Biochemical and Biophysical Research Communications 434 (2013) 634–640

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Apicularen A acetate induces cell death via AIF translocation and disrupts the microtubule network by down-regulating tubulin in HM7 human colon cancer cells

Kang-Sik Seo [a,1], Hoon Kim [d,1], Tae-Hwa Hong [a,1], Jong-Seok Kim [a], Kyoung-Sub Song [a], Eun-Jin Yun [a], Ji-Hoon Park [a], Young-Hoon Jung [e], Jong-Il Park [a], Gi Ryang Kweon [a,c], Wan-Hee Yoon [a], Kyu Lim [a,b,c,*], Byung-Doo Hwang [a,*]

[a] Department of Biochemistry, College of Medicine, Chungnam National University, Daejeon, Republic of Korea
[b] Cancer Research Institute, Chungnam National University, Daejeon, Republic of Korea
[c] Infection Signaling Network Research Center, Chungnam National University, Daejeon, Republic of Korea
[d] Dental Surgery, Eulji University Hospital, Daejeon, Republic of Korea
[e] School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea

ARTICLE INFO

Article history:
Received 20 March 2013
Available online 10 April 2013

Keywords:
Apicularen A acetate
Caspase-independent apoptosis
Apoptosis-inducing factor
Microtubule disruption

ABSTRACT

Apicularen A is a novel antitumor agent and strongly induces death in tumor cells. In this study, we synthesized apicularen A acetate, an acetyl derivative of apicularen A, and investigated its antitumor effect and mechanism in HM7 colon cancer cells. Apicularen A acetate induced apoptotic cell death and caspase-3 activation; however, the pan-caspase inhibitor Z-VAD-fmk could not prevent this cell death. Apicularen A acetate induced the loss of mitochondrial membrane potential and the translocation of apoptosis-inducing factor (AIF) from mitochondria. In addition, apicularen A acetate significantly decreased tubulin mRNA and protein levels and induced disruption of microtubule networks. Taken together, these results indicate that the mechanism of apicularen A acetate involves caspase-independent apoptotic cell death and disruption of microtubule architecture.

1. Introduction

Numerous antitumor agents exist, but their uses are restricted due to their side effects and insufficient clinical efficacy. Therefore, safer and more effective derivatives of antitumor agents are needed, and efforts to develop new agents are continuously underway. Apicularen A, a macrolide from the marine myxobacterial genus *Chondromyces*, causes growth inhibition and apoptosis in several types of cell lines [1–4]. In addition, in our previous study, we observed that apicularen A strongly induced cell death in HM7 colon cancer cells by up-regulating Fas ligand expression and disrupting microtubule architecture [5]. These results led us to investigate whether a synthetic derivative of apicularen A might have a more potent antitumor activity or induce cell death by a distinct mechanism.

Programmed cell death, especially apoptosis, is required for maintenance of cellular homeostasis [6]. In cancer cells, the ability and propensity to undergo apoptosis is critical in determining drug sensitivity or resistance [7]. Caspases, a family of cysteine proteases, play a major role in apoptosis by promoting intrinsic or extrinsic cell death pathways [8]. However, caspase-independent inducers such as Endonuclease G (Endo G), Smac/Diablo, HtrA2/Omi, and apoptosis-inducing factor (AIF) are also involved in apoptotic cell death via release of these factors from the mitochondria [9]. One of these proteins, AIF, is translocated to the nucleus after being released from the mitochondria, and induces cell death by promoting DNA degradation and chromatin condensation [9].

Microtubules are highly dynamic cytoskeletal components of eukaryotic cells [10]. They are essential for cellular motility, movement of vesicles, organization of intracellular organelles, and orderly segregation of chromosomes during mitosis [11]. Because cancer cells are rapidly growing and dividing, microtubules are considered to be important targets for the development of cancer chemotherapeutic agents [12]. Indeed, a large number of drugs induce death of cancer cells by interfering with microtubule assembly. For example, colchicines and vinblastine inhibit the assembly of microtubules by binding to soluble α- and β-tubulin heterodimers, whereas paclitaxel and epothilones inhibit microtubule disassembly by binding to polymerized tubulins [11,13]. However, apicularen A is the only known agent that disrupts microtubule networks by down-regulating tubulin synthesis.

* Corresponding authors. Address: Department of Biochemistry, College of Medicine, Chungnam National University, Daejeon, Republic of Korea. Fax: +82 42 580 8121 (K. Lim), fax: +82 42 580 8121 (B.D. Hwang).
E-mail addresses: kyulim@cnu.ac.kr (K. Lim), bdhwang@cnu.ac.kr (B.-D. Hwang).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.03.133

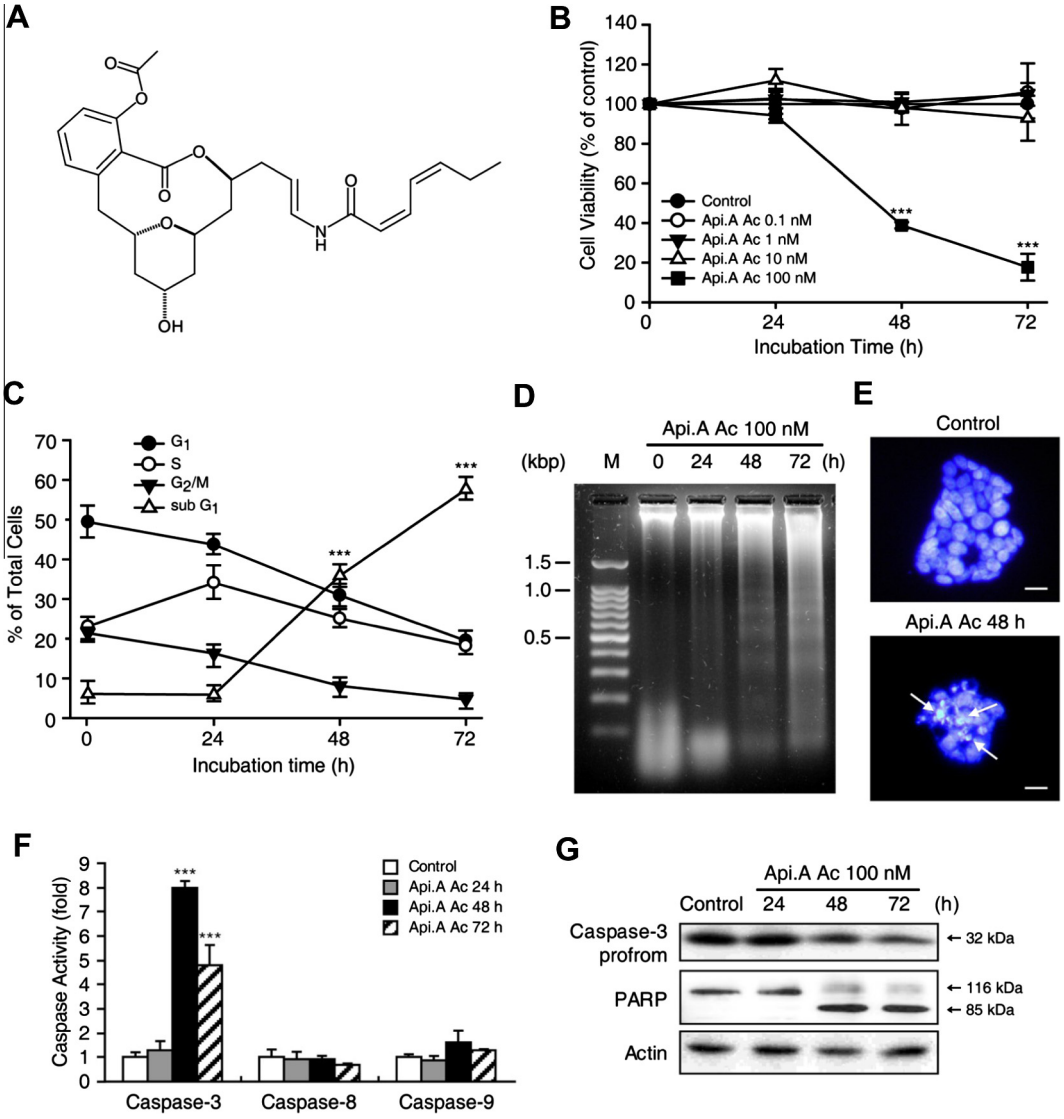

Fig. 1. Effect of apicularen A acetate on cell viability and apoptotic cell death in HM7 cells. (A) Chemical structure of apicularen A acetate (11-(3-hepta-2,4-dienoylaminoallyl)-15-hydroxy-9-oxo-10,17-dioxatricyclo[11.3.1.0^{3,8}] heptadeca-3,5,7-triene-7-yl acetate). (B) HM7 cells were treated with various concentrations of apicularen A acetate for the indicated times. Cell viability was determined using the MTT assay. (C) DNA contents in apicularen A acetate-treated HM7 cells. HM7 cells were incubated for the indicated times in the presence or absence of 100 nM apicularen A acetate. DNA contents were analyzed by flow cytometry. (D) Apicularen A acetate induced DNA laddering, i.e., fragmentation. HM7 cells were incubated for the indicated times in the presence or absence of 100 nM apicularen A acetate. Total genomic DNA was extracted and resolved by electrophoresis on a 1.2% agarose gel. DNA laddering was visualized by ultraviolet transillumination. (E) Cells were treated with or without 100 nM apicularen A acetate for 48 h, and nuclei were stained with Hoechst 33342 and visualized using fluorescence microscopy (scale bar = 20 μm). (F) Activities of caspases were determined using fluorogenic peptide substrates of each caspase. (G) HM7 cells were incubated for the indicated times in the presence or absence of 100 nM apicularen A acetate. Lysates of cells were subjected to Western blotting analysis with antibodies against caspase-3 and PARP. All error bars indicate ± SEM. $^{***}P < 0.001$.

In this study, we synthesized an acetyl derivative of the phenolic hydroxyl group of apicularen A, apicularen A acetate (Fig. 1A), and investigated its cytotoxic and apoptotic activity in HM7 cells. Our findings demonstrate that apicularen A acetate induces apoptotic cell death in these cells by releasing AIF from mitochondria and disrupting microtubule networks.

2. Materials and methods

2.1. Reagents

Apicularen A acetate (11-(3-Hepta-2,4-dienoylaminoallyl)-15-hydroxy-9-oxo-10,17-dioxatricyclo [11.3.1.0^{3,8}] heptadeca-3,5,7-triene-7-yl acetate) was provided by Dr. Jung (Lab. of Organomedicinal Chemistry, College of Pharmacy, Sungkyunkwan University). Enhanced chemiluminescence kits (ECL system) were purchased from Amersham Biosciences. Anti-caspase-3 antibody was purchased from R & D Systems. Anti-PARP, anti-actin, anti-COX IV, anti-Cu/Zn SOD and anti-AIF antibodies were purchased from Santa Cruz Biotechnology. Z-VAD-fmk was purchased from Calbiochem. Anti-α-tubulin antibody and other reagents were purchased from Sigma.

2.2. Cell culture

The human colon carcinoma cell line HM7 was a gift from Young S. Kim (Gastrointestinal Research Laboratory, University of California, San Francisco, CA). Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics. Cells were maintained in an incubator at 37 °C, 5% CO_2, and 95% air.

2.3. Measurement of cell viability

Exponentially growing cells were treated with a series of concentrations of apicularen A acetate for 24, 48, and 72 h. Thiazolyl blue tetrazolium blue (MTT) solution was added to each well (0.5 mg/ml) and incubated for 2 h. Cell survival was assessed by measuring the absorbance at 570 nm in an ELISA plate reader.

2.4. Flow cytometric analysis for cell cycle distribution

After treatment with apicularen A acetate, HM7 cells were washed and fixed in 70% ethanol. Before analysis, cells were centrifuged and incubated with propidium iodide (50 μg/ml) supplemented with RNase A (1 mg/ml) for 30 min at room temperature. Quantification of the relative DNA contents was measured by flow cytometry analysis using a Becton–Dickinson FACSort and manual gating using the CellQuest software.

2.5. DNA fragmentation assay

DNA fragmentation assay of cells treated with apicularen A acetate was examined as previously described [5].

2.6. Hoechst 33342 staining

For analysis of condensed apoptotic nuclei, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS, incubated with Hoechst 33342 (5 μg/ml) for 20 min at room temperature in the dark, and washed with PBS. The cells were then analyzed by fluorescence microscopy.

2.7. Caspase activity assays

The extracts were prepared by suspending 2×10^6 HM7 cells in 100 μl of TTE buffer [10 mM Tris–HCl (pH 8.0), 0.5% Triton X-100, and 10 mM EDTA] on ice for 30 min, followed by centrifugation at 15,000*g* for 10 min at 4 °C. For each sample, 10 μl of lysate containing 30 μg of total protein was mixed with 90 μl of assay buffer [20 mM HEPES (pH 7.5), 10% glycerol, and 2 mM DTT], containing 40 μM of Ac-DEVD-AFC, Ac-IETD-AFC, and Ac-LEHD-AFC (where DEVD is Asp-Glu-Val-Asp, IETD is Ile-Glu-Thr-Asp, LEHD is Leu-Glu-His-Asp, and AFC is 7-amino-4-trifluoromethyl coumarin). Activities of caspase-3, -8, and -9 were measured at 37 °C using a spectrofluorometric plate reader (Perkin–Elmer LS-50B) in kinetic mode, using excitation and emission wavelengths of 400 nm and 505 nm, respectively.

2.8. Western blotting analysis

Western blotting analysis of caspase-3, PARP, Fas, FasL, AIF, COX IV, Cu/Zn SOD, and α-tubulin was performed as previously described [5].

2.9. Mitochondrial membrane potential assay

Cells were trypsinized and incubated for 20 min in 1 ml of room temperature media containing 5 μM Rhodamine 123. The cells were then washed and analyzed on a FACscan flow cytometer. Reduction in Rhodamine 123 fluorescence indicated loss of mitochondrial membrane potential.

2.10. Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA levels of α-tubulin and GAPDH were examined by RT-PCR analysis as previously described [5].

2.11. Subcellular fractionation

Cells were washed in cold PBS and then resuspended in 1 ml cold homogenizing buffer containing 10 mM HEPES (pH 7.5), 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and protease inhibitor cocktail tablets. A homogenate was prepared using a glass Dounce homogenizer type-B pestle (50 strokes). After centrifugation at 760*g* at 4 °C for 10 min, the supernatant was collected and centrifuged at 12,000*g* at 4 °C for 10 min. The pellet was washed twice, resuspended in homogenizing buffer, and used as the mitochondrial fraction. The supernatant was centrifuged at 100,000*g* at 4 °C for 1 h, and the cleared supernatant was used as the cytosolic fraction.

2.12. Transfection of siRNA

The siRNAs targeting human AIF (siRNA I target sequence: 5′-CUUGUUCCAGCGAUGGCAU-3′, siRNA II target sequence: 5′-GAUCCUCCCCGAAUACCUC-3′) mRNA (20 nM), or scrambled negative control siRNA (20 nM) were transfected into HM7 cells using G-fectin (Genolution, Korea).

2.13. Immunofluorescence assay

The cells were washed twice with PBS, and then permeabilized with 0.25% Triton X-100 and 0.5% glutaraldehyde for 1 min at room temperature. Then cells were fixed with 1% glutaraldehyde for 10 min before overnight incubation with anti-AIF or anti-α-tubulin antibody, diluted 1:500. After washing three times in PBS containing 0.1% Tween-20 (PBS/T), cells were incubated for 1 h with the secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit IgG, diluted 1:400) in the dark. After washing five times, cells were stained with 20 μg/ml propidium iodide and 1 mg/ml RNase A for 20 min at room temperature. Fluorescent signals were visualized, and digital images were obtained, using an Olympus FV-500 fluorescence microscope.

2.14. Statistical analyses

Results are expressed as the mean ± SD or mean ± SEM. The statistical significance of differences between groups was analyzed using Student's *t* test and analysis of variance (ANOVA). Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Apicularen A acetate induces cytotoxicity and apoptotic cell death in HM7 cells

The effect of apicularen A acetate on viability of HM7 cells was determined using an MTT assay. As shown in Fig. 1B, 100 nM apicularen A acetate significantly inhibited the viability of HM7 cells. Although the MTT assay is suitable for the determination of changes in the metabolic activity of cells under toxic stress, it does not necessarily yield specific information on the detailed events associated with cell death [14]. Therefore, flow cytometry was performed to determine the effects of apicularen A acetate on the cell cycle and cell death in HM7 cells. Apicularen A acetate decreased the populations of G_1, S, and G_2/M phase cells, whereas it increased the population of sub-G_1 phase cells in a time-dependent manner (Fig. 1C). To determine whether the increase of the sub-G_1 population in apicularen A acetate-treated cells was associated with apoptotic cell death, DNA fragmentation assays were performed. Consistent with the increase in sub-G_1 cells, apicularen A acetate induced DNA fragmentation at 48 and 72 h (Fig. 1D). In addition,

condensed apoptotic nuclei in apicularen A acetate-treated cells were observed by staining with Hoechst 33342 (Fig. 1E). Taken together, these results demonstrate that apicularen A acetate strongly induced cytotoxicity and apoptotic cell death in HM7 cells.

Caspase-3 acts as a major executor of apoptotic cell death by cleaving poly (ADP-ribose) polymerase (PARP) to block the cellular DNA repair system [15]. To determine whether apicularen A acetate induces caspase-3 activation, we monitored caspase-3 activity in apicularen A acetate-treated HM7 cells using a spectrofluorometric assay. As shown in Fig. 1F, caspase-3 activity increased in cells treated with treating apicularen A acetate, whereas the activities of the apoptosis initiator caspases, caspase-8 and -9, were not changed [16]. Moreover, apicularen A acetate decreased the level of the proform of caspase-3 and increased PARP cleavage in a time-dependent manner (Fig. 1G), indicating that apicularen A acetate treatment activated caspase-3 in HM7 cells.

3.2. Fas–FasL signaling is not required for apicularen A acetate-induced cell death

We previously reported that apicularen A induces apoptotic cell death by up-regulating Fas ligand (FasL), and that the anti-FasL antibody NOK-1 blocks apicularen A-induced apoptosis [5]. To investigate whether the Fas and FasL pathway is involved in apicularen A acetate-induced cell death, we analyzed the protein levels of Fas and FasL. Apicularen A acetate induced increases in FasL protein levels, but did not change the protein levels of Fas (Fig. 2A). Next, we treated cells with NOK-1 antibody to determine whether apicularen A acetate-induced cytotoxicity and cell death could be blocked. Interestingly, NOK-1 antibody failed to prevent cytotoxicity, caspase-3 activation, and PARP cleavage resulting from treatment with apicularen A acetate (Fig. 2B and C), indicating that Fas–FasL signaling is not involved in apicularen A acetate-induced cell death.

3.3. Apicularen A acetate induces apoptotic cell death in a caspase-3-independent manner

To assess the contribution of caspase-3 activation to the lethality of apicularen A acetate, we added the pan-caspase inhibitor Z-VAD-fmk to HM7 cells. As shown in Fig. 2D and E, apicularen A acetate-induced caspase-3 activation and PARP cleavage were completely blocked by pretreatment with Z-VAD-fmk. Next, we examined the effect of Z-VAD-fmk on cell viability after treatment with apicularen A acetate. Strikingly, in contrast to the inhibition of caspase-3 activity, apicularen A acetate-induced cytotoxicity was not blocked by Z-VAD-fmk (Fig. 2F). Furthermore, the apicularen A acetate-induced increase in the population of sub-G_1 cells was not prevented by pretreatment with Z-VAD-fmk (Fig. 2G), suggesting that apicularen A acetate induced apoptosis via a caspase-3-independent pathway.

3.4. Apicularen A acetate promotes nuclear translocation of AIF

AIF is a mitochondrial protein that may mediate caspase-independent cell death by inducing DNA fragmentation following its translocation to the nucleus [17]. We hypothesized that the release of AIF from mitochondria might be related to the caspase-independent apoptosis in apicularen A acetate-treated HM7 cells. Therefore, we first examined the effect of apicularen A acetate on mitochondrial membrane potential ($\Delta\Psi$m). As shown in Fig. 3A, HM7 cells treated with apicularen A acetate exhibited a time-dependent decrease in $\Delta\Psi$m. Next, we investigated whether AIF release from mitochondria is induced by apicularen A acetate. Mitochondrial AIF was released to the cytosol after treatment with apicularen A acetate for 48 h (Fig. 3B). This finding was supported by the results of immunofluorescence assays using an anti-AIF antibody and propidium iodide, which revealed that AIF indeed translocated to the nuclei in apicularen A acetate-treated cells (Fig. 3C). To further examine AIF dependency, we used two siRNAs against AIF, and found that AIF knockdown partially blocked the cytotoxicity induced by apicularen A acetate (Fig. 3D and E). Taken together, these results suggest that AIF is involved in apicularen A acetate-induced apoptotic cell death.

3.5. Apicularen A acetate decreases tubulin protein levels and disrupts the microtubule network

We previously reported that apicularen A decreases the protein levels of α/β-tubulin and disrupts intracellular microtubule networks, and that these effects of apicularen A cause cell death in HM7 colon cancer cells [5]. To examine whether apicularen A acetate also induces down-regulation of tubulin, we determined the protein and mRNA levels of tubulin after apicularen A acetate treatment. The α-tubulin protein levels were decreased by apicularen A acetate exposure in a time-dependent manner (Fig. 4A). In addition, treatment with apicularen A acetate led to an decrease in α-tubulin mRNA levels (Fig. 4B). To investigate the microtubule architecture in more detail, we performed immunofluorescence assays. Apicularen A acetate induced changes in the microtubule networks of the cytosol and reduced the density of tubulin-associated fluorescence (Fig. 4C), indicating that apicularen A acetate induces down-regulation of tubulin synthesis and disrupts microtubule networks in HM7 cells.

4. Discussion

In this study, we showed that apicularen A acetate induces caspase-independent cell death in HM7 colon cancer cells. Apicularen A acetate-induced cell death was not reduced by caspase inhibitor Z-VAD-fmk, but AIF release from mitochondria was observed. Because apicularen A-induced cell death is mediated by caspase-3 and -8 [5], the cell death pattern induced by apicularen A acetate indicates that acetyl substitution of the functional group of apicularen A contributes to its caspase-independent mechanism.

Enhanced Fas–FasL signaling is associated with apoptotic cell death by triggering activation of caspase-3 and -8 signaling cascades [18]. By contrast, Fas–FasL signaling can promote cell proliferation, differentiation, and tumor progression [19]. In this study, the anti-FasL antibody NOK-1 failed to block apicularen A acetate-induced cell death, although apicularen A acetate increased FasL in a time-dependent manner. Because caspase inhibition did not block cell death induced by apicularen A acetate, it is possible that Fas–FasL signaling was attenuated by a decrease in caspase-mediated apoptosis. This is reinforced by the observation that caspase-8 activity was also not changed by treatment with apicularen A acetate. Therefore, apicularen A acetate-induced FasL up-regulation may involve cellular processes other than apoptotic cell death.

Many tumor cells are not susceptible to traditional chemotherapeutic agents because they have mutated or down-regulated key apoptotic genes such as p53, proapoptotic Bcl proteins, and effector caspases [17,20]. In this context, the executor proteins of caspase-independent cell death become attractive molecular targets for cancer therapeutics. AIF is a major effector of caspase-independent cell death. Apoptotic stimuli can trigger loss of $\Delta\Psi$m, as well as AIF translocation from mitochondria to the nucleus. In the nucleus, AIF interacts with DNase endo G to promote DNA fragmentation and induce cell death [17]. Our results showed that apicularen A acetate disrupts $\Delta\Psi$m and induces AIF release from mitochondria. In addition, AIF knockdown blocked the cytotoxicity

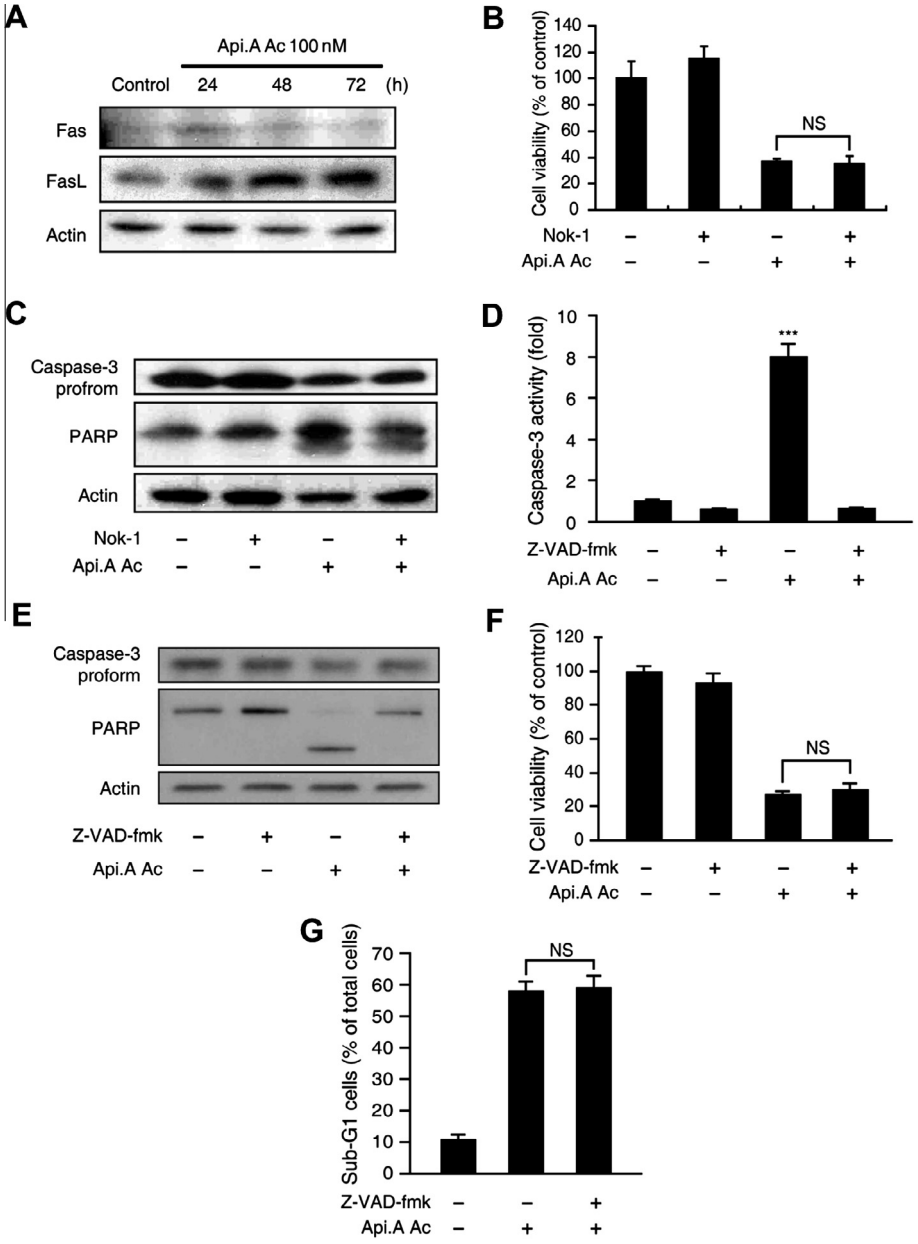

Fig. 2. Effect of caspase inhibitor or anti-FasL antibody on apicularen A acetate-induced apoptosis in HM7 cells. (A) HM7 cells were incubated for the indicated times in the presence or absence of 100 nM apicularen A acetate. The protein levels of Fas and FasL were analyzed by Western blotting. (B) HM7 cells were incubated for 48 h in the presence or absence of both 0.1 μg/ml NOK-1 antibody and 100 nM apicularen A acetate. Cell viability was determined using MTT assays. (C) HM7 cells were incubated with 100 nM apicularen A acetate for 48 h in the presence or absence of 0.1 μg/ml NOK-1 antibodies. The protein levels of caspase-3 and PARP were analyzed by Western blotting. (D–G) HM7 cells were pretreated with 20 μM of Z-VAD-fmk for 1 h, and then treated with 100 nM apicularen A acetate for 48 h. (D) Cells were lysed, and caspase-3 activity was determined using fluorogenic peptide substrates of caspase-3. (E) Cells were lysed, and the protein levels of caspase-3 and PARP were analyzed by Western blotting with anti-caspase-3 and anti-PARP antibodies, respectively. (F) The viability of apicularen A acetate-treated cells was determined using an MTT assay. (G) Sub-G_1 population of apicularen A acetate-treated cells were analyzed by flow cytometry. All error bars indicate ± SEM. $^{***}P < 0.001$.

induced by apicularen A acetate. These are distinct from the mechanism of apicularen A-induced cell death, which does not change mitochondrial membrane potential or the protein levels of mitochondrial apoptosis effectors such as Bcl-2, Bcl-xL, and cytochrome C [5]. Although it is possible that cytochrome C is released as a result of apicularen A acetate-induced disruption of $\Delta\Psi m$, this could not be related to cell death because the activity of caspase-9 is unchanged. Taken together, our data demonstrate that the acetyl modification of the phenolic hydroxyl group in apicularen A changes the mechanism of cell death to caspase-independent apoptosis via AIF translocation. Therefore, this derivative may be useful in overcoming drug resistance in cancer cells that are resistant to caspase-dependent cell death.

A wide variety of drugs block the dynamic activity of microtubules, thereby preventing mitosis, and such drugs are continuously being developed for use in cancer treatment [12]. Here, we showed that apicularen A acetate decreased tubulin protein levels and disrupted microtubule networks, consistent with similar findings for apicularen A. Because microtubules form a cytoskeletal scaffold for organization of organelles [21], apicularen A acetate-induced

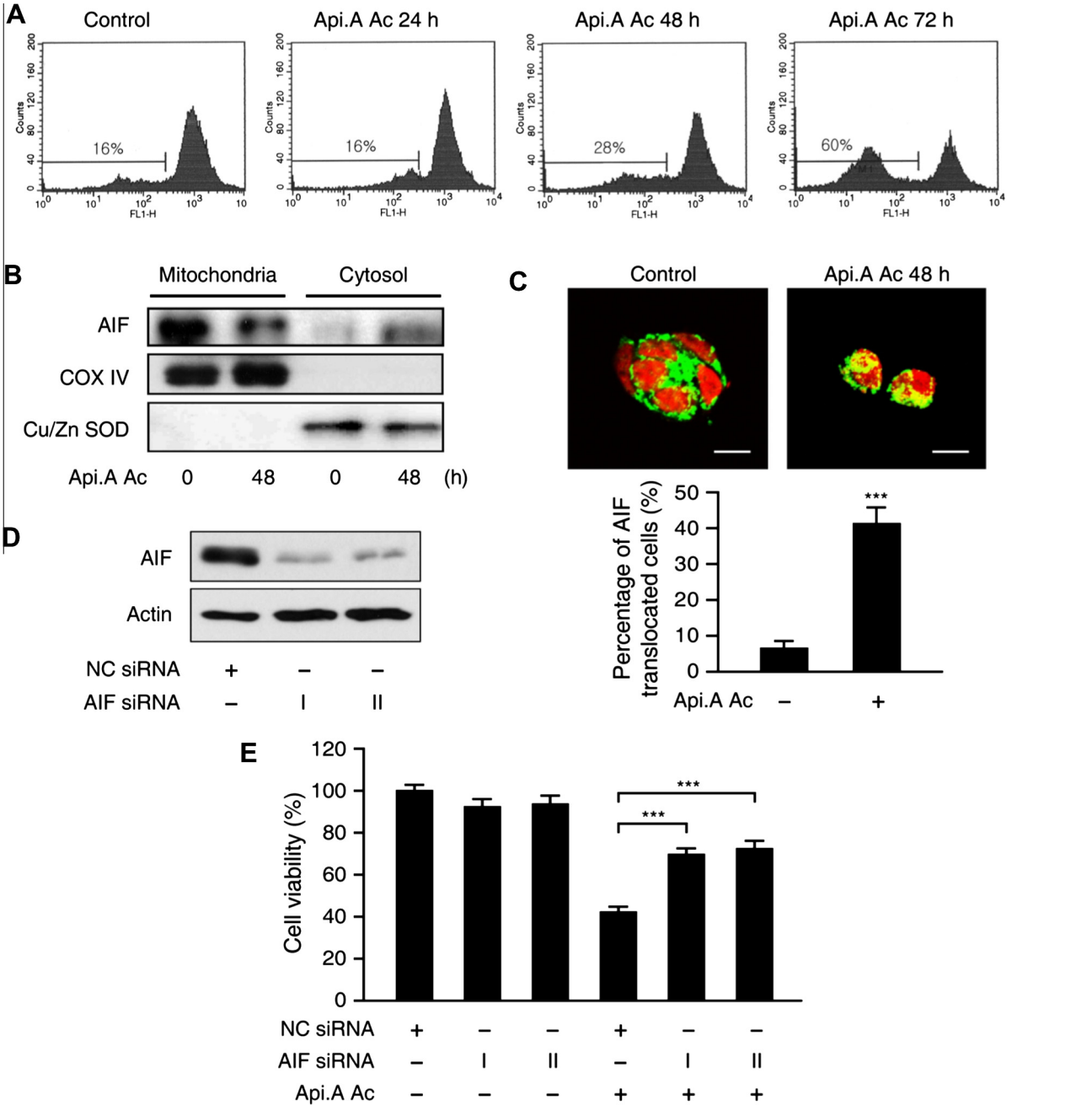

Fig. 3. Disruption of mitochondrial membrane potential and release of AIF by apicularen A acetate. (A) HM7 cells were treated with 100 nM apicularen A acetate for the indicated times, and then cells were incubated with 10 μM Rhodamine 123 for 20 min and immediately scored by flow cytometry. (B) HM7 cells were treated with apicularen A acetate for 48 h, and then subjected to subcellular fractionation. Translocation of AIF from mitochondria to cytosol was analyzed by Western blotting using an anti-AIF antibody. Cytochrome c oxidase subunit IV (COX IV) and Cu/Zn superoxide dismutase (SOD) were used as controls of purify of the mitochondrial and cytosolic fractions, respectively. (C) (Top) Subcellular distribution of AIF was determined by immunofluorescence assay using an anti-AIF antibody (green). Nuclei were counterstained with propidium iodide (red). Scale bar = 20 μm. (Bottom) The percentage of AIF translocated cells relative to total cells under each of the indicated conditions are shown in the histograms. (D) HM7 cells were transfected with negative control (NC) siRNA or AIF siRNA sets (I and II). The protein levels of AIF were determined by Western blotting. (E) HM7 cells were transfected with NC siRNA or AIF siRNAs, followed by treatment with 100 nM apicularen A acetate. Cell viability was determined using MTT assays. All error bars indicate ± SEM. $^{***}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

disorganization of microtubule networks may contribute to irregular localization and shape of nuclei. In addition, intracellular tubulin levels are closely associated with cell fate. For example, a decrease in tubulin protein level of 60–70% inhibits cell survival [11], and reduction in tubulin polymerization by ethanol treatment results in cell death [22]. In this study, apicularen A acetate strongly decreased protein levels of tubulin relative to those in non-treated cells. Therefore, the apicularen A acetate-induced decrease in tubulin protein may partly contribute to apoptotic cell death and cytotoxicity in HM7 cells.

In conclusion, we have shown that apicularen A acetate induces cell death through a caspase-independent pathway, causing AIF translocation from mitochondria and disrupting microtubule networks via down-regulation of tubulin synthesis. Thus, apicularen A acetate would appear to have potential as a novel anticancer drug. Moreover, our results suggest that substitution of functional

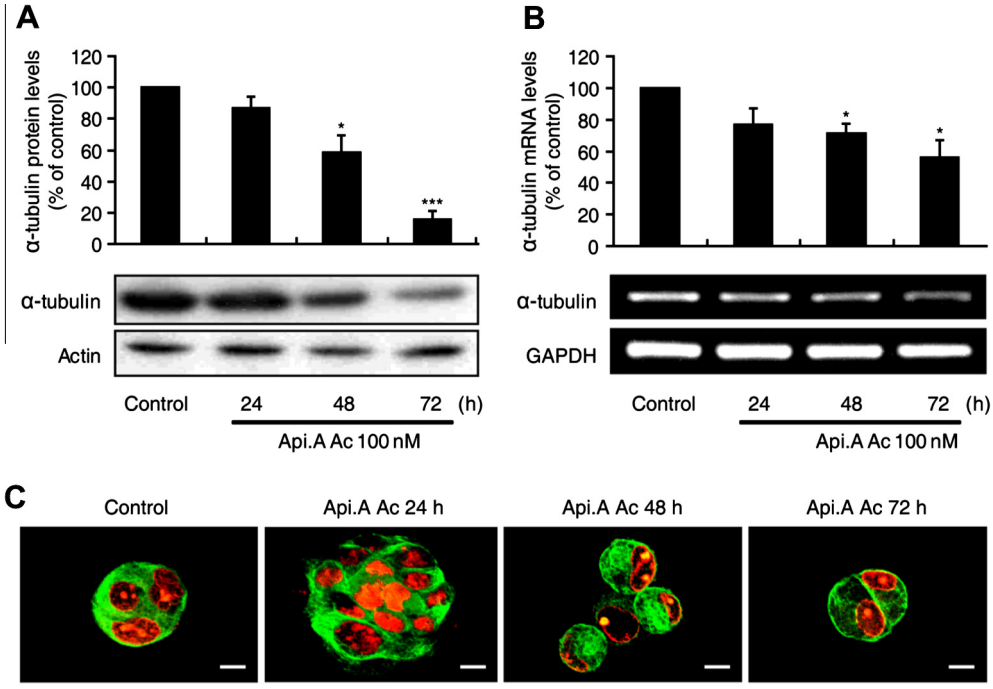

Fig. 4. Effect of apicularen A acetate on α-tubulin in HM7 cells. (A) HM7 cells were treated with 100 nM apicularen A acetate for the indicated times. The protein levels of α-tubulin were analyzed by Western blotting. The normalized protein levels of α-tubulin are indicated as a ratio against actin, used as a loading control (upper panel). (B) HM7 cells were treated with 100 nM apicularen A acetate for the indicated times. The α-tubulin mRNA levels were measured by RT-PCR. PCR products were resolved on 1.2% agarose gel containing ethidium bromide. The normalized mRNA levels of α-tubulin are indicated as a ratio against GAPDH, used as a loading control (upper panel). (C) HM7 cells were treated with 100 nM apicularen A acetate for the indicated times. The intracellular microtubule networks were determined by immunofluorescence assay using an anti-α-tubulin antibody (green). Nuclei were counterstained with propidium iodide (red). Scale bar = 10 μm. All error bars indicate ± SD. $^{*}P < 0.05$; $^{***}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

groups in anticancer drugs might be a good therapeutic strategy for the development of treatments for cancer cells that are insusceptible to traditional anticancer drugs.

Acknowledgments

This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (2007-0054932).

References

[1] B. Kunze, R. Jansen, F. Sasse, G. Hofle, H. Reichenbach, Apicularens A and B, new cytostatic macrolides from Chondromyces species (myxobacteria): production, physico-chemical and biological properties, J. Antibiot. (Tokyo) 51 (1998) 1075–1080.
[2] J. Hong, K. Ishihara, O. Zee, K. Ohuchi, Induction of apoptosis by apicularen A in human promyelocytic leukemia cell line HL-60, Planta Med. 71 (2005) 306–312.
[3] J. Hong, K. Yamaki, K. Ishihara, J.W. Ahn, O. Zee, K. Ohuchi, Induction of apoptosis of RAW 264.7 cells by the cytostatic macrolide apicularen A, J. Pharm. Pharmacol. 55 (2003) 1299–1306.
[4] J. Hong, A. Yokomakura, Y. Nakano, H.S. Ban, K. Ishihara, J.W. Ahn, O. Zee, K. Ohuchi, Induction of nitric oxide production by the cytostatic macrolide apicularen A [2,4-heptadienamide, N-[(1E)-3-[(3S,5R,7R,9S)-3,4,5,6,7,8,9,10-octahydro-7,14 dihydroxy-1-oxo-5,9-epoxy-1H-2-benzoxacyclododecin-3-yl]-1 propenyl]-, (2Z,4Z)-(9CI)] and possible role of nitric oxide in apicularen A-induced apoptosis in RAW 264.7 cells, J. Pharmacol. Exp. Ther. 312 (2005) 968–977.
[5] J.S. Kim, Y.C. Lee, H.T. Nam, G. Li, E.J. Yun, K.S. Song, K.S. Seo, J.H. Park, J.W. Ahn, O. Zee, J.I. Park, W.H. Yoon, K. Lim, B.D. Hwang, Apicularen A induces cell death through Fas ligand up-regulation and microtubule disruption by tubulin down-regulation in HM7 human colon cancer cells, Clin. Cancer Res. 13 (2007) 6509–6517.
[6] R. Kim, Recent advances in understanding the cell death pathways activated by anticancer therapy, Cancer 103 (2005) 1551–1560.
[7] W. Zhang, C. Zhang, N. Narayani, C. Du, K.C. Balaji, Nuclear translocation of apoptosis inducing factor is associated with cisplatin induced apoptosis in LNCaP prostate cancer cells, Cancer Lett. 255 (2007) 127–134.
[8] G. Kroemer, S.J. Martin, Caspase-independent cell death, Nat. Med. 11 (2005) 725–730.
[9] S.W. Tait, D.R. Green, Caspase-independent cell death: leaving the set without the final cut, Oncogene 27 (2008) 6452–6461.
[10] J. Zhou, H.B. Shu, H.C. Joshi, Regulation of tubulin synthesis and cell cycle progression in mammalian cells by gamma-tubulin-mediated microtubule nucleation, J. Cell. Biochem. 84 (2002) 472–483.
[11] S.B. Barlow, M.L. Gonzalez-Garay, F. Cabral, Paclitaxel-dependent mutants have severely reduced microtubule assembly and reduced tubulin synthesis, J. Cell Sci. 115 (2002) 3469–3478.
[12] R. Aneja, J. Zhou, S.N. Vangapandu, B. Zhou, R. Chandra, H.C. Joshi, Drug-resistant T-lymphoid tumors undergo apoptosis selectively in response to an antimicrotubule agent, EM011, Blood 107 (2006) 2486–2492.
[13] Y. Wang, F. Cabral, Paclitaxel resistance in cells with reduced beta-tubulin, Biochim. Biophys. Acta 1744 (2005) 245–255.
[14] T.R. Zilz, H.R. Griffiths, M.D. Coleman, Apoptotic and necrotic effects of hexanedione derivatives on the human neuroblastoma line SK-N-SH, Toxicology 231 (2007) 210–214.
[15] L. Tyas, V.A. Brophy, A. Pope, A.J. Rivett, J.M. Tavare, Rapid caspase-3 activation during apoptosis revealed using fluorescence-resonance energy transfer, EMBO Rep. 1 (2000) 266–270.
[16] E.M. Velazquez-Delgado, J.A. Hardy, Phosphorylation regulates assembly of the caspase-6 substrate-binding groove, Structure 20 (2012) 742–751.
[17] S.P. Cregan, V.L. Dawson, R.S. Slack, Role of AIF in caspase-dependent and caspase-independent cell death, Oncogene 23 (2004) 2785–2796.
[18] D. Mandal, A. Mazumder, P. Das, M. Kundu, J. Basu, Fas-, caspase 8-, and caspase 3-dependent signaling regulates the activity of the aminophospholipid translocase and phosphatidylserine externalization in human erythrocytes, J. Biol. Chem. 280 (2005) 39460–39467.
[19] C. Lambert, A.M. Landau, J. Desbarats, Fas-beyond death: a regenerative role for Fas in the nervous system, Apoptosis 8 (2003) 551–562.
[20] E. Devarajan, A.A. Sahin, J.S. Chen, R.R. Krishnamurthy, N. Aggarwal, A.M. Brun, A. Sapino, F. Zhang, D. Sharma, X.H. Yang, A.D. Tora, K. Mehta, Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance, Oncogene 21 (2002) 8843–8851.
[21] C.E. Keller, B.P. Lauring, Possible regulation of microtubules through destabilization of tubulin, Trends Cell Biol. 15 (2005) 571–573.
[22] A. Banan, G.S. Smith, C.L. Rieckenberg, E.R. Kokoska, T.A. Miller, Protection against ethanol injury by prostaglandin in a human intestinal cell line: role of microtubules, Am. J. Physiol. 274 (1998) G111–121.