mounting evidence supporting the more general utilization of PARPi in advanced sporadic cancers. In this study, we demonstrate that HDAC inhibitors can sensitize prostate cancer cells to the PARPi, olaparib. HDAC inhibitor and PARPi as single agents were minimally effective in killing prostate cancer cells at the concentration utilized, but the combination resulted in synergistic loss of cell viability. For DU145 cells in particular, we observed significantly higher...
A

Vehicle

SAHA

Olaparib

SAHA + Olaparib

B

DU145

\% γH2AX* cells

Vehicle SAHA OLA SAHA + OLA

RWPE1

\% γH2AX* cells

Vehicle SAHA OLA SAHA + OLA

C

24 h

SAHA: - + - +
Olaparib: - + - +
γH2AX
Lamin A/C

48 h

24 h

Fold-change in expression

Vehicle SAHA OLA SAHA + OLA

48 h

Fold-change in expression

Vehicle SAHA OLA SAHA + OLA

D

\% TAIL DNA

Vehicle SAHA OLA SAHA + OLA

Olaparib SAHA + Olaparib
apoptotic cell death in the combined treatment which correlated with enhanced DSBs at 48 hours which has been associated with DNA damage-induced apoptosis (29). Moreover, normal prostate epithelial cells are less sensitive than prostate cancer cells to the combined effects of HDAC and PARP inhibition. Similarly, in human fibroblasts, resistance to HDAC inhibitors was attributed to their ability to resolve DSBs compared with transformed cells, in which DNA repair proteins like Rad50 and Mre11 were suppressed by the inhibitor (30). Apoptosis was not detected in PC-3 treated with SAHA \(\text{+}\) olaparib suggesting a different mode of cell death is responsible for the loss of cell viability. One possible mechanism of cell death is autophagy, a nonapoptotic programmed cell death. Autophagy has been reported to play a role in HDAC inhibitor–induced cell death of PC-3M prostate cancer cells (31) and MCF-7 breast cancer cells (32). However, induction of autophagy by HDAC inhibitors was shown to have both pro-cell death and prosurvival functions in cancer cells (33). Further investigation into the modes of cell death would help in the understanding of the molecular events leading to HDAC and PARP inhibition–induced cell death.

With respect to the DNA damage produced by the combination of HDAC and PARP inhibition, we show that

Figure 5. SAHA and olaparib decrease HR DNA repair proteins. DU145 cells were treated with increasing concentration of (A and B) SAHA (0.25–2.0 \(\mu\)mol/L) or (C and D) olaparib (OLA; 5–20 \(\mu\)mol/L), and BRCA1 and RAD51 nuclear and cytoplasmic protein expression was assessed by Western blotting. Fold change in protein expression over untreated control normalized to either lamin A/C or \(\alpha\)-tubulin for nuclear and cytoplasmic fraction, respectively, is shown in Supplementary Fig. S2. Western blot analysis and fold change in protein expression over untreated control (vehicle) for nuclear RAD51 (E and F) and BRCA1 (G and H) after 48-hour treatment with SAHA (1 \(\mu\)mol/L), olaparib (20 \(\mu\)mol/L), and the combination is shown. *, \(P < 0.01\) (SAHA vs. SAHA \(\text{+}\) olaparib); **, \(P < 0.03\) (olaparib vs. SAHA \(\text{+}\) olaparib).

Figure 4. Cotreatment of SAHA and olaparib (OLA) leads to accumulation of unrepaired DNA damage. A, immunofluorescence staining of \(\gamma\)H2AX foci (red punctate staining) at the site of DSBs in DU145 and RWPE1 cells treated with SAHA (1 \(\mu\)mol/L), olaparib (20 \(\mu\)mol/L), and the combination after 48 hours. Nuclei were counterstained with DAPI. Z-stack images were acquired with a confocal laser scanning microscope and maximum intensity projection images of \(\gamma\)H2AX and foci immunofluorescence are shown. B, percentage of \(\gamma\)H2AX \(\text{+}\) cell (\(\geq\)10 foci/cell) for DU145 and RWPE1 cells under different treatment conditions. C, Western blot analysis and corresponding densitometry plot showing increase in nuclear \(\gamma\)H2AX protein expression of DU145 cells treated with SAHA and olaparib and the combination at 24 and 48 hours. D, mean of % tail DNA \(\pm\) SEM of DU145 cells subjected to alkaline comet assay 24 hours after treatment. Representative comet images for each treatment are shown. *, \(P < 0.001\) (compared with SAHA \(\text{+}\) olaparib). **, \(P < 0.001\) (SAHA vs. SAHA \(\text{+}\) olaparib).
proteins involved in HR repair of DSBs, BRCA1, and RAD51, were downregulated by both inhibitors. This is consistent with several studies that demonstrated HDAC inhibitors suppress HR gene expression through the E2F1 transcriptional regulator (17), translational repression via miRNAs such as miR182 (34), as well as posttranslational degradation through hyperacetylation of Hsp90 (35). Nonetheless, antineoplastic mechanism of action for HDAC inhibitors has not been well elucidated, and due to their broad range of possible targets, it is likely the SAHA may have affected the function of other proteins involved in DNA damage response. Notably, HDAC inhibitors have also been shown to target proteins involved in NHEJ. In these studies, NHEJ-related proteins, KU70, KU86, and DNA-PK were downregulated in irradiated melanoma cells, whereas RAD51 and DNA-PK were shown to be downregulated in irradiated prostate cancer cells upon HDAC inhibition, resulting in radiosensitization of the cells (16, 36). However, in another study, microarray data analysis of DU145 and PC-3 cells treated with 1 μmol/L SAHA or 1 mmol/L valproic acid, levels of KU, and DNA-PK were not affected, whereas there was downregulation of RAD51 and related genes, including BRCA1 (17). Therefore, functional assessment of the specific DNA repair pathways as well as the mechanism underlying the downregulation of specific repair genes upon HDAC inhibition will help in elucidating the role of HDAC inhibitors in modulating DNA damage response.

Interestingly, PARP inhibition was also independently found to downregulate BRCA1 and RAD51 protein levels in prostate cancer cells. This suppression of BRCA1 and RAD51 by PARPi was previously reported in a study using 6(5H)-phenanthridinone (PHEN) in colon and lung cancer cell lines (37) whereby PHEN increased the occupancy of the repressive transcription regulators E2F4/p130 complex on the promoters of BRCA1 and RAD51. This mechanism has also been attributed the downregulation of BRCA1 and RAD51 by hypoxia (38, 39) and is associated with specific histone modifications (40). Under hypoxic conditions, both E2F1 and E2F4 bind to BRCA1 promoter at two adjacent E2F sites to repress gene transcription. In our system, the cotreatment of HDAC and PARPi led to a robust downregulation of RAD51 compared with the modest effect by the inhibitors individually. The involvement of E2F transcription regulators in both HDAC- and PARP-mediated regulation of BRCA1 and RAD51 expression present a possible cooperation/interaction between the two mechanisms. Undoubtedly, there are other mechanisms whereby HDACs and PARPs regulate protein expression, complementing an ability to promote an active chromatin structure that is permissive for transcription (41).

Knockdown experiments showed loss of RAD51 led to sensitivity to SAHA and olaparib as single agents, as well as enhanced apoptotic cell death in the SAHA + olaparib cotreatment. These results support a critical role of RAD51 in preventing DNA-damage induced apoptosis in prostate cancer.
cancer cells. Comparative analysis of LNCaP, DU145, PC-3, and normal prostate epithelial cell line, PrEC, demonstrated significantly elevated levels of RAD51 mRNA and protein expression in the malignant cell lines (4). This genetic alteration seems to indicate that RAD51 or HR is important for their survival. Indeed, stable cell lines of LNCaP engineered to have lower levels RAD51 demonstrated increased radiosensitivity (42). In addition, down-regulation of RAD51 by imatinib, a receptor tyrosine kinase inhibitor, sensitized PC-3 cells to ionizing radiation, mitomycin C, and gemcitabine (43). RAD51 has been reported to be overexpressed in high-grade prostate cancer (Gleason score >7), both in sporadic and those with germline BRCA mutations (44). Overexpression of RAD51 is seen in many forms of human cancers (45) and is clinically correlated with a more aggressive phenotype and decreased patient survival (44, 46, 47). Tumors with increased levels of RAD51 typically have higher HR activity which likely contributes to drug resistance. Our proposed combination HDAC and PARP inhibition downregulates RAD51 and therefore may be beneficial for prostate cancer overexpressing RAD51. On the other hand, loss of BRCA1 increased sensitivity to olaparib slightly but not to SAHA, and conferred some resistance to apotosis in SAHA + olaparib–treated cells, implying that BRCA1 is not critical in preventing DNA-damage induced apoptosis and suggests redundancy in its role in HR repair in prostate cancer cells. In certain BRCA-1–deficient breast tumors, overexpression of RAD51 has been shown to override the lack of BRCA1 in terms of its impact on cell proliferation, DNA damage HR repair, and survival (48).

A recent clinical trial with niraparib/MK4827 was the first study to report clinical activity of a PARPi in sporadic CRPC without BRCA mutations, where 9 of 21 (43%) patients experienced disease control (49), suggesting that BRCA-proficient patients with HR defects are susceptible to PARP inhibition. Our data indicate that HDAC inhibitor in combination with PARPi can additively downregulate HR proteins, especially RAD51, and induce apoptosis in prostate cancer cells. These findings support further evaluation of the therapeutic potential of combined PARP and HDAC inhibition for the treatment of prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest were disclosed.

Authors' Contributions
Conception and design: O.S. Chao, O.B. Goodman
Development of methodology: O.S. Chao, O.B. Goodman
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O.S. Chao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O.S. Chao
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Synergistic Loss of Prostate Cancer Cell Viability by Coinhibition of HDAC and PARP

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