

# DNA Methyltransferase Inhibition Enhances Apoptosis Induced by Histone Deacetylase Inhibitors<sup>1</sup>

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## Abstract

Histone acetylation has long been associated with transcriptional activation, whereas conversely, deacetylation of histones is associated with gene silencing and transcriptional repression. Here we report that inhibitors of histone deacetylase (HDAC), depsipeptide and trichostatin A, induce apoptotic cell death in human lung cancer cells as demonstrated by DNA flow cytometry and Western immunoblot to detect cleavage of poly(ADP-ribose) polymerase. This HDAC inhibitor-induced apoptosis is greatly enhanced in the presence of the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (DAC). The HDAC inhibitor-induced apoptosis appears to be p53 independent, because no change in apoptotic cell death was observed in H1299 cells that expressed exogenous wild-type p53 (H1299 cells express no endogenous p53 protein). To further investigate the mechanism of DAC-enhanced, HDAC inhibitor-induced apoptosis, we analyzed histone H3 and H4 acetylation by Western immunoblotting. Results showed that depsipeptide induced a dose-dependent acetylation of histones H3 and H4, which was greatly increased in DAC-pretreated cells. By analyzing the acetylation of specific lysine residues at the amino terminus of histone H4 (Ac-5, Ac-8, Ac-12, and Ac-16), we found that the enhancement of HDAC inhibitor-induced acetylation of histones in the DAC-pretreated cells was not lysine site specific. These results demonstrate that DNA methylation status is an important determinant of apoptotic susceptibility to HDAC inhibitors.

## Introduction

The  $\epsilon$ -amino group of specific lysine residues of histones H3 and H4 is modified by histone acetyltransferases and HDAC<sup>3</sup> enzymes. The net level of histone acetylation depends on the balance of activity of these enzymes (1, 2). Acetylation of lysine residues on the NH<sub>2</sub>-terminal tails of the histones neutralizes the positive charge of the histone tail and decreases its affinity to negatively charged DNA (3, 4). As a consequence, it is thought that histone acetylation leads to changes of nucleosomal conformation and allows DNA to become more accessible to transcription factors (5, 6). As such, histone acetylation has been associated with transcriptionally active regions of the genome, whereas silenced regions are associated with hypoacetylated histones.

Because the acetylation state of histones is associated with transcriptional activity, the role of histone acetylation in regulating reexpression of silenced tumor suppressor genes has been studied widely (7–10). Growing evidence suggests a relationship between alterations

in chromatin structure by histone hyperacetylation or deacetylation and the development of cancer (7, 8, 11, 12). The short chain fatty acid butyrate (which is a breakdown product of fiber fermentation in the colon) appears to inhibit HDAC and suppresses the progression of colon cancer (8, 11). P300/CBP and pCAF, which are transcriptional coactivators, have been shown to have histone acetyltransferase activity and are disrupted by E-1a, a viral oncoprotein (12). The RB, which plays an important role in controlling cell cycle progression by regulating E2F activity, is a transcriptional repressor that was recently reported to recruit HDAC1 to the E2F-regulated cyclin E promoter (7). The RB pathway of G<sub>1</sub>-S cell cycle control is altered in most, if not all, cancers (8). Additional evidence suggesting a link between histone acetylation and cancer is the functional link between DNA methylation and histone acetylation through the family of methyl DNA binding proteins. Taken together, these data indicate that the development of cancer is associated with histone acetylation.

To study histone acetylation, specific inhibitors of histone deacetylase have been used. TSA, a microbial metabolite, is a potent reversible inhibitor of HDAC (13). TSA is reported to induce hyperacetylation of histones (14), cell cycle arrest, and apoptosis (15, 16). Depsipeptide (FR901228) is another inhibitor of HDAC, which has a structure unrelated to TSA (17). Depsipeptide strongly inhibits proliferation of tumor cells by arresting cell cycle transition at the G<sub>0</sub>-G<sub>1</sub> and G<sub>2</sub>-M phases (18) and induces apoptotic cell death in human breast cancer cells (19). TSA and depsipeptide target the same pathway; however, the antiproliferative effect of depsipeptide is 10-fold greater than that of TSA, and the IC<sub>50</sub> of depsipeptide on histone acetylation is much lower than TSA (17).

Cytosine methylation of CpG islands within the promoter regions of tumor suppressor genes can cause transcriptional silencing (20, 21). This epigenetic process is characterized by reversible transcriptional silencing without structural genetic alterations and can be reversed *in vitro* through the use of DNA methyltransferase inhibitors such as DAC. Recent reports have linked the process of DNA methylation and histone acetylation through the family of methyl DNA binding proteins, as characterized by MeCP2 (21, 22). In addition, Dnmt1 is able to directly bind to HDAC enzymes. We hypothesize here that the antiproliferative and proapoptotic activities of the DNA methyltransferase inhibitors and HDAC inhibitors are linked. We performed experiments in human lung cancer cell lines H23 and H719. Both cell lines were treated with HDAC inhibitors alone or in combination with DAC to investigate whether apoptotic cell death occurs. We also analyzed the level of HDAC inhibitor-induced histones H3 and H4 acetylation with or without DAC treatment. The results of our experiments show that TSA and depsipeptide induced a p53-independent apoptosis that was greatly increased in the presence of the DNA methyltransferase inhibitor, DAC. Furthermore, the mechanism by which DAC enhances HDAC inhibitor-induced apoptosis may be related to an enhancement of depsipeptide-induced hyperacetylation of histones in the presence of DAC.

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<sup>3</sup> The abbreviations used are: HDAC, histone deacetylase; ATCC, American Type Culture Collection; DAC, 5-aza-2'-deoxycytidine; PARP, poly(ADP-ribose) polymerase; RB, retinoblastoma protein; TSA, trichostatin A; wt, wild type.

## Materials and Methods

**Cell Culture and Chemical Treatment.** Human lung cancer cell lines H23, H719, and H1299 were obtained from the ATCC and grown in RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin at 37°C (5% CO<sub>2</sub>; Ref. 23). Twenty-four h prior to experiments,  $0.5 \times 10^6$  cells were plated on 100-cm<sup>2</sup> plates. DAC (Sigma Chemical Co., St. Louis, MO) was added to cells at 1  $\mu$ M for 12–72 h. Fresh DAC was changed every 24 h. Depsipeptide was kindly provided by Dr. Kenneth K. Chan (The College of Pharmacy, The Ohio State University). TSA was purchased from Sigma. DAC, depsipeptide, and TSA were dissolved in DMSO. The maximum concentration of DMSO used in all experiments was <0.01%. All untreated cells were treated with 0.01% of DMSO as a control.

**Trypan Blue Assay.** Cells that were treated with different drugs at different concentrations were harvested and stained with trypan blue (final concentration, 0.02%; Life Technologies, Inc., Gaithersburg, MD). The stained cells were then counted immediately under a microscope. At least 200 cells were counted for each time point. Stained black cells were considered as dead cells and unstained bright cells as viable cells.

**Protein Extraction and Western Immunoblotting.** Cells were harvested, and proteins were extracted as described previously (23). Cells were lysed with lysis buffer [50 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% Igepal CA-630, and a mixture of protease inhibitors (Roche Diagnostics, Mannheim, Germany)]. Equivalent amounts of proteins (150–250  $\mu$ g) were size fractionated on SDS-PAGE (9% for p53 and  $\alpha$ -tubulin and 7.5% for PARP). Proteins on the gels were transferred onto nitrocellulose membranes and blocked [5% nonfat milk, 200 mM NaCl, 25 mM Tris (pH 7.5), and 0.05% Tween 20]. The membranes were incubated with primary antibody at 4°C overnight with rocking. After washing with TBS-T (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20) six times at 5 min each, membranes were incubated with the appropriate secondary antibody (antimouse 1:2000 for p53, 1:5000 for  $\alpha$ -tubulin, and 1:2500 for PARP) at room temperature for 1–2 h. Protein bands were detected using a chemiluminescent detection system (Amersham Pharmacia Biotech, Uppsala, Sweden). The antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; anti-p53, DO-1; 1  $\mu$ g/ml), PharMingen (San Diego, CA; anti-PARP; 1  $\mu$ g/ml), and Oncogene Research Products (Darmstadt, Germany;  $\alpha$ -tubulin, Ab-1; 0.3  $\mu$ g/ml).

**Acid Extraction of Histones.** The acid extraction of histones was performed as described previously (2, 13) with modifications. Briefly, cells (70% confluence) were treated with depsipeptide alone or in combination with DAC. After treatment, cells were scraped and centrifuged at  $200 \times g$  for 10 min and then suspended in 10 volumes of PBS. Cells were centrifuged again at  $200 \times g$  for 10 min. Cells were suspended with five volumes of lysis buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, and 1.5 mM phenylmethylsulfonyl fluoride] and 0.4 N sulfuric acid and then lysed on ice for 30 min. After centrifugation at  $11,000 \times g$  for 10 min at 4°C, the cell supernatant fraction that contained acid-soluble proteins was retained. Supernatant was dialyzed against 200 ml of 0.1 N acetic acid twice for 1–2 h each and then dialyzed against 200 ml of H<sub>2</sub>O for 1 h, 3 h, and overnight. Dialysis was performed using a Slide-A-Lyzer cassette (Pierce, Rockford, IL) as instructed by the manufacturer. Proteins (20  $\mu$ g for each lane) were quantitated and size fractionated on SDS-PAGE (15%) for Western immunoblotting. The antibodies were purchased from Serotec, Ltd. (Oxford, United Kingdom; anti-acetylated histone H4, 1:1250; anti-Ac-5, Ac-8, Ac-12, and Ac-16 of acetylated histone H4, 1  $\mu$ g/ml) and Upstate Biotechnology (Lake Placid, NY; anti-acetylated histone H3, 1  $\mu$ g/ml).

**Flow Cytometry.** Cells were trypsinized and washed with cold PBS once and then fixed with 70% ethanol and stored at –20°C overnight. Fixed cells were then washed with PBS and suspended in 200  $\mu$ l of propidium iodide (10  $\mu$ g/ml; Sigma). Stained cells were incubated at room temperature for 30 min in the dark. A Beckton-Coulter Elite (Miami, FL) Fluorescence Activated Cell Sorter was used to analyze cellular DNA content. Apoptosis was assayed by the appearance of a sub-G<sub>1</sub> (<2N ploidy) population by the computer program Lysis II.

**Plasmid Construction and Transient Transfection.** Wild-type p53 DNA was inserted into the vector PCIneo (Promega Corp., Madison, WI). Ten  $\mu$ g of PCIneo wt p53 or empty PCIneo vector was transfected into p53 null H1299 cells using a calcium phosphate method (24). After incubation at 37°C for 8–10 h, cells were washed with cold PBS twice and replaced with fresh

medium. Twenty-four h after transfection, fresh medium was added, and cells were treated with DAC (1  $\mu$ M) or depsipeptide (0.05  $\mu$ M), singly or in combination. Cells were lysed, and proteins were assayed with Western immunoblotting as described above.

## Results

**HDAC Inhibitor-induced Apoptosis Is Enhanced in the Presence of DAC.** HDAC inhibitors, depsipeptide and TSA, have been shown to have potential antitumor activity in human cancer cells (14, 17, 19). We used two lung cancer cell lines, H719 and H23, as target cells to study the effects of depsipeptide and TSA. Both H719 and H23 were chosen because they are known to lack the cyclin dependent kinase inhibitor p16<sup>INK4</sup>, on the basis of promoter hypermethylation (25). We were interested in evaluating the effects of combining HDAC inhibitors and DNA methyltransferase inhibitors on p16<sup>INK4</sup> protein expression. Cells were exposed to TSA or depsipeptide at different concentrations for 6 h, washed and placed in fresh medium, and then incubated for 24 h to measure cell viability with trypan blue assay. Fig. 1A shows that TSA and depsipeptide decreased cell viability in both cell lines in a dose-dependent fashion. Cell viability, for example, decreased in a graded fashion from  $93.5 \pm 3.2\%$  to  $58.2 \pm 2\%$  in H719 cells treated with increasing doses of TSA for 6 h (Fig. 1A). Similarly, the cell viability decreased from  $91.5 \pm 3.2\%$  to  $41 \pm 3.2\%$  in H719 cells when treated with depsipeptide at doses ranging from 0.05 to 5  $\mu$ M (Fig. 1A). Almost identical results were obtained with H23 cells. DAC-induced changes in cell viability were slight when cells were exposed to DAC (1  $\mu$ M) for up to 72 h (Fig. 1, B and C). When TSA or depsipeptide (at the lowest concentrations tested in Fig. 1A) was introduced into DAC-pretreated cells, a significant enhancement of cytotoxicity was observed (Fig. 1, B and C). Cells were pretreated with DAC (1  $\mu$ M) for 24, 48, and 72 h, respectively, and TSA (0.5  $\mu$ M) or depsipeptide (0.05  $\mu$ M) was added to the DAC-pretreated cells for the final 6 h of drug exposure (at 18–24, 42–48, or 66–72 h, respectively). Cell viability decreased significantly, with increased toxicity noted in cells that had longer times of exposure to DAC (Fig. 1, B and C). For example, in H719 cells (Fig. 1B), treatment with an identical dose of depsipeptide (0.05  $\mu$ M) demonstrated greater toxicity, depending on the duration of DAC pretreatment. In depsipeptide-treated cells, we observed cell viability of  $91.5 \pm 3.2\%$  with no DAC pretreatment,  $67.7 \pm 2.2\%$  with 24 h of DAC pretreatment,  $51.2 \pm 1.7\%$  after 48 h of DAC pretreatment, and  $38.2 \pm 3.2\%$  with 72 h of DAC pretreatment. Similar results were obtained with H23 cells (Fig. 1C) and with TSA alone and in combination with DAC, although the potency of TSA was less than depsipeptide (Fig. 1).

To determine whether apoptotic cell death is responsible for the HDAC inhibitor-induced decreases in cell viability, we performed flow cytometry analysis to examine cellular DNA content and Western immunoblotting to detect PARP cleavage. Fig. 2 shows the results of the flow cytometry data, demonstrating changes in cell cycle and accumulation of sub-G<sub>0</sub>-G<sub>1</sub> (apoptotic) DNA in the drug-treated cells. Cells treated with DAC alone showed cell cycle changes that were dependent on the duration of DAC treatment as shown in Fig. 2, B and C. Twelve h of DAC treatment did not induce obvious changes in cell cycle, although 72 h induced a significant increase in G<sub>2</sub>-M phase cells and a decrease in G<sub>0</sub>-G<sub>1</sub> cells, with no significant increase in apoptotic DNA (Fig. 2C compared with 2A, control panel). TSA (0.5  $\mu$ M) and depsipeptide (0.05  $\mu$ M) also induced a G<sub>2</sub>-M block and a decrease in G<sub>0</sub>-G<sub>1</sub> phase cells (Fig. 2, D and F). Depsipeptide alone induced an increase in sub-G<sub>0</sub>-G<sub>1</sub> DNA (Fig. 2D). Clearly, the treatment of depsipeptide in combination with DAC for 12 h induced a significant increase in apoptotic DNA (Fig. 2E). Also, the HDAC inhibitor-induced apoptosis was dependent on the duration of DAC

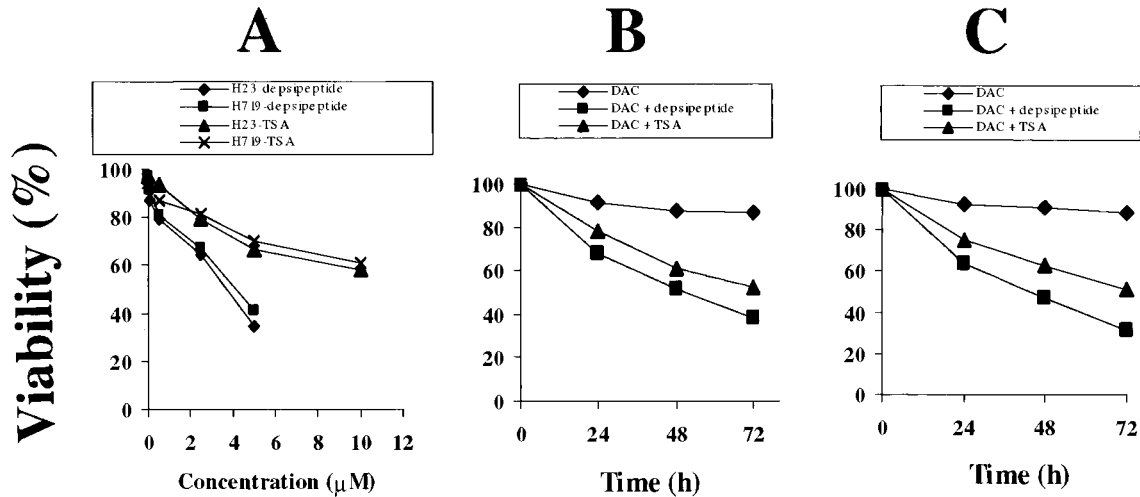


Fig. 1. Effects of depsipeptide or TSA alone at various concentrations, or in combination with DAC, on cell viability of H719 and H23 cells. A, H719 or H23 cells were treated with depsipeptide (0.05, 0.5, 2.5, or 5  $\mu\text{M}$ ) or TSA (0.5, 2.5, 5, or 10  $\mu\text{M}$ ) for 6 h and then incubated in fresh medium for 24 h. Depsipeptide (0.05  $\mu\text{M}$ ) or TSA (0.5  $\mu\text{M}$ ) was introduced into DAC (1  $\mu\text{M}$ ; 24, 48, or 72 h)-pretreated H719 cells (B) or H23 cells (C) for 6 h and then incubated for 24 h and collected for trypan blue assay.

treatment time. For example, only 3.4% apoptotic DNA was observed when TSA (0.5  $\mu\text{M}$ ; 12 h) was added into 12-h DAC-treated cells (1  $\mu\text{M}$ ; Fig. 2G). However, when TSA (0.5  $\mu\text{M}$ ; 12 h at 60–72 h) was added into 72-h DAC-treated cells (1  $\mu\text{M}$ ), apoptotic DNA was increased to 43% (Fig. 2H). Similar results were observed in H23 cells (data not shown). These flow cytometric data suggest that the alterations in cell viability demonstrated in Fig. 1 are related to programmed cell death in the cells treated with DNA methyltransferase inhibitors in combination with HDAC inhibitors.

The apoptotic cleavage of PARP was analyzed with Western immunoblotting as described previously (26). When both of the cell lines were treated with depsipeptide (0.05  $\mu\text{M}$ ) alone for 6 h and then incubated in fresh medium for 12–48 h, we observed the  $M_r$  89,000

cleaved PARP fragment consistent with apoptotic cell death (Fig. 3A, H719 cells and Fig. 3B, H23 cells). However, an increase in cleaved  $M_r$  89,000 fragment was observed when cells were treated with DAC (1  $\mu\text{M}$ ) and depsipeptide (0.05  $\mu\text{M}$ ; Fig. 3). The increase in cleaved PARP fragment was dependent on the duration of DAC treatment (Fig. 3). These data are consistent with that from the flow cytometric analysis. We noted the apparent basal level of PARP cleavage in the H23 cells (Fig. 3B, Lane 1) and found this to be reproducible. This appears to be attributable to an increased level of basal apoptosis in these cells. The flow cytometry data of control H23 cells seems to support this because there is increased sub- $G_0$ - $G_1$  DNA content in these cells [ $\sim$ 3% compared with  $\sim$ 1% noted in the H719 cells (Fig. 2A and data not shown)].

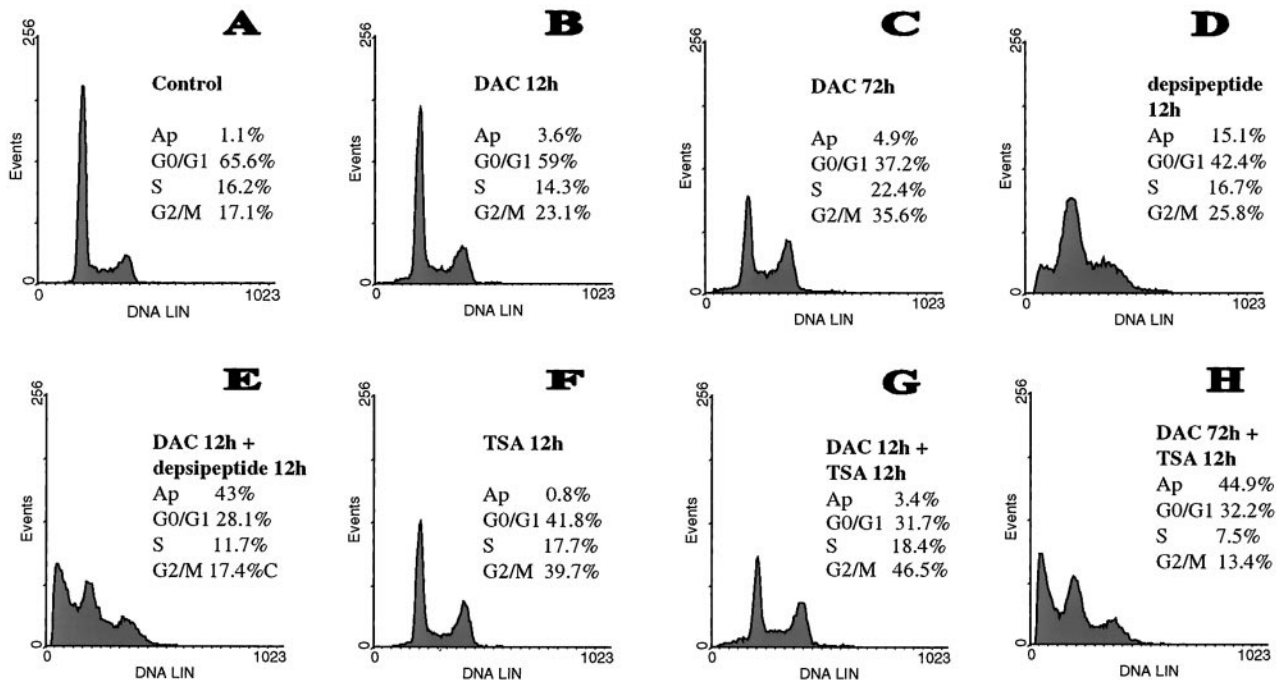


Fig. 2. The analysis of cell cycle changes and apoptotic DNA after treatments with depsipeptide and TSA alone, or in combination with DAC, in H719 cells by flow cytometry. A, control cells exhibit two peaks,  $G_0$ - $G_1$  and  $G_2$ -M. DNA that is smaller than  $G_0$ - $G_1$  is consistent with apoptotic DNA. H719 cells were treated with DAC (1  $\mu\text{M}$ ) for 12 h (B) or 72 h (C). Cells were exposed to depsipeptide at 0.05  $\mu\text{M}$  (D) or TSA at 0.5  $\mu\text{M}$  alone (F) for 12 h. Cells were treated with DAC (1  $\mu\text{M}$ ) for 12 h and followed by depsipeptide treatment (0.05  $\mu\text{M}$ ) for 12 h (E). Cells were treated with DAC (1  $\mu\text{M}$ ) for 12 h and TSA (0.5  $\mu\text{M}$ ) for 12 h (G) or treated with DAC (1  $\mu\text{M}$  for 72 h) and TSA (0.5  $\mu\text{M}$  for 12 h at 60–72 h; H). In each case, samples were incubated in fresh, drug-free medium for 24 h after the removal of drug.

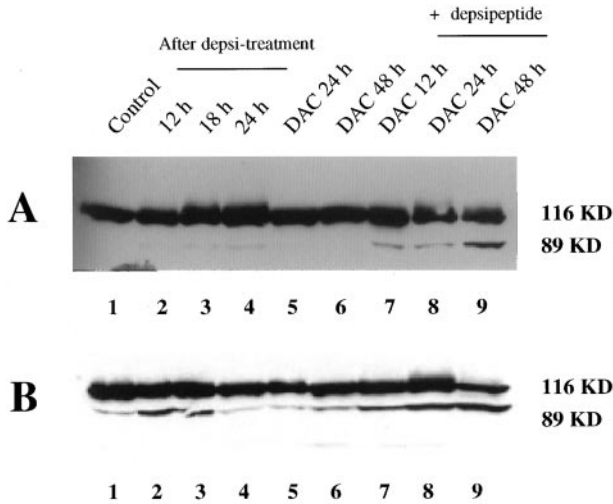


Fig. 3. The cleavage of PARP induced by desipiptide alone or desipiptide combined with DAC. H719 cells (A) or H23 cells (B) were treated with desipiptide (0.05  $\mu\text{M}$ ) alone for 6 h and then washed and incubated for 12, 18, and 24 h (Lanes 2, 3, and 4). Cells were exposed to DAC (1  $\mu\text{M}$ ) for 24 or 48 h and then washed and incubated for 24 h (Lanes 5 and 6). For the treatment with DAC and desipiptide, cells were treated with DAC (1  $\mu\text{M}$ ) for 12, 24, or 48 h, and then desipiptide (0.05  $\mu\text{M}$ ) was added into the DAC-treated cells for the final 6 h (at 6–12, 18–24, or 42–48 h; Lanes 7, 8, and 9, respectively). The DAC plus desipiptide-treated cells were then washed and incubated for 24 h.

**HDAC Inhibitor-induced Apoptosis Is p53 Independent.** H23 cells have a missense mutation in the p53 DNA that alters codon 246 from ATG to ATC and the amino acid from methionine to isoleucine (ATCC, National Cancer Institute), whereas no p53 mutation has been described in H719 cells (ATCC). To investigate whether p53 is involved in the desipiptide-induced apoptosis, we analyzed p53 expression using Western immunoblotting. Fig. 4A shows no change in p53 protein when cells were treated with either DAC or desipiptide alone or in combination in either cell line. To further evaluate the role of p53 in the HDAC inhibitor-induced apoptosis, we performed transfection experiments. H1299 cells are derived from a patient with lung cancer and express no endogenous p53 protein. These cells express wt RB and have undetectable p16<sup>INK4</sup>, although it is not known whether this is from homozygous deletion or promoter hypermethylation (27). wt p53 was inserted into the eukaryotic expression vector PCIneo and transfected into H1299 cells. Fig. 4B shows that transient transfection of these cells with PCIneo-wt p53 produced abundant p53 protein expression as measured by Western immunoblotting. No obvious changes in p53 level were found after DAC (1  $\mu\text{M}$ ; 48 h), desipiptide (0.05  $\mu\text{M}$ ; 6 h), or DAC plus desipiptide treatment (Fig. 4B). We saw no PARP cleavage in H1299 cells treated with DAC (1  $\mu\text{M}$ ; 48 h), desipiptide (0.05  $\mu\text{M}$ ; 6 h) or DAC and desipiptide together, regardless of whether the cells expressed wt p53 protein or not (Fig. 4C). These data suggest that p53 is not involved in HDAC inhibitor-induced apoptosis in human lung cancer cells. The resistance of H1299 cells to this apoptotic pathway is unexplained. We do not believe that the differences in susceptibility to treatment with HDAC inhibitors and DNA methyltransferase inhibitors is attributable to differences in *CDKN2* promoter methylation. As noted above, both H23 and H719 cells are characterized by lack of p16<sup>INK4</sup> protein expression because of *CDKN2* hypermethylation. Although we have not characterized the reason for p16<sup>INK4</sup> loss in H1299 cells, we have tested other lung cancer cells that lack p16<sup>INK4</sup> on the basis of homozygous deletions and found that these cells retain susceptibility to HDAC-induced (and DAC-enhanced) apoptosis. (data not shown).<sup>4</sup>

<sup>4</sup> W-G. Zhu and G. A. Otterson, unpublished observations.

**DNA Methyltransferase Inhibitor Enhances Histone Acetylation Induced by Desipiptide.** To further study the mechanism by which desipiptide-induced apoptosis is enhanced in the presence of the DNA methyltransferase inhibitor DAC, we analyzed histone H3 and histone H4 acetylation with Western immunoblotting. Because histones are tightly associated with nuclear DNA (1, 2, 28), we isolated nuclear histones with acid extraction, as described in “Materials and Methods.” Fig. 5, A and B, shows that desipiptide induced significant acetylation of histone H3 and H4 in H719 cells. The desipiptide-induced histone acetylation was dose dependent. For example, acetylated histone H3 was barely detectable, and acetylated histone H4 was not seen when cells were treated with desipiptide alone at 0.05  $\mu\text{M}$  for 6 h (Fig. 5, A and B). Desipiptide at 0.125 or 0.25  $\mu\text{M}$  induced a dose-dependent, increased acetylation of histones H3 and H4. DAC alone (1  $\mu\text{M}$  for up to 72 h) did not induce any significant histone acetylation (Fig. 5, A and B). Interestingly, the levels of acetylated histone H3 and H4 were greatly increased when desipiptide (0.05  $\mu\text{M}$ ; 6 h) was introduced into DAC-pretreated cells

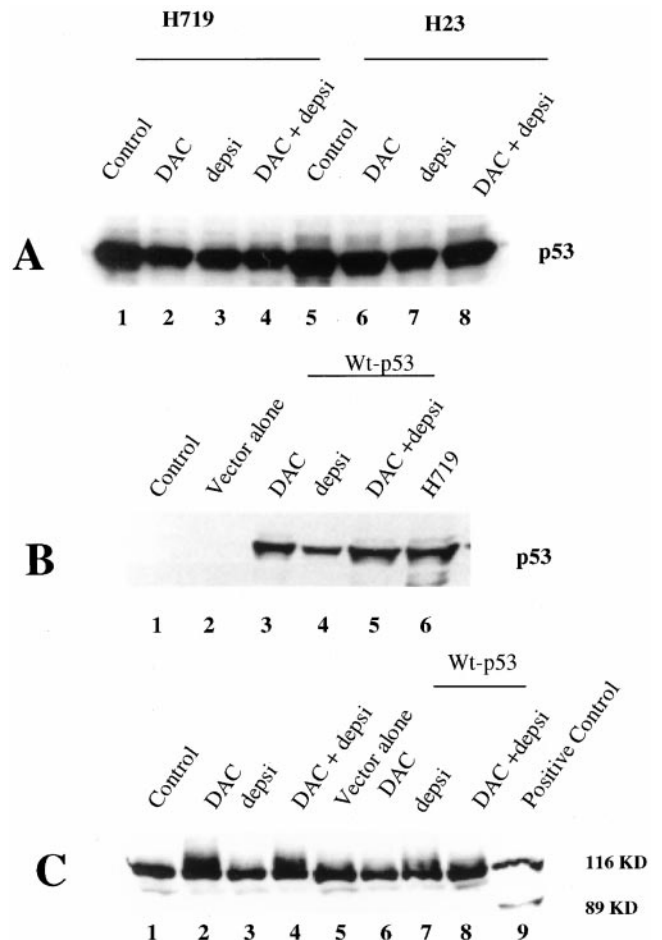


Fig. 4. Changes of p53 level and cleavage of PARP induced by desipiptide or desipiptide combination with DAC. A, H719 or H23 cells were treated with DAC (1  $\mu\text{M}$ ) for 48 h alone (Lanes 2 and 6), desipiptide (0.05  $\mu\text{M}$ ) for 6 h alone (Lanes 3 and 7), or in combination (Lanes 4 and 8), and then washed and incubated for 24 h. Cells were harvested, and the p53 level was assayed with Western immunoblotting. Lane 1 (H719 cells) and Lane 5 (H23 cells) are control samples. B shows p53 expression and C shows cleavage of PARP in H1299 cells. The PCIneo-wt p53 was transiently transfected into H1299 cells. The cells were then treated with DAC (1  $\mu\text{M}$  for 48 h; Lane 3 in B and Lanes 2 and 6 in Fig. C), desipiptide (0.05  $\mu\text{M}$  for 6 h; Lane 4 in B and Lanes 3 and 7 in C), or in combination with both drugs together (Lane 5 in B and Lanes 4 and 8 in C). Lane 6 in B is untreated H719 cells as p53-positive control. Lane 9 in C is a positive control for PARP cleavage that H719 cells were treated with DAC (1  $\mu\text{M}$  for 48 h) and desipiptide (0.05  $\mu\text{M}$  for 6 h). All of the treated cells were washed and incubated for 24 h, followed by protein harvesting for detecting the p53 level or PARP cleavage with Western immunoblotting.

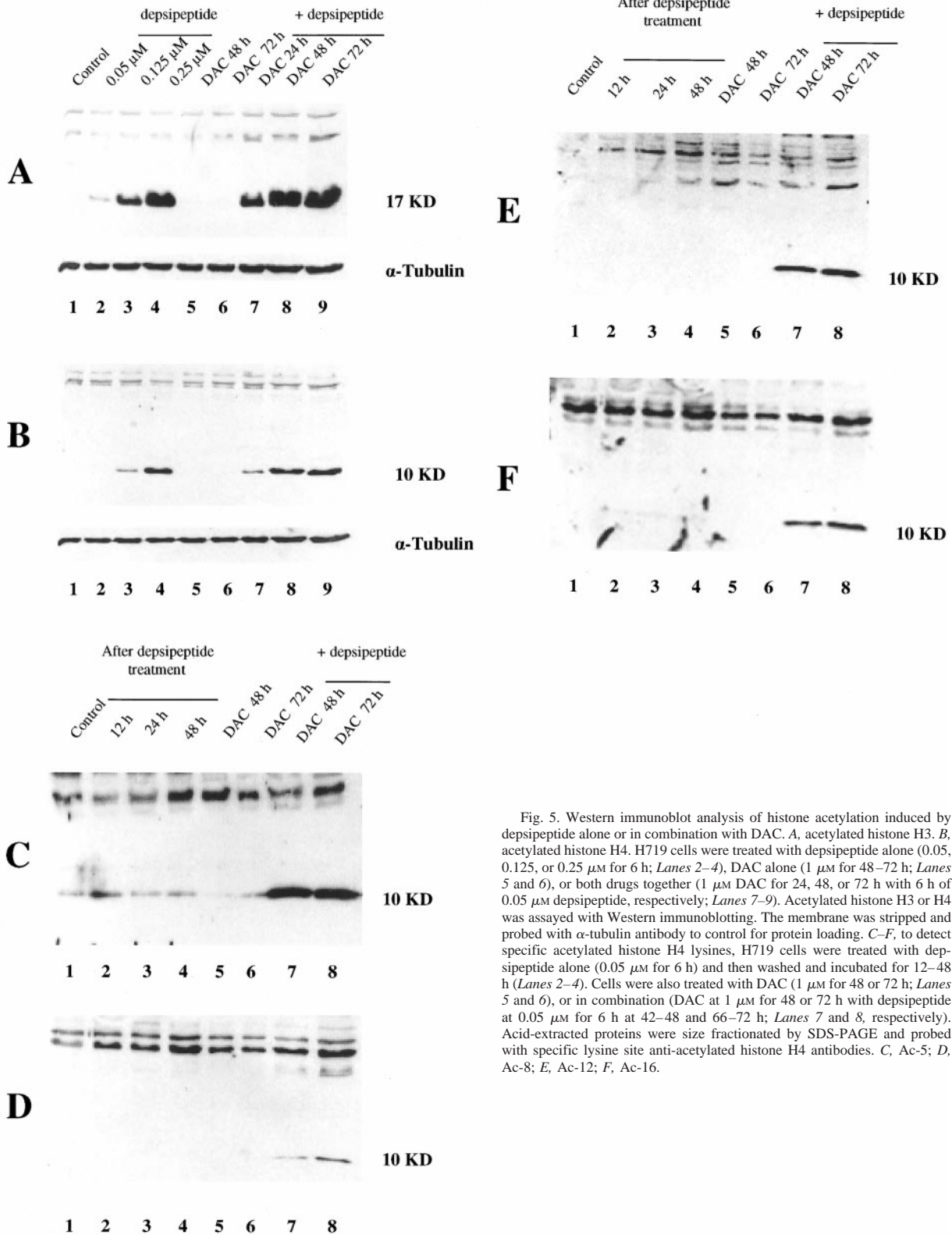


Fig. 5. Western immunoblot analysis of histone acetylation induced by depsipeptide alone or in combination with DAC. *A*, acetylated histone H3. *B*, acetylated histone H4. H719 cells were treated with depsipeptide alone (0.05, 0.125, or 0.25  $\mu$ M for 6 h; *Lanes 2–4*), DAC alone (1  $\mu$ M for 48–72 h; *Lanes 5 and 6*), or both drugs together (1  $\mu$ M DAC for 24, 48, or 72 h with 6 h of 0.05  $\mu$ M depsipeptide, respectively; *Lanes 7–9*). Acetylated histone H3 or H4 was assayed with Western immunoblotting. The membrane was stripped and probed with  $\alpha$ -tubulin antibody to control for protein loading. *C–F*, to detect specific acetylated histone H4 lysines, H719 cells were treated with depsipeptide alone (0.05  $\mu$ M for 6 h) and then washed and incubated for 12–48 h (*Lanes 2–4*). Cells were also treated with DAC (1  $\mu$ M for 48 or 72 h; *Lanes 5 and 6*), or in combination (DAC at 1  $\mu$ M for 48 or 72 h with depsipeptide at 0.05  $\mu$ M for 6 h at 42–48 and 66–72 h; *Lanes 7 and 8*, respectively). Acid-extracted proteins were size fractionated by SDS-PAGE and probed with specific lysine site anti-acetylated histone H4 antibodies. *C*, Ac-5; *D*, Ac-8; *E*, Ac-12; *F*, Ac-16.

(Fig. 5, *A* and *B*). The increase in acetylated histones by depsipeptide was also dependent on the duration of DAC treatment. In Fig. 5, *A* and *B*, the intensity of histone acetylation in the combination treated cells (*Lanes 7–9*) should be compared with 0.05  $\mu$ M depsipeptide alone (*Lane 2*).

To determine whether histone acetylation induced by depsipeptide is lysine site specific, we performed immunoblotting to test acetyla-

tion using antibodies directed against specific acetylated lysine residues at the NH<sub>2</sub> terminus of histone H4. These antibodies recognize only acetylated lysines at residues 5, 8, 12, and 16 (designated Ac-5, Ac-8, Ac-12, and Ac-16). Detectable acetylated histone H4 was found at baseline at lysine 5 (Ac-5), but not at Ac-8, Ac-12, and Ac-16 in untreated control cells (Fig. 5, *C–F*). Depsipeptide (0.05  $\mu$ M; 6 h) induced detectable acetylation of histone H4 at Ac-5 and Ac-16 but

not at Ac-8 and Ac-12. However, depsipeptide induced prominent increases of acetylation of histone H4 at all lysine sites in the DAC-pretreated H719 cells (Fig. 5, C–F). DAC alone could not induce increased acetylation of histone H4 at any lysine site (Fig. 5, C–F).

## Discussion

Our data demonstrate that the HDAC inhibitors TSA and depsipeptide induce a dose-dependent apoptosis in human lung cancer cells (Figs. 2 and 3). The HDAC inhibitor-induced apoptosis is greatly enhanced in the presence of the DNA methyltransferase inhibitor DAC (Figs. 2 and 3), is p53 independent (Fig. 4), and may be related to enhancement of histone acetylation (Fig. 5).

Consistent with our data, HDAC inhibitor-induced apoptosis has been reported in several human cancer cell lines (15, 19, 29, 30). The mechanism by which HDAC inhibitors induce apoptosis in human cancer cells is not fully understood. There are several possible explanations (3, 10, 15, 16, 29–35). It may be that histone acetylation increases chromatin relaxation and enhances the accessibility of DNA to apoptotic endonucleases (3, 16). Consistent with the correlation between histone acetylation and transcriptional activity, newly synthesized protein has been reported to be involved in HDAC inhibitor-induced apoptosis (15, 30). Cycloheximide, an inhibitor of protein synthesis, almost completely prevented the formation of apoptotic bodies and nuclear fragmentation in Jurkat cells treated with butyrate or TSA (15). M-carboxycinnamic acid bishydroxamide is a potent inhibitor of HDAC and is reported to induce apoptosis in human neuroblastoma cells (30). This apoptosis was suppressed in the presence of cycloheximide. In addition, recent findings have indicated that HDAC inhibitor-induced apoptosis may be related to gene expression modulated by HDAC inhibitors (31–35). These genes include *p21* (19, 31, 32), *c-myc* (33, 34), and *gelsolin* (35). The inability, in our study, to reconstitute drug sensitivity in H1299 cells by expressing p53 is consistent with the findings of others who have reported that p53 is not involved in HDAC inhibitor-induced apoptosis (8, 19, 31, 32). The reason for H1299 resistance to these drugs is also enigmatic at this time. We plan to investigate the cellular and molecular differences in susceptibility to HDAC inhibitors and DAC sensitivity in future work.

Interestingly, the depsipeptide- or TSA-induced apoptosis is enhanced greatly in the presence of the DNA methyltransferase inhibitor DAC in this study. The mechanism by which HDAC inhibitor-induced apoptosis in lung cancer cells is enhanced in the presence of DAC is unclear. However, our data suggest a functional connection between DAC-induced unmethylated DNA and HDAC inhibitor-induced acetylation of histones. Other researchers have reported previously that DNA methylation state is functionally connected with chromosome structure (21, 22, 36, 37). For example, the inactive X chromosome in mammals is both hypoacetylated and hypermethylated (38). Methylated DNA is transcriptionally repressed, and in this condition methylated DNA is assembled into nucleosomal structure (39, 40). Recently, two groups provided a substantive clue that a methyl CpG binding protein, MeCP2, forms a complex with HDAC that affects chromatin architecture and gene regulation (21, 22). Eden *et al.* (41) demonstrated that the DNA methylation state influences the local histone acetylation level, and DNA methylation is involved in inducing decreased levels of chromatin acetylation. Cameron *et al.* (9) also reported that the HDAC inhibitor TSA acts synergistically with DAC to restore mRNA expression of several methylated tumor suppressor genes. Hypermethylated *MLH1*, *TIMP3*, *CDKN2B*, and *CDKN2A* cannot be transcriptionally reactivated with TSA alone; however, after minimal demethylation in the presence of DAC, TSA treatment resulted in enhanced expression of each gene (9). These

findings linked DNA methylation to histone deacetylation to explain methylation-mediated gene silencing. In addition to the functional link noted between histone deacetylation and DNA methylation, several labs have recently shown a direct physical link by the copurification or HDAC enzymes with the major DNA methyltransferase enzyme, Dnmt1 (42–44).

On the basis of our experimental data and recent findings of a molecular link between methylated DNA and hypoacetylated histones, we would hypothesize that the susceptibility to HDAC inhibitor-induced apoptosis is from a decondensation of chromatin, allowing for easier access to endogenous proapoptotic endonucleases. The enhancement of apoptosis associated with increased histone acetylation by the DNA methyltransferase inhibitor DAC is supportive of this hypothesis. We have thus far been unable to demonstrate convincingly alterations (in these cells) of known pro- and antiapoptotic proteins.<sup>4</sup> It remains possible that treatment with these agents alters the expression patterns of genes that are relevant for apoptotic cell death that we have not yet tested for. Alternatively, other as yet unknown activities of these agents may conspire to give them their apparent synergistic activity. An additional potential mechanism for this synergistic activity may rely on the physical association of Dnmt1 and HDAC (42–44). One could postulate that the inhibition of Dnmt1 with DAC induces an alteration in the configuration of the enzyme that, in some way, is translated to the associated HDAC, enhancing its susceptibility to inhibition. It may be interesting to study the affinity of HDAC and Dnmt1 with and without the presence of these small molecular inhibitors. The mechanism by which DAC increases HDAC inhibitor-induced acetylation of histones H3 and H4 remains unclear. A further step for our research is to determine whether MeCP2 or other methyl-binding proteins are involved in the DAC-induced enhancement of histone acetylation from HDAC inhibitors. Finally, it will be relevant to examine alterations of gene expression, histone acetylation, and degree of apoptosis in nontransformed “normal” cells. Others (45) have suggested that depsipeptide-induced apoptosis exhibits selectivity toward malignant chronic lymphocytic leukemia cells (as opposed to peripheral blood mononuclear cells from normal volunteers). This has significance for the possibility of introducing agents with this mechanism of action into the clinic.

In conclusion, the data presented in this study show, for the first time, that apoptosis induced by HDAC inhibitors is greatly enhanced in the presence of DAC in human lung cancer cells. However, which gene or genes are involved in this apoptosis is unknown. Future studies include examination of apoptosis-related genes in HDAC inhibitor-induced apoptosis, determination of how DAC modulates HDAC inhibitor-induced acetylation of histones, and evaluation of these effects in nontransformed cells. We have just begun to perform these experiments in normal human bronchial epithelial cells. It is possible that these agents (or analogues of them) may be used either alone or together in the future as cancer chemotherapeutic agents, and indeed, clinical trials are already under way with these and related agents. A more thorough understanding of their mechanism of action is desirable.

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