The Synthesis of *N*-Hydroxy-*N'*-phenyloctanediamide and Its Inhibitory Effect on Proliferation of AXC Rat Prostate Cancer Cells

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We have developed a practical synthesis of *N*-hydroxy-*N'*-phenyloctanediamide from the methyl ester of suberanilic acid. It provides the product in high yield and purity with a simple purification process. We have found that at 10^{-5} M it has a dramatic effect on T/5 AXC/SSh rat prostate cancer cells *in vitro*. It is a potent inhibitor of cell proliferation and it changes the cell morphology to resemble nonmalignant cells.

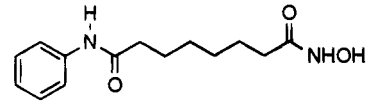
Introduction

As of 1990, adenocarcinoma of the prostate has become the most common malignancy in American men,[1] and approximately 200 000 new cases are anticipated in the United States in 1994.[2] Tumor incidence increases with age and affects greater than 50% of men over age 70.[3] Most tumors remain small and localized, but those that do metastasize are associated with a high mortality rate.[4]

Three-fourths of patients with prostatic carcinoma present with inoperable metastatic tumors.[5] Growth of most prostate tumors depends on androgens during the initial stages of tumor development,[6] and thus hormonal therapy, by surgical or medical suppression of androgen action,[7] remains a major approach to the treatment of prostate cancer. Most patients relapse, however, and further hormonal therapy is of limited value.[8] Tumors arising after hormonal therapy generally are less differentiated and usually lack hormonal dependency, hence the poorer prognosis for treatment of these tumors.[9]

The progression of androgen-dependent, normal prostate cells to androgen-independent prostate cancer cells remains an enigma and has compromised the long-term success of hormonal therapy. Other nonhormonal therapeutic approaches are currently in use against prostate cancer. Most of these, such as radiation and chemotherapy, offer only limited benefit to patients with metastatic disease and frequently cause severe side effects as well. In recent years, the use of differentiation factors has opened a new avenue for the treatment of cancer. It has been postulated that the fundamental lesion in the neoplastic cell is its inability to differentiate.[10] Thus 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 et al. have discovered a series of suberic acid derivatives that are potent cytodifferentiating agents toward leukemia cells.[11] These difunctional tethered molecules are optimally spaced by six CH_2 groups. We selected one of their most potent compounds and have measured the effects of it on AXC rat prostate cancer cells *in vitro*. That compound is *N*-hydroxy-*N'*-phenyloctanediamide, also called suberanilohydroxamic acid (SAHA). Breslow et al. found that at a concentration of



SAHA

2.5 μM in the culture medium it caused 70% of murine erythroleukemia (MELC 745-DS19) cells to become benzidine reactive. At 1 μM it caused 21% of HL-60 cells (from human acute promyelocytic leukemia) to develop the ability to reduce nitroblue tetrazolium.[11]

Results and Discussion

Chemistry. We have devised a practical synthesis of SAHA that gives good yields after a simple purification process. In the patent, Breslow et al. report four general procedures for the preparation of members of this class of hydroxamic acids in yields ranging from 15 to 65%. Their simplest procedure involves combining suberoyl chloride with a mixture of aniline, hydroxylamine, and aqueous KOH to afford SAHA directly in 15–30% yields. The resulting mixture of symmetrical and unsymmetrical products requires chromatography for separation of the SAHA. The dianilide byproduct is particularly difficult to remove because it is the fastest moving component on silica gel while having very low solubility in the eluting solvent. Thus it contaminates most of the fractions. Recrystallization is not helpful because the dianilide appears to be more soluble in the presence of SAHA than without it. We have developed a three-step preparation where that impurity is not present in the last step.

Heating suberic acid with 1.1 equiv of aniline at 185–190 °C for 10 min affords suberanilic acid in 42% yield along with suberic dianilide in 14% yield. These yields are very reproducible; even using 1.5 equiv of aniline gave the same yields. These and the remaining suberic acid are readily separable by solubility differences in aqueous base and hot water. The suberanilic acid was esterified with excess methanol or ethanol (94% yield) and then treated with methanolic hydroxylamine and sodium methoxide. The resulting salt was acidified to afford the SAHA in 90% yield.

It is common practice to prepare hydroxamic acids from esters and hydroxylamine in methanol or ethanol,[12] where the sodium salt of the hydroxamic acid

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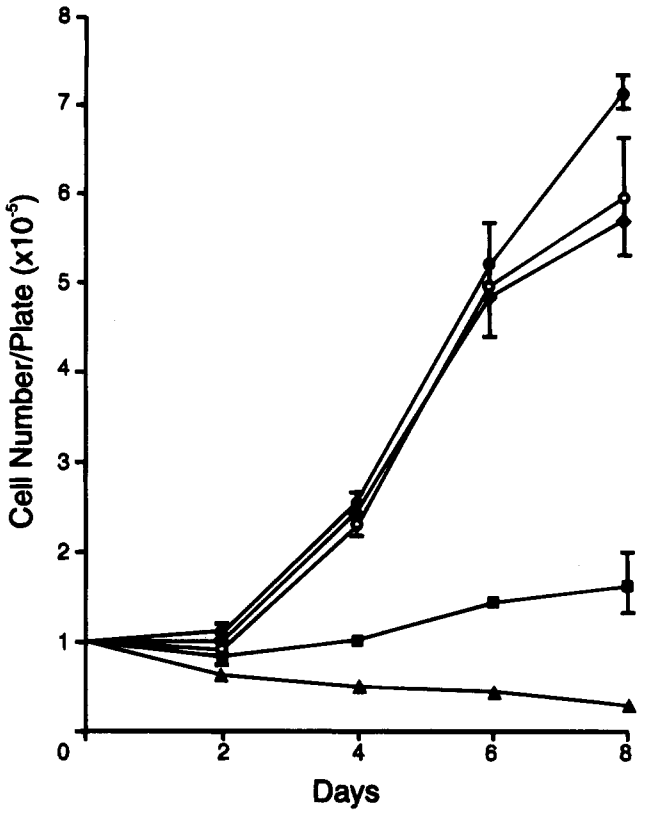

Figure 1. Effect of SAHA on proliferation of T/5 AXC/SSh rat prostate carcinoma cells. Cells were seeded at a concentration of 10^5 cells per 35 mm plate in 2 mL of MEM medium containing 10% FBS and 10^{-7} M testosterone in the presence or absence of SAHA (10^{-4}–10^{-6} M) and/or DMSO (0.1%) as indicated. Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C. All cultures were refed with their appropriate culture medium every 48 h. All data points are the mean and standard deviation of triplicate dishes: (○) control, (●) DMSO control, (▲) 10^{-4} M, (■), 10^{-5} M, (◆) 10^{-6} M.

slowly crystallizes. This is filtered and acidified to free the hydroxamic acid. However, the salt of SAHA has appreciable solubility in ethanol and methanol. On the other hand, SAHA is insoluble in water; therefore, the reaction mixture was simply poured into water and acidified, giving a heavy precipitate of the pure product. None of the ester is hydrolyzed under the reaction conditions; therefore, if the ester is not contaminated with suberanilic acid, the SAHA will likewise be free of it.

Biology. SAHA was a potent inhibitor of T/5 cell proliferation (Figure 1). Cell proliferation was completely inhibited by SAHA at a concentration of 10^{-4} M. The compound was cytotoxic at this concentration, and all cells were killed during the first few days of culture. At a concentration of 10^{-5} M, proliferation was greatly reduced. SAHA caused cells to flatten, spread out, and become less refractile. T/5 cells normally grew as tightly packed cuboidal epithelial cells but resembled squamous epithelial cells in the presence of SAHA. As a result, cells reached confluency at a much lower cell number than the controls. Cells reassumed their usual morphology at an SAHA concentration of 10^{-6} M but proliferated at a slower rate than the controls. These decreases in cell number were not statistically significant, however. Lower concentrations of SAHA were identical to the control cultures and are not depicted for the sake of clarity.

Because DMSO has been shown to alter cell morphology in another line of rat prostate cancer cells,[13,14] the effects of DMSO alone at the concentration used to prepare the highest concentration of SAHA were tested. SAHA has limited solubility in aqueous solution and must be dissolved in DMSO prior to the addition to cell culture medium. At the concentration of DMSO employed (0.1%), no morphological alterations were detected. DMSO-treated cells proliferated as well as the untreated control cells. Thus DMSO does not appear to alter cellular phenotype or proliferative ability of T/5 cells at a concentration of 0.1%. Higher concentrations of DMSO were not examined.

The above data indicate that SAHA is a potent inhibitor of rat prostate cancer cell proliferation. The ability of SAHA to induce cell spreading, loss of refractility, and a greatly reduced cell number at confluency consistent with a more differentiated, normal phenotype would argue that SAHA is acting as a differentiation factor on these cells. This preliminary study suggests that SAHA holds promise as a potential therapeutic agent for the treatment of prostate cancer.

Experimental Section

^{1}H NMR and ^{13}C NMR spectra were recorded at 300.075 and 75.46 MHz, respectively. Coupling constants are given in hertz. Thin layer chromatography was done with Kodak chromatogram sheet silica gel with fluorescent indicator. Hydroxamic acids gave a yellow spot when dipped in aqueous ferric choride. The R_f values using 5% water in THF are as follows: suberic bishydroxamic acid 0.1, suberanilic acid 0.3, SAHA 0.7, suberic dianilide 1.0, and methyl suberanilate 1.0. Infrared spectra were run on mineral oil mulls.

Suberanilic Acid. Freshly distilled aniline (4.09 g, 0.044 mol) and suberic acid (6.96 g, 0.040 mol) were combined in a large test tube and heated at 185–190 °C for 10 min. Vigorous bubbling evolution of water from the melt was evident. This was cooled and dispersed in a solution of 4.0 g of KOH in 50 mL of water with stirring for 20 min. The resulting white suspension was suction filtered. The solid was rinsed with water and dried to afford 1.87 g (14.4%) of suberic dianilide, mp 185–186.5 °C (lit.[15] mp 186–187 °C). The clear filtrate was acidified with aqueous HCl. The resulting heavy white precipitate was filtered, and the solid was stirred with 100 mL of water at 50 °C. This was filtered hot, and the solid was stirred in another 80 mL of hot water, filtered, rinsed with hot water, and dried, giving 4.16 g (41.7%) of white solid: mp 126–128 °C (lit.[15] mp 125–127 °C); ^{1}H NMR (DMSO) δ 11.97 (s, 1), 9.84 (s, 1), 7.57 (d, 2, J = 7.2), 7.26 (t, 2, J = 7.5), 7.00 (t, 1, J = 7.35), 2.27 (t, 2, J = 7.6), 2.18 (2, t, J = 7.3), 1.56 (p, 2, J = 7.1), 1.48 (p, 2, J = 6.9), 1.28 (m, 4); ^{13}C NMR (DMSO) δ 174.69, 171.42, 139.56, 128.84 (2C), 123.11, 119.23 (2C), 36.56, 33.82, 28.61, 28.64, 25.17, 24.59; IR 3322, 1697, 1662, 1599, 1524, 1444 cm^{-1}. Cooling the final filtrate gave 1.83 g (26%, while more remains in solution) of recovered suberic acid.

Methyl Suberanilate. Suberanilic acid (4.14 g, 0.0166 mol) was dissolved in 25 mL of methanol, and 0.5 g of Dowex 50W-X2 acid resin was added. This was heated at reflux with magnetic stirring for 22 h, cooled, filtered, and evaporated to afford a pale yellow solid. The solid was dissolved in 10 mL of chloroform (markedly endothermal dissolution) and passed through a 2 × 3 cm column of silica gel, rinsing with additional chloroform. Evaporation afforded 4.10 g (94% yield): mp 64–65.5 °C; ^{1}H NMR ($CDCl_3$) δ 7.52 (d, 2, J = 8.1), 7.32 (t, 2, J = 8.2), 7.10 (t, 1, J = 7.4), 3.67 (s, 3), 2.35 (t, 2, J = 7.4), 2.32 (t, 2, J = 7.5), 1.74 (p, 2, J = 7.3), 1.64 (p, 2, J = 6.8), 1.38 (m, 4); ^{13}C NMR ($CDCl_3$) δ 174.26, 171.38, 137.98, 128.90 (2C), 124.10, 119.75 (2C), 51.47, 37.50, 33.90, 28.68 (2C), 25.28, 24.63; IR

3301, 1729, 1658, 1599, 1534, 1447, 1173 cm^{-1}. Anal. ($C_{15}H_{21}NO_3$) C, H, N.

Suberanilohyroxamic Acid. Hydroxylamine hydrochloride (2.17 g, 0.0312 mol) was dissolved in 15 mL of methanol in a 50 mL flask equipped with magnetic stirring and an addition funnel. One milligram of phenolphthalein was added. A solution of sodium metal (1.08 g, 0.0468 mol) in 13 mL of methanol was placed in the addition funnel and enough added to reach a pink end point. A precipitate of NaCl appeared. Solid methyl suberanilate (4.10 g, 0.0156 mol) was added, which dissolved readily. The remainder of the sodium methoxide solution was added, and the mixture was stirred for an hour. After a few hours, a thick precipitate had appeared. This was left for a total of 26 h at room temperature and then rinsed into 100 mL of water where most of it dissolved. Glacial acetic acid (4.0 g) was added with stirring. The resulting heavy precipitate was suction filtered, rinsed with water, then slurried with another 75 mL of water, filtered, and rinsed again. The solid was dried at room temperature, affording 3.70 g (90%) of white solid, mp 159–160.5 °C, showing no impurities by thin layer chromatography or NMR: ^1H NMR (DMSO) δ 10.33 (s, 1), 9.84 (s, 1), 8.66 (s, 1), 7.57 (d, 2, J = 7.6), 7.27 (t, 2, J = 7.2), 7.00 (t, 1, J = 7.4), 2.27 (t, 2, J = 7.6), 1.92 (t, 2, J = 7.4), 1.56 (p, 2, J = 6.7), 1.47 (p, 2, J = 6.2), 1.26 (m, 4); ^{13}C NMR (DMSO) δ 171.45, 169.34, 139.58, 128.87 (2C), 123.14, 119.26 (2C), 36.61, 32.48, 28.65 (2C), 25.27 (2C); IR 3312, 3270, 1663, 1618, 1599, 1530, 1444 cm^{-1}. Anal. ($C_{14}H_{20}N_2O_3$) C, H, N.

The product should be manipulated with a porcelain (not metal) spatula. If the compound contacts metal when wet, an orange stain occurs. Above the melting point, slow evolution of bubbles occurs.

Cell Culture. The T/5 cell line is a clonally-derived,[16] androgen-responsive[17] cell line originally obtained from a spontaneous adenocarcinoma of an aged AXC/SSh rat.[18] Cells were cultured in Auto-Pow Eagle's minimal essential medium (ICN Biomedicals, Inc., Aurora, OH) supplemented with L-glutamine (2 mM, ICN), 1 × nonessential amino acids (ICN), sodium pyruvate (1 mM, ICN), heat-inactivated fetal bovine serum (10%, Hyclone Laboratories, Inc., Logan, UT), testosterone (10^{-7} M, Sigma Chemical Corp., St. Louis, MO), gentamycin (50 μg/mL, United States Biochemical, Cleveland, OH), and kanamycin (50 μg/mL, United States Biochemical). Cultures were maintained in a humidified atmosphere of 5% CO_2 in air and were subcultured twice a week by trypsinization at a 1:5 split ratio.

Cell Proliferation Assays. log phase cultures of T/5 cells were harvested by trypsinization, and the cells were pooled and centrifuged at 200g for 5 min. The cell pellet was resuspended in growth medium, and the number of cells present was determined by a Coulter Model ZM Cell Counter (Coulter Electronics, Hialeah, FL). Parallel sets of cultures were then seeded with 10^5 cells per 35 mm cell culture dish in 2 mL of growth medium with or without SAHA at concentrations of 10^{-4}–10^{-9} M in decade intervals. SAHA was prepared as a 10^{-1} M solution in DMSO and then diluted to the indicated concentrations in culture medium. Two sets of control cultures were prepared. One set was exposed to DMSO at the same concentration (0.1% v/v) used as a vehicle to prepare the SAHA-containing culture medium at a concentration of 10^{-4} M. The other set contained neither DMSO nor SAHA. Every 48 h, during a period of 8 days, triplicate dishes from each condition were harvested by trypsinization, and the number of cells per dish was determined by the Coulter counter. Remaining culture dishes were also refed with their appropriate media every 48 h. The experiment was repeated for a total of three times.

Data are expressed as the mean and standard deviation of triplicate dishes. One way analysis of variance was used to analyze the data.

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