Journal of Medicinal Chemistry

Brief Article

A New Simple and High-Yield Synthesis of Suberoylanilide Hydroxamic Acid and Its Inhibitory Effect Alone or in Combination with Retinoids on Proliferation of Human Prostate Cancer Cells

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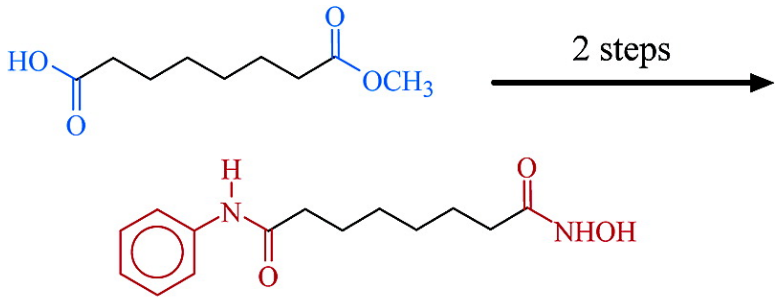
J. Med. Chem., **2005**, 48 (15), 5047-5051• DOI: 10.1021/jm058214k • Publication Date (Web): 25 June 2005



SAHA (79.8%)

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A New Simple and High-Yield Synthesis of Suberoylanilide Hydroxamic Acid and Its Inhibitory Effect Alone or in Combination with Retinoids on Proliferation of Human Prostate Cancer Cells

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Received March 28, 2005

We have developed a procedure for the synthesis of *N*-hydroxy-N^1-phenyloctanediamide (suberoylanilide hydroxamic acid (SAHA)), providing the product in 79.8% yield. SAHA is a potent inhibitor of histone deacetylase, induces differentiation and/or apoptosis in certain transformed cells in culture, and suppressed the growth of human prostate cancer LNCaP and PC-3 cell lines. The combination of SAHA with other compounds inhibited cell proliferation of LNCaP cells in additive fashion and resulted in synergistic growth inhibition.

Introduction

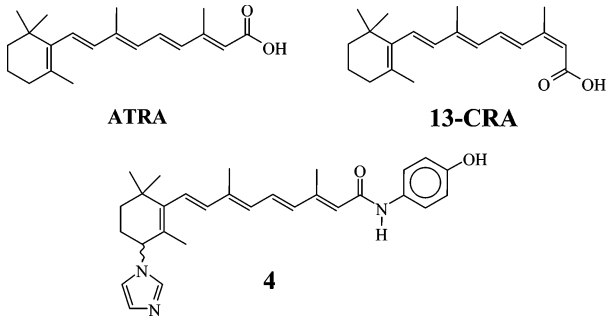
Prostate cancer (PCA) is the most common malignancy and age-related cause of cancer death worldwide. Apart from lung cancer, PCA is the most common form of cancer in men and the second leading cause of death in American men. In the U.S. in 2004, an estimated 230 000 new cases of prostate cancer were diagnosed and about 23 000 men died of this disease.[1a] During the period of 1992−1999, the average annual incidence of PCA among African American men was 59% higher than among Caucasian men and the average annual death rate was more than twice that of Caucasian men.[1b]

The growth of most prostate tumors depends on androgens during the initial stages of tumor development, and thus, antihormonal therapy, by surgical or medical suppression of androgen action, remains a major treatment option of the disease.[2] Although this treatment may be initially successful, most tumors eventually recur because of the expansion of an androgen-refractory population of PCA cells.[3] Metastatic disease that develops even after potentially curative surgery remains a major clinical challenge. Therapeutic treatments for patients with metastatic PCA are limited because current chemotherapeutic and radiotherapeutic regimens are largely ineffective.[4] Hence, there is urgent need to develop new therapeutic agents with defined targets to prevent and treat this disease.

PCA tumors that arise after antihormonal therapy generally are less differentiated, and it is believed that agents that can induce the cells to differentiate would represent a new therapeutic strategy.[5] Hence, the goal of differentiation therapy is to induce malignant cells to pass the block to maturation by allowing them to progress to more differentiated cell types with less proliferative ability.

Breslow and colleagues[6] have led the way in the discovery of agents that inhibit the enzyme histone deacetylase (HDAC), thereby altering chromatin structure and changing gene expression patterns. Histone deacetylase inhibitors (HDACIs) are potent differentiating agents toward a variety of neoplasms, including leukemia and breast and prostate cancers. Combinations of HDACIs with other known therapies including retinoic acids (RAs) have been investigated. RAs exert their effects via a nuclear receptor complex that interacts with promoters of RA-responsive genes.[7] An HDAC subunit is an intergral part of this corepressor complex, which is involved in transcriptional silencing in the absence of ligand.[8] This association provides a rationale for combining HDACIs and RAs/retinoids therapeutically. One of the early HDACIs discovered by Breslow and colleagues is *N*-hydroxy-N^1-phenylactanediamide, also called suberanilide hydroxamic acid (SAHA).[6] This compound has recently (2004) been approved by the U.S. Food and Drug Administration for phase II clinical trials in lymphoma patients.[9]

Chart 1. Structures of ATRA, 13-CRA, and **4**



Recently, we reported on a family of compounds that inhibit the P450 enzyme(s) responsible for the metabolism of all-trans retinoic acid (ATRA, Chart 1).[10] These compounds, also referred to as retinoic acid metabolism blocking agents (RAMBAs), are able to enhance the antiproliferative effects of ATRA in breast and prostate cancer cells in vitro. In addition, the RAMBAs were shown to induce differentiation and apoptosis in these cancer cell lines. However, we also observed that the breast cancer cell lines were exquisitely more sensitive to the RAMBAs.

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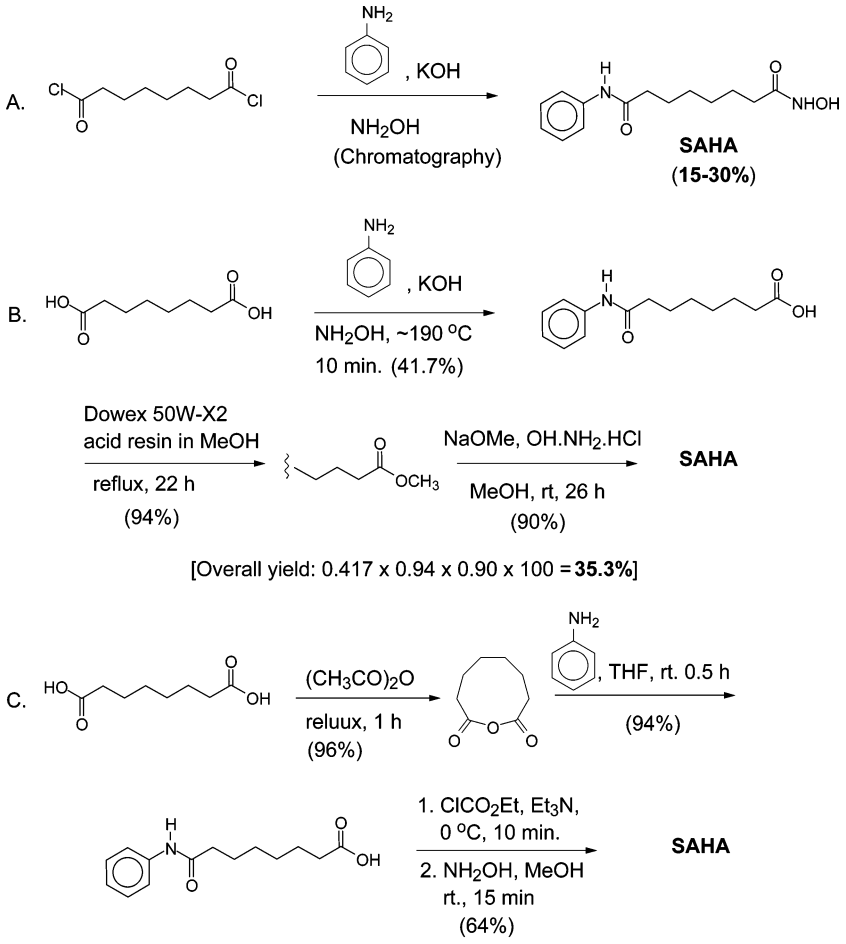
10.1021/jm058214k CCC: $30.25
Published on Web 06/25/2005

Scheme 1. Previous Procedures for Syntheses of SAHA



The present study describes a facile, expeditious, and high-yield synthesis of SAHA. Furthermore, we assessed the effects of combinations of RAs or a RAMBA (**4**) and SAHA on human LNCaP prostate cancer cells in vitro. We show that these combinations of agents lead to enhanced (additive/synergistic) PCA growth inhibition.

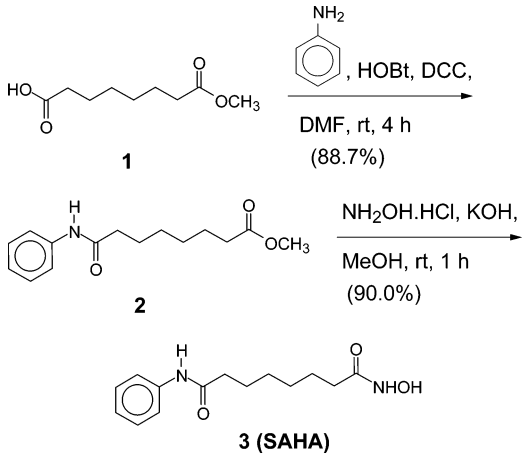
Results and Discussion

Chemistry. To the best of our knowledge, three synthetic procedures for the preparation of SAHA have been reported, and these are presented in Scheme 1. The first, reported in a patent by Breslow and colleagues[6] is a one-pot reaction whereby suberoyl chloride is treated with aniline, hydroxylamine, and aqueous KOH to give SAHA in low 15−30% yield. In addition to the low yield, the procedure also suffers from the formation of a dianilide byproduct that is particularly difficult to remove, thus requiring tedious column chromatography purification. In the second procedure developed by Stowell et al.,[11] the dianilide byproduct was readily separated. However, this three-step procedure starting from suberic acid furnished SAHA in only 35% overall yield. Furthermore, it involves long reaction times (up to 28 h) and harsh reaction conditions (185−190 °C). The most recent approach[12] is also a three-step procedure from suberic acid via suberoyl anhydride, but under mild reaction conditions, it gives SAHA in 57.8% overall yield.

The overall yield of SAHA via the procedure of Stowell et al. was adversely affected by the low 41.7% yield for synthesis of suberanilic acid, while that described by Mai et al. was adversely affected by the relatively low 64% yield of the hydroxamate. Although the yield for the hydroxamate formation step of Stowell et al. was high (90%), it involved the use of sodium metal, which is not recommended especially in large-scale preparations. However, our mild procedure (vide infra) also resulted in a high yield (90%) of the hydroxamate. We have developed a two-step procedure that requires milder reaction conditions and without chromatographic purification to give SAHA of high purity and high overall yield of 79.8% (Scheme 2).

The commercially available suberic acid monomethyl ester (**1**, 5 g costs $112) was condensed with aniline in a typical coupling reaction for amide bond formation using hydroxybenzotriazole (HOBt) and dicyclocarbodiimide (DCC) in DMF. The mixture was stirred at room temperature for 1.5 h. The product was obtained by diluting the mixture with water, and crude product was dissolved in petroleum ether and ethyl acetate (1:1) and then filtered through a short pad of silica gel. Thus, suberanilic acid methyl ester (**2**) was obtained in 88.7% yield. Hydroxylamine was prepared by mixing hydroxylamine hydrochloride with potassium hydroxide in methanol. The freshly prepared hydroxylamine was stirred at room temperature with methyl suberanilate for 1 h. The product was obtained by adding water and neutralizing with acetic acid to obtain SAHA (**3**) in 90.0% yield (99.5% purity as determined by HPLC). **2**

Scheme 2. New Synthesis of **3** (SAHA)



[Overall yield: 0.887 x 0.90 x 100 = **79.8%**]

and SAHA were fully characterized by physical and spectral methods and were consistent with literature data.[5,11,12] The yields of our two-step synthesis of SAHA are highly reproducible because we have repeated these reactions at least three times with a standard deviation of less than 2%. The advantage of our process is its simplicity and mild reaction conditions and that it does not require chromatographic purification. In addition, it gives a higher yield than the alternative procedures.

Biological Studies. In a recent study we observed that some human breast cancer cell lines (MCF-7 and T-47D) were exquisitely sensitive to ATRA and some of our novel RAMBAs.[10] The apparent lack of sensitivity of the breast cancer cells (MDA-MB-213) and two prostate cancer cell lines (LNCaP and PC-3) to ATRA and our novel RAMBAs may be due to inactivation of various genes that are essential for retinoid activity.[7,8] We then asked whether prostate cancer cells that display poor antiproliferative responses to ATRA and our novel RAMBA could be made more sensitive by co-treatment with SAHA. On the basis of previous evidence (common signal transduction pathways and possible modulation at the nuclear receptor levels), we hypothesize that a combination of SAHA and ATRA/RAMBA may have additive or synergistic inhibitory activity on prostate cancer cells and tumors.

The dose−response curves of **4** and SAHA showed that SAHA (IC_{50} = 1.0 μM) was a more potent antiproliferative agent than **4** (IC_{50} = 5.5 μM) against LNCaP prostate carcinoma cells (Figure 1A,B). To evaluate the potential additive/synergistic effect of SAHA and **4** on the growth of LNCaP cells, we determined the effects of SAHA and **4** alone and in combination. For the combination, we chose the concentrations of both compounds that gave cell growth inhibition of approximately 50% (Figure 1C). The inhibition of cell growth by this combination was *additive* in that its effect was equal to the sum of the effects of the two compounds separately, using the Valeriote and Lin analysis.[13] Furthermore, we found that the combination of SAHA (1.0 μM) with either ATRA (10.0 μM, Figure 2A) or 13-CRA (10.0 μM, Figure 2B) resulted in *additive* and *synergistic* LNCaP cell growth inhibition, respectively. When evaluated by ANOVA, the enhanced LNCaP cell growth inhibitions by the combined agents were in all cases significant (see

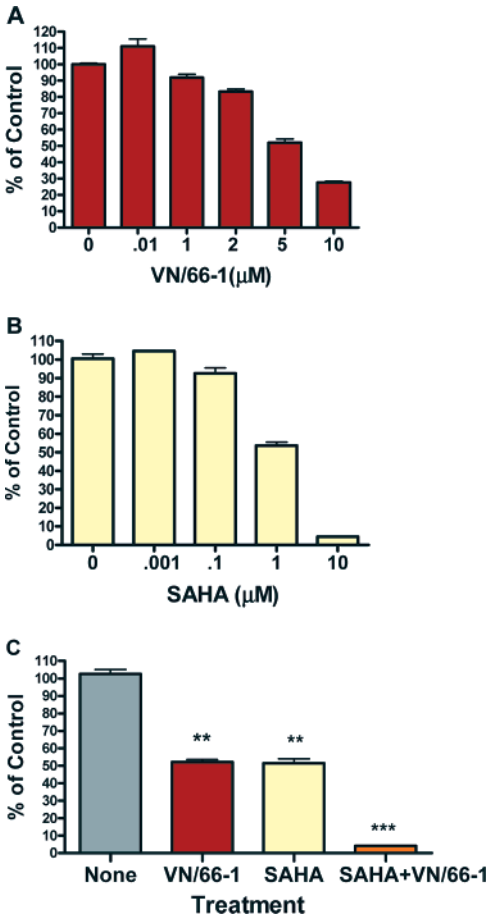


Figure 1. Dose−response curves for growth inhibitory action of **4** (A) and SAHA (B) on LNCaP prostate cancer cells and growth inhibitory action of **4** (5.0 μM) and SAHA (1.0 μM) (C) administered alone and in combination on LNCaP prostate cancer cells. Cells were treated with the compounds for 4 days, and cell proliferation was measured by MTT assay. Values are the mean ± SEM from three experiments: (*) $P < 0.0001$, **4** vs control; (**) $P < 0.009$, SAHA + **4** vs **4**.

Figures 1A and 2B). In all likelihood, these effects of SAHA and retinoids/RAMBAs would lead to suppression of tumor growth. Finally, we also found that SAHA caused a dose-dependent inhibition of PC-3 prostate cancer cell growth inhibition, with IC_{50} values of 6.5 μM (Figure 3). It is most likely that the combination of SAHA with retinoids/RAMBAs would also be effective in the hormone-independent PC-3 cell line.

Conclusion

We have described a novel, highly reproducible, and simple procedure that enabled us to synthesize SAHA in unprecedented high yield and purity. The biological data indicate that SAHA is a potent inhibitor of human prostate cancer cell proliferation. Furthermore, the observation of synergistic/additive interaction of SAHA and RAs or a RAMBA on cell growth inhibition suggests that the combination of retinoids and HDACIs may show potential in the therapy of prostate cancer and possibly other cancers. The mechanism(s) that underlies these observations is currently under investigation.

Experimental Section

Chemistry. General procedures and techniques were identical to those previously reported.[10] Infrared spectra were recorded on a Perkin-Elmer 1600 FTIR spectrometer using

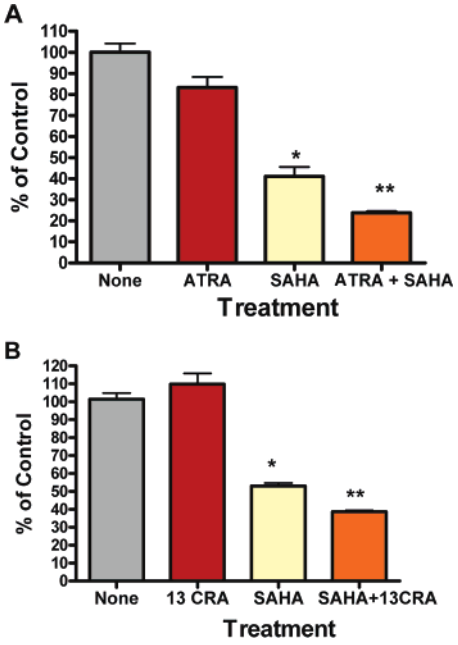


Figure 2. (A) Effects of simultaneous exposure of ATRA (10.0 μM) and SAHA (1.0 μM) and (B) 13-CRA (10.0 μM) on human LNCaP prostate cancer cell proliferation. The indicated concentrations of agents were used alone or in combination. Values are the mean ± SEM from three experiments. ∗ and ∗∗ denote significant difference ($P < 0.05$) between SAHA or ATRA and SAHA + ATRA and between SAHA or 13-CRA and SAHA and 13-CRA, respectively. The results represent the average and standard deviation of three experiments performed in triplicate.

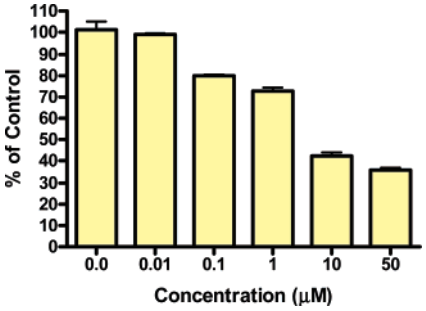


Figure 3. Effects of SAHA on human prostate cancer cell (PC-3) proliferation.

Nujol paste. High-resolution mass spectra (HRMS) were determined on a 3-Tesla Finnigan FTMS-2000 FT mass spectrometer, ESI mode (Department of Chemistry, The Ohio State University). ^{1}H NMR Spectra were performed in $CDCl_3$ and DMSO-d_6 at 500 MHz with Me_4Si as an internal standard using a Varian Inova 500 MHz spectrometer. As a criterion of purity for key target compounds, we have provided (see Supporting Information) high-resolution mass spectral data with HPLC chromatographic data indicating compound homogeneity. Melting points (mp) were determined with a Fischer Johns melting point apparatus and are uncorrected.

Suberanilic Acid Methyl Ester (2). Suberic acid monomethyl ester (**1**, 10 g, 0.0531 mol), 1-hydroxybenzotriazole (8.61 g, 0.0637 mol), and aniline (5.93 g, 0.0637 mol) were dissolved in DMF (60 mL) at room temperature. Dicyclohexylcarbodiimide (DCC) (13.14 gm, 0.0637 mol) was added, and the mixture was stirred at room temperature for 1.5 h. The precipitate of dicyclohexylurea was filtered off and was washed with 5 mL DMF. The filtrate was added in cold stirring water (900 mL). The precipitate was filtered and dried under vacuum. The crude product was placed on a short pad of silica gel and eluted with petroleum ether and ethyl acetate (1:1). The solvent was removed under vacuum to give 12.4 g of product, yield 88.7%: mp 64−65 °C; IR (Nujol) 3300, 1725, 1656, 1598, 1533, 1499, 1418, 1329, 1249, 1228, 1197, 1172, 958, 882, 844, 728, 690, 519 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.52 (d, 2H, J = 7.5 Hz, 2^1- and 6^1-Hs), 7.32 (t, 2H, J = 8.0 Hz, 3^1- and 5^1-Hs), 7.17 (brs, 1H, NH), 7.09 (t,1H, J = 7.5 Hz, 4^1-H), 3.66 (s, 3H, −OCH_3), 2.35 (t, 2H, J = 7.0 Hz, 2- and 7-Hs), 2.31 (t, 2H, J = 7.5 Hz, 2- and 7-Hs), 1.74 (p, 2H, J = 7.5 Hz, 3- and 6-Hs), 1.64 (p, 2H, J = 7.0 Hz, 3- and 6-Hs), 1.38 (m, 4H, 4- and 5-Hs). HRMS calcd 286.1414 ($C_{15}H_{21}NO_3.Na^+$), found 286.1411.

Suberyolanilide Hydroxamic Acid (SAHA, 3). Hydroxylamine hydrochloride (59.86 g, 0.861 mol) in methanol (150 mL) was mixed with KOH (48.26 gm, 0.861 mol) at 40 °C in methanol (280 mL), cooled to 0 °C, and filtered. The suberanilic acid methyl ester (12.3 g, 0.0467 mol) was then added to the filtrate followed by addition (over 30 min) of KOH (3.93 g, 0.07 mol). The mixture was stirred at room temperature for 1 h. The mixture was added to stirring cold water (1500 mL), and the pH was adjusted to 7 by adding acetic acid. The precipitate was filtered off, and the resulting product was dried in a vacuum oven at 40 °C overnight to yield 10.84 g (90.0%) of **3** (SAHA): mp 159−160.5 °C; IR (Nujol) 3310, 3267, 2853, 1660, 1614, 1598, 1530, 1464, 1443, 1376, 1316, 976, 761, 703, 573 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.32 (s, 1H, −OH), 9.83 (s, 1H, −NH), 8.64 (s, 1H, −NH), 7.57 (d, 2H, J = 8.5 Hz, 2^1- and 6^1-Hs), 7.27 (t, 2H, J = 7.5 Hz, 3^1- and 5^1-Hs), 7.0 (t, 1H, J = 7.5 Hz, 4^1-H), 2.28 (t, 2H, J = 7.5 Hz, 2- and 7-Hs), 1.93 (t, 2H, J = 7.5 Hz, 2- and 7-Hs), 1.55 (p, 2H, J = 6.5 Hz, 3- and 6-Hs), 1.48 (p, 2H, J = 6.5 Hz, 3- and 6-Hs), 1.27 (m, 4H, 4- and 5-Hs). HRMS calcd 287.1366 ($C_{14}H_{20}N_2O_3$·Na^+), found 287.1431.

HPLC Analysis. Chromatographic analysis was achieved by a reverse-phase HPLC method on a Waters Novapak C18 column (3.9 mm × 150 mm) protected by Waters guard cartridge packed with pellicle C18 as previously described. Briefly, the HPLC system used in this study consisted of a Waters solvent delivery system, a Waters controller (Milford, MA) coupled to a Waters 717^{plus} autosampler, and a Waters 996 photodiode array detector operating at 240.0 nm. A multilinear gradient solvent system, (i) 50 mM aqueous K_2PO_4 buffer/CH_3CN/Et_3N (85.975:14:0.025) (100% → 0%) and (ii) 50 mM aqueous K_2PO_4 buffer/CH_3CN/Et_3N (69.975:30:0.025) (0% → 100%) at a flow rate of 0.75 mL/min, was used. This solvent system was adapted with modifications from that reported by Kelley et al.[14] The retention time was 13.44 min for SAHA. Mobile phase ii was used for suberanilic acid methyl ester, and the retention time was 15.59 min. The HPLC analysis was performed at ambient temperature, and data acquisition and management were achieved with a Waters millennium chromatography manager.

Cell Growth Inhibition Assay (MTT Colorimetric Assay). LNCAP cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin, and streptomycin, as the complete culture medium. The 2 × 10^4 cells were seeded in 24-well plates and incubated in a 5% CO_2 incubator at 37 °C for 1 day. Cultures were treated with various compounds as listed, alone and in combination on days 2 and 4. Cells were washed on day 2, and media were changed. Mitochondrial metabolism was measured as a marker for cell growth by adding 100 μL/well MTT (5 mg/mL in medium) with 2 h of incubation at 37 °C on day 6. Crystals formed were dissolved in 500 μL of DMSO. The absorbance was determined using a microplate reader at 560 nm. The absorbance data were converted into a cell proliferation percentage. Each assay was performed in triplicate.

PC-3 cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin, and streptomycin, as the complete culture medium. The 2 × 10^4 cells were seeded in 24-well plates and incubated in a 5% CO_2 incubator at 37 °C for 1 day. Cultures were treated with various compounds as listed, alone and in combination on days 2 and 4. Cells were washed on day 2, and media were changed. Mitochondrial metabolism was measured as a marker for cell growth by adding 100 μL/well MTT (5 mg/mL in medium) with

2 h of incubation at 37 °C on day 6. The crystals formed were dissolved in 500 μL of DMSO. The absorbance was determined using a microplate reader at 560 nm. The absorbance data were converted into a cell proliferation percentage. Each assay was performed in triplicate.

Acknowledgment. This research was supported by grants from the U.S. Department of Defense under the Peer Review Medical Research Program (Grant W81XWH-04-1-0101), the U.S. Army Medical Research and Material Command (Grant DAM D17-01-1549), and TEDCO to Vincent C. O. Njar. We thank Dr. Gary Strahan, Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore (UMB), for some of ^{1}H NMR data.

Supporting Information Available: HPLC chromatograms and high-resolution mass spectral data for **2** and SAHA. This material is available free of charge via the Internet at http://pubs.acs.org.

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