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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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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 IC_{50} 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-κB, 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 germacrane skeleton were isolated from *Calea uritisifolia*, 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-κB (NF-κB) 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 H_2O and extracted with $CHCl_3$ three times. The concentrated $CHCl_3$ extract (7.1 g) was subjected to silica gel column chromatography eluted by a $CHCl_3$-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% CH_3CN; 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); juanislamin, 35.1 (138 mg); and 2,3-epoxy-juanislamin, 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-juanislamin) 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% CO_2 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 IC_{50}, the starting cell number was 2×10^5/ml. For evaluating apoptotic cell death, cells were seeded at a density of 2×10^5/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 $MgCl_2$, 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 $MgCl_2$, 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-IκB, 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-IκB-α (Ser32) (p-IκB) (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 inibitor was determined from the dose-response curve for the extent of cell death. Each caspase inhibitor was used at the concentrations 50 – 100 μM. 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 μg 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 μM 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) activety

HeLa cells were cultured in Dulbecco's Eagle's medium (Nissui, Tokyo) containing 10% calf serum (Boehringer Mannheim, Mannheim, Germany) and 100 μM 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-κB-SEAP (0.3 μg/well) (BD Biosciences, CA, USA) or pSV-b-GAL (0.3 μg/well) (Promega) vector was co-transfected with a Effectene™ transfection reagent (QIAGEN, Hilden, Germany). After 16 h, the medium containing plasmid was exchanged to 1000 μl 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

Cytotoxity of sesquiterpene lactones from Calea uritisifolia in SW480 and HL60 cells

We examined the effects of six sesquiterpene lactones including parthenolide (Fig. 1) at various concentrations on the cytotoxity 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, arucanolide 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 arucanolide 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

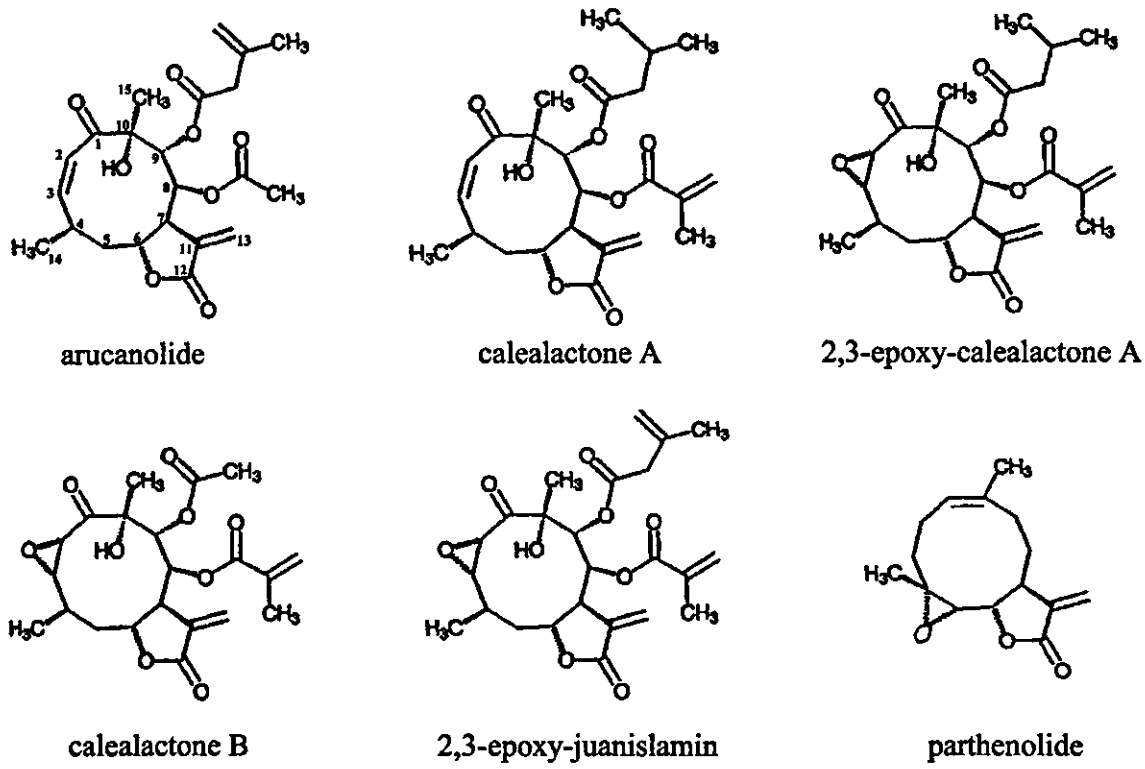

Fig. 1. Chemical structures of sesquiterpene lactones in *Calea urticifolia*. The numbers of the C-atoms are shown for arucanolide.

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

a) SW480 and HL60 cells

Cell	Arucanolide	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	(μM)

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

	Arucanolide	Parthenolide	Resveratrol	
PBLs	2.3 ± 0.3	1.1 ± 0.2	10.0 ± 0.2	(μM)

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

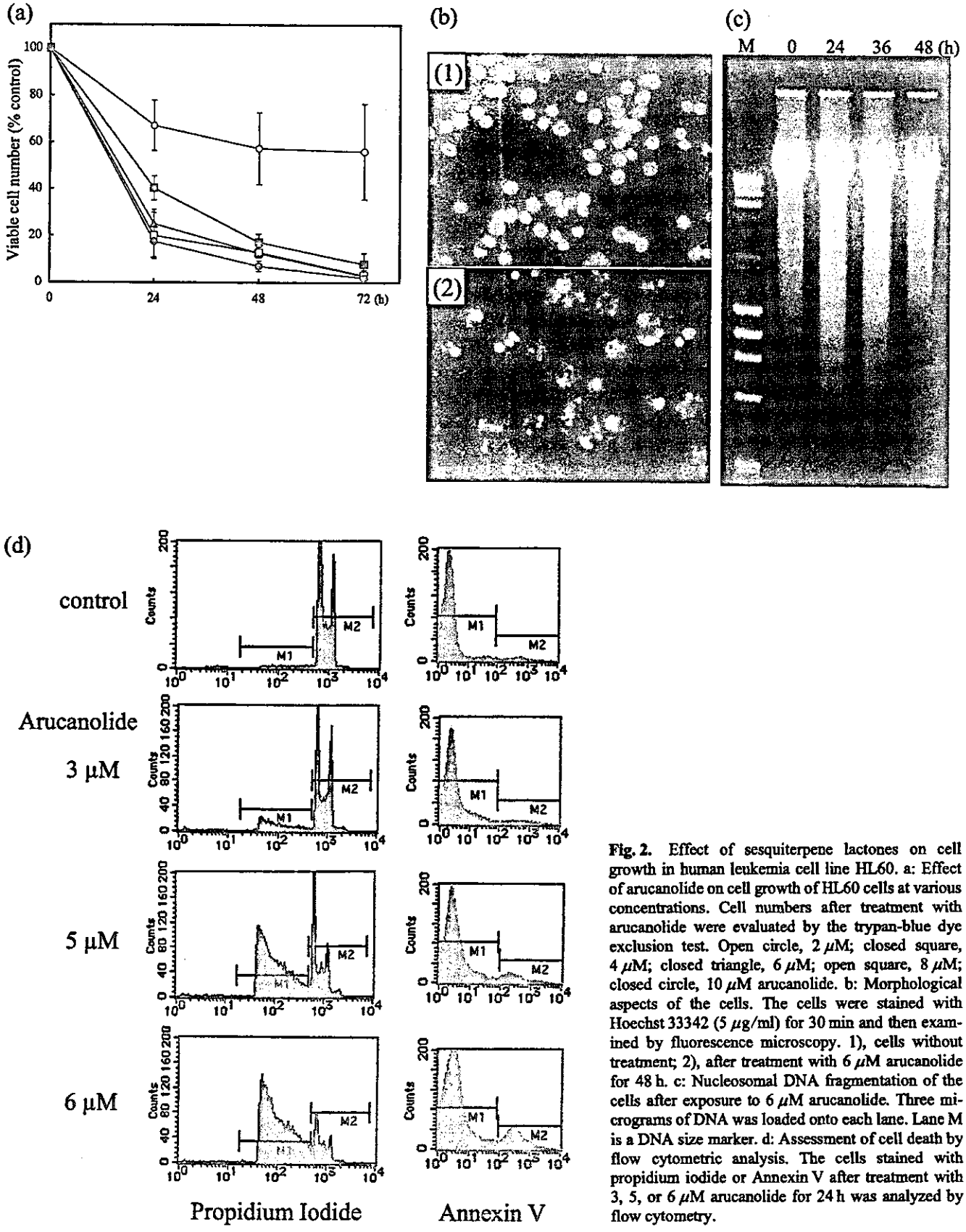

Fig. 2. Effect of sesquiterpene lactones on cell growth in human leukemia cell line HL60. a: Effect of arucanolide on cell growth of HL60 cells at various concentrations. Cell numbers after treatment with arucanolide were evaluated by the trypan-blue dye exclusion test. Open circle, 2 μM; closed square, 4 μM; closed triangle, 6 μM; open square, 8 μM; closed circle, 10 μM arucanolide. b: Morphological aspects of the cells. The cells were stained with Hoechst 33342 (5 μg/ml) for 30 min and then examined by fluorescence microscopy. 1), cells without treatment; 2), after treatment with 6 μM arucanolide for 48 h. c: Nucleosomal DNA fragmentation of the cells after exposure to 6 μM 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 μM 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 μM, as compared with the control without arucanolide treatment (Fig. 2a). In the treatment with 6 μM 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 executionors (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 μM 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

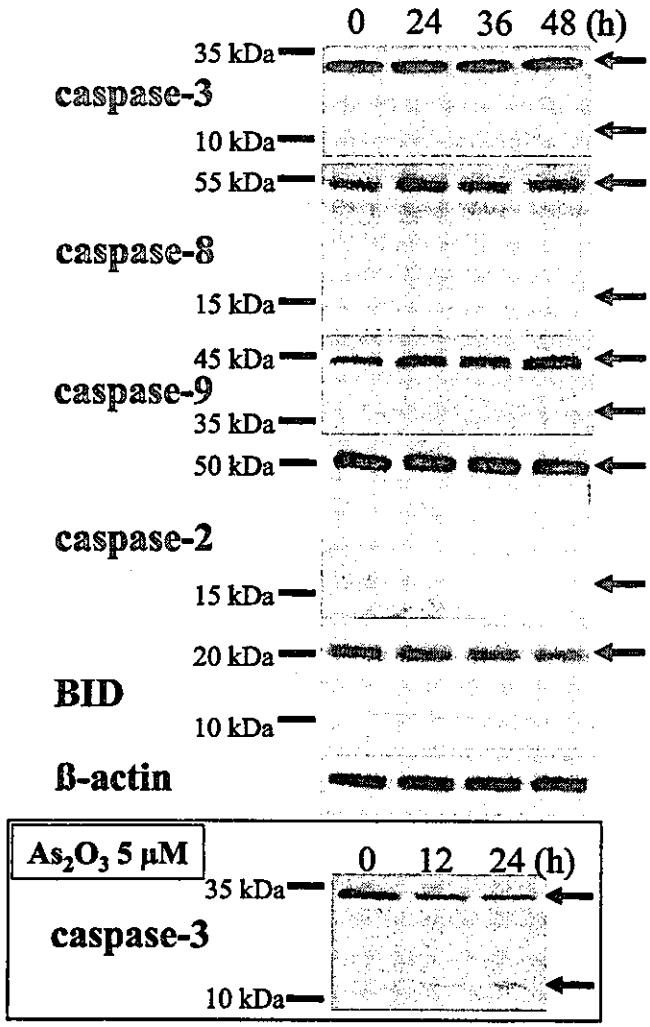

Fig. 3. Arucanolide-induced cell death in HL60. Activation of caspase-3, -8, -9, and -2 and BID after the treatment with 6 μM arucanolide was examined by Western blot analysis. Arsenic trioxide was used as a reference of caspase-dependent apoptosis.

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-κB

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

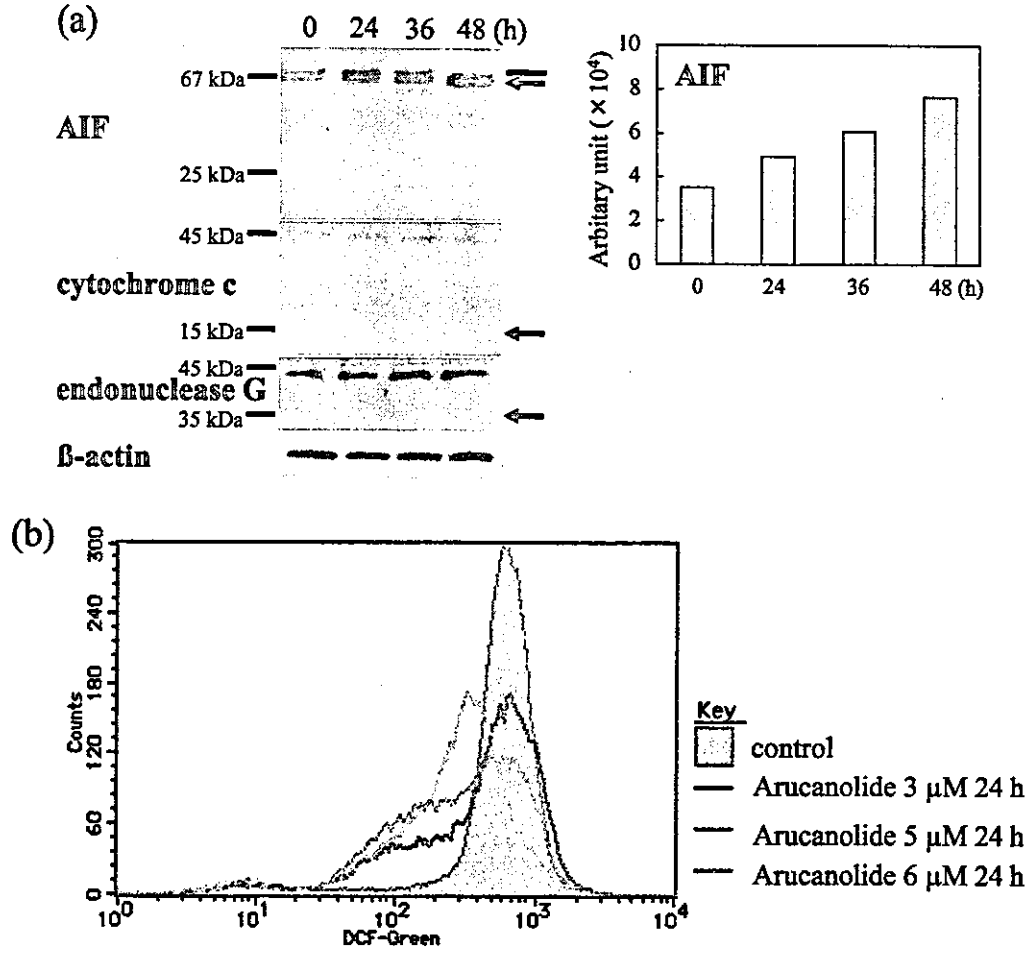

Fig. 4. Arucanolide-induced cell death in HL60. a: Activation of AIF, cytochrome c, and endonuclease G after the treatment with 6 μM arucanolide examined by Western blot analysis. b: Mitochondrial membrane potential after treatment with 3, 5, or 6 μM arucanolide. The relative fluorescence intensity of 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 μM arucanolide.

examined the activation of NF-κB in arucanolide-induced apoptosis (Fig. 5). When apoptotic stimuli including TNF-α cause phosphorylation of IκB, NF-κB is activated and translocates into nuclei. In our study, Western blot analysis showed that the level of phosphorylated IκB was unchanged (Fig. 5a). When the cells transfected with pNF-κB-SEAP vector were exposed to TNF-α, the cis-element for NF-κB 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-κB.

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

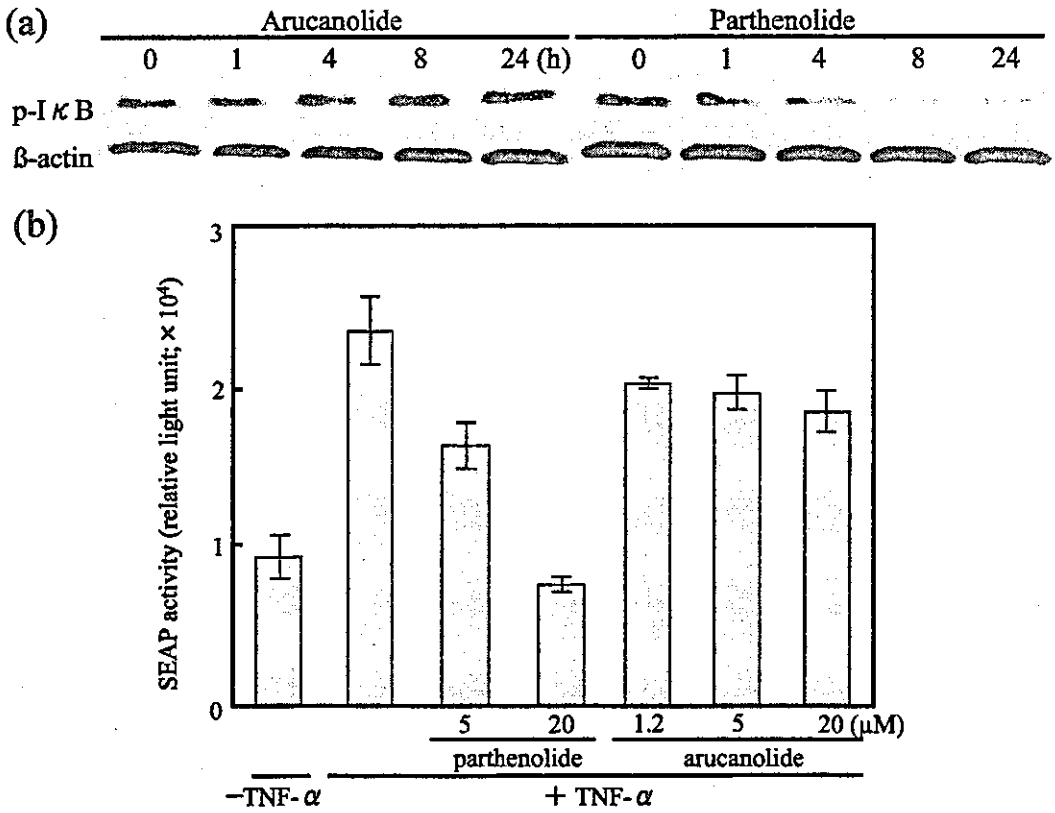

Fig. 5. NF-κB signaling in arucanolide-induced cell death. a: Phosphorylated form of IκB in HL60 cells examined by Western blot analysis compared with those in parthnolide-treated cells. b: Secreted alkaline phosphatase (SEAP) activety in parthenolide- and arucanolide-treated HeLa cells. The method is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

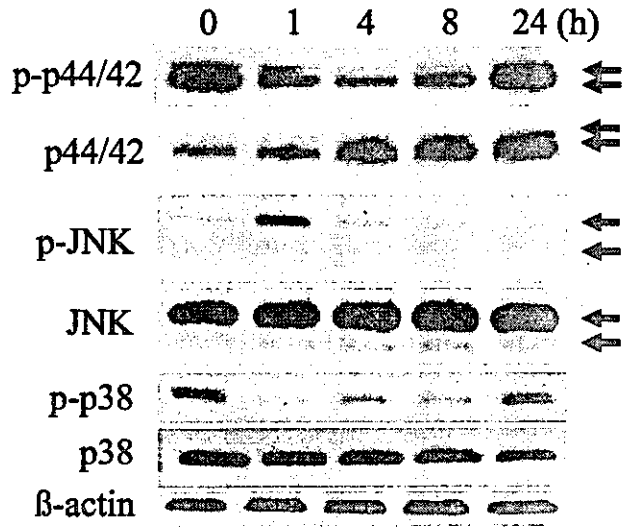

Fig. 6. Death-associated MAP kinase signaling of arucanolide-induced cell death in HL60 cells. p44/42 MAP kinase, p-p44/42 MAP kinase, JNK, p-JNK, p38, and p-p38 were examined by Western blot analysis.

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 μM 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-κB (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 μM (22). In the present study, we have found that arucanolide had a more potent cytotoxic effect compared to parthenolide as reflected in IC_{50}s 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 /effecter 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 monoposphate 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-κB (3 – 5), but arucanolide did not affect NF-κB.

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 stains of *Plasmodium falciparum* (28). Artemisinin inhibits nitric oxide synthesis in cytokine-stimulated human astrocytoma T67 cells through the inhibition of NF-κB 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-κB activity (33, 34). Patel et al. reported that parthenolide mimicked the effects of IκB-α by inhibiting DNA binding activity of NF-κB 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.

IC_{50} of arucanolide in mitogen-stimulated normal peripheral blood lymphocytes was observed at 2.3 μM, but that of parthenolide was 1.1 μM (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-methylbutylric 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 arucanlide, 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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Special Issue

Monoamine Oxidases: Molecular, Pharmacological and Neurotoxicological Aspects

A Tribute to Prof. Merton Sandler

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Dopamine-Derived Salsolinol Derivatives as Endogenous Monoamine Oxidase Inhibitors: Occurrence, Metabolism and Function in Human Brains

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Abstract

Salsolinol, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, is an endogenous catechol isoquinoline detected in humans by M. Sandler. In human brain, a series of catechol isoquinolines were identified as the condensation products of dopamine or other monoamines with aldehydes or keto-acids. Recently selective occurrence of the (R)enantiomers of salsolinol derivatives was confirmed in human brain, and they are synthesized by enzymes in situ, but not by the non-enzymatic Pictet–Spengler reaction. A (R)salsolinol synthase catalyzes the enantio-specific synthesis of (R)salsolinol from dopamine and acetaldehyde, and (R)salsolinol N-methyltransferase synthesizes N-methyl(R)salsolinol, which is further oxidized into 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion by non-enzymatic and enzymatic oxidation. The stepwise reactions, N-methylation and oxidation, induce the specified distribution of the N-methylated and oxidized derivatives in the human nigro-striatum, suggesting that these derivatives may be involved in the function of dopamine neurons under physiological and pathological conditions. As shown by in vivo and in vitro experiments, salsolinol derivatives affect the levels of monoamine neurotransmitters though the inhibition of enzymes related in the metabolism of catechol- and indoleamines. In addition, the selective neurotoxicity of N-methyl(R)salsolinol to dopamine neurons was confirmed by preparation of an animal model of Parkinson's disease in rats. The involvement of N-methyl(R)salsolinol in the pathogenesis of Parkinson's disease was further indicated by the increase in the N-methyl(R)salsolinol levels in the cerebrospinal fluid and that in the activity of its synthesizing enzyme, a neural (R)salsolinol N-methyltransferase, in the lymphocytes prepared from parkinsonian patients. N-Methyl(R)salsolinol induces apoptosis in dopamine neurons, which is mediated by death signal transduction in mitochondria. In addition, salsolinol was found to function as a signal transmitter for the prolactin release in the neuro-intermediate lobe of the brain. These results are discussed in relation to role of dopamine-derived endogenous salsolinol derivatives as the regulators of neurotransmission, dopaminergic neurotoxins and neuro-hormonal transmitters in the human brain.

Keywords: Salsolinol; Monoamine oxidase inhibitor; Parkinson's disease; Neurotoxin; Prolactin

INTRODUCTION

Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, Sal) was detected in urine of parkinsonian patients administered with L-DOPA (Sandler et al., 1973). This finding stimulated the studies on Sal derivatives in the brain, and gave new aspects of the endogenous alkaloids, which had been considered to

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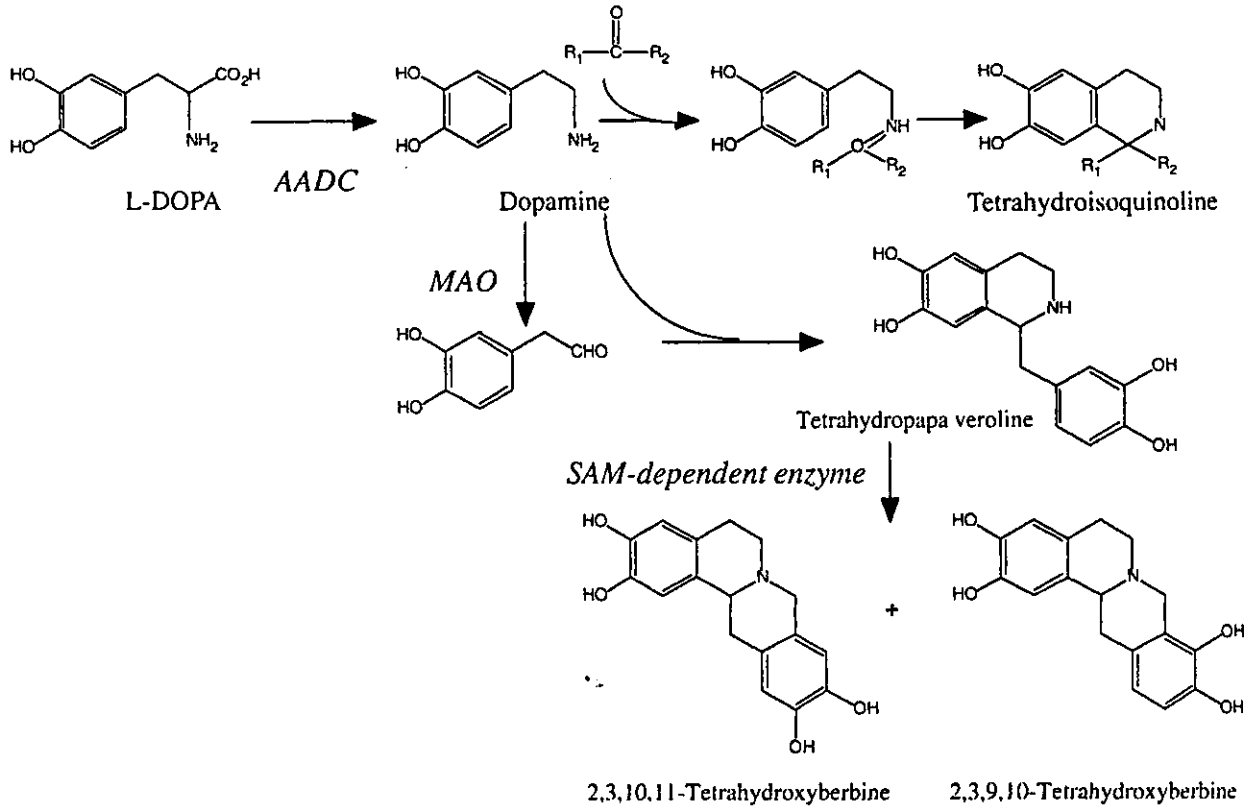

Fig. 1. Chemical synthesis of catechol isoquinolines from dopamine. L-DOPA is decarboxylated into dopamine by aromatic L-amino acid decarboxylase (AADC), which condensed with aldehydes into tetrahydroisoquinolines. Dopamine is oxidized by monoamine oxidase (MAO) into 3,4-dihydroxyphenylacetaldehyde, which generates tetrahydropapaveroline (THP) by condensation with dopamine. 2,3,10,11-Tetrahydroberbine and 2,3,9,10-tetrahydroberbine are produced from THP by *S*-adenosyl-L-methonine (SAM)-dependent enzymes.

occur only in plants. As shown in Fig. 1, the non-enzymatic Pictet–Spengler reaction, which involves the condensation of β-arylethanolamines with carbonyl compounds, produces Sal derivatives from dopamine with aldehydes or keto-acids. In human tissues three classes of monoamine-derived alkaloids have been reported; dopamine-derived catechol 1,2,3,4-tetrahydroisoquinolines (TIQs), and β-phenethylamine-derived TIQs without catechol structure and indoleamine-derived β-carbolines.

In normal non-alcoholic subjects and alcoholics, Sal and *O*-methylated Sal were found in urine, cerebrospinal fluid and brains (Collins, 1980; Sjöquist et al., 1981). A series of Sal-related isoquinolines were identified in human tissues as summarized in Table 1. In addition, in adrenal tissue 1-methyl-4,6,7-trihydroxy-TIQ and 1,2-dimethyl-4,6,7-trihydroxy-TIQ were detected as the condensation product of norepinephrine or epinephrine with acetaldehyde (Cohen and Collins, 1970). In the urine of phenylketonuric children, a TIQ carboxylic acid, 3′,4′-deoxynorlaudanosaline-carboxylic acid (DNLCA) was detected (Lasala and Coscia, 1979), which is derived from dopamine and phenylpyruvic acid. In addition, 3,4-dihydroxyphenylacetaldehyde, an oxidation product of dopamine by monoamine oxidase (monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4, MAO), reacts with dopamine to produce tetrahydropapaveroline (1-(3′,4′-dihydroxybenzyl)-1,2,3,4-tetrahydro-6,7-dihydroxyisoquinoline, norlaudanosoline, THP) (Holtz et al., 1964). 3′-*O*-Methyl norlaudanosolinecarboxylic acid (Coscia et al., 1977) and tetrahydroberberines (THBs), produced from THP by *S*-adenosyl-L-methionine (SAM)-dependent enzymes, 2,3,10,11-THB and 2,3,9,10-THB were also identified in urine of parkinsonian patients receiving L-DOPA therapy (Cashaw et al., 1974).

Until recently isoquinolines had been considered to occur as racemic forms in humans, which are generated by non-enzymatic condensation of monoamines. However, more accurate and simpler chromatographic methods for analysis of Sal enantiomers proved the predominant occurrence of the (*R*)enantiomers in mammalian tissues, suggesting that Sal might be synthesized enzymatically.

This reviews summarizes the recent advances in our understanding on the occurrence, synthesis and

Table 1
Concentrations of salsolinol derivatives in human brain, CSF and IVF

Salsolinol derivatives	Tissue	Content (pmol/g wet weight)	Reference
Salsolinol	CSF: parkinsonian	<5[a]	Moser and Kämpf (1992)
	Striatum: control	19 ± 10	Ung-Chhun et al. (1985)
	Alcoholic	58 ± 21	
	Hypothalamus: control	43 ± 13	Ung-Chhun et al. (1985)
	Alcoholic	87 ± 49	
Salsolinol-1-carboxylic acid	Striatum	161 ± 69	Ung-Chhun et al. (1985)
	Hypothalamus	83 ± 42	
6 or 7-Methyl-salsolinol	CSF: control	1.3 ± 0.3[a]	Dordain et al. (1984)
	Parkinsonian	0.6 ± 0.2	
	Alcoholic	1.1 ± 0.3	
	Striatum: control	9 ± 6	Ung-Chhun et al. (1985)
	Alcoholic	11 ± 6	
	Hypothalamus: control	8 ± 3	Ung-Chhun et al. (1985)
	Alcoholic	2 ± 1	
2(*N*)-Methyl-norsalsolinol	CSF: parkinsonian	15, 26, 60[a]	Moser and Kämpf (1992)
1,2-Dimethyl-6,7-dihydroxyisoquinolinium ion	Substantia nigra	254 ± 59.0	Maruyama et al. (1997a)
	IVF	16.0 ± 16.5 nM	Maruyama et al. (1996b)

The data mentioned here were not determined for the enantiomeric characters.
[a] pmol/ml.

function of monoamine-derived isoquinolines in the human brains.

BIOSYNTHESIS OF (*R*)SALSOLINOL DERIVATIVES IN HUMAN BRAIN

Sal has an asymmetric center at first position and exists as (*R*)- and (*S*)enantiomer. Dostert and his colleagues found that in urine from healthy volunteers the (*R*)enantiomer of Sal is predominant, and proposed the biosynthesis pathway, as shown in Fig. 2 (Dostert et al., 1990). The condensation of dopamine with pyruvic acid yields 1-carboxyl-Sal, which is detected in human urine, CSF and caudate nucleus, but the enantomeric structure was not determined. The decarboxylation of 1-carboxyl-Sal produces 1,2-dehydrosalsolinol, which was also identified in human urine (Dostert et al., 1990). However, the enzymatic reduction of 1,2-dehydrosalsolinol or the decarboxylation of 1-carboxyl-Sal into optically active Sal has never been shown in animals or plants. This hypothesis of (*R*)Sal synthesis had not been fully confirmed.

More recently a quite sensitive method of Sal enantiomers has been developed by use of high-performance liquid chromatography (HPLC) with chiral columns and electrochemical detection (Maruyama et al., 1997a; Naoi et al., 1998b). Table 2 summarizes the results in analyses of Sal derivatives in human materials. Only the (*R*)enantiomers of Sal and *N*-methylated Sal occur in the human brain, cerebrospinal fluid (CSF) and intraventricular fluid (IVF), and the (*S*)enantiomers were not detected (Maruyama et al., 1996a,b, 1997c). Then, we isolated two enzymes involved in the synthesis of optically active Sal derivatives in the human brain. As shown in Fig. 2, a (*R*)salsolinol synthase catalyzes the enantio-selective synthesis of (*R*)Sal and 1-carboxyl(*R*)-Sal from dopamine with acetaldehyde or pyruvic acid (Naoi et al., 1996b). The decarboxylation of 1-carboxyl(*R*)Sal into 1,2-dehydrosalolsinol was confirmed to occur by a non-enzymatic reaction. However, the enantio-selective conversion of the latter into (*R*)Sal was not confirmed by use of human brain samples. The *N*-methylation of (*R*)salsolinol into *N*-methylsalsolinol (*N*MSal) is catalyzed by two *N*-methyltransferases with different optimum pH, at pH 7.0 and 8.4. *N*M(*R*)Sal is enzymatically oxidized into 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion ($DMDHIQ^+$) by an oxidase sensitive to semicarbaside (Naoi et al., 1995) and also non-enzymatically by autoxidation (Maruyama et al., 1995). Table 3 summarizes the enzymatic characteristics of the enzymes participating the synthesis and metabolism of (*R*)Sal derivatives.

More recently, we reported that only the (*S*)enantiomer of THP was detected in human brains (Sango et al., 2000). The concentrations varied from 0.12 to 0.28 pmol/g wet weight of brain tissues, and were much lower than those of dopamine (450–5440 nmol/g wet

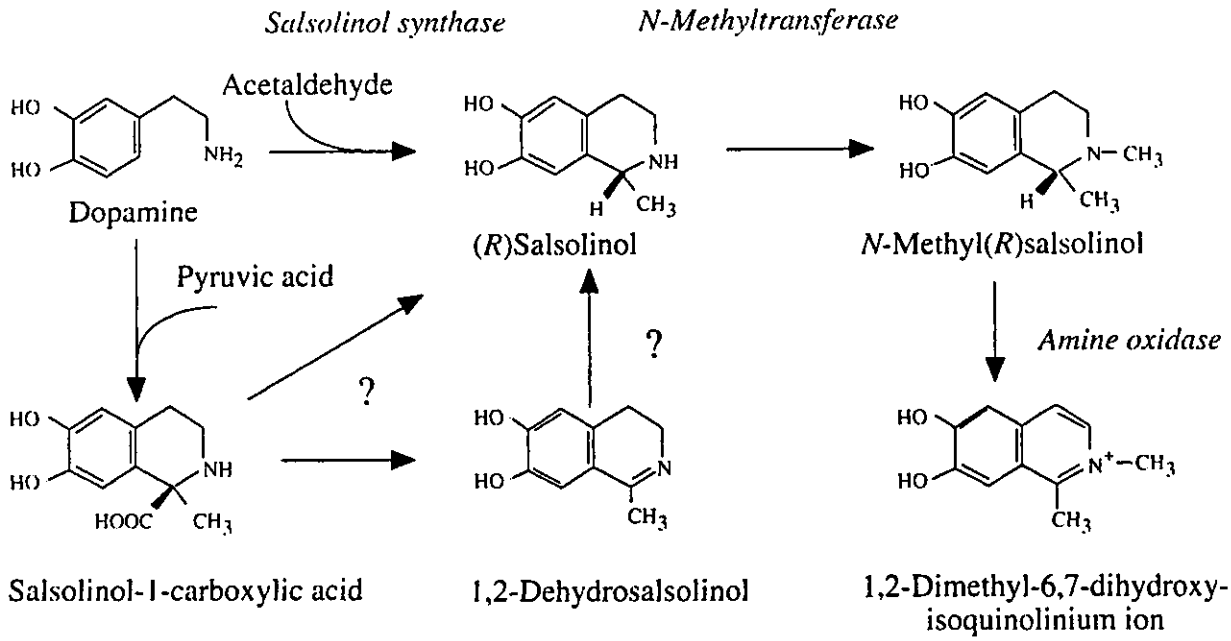

Fig. 2. Biosynthesis pathway of (*R*)salsolinol derivatives in human brain. The enzymatic condensation of dopamine with acetaldehyde or pyruvic acid is catalyzed by (*R*)salsolinol synthase to yield (*R*)Sal and (*R*)Sal-1-carboxylic acid. However, the enantio-selective synthesis of (*R*)Sal from (*R*)Sal-1-carboxylic acid and 1,2-dehydrosalsolinol was not confirmed. *N*-Methyltransferase catalyzes the *N*-methylation of (*R*)Sal into *N*M(*R*)Sal, which is further oxidized into 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion ($DMDHIQ^+$).

Table 2
Concentrations of (*R*)- and (*S*)enantiomer of salsolinol derivatives in human tissues, detected by HPLC

Salsolinol derivatives	Tissue	Concentration	Reference
(*R*)Salsolinol	Urine	1.95–11 ng/ml	Dostert et al. (1989)
		10.2–51.75 μg per day	Strolin Benedetti et al. (1989a)
		16.2–103.3 pmol/ml	Dostert et al. (1990)
	Alcoholic	47.5–231.1 nmol per day	Dostert et al. (1991)
	Plasma	0.397 ng/ml	Baum and Rommelspacher (1994)
		0.4–4.2 pmol/ml	Haber et al. (1996)
	Frontal cortex	134 ± 125 pmol/g	Maruyama et al. (1997a)
	Caudate	73.3 ± 79.9	
	Putamen	37.8 ± 23.0	
	Substantia nigra	94.5 ± 78.7	
	IVF	0.39 ± 0.21 nM	Maruyama et al. (1996b)
(*S*)Salsolinol	Urine	0–23.3 pmol/ml	Dostert et al. (1990)
	Alcoholic	56.1–139.5 nmol per day	Dostert et al. (1991)
	Plasma	0.4–3.1 pmol/ml	Haber et al. (1996)
	Brain regions, CSF, IVF	Not detected	Maruyama et al. (1996b)
N-Methyl(*R*)salsolinol	Caudate	65.7 ± 88.3 pmol/g	Maruyama et al. (1997a)
	Putamen	110 ± 126	
	Substantia nigra	76.6 ± 23.0	
	CSF		
	Parkinsonian	8.32 ± 2.89 nM	Maruyama et al. (1996a)
	Control	4.53 ± 2.08 nM	
	IVF	9.15 ± 9.08 nM	Maruyama et al. (1996b)

Measured by GC-MS.