

Apicidin, a novel histone deacetylase inhibitor, has profound anti-growth activity in human endometrial and ovarian cancer cells

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Abstract. Histone deacetylase inhibitors (HDACIs) can inhibit proliferation, induce cell cycle arrest and stimulate apoptosis of cancer cells. Our purpose was to investigate the antiproliferative effects of a novel HDACI, apicidin, on the Ishikawa endometrial cancer cell line, the SK-OV-3 ovarian cancer cell line and normal human endometrial epithelial cells. Endometrial and ovarian cancer cells were treated with various concentrations of apicidin, and the effects on cell growth, cell cycle, apoptosis and related measurements were investigated. MTT assays showed that all endometrial and ovarian cancer cell lines were sensitive to the growth inhibitory effect of apicidin, although normal endometrial epithelial cells were viable after the treatment with the same doses of apicidin that induced the growth inhibition of endometrial and ovarian cancer cells. Cell cycle analysis indicated that their exposure to apicidin decreased the proportion of cells in S-phase and increased the proportion in G0/G1 and/or G2/M phases of the cell cycle. Induction of apoptosis was confirmed by Annexin V staining of externalized phosphatidylserine and loss of the transmembrane potential of mitochondria. This induction occurred in concert with the altered expression of p21^{WAF1}, p27^{KIP1}, p16, cyclin A, and E-cadherin. Furthermore, apicidin treatment of these cell lines increased acetylation of H3 and H4 histone tails. These results suggest that apicidin exhibits the antiproliferative effects through selective induction of genes related to cell growth, malignant phenotype, and apoptosis. The findings raise the possibility that apicidin may prove particularly effective in the treatment of endometrial and ovarian cancers.

Introduction

Endometrial and ovarian cancers are the most common malignant tumors of the female genital tract. The incidence of endometrial cancer has increased in recent years, accounting for ~13% of female cancers (1). Early stages of ovarian cancer are frequently asymptomatic and difficult to detect, and thus diagnosis usually occurs after the disease has advanced. The search for agents effective in the treatment of either advanced or recurrent gynecological cancer has been disappointing. To date, cisplatin, doxorubicin, and paclitaxel demonstrate the greatest efficacy (2,3). However, although reported response rates have been as high as 70%, the duration of the responses remains brief, from 4 to 8 months. Therefore, innovative approaches are needed for the treatment of gynecological cancer.

Local remodeling of chromatin and dynamic changes in the nucleosomal packing 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 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 N-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 amino-terminal tails. Acetylation of histone proteins neutralizes the positive charge on lysine residues and disrupts nucleosome structure, allowing the unfolding of the associated DNA, subsequent access by transcription factors, and changes in gene expression.

Apicidin is a novel cyclic tetrapeptide with a potent broad spectrum of antiprotozoal activity against Apicomplexan parasites (7). The chemical structure of apicidin is depicted in Fig. 1. Its structure is related to trapoxin, a potent HDACI, and some biological activity including antiproliferative and toxic effects has been shown in some cancer cell lines (8). However, the biological activity of apicidin in endometrial and ovarian cancer cells has not been clarified. In this study, we examined whether apicidin was able to mediate inhibition of cell growth, cell cycle arrest, apoptosis, and the expression of genes related to the malignant phenotype in the

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endometrial cell line Ishikawa and the ovarian cancer cell line SK-OV-3. Furthermore, we compared the sensitivity of apicidin to normal endometrial epithelial cells with that to endometrial cancer cells.

Materials and methods

Materials. Apicidin [cyclo (*N*-*O*-methyl-L-tryptophanyl-L-isoleucyl D-pipecolinyl-L-2-amino-8-oxodecanoyl)] was purchased from Calbiochem (Darmstadt, Germany) and re-suspended in dimethyl sulphoxide (DMSO).

Cell lines. The Ishikawa human cell line was kindly provided by Dr Masato Nishida (Tsukuba University, Ibaraki, Japan), and the SK-OV-3 human cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Cell culture. The human endometrial cancer cell line Ishikawa was grown in DMEM, and the human ovarian cancer cell line SK-OV-3 was maintained in RPMI-1640, each supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (10,000 U/ml), and streptomycin (10 mg/ml) (all from Gibco, Rockville, MD).

Normal endometrial specimens were obtained from ten pre-menopausal patients who had undergone hysterectomies for leiomyoma. All patients had been free of any hormonal treatments before the operation. All of the specimens were diagnosed as being from the late proliferative phase (11-13th day of the menstrual cycle) using a standard histological examination of endometrial tissues. This study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Oita University, and written informed consent was obtained from all patients. Normal endometrial epithelial cells were separated from stromal cells by digesting the tissue fragments with collagenase. Briefly, the tissue was minced into 2- to 3-mm pieces and incubated with collagenase (200 U/ml) (Gibco) in RPMI-1640 medium (Gibco) with stirring for 40 min at 37°C. The suspension was then filtered through a 150- μ m wire sieve to remove mucus and undigested tissue. The filtrate was then passed through an 80- μ m wire sieve, which allowed the stromal cells to pass through while the intact glands were retained. After washing three times with serum-free RPMI-1640, normal human endometrial epithelial cells were transferred to culture flasks (Corning, NY) at a density of 10^6 cells/ml in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), streptomycin (100 U/ml), and penicillin (100 U/ml). After 16 h, the attached cells, which were >98% pure as analysed by immunocytochemical staining with antibodies to keratin, vimentin (V9), factor VIII, and leukocyte common antigen (2B11+PD7/26) (all from Dako, Copenhagen, Denmark), were used for the experiments. The cultures were incubated at 37°C in an atmosphere of 5% CO₂ in air at 100% humidity.

Growth studies. Cells were seeded at an initial density of 10^4 per well in 96-well plates, incubated for 24 h, and treated with various concentrations (1×10^{-7} – 1×10^{-5}) of apicidin. After 3 days, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

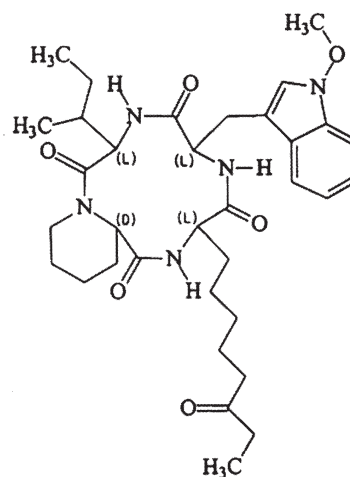


Figure 1. The chemical structure of apicidin.

kit (CHEMICON International, Inc., CA). The cells were stained with 200 μ g/ml MTT and lysed in 5% SDS, and the absorbance was read at 540 nm using a 96-well plate reader. All experiments were performed independently at least three times in triplicate per experimental point.

Cell cycle analysis by flow cytometry. The cells were cultured for 48 h with and without apicidin, trypsinized, washed in PBS and fixed with 70% ethanol/PBS. They were then treated with 0.25 μ g/ml RNase (MP Biomedicals, Inc., OH, USA), and incubated at 37°C for 30 min. Nuclei were stained with 50 μ g/ml propidium iodide, and the relative DNA content was analyzed using a FACScalibur flow cytometer equipped with CellQuest software (Becton-Dickinson Immunocytometry Systems, San Jose, CA).

Annexin V staining. The Annexin V assays were performed according to the manufacturer's protocol (BioVision, Mountain View, CA). Briefly, the cultured cells were collected, washed with binding buffer, and incubated in 500 μ l of a binding buffer containing 5 μ l of Annexin-V-FITC. The nuclei were counterstained with propidium iodide. The percentage of apoptotic cells was determined using the FACScalibur flow cytometer.

Assessment of the mitochondrial transmembrane potential. The mitochondrial transmembrane potential was assayed using the MitoCapture™ mitochondrial apoptosis detection kit (BioVision). Briefly, cells were collected, washed twice with PBS and incubated with 1 ml of the diluted MitoCapture solution at 37°C for 20 min. MitoCapture intensity was determined using the FACScalibur flow cytometer.

Western blot analysis. Expression of specific proteins was detected by Western blotting. Cells were washed twice in PBS, suspended in lysis buffer (50 mM Tris, pH 8.0, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediamine tetraacetic acid, 50 mM NaF, and 0.1% phenylmethylsulfonyl fluoride), and placed on ice for 30 min. After centrifugation at 15,000 rpm for 15 min at 4°C, the supernatant was collected. Protein

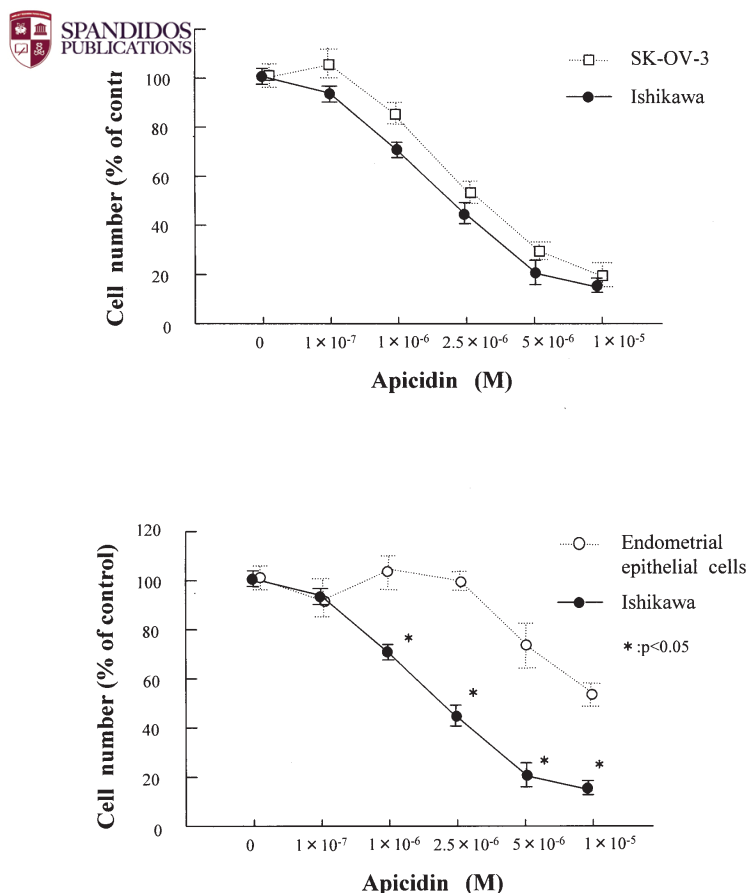


Figure 2. Dose-response effects of apicidin on the survival of human endometrial cancer and ovarian cancer cell lines and normal endometrial epithelial cells in culture. Cells were treated with apicidin (1×10^{-7} – 1×10^{-5}) for 72 h in 96-well plates. The cells were then stained with MTT and lysed, and the absorbency was read in a plate reader using a 540-nm filter. Surviving cells were calculated as percentage control. Results represent the mean \pm SD of three independent experiments with triplicate wells ($p < 0.05$).

concentrations were quantitated using the Coomassie protein assay reagent (Pierce, Rockford, IL). Whole cell protein extract was resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel under reduced conditions. After transfer to Immobilon polyvinylidene transfer membranes (Millipore, Bedford, MA), the protein was stained with Ponceau S (Sigma-Aldrich) to verify uniform loading and transfer. Membranes were blocked with 5% skim milk (Becton-Dickinson) in Tris-buffered saline with Tween-20 (50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20, pH 7.4) (TBS-T) overnight and subsequently incubated with primary antibodies [cyclin A, p16, p27^{KIP1}, E-cadherin (BD Biosciences, San Diego, CA), p21^{WAF1} (Oncogene Research Products, San Diego, CA), glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (Ambion, Austin, TX), and acetyl-histone-H3, acetyl-histone-H4 (Upstate, Lake Placid, NY)] with the appropriate dilutions overnight. The membranes were washed three times with TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the membranes were washed three times with TBS-T and analyzed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Chicago, IL).

Statistical analysis. Data are presented as the means \pm SD and were appropriately analyzed by the Mann-Whitney U-test and Bonferroni/Dunn test with StatView 4.5 (Abacus Concepts, Berkeley, CA, USA). $p < 0.05$ was accepted as statistically significant.

Results

Effect of apicidin on the proliferation of endometrial and ovarian cancer cells and normal endometrial epithelial cells. As shown in Fig. 2, the growth of Ishikawa and SK-OV-3 cells was inhibited by apicidin in a dose-dependent manner. The effective dose of the apicidin that inhibited 50% clonal growth (ED_{50}) of the endometrial and ovarian cancer cell lines ranged between 1.0×10^{-6} and 2.5×10^{-6} M.

The growth of the endometrial cancer cells tested was more sensitive to apicidin than the normal endometrial epithelial cells (Fig. 2). The normal endometrial epithelial cells showed only 70% of control-growth inhibition at 5.0×10^{-6} M apicidin, whereas Ishikawa endometrial cancer cells showed 20% of control-growth inhibition at the same dose.

Cell cycle analysis of endometrial and ovarian cancer cells after exposure to apicidin. The effects of apicidin on the cell cycle of the endometrial and ovarian cancer cells were determined. Ishikawa cells cultured for 48 h in the presence of apicidin showed an accumulation of endometrial cancer cells in both G0/G1 and G2/M phases of the cell cycle at a low concentration of apicidin (5×10^{-7} M), and G2/M phase at a high concentration (1×10^{-6} M) with a concomitant decrease in the proportion of those in S phase (Fig. 3). A total of 58.8% of the untreated Ishikawa cells were in G0/G1 compared with 65.2% of cells cultured with apicidin 5×10^{-7} M, and 13.6% of the Ishikawa untreated cells were in G2/M compared with 21.2% of cells cultured with apicidin 5×10^{-7} M, and 33.7% with 1×10^{-6} M.

SK-OV-3 cells showed an accumulation in the G0/G1 phase at both concentrations (5×10^{-7} and 1×10^{-6} M) with a decrease in the proportion of those in S phase. A total 57.6% of the untreated SK-OV-3 cells were in G0/G1 compared with 63.2% of cells cultured with apicidin 1×10^{-6} M, and 67.4% with 2.5×10^{-6} M (data not shown).

Effect of apicidin on the induction of apoptosis. The strong antiproliferative effect of apicidin on Ishikawa and SK-OV-3 cells observed *in vitro* may be caused in part by the induction of apoptosis. To test this, we used two different methods.

The proportion of apoptotic cells was determined by the Annexin V/FITC kit. As shown in Fig. 4, after 48 h of treatment with 1×10^{-6} M apicidin, ~35.5% of Ishikawa cells and 22.6% of SK-OV-3 cells displayed high Annexin V/FITC and low PI staining, indicating apoptosis. Secondary necrotic cells, which had high Annexin V/FITC and PI staining, included 38.3% of Ishikawa cells and 15.8% of SK-OV-3 cells.

After treatment with apicidin 1×10^{-6} M for 48 h, the number of Ishikawa cells displaying low MitoCapture staining increased to 64.6%, and SK-OV-3 cells to 55.0%, indicating loss of the mitochondrial transmembrane potential that occurs following induction of apoptosis (Fig. 5).

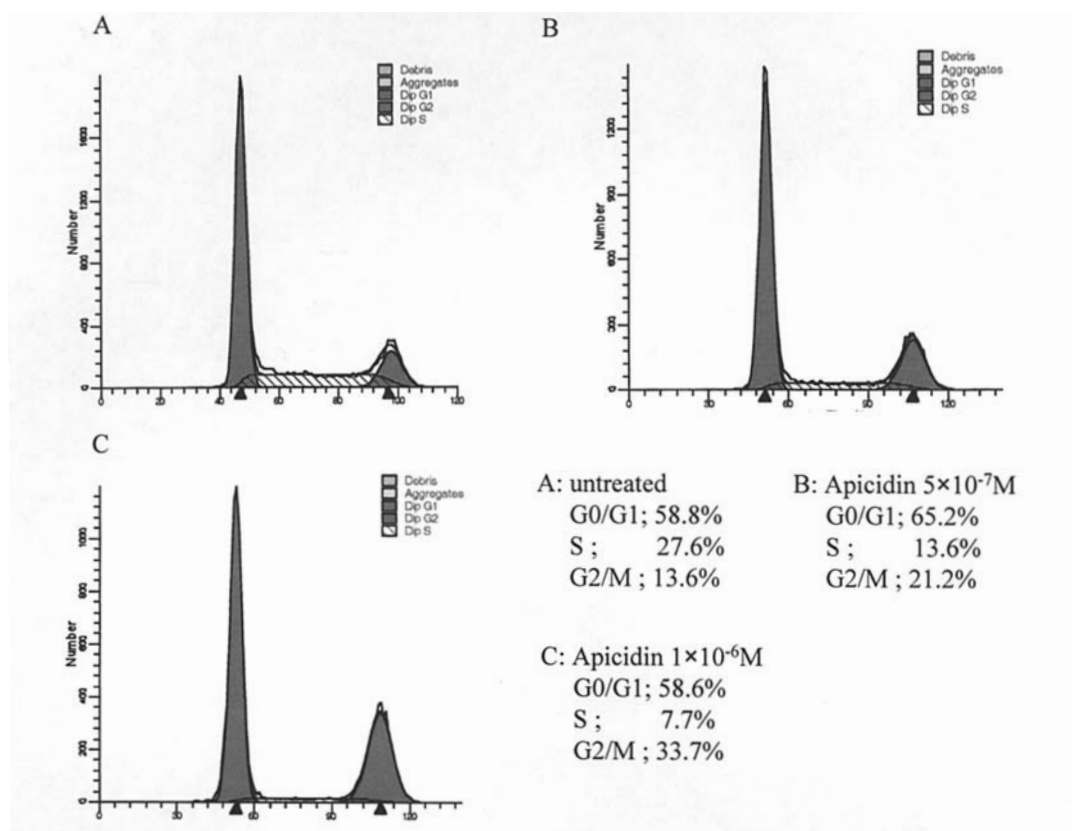


Figure 3. Cell cycle analysis of Ishikawa cells by flow cytometry. Ishikawa cells were cultured with apicidin (5×10^{-7} – 1×10^{-6}) for 48 h, harvested and stained with propidium iodide (PI). Control cells were treated with vehicle alone. Cell cycle analysis was performed by flow cytometry (see Materials and methods). The results of a representative study are shown; two additional experiments yielded similar results.

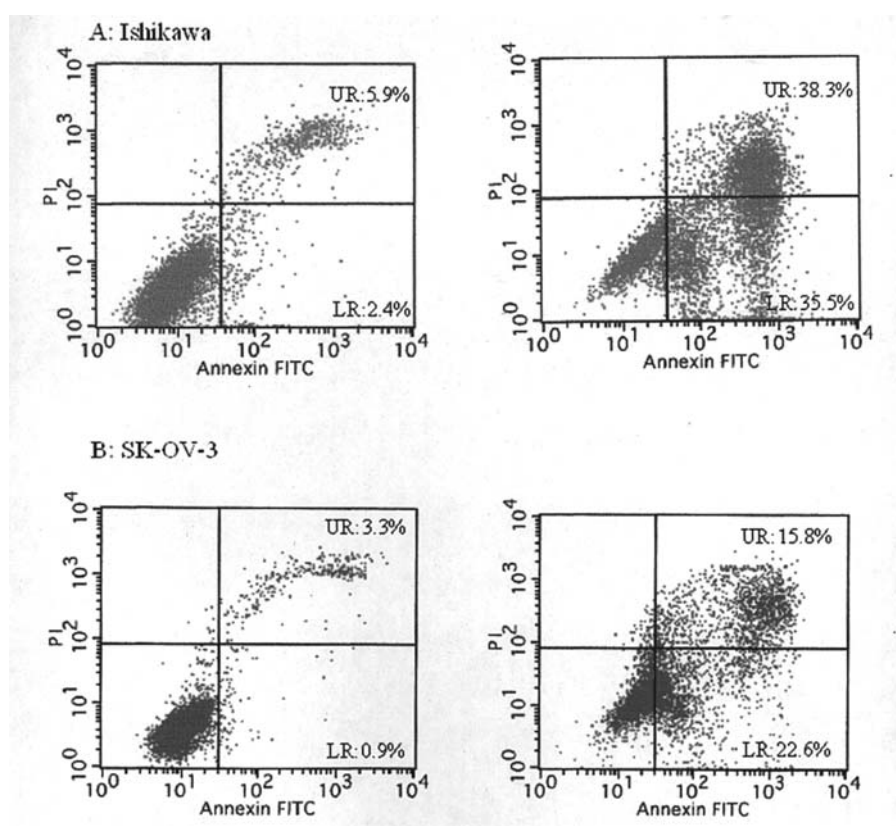


Figure 4. Induction of apoptosis by apicidin in Ishikawa and SKOV-3 cells detected by Annexin V-FITC and PI staining. Cells were cultured with 1×10^{-6} M apicidin for 48 h, stained with Annexin V-FITC and PI, and analysed by flow cytometry. Lower right quadrants, percentage of early apoptotic cells; and upper right quadrants, secondary necrotic cells. Three duplicate experiments were performed with similar results.

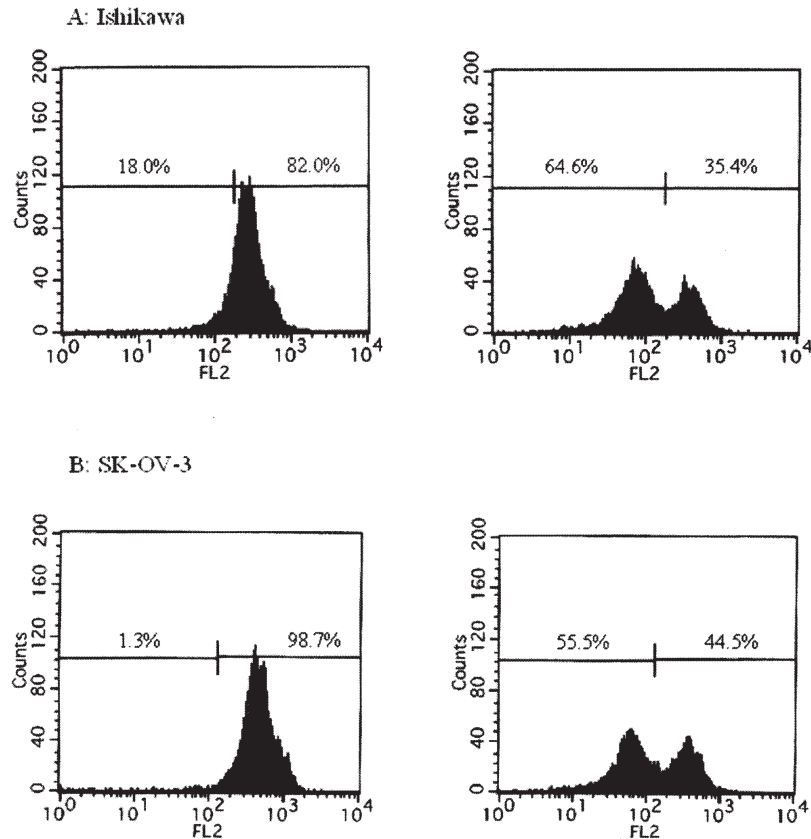


Figure 5. Effect of apicidin treatment on the mitochondrial transmembrane potential. Cells were cultured with 1×10^{-6} M apicidin for 48 h, and the MitoCapture intensity was determined by flow cytometry. FL2 indicates MitoCapture intensity, representing mitochondrial transmembrane potential. Data represent three duplicate experiments with similar results.

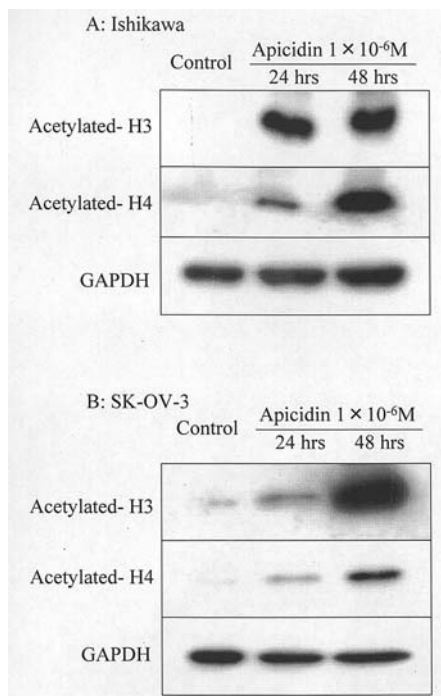


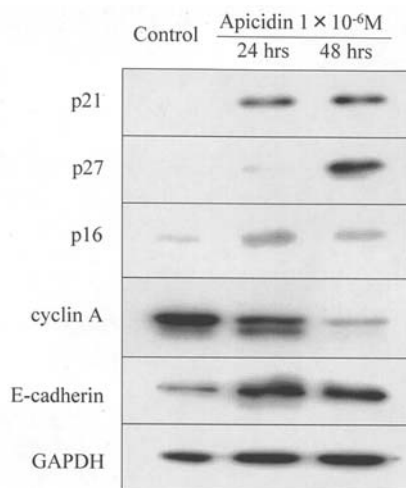
Figure 6. Protein expression of acetylated histone H3 and H4 as measured by Western blot analysis. Ishikawa and SKOV-3 cells were treated with 1×10^{-6} M apicidin and cell lysates were harvested after 24 and 48 h. Western blot analysis was performed with a series of antibodies (acetylated histones H3 and H4). Control cells were treated with vehicle alone. The amount of protein was normalized by comparison to levels of GAPDH.

Effect of apicidin on acetylation of histones. The level of histone acetylation *in vivo* is mainly maintained by the balance between histone acetylase and HDAC. Various HDACs induce acetylation of histones. To examine the relationship between cell death and histone acetylation of Ishikawa and SK-OV-3 cells, we next analyzed the effect of apicidin on the intracellular level of histone H3 and H4. Treatment of cells with apicidin for 24 and 48 h dramatically induced the levels of acetylated H3 and H4 in a time-dependent manner (Fig. 6).

Effect of apicidin on the expression of cell cycle- and apoptosis-related proteins. p21^{WAF1}, p27^{KIP1} and p16 are cyclin-dependent kinase inhibitors (CDKIs) that bind to cyclin-dependent kinase complexes and decrease kinase activity, and may act as key regulators of G0/G1 accumulation. We examined the effect of apicidin on the expression of p21^{WAF1}, p27^{KIP1} and p16 by Western blot analysis (Fig. 7). Apicidin markedly up-regulated the level of p21^{WAF1} and p27^{KIP1} protein, which was expressed at negligible levels in the untreated endometrial and ovarian cancer cell lines. Expression of p16 protein was observed in untreated cancer cells and was slightly up-regulated by apicidin. Conversely, apicidin decreased the level of cyclin A. Cyclin B and cyclin D3 levels were unchanged (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. Apicidin increased

A



B

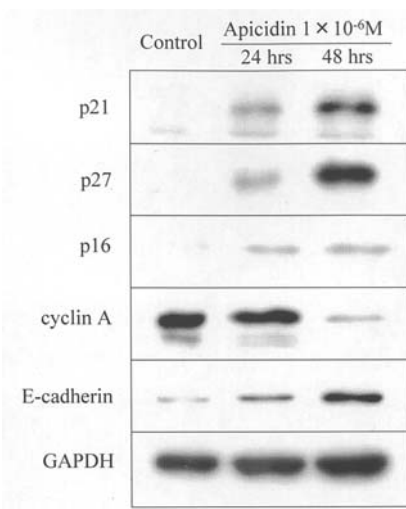


Figure 7. Cell cycle- and apoptosis-related protein expression in Ishikawa cells and SK-OV-3 cells, as measured by Western blot analysis. Ishikawa cells (A) and SK-OV-3 (B) cells were treated with 1×10^{-5} M apicidin, and cell lysates were harvested after 24 and 48 h. Western blot analysis was performed with a series of antibodies: p21^{WAF1}, p27^{KIP1}, p16, cyclin A, and E-cadherin. Control cells were treated with vehicle alone. The amount of protein was normalized by comparison to levels of GAPDH.

the expression level of E-cadherin in Ishikawa and SK-OV-3 cells.

Discussion

Histone acetylation is regulated by a balance between histone acetyltransferase (HATs) and histone deacetylase (HDAC). To date, four families of HAT (P/CAF, p300/CBP, TAF250, and SRC-1) have been identified (9,10). Although all HATs are able to modify histones in free solution, non-histone targets such as the general transcription factor Rb, transcription factor p53, and erythroid Kuppel-like factor, are also substrates for HATs (11,12), suggesting that HATs may regulate transcription by modifying a variety of promoter-bound proteins. 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 (13-16).

Various HDAC inhibitors (HDACIs) such as trichostatin A (17), sodium butyrate (18), trapoxin (8), depudecin, FR901228, oxamflatin, and MS-27-275 inhibit cancer cell growth *in vitro* and *in vivo*. They accumulate acetylated histone in the nucleus, and have been demonstrated to arrest cell growth and reverse neoplastic characteristics in cultured cells. Recent evidence suggests that the induction of histone hyperacetylation by HDACIs is responsible for antitumor effects through selective induction of genes, which play an important role in the cell cycle, cell morphology and apoptosis.

Apicidin, a fungal metabolite, has been shown to exhibit antiparasitic activity against apicomplexan parasites by inhibiting their HDACs (18). Additional studies further demonstrated that apicidin exhibits an anti-proliferative effect in various cancer cell lines, such as cervical cancer, breast cancer, leukemia and prostate cancer (8,19,20). Studies by Takai *et al* indicated that a wide array of HDACIs (SAHA, VPA, TSA and NaB) had a significant growth-suppressing effect on six endometrial (21) and nine ovarian cancer cell lines (22). This stimulated us to examine the effect of apicidin, a novel fungal metabolite on endometrial and ovarian cancer cell lines.

We have demonstrated that apicidin is highly effective in suppressing the growth of human endometrial cancer cell line Ishikawa, and ovarian cancer cell line SKOV-3. The prominent arrest of Ishikawa and SKOV-3 in the G0/G1 phase of the cell cycle is likely to account for this effect. Because acetylation and deacetylation of histone have been shown to play an important role in the regulation of gene expression in eukaryotic cells (23-28), cell cycle arrest at the G1 phase by apicidin might be attributed to alteration of the expression of the genes important to G1-S progression. The expression of p21^{WAF1} and p27^{KIP1}, which are cyclin-dependent kinase inhibitors, has an important role in blocking the cell cycle in the G1 phase (29). Protein levels of both p21^{WAF1} and p27^{KIP1} were found to be increased following treatment of endometrial and ovarian cancer cells with apicidin, supporting their contribution as a possible mechanism by which apicidin inhibits endometrial and ovarian cancer growth. Similar results were obtained with other HDACIs (21,22).

In addition, p16 controls cell cycle proliferation during G1 by inhibiting the ability of cyclin D/CDK4 and cyclin D/CDK6 complexes to phosphorylate retinoblastoma protein (pRb). It can be inactivated via multiple mechanisms including homozygous deletion, point mutation and promoter hypermethylation in various human tumors (30-33). Wong *et al* (34) reported that methylation of the p16 promoter was detected in 20% of endometrial cancers, and this alternation is associated with advanced stages and poor prognosis. Apicidin slightly up-regulated the expression of p16.

The E-cadherin gene is involved in cell-cell adhesion, and the loss of E-cadherin protein function has been associated with enhanced metastatic growth of tumor cells (35). Inactivation of this gene by hypermethylation has been observed in breast carcinoma cells (36) and in primary breast tumors (37). Further, hypermethylation of this gene is associated with tumor differentiation and myometrial invasion



etrial cancer (38). We found that transcription of *p21^{WAF1}* was markedly up-regulated in Ishikawa and SK-OV-3 cells treated with apicidin, suggesting a gain of tumor suppressor function in response to the inhibition of HDAC.

Both hypermethylation and histone deacetylation result in transcriptional silencing. Evidence suggests that these processes are often not independent of each other and result in layers of epigenetic silencing. Theoretically, the reversal of both processes should lead to a greater gene transcription than the reversal of one epigenetic layer alone. Using a microarray-based technique that evaluates gene expression in combination with epigenetic change, histone deacetylase inhibitor alone failed to reactivate the expression of genes that CpG island hypermethylated (39). Although both the p16 and E-cadherin genes are transcriptionally silenced by hypermethylation, apicidin up-regulated both genes in different degrees. In this study, the methylation statuses of these genes were not analyzed, so further analysis may be needed to provide information about this regulation mechanism by apicidin.

Cyclins are defined to be key proteins in the control of cell proliferation. Cyclin A acts from the late G1 phase through the M phase of the cell cycle, and forms a complex with cdk2 in the late G1-S phase and with cdc2 in the G2/M phase (40). Cyclin A expression is involved in the progression to malignancy of the endometrium and is correlated with proliferative activity and prognostic features (41). Apicidin decreased the expression of cyclin A, and thus modulated the activity of the downstream pRb/E2F axis, thereby triggering cell cycle arrest, especially in the G2/M phase at a higher concentration.

MTT assay showed that the growth of Ishikawa was more sensitive to apicidin than the normal endometrial epithelial cells. Total inhibition of HDAC was speculated to be toxic for human use, despite the non-potent effect of this kind of drug when using low concentration or higher sensitivity of cancer cells compared with normal healthy cells. It has not been clearly identified which gene is targeted by HDACIs, however, they seems to alter the expression of a very limited number of genes, contrary to HDACs in chromatin. Concerning the apoptosis and sensitivity of cancer cells, apicidin may control the expression of genes that regulate oncogenesis. This result suggests that the anticancer activity of apicidin may occur with minimal side-effects, raising hopes that apicidin may become a useful adjuvant therapy for gynecological cancers.

In summary, apicidin exhibits antiproliferative activity and potentially induces apoptosis in human endometrial and ovarian cancer cells. These events are accompanied by the induction of p21^{WAF1} and p27^{KIP1} and the down-regulation of several antiapoptotic- and cell cycle-related proteins, such as cyclin A. Furthermore, apicidin significantly inhibited the cell growth of endometrial cancers cell compared with that of normal endometrial epithelial cells. These findings suggest that apicidin may be particularly effective in the treatment of endometrial and ovarian cancer. Although many HDACIs are currently under clinical trial, there are several questions which remained to be addressed, since the understanding of HDAC-class enzyme specificity and the knowledge of drug and tumor specificity are still limited. Optimal correlation assays and optimal gene targets remain to be defined.

Acknowledgements

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