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Apicidin, a Histone Deacetylase Inhibitor, Inhibits Proliferation of Tumor Cells via Induction of p21$^{WAF1/Cip1}$ and Gelsolin[1]

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ABSTRACT

Apicidin [cyclo(*N*-*O*-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)] is a fungal metabolite shown to exhibit antiparasitic activity by the inhibition of histone deacetylase (HDAC). In this study, we evaluated apicidin as a potential antiproliferative agent. Apicidin showed a broad spectrum of antiproliferative activity against various cancer cell lines, although with differential sensitivity. The antiproliferative activity of apicidin on HeLa cells was accompanied by morphological changes, cell cycle arrest at G_1 phase, and accumulation of hyperacetylated histone H4 *in vivo* as well as inhibition of partially purified HDAC *in vitro*. In addition, apicidin induced selective changes in the expression of p21$^{WAF1/Cip1}$ and gelsolin, which control the cell cycle and cell morphology, respectively. Consistent with increased induction of p21$^{WAF1/Cip1}$, phosphorylation of Rb protein was markedly decreased, indicating the inhibition of cyclin-dependent kinases, which became bound to p21$^{WAF1/Cip1}$. The effects of apicidin on cell morphology, expression of gelsolin, and HDAC1 activity *in vivo* and *in vitro* appeared to be irreversible, because withdrawal of apicidin did not reverse those effects, whereas the induction of p21$^{WAF1/Cip1}$ by apicidin was reversible. Taken together, the results suggest that induction of histone hyperacetylation by apicidin is responsible for the antiproliferative activity through selective induction of genes that play important roles in the cell cycle and cell morphology.

INTRODUCTION

Histone acetylation, which is regulated by a balance between HATs[4] and HDACs (1), has been suggested to play an important role in gene expression by affecting the dynamics of chromatin folding during gene expression (2). This hypothesis is supported by the fact that hyperacetylation of lysine residues in the N-terminal tails of histone correlates with active gene loci; in contrast, their hypoacetylation occurs at silent or heterochromatic chromosomal regions (3). Furthermore, accumulating evidence suggests that HATs and HDACs act as transcriptional coactivators and transcriptional co-repressors, respectively (3–6). To date, four families of HAT (P/CAF, p300/CBP, TAF250, and SRC-1) have been identified (7–13). Although all HATs are able to modify histones in free solution, non-histone targets such as the general transcription factors, transcription factor F and transcription factor Eb, transcription factor p53, and erythroid Kruppel-like factor, are also substrates for HATs (14, 15), suggesting that HATs may regulate transcription by modifying a variety of promoter-bound proteins. Since the first HDAC, HDAC1, was purified and cloned as a protein that bound the irreversible inhibitor trapoxin (16), additional human HDACs have been identified and classified into two classes: Rpd3-like proteins HDAC1 (16), HDAC2 (17), and HDAC3 (18); and the recently characterized yeast Hda1-like proteins HDAC4, HDAC5, HDAC6 (19), and HDAC-A (20). Biochemical and molecular biological studies have established that HDACs are components of large multiprotein complexes that target promoter sites through their interaction with sequence-specific transcription factors (4, 21–23). In Sin3 complex (a component common to both mammalian and yeast HDAC complexes), mammalian HDAC is associated with NCo-R and SMRT, which function as co-repressors (24–26). In addition, other transcriptional repressors, such as Mad, nuclear receptors, Rb, YY1, and yeast Ume6 protein, associate with a HDAC complex (17, 24, 26–31). These observations provide a molecular basis for HATs and HDACs as regulators of transcription.

Experiments searching for detransforming activity with oncogene-transformed cells led to the identification of various natural and synthetic compounds, such as NaB (32), trapoxin (33), trichostatin A (34), depudecin (35), FR901228 (36), oxamflatin (37), and MS-27-275 (38). In addition to their ability to revert the cell morphology of various oncogene-transformed cells or cancer cells to apparently normal cells, these compounds have also been shown to exhibit HDAC-inhibitory activity and cell cycle arrest, leading to the suggestion that the morphological reversion of transformed cells was the result of their HDAC inhibitory activity. Although the precise mechanism has not been elucidated, the inhibitory effects of these compounds on cell cycle appears to be the result of selective induction of endogenous genes that play significant roles in G_1-S progression of the cell cycle (37). Therefore, HDAC inhibitors have been considered to be a novel class of cancer treatment agent.

Apicidin is a novel cyclic tetrapeptide with a potent broad spectrum of antiprotozoal activity against apicomplexan parasites (39), and its structure is related to trapoxin, a potent HDAC inhibitor. Therefore, the antiparasitic activity of apicidin appears to be attributable to inhibition of apicomplexan HDAC at low nanomolar concentrations. Indeed, the potent HDAC-inhibitory activity of apicidin prompted us to evaluate apicidin as a potential antiproliferative agent. Here, we report characteristic features of apicidin and its strong antiproliferation efficacy.

MATERIALS AND METHODS

Materials. Apicidin [cyclo(*N*-*O*-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)], a known antiprotozoal agent, was prepared from *Fusarium* sp. strain KCTC 16677 according to the method described previously by us (40).

Cell Culture and Preparation of Cell Extracts. HeLa, a human cervix cancer cell line; v-*ras*-transformed NIH3T3, a mouse fibroblast cell line; Colon 3.1-M26, a mouse colon carcinoma cell line; MG63, a human osteosarcoma cell line; and MCF-7, a human breast cancer cell line, were cultured in DMEM (Life Technologies, Inc.), supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), and 1% penicillin/streptomycin (Life Technologies, Inc.). A2058, a human melanoma cell line; AGS, a human gastric adenocarcinoma cell line; HBL-100, a human breast cancer cell line; and

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[4] The abbreviations used are: HAT, histone acetyltransferase; HDAC, histone deacetylase; CDK, cyclin-dependent kinase; NaB, sodium *n*-butyrate; SRB, sulforhodamine B.

Table 1 *Growth-inhibitory concentrations of apicidin in various cell lines*

Exponentially growing mouse and human cell lines were treated with various concentrations of apicidin for 48 h, and the viable cell numbers were determined by SRB assay, as described in "Materials and Methods." The results are means of triplicates from three separate experiments.

Cell line	IC_{50}, μg/ml
CCD-18Co	2.36
HeLa	0.51
v-*ras*-transformed NIH3T3	0.18
Colon 3.1-M26	0.17
MG63	1.88
MCF7	1.17
HBL-100	0.57
AGS	0.13
A2058	0.55
ZR-75-1	1.17

CCD-18Co, a human normal colon cell line, were cultured in RPMI 1640 (Life Technologies, Inc.), 10% fetal bovine serum, and 1% penicillin/streptomycin. HeLa cells were incubated with 0.01, 0.1, 0.5, 1, and 2 μg/ml apicidin or 1 mM NaB (Sigma Chemical Co., St. Louis, MO) in culture medium for 24 h. In some experiments, proliferating HeLa cells were treated with 1 μg/ml apicidin or 1 mM NaB for 24 h. At this time point, the medium was removed, cells were washed thoroughly with PBS, and medium with no apicidin or NaB was added back. Cells were then incubated further until the end of the 24-, 48-, and 150-h periods. The controls were prepared by incubating cells for the corresponding period in complete medium containing DMSO instead of the agents. After stimulation, cells were rinsed twice with ice-cold wash buffer containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 15 mM sodium PP_i, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride, and were then extracted in the same buffer containing 1% NP40. Cells were collected with a plastic scraper, homogenized, and cleared by centrifugation at 4°C for 15 min at 15,000 × *g*. The protein concentration was measured by the Bradford method, with BSA as the standard. Aliquots of the supernatants were frozen in liquid nitrogen and stored at −70°C.

Cell Growth Inhibition Assay. To examine the effect of apicidin on the proliferation of mouse and human cancer cell lines, cell growth inhibition was assessed with the SRB protein dye assay 48 h after cell seeding at 1×10^5 cells/well in 6-well plates in complete growth medium (41). In brief, exponentially growing cells were treated with 0.01, 0.1, 0.5, 1, and 2 μg/ml apicidin for 48 h, and the culture medium was removed. The cells were then fixed by incubating with 1 ml of 10% trichloroacetic acid at 4°C for 1 h, followed by five washes with distilled water. After complete air-drying of the plate, 0.4% SRB solution in 1% glacial acetic acid was added at room temperature for 30 min to stain the cells. Subsequently, the plate was washed five times with 1% glacial acetic acid and allowed to air-dry overnight. Tris-HCl (1 ml, 10 mM) was then added to each well to dissolve the SRB bound to cellular protein; the SRB absorbance was then measured at 490 nm on an EL 808 ultra microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The absorbance is proportional to the number of cells attached to the culture plate. Therefore, the results of SRB represent the antiproliferative effect of apicidin on mouse and human cancer cell lines.

HDAC Assay. The mammalian HDAC was partially purified from HeLa cells (42) and assayed for HDAC activity as described previously (43) by incubation with [^{3}H]acetyl-labeled histones as the substrate for 20 min at 37°C. The released [^{3}H]acetic acid was extracted with ethyl acetate, quantitated by scintillation counting, and used as a measure of HDAC activity.

Immunoblotting. HeLa cells were incubated with 0.01, 0.1, 0.5, 1, and 2 μg/ml apicidin or 1 mM NaB in culture medium for 24 h. Cell lysates were boiled in Laemmli sample buffer for 3 min, and 30 μg of each total protein were subjected to SDS-PAGE on 15% slab gels for the analysis of gelsolin, cyclin D1, CDK2, $p21^{WAF1/Cip1}$, HDAC1, and p53, except for pRb, which was run on a 7.5% slab gel. Proteins were transferred to polyvinylidene difluoride membranes, and membranes were blocked for 30 min in PBS containing 0.1% Tween 20 (PBS-T) and 5% (w/v) dry skim milk powder, and incubated overnight with antigelsolin (Transduction Laboratories), -cyclin D1 (Upstate Biotechnology Inc.), -CDK2 (Upstate Biotechnology), -$p21^{WAF1/Cip1}$ (Santa Cruz Biotechnologies, Inc.), -HDAC1 (Upstate Biotechnology), -p53 (Upstate Biotechnology), and -pRb (PharMingen) antisera. The membranes were then washed with PBS-T and incubated for 2 h with an antirabbit or an antimouse secondary antibody. Bound antibodies were detected with the enhanced amplified alkaline phosphatase immunoblot system (Bio-Rad).

Histone Isolation and Immunodetection of Acetylated Histone H4. HeLa cells were incubated with 0.01, 0.1, 0.5, 1, and 2 μg/ml apicidin or 0.1% DMSO in culture medium. After 24 h, cells were trypsinized, and histones were isolated by the established techniques (32). Each histone sample (50 μg) was dialyzed and concentrated, using a Microcon-3 (Amicon, Inc), against 0.1 M acetic acid and distilled water, respectively, and the samples were resuspended in Laemmli sample buffer for 3 min and subjected to 10–20% Tricine SDS-PAGE for the determination of acetylated histone H4. Acetylated histone H4 was detected by anti-acetyl histone H4 antiserum (Upstate Biotechnology) at 1:2000 dilution in 5% dry skim milk powder in PBS-T and visualized as described above.

Analysis of DNA Synthesis and Cell Cycle. DNA synthesis was measured by [^{3}H]thymidine incorporation assay as described previously (44). HeLa cells were plated at 20,000 cells/well in 24-well plates in complete growth medium and incubated for 24 h. The medium was removed, and the cells were cultured for an additional 16 h with 1 μg/ml apicidin. Two hundred fifty nl of 1 mCi/ml *methyl*-[^{3}H]thymidine (Amersham Pharmacia Biotech) were added to each well, and the cells were incubated for an additional 8 h. The cells were fixed and washed twice with ice-cold trichloroacetic acid (10%). The precipitated material was solubilized with 0.2 N NaOH, and the incorporated radioactivity was counted by liquid scintillation; results are expressed as percentage of the maximal [^{3}H]thymidine incorporated in the presence of 0.1% DMSO alone. To analyze the effect of apicidin on cell cycle progression, the DNA content profile of a given population was determined by flow cytometry according to the method described by Noguchi and Browne (45). Briefly, after fixation with 70% ethanol and treatment with 0.25 μg/ml RNase, nuclei were stained with 50 μg/ml propidium iodide, and the relative DNA content was measured using a BRYTE HS system (Bio-Rad Laboratories) and a ModFit LT (Verity Software House, Inc., Topsham, ME).

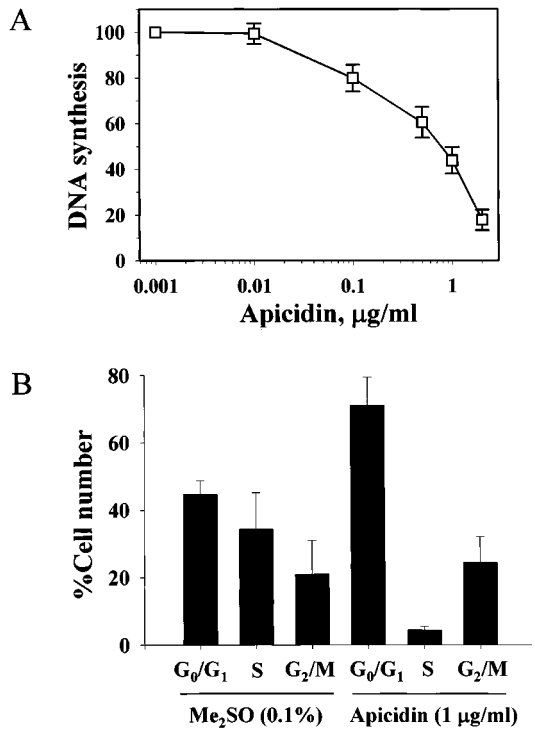

Fig. 1. Effect of apicidin on cell cycle progression of HeLa cells. *A*, HeLa cells (seeded at a density of 2×10^4/well and grown for 24 h in 24-well plates) were treated with various concentrations of apicidin for 24 h, and then DNA synthesis was determined by incubation with 5×10^4 cpm [^{3}H]thymidine/ml of medium; results are presented as percentage of cpm of the control culture. Data are the means of triplicate determinations from three experiments; *bars*, SE. *B*, 1×10^6 HeLa cells were cultured for 24 h, and 0.1% DMSO or 1 μg/ml of apicidin was added to the culture at time 0 h. After incubation for 24 h, cells were collected, and their isolated nuclei were analyzed by flow cytometry. Distribution of cells in the cell cycle was determined using ModFit LT software. Data are the means of triplicate determinations from three experiments; *bars*, SE.

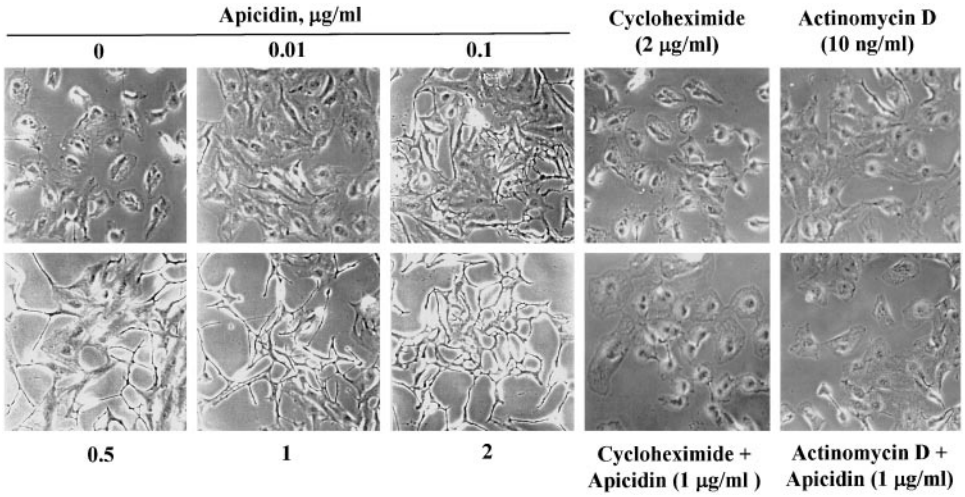

Fig. 2. Morphological changes of HeLa cells induced by apicidin. HeLa cells were treated for 24 h with the indicated concentrations of apicidin. The morphological changes were induced by apicidin and counteracted by simultaneous addition of cycloheximide or actinomycin D (original magnification, ×200).

RESULTS AND DISCUSSION

Antiproliferative Effect of Apicidin. Apicidin, a fungal metabolite, has been shown to exhibit a potent, broad spectrum of antiprotozoal activity against apicomplexan parasites by inhibiting their HDACs (39). Recently, accumulating evidence has suggested that deregulation of HAT and HDAC plays a causative role in the generation of cancer. To evaluate the potential of HDAC inhibitor apicidin as an antiproliferative agent such as trichostatin A (34), trapoxin (33), FR901228 (36), and oxamflatin (37), we first examined the effect of apicidin on the proliferation of mouse and human cancer cell lines *in vitro* using the SRB assay. As shown in Table 1, cell growth was inhibited to various degrees in the presence of apicidin, having half-maximum effects between 1.8 and 0.1 μg/ml. Apicidin showed a marked antiproliferative effect against AGS, a human stomach cancer cell, and v-*ras*-transformed NIH3T3 cells ($IC_{50} < 0.2$ μg/ml), and a moderate effect against MG63, a human osteosarcoma cell line, MCF7, a human breast cancer cell, and ZR-75-1, a human breast carcinoma cell ($IC_{50} > 1$ μg/ml). However, the growth of the cancer cell lines tested was more sensitive to apicidin than the normal cell line, CCD-18Co (a normal human colon cell line), for which the IC_{50} value of apicidin was 2.36 μg/ml. The results indicate that apicidin has a broad spectrum of antiproliferative activity toward various cancer cell lines. To further analyze the antiproliferative effect of apicidin, its effect on the cell cycle progression was next investigated. Thus, HeLa cells, an epithelium-like carcinoma cell line from human cervix, were treated with various concentrations of apicidin for 24 h and were incubated with [^{3}H]thymidine for the last 8 h. Treatment of asynchronous HeLa cell cultures with apicidin resulted in a dose-dependent inhibition of [^{3}H]thymidine incorporation, with 50% inhibition at 1 μg/ml and maximal inhibition at 2 μg/ml, indicating significant inhibition of G_1-S progression (Fig. 1*A*). Further analysis of the effect of apicidin on the distribution of cell population showed effects similar to the above result (Fig. 1*B*). Apicidin treatment increased the number of cells at G_0-G_1 from 45% to 70%, whereas the cells at S phase decreased from 35% to 8%, indicating inhibition of the cell cycle at G_0-G_1. Taken together, the results indicate that the antiproliferative activity of apicidin might be attributed to cell cycle arrest at G_1 phase in HeLa cells.

Morphological Changes of HeLa Cells by Apicidin. Recently, many HDAC inhibitors, including depudecin, FR901228, trapoxin, oxamflatin, and trichostatin A, have been shown to induce morphological changes of v-*ras*-transformed NIH3T3 cells (35, 36, 46) or HeLa cells (34, 37). These observations prompted us to examine the effect of apicidin on the induction of morphological changes of HeLa human tumor cells. As shown in Fig. 2, HeLa cells have an oval or polygonal appearance in the absence of apicidin; however, they were dramatically changed to an elongated shape with filamentous protrusions after treatment with increasing concentrations of apicidin (up to 2 μg/ml). The production of filamentous protrusions increased in a dose-dependent manner. The changes produced by NaB, another HDAC inhibitor, on the morphology of HeLa cells were in appearance similar to those of apicidin, indicating that morphological reversion in HeLa cells by apicidin could result from the inhibition of HDAC. In addition, the morphological change seemed to require *de novo* protein and RNA synthesis because cycloheximide and actinomycin D significantly inhibited the detransforming activity of apicidin (Fig. 2).

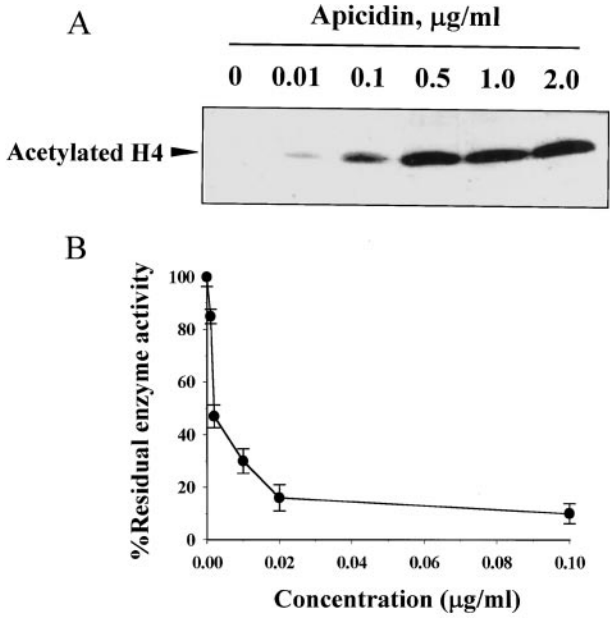

Fig. 3. Apicidin induces accumulation of acetylated histones *in vivo* and inhibits human HDAC activity *in vitro* in a concentration-dependent manner. *A*, proliferating HeLa cells were treated with various concentrations of apicidin. After 24 h, histones were isolated, and 50 μg were run on 10–20% Tricine gel, blotted, and probed with an antibody against acetylated histone H4. *B*, nuclear HDAC was partially purified from cell extracts of HeLa cells, and its enzymatic activity was determined as described in "Materials and Methods." The enzyme preparation was pretreated with various concentrations of apicidin at 4°C, and the residual enzyme activity was determined at the times indicated by measuring the amount of [^{3}H]acetic acid released during incubation for 10 min at 37°C. Data are the means of triplicate determinations from three independent experiments; *bars*, SE.

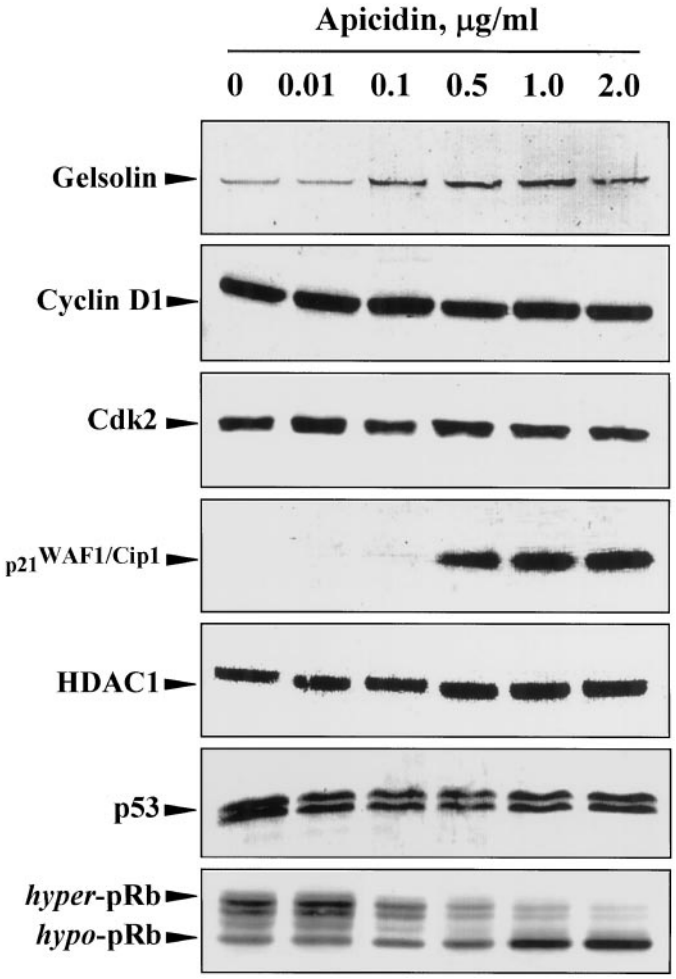

Fig. 4. Effect of apicidin on the expression of endogenous genes in HeLa cells. Lysates (30 μg) of the HeLa cells exposed to 0.01, 0.1, 0.5, 1, or 2 μg/ml apicidin (*Lanes 2–6*, respectively) or 0.1% DMSO (*Lane 1*) were examined by 15% SDS-PAGE and analyzed with immunoblotting using antibodies for gelsolin, cyclin D1, CDK2, $p21^{WAF1/Cip1}$, HDAC1, and p53. The phosphorylation status of pRb was determined by electrophoretic mobility on 7.5% SDS-PAGE. Total pRb was detected in HeLa whole-cell lysates by immunoblot using an antibody that recognizes phosphorylated (*hyper-pRb*) and unphosphorylated (*hypo-pRb*) forms of pRb, after 24 h of treatment with indicated concentrations of apicidin.

The requirements of *de novo* protein and RNA synthesis for morphological changes induced by apicidin are very similar to those of oxamflatin (37). v-*ras*-transformed NIH3T3 cells also reverted from a spindle-like to a normal morphology after treatment with apicidin, similar to that observed with depudecin (Ref. 35; data not shown). The broad spectrum of detransforming activity of apicidin implies possible roles in stress fiber formation and cell growth control.

Effect of Apicidin on HDAC Activity in HeLa Cells. The level of *in vivo* histone acetylation is regulated mainly by the balance between HAT and HDAC. Recently, human HDAC, one of the regulators of histone acetylation, was purified and cloned using a trapoxin-based affinity matrix (16). In addition, apicidin has been shown to inhibit the activity of HDAC partially purified from *Eimeria tenella* as well as to induce hyperacetylation of histone in *Plasmodium falciparum* (39). To examine the sensitivity of HeLa HDAC to apicidin, we first examined the effect of apicidin on partially purified HDAC from HeLa cells by measuring the amount of [^{3}H]acetic acid released from [^{3}H]acetylated histones. As shown in Fig. 3*B*, HDAC activity was inhibited in a concentration-dependent manner, with 50% inhibition at 0.003 μg/ml (5 nM) and maximal inhibition at 0.025 μg/ml (40 nM). The IC_{50} of apicidin is lower than those of NaB (119 μM; Ref. 37), oxamflatin (15.7 nM; Ref. 37), depudecin (4.7 μM; Ref. 35), and MS-27-275 (2 μM; Ref. 38), indicating that the novel cyclic tetrapeptide has potent HDAC inhibitory activity.

We next analyzed the effect of apicidin on the intracellular level of histone H4 acetylation, using a specific antibody against acetylated histone H4. HeLa cells were treated with various concentrations of apicidin for 24 h, and histones extracted from nuclei were subjected to SDS-PAGE and immunoblot analysis (Fig. 3*A*). Proliferating HeLa cells had low levels of acetylated histone H4 in the absence of apicidin. However, the acetylation of histone H4 was increased in a dose-dependent manner, reaching maximum at 0.5 μg/ml of apicidin.

Taken together, the *in vivo* effect of apicidin on nuclear histone acetylation is very closely correlated with the *in vitro* effect of apicidin on HDAC activity, indicating that the induction of histone hyperacetylation by apicidin most likely results from inhibition of histone deacetylase. Although transient histone H4 acetylation by HDAC inhibitors, including NaB and trichostatin A (47), was observed in human keratinocytes, the histone hyperacetylation in HeLa cells by apicidin was persistent up to 48 h (data not shown).

Changes of Endogenous Gene Expression by Apicidin. Apicidin caused not only morphological changes (Fig. 2) but also cell cycle

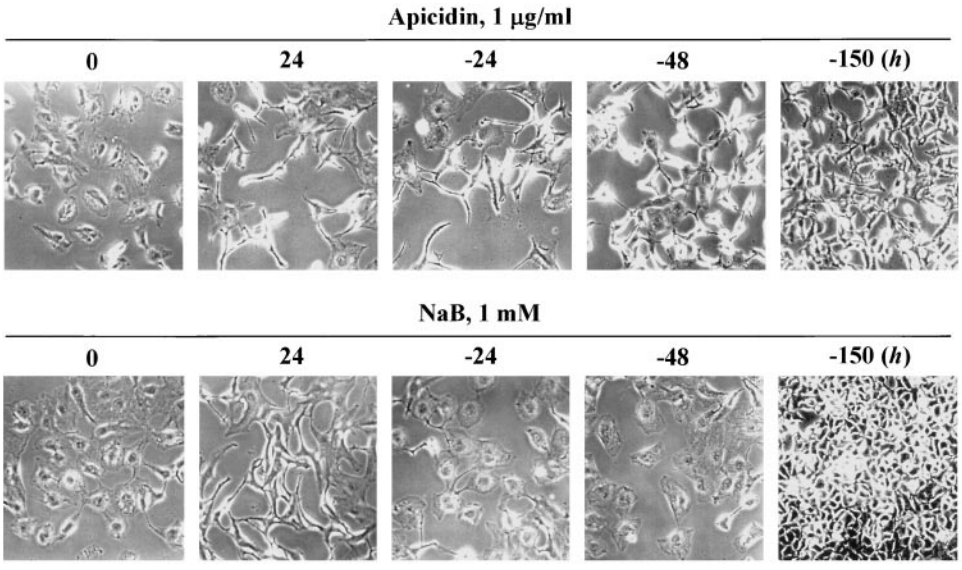

Fig. 5. Reversibility of morphological changes by apicidin. Proliferating HeLa cells were treated with 1 μg/ml apicidin or 1 mM NaB for 24 h. At this time point, media were removed, cells were washed thoroughly with PBS, and medium with no apicidin or NaB was added back. Cells were then incubated further until the end of the 24-, 48-, and 150-h periods, at which time morphological changes were determined.

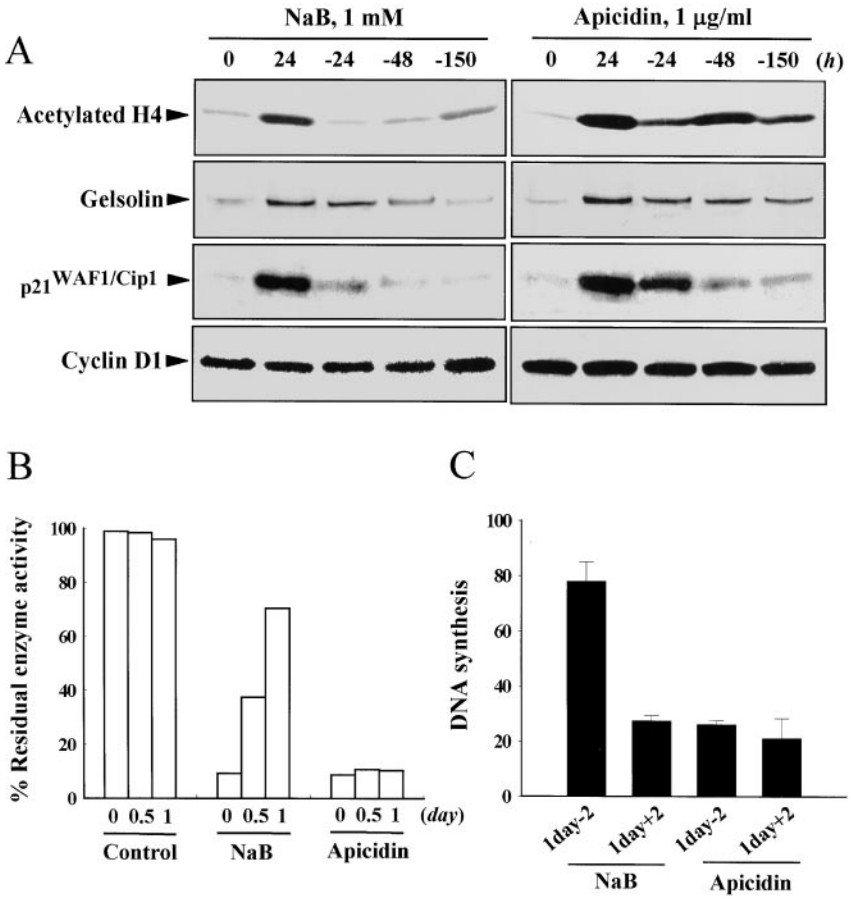

Fig. 6. Reversibility of antiproliferative effect of apicidin. *A*, reversibility of induction of acetylated histone H4, gelsolin, $p21^{WAF1/Cip1}$, and cyclin D1 expression in HeLa cells by apicidin. Proliferating HeLa cells were treated with 1 μg/ml apicidin or 1 mM NaB for 24 h. At this time point, media were removed, cells were washed thoroughly with PBS, and medium with no apicidin or NaB was added back. Cells were then incubated further until the end of the 24-, 48-, and 150-h periods, at which time histones or proteins were extracted, and 50 μg of isolated histones or 30 μg of proteins were separated by 10–20% Tricine gel electrophoresis or 15% SDS-PAGE, respectively, and analyzed by immunoblotting. *B*, reversibility of enzyme inhibition by apicidin. The partially purified HDAC preparations were pretreated with 0.1% DMSO (*Control*), 1 mM NaB, or 0.1 μM apicidin for 12 h at 4°C. The treated enzyme preparations were dialyzed against drug-free buffer for 0.5 or 1 day, and the residual enzyme activity was determined. *C*, reversibility of growth arrest induced by apicidin in HeLa cells. Proliferating HeLa cells were treated with 1 mM NaB or 1 μg/ml apicidin for 1 day. Some cells were then treated for additional 2 days (*1 day + 2*) or were washed with PBS and left untreated for 2 days (*1 day − 2*). Data are the means of three experiments performed in triplicate; *bars*, SE. Data are expressed as the fold increase over the 1 day + 2 sample.

arrest (Fig. 1), similar to other inhibitors [trichostatin A (34), trapoxin (33), FR901228 (36), and oxamflatin (37)]. Specifically, morphological changes of HeLa cells by apicidin required *de novo* protein and RNA synthesis (Fig. 2), indicating the involvement of proteins in the alteration of cellular shape and cytoskeletal architecture. We therefore examined the effect of apicidin on the expressions of gelsolin, a Ca^{2+}-dependent actin filament-severing and capping protein (48) that controls the length of actin filaments and cell shape and motility (49). The expression of gelsolin in HeLa cells was obviously up-regulated by apicidin, which occurred at concentration of 0.5 μg/ml, and no further induction was observed at higher concentrations, indicating a threshold effect (Fig. 4). This effect was very similar to the effect on morphology (compare Fig. 4 with Fig. 2), suggesting that the increase in gelsolin produced by apicidin could be responsible for the apicidin-mediated detransforming activity. This suggestion is supported by the observations that HDAC inhibitor-induced morphological changes were suppressed by microinjection of antigelsolin antibodies (50) and that malignant transformation was correlated with decreased expression of gelsolin (51, 52).

As shown in Fig. 1, apicidin was found to inhibit G_1-S progression in the cell cycle of HeLa cells. Because acetylation and deacetylation of histone have been shown to play an important role in the regulation of gene expression in eukaryotic cells (9–11, 17, 26–28), cell cycle arrest at G_1 phase by apicidin might be attributable to alteration of the expression of genes important to G_1-S progression. To test this possibility, we determined the levels of cyclin D1, CDK2, $p21^{WAF1/Cip1}$, HDAC1, and p53, as well as the phosphorylation of Rb by immunoblot analysis. The protein levels of cyclin D1, CDK2, HDAC1, p53, and Rb were not affected by the addition of apicidin for 24 h (Fig. 4). On the other hand, the expression of $p21^{WAF1/Cip1}$, a CDK inhibitor, was markedly up-regulated by apicidin at 0.5 μg/ml but did not appear to be further induced at higher concentrations, as observed in the expression of gelsolin by apicidin (Fig. 4). In addition, Rb was hyperphosphorylated in the absence of apicidin as monitored by its slower migration in immunoblots of one-dimensional SDS-PAGE; apicidin treatment, however, caused the collapse of the slower migration of a family of bands into a single band, indicating dephospho-

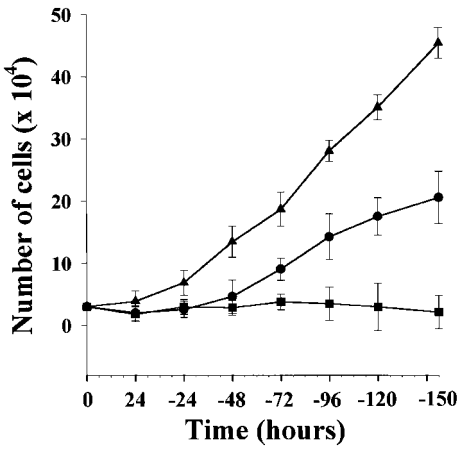

Fig. 7. Effect of the removal of apicidin on the growth of HeLa cells. Proliferating HeLa cells were either treated (●, ■) or not treated (▲) with 1 μg/ml apicidin for 24 h. At this time point, media were removed (●), the cells were washed thoroughly with PBS, and medium with no apicidin was added back. Cells were then incubated further for the indicated times, after which the number of cells was determined by SRB assay, as described in "Materials and Methods." Data are the means of triplicate determinations from three experiments; *bars*, SE.

rylation of Rb protein. Induction of $p21^{WAF1/Cip1}$ by apicidin was well correlated with a decrease in Rb phosphorylation. $p21^{WAF1/Cip1}$ expression is usually controlled at the transcriptional level by both p53-dependent and -independent mechanisms (53). In HeLa cells, $p21^{WAF1/Cip1}$ appeared to be expressed by a p53-independent mechanism because expression of p53 was not paralleled by that of $p21^{WAF1/Cip1}$. The G_1 arrest of HeLa cells may be caused by induction of $p21^{WAF1/Cip1}$, which binds to CDKs and inhibits their activity, leading to hypophosphorylation of Rb protein. This hypothesis is supported by similar results obtained with other HDAC inhibitors such as oxamflatin (37) and trapoxin (54).

Irreversible Effect of Apicidin on HDAC, Expression of $p21^{WAF1/Cip1}$ and Gelsolin, Growth Arrest, and Morphological Reversion. Although trapoxin and trichostatin A exerted almost the same biological effect on the cell cycle and differentiation, their modes of inhibition appear to be different: trichostatin A inhibits HDAC irreversibly, and trapoxin reversibly (33, 55). These observations prompted us to examine the effect of withdrawal of apicidin on morphological reversion, expression of $p21^{WAF1/Cip1}$ and gelsolin, growth arrest, and HDAC activity. HeLa cells were treated with apicidin for 24 h. Apicidin was then withdrawn by exchanging culture medium with fresh medium with no apicidin, and the cells were then allowed to grow further for various times. Morphological reversion of HeLa cells to a characteristically elongated cell with filamentous protrusions had not occurred by 24 h after the withdrawal of apicidin and was sustained until 7 days (Fig. 5). In contrast, after withdrawal of NaB, HeLa cells reverted to a shape with an oval or polygonal appearance, as before the addition of apicidin (Fig. 5).

We next tested the reversibility of apicidin-induced inhibition of HDAC *in vitro* and *in vivo*. As shown in Fig. 6*B*, withdrawal of NaB from the reaction mixture by dialysis resulted in a gradual recovery of HDAC activity during dialysis, with ~70% of the initial enzyme activity recovered. However, when cells were treated with apicidin, the enzyme activity was never recovered even after 1 day of dialysis. Consistent with these data, histone H4 extracted from nuclei was found to be significantly hyperacetylated up to 7 days after the removal of apicidin (Fig. 6*A*), whereas hyperacetylation of histone H4 was markedly decreased shortly after the withdrawal of NaB (Fig. 6*A*). These results suggest that the effect of apicidin on morphological changes and HDAC activity *in vitro* and *in vivo* is irreversible and that the morphological change and inhibition of HDAC are closely associated. However, it appears that the reversibility was not absolute: 7 days after the removal of apicidin, $p21^{WAF1/Cip1}$ levels reverted to those observed in the absence of apicidin, whereas gelsolin apparently did not revert, as determined by immunoblot analysis (Fig. 6*A*). Cyclin D1 concentrations, which were not affected by apicidin (Fig. 4), remained unaltered under any experimental condition (Fig. 6*A*). The number of cells, however, appeared to have increased, as observed microscopically, 7 days after apicidin was removed, although the elongated cell shape with filamentous protrusions remained unaltered (Fig. 5). We thus determined the cell number through 7 days after withdrawal of apicidin. Although the growth of cultures from which apicidin was removed was significantly inhibited up to 48 h after withdrawal of apicidin, growth gradually recovered to 40% of that of the culture not treated with apicidin (Fig. 7). Consistent with these data, DNA synthesis was attenuated up to 48 h after apicidin was removed (Fig. 6*C*), whereas withdrawal of NaB led to a rapid recovery of DNA synthesis, to ~80% of the initial DNA synthesis. Therefore, it seems possible that the irreversible effect of apicidin might be attributed, in part, to the slow dissociation rate of the enzyme-inhibitor complex, resulting in apparent HDAC inhibition. Although the irreversible inhibitory effect of apicidin on the development of intracellular apicomplexan parasites *in vitro* has been observed previously (39), the mechanism by which apicidin inhibits HDAC remains to be explored.

In conclusion, we demonstrated that apicidin, a known antiprotozoal agent, is a potent antiproliferative agent with a broad spectrum of activities against various cancer cell lines. Apicidin treatment induced cell cycle arrest at G_1 phase in HeLa cells, probably through the induction of the CDK inhibitor $p21^{WAF1/Cip1}$, which has been shown to play an important role in the G_1 checkpoint of the cell cycle. This is further supported by the observation that the induction of $p21^{WAF1/Cip1}$ by apicidin was accompanied by decreased Rb phosphorylation. The antiproliferative activity of apicidin paralleled the detransforming activity and inhibition of HDAC activity *in vitro* and *in vivo*. Morphological changes of HeLa cells induced by apicidin appeared to be produced in part by the induction of gelsolin, which has been shown to be involved in the control of cell shape and malignant transformation. Furthermore, the morphological changes, inhibition of HDAC *in vitro* and *in vivo*, and expression of gelsolin but not $p21^{WAF1/Cip1}$ were irreversible. These results suggest that inhibition of HDAC activity by apicidin is closely associated with morphological change, induction of $p21^{WAF1/Cip1}$ and gelsolin, and inhibition of DNA synthesis, thereby leading to inhibition of cell proliferation. Thus, apicidin appears to be a potential therapeutic agent for the treatment of cancer.

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