Histone Deacetylase Inhibitors Have a Profound Antigrowth Activity in Endometrial Cancer Cells

Noriyuki Takai,1,3 Julian C. Desmond,1 Takashi Kumagai,1 Dorina Gui,2 Jonathan W. Said,2 Sadie Whitaker,1 Isao Miyakawa,3 and H. Phillip Koeffler1

1Division of Hematology/Oncology, Cedars-Sinai Medical Center/University of California at Los Angeles School of Medicine and 2Department of Pathology, Center of Health Science/University of California at Los Angeles School of Medicine, Los Angeles, California, and 3Department of Obstetrics and Gynecology, Oita Medical University, Oita, Japan

ABSTRACT

Purpose: HDAC inhibitors (HDACIs) have been shown to inhibit cancer cell proliferation, stimulate apoptosis, and induce cell cycle arrest. Our purpose was to investigate the antiproliferative effects of the HDACIs [suberoyl anilide bishydroxamine, valproic acid (VPA), trichostatin A, and sodium butyrate] against six endometrial cancer cell lines.

Experimental Design: Endometrial cancer cells were treated with a variety of HDACIs, and the effect on cell growth, cell cycle, and apoptosis was measured. The ability of VPA to inhibit the growth of endometrial tumors growing in immunodeficient mice was also assessed.

Results: Clonogenic assays showed that all cancer cell lines were sensitive to the growth inhibitory effect of HDACIs. Cell cycle analysis indicated that treatment with HDACIs decreased the proportion of cells in S phase and increased the proportion of cells in the G0-G1 and/or G2-M phases of the cell cycle. Terminal deoxynucleotidyl transferase-mediated nick end labeling assays showed that HDACIs induced apoptosis. This was concomitant with altered expression of genes related to malignant phenotype, including an increase in p21Waf1, p27Kip1, and E-cadherin, and a decrease in Bcl-2 and cyclin-D1 and -D2. Chromatin immunoprecipitation analysis revealed a remarkable increase in levels of acetylated histones associated with the p21 promoter after suberoyl anilide bishydroxamine treatment. In nude mice experiments, VPA inhibited significantly human uterine tumor growth without toxic side effects.

Conclusions: These results suggest that HDACIs are effective in inhibiting growth of endometrial cancer cells in vitro and in nude mice, without toxic side effects. The findings raise the possibility that HDACIs may prove particularly effective in treatment of endometrial cancers.

INTRODUCTION

Endometrial cancer is the most common malignant tumor of the female genital tract. Its incidence has increased in recent years, making up ~13% of female cancers (1). Furthermore, the search for agents effective in the treatment of either advanced or recurrent endometrial cancer has been disappointing. To date, cisplatin, doxorubicin, and paclitaxel demonstrate the greatest efficacy (2, 3). However, although reported response rates have been ≥70%, the duration of response remains brief, from between 4 and 8 months.

Local remodeling of chromatin and dynamic changes in the nucleosomal packaging of DNA are key steps in the regulation of gene expression, consequently affecting proper cell function, differentiation, and proliferation. Chromatin structure may affect transcriptional activation by blocking the access of trans-activating factors to their target sequences and/or the assembly of the basal transcriptional machinery to form the preinitiation complex (4, 5). One of the most important mechanisms in chromatin remodeling is the post-translational modification of the NH2-terminal tails of histones by acetylation, which contributes to a "histone code" determining the activity of target genes (6). Transcriptionally silent chromatin is composed of nucleosomes in which the histones have low levels of acetylation on the lysine residues of their NH2-terminal tails. Acetylation of histone proteins neutralizes the positive charge on lysine residues and disrupts nucleosome structure, allowing unfolding of the associated DNA, subsequent access by transcription factors, and changes in gene expression.

Acetylation of core nucleosomal histones is regulated by the opposing activities of histone acetyltransferases and deacetyltransferase HDACs. HDACs catalyze the removal of acetyl groups on the NH2-terminal lysine residues of core nucleosomal histones, and this activity is generally associated with transcriptional repression. Aberrant recruitment of HDAC activity has been associated with the development of certain human cancers (7). Transcription factors, such as Mad-1, BCL-6, and ETO, have also been shown to assemble HDAC-dependent transcriptional repressor complexes (8–11).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

Note: H. P. K. holds the Mark Goodson Endowed Chair of Oncology Research and is a member of the Jonsson Cancer Center and Molecular Biology Institute of UCLA.

Requests for reprints: H. Phillip Koeffler, Division of Hematology/Oncology, Cedars-Sinai Medical Center/UCLA School of Medicine, 8700 Beverly Boulevard, Los Angeles, CA 90048. Phone: (310) 423-4609; Fax: (310) 423-0443; E-mail: koeffler@cshs.org.

Received 8/26/03; revised 10/22/03; accepted 12/3/03.

Grant support: NIH grants, A. and S. Schwartz Fund, the Marie and David Green Fund, the Alec Borden Fund, and the Women’s Cancer Research Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

Note: H. P. K. holds the Mark Goodson Endowed Chair of Oncology Research and is a member of the Jonsson Cancer Center and Molecular Biology Institute of UCLA.

Requests for reprints: H. Phillip Koeffler, Division of Hematology/Oncology, Cedars-Sinai Medical Center/UCLA School of Medicine, 8700 Beverly Boulevard, Los Angeles, CA 90048. Phone: (310) 423-4609; Fax: (310) 423-0443; E-mail: koeffler@cshs.org.

Clinical Cancer Research 1141

Vol. 10, 1141–1149, February 1, 2004

Clinical Cancer Research 1141

Vol. 10, 1141–1149, February 1, 2004

Received 8/26/03; revised 10/22/03; accepted 12/3/03.

Grant support: NIH grants, A. and S. Schwartz Fund, the Marie and David Green Fund, the Alec Borden Fund, and the Women’s Cancer Research Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

Note: H. P. K. holds the Mark Goodson Endowed Chair of Oncology Research and is a member of the Jonsson Cancer Center and Molecular Biology Institute of UCLA.

Requests for reprints: H. Phillip Koeffler, Division of Hematology/Oncology, Cedars-Sinai Medical Center/UCLA School of Medicine, 8700 Beverly Boulevard, Los Angeles, CA 90048. Phone: (310) 423-4609; Fax: (310) 423-0443; E-mail: koeffler@cshs.org.

Clinical Cancer Research 1141

Vol. 10, 1141–1149, February 1, 2004

Received 8/26/03; revised 10/22/03; accepted 12/3/03.

Grant support: NIH grants, A. and S. Schwartz Fund, the Marie and David Green Fund, the Alec Borden Fund, and the Women’s Cancer Research Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

Note: H. P. K. holds the Mark Goodson Endowed Chair of Oncology Research and is a member of the Jonsson Cancer Center and Molecular Biology Institute of UCLA.

Requests for reprints: H. Phillip Koeffler, Division of Hematology/Oncology, Cedars-Sinai Medical Center/UCLA School of Medicine, 8700 Beverly Boulevard, Los Angeles, CA 90048. Phone: (310) 423-4609; Fax: (310) 423-0443; E-mail: koeffler@cshs.org.

Clinical Cancer Research 1141

Vol. 10, 1141–1149, February 1, 2004
butyric acid (VPA); (b) organic hydroxamic acids [e.g., TSA and suberoyl anilide bis-hydroxamine (SAHA)]; (c) cyclic tetrapeptides (e.g., trapoxin); and (d) benzamides (e.g., MS-275; Ref. 21). The structure of SAHA is related to that of TSA (22), a natural product isolated from Streptomyces hygroscopicus that was initially used as an antifungal antibiotic. Phenylbutyrate has been used as a single agent in the treatment of β-thalassemia, toxoplasmosis, and malaria.

The inhibition of HDACs by SAHA occurs through a direct interaction with the catalytic site of the enzyme, as shown by X-ray crystallography studies (22). 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. Differential display analysis of transformed lymphoid cell lines revealed that the expression of only 2–5% of transcribed genes is changed significantly after treatment with TSA (23).

SAHA is a potentially new therapeutic approach to cancer treatment and is in Phase I clinical trials for the treatment of a variety of solid and hematological tumors, and VPA is an established drug in the long-term therapy of epilepsy. Some HDACIs (e.g., TSA and trapoxin) are of limited therapeutic use because of poor bioavailability in vivo as well as toxic side effects at high doses. NaB and phenylbutyrate are degraded rapidly after i.v. administration and therefore require high doses exceeding 400 mg/kg/day (24). Furthermore, these compounds are not specific for HDACs because they also inhibit phosphorylation and methylation of proteins, as well as DNA methylation (25).

This study was designed to define the biological and therapeutic effects of HDACIs in treating endometrial cancer. We focused particularly on SAHA and VPA, which are recognized as the least toxic HDACIs. We examined whether these compounds were able to mediate inhibition of cell growth, cell cycle arrest, apoptosis, and expression of genes related to the malignant phenotype in a variety of endometrial cancer cell lines. Furthermore, we examined whether SAHA was able to induce the accumulation of acetylated histones in the chromatin of the p21WAF1 gene in human endometrial carcinoma cells. Finally, we tested the ability of VPA to inhibit the growth of the endometrial cancer cell line HEC-1B in vivo.

MATERIALS AND METHODS

Cell Lines. HEC-1B, RL95–2, KLE, and AN3CA cell lines were obtained from American Type Culture Collection (Rockville, MD). HEC59 cell line was kindly provided by Dr. Timothy J. Kinsella, Case Western University. Ishikawa cell line was kindly provided by Dr. Bruce A. Lessey, University of North Carolina at Chapel Hill. Cells were maintained according to the recommendations of the supplier.

HDACs. SAHA was obtained from Alexis (Lausen, Switzerland). VPA, TSA, and NaB were obtained from Sigma (St. Louis, MO).

Clonogenic Assay in Soft Agar. The effect of HDACIs on clonogenic growth of endometrial cancer cells was determined by dose-response studies in soft agar as described previously (26). Endometrial cancer cells taken from 60–80% confluent liquid cultures were trypsinized. Washed single-cell suspensions of cells were then enumerated and plated into 24-well, flat-bottomed plates using 1 × 10³ cells/well in a total volume of 400 μl/well. The feeder layer was prepared with agar that had been equilibrated at 42°C. Before this step, the HDACI compounds were pipetted into the wells. After 14 days of culture, colonies (>50 cells) were counted using an inverted microscope. All experiments were done independently at least three times in triplicate dishes per experimental point.

Cell Cycle Analysis by Flow Cytometry. Cell cycle was analyzed by flow cytometry after 3 days of culture either with or without HDACIs as described (26). Briefly, endometrial cancer cells were cultured at <60% confluency 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 propidium iodide, 1 mg/ml RNase (Sigma), and 0.1% NP40 (Sigma). Analysis was performed immediately after staining using the CELLFit program (Becton Dickinson & Co.), whereby the S phase was calculated using an RFit model.

Measurement of Apoptosis. DNA strand breaks were identified by terminal deoxynucleotidyltransferase-mediated UTP end labeling technique using the in situ Cell Death Detection Kit as directed (Boehringer Mannheim).

Western Blotting. Expression of specific proteins was detected by Western blot. 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 × 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 lysates (40 μg) were resolved by 4–15% SDS-polyacrylamide gel and transferred to an immobilin polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL). Antiacetylated H3 polyclonal antibody (1:1000; Upstate Biotechnology, Inc., Lake Placid, NY), antiacetylated H4 polyclonal antibody (1:1000; Upstate Biotechnology), anti-p21WAF1 monoclonal antibody (mAb; Ab-1, 1:1000; Oncogene, San Diego, CA), anti-p27KIP1 monoclonal antibody (C-19, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-E-cadherin mAb (G-10, 1:1000; Santa Cruz Biotechnology), androgen receptor (AR) mAb (441, 1:1000; Santa Cruz Biotechnology), anti-β-catenin mAb (A-12, 1:1000; Santa Cruz Biotechnology), anti-cyclin D1 mAb (A-20, 1:1000; Santa Cruz Biotechnology), anti-cyclin D2 polyclonal antibody (M-20, 1:1000; Santa Cruz Biotechnology), and antitryptase 3-phosphatase dehydrogenase mAb (Research Diagnostics, Flanders, NJ) were used. The blots were developed using the enhanced chemiluminescence kit (Amersham). Band intensity was measured using a densitometer “ultra-violet products gel analysis suite,” and fold increase in expression as compared with control, untreated cells was calculated.

Chromatin Immunoprecipitation (ChIP) Assay. Cells were plated at a density of 1 x 10⁶ cells/10-cm dish and incubated overnight at 37°C with 5% CO2. After 12 h, cells were cultured either in 5 x 10⁻⁶ M SAHA or control cultures for 24 h. Formaldehyde was then added to the cells to a final concentration of 1%, and the cells were incubated at 37°C for 10
min. The medium was removed, and the cells were suspended in 1 ml of ice-cold PBS containing protease inhibitors (Complete; Boehringer Mannheim). Cells were pelleted, resuspended in 0.5 ml of SDS lysis buffer [1% SDS/10 mM EDTA/50 mM Tris-HCl (pH 8.1)], and incubated on ice for 10 min. Lysates were sonicated with three 10-s bursts. Debris was removed from samples by centrifugation for 10 min at 15,000 × g at 4°C. An aliquot of the chromatin preparation (200 µl) was set aside and designated as the Input Fraction. Supernatants were diluted 5-fold in immunoprecipitation buffer [0.01% SDS/1.1% Triton X-100/1.2 mM EDTA/16.7 mM Tris-HCl (pH 8.1)/16.7 mM NaCl], and 80 µl of a 50% protein A-Sepharose slurry containing 20 µg of sonicated salmon sperm DNA and 1 mg/ml BSA in TE buffer [10 mM Tris-HCl (pH 8.1)/1 mM EDTA] were added and incubated, rocking for 30 min at 4°C. Beads were pelleted by centrifugation, and supernatants were placed in fresh tubes with 5 µg of anti-acetylated histone H3 antibody (Upstate Biotechnology), anti-acetylated histone H4 antibody (Upstate Biotechnology), or normal rabbit serum and incubated overnight at 4°C. Protein A-Sepharose slurry (60 µl) was added, and samples were rocked for 1 h at 4°C. Protein A complexes were centrifuged and washed five times for 5 min each, according to the manufacturer’s protocol. Immune complexes were eluted twice with 250 µl of elution buffer (1% SDS/0.1 M NaHCO3) for 15 min at room temperature. A total of 20 µl of 5 M NaCl was added to the combined eluates, and the samples were incubated at 65°C for 4 h. EDTA, Tris-HCl (pH 6.5), and proteinase K were then added to the samples at a final concentration of 10 mM, 40 mM, and 0.04 µg/µl, respectively, and the samples were incubated at 45°C for 1 h. Immunoprecipitated DNA (both immunoprecipitation samples and Input) was recovered by phenol/chloroform extraction and ethanol precipitation by phenol/chloroform extraction and ethanol precipitation and analyzed by 2.5% agarose/ethidium bromide gel electrophoresis. The primer pairs used for p21WAF1 ChIP analysis were: 5'-GGT GTC TAG GTG CTC CAG GT-3' (forward), 5'-GCA CTC TCC AGG AGG ACA CA-3' (reverse). The primers used for β-actin ChIP analysis were: 5'-GCC AGC TCT CGC ACT CTG TT-3' (forward), 5'-AGA TCG CAA CCG CCT GGA AC-3' (reverse).

In Vivo Animal Treatment Protocol. Ten female 6-week-old immunodeficient BNX nude mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and were maintained in pathogen-free conditions with irradiated chow. Animals were bilaterally s.c. injected with 5 × 10⁶ of HEC-1B cells in 0.1 ml of Matrigel (Collaborative Biomedical Products, Bedford, MA). A total of 5 × 10⁶ of HEC-1B cells in 0.1 ml of Matrigel (Collaborative Biomedical Products) was bilaterally s.c. injected into the trunk of 10 mice, forming two tumors per mouse. Treatment was started on the day after the injection of these human endometrial cancer cells and discontinued after 5 weeks. Cohorts (5 mice/group) received either diluent only (control group) or VPA (10 mg/day) i.p. for 5 days/week. Tumors were measured every week with vernier calipers. Tumor size was calculated using the formula: A (length) × B (width) × C (height) × 0.5236. At the end of the experiments (after 5 weeks), blood was collected from the orbital sinus for serum chemistries and blood analysis using DuPont Analyst Benchtop Chemistry System (Dade International, Newark, DE) and by Serono-Baker 9000 Diff (Biochem Immuno-Systems, Allen-town, PA), respectively. Animals were sacrificed by carbon dioxide asphyxiation, and tumor weights were measured after their careful resection. Tumors, livers, lungs, spleens, and kidneys were fixed and stained for histological analysis. All animal experiments were performed in compliance with NIH guidelines.

Histology. Tumors and normal organs from sacrificed mice were fixed in 10% neutral buffered formalin and embedded in paraffin wax before histological sectioning. Sections were stained with H&E, and tumor necrosis and fibrosis were evaluated. Normal organs were evaluated for evidence of toxic damage. Controls consisted of tumors and organs from mice not subjected to treatment.

Statistical Analysis. All numerical data were expressed as the average of the values obtained ± SD. Statistical significance of differences between tumors in mice was analyzed by nonparametric Mann-Whitney U test using STAT VIEW software (Abacus Concept, Barkley, CA). For all other experiments, significance was determined by conducting a paired Student’s t test.

RESULTS

Effect of HDACIs on the Clonal Proliferation of Endometrial Cancer Cells in Vitro. To study the effect of the HDACIs SAHA, VPA, TSA, and NaB on the clonogenic growth of endometrial cancer cell lines, a two-layer soft-agar system was performed. As can be seen in Fig. 1, the HDACIs SAHA and VPA inhibited clonal proliferation of all of the endometrial cancer cell lines tested in a dose-dependent manner. Similar results were observed with TSA and NaB (data not shown). The effective dose of the HDACIs that inhibited 50% clonal growth (ED₅₀) of the endometrial cancer cell lines is shown in Table 1 and ranged between 7.8 × 10⁻⁸ M and 3.1 × 10⁻⁶ M for SAHA, 7 × 10⁻⁴ M and 3.8 × 10⁻³ M for VPA, 5.1 × 10⁻⁸ M and 1.9 × 10⁻⁷ M for TSA, and 8.3 × 10⁻⁸ M and 4.1 × 10⁻⁷ M for NaB. The Ishikawa and HEC-1B cells were the most sensitive to the inhibitory effects of HDACIs (Ishikawa = HEC-1B > HEC59 > RL95-2 > KLE > AN3CA; Table 1).

Cell Cycle Analysis of Endometrial Cancer Cells after Exposure to HDACIs. The effect of HDACIs on the cell cycle of the endometrial cancer cells was determined. HEC-1B cells cultured for 3 days in the presence of HDACIs showed an accumulation of endometrial cancer cells in the G₁-G₀, and/or G₂-M phases of the cell cycle, with a concomitant decrease in the proportion of those in S phase (Table 2), e.g., a total of 51% of the untreated HEC-1B cells was in G₀-G₁ compared with 26% of cells cultured with SAHA (5 × 10⁻⁶ M) and 68% of cells cultured with VPA (5 × 10⁻³ M). A total of 11% of the HEC-1B untreated cells was in G₂-M compared with 54% of cells cultured with SAHA (5 × 10⁻⁶ M) and 12% of cells cultured with VPA (5 × 10⁻³ M). This was representative of all of the HDACIs tested (Fig. 2).
in vitro cells observed strong antiproliferative effect of HDACIs on endometrial cancer. To test this, we used the terminal deoxynucleotidyltransferase-mediated UTP end labeling assay on the endometrial apoptosis. To test this, we used the terminal deoxynucleotidyltransferase-mediated UTP end labeling assay on the endometrial apoptosis. To test this, we used the terminal deoxynucleotidyltransferase-mediated UTP end labeling assay on the endometrial apoptosis.

**Effect of HDACIs on Acetylation of Histones.** Treatment of HEC-1B endometrial cancer cells with SAHA and VPA dramatically induced the levels of acetylated H3 and H4 (Fig. 3). This was representative of all of the HDACIs tested, as shown in Table 3.

**Effect of HDACIs on Expression of Cell Cycle and Apoptosis-Related Proteins As Well As The AR and E-Cadherin.** p21<sup>WAF1</sup> and p27<sup>KIP1</sup> are cyclin-dependent kinase inhibitors that bind to cyclin-dependent kinase complexes and decrease kinase activity and may act as key regulators of G<sub>S</sub>-G<sub>1</sub> accumulation (reviewed in Ref. 27). We examined the effect of HDACIs on expression of p21<sup>WAF1</sup> and p27<sup>KIP1</sup> by Western blot analysis (SAHA and VPA; Fig. 4, B and C; TSA and NaB, data not shown). HDACIs markedly up-regulated the level of p21<sup>WAF1</sup> protein, which was expressed at negligible levels in the untreated HEC-1B endometrial cancer cell lines. Expression of p27<sup>KIP1</sup> protein was observed in untreated HEC-1B cells, and this was up-regulated by HDACIs. Conversely, HDACIs decreased the levels of cyclin D1 and cyclin D2.

The effect of HDACIs on the cellular content of bcl-2 protein was measured by Western blot (SAHA and VPA; Fig. 4, B and C; TSA and NaB, data not shown). SAHA decreased Bcl-2 levels by 13%, VPA lowered expression by 10%, TSA decreased levels by 28%, and NaB caused a 35% decrease in bcl-2 expression in the HEC-1B cells, whereas Bax expression was unchanged.

The AR gene has been reported recently to be inactivated by CpG hypermethylation in endometrial cancer (28). Expression of AR was up-regulated after exposure to HDACIs in HEC-1B cells (SAHA and VPA; Fig. 4, B and C; TSA and NaB, data not shown). E-cadherin binds to β-catenin and can act as a tumor suppressor gene; its promoter has CpG islands which are frequently methylated in selected cancers. HDACIs increased the expression level of E-cadherin in HEC-1B cells (SAHA and VPA; Fig. 4, B and C; TSA and NaB, data not shown).

**Effect of HDACIs on the Induction of Apoptosis.** The strong antiproliferative effect of HDACIs on endometrial cancer cells observed in vitro may be caused in part by the induction of apoptosis. To test this, we used the terminal deoxynucleotidyltransferase-mediated UTP end labeling assay on the endometrial cancer cell lines treated with the HDACIs for 4 days. As shown in Fig. 3, SAHA and VPA induced apoptosis in a dose-dependent manner, with 50% (5 × 10<sup>–6</sup> M, 4 days) and 33% (5 × 10<sup>–3</sup> M, 4 days) of HEC-1B cells undergoing apoptosis, respectively. Exposure of the endometrial cancer cells to SAHA (5 × 10<sup>–6</sup> M) for 4 days induced apoptosis in each of the cell lines [Ishikawa (42%), HEC-1B (50%), RL95-2 (33%), HEC59 (30%), AN3CA (25%), and KLE cells (11%)]. This was representative of all of the HDACIs tested, as shown in Table 3.

**Table 1** Inhibition of clonal proliferation (ED<sub>50</sub>) of endometrial cancer cell lines by HDACIs<sup>a</sup>

The numbers indicate the concentration of HDACIs that induced 50% clonal growth inhibition (ED<sub>50</sub>) in each endometrial cancer cell line. The ED<sub>50</sub> was determined by plotting the inhibition of colony formation of the endometrial cancer cells in the presence of increasing concentrations of each HDACI [SAHA, 1 × 10<sup>–8</sup> M to 5 × 10<sup>–6</sup> M; VPA, 1 × 10<sup>–8</sup> M to 5 × 10<sup>–3</sup> M; (see Fig. 1); TSA, 1 × 10<sup>–9</sup> to 3 × 10<sup>–7</sup> M; NaB, 1 × 10<sup>–4</sup> to 5 × 10<sup>–3</sup> M].

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SAHA (M)</th>
<th>VPA (M)</th>
<th>TSA (M)</th>
<th>NaB (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ishikawa</td>
<td>7.8 × 10&lt;sup&gt;–7&lt;/sup&gt; M</td>
<td>7.0 × 10&lt;sup&gt;–4&lt;/sup&gt; M</td>
<td>5.2 × 10&lt;sup&gt;–8&lt;/sup&gt; M</td>
<td>8.3 × 10&lt;sup&gt;–7&lt;/sup&gt; M</td>
</tr>
<tr>
<td>HEC-1B</td>
<td>7.8 × 10&lt;sup&gt;–7&lt;/sup&gt; M</td>
<td>7.5 × 10&lt;sup&gt;–4&lt;/sup&gt; M</td>
<td>5.1 × 10&lt;sup&gt;–8&lt;/sup&gt; M</td>
<td>8.4 × 10&lt;sup&gt;–7&lt;/sup&gt; M</td>
</tr>
<tr>
<td>HEC59</td>
<td>1.2 × 10&lt;sup&gt;–6&lt;/sup&gt; M</td>
<td>8.2 × 10&lt;sup&gt;–4&lt;/sup&gt; M</td>
<td>7.0 × 10&lt;sup&gt;–8&lt;/sup&gt; M</td>
<td>1.8 × 10&lt;sup&gt;–7&lt;/sup&gt; M</td>
</tr>
<tr>
<td>RL95-2</td>
<td>2.4 × 10&lt;sup&gt;–6&lt;/sup&gt; M</td>
<td>2.5 × 10&lt;sup&gt;–3&lt;/sup&gt; M</td>
<td>7.2 × 10&lt;sup&gt;–8&lt;/sup&gt; M</td>
<td>3.0 × 10&lt;sup&gt;–7&lt;/sup&gt; M</td>
</tr>
<tr>
<td>KLE</td>
<td>2.5 × 10&lt;sup&gt;–6&lt;/sup&gt; M</td>
<td>2.3 × 10&lt;sup&gt;–3&lt;/sup&gt; M</td>
<td>9.8 × 10&lt;sup&gt;–8&lt;/sup&gt; M</td>
<td>3.9 × 10&lt;sup&gt;–7&lt;/sup&gt; M</td>
</tr>
<tr>
<td>AN3CA</td>
<td>3.1 × 10&lt;sup&gt;–6&lt;/sup&gt; M</td>
<td>3.8 × 10&lt;sup&gt;–3&lt;/sup&gt; M</td>
<td>1.9 × 10&lt;sup&gt;–7&lt;/sup&gt; M</td>
<td>4.1 × 10&lt;sup&gt;–7&lt;/sup&gt; M</td>
</tr>
</tbody>
</table>

<sup>a</sup> HDACI, HDAC inhibitor; SAHA, suberoylanilide bishydroxamine; VPA, valproic acid; TSA, trichostatin A; NaB, sodium butyrate.
Table 2  Cell cycle changes mediated by HDACIs in endometrial cancer cell lines\textsuperscript{a}

Endometrial cancer cells were plated in triplicate wells and grown in the presence or absence (control) of the HDACIs for 3 days at the indicated concentration, and cell cycle distribution was measured. The numbers indicate % ± SD.

<table>
<thead>
<tr>
<th>Control</th>
<th>SAHA 5 × 10(^{-6}) M</th>
<th>VPA 5 × 10(^{-3}) M</th>
<th>TSA 3 × 10(^{-7}) M</th>
<th>NaB 5 × 10(^{-5}) M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ishikawa</td>
<td>49 ± 10</td>
<td>24 ± 8</td>
<td>72 ± 21</td>
<td>31 ± 13</td>
</tr>
<tr>
<td>G(<em>{1})/G(</em>{0})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>41 ± 4</td>
<td>16 ± 2</td>
<td>18 ± 10</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>G(_{2})/M</td>
<td>10 ± 7</td>
<td>60 ± 5</td>
<td>10 ± 9</td>
<td>53 ± 11</td>
</tr>
<tr>
<td>HEC-1B</td>
<td>51 ± 10</td>
<td>26 ± 4</td>
<td>68 ± 9</td>
<td>37 ± 6</td>
</tr>
<tr>
<td>G(<em>{1})/G(</em>{0})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>38 ± 3</td>
<td>20 ± 10</td>
<td>20 ± 10</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>G(_{2})/M</td>
<td>11 ± 8</td>
<td>54 ± 13</td>
<td>12 ± 5</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>HEC59</td>
<td>45 ± 7</td>
<td>30 ± 9</td>
<td>66 ± 10</td>
<td>38 ± 9</td>
</tr>
<tr>
<td>G(<em>{1})/G(</em>{0})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>40 ± 7</td>
<td>21 ± 11</td>
<td>15 ± 6</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>G(_{2})/M</td>
<td>15 ± 2</td>
<td>49 ± 15</td>
<td>19 ± 11</td>
<td>41 ± 18</td>
</tr>
<tr>
<td>RL95-2</td>
<td>50 ± 9</td>
<td>20 ± 6</td>
<td>74 ± 17</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>G(<em>{1})/G(</em>{0})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>31 ± 10</td>
<td>18 ± 10</td>
<td>11 ± 1</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>G(_{2})/M</td>
<td>19 ± 4</td>
<td>62 ± 19</td>
<td>15 ± 9</td>
<td>59 ± 24</td>
</tr>
<tr>
<td>KLE</td>
<td>41 ± 8</td>
<td>28 ± 3</td>
<td>59 ± 10</td>
<td>35 ± 9</td>
</tr>
<tr>
<td>G(<em>{1})/G(</em>{0})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>49 ± 17</td>
<td>32 ± 2</td>
<td>27 ± 6</td>
<td>31 ± 12</td>
</tr>
<tr>
<td>G(_{2})/M</td>
<td>10 ± 4</td>
<td>40 ± 8</td>
<td>14 ± 5</td>
<td>34 ± 10</td>
</tr>
<tr>
<td>AN3CA</td>
<td>44 ± 8</td>
<td>28 ± 8</td>
<td>62 ± 22</td>
<td>19 ± 6</td>
</tr>
<tr>
<td>G(<em>{1})/G(</em>{0})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>47 ± 11</td>
<td>30 ± 9</td>
<td>25 ± 11</td>
<td>29 ± 17</td>
</tr>
<tr>
<td>G(_{2})/M</td>
<td>9 ± 7</td>
<td>42 ± 17</td>
<td>13 ± 7</td>
<td>52 ± 20</td>
</tr>
</tbody>
</table>

\textsuperscript{a} HDACI, HDAC inhibitor; SAHA, suberoyl anilide bishydroxamine; VPA, valproic acid; TSA, trichostatin A; NaB, sodium butyrate.

ChIP. The effect of HDAC inhibition on the acetylation of histones H3 and H4, which are associated with the p21\textsuperscript{WAF1} gene promoter, was examined using ChIP. Chromatin fragments from HEC-1B cells cultured either with or without SAHA (5 × 10\(^{-8}\) M) for 2 days were immunoprecipitated with antibody to acetylated histone H3 and H4. DNA from the immunoprecipitate was isolated, and a 255-bp fragment of the p21\textsuperscript{WAF1} promoter region was amplified (Fig. 5). After culture with SAHA, ~12-fold more p21\textsuperscript{WAF1} promoter DNA was associated with highly acetylated H3 and H4 histone, compared with the same region isolated from cells cultured without SAHA (Fig. 5).

Antitumor Effect in Vivo. We tested the ability of VPA to inhibit the growth of human HEC-1B endometrial tumors growing in immunodeficient mice during 5 weeks of therapy. HEC1-B cells (5 × 10\(^{6}\)) developed robust tumors in vivo (control mice). As shown in Fig. 6A, administration of VPA remarkably suppressed the growth of the tumors; all of the treatment groups had statistically significantly smaller tumors than the diluent-control groups (P = 0.02). In addition to determining the volume of the tumors over time, they were also carefully dissected at the termination of the study and weighed. The results by weight paralleled the volume measurements (Fig. 6B).

Antitumor Effect in Vivo. We tested the ability of VPA to inhibit the growth of human HEC-1B endometrial tumors growing in immunodeficient mice during 5 weeks of therapy. HEC1-B cells (5 × 10\(^{6}\)) developed robust tumors in vivo (control mice). As shown in Fig. 6A, administration of VPA remarkably suppressed the growth of the tumors; all of the treatment groups had statistically significantly smaller tumors than the diluent-control groups (P = 0.02). In addition to determining the volume of the tumors over time, they were also carefully dissected at the termination of the study and weighed. The results by weight paralleled the volume measurements (Fig. 6B). Tumor weights in the treatment groups were 75% less than those in the control cohort (P < 0.01). During the study, all of the mice were weighed once per week. The body weights of all treated groups were between 91 and 101% of those in the control groups (data not shown). In general, all of the mice of each of the cohorts looked healthy. No significant differences in either the mean weights, histology of internal organs, and mean blood chemistries, including liver parameters and hematopoietic values, were found between diluent-treated mice and those that received 5 weeks of therapy (data not shown). Histological analysis of HEC-1B tumors from untreated mice revealed moderately differentiated carcinomas with small foci of necrosis and fibrosis, which constituted ~20% of the area of the tumor section. Approximately 50–60% of each of the tumor sections from mice treated with VPA revealed necrosis and histological changes of apoptosis, including formation of apoptotic bodies. Fibrosis composed ~30% of the tumor area (data not shown).
**Fig. 3** Induction of apoptosis by HDACIs. HEC-1B cells were treated for 4 days with different concentrations of either VPA or SAHA and then analyzed for apoptosis by terminal deoxynucleotidyl transferase-mediated nick end labeling assay. Rectangles, the mean ± SD of three independent experiments. *P < 0.05 as determined by Student’s *t* test difference compared with the control group.

**DISCUSSION**

HDACIs can prevent proliferation and induce differentiation of numerous transformed cell types, including neuroblastoma, erythroleukemia, acute myelogenous leukemia, and carcinomas of the skin, breast, prostate, bladder, lung, colon, and cervix (29–34). The effect of HDACIs in endometrial cancer, however, has not been fully examined. A recent study by Terao et al. (35) indicated that NaB had a significant growth-suppressing effect on human endometrial and ovarian cancer cells, irrespective of p53 gene status. This stimulated us to examine the effect of a wide array of HDACIs (SAHA, VPA, TSA, and NaB) on six endometrial cancer cell lines (Ishikawa, Hec-1B, Hec59, RL95–2, KLE, and AN3CA) that each express a mutated, nonfunctional p53 protein.

We have demonstrated that each of the HDACIs is highly effective in suppressing the growth of human endometrial cancer cells. The prominent arrest of cancer cells in the G1 phase of the cell cycle is likely to account for this effect. Expression of p21WAF1 and p27KIP1 are cyclin-dependent kinase inhibitors that have important roles in blocking the cell cycle in the G1 phase (27). Protein levels of both p21WAF1 and p27KIP1 were found to be increased after treatment of endometrial cancer cells with HDACIs, supporting their contribution as a possible mechanism by which HDACIs inhibit endometrial cancer growth. The data corroborate recent results showing a possible correlation between G1-G1 arrest and transcriptional induction of p21 in particular cancer cell types (36).

However, other studies have observed G1 cell cycle arrest to occur independently of p21WAF1 gene expression, e.g., in primary mouse embryonic fibroblast derived from p21WAF1 knockout mice, butyrate HDACIs still induced G1 arrest (37). In addition, Kim et al. (38) observed that in p21 (−/−) colon carcinoma, TSA was capable of inducing apoptosis. Thus, p21WAF1 is unlikely to be the sole target gene responsible for TSA- and/or butyrate-induced cell growth arrest. Additional studies are necessary to clarify whether this is also true in endometrial cancer cells.

The enhanced expression of p21WAF1 was accompanied by an accumulation of acetylated histones H3 and H4 associated with the p21WAF1 gene. We and others (37, 41) have shown that HDACIs decrease levels of cyclin D1 and D2, and this appears to occur at the transcription level. Therefore, HDACIs cause decreased expression of D cyclins and increase expression of p21WAF1, which probably combine to modulate the activity of the downstream pRb/E2F axis triggering cell cycle arrest (42).

Apoptosis or programmed cell death is an active gene-directed mechanism of cellular suicide, important for the development and homeostasis of multicellular organisms. Specific therapies have been designed to enhance the susceptibility of human cancers to undergo apoptosis. We show that treatment with HDACIs dramatically and significantly increased the number of apoptotic cells in all of the endometrial cancer cell lines. This effect was associated with a decrease in levels of the antiapoptotic protein Bcl-2.

Control of expression of AR can be controlled by the status of the chromatin in the region of this gene. Forced expression of AR in endometrial cancer cells has been associated with slowing of cell growth. HDAC is involved in the transrepression path-

<table>
<thead>
<tr>
<th>Apoptotic cells (%)</th>
<th>Control</th>
<th>SAHA 5 × 10⁻⁶ M</th>
<th>VPA 5 × 10⁻³ M</th>
<th>TSA 5 × 10⁻⁷ M</th>
<th>NaB 5 × 10⁻⁷ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ishikawa</td>
<td>3 ± 1</td>
<td>42 ± 11</td>
<td>40 ± 19</td>
<td>48 ± 20</td>
<td>39 ± 15</td>
</tr>
<tr>
<td>HEC-1B</td>
<td>2 ± 1</td>
<td>50 ± 21</td>
<td>33 ± 18</td>
<td>41 ± 14</td>
<td>39 ± 23</td>
</tr>
<tr>
<td>HEC59</td>
<td>0</td>
<td>33 ± 15</td>
<td>29 ± 12</td>
<td>50 ± 24</td>
<td>35 ± 18</td>
</tr>
<tr>
<td>RL95-2</td>
<td>0</td>
<td>30 ± 10</td>
<td>22 ± 9</td>
<td>55 ± 31</td>
<td>33 ± 14</td>
</tr>
<tr>
<td>KLE</td>
<td>0</td>
<td>11 ± 8</td>
<td>25 ± 14</td>
<td>38 ± 19</td>
<td>28 ± 10</td>
</tr>
<tr>
<td>AN3CA</td>
<td>2 ± 0</td>
<td>25 ± 6</td>
<td>28 ± 8</td>
<td>35 ± 18</td>
<td>30 ± 17</td>
</tr>
</tbody>
</table>

*HDACI, HDAC inhibitor; SAHA, suberoyl anilide bishydroxamine; VPA, valproic acid; TSA, trichostatin A; NaB, sodium butyrate.*

Table 3 Proportion of cells undergoing apoptosis produced by HDACIs in endometrial cancer cell lines*
way of the AR (43). Although the results in this study suggest that AR is up-regulated after HDACI treatment, synergistic decrease of clonal proliferation was not seen after combined treatment with HDACIs and dihydrotestosterone in endometrial cancer cells (data not shown).

The E-cadherin gene is involved in cell–cell adhesion, and loss of function has been associated with enhanced metastatic growth of tumor cells (44). Inactivation of this gene by hypermethylation has been observed in breast carcinoma cells (45) and primary breast tumors (43). We find that transcription of E-cadherin is up-regulated in endometrial cancer cells treated with HDACIs, suggesting a gain of tumor suppressor function in response to inhibition of histone deacetylase.

Although HDACIs have been shown to have an antiproliferative effect in vitro, a number of limitations hamper their clinical use. NaB, a low-potency HDACI, has been extensively studied; it has antitumor activity and can induce differentiation of some cancer cell lines, but clinical utility has been restricted by its short half-life (5 min), limiting the ability to achieve therapeutic plasma level. TSA is of limited therapeutic use because of toxic side effects in vivo.

SAHA and VPA, however, are relatively safe and nontoxic in vivo; for this reason, they are the two HDACIs that we focused on in this study. SAHA is currently in Phase I clinical trials for the treatment of solid and hematological tumors, and VPA has been used in the treatment of epilepsy for almost 30 years. Our in vivo studies show that VPA at 0.3–1.5 mM inhibited cell proliferation, induced cell cycle arrest, and stimulated apoptosis in HEC-1B endometrial cancer cells. This range of concentrations of VPA can be achieved in a patient’s serum when receiving a daily dose of 20–30 mg/kg for epilepsy. These data are also consistent with our in vitro data. Our histological data showed that the HEC-1B cells formed moderately differentiated adenocarcinomas in the nude mice. Extensive necrosis, apoptosis, and fibrosis involving ~30–60% of the tumor area was observed in the mice treated with VPA. This anticancer activity occurred without any major side effects, raising hopes that VPA may become a useful adjuvant therapy for endometrial cancers. This may be particularly true for the individuals who have minimal residual disease after curative attempt by surgery,
Antigrowth Activity of HDACIs in Endometrial Cancer

In summary, HDACIs exhibit antiproliferative activity and potently induce apoptosis in human endometrial cancer cells. These events are accompanied by induction of p21WAF1, p27KIP1, and down-regulation of several antiapoptosis and cell cycle-related proteins, bel-2, cyclin D1, and cyclin D2. Furthermore, VPA significantly inhibited tumor growth in nude mice without apparent toxic side effects. The present findings raise the possibility that HDACIs may prove particularly effective in the treatment of endometrial cancers.

REFERENCES


Histone Deacetylase Inhibitors Have a Profound Antigrowth Activity in Endometrial Cancer Cells

Noriyuki Takai, Julian C. Desmond, Takashi Kumagai, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/3/1141

Cited articles
This article cites 43 articles, 18 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/3/1141.full.html#ref-list-1

Citing articles
This article has been cited by 31 HighWire-hosted articles. Access the articles at:
/content/10/3/1141.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.