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The Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid Induces Differentiation of Human Breast Cancer Cells[1]

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ABSTRACT

Histone deacetylase (HDACs) regulate histone acetylation by catalyzing the removal of acetyl groups on the NH_2-terminal lysine residues of the core nucleosomal histones. Modulation of the acetylation status of core histones is involved in the regulation of the transcriptional activity of certain genes. HDAC activity is generally associated with transcriptional repression. Aberrant recruitment of HDAC activity has been associated with the development of certain human cancers. We have developed a class of HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA), that were initially identified based on their ability to induce differentiation of cultured murine erythroleukemia cells. Additional studies have demonstrated that SAHA inhibits the growth of tumors in rodents. In this study we have examined the effects of SAHA on MCF-7 human breast cancer cells. We found that SAHA causes the inhibition of proliferation, accumulation of cells in a dose-dependent manner in G_1 then G_2-M phase of the cell cycle, and induction of milk fat globule protein, milk fat membrane globule protein, and lipid droplets. Growth inhibition was associated with morphological changes including the flattening and enlargement of the cytoplasm, and a decrease in the nuclear: cytoplasmic ratio. Withdrawal of SAHA led to reentry of cells into the cell cycle and reversal to a less differentiated phenotype. SAHA induced differentiation in the estrogen receptor-negative cell line SKBr-3 and the retinoblastoma-negative cell line MDA-468. We propose that SAHA has profound antiproliferative activity by causing these cells to undergo cell cycle arrest and differentiation that is dependent on the presence of SAHA. SAHA and other HDAC inhibitors are currently in Phase I clinical trials. These findings may impact the clinical use of these drugs.

INTRODUCTION

Breast cancer is the most common cause of cancer and the second most common cause of cancer deaths in women in the United States (1). Despite major recent advances in therapy, more effective approaches to the treatment and prevention are necessary. HDAC[3] inhibitors such as SAHA[4] may provide an alternative therapeutic approach for the treatment of breast cancer. SAHA has been shown to induce growth arrest, differentiation, and/or apoptosis in a variety of transformed cell lines and inhibits tumor development in rodents, including the development of *N*-methylnitrosourea-induced mammary tumors in rats (2).

HDACs regulate histone acetylation by catalyzing the removal of acetyl groups on the NH_2-terminal lysine residues of the core nucleosomal histones. Modulation of the acetylation status of core histones is involved in the regulation of the transcriptional activity of certain genes. HDAC activity is generally associated with transcriptional repression. Aberrant recruitment of HDAC activity has been associated with the development of certain human cancers. For example, in acute promyelocytic leukemia, the oncoprotein produced by the fusion of PML and retinoic acid receptor α appears to suppress specific gene transcription through the recruitment of HDACs (3). In this manner, the neoplastic cell is unable to undergo differentiation and leads to excess proliferation.

The inhibition of HDAC by SAHA occurs through a direct interaction with the catalytic site of the enzyme as shown by X-ray crystallography studies (4). Inhibition of HDAC activity results in the accumulation of acetylated histones H2a, H2b, H3, and H4. The result of HDAC inhibition is believed not to have a generalized effect on the genome but rather only effects the transcription of a small subset of the genome (5). Evidence provided by DNA microarrays using malignant cell lines cultured with a HDAC inhibitor shows that there are a small (1–2%) number of genes of which the products are altered.[5]

In this study we have investigated the effect of SAHA on the proliferation and differentiation of human transformed breast cancer cell lines. We found that SAHA inhibits proliferation at low μM concentrations in both ER-positive and ER-negative cell lines. SAHA, at these concentrations, induces the expression of the components of milk, including MFGM protein and milk fat globule protein, and the accumulation of neutral lipids. These results indicate that SAHA induces differentiation of human breast cancer cell lines regardless of ER status and that HDAC inhibitors such as SAHA may be new therapeutic agents for the treatment of breast cancer.

MATERIALS AND METHODS

Chemicals and Antibodies. SAHA was synthesized as described previously (6). For experiments SAHA was prepared in a 10 mM stock solution in DMSO and stored at −20°C. Antibodies against the human MFG protein (polyclonal) and MFGM protein (monoclonal) were purchased from Chemicon. Bis-benzimide trihydrochloride (Hoechst #33258; DAPI) from Hoechst. Nile Red Powder (9-diethylamino-5H-benzo(α) phenoxazine-5-one) was purchased from Sigma Chemical Co. and dissolved in acetone as a stock solution of 1 mg/ml. All of the other reagents were of analytical grade and purchased from standard suppliers.

Cell Culture. The human breast cancer cell lines SKBr-3, MCF-7, and MDA-MB-468 (MDA-468) were obtained from the American Type Culture Collection, Rockville, MD. Cell lines were maintained in medium consisting of DMEM/F2/1 (1:1) supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, and 50 units/ml of both penicillin and streptomycin, in a humidified 5% CO_2/air atmosphere at 37°C.

Antiproliferative Index. Cells (2×10^5) were plated onto six-well dishes and treated 48 h after plating with the indicated drug concentrations or DMSO vehicle for 120 h. Drug and medium were exchanged every 48 h. Cells were counted daily. Cells were harvested by trypsinization and counted on a Coulter counter. Experiments were repeated in triplicates. Dose curves were plotted as a logarithmic function of cell number *versus* time.

Drug Treatment of Cell Lines. Cells were plated onto 100-mm tissue culture plates at a density of 2×10^6 for 48 h and then treated with SAHA or equal concentrations of the vehicle. For longer drug exposure times, medium

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[3] The abbreviations used are: HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid; MFG, milk fat globulin; DAPI, 4′,6-diamidino-2-phenylindole; FACS, fluorescence-activated cell sorter; MFGM, milk fat globule membrane; ER, estrogen receptor; RB: retinoblastoma; IF, immunofluorescence.

[4] Phase I trial of SAHA: K. Kelly, P. N. Munster, H. Scher, Memorial Sloan-Kettering Cancer Center IRB #99-059 protocol.

[5] Unpublished observation.

with drug or vehicle were exchanged every 48 h. For wash-out experiments, cells were treated with SAHA daily for 60–72 h (drug and medium were exchanged at 48 h, see above), then SAHA was washed out and replaced with 10% FCS containing DMEM.

Cell Cycle Analysis. Cell cycle distribution was assayed according to Nusse *et al.* (7) with a Becton Dickinson FACS and analyzed by Cell Cycle Multicycle system. (Phoenix Flow System, San Diego, CA). In brief, adherent and supernatant cells were harvested, and cells were resuspended in staining solution containing ethidium bromide (25 μg/ml), sodium citrate (0.1%), NP40 (0.03%), and RNase (10 μg/ml), followed by isolation of nuclei using citric acid (0.01 M) and sucrose (0.25 M).

Quantitative and Qualitative Assessment of Differentiation. For Nile Red staining, we used a protocol described by Greenspan *et al.* (8). In brief, live cells were washed with HEPES buffer and resuspended in a 1:200 dilution of Nile Red in HEPES buffer (stock solution 1 mg/ml Nile Red in acetone) for 5 min. Fluorescent intensity was assessed in the green, orange, and red spectra with a Becton Dickinson fluorescence analyzer. Fluorescent intensity was compared with that of untreated cells. Nonpolar or neutral lipids stained bright green-yellow; polar lipids stained dark red when assessed with a broad band GFP filter. For quantitative analysis, the number of treated cells with increased intensity in fluorescence was compared with that of untreated cells. The fluorescent intensity seen in the 5% of untreated cells with the strongest fluorescent signal was used as an arbitrary baseline. The percentage of treated cells exhibiting equal or greater fluorescent intensity was then scored. Experiments were repeated three times and error bars denote the SE.

H&E Stain. Cell monolayers grown on Lab-Tek chamber slides were fixed with paraformaldehyde (4%) for 10 min at room temperature and stained according to standard H&E staining protocols (9).

Immunoblotting and IF. For IB, cells were harvested in medium, washed twice in PBS, and then dissolved in NP-40 lysis buffer [50 mM Tris-Cl (pH 7.4), 1% NP40, 40 mM NaF, 150 mM NaCl, 1 μM of each NA_3VO_4, phenylmethylsulfonyl fluoride, and DTT, leupeptin, aprotinin, and soybean trypsin inhibitor]. Lysates (25 μg protein) were loaded onto SDS-PAGE minigels. As described previously (10), proteins were transferred to nitrocellulose membranes and incubated with primary and secondary antibodies. Proteins were visualized by chemiluminescence (Amersham Corp.) on Bio-Max film (Eastman Kodak).

For IF, 5×10^3 cells were plated onto fibronectin-coated Lab-Tek two-well chamber slides (Fisher Scientific). After drug treatment, slides were washed twice with ice-cold PBS and fixed with a methanol and acetone solution (1:1) for 15 s. Fixed monolayers were then washed with distilled water and blocked with 5% BSA in PBS solution. After blocking, cells were incubated with the primary antibody (1:100 in 5% BSA in PBS) at 37°C for 1 h and washed three times with 1% BSA in PBS. Then, monolayers were incubated with fluorescein- and rhodamine-conjugated secondary antibodies at 37°C for 1 h. Nuclei were stained with DAPI at concentration of 0.5 μg/ml. Cells were visualized and imaged by confocal microscopy (10).

Histone Acetylation. MCF-7 cells were cultured with 5 μM SAHA or vehicle for 4 h and 24 h in 15-cm dishes at a density of 5×10^6 cells/dish. Histones were then isolated by rising and harvesting cells with ice-cold PBS. Cells were centrifuged at 1000 rpm for 5 min and resuspended in 1 ml of histone lysis buffer [8.6% sucrose, 1% Triton X-100, 50 mM sodium bisulfite, 10 mM Tris-HCl (pH 6.5), and 10 mM $MgCl_2$] and Dounce homogenized. Cell lysates were centrifuged at 700 rpm for 5 min. The nuclear pellet then was washed three times with the lysis buffer and once with 10 mM Tris-HCl (pH 7.4) and 13 mM EDTA. Histones were acid extracted from the nuclear pellets as described previously (11). Acetylation of core histones in the cells or tumors was determined by Western blotting using rabbit polyclonal antibodies against acetylated histone H3 (Upstate Biotechnology, Lake Placid, NY) and visualized using the Super Signal chemiluminescence system (Pierce, Rockford, IL), as described previously (12).

RESULTS

SAHA Inhibits Cell Proliferation in Mammary Epithelial Cells. The ER-positive cell line MCF-7 was cultured with increasing concentrations of the HDAC inhibitor SAHA (1.25–20 μM). As seen in Fig. 1*A*, concentrations of SAHA of $\geq$2.5 μM caused profound growth

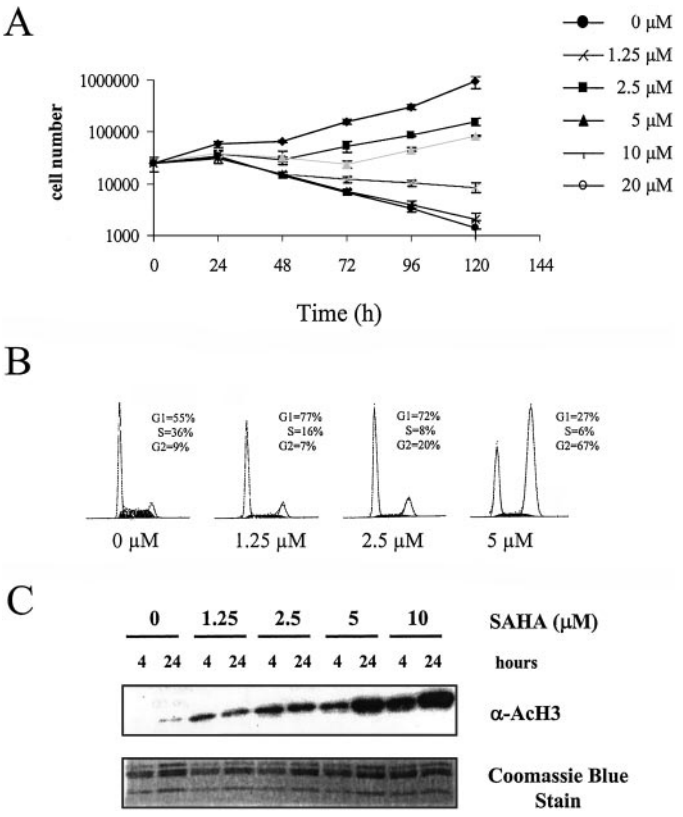

Fig. 1. SAHA inhibits proliferation of MCF-7 cells in a dose-dependent manner. MCF-7 cells were cultured with the indicated concentrations of SAHA. *A*, cell density was determined after culture with SAHA from 0–120 h; *bars*, ± SD. *B*, cell cycle distribution was determined by FACS analysis of ethidium bromide-stained cells before and 48 h after culture with SAHA. *C*, cells were harvested, and histones were prepared as described in "Materials and Methods." Histone acetylation was detected by Western blot using a polyclonal antibody against acetylated H3. The *top panel* contains acetylated histone H3, the *bottom panel* shows a Coomassie blue-stained polyacrylamide gel of the total histones extracted from the cells.

arrest. The IC_{50} of 0.75 μM was calculated as 50% reduction in cell number after 120 h of continuous exposure to SAHA. Cell cycle analysis evaluated after 48 h exposure to SAHA showed an accumulation of cells predominantly in G_1 at low concentrations (1.25 and 2.5 μM). However, higher concentrations ($\geq$5 μM) caused cell cycle arrest predominantly in G_2-M (Fig. 1). Microscopic assessment of chromosomal DNA by Hoechst staining showed that cells were not arrested in mitosis and, therefore, the cell cycle arrest occurred in G_2. The effects of SAHA on the acetylation of histones were assessed for increasing concentrations of SAHA at 4 h and 24 h. Untreated MCF-7 cells had very low levels of acetylated histones (H3; Fig. 1*C*). An increase in H3 acetylation was seen with increasing SAHA concentrations.

Induction of Morphological and Biochemical Differentiation by SAHA in MCF-7 Cells. We next evaluated the phenotypic effects on SAHA at concentrations that caused complete growth arrest (5 μM) Fig. 2. Treatment with SAHA resulted in significant changes in morphology. Untreated cells were round with scanty eosinophilic cytoplasm and indistinct cell margins. Cells had the large basophilic nuclei and prominent nucleoli characteristic of carcinoma cells. Whereas these cells originated from a mammary background, they did not express significant features of mammary differentiation such as milk fat proteins or fat-containing vacuoles. In addition, considerable mitotic activity was present (Fig. 2*a*).

Cells treated with SAHA underwent significant changes (Fig. 2*b*). The cells increased in size and flattened. The originally round cells

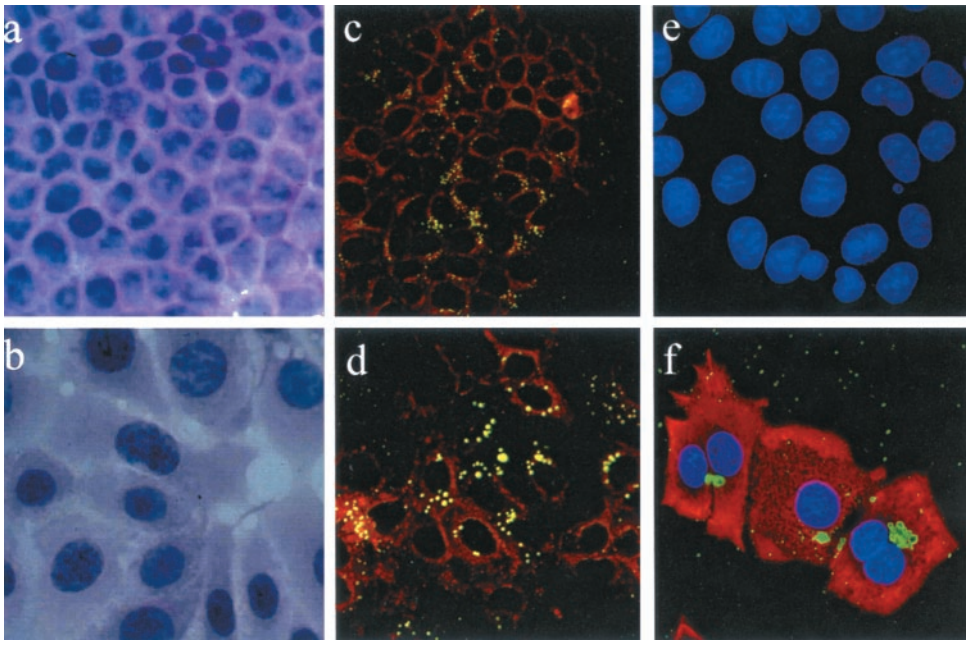

Fig. 2. SAHA induces morphological and biochemical differentiation. MCF-7 cells were grown on Lab Tek cover culture chambers and cultured without (*a*, *c*, and *e*) or with (*b*, *d*, and *f*) SAHA (5 μM) for 48 h. Morphological changes were evaluated by light microscopy (×40) using H&E-stained tissue sections (*a* and *b*). Induction of biochemical differentiation was determined using Nile Red stain of live cells unfixed cells for lipid droplets (*c* and *d*). MFG was detected using a polyclonal rabbit primary and a fluorescein (*green*) labeled secondary antibody. MFMG protein was detected with a mouse primary and rhodamine (*red*)-labeled secondary antibody. Nuclear DNA was stained with DAPI (*e* and *f*). Images were acquired by confocal microscopy (×63).

appeared more columnar and had distinct cellular boundaries. The increase in cell size was predominantly attributable to an abundance of cytoplasm, which led to a decrease in the nuclear:cytoplasmic ratio. Changes were also noted in the nuclear morphology. Nuclear borders became more distinct, and the nucleoli were less visible. Treated cells became more epithelial in appearance, with intercellular digitations and bridges. Many cells contained perinuclear vacuoles; however, these were more evident when using special stains. Overall these morphological changes were suggestive of epithelial mammary differentiation. Only minimal mitotic activity was notable in cells treated with SAHA.

These morphological changes began at 24 h after drug treatment, with induction of increasing numbers of intracellular lipid droplets. At that time only minimal flattening and enlarging of cells was evident, but this became clearly visible by 48 h and persisted throughout the time course of 120 h in the presence of drug.

Induction of Lipid Droplets. One of the markers of functional differentiation of mammary tissue is the induction of lipid droplets. Lipid droplets are composed mainly of triglycerides and comprise an important component of milk. Nile Red dye is a vital stain that distinguishes the components of intracellular lipid droplets. It is fluorescent in a hydrophobic environment. Polar lipids such as phospholipids fluoresce dark orange in the presence of Nile Red, whereas neutral lipids fluoresce brilliant yellow-gold. Untreated MCF-7 cells expressed only minimal detectable lipid vacuoles (Fig. 2*c*). Lipid droplets accumulated in MCF-7 cells cultured with SAHA. Triglycerides are neutral lipids. The lipid droplets located in the perinuclear area of treated MCF-7 cells fluoresced brilliant yellow-gold suggesting that these droplets contained neutral lipids and may be milk fat triglycerides. The cell membranes, which contain phospholipids, appeared dark orange. Pretreatment of cells with isopropyl alcohol before Nile Red staining in untreated or SAHA treated cells extracted the bright green-yellow neutral fat droplets but not the dark orange staining polar lipids present in cellular structures and cytoskeleton (data not shown).

In contrast to other lipid stains such as Oil Red O and Sudan Black, Nile Red stain fluorescence can be quantified. Culturing MCF-7 cells with SAHA (5 μM) for 48 h resulted in a 6.7-fold ($\pm$1.34) induction of neutral lipids. Nile Red fluorescence intensity was significantly reduced when cells were exposed to isopropyl alcohol before staining (Fig. 3*A*).

Induction of Milk Fat Proteins by SAHA. Milk contains a mixture of lipids and proteins. The intracellular milk fat droplets are surrounded by MFGM proteins. Other protein components of milk are MFGs. Expression of these proteins was assessed in MCF-7 cells before and after exposure to SAHA (5 μM). IF and Western blot analysis (Figs. 2*e* and 3*B*) of untreated cells revealed low levels of MFG protein and MFGM protein expression. Expression of both examined proteins was markedly induced by SAHA. Whereas the MFG proteins were detected as droplets, expression of MFGM proteins were more evenly distributed throughout the cytoplasm (Figs. 2*f* and 3*B*, 24 h and 48 h).

Induction of Morphological and Functional Differentiation Is Dependent on Presence of SAHA. We found that culture of MCF-7 cells with SAHA at concentrations that caused complete growth arrest resulted in cell cycle arrest and differentiation. We then studied whether these observations were dependent on the presence of the drug. As described above, we cultured MCF-7 cells with increasing

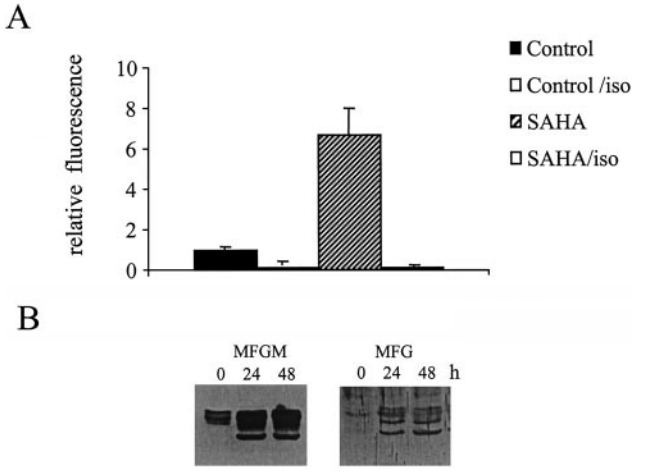

Fig. 3. SAHA induces components of milk. *A*, quantitative analysis of lipid induction. MCF-7 cells were cultured with SAHA (5 μM), and live cells were stained with Nile Red. Cells with increased fluorescent intensity were scored by FACS as described in "Materials and Methods." Bar histogram of relative increase in fluorescence intensity. Exposure to 100% isopropyl alcohol (*iso*) for 10 min before Nile Red staining eliminated perinuclear lipid droplets that were not bound to cellular structures. *B*, time course of induction of MFMG and MFG determined by Western blot analysis. Samples of NP40 lysates (50 μg protein) were loaded in each *Lane*.

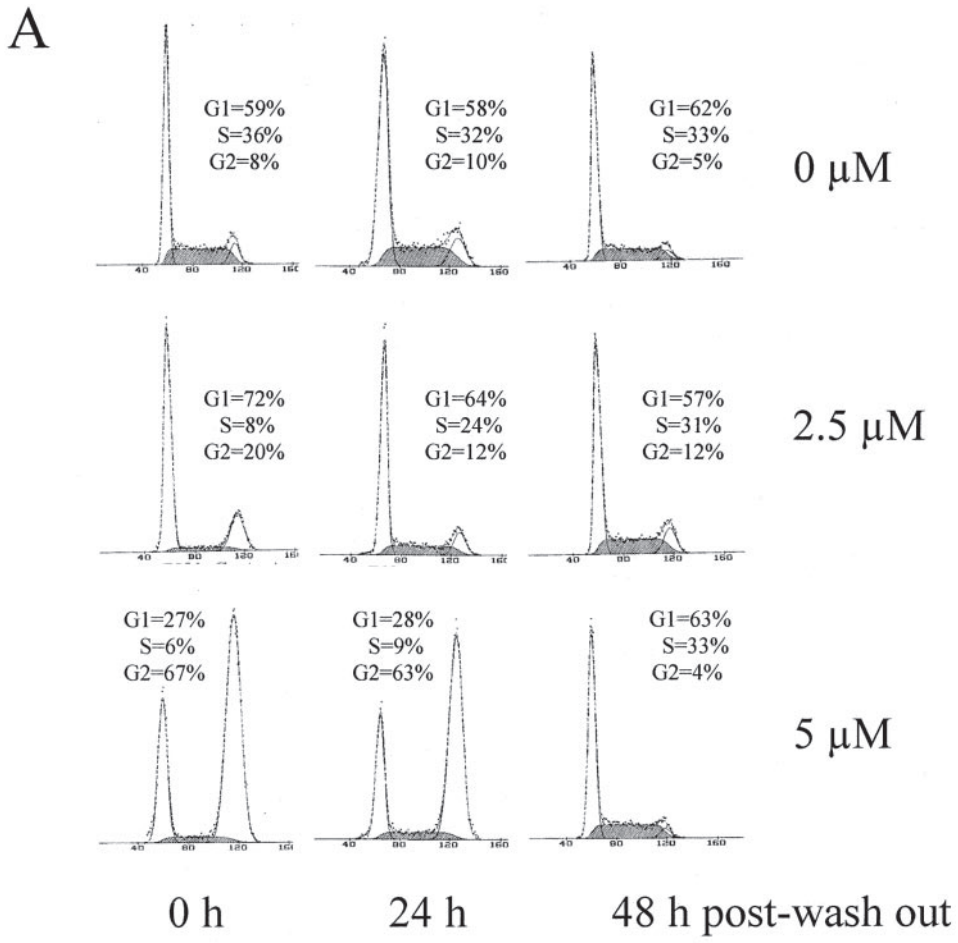

Fig. 4. Antiproliferative effect and differentiation induced by SAHA are reversible on withdrawal of drug. *A*, MCF-7 cells were cultured with the indicated concentrations of SAHA for 48 h. After 48 h, SAHA was removed, and cell cycle was analyzed by FACS analysis at 0, 24, and 48 h after drug withdrawal. *B*, morphological changes and differentiation were evaluated in MCF-7 cells grown on Lab Tek chamber slides before (*a*), 60 h after SAHA (5 μM) exposure (*b*), and 48 h after SAHA was withdrawn (*c*). MFG was detected using a polyclonal rabbit primary and a fluorescein (*green*) labeled secondary antibody. MFMG protein was detected with a mouse primary and rhodamine (*red*) labeled secondary antibody. Nuclear DNA was stained with DAPI. Images were acquired by confocal microscopy (×63).

concentrations of SAHA (2.5 and 5 μM) for 72 h. After 72 h, SAHA was washed out. Cell cycle was analyzed at 0, 24, and 48 h after SAHA removal (Fig. 4*A*) Within 24 h of drug withdrawal, many of the cells resumed progression through the cell cycle. In these cells we also found a decrease in milk fat proteins and lipid droplets after drug withdrawal (Fig. 4*B*). Furthermore, 48 h after drug removal, these cells more closely resembled the untreated MCF-7 cells.

Induction of Differentiation by SAHA Is Not Dependent on Expression of ER, RB, or HER-2. We have demonstrated that SAHA induced differentiation in MCF-7 cells. These cells express ER, intact RB, and low levels of HER-2. To determine whether the effects of SAHA are limited cells with these characteristics, we extended our studies to additional cell lines with different features. We evaluated a cell line with high expression of HER-2/*neu* and low ER expression (SKBr-3), and a cell line that lack functional RB and ER (MDA-468; Refs. 10, 13). In both cell lines SAHA induced the lipid droplets, milk fat proteins, and morphological changes (Fig. 5, *A* and *B*, and data not shown). Cell cycle arrest in SKBr-3 and MDA-468 cells was comparable with that observed in MCF-7, as shown in Fig. 1*B*. A dose-dependent G_1 arrest was followed by G_2 arrest at higher concentrations, suggesting that the cell cycle arrest induced by SAHA is not dependent or qualitatively altered in the absence of RB. These results indicate that ER, RB, and HER-2 expression is not required for these phenotypic responses to SAHA.

DISCUSSION

This study describes the effects of SAHA on breast cancer cell lines. Cells treated with SAHA undergo a dose-dependent cell cycle arrest in G_1 and at higher concentrations in G_2-M. The growth arrest is accompanied by morphological changes suggestive of mammary

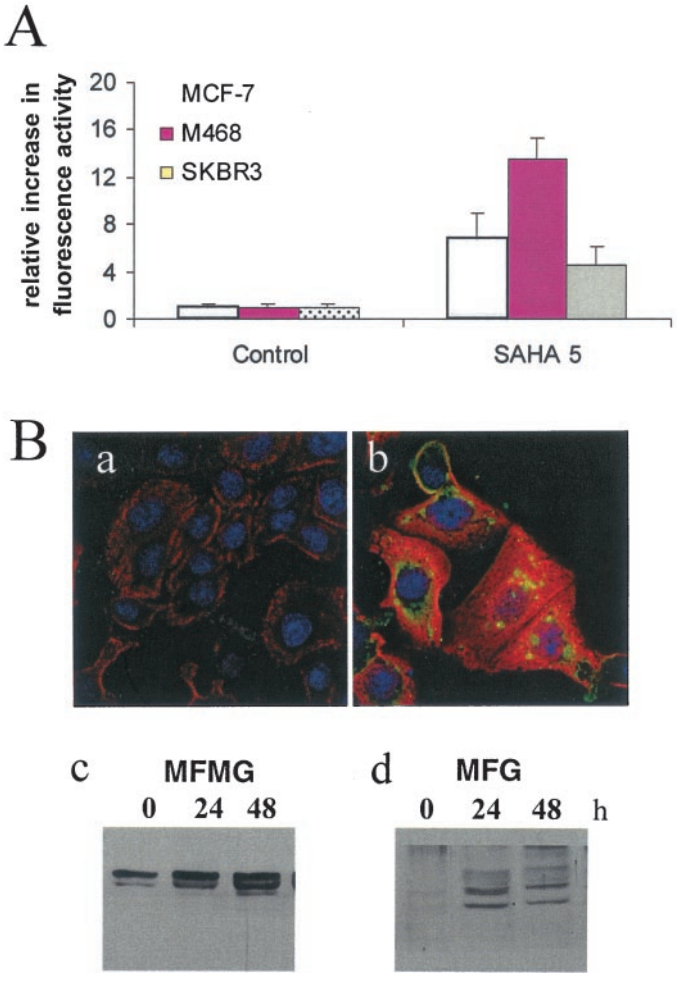

Fig. 5. SAHA-induced differentiation is not dependent on expression of ER, RB, or HER-2 status. *A*, MCF-7, SKBr-3 cells, and MDA-468 cells were cultured with SAHA (5 μM) for 48 h and stained with Nile Red. Quantitative analysis of lipid induction was determined by FACS analysis scoring cells with increased fluorescence intensity as described in "Materials and Methods." Bar histogram of increase in relative fluorescence intensity. *B*, induction of morphological changes and milk fat proteins in SKBr-3 cells before (*a*) and 48 h after (*b*) exposure to SAHA (5 μM). MFG and MFGM were detected as described in Fig. 2*e*. Time course of induction of MFMG (*c*) and MFG (*d*) determined by Western blot analysis. Samples of 50 μg NP40 lysates were loaded on each *Lane*.

differentiation. The morphological changes are characterized by a flattening and enlarging of the cytoplasm and a decrease in the nuclear-cytoplasmic ratio, as well as the number of nucleoli and mitotic figures. Exposure to SAHA causes induction of perinuclear fat vacuoles, the development of MFGMs, and the induction of MFG proteins. These effects were seen independent of the expression of ER, RB, or HER-2 in the examined breast cancer cell lines. Both morphological and biochemical differentiation is reversible and requires the continuous presence of drug in all of the cell lines examined.

These findings suggest that the induction of differentiation by inhibition of HDAC is not limited to hematopoietic cell lines but also occurs in solid tumors. In the murine erythroleukemia cell lines SAHA induces commitment to terminal differentiation, which is not reversible. Differentiation in mammary tissues has been described in response to agents such as retinoic acid, phorbol esters, hexamethylene bisacetamide, ansamycins (10, 14–16), and other HDAC inhibitors such as butyrates and trichostatin A (17–19). In these reports characterization of differentiation is limited to the description of morphological changes and lipid induction or markers that are not breast specific. In this study we extended the description of differentiation markers and performed assays to quantify the expression of differentiation markers such as lipids and milk fat proteins, markers that are characteristic for a differentiated mammary phenotype.

We found that SAHA induces differentiation irrespective of the presence or absence of the RB protein. Whereas RB is required for differentiation in many systems (20–26), our findings suggest that other regulators may be sufficient for differentiation induced by SAHA. We have shown previously that unlike SAHA, treatment of cells with ansamycins resulted in G_1 arrest, the induction of lipid droplets, milk fat proteins, and morphological changes in the presence of functional RB; cells that are deficient in RB underwent growth arrest in mitosis followed by apoptosis (10). Sodium butyrate, a low-potency HDAC inhibitor that has been extensively studied, has antitumor activity and has shown to induce differentiation in breast cancer cell lines; however, the clinical development has been hampered by the short half-life (5 min) and the inability to achieve therapeutic plasma level. To increase bioavailability, sodium butyrates have been linked to hyaluronic acid (17, 27, 28). An analogue of sodium butyrate, phenyl butyrate, is currently undergoing early clinical trial either alone or in combination in patients with hematological or solid tumors. In addition to inducing differentiation, we found that at concentrations (2.5–5 μM) that caused complete growth arrest and differentiation $<$20% of cells were apoptotic (data not shown). However, higher concentrations showed an increase in the number of apoptotic cells. The induction of apoptosis in breast cancer cell lines by SAHA has also been described by other investigators (15). Huang *et al.* (15) found that 48-h exposure to 4 μM of SAHA resulted in 20% apoptotic nuclei. Cell cycle and apoptosis was reported to be associated with up-regulation of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 via a p53-independent mechanism.

These findings suggest that for therapeutic effects, SAHA plasma levels in patients either must reach concentrations sufficient to induce apoptosis, *e.g.*, concentrations higher than those required for differentiation, or patients have to be exposed to SAHA on a continuous basis. In addition, SAHA in combination with other drugs may be effective at concentrations that are sufficient to induce cell cycle blocks in either G_1 or G_2-M. In summary, our studies demonstrate that SAHA has antiproliferative effects on breast cancer cells independent of the expression of RB, ER, or HER-2. These effects are associated with the induction of differentiation markers and morphological changes in the presence of drug. These findings may be relevant for the clinical design of these drugs and the development of surrogate markers for response.

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