SPANDIDOS PUBLICATIONS

SAHA, a HDAC inhibitor, has profound anti-growth activity against non-small cell lung cancer cells

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Abstract. Current chemotherapy of advanced non-small cell lung cancer (NSCLC) produces only a modest increase in survival time. New approaches are needed for this disease. The development of lung cancer is associated with silencing tumor suppressor genes that can occur not only by deletion or mutation, but also by epigenetic changes including histone deacetylation of key lysines. Histone deacetylase inhibitor (HDACI) increases histone acetylation, resulting in DNA with a more open chromatin that favors transcription. We found that the HDACI, suberoylanilide hydroxamic acid (SAHA), suppressed cell growth of five non-small cell lung cancer cell lines in a dose-dependent manner (50% growth inhibition ≈2 μM). Cell cycle assay by fluorescence-activated cell sorting (FACS) demonstrated that SAHA induced a significant G0-G1 growth arrest of NSCLC cells. Protein assay by Western blot analysis showed that SAHA induced expression of $p21^{WAF1}$. These results demonstrated that administration of SAHA may be a novel approach to the treatment of non-small cell lung cancer.

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Abbreviations: NSCLC, non-small cell lung cancer; HDACI, histone deacetylase inhibitor; SAHA, suberoylanilide hydroxamic acid; FACS, fluorescence activated cell sorting; EGFR, epidermal growth factor receptor; HAT, histone acetyltransferase; HDAC, histone deacetylase; NaB, sodium butyrate; VPA, valproic acid; TSA, trichostatin A; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDS, sodium dodecyl sulfate; PI, propidium iodine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Key words: histone deacetylase inhibitor, MTT assay, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay, cell cycle, cyclin-dependent kinase inhibitor, $p21^{WAF1}$

Introduction

Lung cancer is the leading cause of cancer deaths in most countries, and 1.2 million new cases per year are diagnosed in the world (1). Chemotherapy against NSCLC is one therapeutic approach for patients who have unresectable tumors in an advanced stage. But treatment outcomes are still poor. Although newer chemotherapeutic reagents (paclitaxel, docetaxel, gemcitabine, vinorelbine and irinotecan) are more active for lung cancer than prior drugs and do provide a moderate therapeutic benefit in the adjuvant setting, they still have little effect on recurrent disease (2). Another approach has been the use of anti-EGFR molecules [for example, Gefitinib (ZD1839, Iressa) and Erlotinib], which has effectiveness in about 10-20% of individuals, particularly for patients whose cancer is associated with prominent EGFR activity often associated with an activation mutation of EGFR (3,4). Clearly, additional therapies are needed.

Chromatin remodeling is a key step in the regulation of gene expression, consequently affecting cell function, differentiation, and proliferation. Chromatin structure affects transcription by opening or closing the access of transcriptional factors to their target sequences (5,6). The key mechanism in chromatin remodeling is thought to be the modification of NH_2-terminal tails of histones, which contributes to a 'histone code' determining the transcription of target genes (7). 'Closed' chromatin is not transcribed and consists of nucleosomes in which the lysine residues of the histone tails becomes deacetylated. Acetylation of those regions neutralizes the positive charge on lysine residues and changes the nucleosome structure, leading to 'opened' chromatin in which transcription factors have easy access, and resulting in gene expression.

Acetylation of histone tails is regulated by the opposing activities of HATs and HDACs, and aberrant deacetylation due to HDACs is associated with certain types of human cancer (8). Transcription factors, such as Mad-1, BCL-6, and ETO, have also been shown to assemble HDAC-dependent transcriptional repressor complexes (9-12).

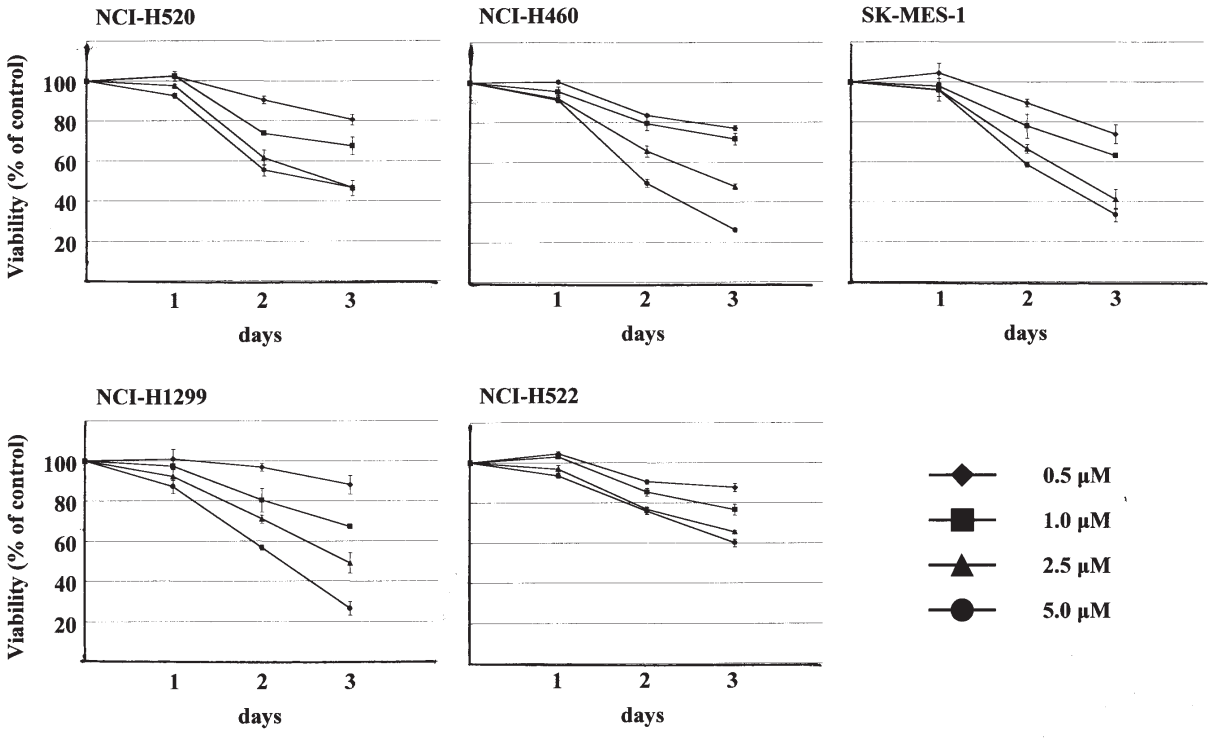

Figure 1. Dose-response effects of SAHA on cell viability of a variety of human NSCLC cell lines. Five NSCLC cell lines were treated with SAHA (0.5, 1.0, 2.5 and 5.0 μM) for 1, 2 and 3 days. Cell viability was determined by MTT assay and plotted as a percentage of viable untreated cells (control). Results represent the mean ± SD of three independent experiments with triplicate wells.

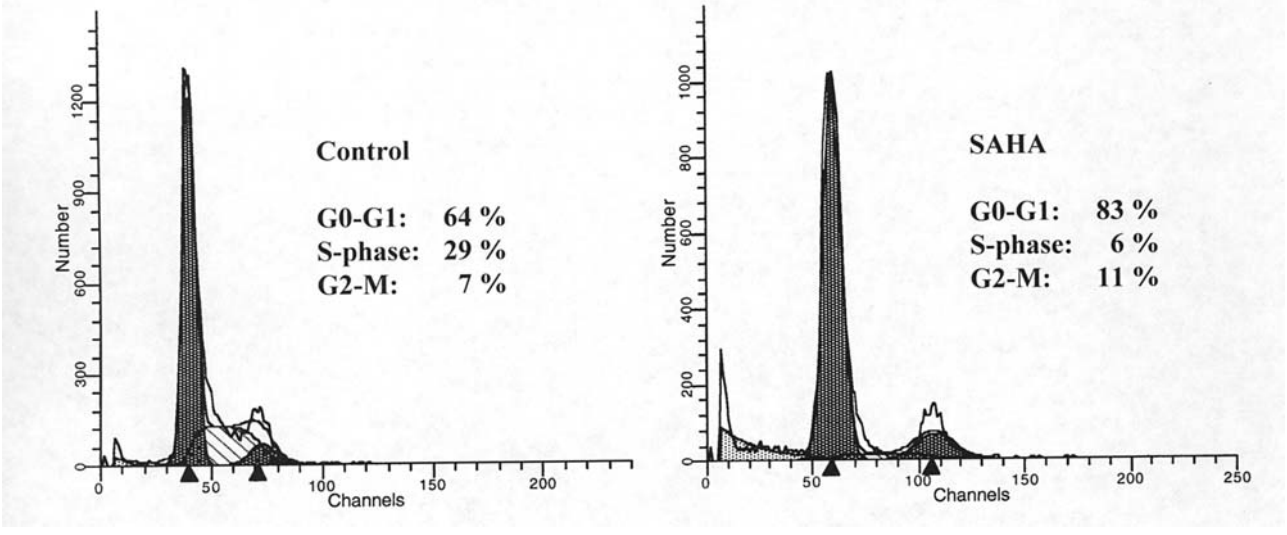

Figure 2. Cell cycle analysis of NCI-H520 cells by flow cytometry. NCI-H520 cells were cultured with SAHA (2.5 μM) for 72 h, harvested, and stained with PI. Control cells were treated with vehicle alone, and cell cycle analysis was performed by flow cytometry (see Materials and methods).

HDACIs can induce apoptosis (13) and enhance cell differentiation (14,15) resulting in slowing growth of cancer cells *in vitro* (16,17) and *in vivo* (18,19). HDACIs are categorized in subgroups including: (a) short-chain fatty acids (NaB and VPA), (b) organic hydroxamic acids (TSA and SAHA), (c) cyclic tetrapeptides (trapoxin), and (d) benzamides (MS-275) (20).

The effect of HDAC inhibition is believed to not be a generalized one on the genome, but a rather limited effect. Differential display analysis of transformed lymphoid cell lines revealed that the expression of only 2-5% of genes was changed significantly after treatment with TSA (21). Several HDACIs (TSA and trapoxin) are not therapeutically useful because of poor bioavailability and/or toxic side effects at high doses. Another HDACI, NaB is degraded rapidly after intravenous administration and very high doses are therefore required, but associated with toxicity (22). The inhibition of HDACs by SAHA occurs through a direct interaction with

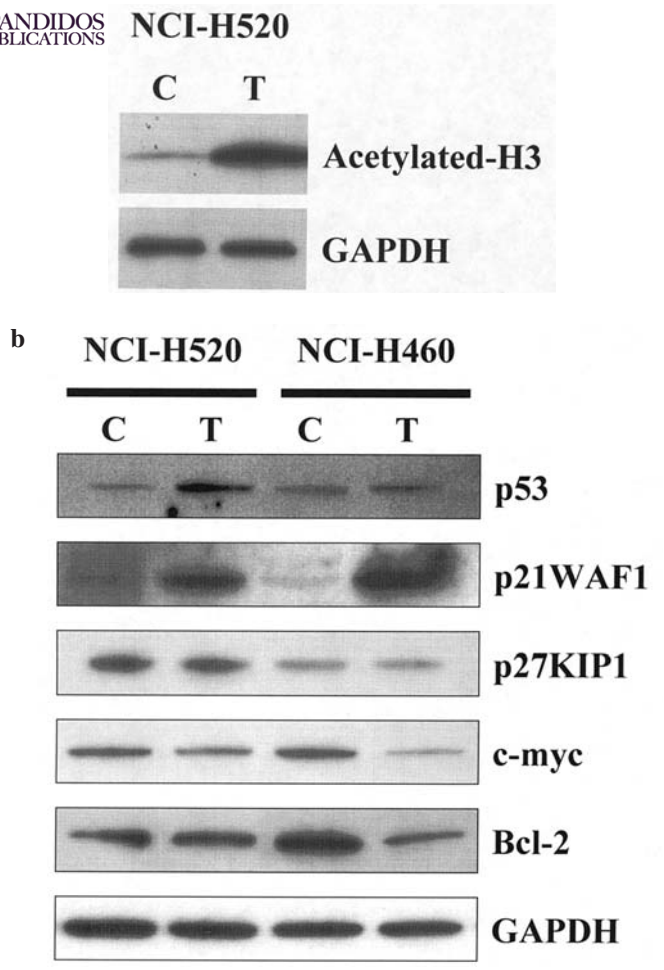

Figure 3. Protein expression by Western blot analysis. NCI-H520 and NCI-H460 lung cancer cells were either treated with SAHA (T) or vehicle alone (C), and harvested after 1 or 3 days. Western blot was analyzed for acetylated histone H3 (panel a, NCI-H520 cells), as well as, for p53, $p21^{WAF1}$, $p27^{KIP1}$, c-myc, and bcl-2 (panel b). The amount of proteins was normalized by comparison with levels of GAPDH.

the catalytic site of the enzyme (23). It has been studied in several phase I/II trials and has potential as a new therapeutic drug for cancer treatment.

In this study, we focused on SAHA, recognized as one of the least toxic HDACI, in order to determine if it is able to inhibit cell growth and cause cell cycle arrest, apoptosis, and expression of genes related to decreased cell growth in NSCLC.

Materials and methods

Cell lines. Non-small cell lung cancer cell lines, NCI-H520 (squamous cell carcinoma), NCI-H460 (large cell carcinoma), NCI-H522 (adenocarcinoma), NCI-H1299 (large cell carcinoma), SK-MES-1 (squamous cell carcinoma) were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal calf serum.

SAHA. SAHA was generously provided by Victoria Richon at Merck (Boston, MA) and dissolved in DMSO (Sigma, St. Louis, MO) at a concentration of 50 mM, which was diluted into PBS at a concentration of 5 mM prior to use. The diluent, DMSO, was added alone to culture media as a control.

MTT assays for cell proliferation and viability. To measure cell proliferation and viability, MTT (Sigma) was dissolved in PBS at 5 mg/ml. Approximately $1x10^4$ cells per well were incubated in culture medium in 96-well plates for various durations, followed by the addition of 10 μl of MTT solution. After 4 h incubation, 50 μl of solubilization solution (20% SDS) was added, and the mixture was incubated at 37˚C for 16 h. In this assay, MTT is cleaved to an orange formazan dye by metabolically active cells. The absorbance of the formazan product was measured with an enzyme-linked immunosorbent assay reader at 595 nm.

Cell cycle analysis by flow cytometry. Cell cycle was analyzed by flow cytometry after 3 days culture with or without SAHA. Briefly, lung cancer cells were cultured for 3 days, trypsinized, washed in Dulbecco's phosphate-buffered saline, fixed in methanol, and incubated for 30 min at 4˚C in the dark with a solution of 5 μg/ml PI, 1 mg/ml RNase (Sigma), and 0.1% NP40 (Sigma). Analysis was performed immediately after staining using the CellFit program (Becton-Dickinson, Franklin Lakes, NJ), whereby the S phase was calculated using an RFit model.

TUNEL assay for measuring apoptosis. DNA strand breaks were identified by terminal deoxynucleotidyltransferase-mediated UTP end labeling technique using the In Situ Cell Death Detection kit as directed (Roche, Indianapolis, IN).

Western blot analysis. Expression of specific proteins was detected by Western blot analysis. Cells were washed twice in PBS, suspended in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin], and placed on ice for 30 min. After centrifugation at 15,000 x g for 20 min at 4˚C, the supernatant was collected. Protein concentrations were quantitated using the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Whole cell lysates (50 μg) were resolved with 4-15% SDS-polyacrylamide gel and transferred to an immobilon polyvinylidene difuride membrane (Amersham, Arlington Heights, IL). Antibodies to acetyl-histone H3 (Upstate Biotechnology, Inc., Lake Placid, NY), p53 (DO-1, Santa Cruz Biotechnology, Inc. Santa Cruz, CA), $p21^{WAF1}$ (Oncogene, San Diego, CA), $p27^{KIP1}$ (C-19; Santa Cruz Biotechnology), c-myc (9E10; Santa Cruz Biotechnology), bcl-2 (100; Santa Cruz Biotechnology), and anti-GAPDH (Research Diagnostics, Flanders, NJ) were used to detect these proteins. The blots were developed using the enhanced chemiluminescence kit (Amersham).

Results

SAHA inhibits proliferation of lung cancer cells. To study the effect of SAHA on cell proliferation of NSCLC cell lines, the cells were cultured in the presence of various concentrations of SAHA. SAHA effectively inhibited cell growth of 4 of 5 cell lines in a dose-dependent manner (Fig. 1). The

effective dose of SAHA that inhibited cell growth by 50% at 3 days was 1.9 μM for SK-MES-1, 2.2 μM for NCI-H520, 2.3 μM for NCI-H460, and 2.4 μM for NCI-H1299. An ED_{50} was not reached for NCI-H522 cell line.

SAHA inhibits S phase of cell cycle in lung cancer cells. The sensitivity of NCI-H520 cells to SAHA was comparable to the other three sensitive cell lines; therefore, it was used as a representative cell line in further studies. After culture in the presence of SAHA (2.5 μM, 3 days), the cells accumulated in the G0/G1 and G2/M phases of the cell cycle with a proportional decrease of cells in S phase (Fig. 2).

Effect of SAHA on acetylation of histone H3. Treatment of NCI-H520 NSCLC cells with SAHA (2.5 μM, 1 day) resulted in the prominent acetylation of histone H3 (Fig. 3a).

Effect of SAHA on expression of cell cycle and apoptosis-related proteins. The effect of SAHA (2.5 μM, 3 days) on cell cycle and apoptosis-related gene expression in both NCI-H520 and -H460 SAHA-sensitive NSCLC cells was examined by Western blot analysis (Fig. 3b). The level of wild-type p53 prominently increased in NCI-H520, and slightly increased in NCI-H460. The $p21^{WAF1}$ protein level markedly increased in both cell lines. Also, c-myc decreased in both cell lines, and Bcl-2 decreased in NCI-H460 after exposure to SAHA.

Discussion

Progress in the understanding of molecular events that occur during tumorigenesis can be used to design novel therapies. The inactivation of tumor suppressor genes is an important event in this process. These genes can be inactivated by mutations, deletions and, as demonstrated more recently, epigenetic events, such as aberrant methylation of promoter regions of genes (24-26) and changes in chromatin structure by histone modification (13).

HDACIs are a promising new class of drugs currently in early phase clinical trials. A large number of structurally diverse HDACI have been synthesized that often inhibit the activity of all eleven class I and II HDACs. While these agents demonstrate many features required for anti-cancer activity, such as low toxicity against normal cells and an ability to inhibit tumor cell growth, their mechanisms of action are largely unknown (27).

We found that SAHA was able to inhibit the growth of lung cancer cell lines, NCI-H520, NCI-H460, NCI-H522, NCI-H1299 and SK-MES-1. Cell cycle analysis by FACS showed that SAHA caused on accumulation of NSCLC cells in G0/G1 and G2/M with a markedly decrease percent of cells in S phase (Fig. 2).

HDACs are ubiquitously distributed throughout the chromatin. Nevertheless, HDACIs, such as SAHA, selectively alter transcription of as few as 2-5% of genes in various transformed cells. For example, $p21^{WAF1}$ is one of the most commonly induced genes in cells cultured with SAHA (28). To explore the mechanism by which SAHA slows growth, we examined changes of several key proteins related to the cell cycle and apoptosis. The cyclin-dependent kinase inhibitor $p21^{WAF1}$ was up-regulated in NCI-H520 and -H460 lung cancer cells treated with SAHA. Levels of p53 increased in NCI-H520 cells that may in part explain the increase of $p21^{WAF1}$ since it is a transcriptional target of p53. Levels of c-myc decreased in both cell lines, which is consistent with the anti-proliferative activity of SAHA.

Apoptosis is also an important factor leading to cellular suicide, and we showed that SAHA decreased expression of the anti-apoptotic protein, bcl-2 in NCI-H460 cells. We could not detect apoptosis in either cell line using TUNEL assay (data not shown). Another study of NSCLC cell lines also showed that HDACIs (TSA and NaB) had limited abilities in inducing apoptosis despite their capacity to effectively inhibit deacetylase activity (29). These investigators thought that the lack of apoptosis reflected an active NF-κB signaling pathway because the addition of a proteosome inhibitor with an HDAC inhibitor induced prominent apoptosis in NSCLC cells (30,31).

In conclusion, our data indicate that SAHA exhibits an anti-proliferative activity and potently induces G0-G1 cell cycle arrest of human lung cancer cells, which is associated with the prominent induction of $p21^{WAF1}$ expression. Clinical trials of SAHA for lung cancer in an adjuvant setting or in combination with other drugs may provide an interesting therapeutic niche for this therapeutically refractory disease.

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