Full Paper

A Potent Apoptosis-Inducing Activity of a Sesquiterpene Lactone, Arucanolide, in HL60 Cells: a Crucial Role of Apoptosis-Inducing Factor

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Received July 9, 2004; Accepted December 16, 2004

Abstract. Six main sesquiterpene lactones (germacranolides) from *Calea urticifolia* were evaluated for in vitro cytotoxicity against human tumor cell lines HL60 and SW480 cells. Among them, arucanolide and parthenolide displayed marked cytotoxicity against both cell lines. Arucanolide exhibited a low IC₅₀ in HL60 cells. The cytotoxic activity of arucanolide was observed at lower concentrations compared to that of parthenolide, which has been reported to be a typical and simple germacranolide. The activity was found to be mainly due to apoptosis that was assessed by morphological findings, DNA ladder formation (24–36 h), and flow cytometric analysis in HL60 cells. Western blotting and an apoptosis inhibition assay using caspase inhibitors did not demonstrate the activation of any caspases tested. However, the mitochondrial membrane potential of HL60 cells was lost after 24-h treatment with arucanolide, and concurrently apoptosis-inducing factor (AIF) released from mitochondria was detected by Western blot analysis. The inactivation of nuclear factor- κ B, which has been commonly shown in parthenolide-induced apoptosis, did not occur in arucanolide-induced apoptosis. Taken together, the findings presented here indicate that arucanolide induced marked apoptosis in HL60 cells mainly by dissipating mitochondrial membrane potential, which would trigger AIF-induced apoptosis.

Keywords: arucanolide, parthenolide, sesquiterpene lactone, apoptosis, apoptosis-inducing factor

Introduction

Increasing attention has been paid to primitive medicinal plants to find new substances with potentially useful biological activities. Recently, sesquiterpene lactones with a germacran skeleton were isolated from *Calea urticifolia*, which has been used in El Salvador as a traditional medicinal plant (1), and examined for effects on cytotoxicity. With relation to sesquiterpene lactones, parthenolide, which is the basic germacranolide, is regarded as one of the tumor suppressive agents (2). The bioactive effect of parthenolide is mediated by preventing nuclear factor- κ B (NF- κ B) signaling (3–5).

In the course of our phytochemical study to search for biologically active compounds, five main sesquiterpene lactones from *Calea urticifolia* were subjected to cytotoxic screening. In the present study, we have demonstrated the cytotoxicity in a series of the sesquiterpene lactones, particularly germacranolides against human cultured cancer cells. In addition, we have discussed the structure-activity relationship in these compounds. Furthermore, it was shown that the apoptosis-inducing effect of arucanolide on HL60 cells was mainly caused by loss of the mitochondrial membrane potential and a concurrent apoptosis-inducing factor (AIF) activation, indicating a different mechanism from that in parthenolide-induced apoptosis.

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Materials and Methods

Plant material

Leaves of *Calea urticifolia* (Miller) DC. were purchased at medicinal market in San Salvador in 1998 and identified by M.I. The voucher specimen has been kept at his laboratory in Gifu Pharmaceutical University (No. EL-091).

Extraction and isolation

The air-dried leaves (170 g) were extracted with acetone by reflux to obtain a crude extract (9.8 g). The extract was suspended in H₂O and extracted with CHCl₃ three times. The concentrated CHCl₃ extract (7.1 g) was subjected to silica gel column chromatography eluted by a CHCl₃-MeOH solvent system from the ratio of 30:1 to 10:1 to give 10 fractions. Fraction 2 (2.1 g) was separated by reversed-phase silica gel column chromatography eluted with 40 and 65% MeOH. A concentrated syrup of 65% MeOH elution was further purified with reversed phase HPLC (Mightysil RP-18 250-20; Kanto Chemical Co., Inc., Tokyo). The HPLC conditions were as follows: flow rate, 11.3 ml/min; detector, 210 nm; solvent, 42% CH₃CN; column oven temperature, 40°C. The values of retention time in min (yield) were as follows: calealactone A, 38.3 (52 mg); 2,3-epoxy-calealactone A, 25.3 (91 mg); calealactone B, 13.5 (30 mg); calealactone C, 20.2 (28 mg); arucanolide, 18.1 (173 mg); juanislinamin, 35.1 (138 mg); and 2,3-epoxy-juanislinamin, 23.3 (195 mg). All isolated compounds have been kept at the laboratory of one of authors (N.M.) in Okayama University of Science (1).

Reagents

Several sesquiterpene lactones with a germacrane skeleton were isolated from leaves of *Calea urticifolia* (Compositae), and their structures were determined (1). Five compounds among them (arucanolide, calealactone A, 2,3-epoxy-calealactone A, calealactone B, and 2,3-epoxy-juanislinamin) and parthenolide as a control were examined against two tumor cell lines. They were prepared in DMSO at the concentration of 10 mM and then further diluted to the working concentration before use. Resveratrol (Sigma-Aldrich Co., St. Louis, MO, USA) was used as a reference because resveratrol, which is a kind of natural polyphenol, is well-known to have strongly cytotoxic activity against cancer cells. Furthermore, arsenic trioxide (Sigma-Aldrich Co.) was used as a reference because it induced caspase-dependent apoptosis in HL60 cells (6, 7).

Cell culture, morphological study, and cell viability

We used two human tumor cell lines. The SW480 cell

line was used as a representative of solid tumor cells (colon cancer) and the HL60 cell line was used as a representative of hematopoietic tumor (leukemia). They were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma, Tokyo) and 2 mM L-glutamine under an atmosphere of 95% air and 5% CO₂ at 37°C. Human peripheral blood lymphocytes (PBL) were isolated from blood of healthy donor by centrifuge with Ficoll-Paque Plus (Amasham Biosciences, Uppsala, Sweden). PBL were stimulated with concanavalin-A (15 µg/ml) for 48 h and used for growth suppression of arucanolide or other compounds. The evaluation of cell growth was determined by the trypan blue dye-exclusion assay. For evaluating IC₅₀, the starting cell number was 2 × 10⁵/ml. For evaluating apoptotic cell death, cells were seeded at a density of 2 × 10⁵/ml in 15-mm-diameter wells and cultured for 12 h, and then DNA ladder formation was examined at various times after the start of treatment with the compounds. For morphological examination of apoptotic changes, cells were stained with Hoechst 33342 (5 µg/ml) at 37°C for 30 min, washed twice with phosphate-buffered saline (PBS), pipetted dropwise onto a glass slide, and examined by fluorescence microscopy using a microscope (Olympus, Tokyo) equipped with an epi-illuminator and appropriate filters.

Analysis of DNA fragmentation by agarose gel electrophoresis

Cellular DNA was extracted from whole cells by the procedure described previously (8). RNase was added to the DNA solution at the final concentration of 20 µg/ml, and the mixture was incubated at 37°C for 30 min. After electrophoresis on a 2.5% agarose gel, DNA was visualized by ethidium bromide staining.

Western blot analysis

Before and after treatment with arucanolide, HL60 cells were washed twice with PBS; lysed in lysis buffer A, B, or C, depending on the preparation; and then homogenized. Lysis buffer A (2 × PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 25 × Complete[®], a mixture of protease inhibitors (Roche, Penzberg, Germany)) was used to analyze caspase-3, -8, -9, -2, and BID. Lysis buffer B (250 mM sucrose, 20 mM Hepes-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 25 × Complete[®]) was used to analyze AIF, cytochrome c, and endonuclease G. Lysis buffer C (250 mM sucrose, 20 mM Hepes-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% Nonidet P-40, 25 × Complete[®], and Phosphatase Inhibitor Cocktail[®] 1 and 2 (Sigma-Aldrich Co.)) was

used to analyze MAP kinases, p-I κ B, Bad, p-Bad, and Bcl-xL. The mitochondrial and cytosolic fractions were prepared as reported previously (9). Ten micrograms of protein of each cell lysate was separated by SDS-PAGE by using an adequate percent of polyacrylamide in the gel and electroblotted onto a PVDF membrane (Du Pont, Boston, MA, USA). After blockage of nonspecific binding sites for 1 h by 5% nonfat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated overnight at 4°C with various antibodies. They include anti-human caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-human caspase-8 (MBL, Nagoya); anti-human caspase-9 (Novus Biologicals Inc., Littleton, CO, USA); anti-human caspase-2 (MBL) and anti-human Bid (Cell Signaling Technology Inc., Beverly, MA, USA); anti-human AIF (ProSci Inc., Poway, CA, USA); anti-human cytochrome c (Upstate Biotechnology Inc., Lake Placid, NY, USA); anti-human endonuclease G (Sigma-Aldrich Co.); anti-human p44/42 MAP kinase (p44/42), anti-human phospho-p44/42 MAPK (Thr202/Tyr204) (p-p44/42), anti-human SAPK/JNK (JNK), anti-human phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK), anti-human p38 MAP kinase (p38), anti-human phospho-p38 MAP kinase (Thr180/Tyr182) (p-p38), anti-human Bad, anti-human phospho-Bad (Ser112) (p-Bad) (Cell Signaling Technology Inc.); anti-human Bcl-xL (H-5) (Santa Cruz Biotechnology); anti-human phospho-I κ B- α (Ser32) (p-I κ B) (Cell Signaling Technology Inc.); and anti-human β -actin (Sigma-Aldrich Co.). The membrane was then washed three times with TPBS, incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega, Madison, WI, USA), or anti-rabbit antibody (New England Biolabs, Beverly, MA, USA) at room temperature, and then washed three times with TPBS. The immunoblot was visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs).

Inhibition of apoptosis by caspase inhibitors

For the study of inhibition of apoptosis, the tripeptide pan-caspase inhibitor Z-VAD-FMK (MBL), caspase-3 inhibitor Z-DEVD-FMK (MBL), caspase-8 inhibitor Z-IETD-FMK (MBL), or caspase-2 inhibitor Z-VDVAD-FMK (MBL) was added in the culture medium 12 h before treatment with arucanolide. Optimal concentration of the inhibitor was determined from the dose-response curve for the extent of cell death. Each caspase inhibitor was used at the concentrations 50–100 μ M. Inhibition of apoptosis by the inhibitors was evaluated by the blockage of the process of nucleosomal DNA fragmentation, which was observed as ladder formation.

RT-PCR

Before and after treatment with arucanolide, HL60 cells were washed twice with PBS. RT-PCR was performed as described previously (10). In brief, total cellular RNA of HL60 cells was isolated by the phenol/guanidium thiocyanate method with DNase I treatment. By reverse transcription of 2 μ g of total RNA, cDNAs were obtained, and amplification of the respective cDNA region was conducted by PCR. PCR primers were used ApoPrimer Set (Bcl-2 family) (Takara Bio, Inc., Shiga). β -Actin cDNA was used for an internal standard. The PCR reaction consisted of 30 cycles (94°C for 30 s, 57.5°C for 1 min, 72°C for 1 min) after an initial denaturation step (95°C for 1 min). PCR products were analyzed by electrophoresis on 2% agarose gels.

Measurement of mitochondrial membrane potential and assessment of cell death by FACS

Mitochondrial membrane potential was measured by use of a fluorescent dye, Mito-Tracker Green (#M-7514; Molecular Probes, Eugene, OR, USA) that estimates the mitochondrial volume and Mito-Tracker Orange (#M-7511, Molecular Probes) that accumulates selectively in active mitochondria and becomes fluorescent when oxidized. Assessment of cell death was made by using propidium iodide (PI) (MBL) and Annexin V (MBL). The cells were treated with 3, 5, or 6 μ M arucanolide for 24 h. After the cells were washed twice with RPMI-1640 medium, the arucanolide-treated or untreated cells were incubated with Mito-Tracker fluorescent probes, PI, or Annexin V (100 nM each) for 30 min at 37°C. After the cells were collected and washed twice with PBS, the cells were resuspended in PBS. The fluorescence of Mito-Tracker Orange, Green, PI, and Annexin V was analyzed by flow cytometry, respectively (Becton Dickinson, San Jose, CA, USA) (10).

Secreted alkaline phosphatase (SEAP) activity

HeLa cells were cultured in Dulbecco's Eagle's medium (Nissui, Tokyo) containing 10% calf serum (Boehringer Mannheim, Mannheim, Germany) and 100 μ M non-essential amino acid (Invitrogen, Carlsbad, CA, USA) at 37°C. The day before transfection, 5.5×10^4 of HeLa cells were seeded on a 12-well tissue culture plate. pNF- κ B-SEAP (0.3 μ g/well) (BD Biosciences, CA, USA) or pSV-b-GAL (0.3 μ g/well) (Promega) vector was co-transfected with a EffecteneTM transfection reagent (QIAGEN, Hilden, Germany). After 16 h, the medium containing plasmid was exchanged to 1000 μ l of fresh growth medium without serum for 24 h. Arucanolide or parthenolide was added into each well,

and 1-h later, recombinant human tumor necrosis factor (TNF)- α (PeproTech House, London, UK) was added into the wells. After 24 h, the medium of each well was collected and assayed for the SEAP activity (11, 12). Measurement of β -galactosidase activity was performed by the method of Hall et al. (13).

Results

Cytotoxicity of sesquiterpene lactones from Calea urticifolia in SW480 and HL60 cells

We examined the effects of six sesquiterpene lactones

including parthenolide (Fig. 1) at various concentrations on the cytotoxicity in two different human tumor cell lines, SW480 and HL60, and the results are shown in Table 1. All compounds caused cytotoxicity against both cell lines, as judged by the trypan blue-exclusion test. Of these sesquiterpene lactones, arucanolid was found to induce greater cytotoxicity in both cell lines compared with other compounds. It was noted that the susceptibility of SW480 and HL60 cells to arucanolid was higher than that to parthenolide (Table 1). HL60 cells are more susceptible to these compounds than SW480 cells. Then we examined the mechanism for the

Fig. 1. Chemical structures of sesquiterpene lactones in *Calea urticifolia*. The numbers of the C-atoms are shown for arucanolid.

Table 1. Growth inhibitory effect (IC_{50}) of the sesquiterpene lactones

a) SW480 and HL60 cells

Cell	Arucanolid	Calealactone A	2,3-Epoxy-calealactone A	Calealactone B	2,3-Epoxy-juanislamin	Parthenolide
SW480	5.6 ± 0.1	10.0 ± 0.8	26.3 ± 2.9	27.5 ± 3.1	12.4 ± 1.4	8.4 ± 0.9
HL60	3.1 ± 0.3	9.1 ± 0.7	6.2 ± 0.7	4.2 ± 0.5	2.9 ± 0.3	3.8 ± 1.9

(μ M)

b) Concanavalin A-stimulated normal peripheral blood lymphocytes (PBLs)

	Arucanolid	Parthenolide	Resveratrol
PBLs	2.3 ± 0.3	1.1 ± 0.2	10.0 ± 0.2

(μ M)

PBLs used were cultured for 48 h in the presence of 15μ g/ml concanavalin A. The starting number of cells was 2×10^5 /ml. The values represent the mean $\pm$ S.D.

Fig. 2. Effect of sesquiterpene lactones on cell growth in human leukemia cell line HL60. a: Effect of arucanolide on cell growth of HL60 cells at various concentrations. Cell numbers after treatment with arucanolide were evaluated by the trypan-blue dye exclusion test. Open circle, 2 μ M; closed square, 4 μ M; closed triangle, 6 μ M; open square, 8 μ M; closed circle, 10 μ M arucanolide. b: Morphological aspects of the cells. The cells were stained with Hoechst 33342 (5 μ g/ml) for 30 min and then examined by fluorescence microscopy. 1), cells without treatment; 2), after treatment with 6 μ M arucanolide for 48 h. c: Nucleosomal DNA fragmentation of the cells after exposure to 6 μ M arucanolide. Three micrograms of DNA was loaded onto each lane. Lane M is a DNA size marker. d: Assessment of cell death by flow cytometric analysis. The cells stained with propidium iodide or Annexin V after treatment with 3, 5, or 6 μ M arucanolide for 24 h was analyzed by flow cytometry.

activity in HL60 cells exposed to arucanolide.

The growth of the HL60 cells was markedly suppressed by the arucanolide treatment at more than 4 μ M, as compared with the control without arucanolide treatment (Fig. 2a). In the treatment with 6 μ M arucanolide for 48 h, we observed apoptotic changes, which were assessed by morphological parameters (nuclear condensation and fragmentation) (Fig. 2b) and DNA ladder formation (24–36 h) in HL60 cells (Fig. 2c). These findings were further confirmed by the analysis of FACS using PI and Annexin V staining, as shown in Fig. 2d. Taken together, it was indicated that the marked cytotoxicity by arucanolide was attributed to apoptotic cell death.

Mechanism of arucanolide-induced apoptosis in HL60 cells

Apoptosis has been well known to be executed by the cascade activation of caspases initiators (e.g., caspase-8 and -9) and executioners (e.g., caspase-3 and -7). To determine which caspase(s) is involved in arucanolide-induced apoptosis, we examined formation of active forms of caspases in cell lysate by Western blot analysis. No processed active forms of the caspases tested were observed after the treatment with 6 μ M arucanolide, whereas the active form of caspase-3 was observed in HL60 cells after the treatment with arsenic trioxide (Fig. 3) (6, 7). Furthermore, pretreatment with the pan-caspase-like protease inhibitor Z-VAD-FMK, caspase-3 inhibitor Z-DEVD-FMK, caspase-8 inhibitor Z-IETD-FMK, or caspase-2 inhibitor Z-VDVAD-FMK did not cause inhibition of DNA ladder formation by arucanolide (data not shown). These results indicate that caspase may not be involved in the apoptosis.

Next we examined the mitochondrial pathway that plays a crucial role in propagation and determination of cell death. The mitochondrial membrane potential and the release of cytochrome c were examined in HL60 cells after the arucanolide treatment. In 24-h-treated cells, the mitochondrial membrane potential was markedly decreased in a concentration-dependent manner when examined by FACS analysis using the Mito-Tracker fluorescent probe (Fig. 4b). On the other hand, Western blot analysis did not show the apparent band of cytochrome c after the arucanolide treatment (Fig. 4a). Consequently, caspase-9, which is known to bind to the cytochrome c/Apaf-1 complex, was not activated after the treatment (Fig. 3). Then, we examined two other factors, AIF and endonuclease G, released from mitochondria in the apoptosis. Western blot analysis showed that the amount of released active AIF following the arucanolide treatment was increased in a time-dependent manner, but the active form of endonuclease G was not

Fig. 3. Arucanolide-induced cell death in HL60. Activation of caspase-3, -8, -9, and -2 and BID after the treatment with 6 μ M arucanolide was examined by Western blot analysis. Arsenic trioxide was used as a reference of caspase-dependent apoptosis.

detected (Fig. 4a). We found no concentration-dependency in released AIF at 36 h after the treatment with arucanolide (data not shown).

Bcl-2 protein exists on the mitochondrial membrane and inhibits the loss of mitochondrial membrane potential induced by apoptotic signals. On the other hand, the proapoptotic protein BAX in the cytosol translocates to mitochondria and promotes the loss of mitochondrial membrane potential (14). However, RT-PCR showed that the expression of Bcl-2 and BAX remained unchanged in the arucanolide-induced apoptosis (Fig. 4c).

Effect of arucanolide on signaling via NF- κ B

Parthenolide has been shown to inhibit NF- κ B (3–5), and the inhibition has induced cell death (4). We

Fig. 4. Arucanolide-induced cell death in HL60. a: Activation of AIF, cytochrome c, and endonuclease G after the treatment with 6 μ M arucanolide examined by Western blot analysis. b: Mitochondrial membrane potential after treatment with 3, 5, or 6 μ M arucanolide. The relative fluorescence intensity of Mito-Tracker Orange was measured by flow cytometry. The patterns of fluorescence intensity of Mito-Tracker Green were almost similar in the samples tested. c: Time course of Bcl-2 and BAX mRNA expression after the treatment with 6 μ M arucanolide.

examined the activation of NF- κ B in arucanolide-induced apoptosis (Fig. 5). When apoptotic stimuli including TNF- α cause phosphorylation of I κ B, NF- κ B is activated and translocates into nuclei. In our study, Western blot analysis showed that the level of phosphorylated I κ B was unchanged (Fig. 5a). When the cells transfected with pNF- κ B-SEAP vector were exposed to TNF- α , the cis-element for NF- κ B was functioning and the mRNA and protein expression of alkaline phosphatase was increased (11, 12). We examined the SEAP activity in the culture medium

after TNF- α treatment in the presence of parthenolide or arucanolide. Although parthenolide prevented the increase in SEAP activity, arucanolide did not (Fig. 5b), indicating that arucanolide did not block activation of NF- κ B.

Effect of arucanolide on MAP kinases

We examined the activation of MAP kinases (Fig. 6) in arucanolide-induced apoptosis. The level of p-p44/42 MAP kinase decreased within 4 h after the arucanolide treatment (Fig. 6). p-JNK was transiently increased at

Fig. 5. NF- κ B signaling in arucanolide-induced cell death. a: Phosphorylated form of I κ B in HL60 cells examined by Western blot analysis compared with those in parthenolide-treated cells. b: Secreted alkaline phosphatase (SEAP) activity in parthenolide- and arucanolide-treated HeLa cells. The method is described in Materials and Methods.

Fig. 6. Death-associated MAP kinase signaling of arucanolide-induced cell death in HL60 cells. p44/42 MAP kinase, p-p44/42 MAP kinase, JNK, p-JNK, p38, and p-p38 were examined by Western blot analysis.

1 h after the treatment (Fig. 6). p-p38 was transiently decreased at 1 h after treatment (Fig. 6). p44/42 MAP kinase has been known to inhibit apoptosis by phosphorylating Bad (Ser 112/155) (15–18); phosphorylated Bad (Ser 155) does not dimerize with Bcl-xL, leading to inhibition of apoptosis (18, 19). We did not observe significant changes in phosphorylated Bad (Ser 112) and Bcl-xL levels after the arucanolide treatment (data not shown).

Discussion

This study was undertaken to explore biological activities of the sesquiterpene lactones in *Calea urticifolia*. Six sesquiterpene lactones, including parthenolide were examined for effects on cytotoxicity. All compounds tested exhibited significant cytotoxic activity in HL60 and SW480 cells. Especially, arucanolide exerted a marked cytotoxic effect at less than 10 μ M against both cell lines and its cytotoxic activity is greater than parthenolide, which has been reported to have a potent anticancer effect (20–22).

Parthenolide, which is one of the most important

active ingredients in the herb European and American feverfew (*Tanacetum parthenium*), has been shown to inhibit NF- κ B (3–5). Moreover, parthenolide induces apoptosis through the activation of caspase-3 and necrosis through the disruption of the cell membrane in HL60 cells (20, 21). Parthenolide has been thought to be one of the promising chemotherapeutic agents because parthenolide triggers apoptosis in sarcomatoid hepatocellular carcinoma SH-J1 cells and also other hepatoma cell lines at low doses of 5–10 μ M (22). In the present study, we have found that arucanolide had a more potent cytotoxic effect compared to parthenolide as reflected in IC₅₀s against HL60 or SW480 cell lines. Furthermore, we found that the cytotoxicity was due to apoptosis mediated by loss of mitochondrial membrane potential and the concurrent AIF release in HL60 cells. Many chemopreventive agents act through induction of apoptosis which would result in inhibition of the carcinogenesis process. During the last several years, it has become increasingly clear that mitochondria play a major rate-limiting role in apoptosis. The decision /effector phase of the apoptotic process converges on mitochondria, where permeabilization of mitochondrial membranes is triggered; and apoptosis inducing factors such as cytochrome c, AIF, and endonuclease G are released. It has been reported that released AIF was gathered around the nuclei in the cytoplasm and partly translocated into nuclei after the treatment of the apoptogenic dolichyl monophosphate in U937 cells and that both caspase-3 and 8 inhibitors blocked the DNA fragmentation (23). AIF causes chromatin condensation and large scale DNA fragmentation of approximately 50 kb (24). Surely, it is not clear whether AIF directly contributes to DNA ladder formation. In our present study, it was shown that arucanolide-induced apoptosis was mediated by the mitochondrial pathway, and that the released AIF level was time-dependently increasing after exposure to arucanolide. Accordingly, our data raise the possibility that AIF, but not caspases, may play a crucial role in DNA ladder formation by one or more yet undefined mechanisms. It was also to be noted that the mechanism of apoptosis induced by arucanolide was different from that of parthenolide. Parthenolide inhibits the activation of NF- κ B (3–5), but arucanolide did not affect NF- κ B.

Traditionally, sesquiterpene lactones have been used as folk medicines because of their various bioactivities. For example, Tenulin and Helenalin were reported to have an ability to inhibit of DNA synthesis of Ehrlich ascites cells and P-388 cells (25–27). Artemisinin, a sesquiterpene lactone with an endoperoxide group, was used as an anti-malarial drug and was effective against both drug-resistant and cerebral malaria-causing strains

of *Plasmodium falciparum* (28). Artemisinin inhibits nitric oxide synthesis in cytokine-stimulated human astrocytoma T67 cells through the inhibition of NF- κ B activation (29). Costunolide, a naturally occurring sesquiterpene lactone, reduced the frequency of colonic aberrant crypt foci induced by azoxymethane (30) and was reported to cause a strong growth inhibition against HL60 cells with apoptotic chromatin condensation (31). Furthermore, costunolide suppresses gene expression of hepatitis B virus surface antigen in human hepatoma cells (32).

Parthenolide has been shown to improve endotoxic shock by reducing plasma nitrate/nitrite level and to reduce lung neutrophil infiltration in the rat sepsis model because it attenuated inducible nitric oxide synthase by inhibiting NF- κ B activity (33, 34). Patel et al. reported that parthenolide mimicked the effects of I κ B- α by inhibiting DNA binding activity of NF- κ B and manganese superoxide dismutase (Mn-SOD) expression, which leads to an enhancing paclitaxel-induced apoptosis of breast cancer cells (35). It was also reported that apoptosis by germacranolides such as tatridine A diacetate and ineupatorolide A was accompanied by an early release of cytochrome c from mitochondria, followed by both activation of caspase-3 and fragmentation of poly (ADP-ribose) polymerase-1 (36). Thus, the mechanism of arucanolide-induced apoptosis was found to be clearly distinct from that of other sesquiterpene lactones.

The levels of p-p44/42, p-JNK, and p-p38 were changed within 24 h after the arucanolide-treatment; however, their levels were restored over 24-h after treatment. We did not conclude that these changes were related to the apoptotic signals. We are making ongoing investigations about this.

IC₅₀ of arucanolide in mitogen-stimulated normal peripheral blood lymphocytes was observed at 2.3 μ M, but that of parthenolide was 1.1 μ M (Table 1b), suggesting that arucanolide is more plausible than parthenolide for application as a chemopreventive agent.

As to the structure-activity relationship of germacranolides tested, the cytotoxic activity observed in the present study was as follows: arucanolide > (parthenolide) > calealactone A > 2,3-epoxy-juanislamin > 2,3-epoxy-calealactone A = calealactone B. The presence of a double bond at C-2 and C-3 seems to be preferable to an epoxyl group. Esterification sometimes enhances the bioactivity as observed for aconitine. Arucanolide is esterified at C-8 and C-9 with two organic acids, which influences the cytotoxic activity. Hydrogenation of a side chain moiety often reduces the bioactivity, which is applied to a 2-methylbutyric acid moiety at C-9 in calealactone A and 2,3-epoxy-calealactone A. The carbon chain length of the organic acid is also important.

As seen in arucanolide, the acetyl group at C-8 may be more efficient in terms of the activity than a 2-methylacryl group.

We would conclude that arucanolide is more advantageous as a chemopreventive agent than parthenolide. Further experiments are required to assess the anticancer effect of arucanolide in an animal model and also to define the detailed mechanisms at the molecular level, which are under current progress in our laboratory.

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