Apicularen A Induces Cell Death through Fas Ligand Up-Regulation and Microtubule Disruption by Tubulin Down-Regulation in HM7 Human Colon Cancer Cells

Jong-Seok Kim,1 Young-Chul Lee,4 Ho-Tak Nam,1 Ge Li,1 Eun-Jin Yun,1 Kyung-Sub Song,1 Kang-Sik Seo,1 Ji-Hoon Park,1 Jong-Woong Ahn,5 OkPyo Zee,6 Jong-II Park,1,2 Wan-Hee Yoon,1 Kyu Lim,1,2 and Byung-Doo Hwang1,3

Abstract

Purpose: Apicularen A has been shown to cause growth inhibition and apoptosis in several cancer cell lines. However, the mechanisms of apicularen A–induced cell death and in vivo effects remain unclear. In this study, we investigated the molecular mechanisms of apicularen A–induced cell death in HM7 human colon cancer cells in vitro and anticancer activity in vivo.

Experimental Design: We tested cytotoxicity with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, apoptosis with DNA fragmentation assay, mitochondrial membrane potential, and cell cycle with fluorescence-activated cell sorting. Caspase activation was done by fluorometry. Alterations of microtubule structure, tubulin protein, and mRNA level were assessed by immunofluorescence, Western blot, and reverse transcription-PCR. In vivo studies were assessed using nude mice tumor cell growth in xenograft model and liver colonization assay.

Results: Apicularen A treatment of HM7 cells inhibited cell growth and this inhibition was partially rescued by z-VAD-fmk. Apicularen A caused accumulation of sub-G1-G0, DNA fragmentation, Fas ligand induction, and activation of caspase-8 and caspase-3, but mitochondrial membrane potential was not changed. Furthermore, β-tubulin protein and mRNA were decreased by apicularen A, but in vitro polymerization of tubulin was not affected. Concurrently, apicularen A–treated cell showed disruption of microtubule architecture. In in vivo studies, apicularen A reduced tumor volume by ~72% at the end of a 15-day treatment. Moreover, apicularen A reduced liver colonization as much as 95.6% (50 μg/kg/d).

Conclusion: Apicularen A induces cell death of HM7 cells through up-regulating Fas ligand and disruption of microtubule architecture with down-regulation of tubulin level. These findings indicate that apicularen A is a promising new microtubule-targeting compound.

Apicularen A was initially isolated from myxobacterial genus Chondromyces (1). It is a macrolide, which is characterized by a salicylic residue as part of a 10-membered lactone bearing an acylenamine side chain (1). Apicularen A is highly cytotoxic to several cells with IC50 values ranging between 0.1 and 3 ng/mL and is considered a potent anticancer lead compound due to its strong cytotoxicity (1). Recently, it was reported that apicularen A is able to induce apoptosis in murine macrophage-like RAW 264.7 cells (2) and in HL-60, a human promyelocytic leukemia cell line (3). The nitric oxide, produced by the apicularen A treatment, has also been reported to participate in apicularen A–induced apoptosis (4). In addition, apicularen A has been reported to be a novel specific vacuolar ATPase inhibitor (5, 6). However, the mechanisms that lead to cell death by apicularen A and in vivo studies on apicularen A are limited.

Apoptosis is dependent on the activities of caspase proteases (7–9) and is accompanied by characteristic morphologic changes, such as chromatin compaction, membrane blebbing, and cell shrinkage (10). The apoptotic cascade can be initiated via the death receptor or mitochondrial pathways (7–9).
death receptor pathway is triggered by members of the death receptor superfamily, that is, a binding of CD95 ligand to CD95 induces receptor clustering and death-inducing signaling complex formation, which recruits via the adaptor molecule FADD, multiple caspase-8 molecules resulting in caspase-8 activation \( (7–9) \). On the other hand, the mitochondrial pathway is triggered by cytochrome \( c \) and released from mitochondria, which then associates with Apaf-1 followed by procaspase-9 to form apoptosome \( (7–9, 11, 12) \). These two pathways converge at the level of caspase-3 activation. The majority of chemotherapeutic agents trigger the mitochondrial pathway \( (13–15) \), although the death receptors have also been reported to be involved in chemotherapy-induced apoptosis \( (16–20) \).

Microtubules are dynamic polymers that play crucial roles in various cellular functions \( (21) \). Especially, their pivotal role in mitosis makes them a target for the development of anticancer drugs \( (22) \). A large number of chemically diverse compounds are able to bind tubulin or microtubules and inhibit polymerization by acting on mitotic spindles. Some of these compounds (Vinca alkaloids and colchicines) inhibit microtubule polymerization, whereas others (taxanes) stabilize microtubules \( (21, 23, 24) \). However, although these compounds exert opposite effects on microtubules, both types of microtubule-interfering agents share the common property of suppressing microtubule dynamics and thereby microtubule function, which leads to mitotic spindle function disruption and the blocking of cell cycle progression at the transition from prometaphase/metaphase to anaphase \( (22) \). However, there have not been any published studies on agents that disrupt microtubule networks by down-regulating tubulin synthesis.

In this study, we investigated the mechanism underlying the cytotoxic effect of apicularen A on the HM7 cells in vitro and in vivo. The investigation showed that apicularen A induces apoptosis and that apoptosis is involved in the induction of Fas ligand (Fas-L), activations of caspase-8, and downstream caspase-9 and -3 contents less than the G1 amount of untreated cells were considered apoptotic.

Mitochondria membrane potentials \( (\Delta \psi_m) \) were measured by rhodamine123 fluorescence \( (26) \). Cells were trypsinized and incubated for 20 min at room temperature in 1 mL DMEM containing 5 \( \mu \)g/mL rhodamine123. The cells were then washed once in PBS and analyzed on a FACSscan flow cytometer for reduced rhodamine123 fluorescence.

DNA fragmentation assays. Cells were rinsed with ice-cold PBS and harvested. The cell pellets were then resuspended and incubated at 60°C for overnight in a solution containing 100 mmol/L Tris-HCl (pH 8.0), 25 mmol/mL EDTA, 0.5% SDS, and 0.1 \( \mu \)g/mL proteinase K. DNA was then extracted with phenol/chloroform \((1:1)\) and chloroform/isomyl alcohol \((1:24)\). The extracted DNA was precipitated and digested in 10 mmol/L Tris-HCl (pH 5.0) containing 10 \( \mu \)g/mL RNase A for 1 h at 37°C. DNA \((10 \mu \)g per sample\) was resolved by electrophoresis in a 1.5% agarose gel impregnated with ethidium bromide \((0.5 \mu \)g/mL\) and DNA patterns were examined by UV transillumination.

**Materials and Methods**

**Reagents.** Apicularen A \((2,4\text{-heptadienamide, N-[(1E)-3-[(3S,5R,7R,9S)-3,4,5,6,7,8,9,10-octahydro-1-oxo-5,9-epoxy-1H-2-benzoacyclododecin-3-yl]-1-propenyl]-(2Z,4Z)-(9CI)]\) was provided by Dr. Ahn (Division of Ocean Science, Korea Maritime University, Busan, Republic of Korea). It was dissolved at \( 100 \text{ mmol/L} \) and stored at -70°C. Goat anti-caspase-3 polyclonal antibody was purchased from R&D Systems. Rabbit anti-Bcl-2, phosphorylated Bcl-2 \((\text{Ser}^{155})\), and Bcl-X \(_\text{L}\) polyclonal antibodies were purchased from Cell Signaling Technology. Goat anti-poly(ADP-ribose) polymerase (PARP), rabbit anti-Fas, and rabbit anti-Fas-L polyclonal antibodies were purchased from Santa Cruz Technology. Anti-\( \beta \)-tubulin monoclonal antibody \((\text{mAb}; \text{TUB2.1})\) and other agents were purchased from Sigma Chemical.

**Cell line and cell culture.** Human colon carcinoma cell line HM7 was a gift from Young S. Kim (Gastrointestinal Research Laboratory, University of California, San Francisco, CA). Cells were cultured in DMEM (Life Technologies) supplemented with 10% heat-inactivated \((56\degree C, 30 \text{ min})\) fetal bovine serum \((\text{Life Technologies})\), 100 units/mL penicillin, and 100 \( \mu \)g/mL streptomycin at 37°C in a humidified incubator containing 5% \( \text{CO}_2 \). The cells used in experiments were no older than 15 passages.

**Measurement of cell survival.** To measure cell survival, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide assays were used \((11)\). Briefly, \( 5 \times 10^4 \) cells were plated in a 96-well microtiter plate and incubated for 24 h. The cultured cells were treated with various concentrations of apicularen A for 0, 6, 12, 24, 36, and 48 h. Then, the treated cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide \((0.5 \text{ mg/mL})\) at 37°C for 4 h. After dissolving the resulting crystals in DMSO, plates were read in a microplate reader at 570 nm with a SpectraMax Plus (Molecular Devices). Control contained the same concentration of DMSO as the apicularen A-treated cells.

**Flow cytometry.** Apoptosis was measured by determining cellular DNA content by propidium iodine staining and flow cytometry \((25)\). Briefly, \( 1 \times 10^4 \) cells were plated in a six-well microtiter plate and cultured for 24 h. The cultured cells were treated with 10 nmol/L apicularen A for 48 h. At the end of each treatment, the cells were trypsinized and fixed in 70% ethanol at -20°C for at least 30 min. Before analysis, cells were washed once in PBS and stained with PBS containing 50 \( \mu \)g/mL propidium iodide and 50 \( \mu \)g/mL RNase A for 30 min at 37°C. Analyses were done by FACSscan flow cytometry \((\text{FACSCalibur, Becton Dickinson Immunocytometry System})\) using CellQuest software provided by Becton Dickinson. Cells with DNA contents less than the G1 amount of untreated cells were considered apoptotic.

Western blot analysis and subcellular fractionation. After treatment with 10 nmol/L apicularen A, adherent cells were washed twice with PBS, gently scraped from dishes, centrifuged, lysed in ice-cold lysis buffer \((50 \text{ mmol/L Tris-HCl (pH 7.4), 0.8 mol/L NaCl, 5 mmol/L MgCl}_2, 0.5% NP40, protease inhibitor cocktail (Roche)}) and clarified by microcentrifugation. Protein levels were measured using the Bradford method \((27)\), and equal amounts of protein were subjected to electrophoresis on 8% to 15% polyacrylamide gels containing SDS under reducing conditions. Separated proteins were electroblotted onto nitrocellulose membranes, and blots were incubated with 5%...
nonfat dry milk (w/v) for 1 h and then washed in TBS containing 0.1% Tween 20. Membranes were then incubated with primary antibody, and antibody binding was detected using the appropriate secondary antibody coupled with horseradish peroxidase as described by the manufacturer. Enhanced chemiluminescence was used to detect relevant proteins, again by following the manufacturer’s instructions (Amersham Life Science, Inc.).

Cytosolic extracts free of nuclei and mitochondria were prepared as described previously (29). Briefly, cells were washed in ice-cold PBS and then in hypotonic extraction buffer [50 mmol/L PIPES (pH 7.4), 50 mmol/L KCl, 5 mmol/L EGTA, 2 mmol/L MgCl₂, 1 mmol/L DTT, 0.1 mmol/L phenylmethylsulfonyl fluoride] and centrifuged. Pellets were resuspended in hypotonic extraction buffer and lysed in a Dounce homogenizer and centrifuged at 16,000 × g for 30 min at 4°C, and the supernatants were either fractionated immediately or quickly frozen in cold 100% ethanol and stored at -80°C. To prepare mitochondrial and nuclear fractions, cells were washed in ice-cold PBS and then resuspended in ice-cold buffer A (250 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L DTT, protease inhibitor cocktail). Cells were homogenized using a Potter-Thomas homogenizer, and nuclear pellets were obtained by spinning at 750 × g for 10 min at 4°C. The supernatants obtained were spun at 10,000 × g for 45 min, and mitochondrial fractions were resuspended in buffer A. Aliquots were quickly frozen in cold 100% ethanol and stored at -80°C.

Reverse transcription-PCR analysis. The mRNA expression levels of β-tubulin were determined by semiquantitative PCR as described previously (29). The primer sequences used were as follows: β-tubulin, 5′-TGCATGCAACACGACGGCC-3′ (sense) and 5′-CTGTTCCTGACACATTGTCG-3′ (antisense). PCR mixtures consisted of cDNA derived from 50 ng mRNA, 5 pmol of each of the sense and antisense primers, 200 μmol of deoxynucleotide triphosphates, and 0.625 unit of TaKaRa Taq DNA polymerase with reaction buffer (TaKaRa Shuzo) in a final volume of 25 μL. PCR cycles were 30-s denaturation at 94°C, 60-s annealing at 56°C, and a 60-s extension at 72°C. PCR products were separated on 1.2% agarose gels with ethidium bromide (0.5 μg/mL), and patterns were examined by UV transillumination.

Isolation of microtubule and in vitro tubulin polymerization assay. Microtubule proteins were prepared from pig brain as described previously (30). All procedures were done at 4°C, except polymerization and centrifugation, which were done at 37°C and 20°C, respectively. Tubulin polymerization was monitored by turbidimetry. Changes in absorbance at 350 nm were measured using a Gilford 560 spectrophotometer with a thermostat cuvette attachment. The sample used was 0.25 mL in a cuvette with a 1-cm light path. Solutions were prepared by sequentially adding 0.1 mol/L MES buffer [0.1 mol/L MES, 1 mmol/L EGTA, 1 mmol/L MgCl₂ (pH 6.8)], 1 mg/mL tubulin, and 2 mmol/L GTP. Turbidity changes were measured and centrifuged, which were done at 37°C, except polymerization and centrifugation, which were done at 37°C and 20°C, respectively. Tubulin polymerization was monitored by turbidimetry. Changes in absorbance were measured at 350 nm using a Gilford 560 spectrophotometer with a thermostat cuvette attachment.

In vivo tubulin polymerization assays. The separation of insoluble polymerized microtubules from soluble tubulin dimers and the analysis of the effect of apicularen A on tubulin polymerization in vivo were done as described previously (31). In brief, HM7 cells on 1 × 10⁶/100-mm² dish were treated with 10 nmol/L apicularen A for 0, 24, and 48 h. Cells were washed with PBS thrice before adding microtubule-stabilizing lysis buffer [20 mmol/L Tris-HCl (pH 6.8), 1 mmol/L MgCl₂, 2 mmol/L EGTA, 20 mg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L orthovanadate, 0.5% NP40]. Supernatants were collected after centrifugation at 15,000 × g for 10 min at 4°C. Pellets were dissolved in SDS-PAGE sample buffer and heated at 95°C for 10 min to dissolve the pellets. Then, we did typical Western blot methods explained above.

Immunofluorescence assays. Cells were grown on glass coverslips in the presence of 10 mmol/L apicularen A for 24 and 48 h. Cells were fixed in 3.7% paraformaldehyde, permeabilized in 0.1% Triton X-100, and then blocked with 2% bovine serum albumin in PBS containing 0.1% Tween 20 (PBS/T) for 2 h before being incubated with 2% bovine serum albumin in PBS/T containing anti-β-tubulin mAb for 2 h at room temperature. After washing with PBS/T, cells were reincubated with FITC-conjugated secondary antibody in the dark room for 1 h, and then cells were stained with 5 μg/mL propidium iodide and 1 mg/mL RNase A for 30 min at room temperature. Cellular microtubules and nucleic acid were observed using an Olympus FV-500 fluorescence microscope.

Tumor cell growth in nude mice. HM7 cell cultures were harvested by brief trypsinization, washed thrice with calcium- and magnesium-free PBS, and resuspended at a final concentration of 10⁶ cells/mL in serum-free DMEM. Single-cell suspensions were contaminated by phase-contrast microscopy, and cell viabilities were determined by trypsin blue exclusion; only single-cell suspensions with a viability of >90% were used. Pathogen-free female BALB/cAnN.Cg- nu athymic nude mice (4 weeks old; Charles River Laboratories) were anesthetized with diethyl ether by inhalation, and 10⁶ HM7 cells in 100 μL of serum-free DMEM were inoculated s.c. into the right flanks. Nine days after tumor cell inoculation, mice received an intratumoral injection of apicularen A (5, 25, and 50 μg/kg/100 μL saline, once daily) or the same amount of physiologic saline for 15 days. Mice were surveyed regularly, and tumors were measured using a caliper. Tumor volumes were determined using the following formula: volume = 0.5 × (width)² × length (32). Each experimental group consisted of six animals, and P < 0.05 was considered statistically significant (33).

Liver colonization. The inhibitory effect of apicularen A on the ability of HM7 cells to colonize the liver after entry into the hepatic portal system was tested in a splenic injection model (34). Tumor cells were resuspended at a final concentration of 10⁶ cells/mL in serum-free DMEM. Athymic nude mice were then anesthetized with diethyl ether, and spleens were exteriorized via a flank incision. One million cells in 100 μL were then slowly injected into the lower polar side of the splenic pulp through a 27-gauge needle, and this was followed by splenectomy 1 min later. Apicularen A (25 or 50 μg/kg/100 μL saline) was injected i.p. once daily for 14 days from the day of operation. Animals were sacrificed 4 weeks postoperatively, liver and spleens were removed and weighed, and tumor metastatic nodules in livers were counted. Six animals were assigned to each experimental group. P values of <0.05 were considered statistically significant. All experiments were done in accordance with the guidelines issued by our institution about the ethics of animal experimentation.

Statistical analysis. Statistical analyses were done as recommended by an independent analyst. These included the unpaired Student's t test (cell proliferation, caspase activation assay, liver weight, and size of s.c. tumors) and the Mann-Whitney test (number of tumor nodules after spleen injection). The number of cells/pellets after the use of phase-contrast microscopy was two times (width)² × length (32). Each experimental group consisted of six animals, and P values of <0.05 were considered statistically significant. All experiments were done in accordance with the guidelines issued by our institution about the ethics of animal experimentation.

Results

Apicularen A inhibited the growth of HM7 colon cancer cell lines. The effect of apicularen A on the growth of HM7 cells was examined at doses between 0.1, 1, 10, and 100 nmol/L for treatment durations between 0, 6, 12, 24, and 48 h. Exposure to 10 or 100 nmol/L of apicularen A suppressed HM7 cell growth in a dose- and time-dependent manner (Fig. 1A; Supplementary Fig. S1). Thus, 10 nmol/L apicularen A was chosen to examine changes in molecular events in the following experiments.

Apicularen A induced apoptosis in HM7 cells and this apoptosis was partially recovered by a pan-caspase inhibitor. It is useful to classify anticancer drugs as cell cycle specific or cell cycle non-specific. Cell cycle–specific agents can be further subdivided into phase-specific agents. To examine whether apicularen A–induced cell growth inhibition was associated with cell cycle...
regulation, cell cycle distributions were analyzed by flow cytometry. As shown in Fig. 1B, apicularen A did not change the cell cycle in 24 h and cell numbers in each phase decreased, whereas numbers of hypodiploid cells later increased at 36 to 48 h, indicating apoptosis.

To further access the hypodiploid population in apicularen A–treated HM7 cells, DNA fragmentation assays were done. Figure 1C shows that exposure to 10 nmol/L apicularen A for ≥24 h induced DNA fragmentation, which is regarded as a hallmark of apoptosis in HM7 cells. These results show that apicularen A induced apoptosis independent of cell cycle progression. On the other hand, growth inhibition occurred as early as 12 h after drug exposure compared with apoptosis (Supplementary Fig. S1). These results suggest that both mechanisms observed could be the subsequent event of the actions of apicularen A that is not identified yet.

Regulators of apoptosis directly or indirectly control the activity of caspases-cysteine proteases, which are the key executors of apoptosis, cleaving their substrates following specific aspartate residue (35). Therefore, the effect of pan-caspase inhibitor (z-VAD-fmk) on the apicularen A–induced apoptosis was examined. The results showed that apicularen A–induced apoptosis was significantly reduced from 70% to 40% in the presence of 50 μmol/L z-VAD-fmk (P < 0.001; Fig. 1D).

The initiator and effector caspases of apicularen A–induced apoptosis in HM7 cells were caspase-8 and caspase-3. The morphologic features of apoptosis resulted from the activation of caspases (7–9) by either death receptor ligation (16–18) or the release of apoptotic mediator from mitochondria (13–15, 19). To determine the specific caspases involved in apicularen A–induced apoptosis in HM7 cells, caspase activity assays for caspase-8, caspase-9, and caspase-3 and Western blot analysis for caspase-3 were done. As shown in Fig. 2A, incubation of HM7 cells with apicularen A resulted in the activation of caspase-3, as evidenced by the conversion of the proenzyme form of caspase-3 (p32) to the catalytically active effector protease (p17). Activation of caspase-3 during apicularen A–induced apoptosis was also confirmed by examining PARP cleavage (Fig. 2A).

Figure 2B shows the effect of apicularen A on the activities of caspase-8 and caspase-9, the upstream activators of caspase-3. On treatment of HM7 cells with apicularen A, about a 4-fold increase in caspase-8 activity toward its specific Ac-IETD-AFC fluorogenic tetrapeptide substrate was detected after 24 h of treatment. This activity increased with time. In contrast to the high level of caspase-8–specific activity, weak caspase-9–specific activity was detected, with only a 1.9-fold increase in caspase-9 activity after treatment with apicularen A for 48 h.

A previous report showed that drug-induced caspase-8 activation is controlled by mitochondria in the non–small cell lung cancer cell line NCI-H460 (36). To rule out mitochondrial involvement in apicularen A–induced caspase-8 activation in HM7 cells, we examined Bcl-2, phosphorylated Bcl-2 (Ser70), Bcl-XL, and cytochrome c by Western blotting and changes in mitochondria membrane potential by rhodamine123 staining. The Western blot analysis presented in Fig. 2C shows that HM7 cells treated with apicularen A showed unchanged Bcl-2, phosphorylated Bcl-2 (Ser70), Bcl-XL, and cytochrome c in
cytosol, and this was confirmed by rhodamine123 staining (Fig. 2D).

**Induction of apoptosis by apicularen A was mediated by the up-regulation of Fas-L.** The results obtained previously suggest that caspase-8 may function as an apical caspase in the pathway that triggers apoptosis in HM7 cells. Caspase-8 is the classic initiator caspase of the Fas (CD95/APO-1) pathway, and this pathway has also been shown to contribute to chemotherapeutic agent-induced apoptosis in various cellular systems (37, 38). Therefore, we assessed the expressions of Fas and Fas-L by Western blotting.

As shown in Fig. 3A, apicularen A significantly induced the level of Fas-L after 12 h of treatment. In contrast to Fas-L, no appreciable change was detected in the expression of Fas in HM7 cells treated with the same concentration of apicularen A from 6 to 48 h. This finding suggests that Fas-L expression, induced by apicularen A, is independent of Fas receptor (Fig. 3A).

Based on the increased early expression of Fas-L by apicularen A (Fig. 3A), it is hypothesized that the secretion and autocrine/paracrine engagement of Fas-L with Fas at the cell surface was associated with the induction of apoptosis via caspase-8 to caspase-3 by apicularen A. To test this hypothesis, HM7 cells were incubated with NOK-1 mAb that interferes with the Fas-L interaction (39) and, thus, inhibits Fas signaling. It was found that preincubation with NOK-1 mAb partially blocked apicularen A–induced apoptosis and PARP cleavage (Fig. 3B and C).

**Apicularen A reduced tubulin levels and disrupted the microtubule network.** For Western blot analysis of apoptosis-related proteins at the beginning of this study, β-tubulin was used as a loading control. In that experiment, β-tubulin levels were reduced by apicularen A. This finding led us to examine changes in tubulin levels and microtubule networks as a function of apicularen A treatment time in HM7 cells. As shown in Fig. 4A, β-tubulin levels reduced with time-dependent manner and dropped dramatically after 36 h of treatment and were almost completely depleted at 48 h. In addition, α-tubulin levels were decreased with the same pattern (data not shown). To verify whether down-regulation of tubulin caused by apicularen A was cell type specific (HM7), we examined NIH3T3 mouse fibroblasts cells, HT-29 human colon cancer cells, and MDA-MB-231 human breast cancer cell lines. As shown in Supplementary Fig. S2, α-tubulin and β-tubulin levels of all tested cell lines were reduced by apicularen A. These data suggest that down-regulation of tubulin caused by apicularen A was not cell type specific.

To examine the mechanism of the apicularen A–induced down-regulation of β-tubulin protein levels, we examined the effect of apicularen A on β-tubulin mRNA levels by reverse transcription-PCR. Apicularen A treatment was found to affect reduction of β-tubulin mRNA levels in a time-dependent manner (Fig. 4A). Moreover, if microtubules were depolymerized by apicularen A, free tubulin level would increase, and this would lead to a decrease in the amounts of α-tubulin and

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**Fig. 2.** Activations of caspase-3 and caspase-8 by apicularen A in HM7 cells. A, at the indicated times after treatment with apicularen A (10 nmol/L), cells were lysed and 30 µg proteins was subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with caspase-3 or PARP antibodies. B, caspase-3, caspase-8, and caspase-9 activities were determined as described in Materials and Methods using the caspase-3, caspase-8, or caspase-9 fluorogenic peptide substrates DEVD-AFC, IETD-AFC, and LETD-AFC, respectively. Columns, mean of analyses done in triplicate; bars, SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001, Student’s t test, significantly different from the control. C, effect of apicularen A on Bcl-2 family members and on cytochrome c release. At the indicated time points after treatment with apicularen A (10 nmol/L), cells were lysed and 30 µg protein was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with Bcl-2 or Bcl-XL antibodies. Subcellular fractions were prepared as described in Materials and Methods. Cytochrome c release from mitochondria to cytosol was analyzed using anti–cytochrome c antibody. M, mitochondria fraction; C, cytosol fraction. D, effect of apicularen A on mitochondrial transmembrane potentials (ΔΨm) in HM7 cells. Cells were treated with and without apicularen A at 10 nmol/L. After the indicated times, cells were incubated with 10 µmol/L rhodamine123 for 20 min and scored immediately by flow cytometry. X axis, rhodamine123 fluorescence; Y axis, cell numbers.
on polymerized tubulin by apicularen A might shift the equilibrium between tubulin polymers and their proteins (40). To investigate this possibility, an in vitro microtubule polymerization assay was done. As shown in Fig. 4B, apicularen A was found to have no effect on microtubule polymerization kinetics.

Because apicularen A caused a marked decrease in tubulin levels, it was considered that treating HM7 cells with apicularen A might shift the equilibrium between tubulin polymers and promoter. Therefore, we investigated the effects of apicularen A on polymerized tubulin levels and on microtubule organization in human HT1080 fibrosarcoma cells, which have clearly defined microtubule networks. It was also found that apicularen A reduced β-tubulin levels (Fig. 4D) in this cell line. Normal microtubule distribution in untreated HT1080 cells is shown in Fig. 4C. Treatment with apicularen A reduced the density and disorganization of microtubules in cytosol and elongated eccentric nuclei (Fig. 4C, d). In vivo assays of tubulin polymerization also showed a marked decrease in free tubulin levels and the complete depletion of polymerized tubulin by apicularen A (Fig. 4D).

**Effect of apicularen A on HM7 cell growth in vivo.** The potential antitumoral effect of apicularen A in vivo was assessed in human tumor xenografts in mice. HM7 cells were implanted s.c into nude mice, and 9 days later, when well-established HM7 xenografts were palpable with tumor sizes of ~75 mm³, mice were randomized into vehicle control and treatment groups containing six animals each. Treated mice received 5, 25, or 50 μg/kg once daily of apicularen A intratumorally for 15 days. A dose-dependent decrease in tumor volume was observed in mice treated with apicularen A. In particular, a 72% decrease in tumor volume was achieved by animals treated daily with 50 μg/kg apicularen A for 15 days versus saline-treated controls (P < 0.001; Fig. 5A).

**Effect of apicularen A on liver colonization by HM7 cells.** The ability of apicularen A to inhibit the development of liver colonization by HM7 cells injected into the spleen of nude mice was examined. Animals were treated with repeated i.p. injections of apicularen A (25 and 50 μg/kg, once daily) over a 14-day period as described above. Under these experimental conditions, massive hepatic tumor burden, ascites, and severe cachexia were evident 4 weeks after this inoculation into the portal system in 100% of the saline-treated control mice, whereas the apicularen A–treated mice seemed normal (Fig. 5B). Moreover, the mean liver weight of the control mice was 2.87 times greater than that of 50 μg/kg apicularen A–treated mice due to massive liver colonization (P < 0.01; Fig. 5C). In addition, the formation of metastatic nodules in livers was inhibited by 95.6% in 50 μg/kg apicularen A–treated animals (P < 0.001; Fig. 5D).

**Discussion**

This investigation presents evidence that the strong anticancer effect of apicularen A against HM7 cells occurs in a mitochondria-independent manner via the Fas-L/caspase-8/ caspase-3 cascade and the disruption of microtubule cytoskeletal organization via down-regulation of tubulin synthesis. Furthermore, this study shows that apicularen A inhibits the primary tumor growth and liver colonization of HM7 cells in vivo.

Emerging evidences indicate that evasion from apoptosis is a hallmark of cancer, and the antitumor effects of many chemotherapeutic agents have been associated with the apoptotic pathway (14, 41–43). Caspase-3, which causes the cleavage and inactivation of key cellular proteins, such as PARP and CAD, is thought to play a key role in the apoptosis of many cells (7–9, 35). In the present study, PARP cleavage was observed and accompanied by procaspase-3 cleavage intermediates, suggesting the activation of caspase-3, and this was confirmed using caspase-3 activation assays using caspase-3 fluorogenic peptide substrate DEVD-AFC (44).

Previous reports have shown that caspase-8 and caspase-9 are upstream initiator caspases that can directly activate caspase-3 (16). Using IETD-AFC as a substrate to measure caspase-8 activity, it was observed that its activity was markedly elevated in HM7 cells exposed to apicularen A. In addition, apicularen A–induced caspase-8 activation showed a similar pattern in a timely manner as that of caspase-3. These results show that apicularen A–triggered apoptosis is crucially dependent on the activation of the caspase-8 cascade. Several drugs trigger the caspase-9/mitochondrial pathway (13–15) and caspase-8 can
be activated in a mitochondria-controlled, caspase-9–independent manner (36). However, our results show that caspase-9 was not activated in response to apicularen A treatment and that mitochondrial membrane potentials did not change. Furthermore, our results show that apicularen A did not affect Bcl-2 and its phosphorylation and Bcl-XL levels, and cytochrome c was not detected in cytosol. These findings indicate that caspase-8 is an initiator caspase and is not activated in a mitochondria-controlled manner.

Caspase-8 is the most apical caspase in Fas (CD95/APO-1)-mediated apoptosis (19, 44); therefore, changes in Fas (CD95/APO-1) and Fas-L expressions were assessed by Western blotting. The results obtained showed that Fas-L expression was markedly induced by apicularen A but Fas was not. Therefore, we conclude that apicularen A mediated apoptosis via the Fas-L/caspase-8/caspase-3 cascade, which is mitochondria independent. However, z-VAD-fmk and NOK-1 treatment only partially rescued apicularen A–induced apoptosis. Moreover, as shown in Supplementary Fig. S3, NOK-1 does not prevent apicularen A–induced down-regulation of β-tubulin. This implies that apicularen A–induced HM7 cell death may not be related to mechanistic linkage between the induction of Fas-L and down-regulation of β-tubulin.

Antimicrotubule drugs suppress microtubule dynamics, disrupt mitotic spindles in dividing cells, and induce cell cycle arrest at the G2-M phase and late apoptosis (21–24). Western blotting and immunocytochemical results in the present study showed that apicularen A decreases tubulin protein levels and disrupts intracellular microtubule networks. However, apicularen A did not affect in vitro microtubule polymerization and did not alter Bcl-2 family or block the G2-M phase. But, β-tubulin mRNA levels were reduced by apicularen A. According to Caudron et al. (45), decreased amounts of tubulin decrease microtubule polymerization because a critical tubulin concentration is required for its polymerization. It has been reported that a 60% to 70% reduction in tubulin levels is too severe to allow cell survival (46), and decreased tubulin polymerization by ethanol in intestinal mucosal cells causes cell death (47, 48), which illustrates the importance of polymerized tubulin for cell survival. As shown in Fig. 4D, β-tubulin is still presented for 24 h in the supernatant and total fraction but not detectable in the pellet because tubulin protein level might be decreased to below the critical concentration for microtubule polymerization. These results indicate that decreased tubulin protein levels and microtubule network disruption by apicularen A are caused by reduced tubulin synthesis but not by altered microtubule dynamics.

We further investigated the ability of apicularen A to inhibit primary tumor growth and liver colonization by HM7 cells. Astonishingly, apicularen A reduced tumor volumes by 72% at the end of 15 days of treatment. The effects of apicularen A on in vitro cell proliferation seemed to be corroborated by the in vivo tumor growth studies. Moreover, apicularen A was found to markedly reduce liver colonization by as much as 95.6% at 50 μg/kg/d after 14 days of treatment. Possible explanations for inhibition of colonization to liver are as follows. First, microtubule-destablizing agents disrupt rapidly proliferating and immature tumor endothelial cells due to their...
reliance on the microtubule cytoskeleton in terms of cell shape maintenance. Tubulin-interfering agents have both antimitotic and antivascular effects, which respectively lead to spindle formation inhibition and reduced tumor blood flow (49, 50). Apicularen A also disrupted the formation of the normal microtubule architecture by down-regulating tubulin synthesis and thus might inhibit tumor colonization to the liver. Second, inhibition of colonization to the liver by apicularen A might be due to a direct cytotoxic effect on cancer cells.

Taken together, our results indicate that apicularen A induces cell death via the death receptor pathway by inducing Fas-L and disrupting the microtubule architecture by down-regulating tubulin synthesis independently. Furthermore, apicularen A was found to exhibit antitumoral and anticolonization activity in vivo. These finding suggest that apicularen A is a promising new microtubule-targeting compound and that it has potential for the management of various malignancies, including metastases.

References


28. Apicularen A Induced Cell Death


Apicularen A Induces Cell Death through Fas Ligand Up-Regulation and Microtubule Disruption by Tubulin Down-Regulation in HM7 Human Colon Cancer Cells

Jong-Seok Kim, Young-Chul Lee, Ho-Tak Nam, et al.


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