FEBS Letters 580 (2006) 2723–2730

Inhibition of vacuolar-type (H^+)-ATPase by the cytostatic macrolide apicularen A and its role in apicularen A-induced apoptosis in RAW 264.7 cells

JangJa Hong[a,b], Aya Yokomakura[a], Yasuhiro Nakano[a], Kenji Ishihara[a], Makoto Kaneda[c], Mitsue Onodera[c], Ken-ichi Nakahama[c], Ikuo Morita[c], Kazuaki Niikura[d], Jong-Woong Ahn[e], OkPyo Zee[b], Kazuo Ohuchi[a,*]

[a] *Laboratory of Pathophysiological Biochemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aoba Aramaki, Aoba-ku, Sendai, Miyagi 980-8578, Japan*
[b] *Laboratory of Pharmacognosy, Graduate School of Pharmacy, Sungkyunkwan University, 300 Chunchun-dong, Suwon, Kyungi-do 440-746, Republic of Korea*
[c] *Department of Cellular Physiological Chemistry, Graduate School, Tokyo Medical and Dental University, Yushima, Tokyo 113-8549, Japan*
[d] *Pharmacology Research Laboratories, Astellas Pharma Inc., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-2698, Japan*
[e] *Division of Ocean Science, Korea Maritime University, 1 Dongsam-dong, Youngdo-ku, Busan 606-791, Republic of Korea*

Received 12 March 2006; revised 31 March 2006; accepted 4 April 2006

Available online 21 April 2006

Edited by Stuart Ferguson

Abstract Apicularen A and the known vacuolar-type (H^+)-ATPase (V-ATPase) inhibitor bafilomycin A_1 induced apoptosis of RAW 264.7 cells, while apicularen B, an *N*-acetyl-glucosamine glycoside of apicularen A, was far less effective. Apicularen A inhibited vital staining with acridine orange of the intracellular organelles of RAW 264.7 cells, inhibited the ATP-dependent proton transport into inside-out microsome vesicles, and inhibited the bafilomycin A_1-sensitive ATP hydrolysis. The IC_{50} values of the proton transport were 0.58 nM for apicularen A, 13 nM for apicularen B, and 0.95 nM for bafilomycin A_1. Furthermore, apicularen A inhibited the bafilomycin A_1-sensitive ATP hydrolysis more potently than apicularen B. F-ATPase and P-ATPase were not inhibited by apicularen A. We concluded that apicularen A inhibits V-ATPase, and thus induces apoptosis in RAW 264.7 cells.

Keywords: Apicularen A; Apicularen B; Vacuolar-type (H^+)-ATPase; Apoptosis; Bafilomycin A_1; RAW 264.7

1. Introduction

The cytostatic macrolide apicularens A and B have been isolated from a variety of strains of the myxobacterial genus *Chondromyces* (i.e., *C. apiculatus*, *C. lanuginosus*, *C. pediculatus*, and *C. robustus*) [1], and the total synthesis of apicularen A has recently been reported [2]. Structurally, apicularen A features a *trans*-hydroxypyran with a salicylic acid residue within a 10-membered lactone, which bears a highly unsaturated enamide side chain (Fig. 1A). This natural product is usually found with varying amounts of its glycoconjugate with *N*-acetyl glucose, apicularen B (Fig. 1A). Apicularen A is reported to be highly cytostatic in human cancer cells, including ovarian, prostate, lung, kidney and cervix cancer cells, leukemia cells, histocytic cell lines, and most importantly, the multi-drug-resistant cell line KB-VI, with IC_{50} values ranging between 0.227 and 22.7 nM, while apicularen B is distinctly less cytostatic than apicularen A, with IC_{50} values of between 0.317 and 1.8 μM [1,3]. The chemical structures of apicularens A and B resemble those of salicylihalamide A and lobatamide B (Fig. 1B and C). These compounds including salicylihalamide B, lobatamides A, C, D, E and F, CJ-12950, CJ-13357, and oximidines I and II are classified as benzolactone enamides [4]. It is reported that salicylihalamide A, lobatamides A–F and oximidines I and II commonly inhibit mammalian vacuolar-type (H^+)-ATPase (V-ATPase) with IC_{50} values in the order of nM [4]. Therefore, it was speculated that apicularens A and B also inhibit V-ATPase.

We previously reported that apicularen A but not apicularen B at 10 and 100 nM induced apoptosis in RAW 264.7 cells, a mouse leukemia monocytic cell line, as evidenced by the formation of a DNA ladder, the increase in the percentage of sub-G_1 cells and annexin V-binding cells, and the activation of caspase [5]. In addition, apoptosis of HL-60 cells, a human leukemia cell line, was also induced by apicularen A [6]. Consequently, we suggested that apicularen A is a candidate for an anti-leukemic drug. However, the mechanism for the induction of apoptosis by apicularen A remains to be elucidated. V-ATPase plays an important role in the regulation of the activity in organelles of the central vacuolar system, and the internal acidification of intracellular compartments such as lysosomes, endosomes, Golgi complexes, and secretary granules has been suggested to play a critical role in the mechanism of cell survival [7]. The V-ATPase inhibitors bafilomycin A_1 [8] and concanamycin A [9], which are structurally different from these benzolactone enamide class compounds, induce apoptosis in several kinds of cancer cells [10,11]. Therefore, we speculated that the apicularen A-induced apoptosis of RAW 264.7 cells might be induced by inhibition of V-ATPase. In addition to the treatment of cancer, V-ATPase inhibitors hold consider-

*Corresponding author. Fax: +81 22 795 6859.
E-mail address: ohuchi-k@mail.pharm.tohoku.ac.jp (K. Ohuchi).

Abbreviations: V-ATPase, vacuolar-type (H^+)-ATPase

0014-5793/$32.00
doi:10.1016/j.febslet.2006.04.031

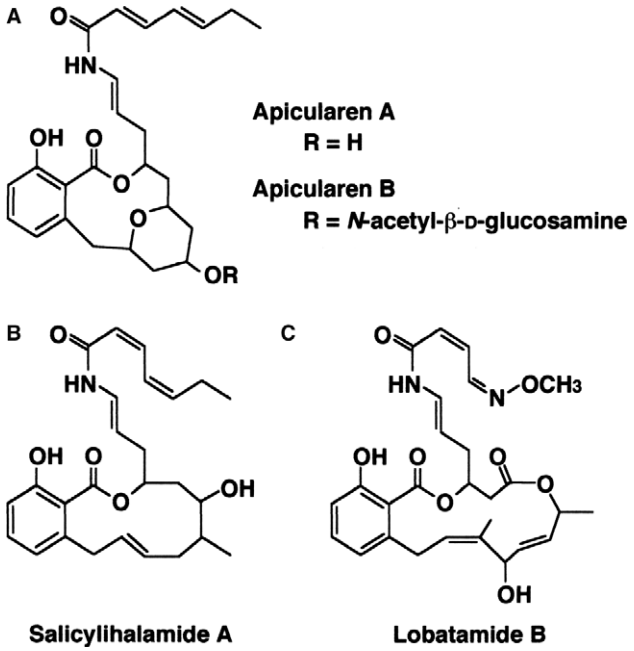

Fig. 1. Chemical structures of apicularens A and B (A), salicylihalamide A (B) and lobatamide B (C).

able promise for the treatment of a number of other diseases, including diabetes, Alzheimer's disease, cardiovascular disorders, and osteoporosis [12–14]. This study was aimed at clarifying whether apicularen A inhibits V-ATPase to explain the mechanism of action of apicularen A for the induction of apoptosis in RAW 264.7 cells.

2. Materials and methods

2.1. Reagents

Apicularen A (2,4-heptadienamide,*N*-[(1*E*)-3-[(3*S*,5*R*,7*R*,9*S*)-3,4,5,6,7,8,9,10-octahydro-7,14-dihydroxy-1-oxo-5,9-epoxy-1H-2-benzoxacyclododecin-3-yl]-1 propenyl]-, (2*Z*,4*Z*)-(9CI)) and apicularen B (2,4-heptadienamide,*N*-[(1*E*)-3-[(3*S*,5*R*,7*R*,9*S*)-7-[[2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]oxy]-3,4,5,6,7,8,9,10-octahydro-14-hydroxy-1-oxo-5,9-epoxy-1H-2-benzoxacyclododecin-3-yl]-1 propenyl]-, (2*Z*,4*Z*)-(9CI)) were purified from culture medium of *Chondromyces* apiculatus JW 184 according to the method described by Jansen et al. [3]. The compounds isolated were identified by a comparison of spectral data (NMR, IR, UV) and $[\alpha]_D$ using authentic compounds, and the purity of each isolated compound was confirmed to be more than 99%. Bafilomycin A_1 was purchased from Wako Pure Chemical Inc., Osaka, Japan. These drugs were dissolved in dimethyl sulfoxide (DMSO) and added to the cell culture medium.

2.2. Cell culture

RAW 264.7 cells (RIKEN Gene Bank, Tsukuba, Japan), a mouse leukemia monocytic cell line, were cultured in Eagle's minimal essential medium (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal bovine serum (FBS, Dainippon Pharmaceutical, Osaka, Japan) and 1% non-essential amino acid solution (Sigma Chemical Co., St. Louis, MO, USA). A431 cells (RIKEN Gene Bank), a human epidermal carcinoma cell line, were cultured in Dulbecco's modified Eagle's medium (Nissui Seiyaku) containing 10% FBS. The cells were incubated at 37 °C under 5% CO_2–95% air.

2.3. Analysis of morphological changes of nuclei

RAW 264.7 cells (1×10^6 cells) were incubated for 24 h at 37 °C in 2 ml of medium. The cells were then washed three times with medium, and further incubated for 24 h at 37 °C in the presence or absence of apicularen A (100 nM), apicularen B (100 nM) or bafilomycin A_1 (100 nM). After incubation, the cells were washed with phosphate-buffered saline (PBS), fixed with methanol and stained with Hoechst dye 33258 (Molecular Probes, Eugene, OR, USA) (100 μg/ml in PBS) for 10 min. The stained cells were observed under a fluorescence microscope (IX70, Olympus, Tokyo, Japan).

2.4. Detection of mitochondrial membrane potential by flowcytometry

RAW 264.7 cells (1×10^6 cells) were incubated for 24 h at 37 °C in 2 ml of medium. The cells were then washed three times with medium, and further incubated for 24 h at 37 °C in the presence or absence of apicularen A (100 nM), apicularen B (100 nM) or bafilomycin A_1 (100 nM). After incubation, the cells were washed three times with PBS, scraped off the plate and stained using a DePsipher™ Kit (Trevigen Inc., Gaithersburg, MD, USA). Subsequently, the intensities for green fluorescence (FL1; a maximal emission at 530 nm) and red fluorescence (FL2; a maximal emission at 590 nm) were analyzed by flowcytometry using FACScan (Becton Dickinon, San Jose, CA, USA), and the percentage of the cells with decreased mitochondrial membrane potential ($\Delta\psi_m$) was calculated.

2.5. Detection of V-ATPase by Western blotting

RAW 264.7 cells and A431 cells were sonicated for 15 s on ice in a sample buffer (62.5 mM Tris, pH 6.8, 6 M urea, 10% glycerol, 2% sodium dodecylsufate (SDS), 0.00125% bromophenol blue and 5% mercaptoethanol) using a Handy Sonic Disrupter (Tomy Seiko Co., Tokyo, Japan) and incubated for 15 min at 65 °C. After centrifugation at $15000 \times g$ and 4 °C for 5 min, an aliquot of the supernatant was loaded on a 7.5% SDS–polyacrylamide gel. After electrophoresis, the gel was transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Immunoblotting for V-ATPase was carried out using the antibody to the C subunit of V-ATPase (V-ATPase C (N-20), Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). As an internal control, actin was detected using a goat anti-actin polyclonal antibody (actin (I-19), Santa Cruz Biotechnology Inc.). The membrane was incubated for 3 h at 4 °C with biotinylated anti-goat IgG (Vector Laboratories, Buringame, CA, USA). The reaction products were incubated for 30 min at room temperature with Vectastatin ABC reagent (Vector Laboratories) and visualized using the Chemiluminescence Detection System (Western Lightning Chemiluminescence Reagent Plus, Perkin–Elmer Life Sciences, Boston, MA, USA). The membrane was exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY, USA).

2.6. Vital staining with acridine orange

Cells were incubated for 24 h at 37 °C in a glass bottom dish (NonCoat 35 mm dish, Matsunami Glass, Tokyo Japan). After three washes with medium, cells were incubated for 4 h at 37 °C in medium containing apicularen A, apicularen B or bafilomycin A_1. The cells were then washed three times with Hanks' solution, and incubated for 10 min at 37 °C in Hanks' solution containing acridine orange (very high purity, Polysciences Inc., Warrington, PA, USA) at a concentration of 5 μg/ml [15]. After being washed with Hanks' solution, the cells were observed with a laser-scanning confocal microscope (LSM510META, Olympus) under fluorescence emission at 568, 579, 589, 600, 611, 632 and 643 nm with excitation at 488 nm.

2.7. Proton transport assay using microsome vesicles prepared from mouse peritoneal macrophages

Eight-week-old ddY male mice (Nihon SLC, Hamamatsu, Japan) were injected intraperitoneally with 2 ml of a 3% thioglycolate medium (Difco, Detroit, MI, USA). After 4 days, peritoneal macrophages were harvested by peritoneal lavage with 6 ml Hanks' balanced salt solution. The cells were washed twice with PBS, and were homogenized at 4 °C using a Dounce homogenizer (20 strokes) in a buffer (5 mM Tris, pH 7.0, 250 mM sucrose, 1 mM EGTA, 1 mM $KHCO_3$ and 1 mM dithiothreitol). After an initial centrifugation ($1000 \times g$ for 5 min at 4 °C), the supernatant was centrifuged at $6000 \times g$ for 15 min at 4 °C. The resulting supernatant was centrifuged at $42000 \times g$ for 30 min at 4 °C and the final pellet, a microsomal fraction, was stored at −80 °C.

Proton transport by the isolated macrophage microsome was assayed in a dual wavelength spectrophotometer (UV-3000, Shimadzu, Kyoto, Japan) by measuring uptake of acridine orange in a reaction

medium (10 mM bis-Tris-propane, pH 7.0, 10 μM acridine orange, 150 mM KCl, 2 mM $MgCl_2$, 1 μM valinomycin and 5 μg/ml oligomycin) [16]. The reaction was initiated by adding ATP (final concentration 1 mM). Different concentration of test compounds were added 10 min before the addition of ATP. Proton transport was monitored by measuring absorption change of acridine orange at 492–540 nm (an index of the amount of acridine orange in a reaction medium). The initial rate of change in the absorbance of acridine orange was used for the calculation of IC_{50} values.

2.8. Determination of ATP hydrolysis activity by V-, F- and P-ATPases

Hydrolysis of ATP by V-ATPase was determined by the bafilomycin A_1-sensitive assay method. The microsome fraction prepared from mouse peritoneal macrophages (3.7 μg protein) was incubated for 30 min at 37 °C in 1 ml of the reaction buffer (10 mM HEPES–Tris, pH 7.0, 1 mM ATP, 5 mM $MgCl_2$, 50 mM KCl, 5 μM valinomycin, 5 μM nigericin, 1 mM orthovanadate, 10 μg/ml oligomycin, 10 mM NaN_3, 1 mM levamisole and 10 mM NaF) in the presence or absence of 10 nM apicularen A, apicularen B or bafilomycin A_1. The reaction was stopped by the addition of 2 ml of the ice-cold stop solution containing 3.6% ammonium molybdate and 12% perchloric acid. Then, 6 ml of *n*-butyl acetate was added, mixed vigorously and the mixture was centrifuged at $1500 \times g$ for 10 min. Inorganic phosphate in the supernatant was measured from the absorbance at 350 nm.

For the determination of F-ATPase, mouse liver mitochondria was prepared according to the method described Wlodawer et al. [17] from 9-week-old ddY mice (Nihon SLC). Hydrolysis of ATP by F-ATPase was determined by the oligomycin-sensitive assay method. The mitochondria fraction prepared from mouse liver (0.13 μg protein) was incubated for 30 min at 37 °C in 1 ml of the reaction buffer (10 mM HEPES–Tris, pH 7.0, 1 mM ATP, 5 mM $MgCl_2$, 50 mM KCl, 5 μM valinomycin, 5 μM nigericin, 1 mM orthovanadate, 10 nM bafilomycin A_1, 1 mM levamisole and 10 mM NaF) in the presence or absence of 10 nM apicularen A, apicularen B or 0.1 μg/ml of oligomycin A. Inorganic phosphate released was determined as described above.

For the determination of P-ATPase, rabbit stomach mucus membrane microsome fraction was prepared according to the method described by Wolosin and Forte [18] from 13-week-old Japanese white rabbits (Nihon SLC). Hydrolysis of ATP by P-ATPase was determined by the orthovanadate-sensitive assay method. The stomach mucus microsome (0.21 μg protein) was incubated for 30 min at 37 °C in 1 ml of the reaction buffer (150 mM Tris–HCl, pH 7.4, 1 mM ATP, 2 mM $MgCl_2$, 100 μM valinomycin, 20 mM KCl) in the presence or absence of 10 nM apicularen A, apicularen B or 10 μM of sodium orthovanadate according to the method described by Yoda and Hokin [19]. Inorganic phosphate released was determined as described above. The mice and rabbits were treated in accordance with the procedures approved by the Animal Ethics Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan.

3. Results and discussion

On treatment with apicularen A at 100 nM, the condensation of nuclei and chromatin, a morphological change characteristic of apoptosis [20], was observed at 24 h (Fig. 2B), while apicularen B at 100 nM had no such effect (Fig. 2C). The specific V-ATPase inhibitor bafilomycin A_1 [7] at 100 nM also induced morphological changes in the nuclei at 24 h (Fig. 2D). The inset in each panel in Fig. 2 shows a magnified nucleus. These morphological changes in nuclei at 24 h were also observed at 10 nM of apicularen A and bafilomycin A_1, but not at 1 nM (not shown in Fig. 2). In accordance with the mor-

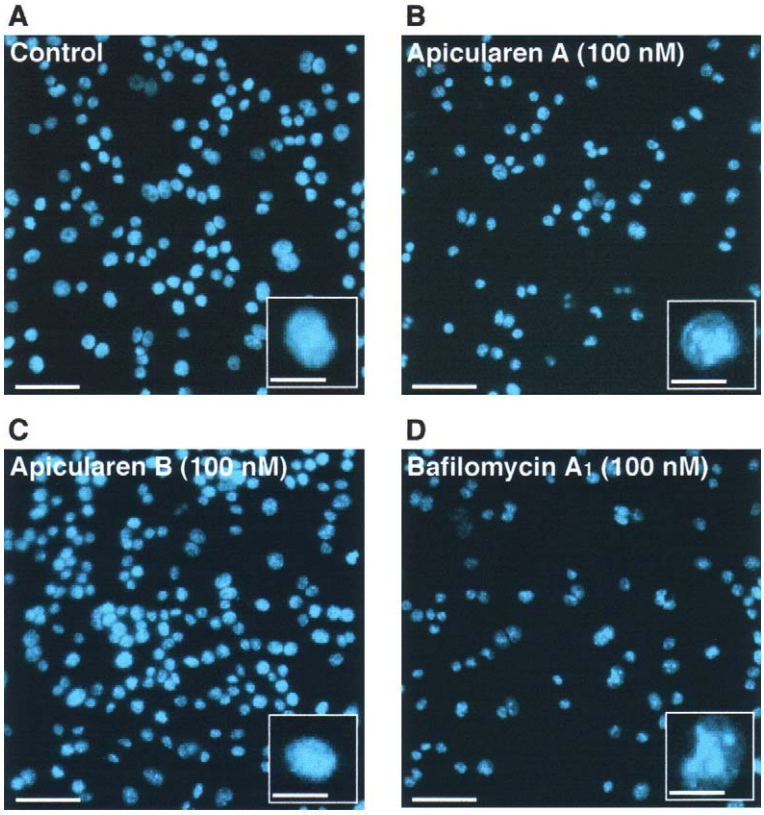

Fig. 2. Effects of apicularen A, apicularen B and bafilomycin A_1 on morphological changes of nuclei. After incubation in the presence or absence (A) of apicularen A (B), apicularen B (C) or bafilomycin A_1 (D) each at 100 nM for 24 h at 37 °C, the cells were stained with the DNA-specific fluorochome Hoechst dye 33258. The bar represents 50 μm. The inset in each panel represents a typical magnified nucleus. The bar in the inset represents 5 μm.

phological changes of nuclei on treatment with apicularen A (100 nM) for 24 h, disruption of the mitochondrial membrane potential was observed at 24 h in cells treated with apicularen A (100 nM) (Fig. 3A and B). In addition, as reported previously, the percentage of annexin V-positive cells and propidium iodide-negative cells at 24 h was increased by apicularen A (100 nM) [5]. The percentage of early apoptotic cells (annexin V-positive and propidium iodide-negative cells) and necrotic cells (annexin V-positive and propidium iodide-positive cells) was increased time-dependently by apicularen A at 100 nM (data not shown). In contrast, apicularen B at 100 nM had no effect on the mitochondrial membrane potential (Fig. 3A and B). On the other hand, the V-ATPase inhibitor bafilomycin A_1 at 100 nM also disrupted the mitochondrial membrane potential (Fig. 3A and B). At 10 nM, apicularen A and bafilomycin A_1 induced disruption of the mitochondrial membrane potential, but no significant change was observed at 1 nM (not shown in Fig. 3). The potency of apicularen A for the induction of apoptosis was almost the same as that of bafilomycin A_1. Induction of apoptosis in RAW 264.7 cells by bafilomycin A_1 has already been reported [21]. Previously, we reported that apicularen A induces apoptosis of RAW 264.7 cells as evidenced by the formation of a DNA ladder, an increase in the percentage of sub-G_1 cells and annexin V-binding cells, and the activation of caspase [5]. The morphological changes in nuclei (Fig. 2) and the disruption of mitochondrial membrane potential (Fig. 3) by apicularen A further support our previous findings that apicularen A induces apoptosis in RAW 264.7 cells more potently than apicularen B [5].

By Western blotting analysis using an antibody to the C subunit of V-ATPase, we examined whether RAW 264.7 cells have V-ATPase. V-ATPase is composed of a peripheral V_1 domain having 8 subunits (A–H) responsible for ATP hydrolysis, and an integral Vo domain having 5 subunits (a, d, c, c′ and c″) responsible for proton translocation [22]. The C subunit of V-ATPase, the molecular weight of which is 42 kDa, is an auxiliary subunit with ubiquitous expression [23,24]. As shown in Fig. 4, a band for the C subunit of V-ATPase was detected in RAW 264.7 cells. The band was also detected in A431 cells which have been reported to have V-ATPase as determined by vital staining with acridine orange [15,25]. These results indicate that RAW 264.7 cells also have V-ATPase. V-ATPase has been identified in cellular organelles belonging to the central vacuolar system [22], and are the most probable cause of the acidity in the central vacuolar system. The internal acidification of intracellular compartments has been suggested to play a critical role in the mechanism of cell survival [7]. Inhibition of V-ATPase by bafilomycin A_1 [8] and concanamycin A [9] induced apoptosis in several kinds of cancer cells [10,11],

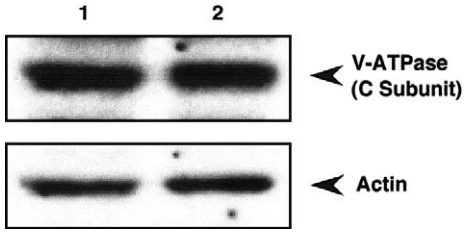

Fig. 4. Detection of V-ATPase in RAW 264.7 cells and A431 cells. The cells were sonicated in a sample buffer, centrifuged at $15000 \times g$ and 4 °C for 5 min, and 5 μg protein of the supernatant was loaded on a 7.5% SDS–polyacrylamide gel. After electrophoresis, the C subunit of V-ATPase and actin were detected by Western blotting, respectively. Lane 1: RAW 264.7 cells and lane 2: A431 cells.

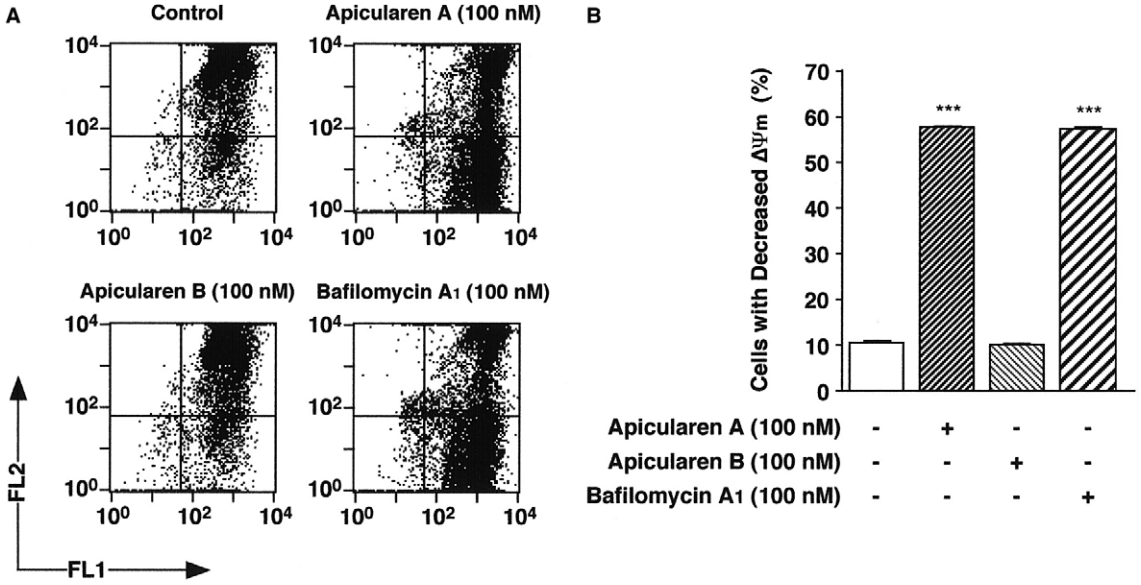

Fig. 3. Effects of apicularen A, apicularen B and bafilomycin A_1 on the mitochondrial membrane potential. RAW 264.7 cells (1×10^6 cells) were incubated for 24 h at 37 °C in 2 ml of medium. The cells were then washed three times with medium, and further incubated for 24 h at 37 °C in the presence (+) or absence (−) of apicularen A, apicularen B or bafilomycin A_1 each at 100 nM. After incubation, the cells were washed three times with PBS, scraped off the plate and stained using a DePsipher™ Kit. Subsequently, the intensities for green fluorescence (FL1; a maximal emission at 530 nm) and red fluorescence (FL2; a maximal emission at 590 nm) were analyzed by flowcytometry (A), and the percentage of the cells with decreased mitochondrial membrane potential ($\Delta\psi_m$) in the lower right quadrant was calculated (B). Values are the means from four samples with the S.E.M. shown by vertical bars. Statistical significance: $^{***}P < 0.001$ vs. the non-stimulated control.

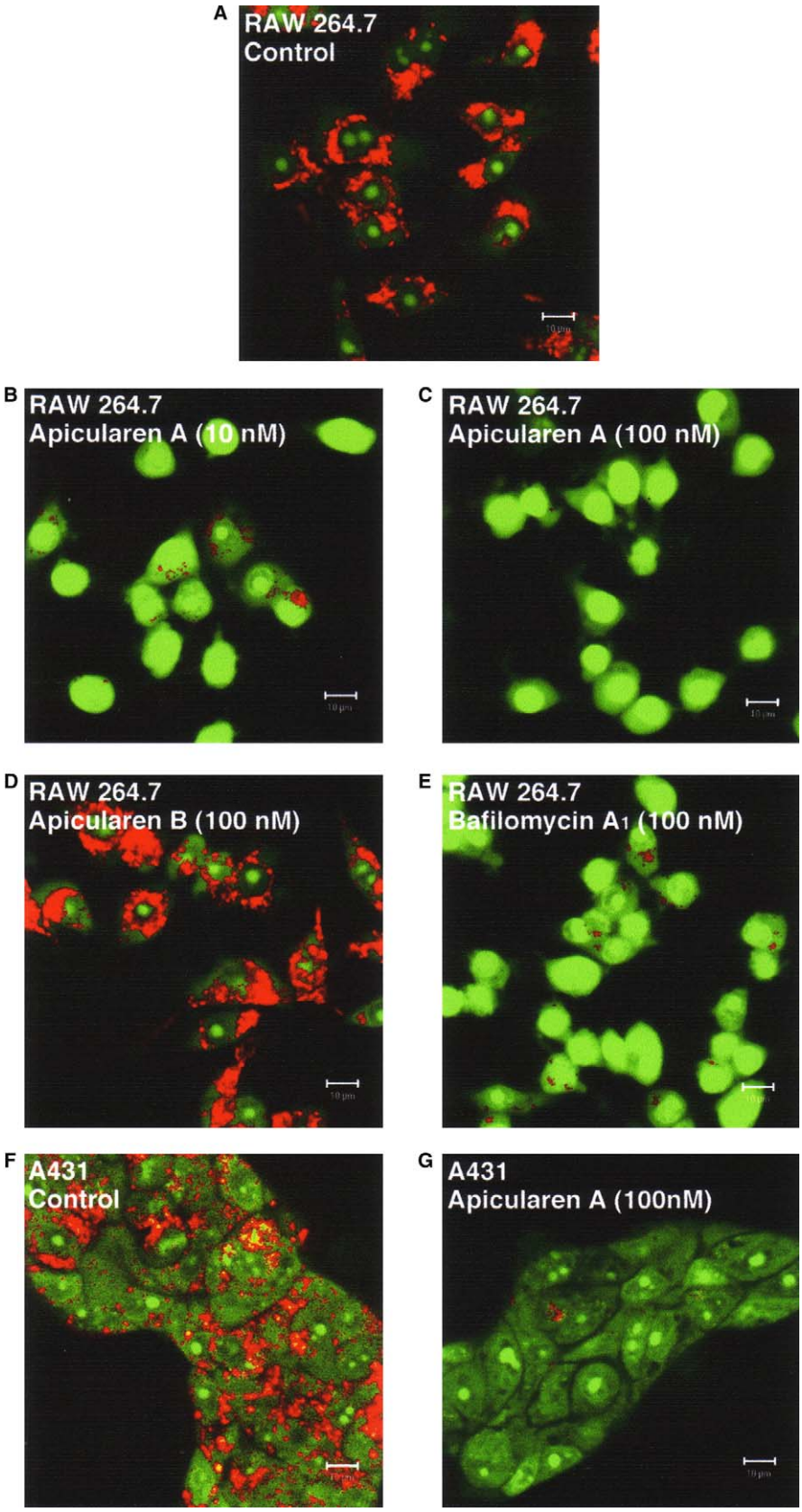

Fig. 5. Vital staining with acridine orange. RAW 264.7 cells were incubated for 4 h at 37 °C in medium containing apicularen A at 0 nM (A), 10 nM (B) and 100 nM (C), apicularen B at 100 nM (D), and bafilomycin A_1 at 100 nM (E). A431 cells were incubated for 4 h at 37 °C in medium containing apicularen A at 0 nM (F) and 100 nM (G). The cells were then stained with acridine orange for 10 min, washed with Hanks' solution, and observed with a laser-scanning confocal microscope. Each bar indicates 10 μm.

and caspase-3 is activated during the process of apoptosis induced by concanamycin A in B cell hybridoma HS-72 cells [11].

Incubation of RAW 264.7 cells with acridine orange stained intracellular organelles in orange fluorescence (Fig. 5A). Acridine orange is an acidophilic weak base and taken up by living cells and accumulates in acidified compartments [15,25,26]. In cells, fluorescence of acridine orange is green at low concentrations, but changes to orange at high concentrations [15,25]. Upon treatment with apicularen A at 10 and 100 nM for 4 h, the orange fluorescence in the intracellular organelles was almost completely diminished (Fig. 5B and C, respectively), however, apicularen A at 1 nM had no effect (not shown in Fig. 5). The effect of apicularen A at 10 and 100 nM on the vital staining was observed even 2 h after incubation (not shown in Fig. 5). Treatment with the V-ATPase inhibitor bafilomycin A_1 at 100 nM for 4 h also decreased the orange fluorescence in the intracellular organelles in RAW 264.7 cells (Fig. 5E). The dose–response analysis in the vital staining with acridine orange revealed that apicularen A was almost as potent as bafilomycin A_1 (not shown in Fig. 5). Also in A431 cells, treatment with apicularen A at 100 nM for 4 h decreased the orange fluorescence (Fig. 5F and G). It is reported that the V-ATPase inhibitors bafilomycin A_1 and concanamycin F decreased the orange fluorescence in A431 cells [15,25]. Recently, Huss et al. [27] have shown that the acidity of lysosomes in PtK2 cells was decreased by apicularen A at 110 nM using the acidotropic reagent LysoTracker. According to Yoshimori et al. [15], bafilomycin A_1 decreased the orange fluorescence almost completely at 1 μM in A431 cells, while it reduced partially at 10 nM and almost completely at 100 nM in BNL CL.2 cells, a mouse normal embryonic liver cell line, indicating that the response to bafilomycin A_1 seems to differ with the type of cell. In a cell-free system, bafilomycin A_1 inhibited V-ATPase at much lower concentrations than cell culture system; the IC_{50} values were 0.04 nM for a human kidney cortex membrane preparation, 0.36 nM for a human liver membrane preparation, and 0.06 nM for a human osteoclastic tumor cell membrane preparation [4]. In our cell culture system, the IC_{50} value of bafilomycin A_1 and apicularen A in RAW 264.7 cells and A431 cells was estimated to be between 1 and 10 nM. However, in the cell-free system using the inside-out microsome vesicles prepared from mouse peritoneal macrophages, the IC_{50} values for ATP-dependent proton transport into the

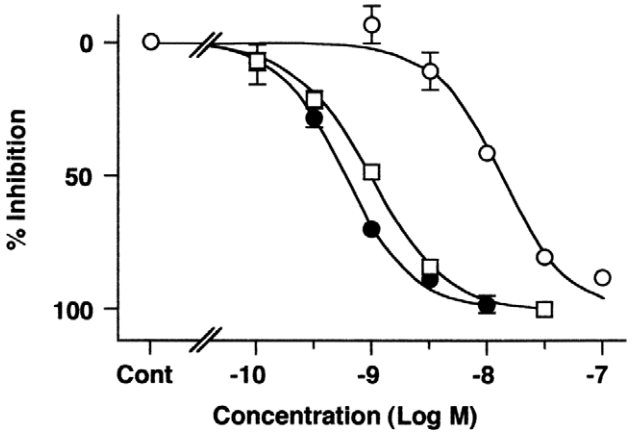

Fig. 6. Effects of apicularens A and B and bafilomycin A_1 on ATP-dependent protone transport using microsome vesicles. Protone transport was assayed by monitoring absorption changes of acridine orange at 492–540 nm (an index of the amount of acridine orange in the buffer). The initial rate of change in the absorbance of acridine orange was used for calculation of the IC_{50} value. Apicularen A (closed circle), apicularen B (open circle) or bafilomycin A_1 (open square) was added 10 min before the addition of ATP. Values are the means from triplicate determinations with the S.E.M. shown by vertical bars.

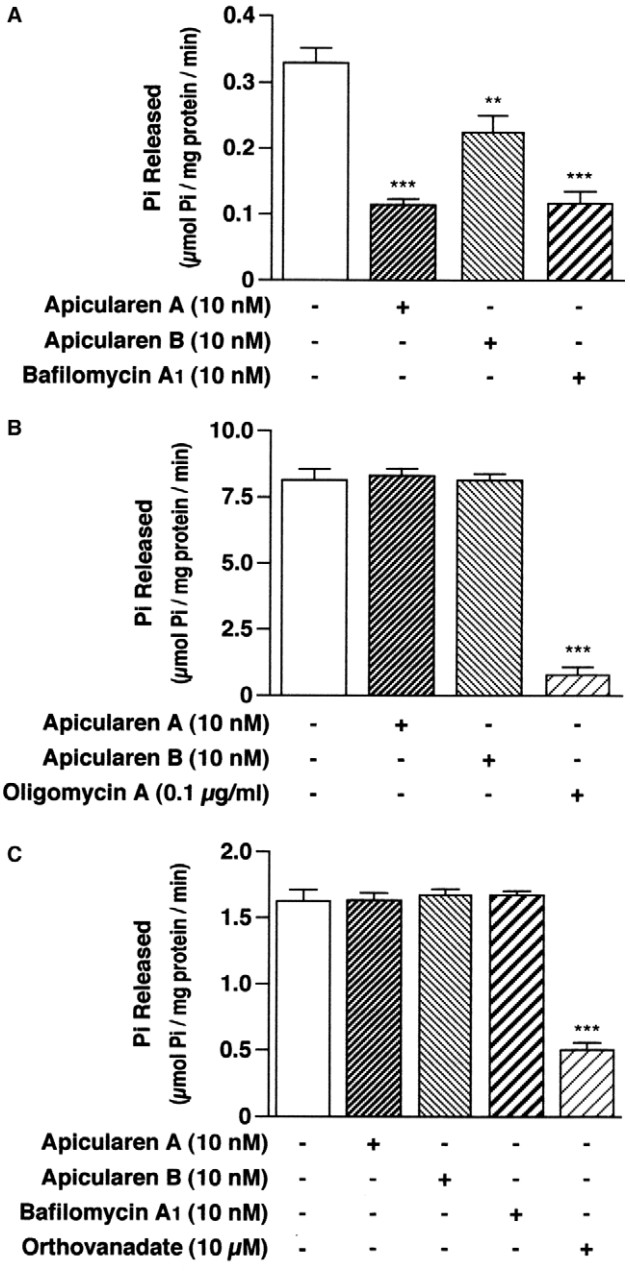

Fig. 7. Effects of apicularens A and B on V-, F- and P-ATPases. Effects of apicularens A and B on ATP hydrolysis activity were determined in the bafilomycin A_1-sensitive assay system for V-ATPase using mouse peritoneal macrophage microsome (A), the oligomycin A-sensitive assay system for F-ATPase using mouse liver mitochondria (B), and the orthovanadate-sensitive assay system for P-ATPase (H^+, K^+-ATPase) using rabbit stomach mucus membrane microsome (C). ATPase activity is expressed as μmol Pi/mg protein/min. Values are the means with S.E.M. shown by vertical bars. Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the control group.

vesicles were 0.58 nM for apicularen A, 0.95 nM for bafilomycin A_1 and 13 nM for apicularen B (Fig. 6), indicating that the concentration required for the inhibition of V-ATPase is less in the cell-free system than in the cell culture system. Based on the IC_{50} values for the ATP-dependent proton transport into the vesicles, apicularen A is more potent than bafilomycin A_1 and about 20 times more potent than apicularen B. The difference in the IC_{50} value between the cell-free system and the cell culture system might be due to the difference in the assay system; in the cell culture system, a higher concentration of the inhibitor might be necessary to penetrate the cells and inhibit the V-ATPase. To further confirm that apicularens A and B inhibit V-ATPase, we analyzed the effect of apicualrens A and B on ATP hydrolysis activity in the V-ATPase inhibitor bafilomycin A_1-sensitive assay system. As shown in Fig. 7A, apicularen A inhibited Pi release from ATP to the same extent with that by bafilomycin A_1 at 10 nM, while apicularen B at 10 nM showed less inhibitory activity than apicularen A. Apicularens A and B did not inhibit Pi release from ATP by rat liver mitochondrial F-ATPase, which was inhibited by the F-ATPase inhibitor oligomycin A at 0.1 μg/ml (Fig. 7B). In addition, apicularens A and B at 10 nM did not inhibit Pi release from ATP by rabbit stomach mucus membrane P-ATPase, which was inhibited by the P-ATPase inhibitor sodium vanadate at 10 μM (Fig. 7C). These findings further supported that apicualrens A and B specifically inhibit V-ATPase; the potency of apicularen A is higher than that of apicularen B. Recently, Huss et al. [27] have reported that apicularens A and B inhibit V-ATPase purified from the midgut of the tobacco hornworm, *Manduca saxta*, with IC_{50} values at 20–60 nM. Taken together, apicularens A and B inhibit mammalian V-ATPase and worm V-ATPase.

In culture of RAW 264.7 cells, our findings indicated that apicularen A inhibited V-ATPase, thus the pH in the intracellular organelles was increased and the uptake of acridine orange into the cellular organelles was decreased, resulting in a change from orange fluorescence to green fluorescence. In contrast, apicularen B at 100 nM had no effect on the vital staining of acridine orange in RAW 264.7 cells (Fig. 5D) or A431 cells (not shown in Fig. 5) although it has a benzolactone enamide core, suggesting that apicularen B exhibits very little inhibitory activity toward V-ATPase in the cell culture system. These findings suggested that apicularen A inhibits V-ATPase, as does bafilomycin A_1, much more potently than apicularen B, and thus inhibits the vital staining with acridine orange in the intracellular organelles. Less cytostatic activity of apicularen B than apicularen A is also reported in human cancer cell lines [1]. The weaker biological activity of apicularen B than apicularen A might be explained by the glycosylation of apicularen A. For example, the glycosylation of isoflavones reduced the inhibitory effect of isoflavones on 12-*O*-tetradecanoylphorbol 13-acetate-induced prostaglandin E_2 production in rat peritoneal macrophages [28]. The very weak inhibitory activity of apicularen B toward vital staining with acridine orange compared with that of apicularen A (Fig. 5) was consistent with the effect on morphological changes in nuclei (Fig. 2C) and the mitochondrial membrane potential (Fig. 3A and B). Our findings suggested that the inhibition of V-ATPase participates in apicularen A-induced apoptosis in RAW 264.7 cells. Previously, Huss et al. [29] reported that the pleomacrolidic type V-ATPase inhibitors bafilomycin A_1 and concanamycin A bind to subunit c in the V_0 domain of V-ATPase, while salicylihalamide, a V-ATPase inhibitor having a benzolactone enamide core, did not block the binding of concanamycin A to the subunit c. Recently, they have reported that apicularen A also did not bind to subunit c [27]. Xie et al. [30] also reported that salicylihalamide A inhibits V-ATPase through a mechanism distinct from bafilomycin A_1. Therefore, the mechanism of action of apicularen A for the inhibition of V-ATPase seems to differ from that of the pleomacrolidic type V-ATPase inhibitors bafilomycin A_1 and concanamycin A.

We conclude that apicularen A inhibits V-ATPase in RAW 264.7 cells, thus inducing the apoptosis of RAW264.7 cells. The potency of apicularen A for the inhibition of V-ATPase is greater than that of bafilomycin A_1 and apicularen B as determined by ATP-dependent proton transport into inside-out microsome vesicles.

Acknowledgements: This work was supported in part by a fund from the Ministry of Health and Social Welfare of Korea, and by a Grant-in-Aid for Scientific Research (11470481) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. JangJa Hong is a visiting researcher from the Graduate School of Pharmacy, Sungkyunkwan University, Korea.

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