

Suberoylanilide hydroxamic acid (vorinostat) represses androgen receptor expression and acts synergistically with an androgen receptor antagonist to inhibit prostate cancer cell proliferation

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Abstract

Growth of prostate cancer cells is initially dependent on androgens, and androgen ablation therapy is used to control tumor growth. Unfortunately, resistance to androgen ablation therapy inevitably occurs, and there is an urgent need for better treatments for advanced prostate cancer. Histone deacetylase inhibitors, such as suberoylanilide hydroxamic acid (SAHA; vorinostat), are promising agents for the treatment of a range of malignancies, including prostate cancer. SAHA inhibited growth of the androgen-responsive LNCaP prostate cancer cell line at

low micromolar concentrations and induced caspase-dependent apoptosis associated with chromatin condensation, DNA fragmentation, and mitochondrial membrane depolarization at higher concentrations ($\geq 5 \mu\text{mol/L}$). Gene profiling and immunoblot analyses showed a decrease in androgen receptor (AR) mRNA and protein in LNCaP cells cultured with SAHA compared with control cells, with a corresponding decrease in levels of the AR-regulated gene, prostate-specific antigen. Culture of LNCaP cells in steroid-free medium markedly sensitized the cells to SAHA. Moreover, a combination of low, subeffective doses of SAHA and the AR antagonist bicalutamide resulted in a synergistic reduction in cell proliferation and increase in caspase-dependent cell death. Addition of exogenous androgen prevented the induction of cell death, indicating that suppression of androgen signaling was required for synergy. At the subeffective concentrations, these agents had no effect, alone or in combination, on proliferation or death of AR-negative PC-3 prostate cancer cells. Our findings indicate that SAHA is effective in targeting the AR signaling axis and that androgen deprivation sensitizes prostate cancer cells to SAHA. Consequently, combinatorial treatments that target different components of the AR pathway may afford a more effective strategy to control the growth of prostate cancer cells. [Mol Cancer Ther 2007;6(1):51–60]

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Introduction

Prostate cancer is a serious health concern in Western countries, being the second most common cause of deaths from cancer in men (1). For patients with biochemical relapse following primary treatment with surgery and/or radiation, or who present with metastatic disease, androgen ablation is the standard first-line treatment. Although the majority of these patients respond initially to androgen ablation, virtually all tumors eventually progress with castration-resistant disease (2). Although there have been recent reports of significant responses with the use of cytotoxic agents in the treatment of castration-resistant disease (3), there remains an urgent need for new therapeutic strategies in both an adjuvant setting and following failure of androgen ablation therapy. Histone deacetylase (HDAC) inhibitors are promising novel agents for the treatment of solid tumors (4–8). Antiproliferative and proapoptotic effects of several HDAC inhibitors, including trichostatin A, depsipeptide, MS-275, sodium butyrate, valproic acid, pyroxamide, and suberoylanilide hydroxamic acid (SAHA), have been reported for prostate cancer cell lines (9–12). These agents also have efficacy in

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xenograft models (13–16) and are currently being investigated in clinical trials for the treatment of solid tumors (17–19).

We have shown that SAHA can inhibit the growth of prostate cancer cell lines *in vitro* and suppresses the growth of the androgen-dependent human prostate cancer xenograft, CWR22, at high doses (16). Moreover, we found that androgen-independent cell lines were less sensitive to growth inhibition and cell death induced by SAHA than the androgen-responsive LNCaP cells (16). Therefore, in this study, we investigated whether the sensitivity of the LNCaP prostate cancer cell line to SAHA is dependent on the presence of a functional androgen signaling axis and whether the combination of low doses of both SAHA and a specific androgen receptor (AR) antagonist would result in enhanced growth suppression and/or apoptosis.

Materials and Methods

Cells and Reagents

LNCaP and PC-3 human prostate carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 supplemented with 10% or 5% fetal bovine serum, respectively. SAHA (now designated by the generic name vorinostat) was synthesized as described previously (20) and dissolved in DMSO. Bicalutamide was obtained from Sigma (St. Louis, MO) and dissolved in ethanol. Anti-AR (C-19), anti-prostate-specific antigen (PSA; C-19), anti-cyclin D1 (A-12), and anti-p21^{WAF1} (AB-3) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-calnexin (MA3-027) antibody was obtained from Affinity Bioreagents (Golden, CO). Anti-Her2/*neu* (Ab-15) was obtained from Lab Vision (Fremont, CA). Horseradish peroxidase-conjugated secondary antirabbit and anti-mouse antibodies were obtained from DAKO (Carpinteria, CA), and secondary antisheep antibody was obtained from Chemicon (Melbourne, Victoria, Australia). The tetrapeptide caspase inhibitor z-VAD-fmk was obtained from Calbiochem (Alexandria, New South Wales, Australia).

Measurement of Cell Viability

Cells were seeded in triplicate in 24-well dishes at a density of 2.5×10^4 per well. Cells were allowed to attach for 48 h before incubation with fresh medium containing SAHA (0, 0.5, 1, 2.5, 5, 7.5, or 10 $\mu\text{mol/L}$) or bicalutamide (0, 1.25, or 2.5 $\mu\text{mol/L}$). Cells were counted using a hemocytometer at 24- or 48-h intervals after initiation of treatment, and cell viability was assessed by trypan blue dye exclusion as described previously (16). Data are expressed as the mean \pm the SE of triplicate wells and are representative of at least three independent experiments.

4',6-Diamidino-2-Phenylindole Staining of Nuclei

Cells were seeded on plastic chamber slides and treated as indicated. After two washes with PBS, cells were fixed in methanol for 5 min, washed again with PBS, and incubated with 0.8 mg/mL 4',6-diamidino-2-phenylindole (DAPI, Roche Diagnostics, Castle Hill, New South Wales,

Australia) in PBS for 15 min at 37°C. After several washes in PBS, the coverslips were mounted on PBS/glycerin. DAPI staining was visualized by fluorescence microscopy.

Measurement of Mitochondrial Membrane Potential

Cells (5×10^5) seeded in 10-cm plates were treated as indicated for up to 48 h. Cells were harvested by trypsinization and resuspended in complete tissue culture medium containing 2 $\mu\text{g/mL}$ rhodamine 123 dye (kindly provided by Prof. Sharad Kumar, Hanson Institute, Adelaide, South Australia, Australia). Cells were incubated at 37°C for 20 min in the dark and then washed twice in PBS. Cells were resuspended in 1 mL PBS containing the viability dye 7-aminoactinomycin D (2 $\mu\text{g/mL}$) and incubated at room temperature for 10 min. Cell fluorescence was analyzed by flow cytometry, with green fluorescence (rhodamine 123) measured in cells with intact cellular membranes (i.e., 7-aminoactinomycin D negative).

Cell Cycle Analysis

Fluorescence-activated cell sorting analysis of cell cycle distribution and sub-G₁ fraction was done on untreated and SAHA-treated cells after 24 to 96 h of culture, using standard histogram analysis as described previously (12).

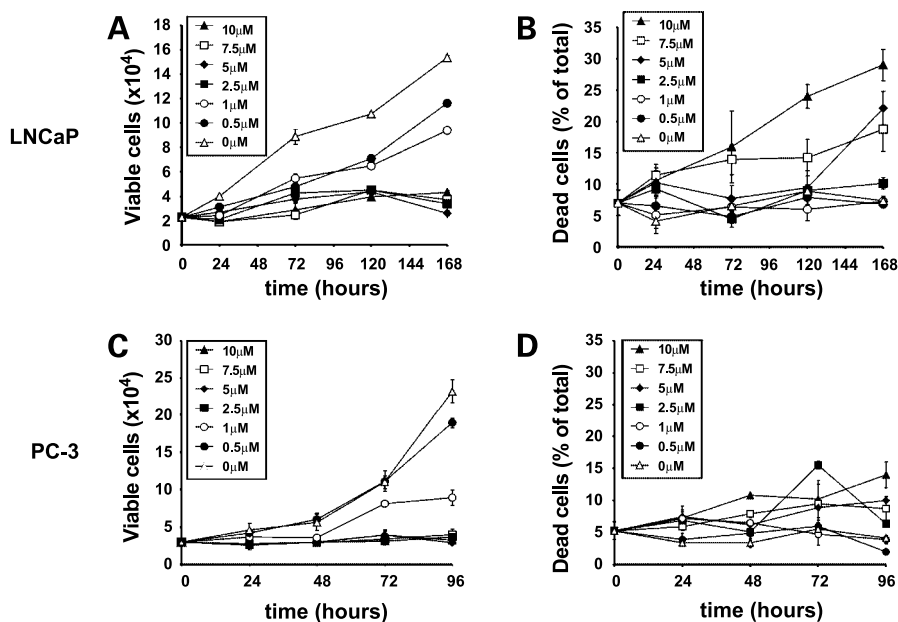
Measurement of DEVD-Caspase Activity

DEVD-caspase activity was assayed by cleavage of z-asp-glu-val-asp-7-amino-4-trifluoro-methyl-coumarin, a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of poly(ADP-ribose) polymerase. Cells (1.5×10^4) seeded in 96-well plates were cultured with SAHA (0, 2.5, 5, or 7.5 $\mu\text{mol/L}$). After 48 h, the cells were lysed in 50 μL NP40 lysis buffer [5 mmol/L Tris-HCl, 5 mmol/L EDTA, 0.5% NP40 (pH 7.5)] and stored at -70°C until assayed. Cell lysates (20 μL) were added to each assay tube containing 8 $\mu\text{mol/L}$ substrate in 200 μL fluorometric caspase assay buffer [50 mmol/L HEPES, 10% sucrose of substrate in 1 mL protease buffer (50 mmol/L HEPES, 10% sucrose, 10 mmol/L DTT, 0.1% CHAPS (pH 7.4))]. After incubation for 4 h at room temperature, fluorescence was quantified (excitation of 400 nm and emission of 505 nm) in a Perkin-Elmer LS50 spectrofluorometer (Wellesley, MA). One unit of caspase activity was taken as one fluorescence unit (at slit widths of 10 nm) per 4 h of incubation with substrate. The tetrapeptide caspase inhibitor z-VAD-fmk was dissolved in DMSO and added to cells at a final concentration of 50 $\mu\text{mol/L}$, 30 min before addition of SAHA. Control cells were incubated with DMSO at the same concentration.

Microarray Analysis

LNCaP cells were cultured with 2.5 or 7.5 $\mu\text{mol/L}$ SAHA for 6 h. Total RNA was isolated from the cells using Trizol reagent (Invitrogen, Carlsbad, CA). Poly(A)⁺ mRNA was isolated from the total RNA using Oligotex columns (Qiagen, Valencia, CA). Poly(A)⁺ mRNA from cells cultured with SAHA was compared with mRNA from cells cultured in the absence of SAHA, using the UniGEM human cDNA version 2.0 array, which contained 9,182 cDNA probes representing 8,372 individual genes/

Figure 1. Inhibition of LNCaP and PC-3 prostate cancer cell growth by SAHA. LNCaP and PC-3 cells (2.5×10^4 per well in 24-well plates) were cultured with SAHA (0, 0.5, 1, 2.5, 5, 7.5, or 10 $\mu\text{mol/L}$) in RPMI 1640 containing 10% FCS, for up to 7 d. Cells were counted every day (PC-3) or every second day (LNCaP) using a hemocytometer, and cell viability was assessed by trypan blue dye exclusion (A and C). The number of dead cells is expressed as a percentage of total cells counted (B and D). Representative of at least three independent experiments. Points, mean of triplicate wells; bars, SE.



expressed sequence tags and 192 internal controls (Incyte, St. Louis, MO). The results were analyzed using GEM Tools image and data analysis software, and a 2-fold change was considered as a threshold for regulation of gene expression.

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay lysis buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L

EDTA, 1% Triton X-100) containing mini-complete protease inhibitor pellets (Roche, Mannheim, Germany). Lysates (20 μg) were electrophoresed through 7.5% or 15% polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, England), and blocked in 3% nonfat milk powder in TBS containing 0.05% Tween 20 overnight.

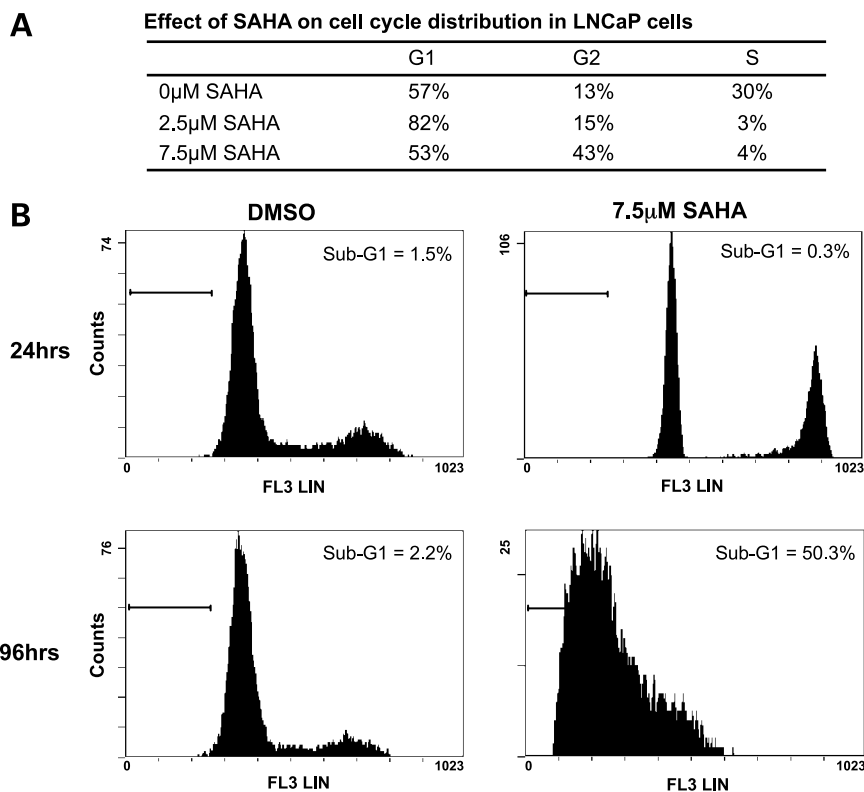


Figure 2. Effect of SAHA on cell cycle distribution in LNCaP cells. **A**, cells cultured with SAHA (0, 2.5, or 7.5 $\mu\text{mol/L}$) for 24 h were harvested by trypsinization, fixed in 70% ethanol, and stained with propidium iodide. Cell cycle distribution was determined by flow cytometry. **B**, cells cultured in the absence or presence of SAHA (7.5 $\mu\text{mol/L}$) for 24 h or 4 d were harvested and fixed as described above and stained with propidium iodide. The fraction of hypodiploid (sub-G₁) nuclei was measured by flow cytometry using standard histogram analysis.

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Immunodetection was done for 1 h at room temperature in 1% nonfat milk powder in TBS containing 0.05% Tween 20, using anti-AR, anti-PSA, anti-Her2/*neu*, anti-p21^{WAF1}, and anti-cyclin D1 antibodies diluted according to the manufacturer's instructions. Membranes were incubated with anti-calnexin as a loading control. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and visualized on X-ray film using the enhanced chemiluminescence detection system (Amersham Biosciences) as described previously (21).

Evaluation of Interaction between SAHA and the Anti-Androgen Bicalutamide

Dose-response curves were generated for bicalutamide and SAHA, both independently and in combination. The effect of combining the two drugs was assessed by the isobole method (22), using the equation $A_c / A_e + B_c / B_e = D$, to determine if their activity was synergistic. A_c and B_c represent the concentration of drug A and drug B used in the combination, and A_e and B_e represent the concentration of drug A and B that produced the same magnitude of effect when administered alone. If D , the combination index, is <1 , then the drugs are considered to act synergistically. If the combination index is ≥ 1 , then the drugs act in an antagonistic or additive manner, respectively.

Results

SAHA Suppresses Growth and Induces Cell Cycle Arrest and Death of LNCaP Prostate Cancer Cells

A dose-dependent inhibition of LNCaP cell growth was observed with concentrations of SAHA ranging from 0.5 to 10 $\mu\text{mol/L}$ (Fig. 1A). Although growth inhibition was observed with 0.5 and 1 $\mu\text{mol/L}$ doses of SAHA over the 7 days of experimental period (Fig. 1A), maximal reduction of cell number was achieved with 2.5 $\mu\text{mol/L}$ and greater concentrations of SAHA. Minimal cell death was observed with 0.5 to 2.5 $\mu\text{mol/L}$ of SAHA, but 5 to 10 $\mu\text{mol/L}$ markedly induced LNCaP cell death at 7 days posttreatment (Fig. 1B). Whereas a dose-dependent inhibition of PC-3 cell growth was also observed with SAHA (Fig. 1C), there was little effect on PC-3 cell death, even with the highest concentration of SAHA (10 $\mu\text{mol/L}$; Fig. 1D).

To further investigate the mechanism of SAHA-induced suppression of cell growth, we analyzed cell cycle distribution in control- and SAHA-treated cells. After 24-h culture with 2.5 $\mu\text{mol/L}$ SAHA, LNCaP cells were arrested in the G_1 phase of the cell cycle (Fig. 2A). This block in cell cycle progression was maintained for at least 72 h (data not shown). In contrast, LNCaP cells cultured with 7.5 $\mu\text{mol/L}$ SAHA for 24 h showed a shift in cell cycle from S phase to the G_2 -M phase, with no change in the proportion of cells in G_1 (Fig. 2A and B). Whereas there was no significant change in the sub- G_1 nuclear fraction in cells cultured with 7.5 $\mu\text{mol/L}$ SAHA for 24 h (Fig. 2B, top), an increased sub- G_1 fraction, indicative of DNA fragmentation, was observed in cells cultured with 7.5 $\mu\text{mol/L}$ SAHA for 4 days (Fig. 2B, bottom).

Cell Death Induced by SAHA Is Characteristic of Apoptosis

SAHA induced caspase-3 activity in LNCaP cells in a dose-dependent manner (Fig. 3A). Caspase-3 activity was blocked when cells were cocultured with the z-VAD-fmk caspase inhibitor. Moreover, addition of the caspase inhibitor prevented cell death induced by either 2.5 or 7.5 $\mu\text{mol/L}$ SAHA (Fig. 3B). Staining of cellular DNA with DAPI showed that LNCaP cells cultured with SAHA (7.5 $\mu\text{mol/L}$) for 24 h exhibited chromatin condensation and nuclear fragmentation (Fig. 3C), characteristic of apoptosis. Mitochondrial membrane potential was measured in

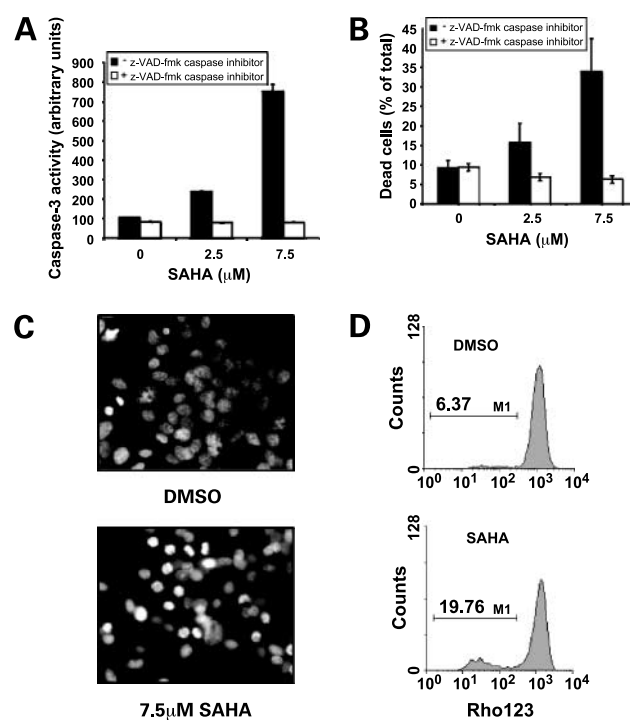
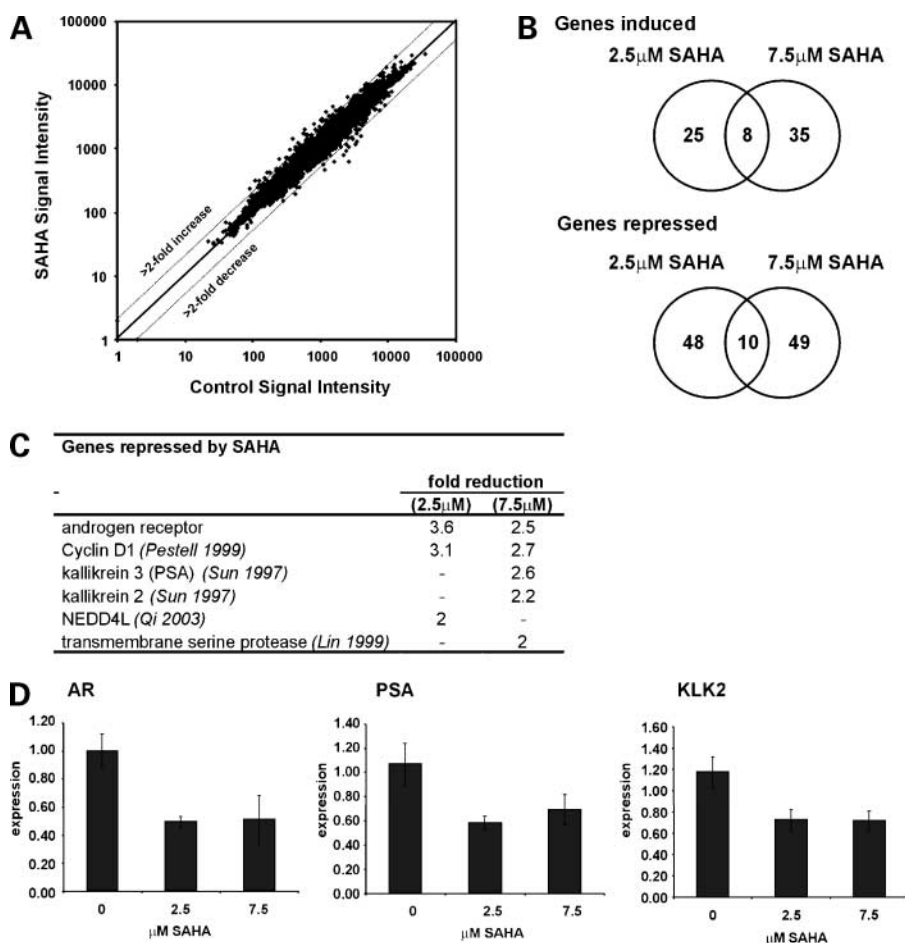


Figure 3. Induction of LNCaP cell death by SAHA. **A**, LNCaP cells (2.5×10^4 per well in 24-well plates) were cultured with SAHA (0, 2.5, or 7.5 $\mu\text{mol/L}$) with or without the z-VAD-fmk caspase inhibitor for 48 h and assayed for caspase-3 activity. **B**, LNCaP cells (2.5×10^4) were cultured with SAHA (0, 2.5, or 7.5 $\mu\text{mol/L}$) with or without the z-VAD-fmk caspase inhibitor for 5 d. Cells were counted at day 5 using a hemocytometer, and cell viability was assessed by trypan blue exclusion. The number of dead cells expressed as a percentage of total cells was counted. Columns, mean of triplicate wells in a representative experiment; bars, SE. **C**, DAPI staining of cell nuclei. LNCaP cells were seeded onto plastic coverslips and cultured with vehicle alone (DMSO) or SAHA (7.5 $\mu\text{mol/L}$) for 24 h. Cells were fixed with methanol and incubated with DAPI, before washing in PBS and mounting on PBS/glycerin. DAPI staining was visualized by fluorescence microscopy. **D**, effect of SAHA on mitochondrial membrane potential in LNCaP cells. Cells were cultured with vehicle alone (DMSO) or SAHA (7.5 $\mu\text{mol/L}$) for 48 h. Cells were harvested by trypsinization, resuspended in RPMI 1640 containing 10% FCS, and incubated with the mitochondrial dye rhodamine 123 (Rho123; 2 $\mu\text{g/mL}$) for 20 min at 37°C. Cells were washed and incubated in PBS containing 2 $\mu\text{g/mL}$ of the viability dye 7-aminoactinomycin D at room temperature for 10 min and then analyzed by flow cytometry. Results shown indicate the level of rhodamine 123 fluorescence in the 7-aminoactinomycin D-negative population.

Figure 4. Summary of gene expression changes in LNCaP cells following SAHA treatment. **A**, scatter plot of control intensity versus SAHA (2.5 $\mu\text{mol/L}$) intensity for all elements on the UniGEM version 2.0 human cDNA microarray, which contains 8,372 unique gene sequences. **B**, Venn diagrams depicting the number of genes with ≥ 2 -fold changes in mRNA levels. *Top*, the number of genes induced; *bottom*, the number of genes repressed by 2.5 and/or 7.5 $\mu\text{mol/L}$ SAHA. **C**, summary of SAHA-induced alterations in expression of genes involved in androgen signaling. **D**, real-time PCR analysis of AR, PSA, and kallikrein 2 (*KLK2*) mRNA levels 2 h following treatment with SAHA.



cells cultured in the presence or absence of SAHA (7.5 $\mu\text{mol/L}$), using the lipophilic, green fluorescent dye rhodamine 123. A 3-fold increase in rhodamine-negative cells, indicating loss of mitochondrial membrane potential, was observed in cells cultured with SAHA for either 24 h (data not shown) or 48 h compared with cells cultured with vehicle alone (vehicle control, 6.4%, versus SAHA, 19.7%; Fig. 3D).

Gene Expression Profiles in LNCaP Prostate Cancer Cells Cultured with SAHA

Gene expression profiles were examined following culture of LNCaP cells for 6 h with low (2.5 $\mu\text{mol/L}$) or high (7.5 $\mu\text{mol/L}$) concentrations of SAHA. Scatter plots of control hybridization intensity versus SAHA hybridization intensity showed that the expression of the majority of genes either was not altered or was altered by < 2 -fold (Fig. 4A). As shown by the Venn diagrams (Fig. 4B), a > 2 -fold change in gene expression was observed for only $\sim 1\%$ of the total number of genes analyzed following culture with 2.5 or 7.5 $\mu\text{mol/L}$ SAHA. The majority of genes identified as being modulated by SAHA in LNCaP cells are involved in signal transduction, transcription, or translation (see Supplementary Tables). The expression levels of the known SAHA target genes, *cyclin D1* and *thioredoxin-*

binding protein-2/VDUP1, were decreased and increased, respectively (see Supplementary Tables).⁶ Interestingly, a subset of genes involved in AR signaling was reduced in expression by at least 2-fold following culture with SAHA (Fig. 4C; refs. 23–28). Of particular interest, the mRNA levels of the AR and the AR-regulated genes, *PSA* (*kallikrein 3*) and *kallikrein 2*, were reduced in cells cultured with SAHA. Whereas expression of AR was decreased by both 2.5 and 7.5 $\mu\text{mol/L}$ SAHA, the expression of PSA and kallikrein 2 was only decreased by 7.5 $\mu\text{mol/L}$ SAHA. Real-time reverse transcription PCR confirmed that the levels of AR, PSA, and kallikrein 2 mRNA were reduced in cells cultured with 7.5 $\mu\text{mol/L}$ SAHA for 2 h (Fig. 4D).

To determine whether the gene expression data identified by the microarray and mRNA expression analyses resulted in biologically meaningful changes in protein expression in the cell, the effects of SAHA on AR and PSA expression were evaluated by immunoblot analysis (Fig. 5). AR steady-state protein levels were markedly reduced in a dose-dependent manner for up to 48 h, with a

⁶ Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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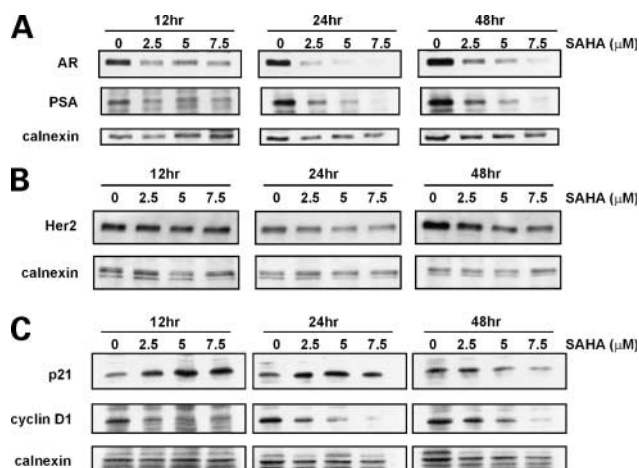


Figure 5. Protein expression changes in LNCaP cells following culture with SAHA. Lysates from cells cultured in the absence or presence of SAHA (2.5, 5, or 7.5 $\mu\text{mol/L}$) for 12, 24, or 48 h were analyzed by immunoblotting for expression of AR and PSA (A), Her2/*neu* (B), and cyclin D1 and p21^{WAF1} (C). For each immunoblot, detection of calnexin was used as a loading control (bottom).

corresponding decrease in the steady-state level of PSA (Fig. 5A). In addition, steady-state protein levels of the heat shock protein 90 (hsp90) client protein, Her2/*neu*, were reduced 48 h following SAHA treatment (Fig. 5B). Protein levels of cyclin D1 and p21^{WAF1}, which were used as controls for SAHA activity (29, 30), were decreased and increased, respectively, in a dose-dependent manner by SAHA (Fig. 5C).

Effects of SAHA on AR Expression and Cell Viability Are Enhanced by Culture in Steroid-Free Medium

Culture of LNCaP cells in charcoal-stripped serum (i.e., under conditions of androgen withdrawal) increased the sensitivity of the cells to SAHA. AR levels were markedly reduced in cells cultured with either 2.5 or 7.5 $\mu\text{mol/L}$ SAHA for 24 h in medium containing charcoal-stripped serum (Fig. 6A). In cells cultured with SAHA in medium with normal serum, only 7.5 $\mu\text{mol/L}$ SAHA caused an equivalent reduction in AR levels (Fig. 6A). Irrespective of whether the cells were grown in medium containing normal or stripped FCS, pretreatment with 5 α -dihydrotestosterone (1 nmol/L) had no effect on the ability of SAHA to reduce AR levels (Fig. 6A), indicating that activation and/or stabilization of the AR by 5 α -dihydrotestosterone did not prevent the reduction in AR levels caused by SAHA. When cultured in medium containing charcoal-stripped serum, cell growth was completely suppressed by 0.5 $\mu\text{mol/L}$ SAHA (Fig. 6B), which is substantially lower than was observed in cells cultured in normal medium (2.5 $\mu\text{mol/L}$; Fig. 1A). An increased percentage of dead cells was also seen with all doses of SAHA, with a maximum cell death of 60% being attained with 10 $\mu\text{mol/L}$ SAHA (Fig. 6B).

SAHA and the AR Antagonist Bicalutamide Act Synergistically to Suppress LNCaP Cell Proliferation and Increase Apoptosis

The enhanced sensitivity of LNCaP cells to SAHA in steroid-free medium suggested that combining SAHA with other inhibitors of androgen signaling may result in increased cell lethality. Culture of LNCaP prostate cancer cells with low, subeffective doses of SAHA (0.5 $\mu\text{mol/L}$) or

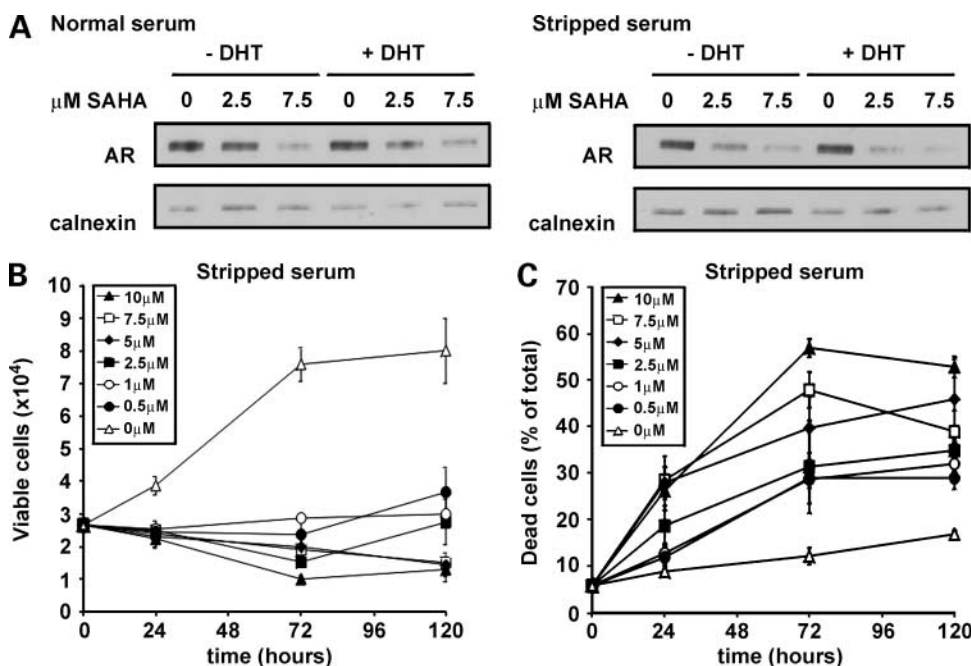
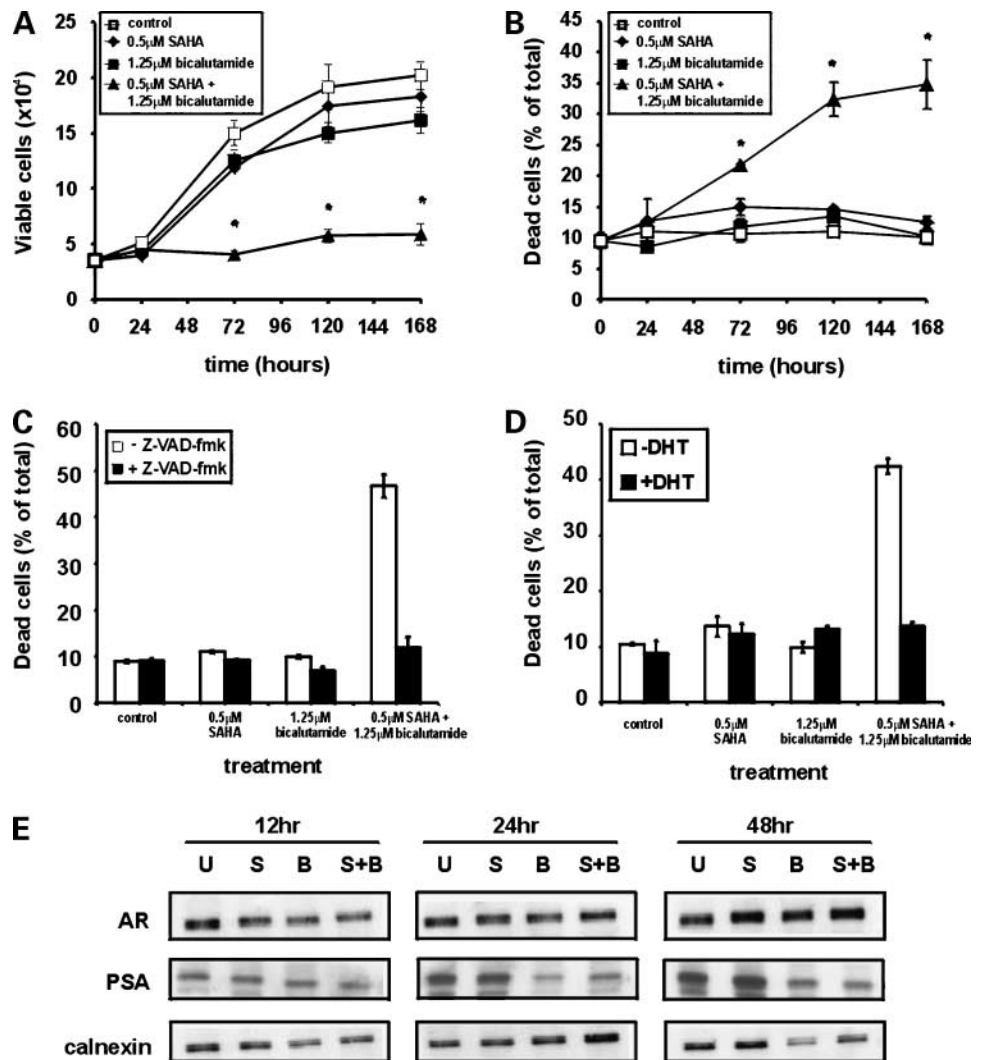


Figure 6. Effect of SAHA in combination with androgen withdrawal on AR expression, cell proliferation, and cell death. A, LNCaP cells (5×10^5 per well in six-well plates) were cultured for 24 h with SAHA (0, 2.5, or 7.5 $\mu\text{mol/L}$) in the presence or absence of 5 α -dihydrotestosterone (DHT; 1 nmol/L), in either regular RPMI 1640 with 10% FCS (left) or phenol red-free RPMI 1640 with 10% charcoal-stripped FCS (right). Cells were lysed and analyzed by immunoblotting for expression of AR, and calnexin was used as a loading control. B and C, LNCaP cells (2.5×10^4 per well in 24-well plates) were cultured in medium containing charcoal-stripped FCS in the absence or presence of SAHA for up to 7 d. B, cells were counted every 24 h using a hemocytometer, and cell viability was assessed by trypan blue exclusion. C, the number of dead cells is expressed as a percentage of total cells counted. Points, mean of triplicate wells in a representative experiment; bars, SE.

Figure 7. Effect of SAHA in combination with the AR antagonist bicalutamide on LNCaP cell proliferation and cell death. **A** and **B**, cells (2.5×10^4 per well in 24-well plates) were cultured in the absence or presence of 0.5 $\mu\text{mol/L}$ SAHA and 1.25 $\mu\text{mol/L}$ bicalutamide, either alone or in combination. **A**, cells were counted every 2nd day using a hemocytometer, and cell viability was assessed by trypan blue exclusion. **B**, the number of dead cells is expressed as a percentage of total cells counted. *Points*, mean of triplicate wells in a representative experiment; *bars*, SE. **C** and **D**, LNCaP cells (2.5×10^4 per well in 24-well plates) were cultured with 0.5 $\mu\text{mol/L}$ SAHA and 1.25 $\mu\text{mol/L}$ bicalutamide, alone or in combination, in the presence or absence of (**C**) the z-VAD-fmk caspase inhibitor or (**D**) the androgen 5 α -dihydrotestosterone (10 nmol/L) for 5 d. Cell viability was assessed as described above. **E**, lysates from untreated LNCaP cells (*U*) and cells cultured with 0.5 $\mu\text{mol/L}$ SAHA (*S*), 1.25 $\mu\text{mol/L}$ bicalutamide (*B*), or SAHA and bicalutamide (*S+B*) were analyzed by immunoblotting for expression of AR and PSA. Calnexin was used as a loading control (*bottom*).



the specific AR antagonist bicalutamide (1.25 $\mu\text{mol/L}$) alone, in normal medium supplemented with 10% serum, had minimal effects on LNCaP cell growth (Fig. 7A) and the percentage of dead cells (Fig. 7B). However, when combined, at the lower doses, these agents caused complete suppression of LNCaP cell growth and induction of cell death (Fig. 7A and B). The action of SAHA and bicalutamide on LNCaP cells was synergistic according to the isobole method of Berenbaum (22), the *D* values for cell proliferation and cell death being 0.2 and 0.8, respectively. An equivalent effect on cell viability was only observed with 50 $\mu\text{mol/L}$ bicalutamide treatment or 7.5 $\mu\text{mol/L}$ SAHA when these agents were used individually. Addition of the z-VAD-fmk caspase inhibitor (Fig. 7C), or excess exogenous androgen (Fig. 7D), completely prevented the synergistic effect of 0.5 $\mu\text{mol/L}$ SAHA and 1.25 $\mu\text{mol/L}$ bicalutamide on cell death. There was no difference in the levels of AR protein in LNCaP cells treated with 0.5 $\mu\text{mol/L}$ SAHA alone or in combination with 1.25 $\mu\text{mol/L}$ bicalutamide (Fig. 7E), indicating that this synergy was not due to altered AR

levels. Although there was a modest decrease in PSA expression at 24 and 48 h posttreatment with bicalutamide, this was not enhanced with the addition of SAHA (Fig. 7E). Consistent with a specific effect of this combination of agents at the subeffective doses on AR-dependent cells, there was no effect of either agent alone or in combination on cellular viability of androgen-independent, AR-negative PC-3 prostate cancer cells (Fig. 8A and B).

Discussion

SAHA is a promising agent currently in clinical trials for treatment of hematologic malignancies and solid tumors. Although previous studies suggest that SAHA can effectively inhibit the growth of prostate cancer cells (16), the mechanism of growth inhibition is not well understood. Moreover, it was evident from our previous studies that SAHA is more efficacious in terms of growth inhibition and induction of cell death in androgen-responsive cells (e.g., LNCaP and CWR22) than in cells

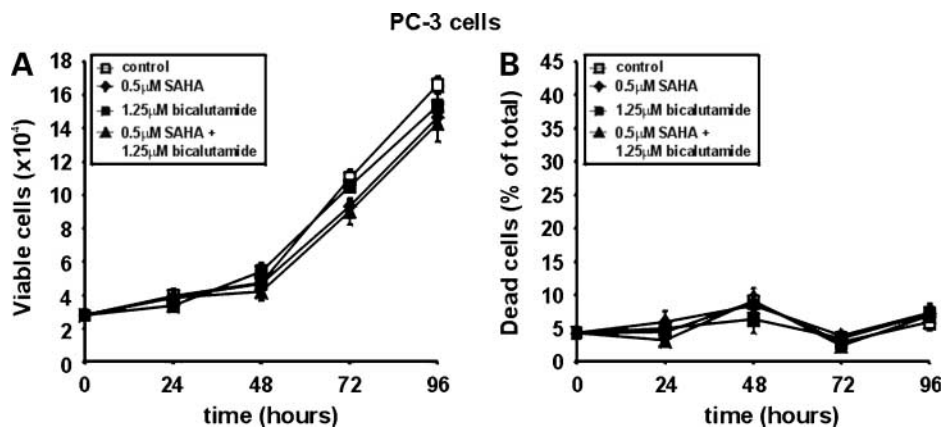


Figure 8. Effect of SAHA in combination with bicalutamide on PC-3 cell proliferation and cell death. PC-3 cells (2.5×10^4 per well in 24-well plates) were cultured in the absence or presence of 0.5 $\mu\text{mol/L}$ SAHA and 1.25 $\mu\text{mol/L}$ bicalutamide, alone or in combination. **A**, cell viability was assessed as described above. **B**, the number of dead cells is expressed as a percentage of total cells counted. Points, mean of triplicate wells in a representative experiment; bars, SE.

that lack AR (PC-3; ref. 16), suggesting that a component of the activity of SAHA in prostate cancer cells relates to the presence of a functional androgen signaling axis. Our current data, showing the modulation of the AR and genes involved in AR signaling, including its direct target genes PSA and kallikrein 2, as well as genes reported to be androgen regulated, such as transmembrane serine protease and NEDD4L, provide direct evidence of an effect of SAHA on AR signaling. In addition, our demonstration that both androgen withdrawal (steroid-free cell culture) and blocking the effects of androgens with an AR antagonist, bicalutamide, result in markedly enhanced sensitivity to SAHA, suggests that HDAC inhibitors, in combination with agents that target androgen signaling, may be a more effective strategy for the treatment of prostate cancer.

Due to the dependence of prostate cells on androgens for growth, current hormonal therapies for advanced prostate cancer aim to inhibit androgen signaling by removing or blocking the effect of bioavailable androgens (31). However, the AR in prostate cancer cells may continue to signal even in the presence of low levels of androgens due to up-regulation of AR expression or activation by alternate mechanisms, including nonclassic ligands or ligand-independent mechanisms (32). The consequence of this continued androgen signaling in prostate cancer is patient relapse with castration-resistant disease (31). Agents that are able to reduce AR expression or function are therefore potentially effective therapies for prostate cancer, especially in conjunction with other agents that target different components of the AR signaling pathway. Previous microarray studies have shown that culture of prostate cancer cells with the AR antagonist bicalutamide results in a reduction in levels of the AR-regulated genes, PSA and kallikrein 2 (33). In the present study, we found that treatment with SAHA also reduced expression of these AR-regulated genes but there was minimal overlap in any other genes regulated by SAHA or bicalutamide in microarray studies. Decreasing the level of AR within prostate cancer cells using HDAC inhibitors could enhance the ability of hormonal agents, such as AR antagonists, to inhibit the growth of prostate cancer and possibly delay the emergence of castration-resistant

disease. Alternatively, the reduction in AR signaling caused by bicalutamide or androgen withdrawal may be sufficient to sensitize the LNCaP cells to SAHA-induced apoptosis. Taken together with the observation that the androgen-independent, AR-negative PC-3 prostate cancer cell line was not affected by the combination of SAHA and bicalutamide and that addition of exogenous androgen could prevent cell death induced by this combination, our data strongly suggest that a functional androgen signaling axis is required for this synergistic effect on LNCaP cell death.

Our data suggest that the reduction in AR level induced by SAHA in LNCaP cells may involve translational as well as transcriptional mechanisms. Acetylation of histones in the AR gene promoter could result in direct repression of the AR gene through changes in chromatin structure, analogous to the regulation of the *cyclin D1* gene. An AR transcriptional repressor complex has been identified in LNCaP prostate cancer cells that can be activated by treatment with a HDAC inhibitor, in turn decreasing AR levels (34). Although our microarray and real-time RT-PCR analyses indicate that expression of AR is reduced by SAHA at the level of mRNA, acetylation of non-histone proteins by SAHA (4), such as the chaperone protein hsp90, or the AR itself, could also influence steady-state AR protein levels. Inhibition of hsp90 activity causes degradation of its client proteins, including the AR (35–37). The HDAC inhibitors depsipeptide and LAQ824 have been shown to cause hsp90 acetylation, thereby inhibiting normal protein-protein interactions and leading to degradation of client proteins (including the AR), in a similar manner to that seen following treatment with the specific hsp90 inhibitor 17-allylaminogeldanamycin (38–40). This mechanism may explain the synergistic activity of HDAC inhibitors and the hsp90 inhibitor 17-allylaminogeldanamycin for inhibition of cancer cell growth (41–43). Our data, showing a reduction in the hsp90 client protein Her2/*neu* in LNCaP cells cultured with SAHA, are consistent with an effect of SAHA on hsp90-dependent protein maturation, which may contribute to the reduction in AR levels.

In summary, we have shown that SAHA decreases AR protein levels in prostate cancer cells and results in cell

cycle arrest and apoptosis. Furthermore, when used at a low dose that has no effect on prostate cancer cell growth, SAHA acts synergistically with a traditional hormonal agent, bicalutamide, to induce apoptosis. Taken together, our results suggest that inhibition of androgen signaling is an important component of SAHA activity in prostate cancer cells and that androgen withdrawal or blockade may sensitize prostate cancer cells to undergo cell death in response to HDAC inhibitors. Given that the majority of clinical prostate tumors express the AR, including those that fail hormonal therapy, further investigation into the use of SAHA for the treatment of prostate cancer is warranted, particularly in the context of combination therapy with conventional androgen ablation therapies.

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