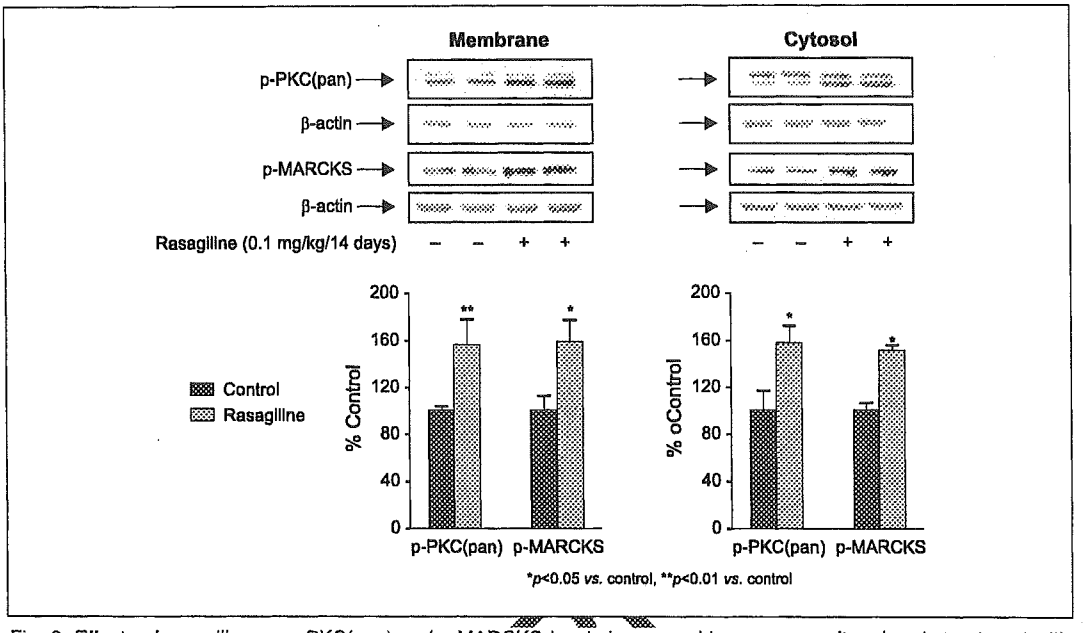

Fig. 9. Effects of rasagiline on p-PKC(pan) and p-MARCKS levels in mouse hippocampus after chronic treatment with rasagiline (ref. 130).

dent on the propargylamine moiety of rasagiline and its derivative, since propargylamine itself is as effective in these mechanisms (102). Indeed, the neuroprotective activity of these compounds is also dependent on the propargylamine moiety (81, 104–106). Considering these findings, the effect of rasagiline and its derivatives on the regulation of PKC-dependent mechanisms and APP processing under *in vivo* conditions was investigated.

Evidence has been provided that rasagiline and its cholinesterase inhibitor derivatives TV3326 and TV3279 induce significant increases in p-PKC levels and in the expression of α and ε PKC isoforms in the mouse and rat hippocampus (86). Additionally, rasagiline treatment significantly elevated the levels of p-MARCKS, a major substrate for PKC, as well as levels of RACK1 (Fig. 9). Similar results have been obtained with propargylamine alone, indicating that the PKC activation by rasagiline and its derivatives is the pharmacological property of the propargylamine moiety (104, 130).

Studies investigating the role of the PKC family in the regulation of cell death have suggested that activation of PKC can prevent apoptosis via two main survival pathways: 1) the antiapoptotic protein BCL-2; and 2) the MAPK/extracellular regulated kinase (ERK) cascades. PKC can phosphorylate Bcl-2 at a site that increases its antiapoptotic function (131), and overexpression of PKC results in increased expression of Bcl-2 (132, 133). Furthermore, MAPK/ERK cascades, which have been shown to inhibit apoptosis in a number of systems, can be activated by PKC. For example, PKC phosphorylates and activates raf-1, an upstream kinase in the MAPK/ERK pathway (134), and pharmacological inhibition of MAPK/ERK signaling blocks phorbol ester-induced protection of neuronal cells against glutamate toxicity (135). Indeed, both of these pathways have been identified in the neuroprotective mechanism of action of rasagiline *in vitro*, where treatment in PC-12 cells with the ERK inhibitor PD98059 and the PKC inhibitors GF109203X and calphestin c (submitted for publication) prevented the neuroprotective activity of rasagiline (and its derivatives, unpublished data) in serum- and nerve growth factor-free system-induced apoptosis (130). Rasagiline induced cytoprotective gene expression (*e.g.*, Bcl-2 and Bcl-xl), which may rescue neurons from apoptosis, as mediated by the mitochondrial death cascade, and therefore protect declining neurons in neurodegenerative disorders and aging (136). Ad-

ditionally, rasagiline dose-dependently (0.1–10 μM) increases the immunoreactivity of the phosphorylated MAPK in PC12 cells. The MEK inhibitor, PD98059, antagonized rasagiline-induced MAPK activation, indicating that MEK phosphorylated MAPK in the presence of rasagiline (104, 129). Thus, the activation of PKC by rasagiline may play a crucial role in its neuroprotective activity, which also involves the regulation of Bcl-2/Bcl-Xl/Bad and Bax.

One of the major downstream substrates of PKC is the MARCKS, which has been implicated in cell motility, cell adhesion, membrane traffic and mitogenesis. PKC phosphorylates serines 152, 156 and 163 of MARCKS (137, 138), which regulates MARCKS's calcium/calmodulin binding activity and filamentous actin cross-linking activity (137, 139). MARCKS is developmentally regulated, being highly expressed in select regions of the developing and adult rat brain (140), and it plays a significant role in spatial learning processes (141). The location of MARCKS in senile plaques along with PKC and Aβ fibrils points to a possible involvement in Aβ-induced neuronal dysfunction (142). Since the phosphorylation of MARCKS has been used as a marker for activation of PKC, the effect of rasagiline on p-MARCKS levels was investigated. The results of these studies showed that p-MARCKS levels were elevated in the hippocampus of rasagiline-treated mice as compared to controls, further supporting the induction of PKC activation by rasagiline. Similar results were also obtained with TV3326 and TV3279 (86).

Owing to the reputed role of RACK1 (receptor for activated C kinase 1) in PKC-mediated events in different cellular systems, the effect of rasagiline treatment on RACK1 levels was also determined. Rasagiline treatment increased the levels of RACK1 in the hippocampus, indicating that this compound may also affect other factors controlling PKC activation. RACK1 is emerging as important in targeting activated PKC to different intracellular sites, where substrates can be phosphorylated and thus modulate PKC-dependent functions (143). In brain, RACK1 mRNA and protein show developmental changes; a parallel in RACK1 and PKC protein ontogenesis suggests that they are interdependent and involved in synaptogenesis and myelination (144). In addition, RACK1 immunoreactivity is reduced in brain cortex of the aged rat, concomitantly with an impaired translocation in PKC activity and immunoreactivity for βII, indicating that RACK1 deficit contributes to age-dependent impairment in PKC activation/compartmentalization (144). Interestingly, it has been shown that in Alzheimer's disease, a reduced level of RACK1 protein can be observed in the brain of affected patients (145), a finding that links to previous observations concerning defective PKC machinery in Alzheimer's brain and peripheral tissues (144, 146). Thus, it can be suggested that upregulation of PKC by rasagiline may maintain appropriate PKC activity, an important task in the management of Alzheimer's disease and in parkinsonian subjects with dementia pathophysiology.

In Alzheimer's disease, a growing body of evidence suggests that increased expression and/or altered processing of APP and the ensuing increase in generation of β-amyloid peptides play a central role in amyloidogensis processes (147). Thus, the observation that rasagiline markedly decreased the levels of cell-associated, full-length APP in the mouse hippocampus could be of value towards accelerating nonamyloidogenic APP processing, thereby reducing β-amyloid levels. Since we have recently demonstrated the involvement of PKC in the release of sAPPα by rasagiline and its cholinesterase inhibitor derivatives (TV3326 and TV3279) in PC-12 cells and SH-SY5Y neuroblastoma cells (104, 129), it is possible that the mechanism by which these drugs affect APP processing *in vivo* may be related to PKC-associated signaling. Indeed, among the various signaling cascades known to participate in Alzheimer's disease pathophysiology and APP processing, PKC has received much attention. *In vivo*, it was shown that specific cholinergic lesions within the basal forebrain of rats lead to reduced secretory APP processing in the cholinergically deafferented neocortex, which strongly correlates with reduced activation of PKC-coupled M1 mAChR (125). Also, Lin *et al.* (126) observed reduced secretory APP processing in neocortex after specific cholinergic lesions of the basal forebrain and increased APP secretion (*i.e.*, less cell-associated APP but higher levels of cerebrospinal fluid secretory APP) after muscarinic agonist treatment. In an animal model of permanent hyperactivation of PKC in neocortex and hippocampus (*i.e.*, in the offspring of rats receiving *in utero* methylazoxymethanol acetate treatment), secretory APP processing was increased (124).

It is currently unknown which isoenzyme of PKC plays a major role in modulating APP processing. Nevertheless, several lines of evidence suggest the involvement of PKCε and PKC in APP processing. Thus, it was demonstrated that the EC_{50} for

PMA regulation of sAPP release was lower in Swiss 3T3 fibroblast cells overexpressing PKCα (122), and a specific inhibitor of PKCα, GO-6976, reduced constitutive and phorbol ester regulation of sAPP in human fibroblasts (148). Furthermore, in a rat fibroblast cell line, sAPP was increased after stable overexpression of PKCα and PKCε isoenzymes (149). It was also shown that blockade of PKCε activation attenuated phorbol ester-induced increase of α-secretase-derived sAPP (150). This finding further supports previous studies on brains of patients with Alzheimer's disease, where PKCε activity in the membrane fraction was reduced (117). In accordance with these findings, we have shown that chronic administration of rasagiline for 14 days causes significant increases in the protein expression of PKC isoenzymes α and ε in the mouse and rat hippocampus. These results are consistent with our recent finding demonstrating induction of PKCα and PKCε mRNAs in PC-12 cells by rasagiline (manuscript in preparation).

In summary, rasagiline, similar to its novel carbamate-containing cholinesterase inhibitor analogues, TV3326 and TV3279, reduced the levels of APP and upregulated the levels of p-PKC, PKCα and PKCε in the mouse hippocampus and in PC-12 cells (130). Furthermore, the presence of a propargylamine group in these compounds is essential for their APP processing to neuroprotective-neurotrophic sAPPα through α-secretase regulation and PKC activation-dependent neuroprotective activities (53). Moreover, studies on structure-activity relationships among rasagiline-related compounds have shown the crucial role of the propargyl moiety in these molecules with respect to these processes. This is supported by the recent observation that propargylamine itself was neuroprotective and able to regulate APP processing and MAPK phosphorylation with similar potency to that of rasagiline and its derivatives (104). Rasagiline and its derivatives TV3326 and TV3279 are now being developed as antidementia drugs in Alzheimer's disease, Lewy body disease and parkinsonian dementia.

Neurotrophic activity of rasagiline: The involvement of GDNF, nerve growth factor and NF-κB

Rasagiline increases the mRNA and protein levels of GDNF (Fig. 10) (151), in addition to those of antiapoptotic Bcl-2 and Bcl-xL, as previously reported (102) in SH-SY5Y cells in culture. Selegiline and desmethylselegiline, propargylamines structurally related to rasagiline, were previously shown to increase mRNA levels of neuroprotective proteins,

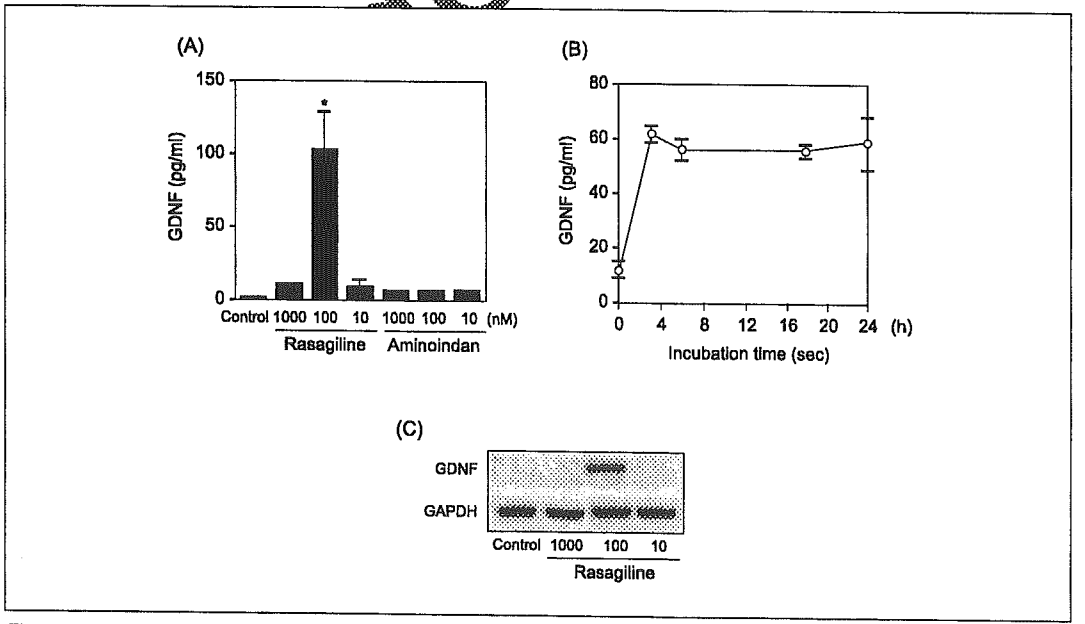

Fig. 10. Induction of GDNF synthesis and release by rasagiline in SHSY-5Y human dopamine neuroblastoma cells (ref. 151).

bcl-2, SOD, glutathione peroxides and GDNF in PC-12 cells (80). It is apparent that the activation of nuclear factor (NF)-κB by rasagiline may mediate the increase in the transcription of pro-survival genes.

In Parkinson's and Alzheimer's diseases, particular neurons deteriorate in a slow and continuous process, in which not only apoptotic, but also pro-survival factors may be activated. NF-κB may be a common regulator collecting information regarding upstream signal transduction events to determine the survival or death of the cells (152). Increased levels of NF-κB were detected in the brains of patients with Alzheimer's disease (153) and Parkinson's disease (154). Apoptosis, as induced by excitotoxicity (glutamate or NMDA) and oxidative stress, activates NF-κB (155), whereas cytotoxicity can be mediated by the activation of pro-apoptotic members of the Bcl-2 family (156). On the contrary, NF-κB has been reported to be cytoprotective against apoptosis as induced by oxidative stress and excitotoxic insults (157). These opposing results suggest that the cell types, the conditions of cytotoxic stimuli and the concomitant activation of other transcription factors may determine whether activated NF-κB promotes cell survival or cell death. The molecular mechanisms of NF-κB activation have been elucidated by Karin and Ben-Neriah (158). NF-κB is activated by IκB phosphorylation by kinase complex (I-B kinase, IKK) composed of IKK-α, -β and -γ. When the IKK complex is phosphorylated, IκB and IκBβ are cleaved at two serine residues in the *N*-terminal region and dissociated, resulting in the activation of NF-κB. Sulfasalazine, an inhibitor of IKK-α and -β (159), inhibits the rasagiline-induced NF-κB activation and the NCI 1420 1–8 induced GDNF protein simultaneously (151). These results indicate that rasagiline may activate NF-κB through the IKK pathway, resulting in GDNF synthesis. Whether rasagiline directly or indirectly affects the IKK components through activation of upstream kinases in the cells remains to be elucidated.

Rasagiline and its various derivatives, but not its aminoindan metabolite, activate PKC and Erk1/2 MAP kinase (104, 129) in a time- and concentration-dependent manner, a property directly dependent on the propargylamine moiety of these drugs, since propargylamine itself had an identical effect with the same potency (104). Activation of PKC by rasagiline is linked to its neuroprotective activity, since inhibitors of ERK and PKC prevent PKC pathway-dependent Bcl-2-induced neuroprotection, thus eliminating the neuroprotective activity of rasagiline (86, 130). This would be compatible with the recent finding indicating a link between PKC and Bcl-2. The results suggest that rasagiline targets the Bcl-2 antiapoptotic family and the protein regulating signal transduction in the MAP kinase system, as well as transcription factors. The mechanism of this these interactions is not yet fully known, but PKC activation may have a role that needs to be investigated. Although the ability of rasagiline to activate NF-κB and increase GDNF is concentration dependent, this action, as well as its ability to be neuroprotective and activate Bcl-2 and PKC, exhibits biphasic curves. Rasagiline has also been shown to exhibit an inverted U-shaped relationship in its neuroprotective function (44, 69) and in its induction of Bcl-2 (102). Tumor necrosis factor (TNF)-α, reactive oxygen species H_2O_2 and β-amyloid all activate NF-κB according to an inverted U-shaped dose-response-dependent curve. While at the low concentration, TNF-α is neuroprotective in correlation with NF-κB activation, in contrast, at the high concentration it is neurotoxic (160). The mechanism behind the inverted U-shaped type of concentration-activity relationship concerning NF-κB remains to be fully elucidated. Such phenomena are not unusual since dopamine, apomorphine, melatonin and green tea polyphenol exhibit exactly the same type of action in SHSY-5Y cells. For these compounds, it has been shown that at low concentrations, their neuroprotective activity is related to activation of the antiapoptotic bcl-2 family genes and proteins. In contrast, at high concentrations—when they are pro-apoptotic and induce cell death—they activate pro-apoptotic Bad, Bax and other cell-inducing genes (103). GDNF is now proposed as an agent for rescuing declining dopamine neurons in Parkinson's disease, as supported by its effectiveness in animal and cellular models (161). Lentiviral-delivered GDNF was reported to rescue dying dopamine neurons in a monkey model of Parkinson's disease prepared with *N*-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) (162). Infusion of GDNF protein by an implanted intracerebroventricular catheter failed to improve symptoms in parkinsonian patients (163). However, it was recently reported that the direct infusion of GDNF into the putamen of parkinsonian patients improved clinical symptoms and fluorodopa uptake to the dopamine terminal (164). These results indicate that GDNF supplemental therapy requires further technical improve-

ment for its effective administration. If rasagiline, which is currently undergoing FDA revision for approval as an antiparkinsonian drug (26), can also increase endogenous GDNF in the human brain, as shown here with SH-SY5Y cells, it may be a more practical therapy to prevent dopamine neuron cell death. In conclusion, rasagiline and related propargylamines are possible neuroprotective agents, and a part of their pharmacological action may be due to the induction of pro-survival genes through NF-κB activation, antiapoptotic Bcl-2 family protein production and inhibition of GAPDH translocation from cytoplasm into the nucleus (69). The activation of transcription factors related to antiapoptotic proteins, GDNF and bcl-2 (Maruyama *et al.*, in preparation) by propargylamines might enable us to suppress neuronal death in neurodegenerative disorders in general. Clinical trials with rasagiline are awaiting final evaluation of the efficacy data to determine the compound's ability to protect specified neurons from degeneration.

Conclusion

Pharmacologically and biochemically, rasagiline is demonstrating neuroprotective activity *in vitro* and *in vivo*. The molecular aspect of this neuroprotective property has now been demonstrated by conventional methods and by genomic and proteomic analysis to involve several factors, including downregulation of pro-apoptotic Bcl-2 family proteins that regulate the mitochondrial-dependent cell survival mechanism on the one hand, and activation of the transcription factor NF-κB and the PKC-dependent MAP kinase pathway that regulate production of neurotrophic factors, including GDNF, BDNF and nerve growth factor (86, 130, 151). Thus, rasagiline may have pharmacological activities that initiate neuroprotection, as well as neuronal plasticity related to the activation of Bcl-2 and PKC. It is apparent that Bcl-2 and PKC pathway-dependent neuroprotective and neurotrophic activities of rasagiline are closely linked, since both ERK and PKC inhibitors prevent these activities (86, 102). Whether this neuroprotective activity can be translated into the treatment of parkinsonian and Alzheimer's disease subjects remains to be determined by PET and SPECT in clinical studies. However, it should be noted that results from recent long-term double-blind controlled studies with rasagiline suggest that it may exert disease-modifying action (27).

Acknowledgment

The support of Technion Research and Development (Haifa, Israel), the National Parkinson's Foundation (Miami, Florida, USA), Teva Pharmaceutical Co. (Netanya, Israel), the Stein Foundation and the Friedman Foundation (Philadelphia, Pennsylvania, USA) are gratefully acknowledged. Permission to use figures from Golding Parkinson's Research Fund and from Technion, as well as from articles appearing in *Neuropharmacology*, *Biochemical Pharmacology*, *British Journal of Pharmacology* and *Expert Reviews of Neurotherapeutics*, are gratefully acknowledged.

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J Pharmacol Sci 97, 242 – 252 (2005)

Journal of Pharmacological Sciences

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 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).

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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.

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

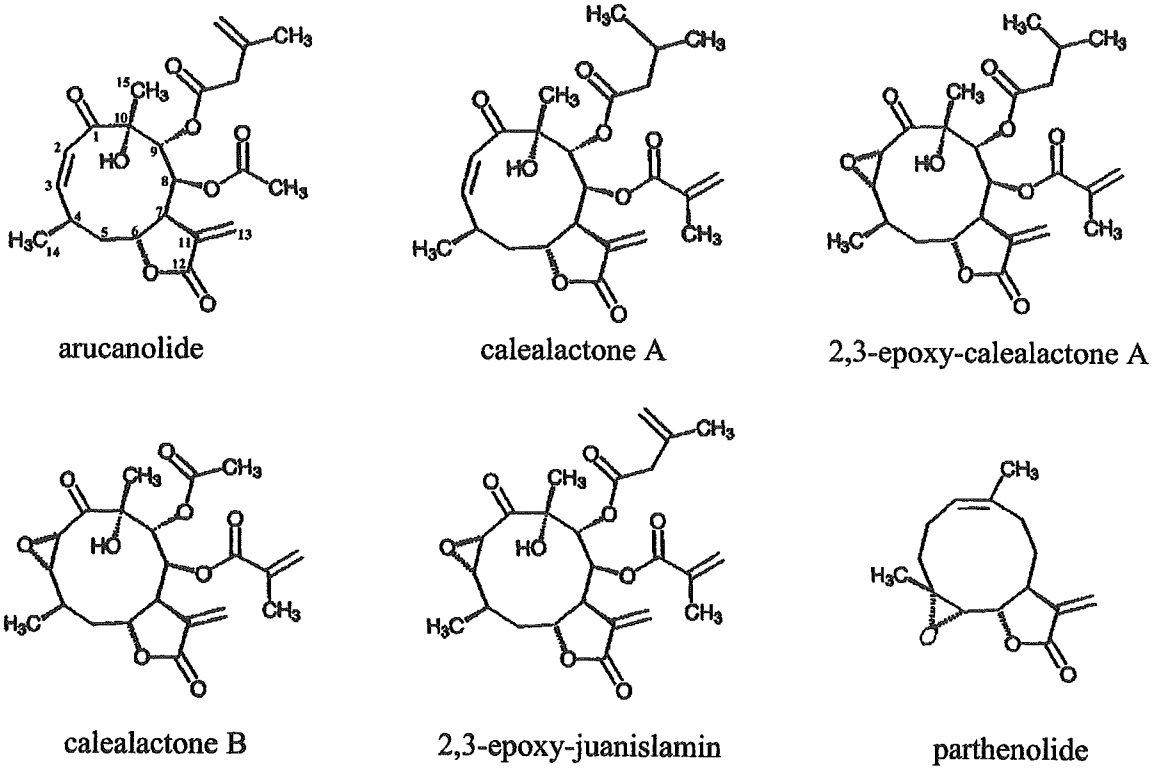

Fig. 1. Chemical structures of sesquiterpene lactones in *Calea urticifolia*. The numbers of the C-atoms are shown for arucanolide.

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.

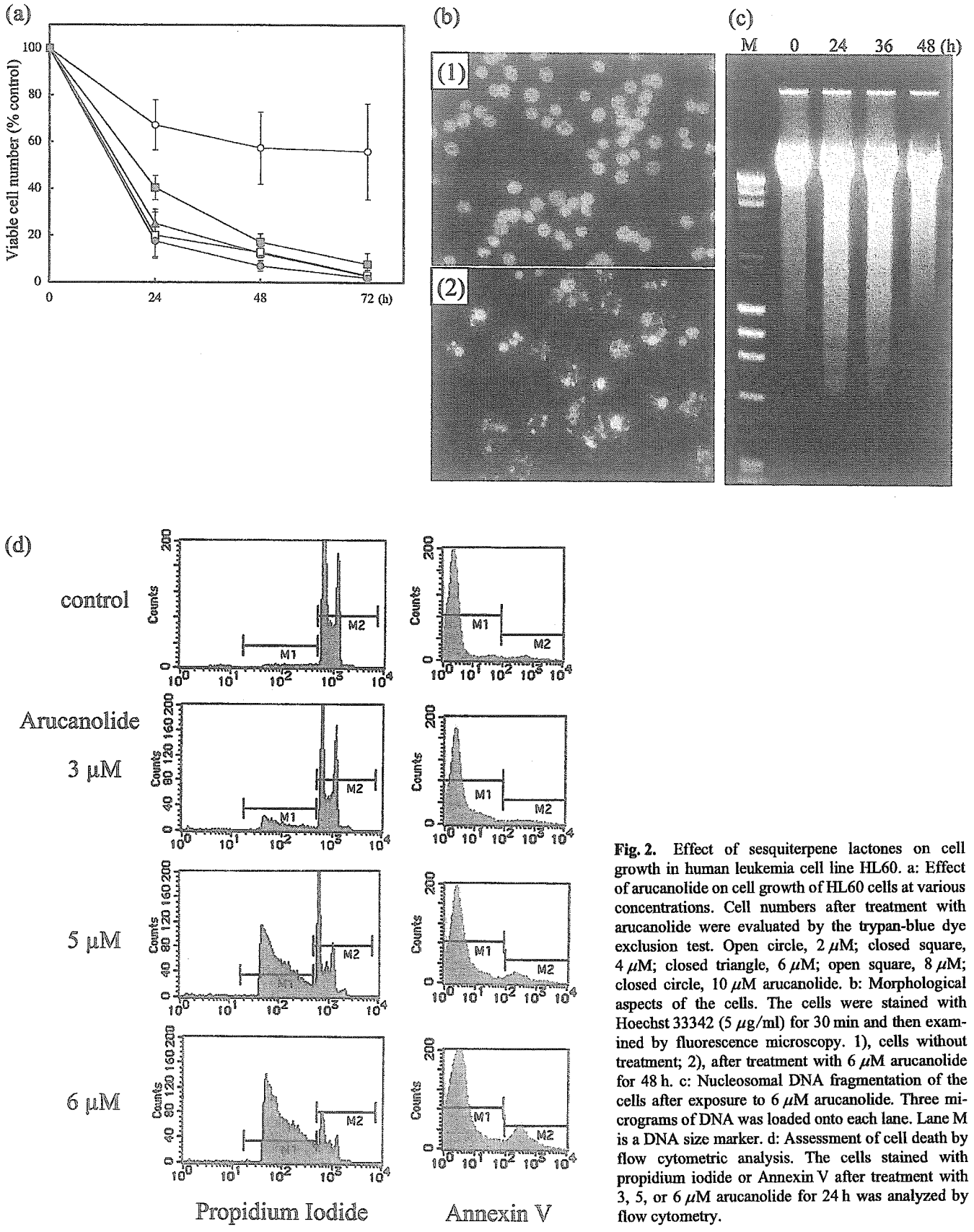

Fig. 2. Effect of sesquiterpene lactones on cell growth in human leukemia cell line HL60. a: Effect of arucanolide on cell growth of HL60 cells at various concentrations. Cell numbers after treatment with arucanolide were evaluated by the trypan-blue dye exclusion test. Open circle, 2 μM; closed square, 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

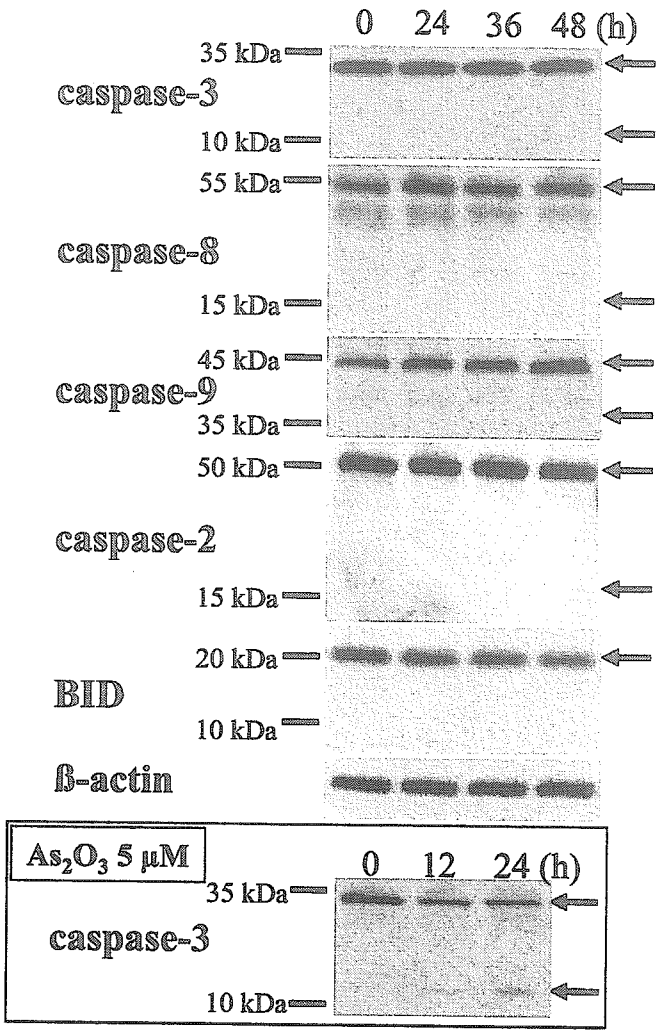

Fig. 3. Arucanolide-induced cell death in HL60. Activation of caspase-3, -8, -9, and -2 and BID after the treatment with 6 μM arucanolide was examined by Western blot analysis. Arsenic trioxide was used as a reference of caspase-dependent apoptosis.

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

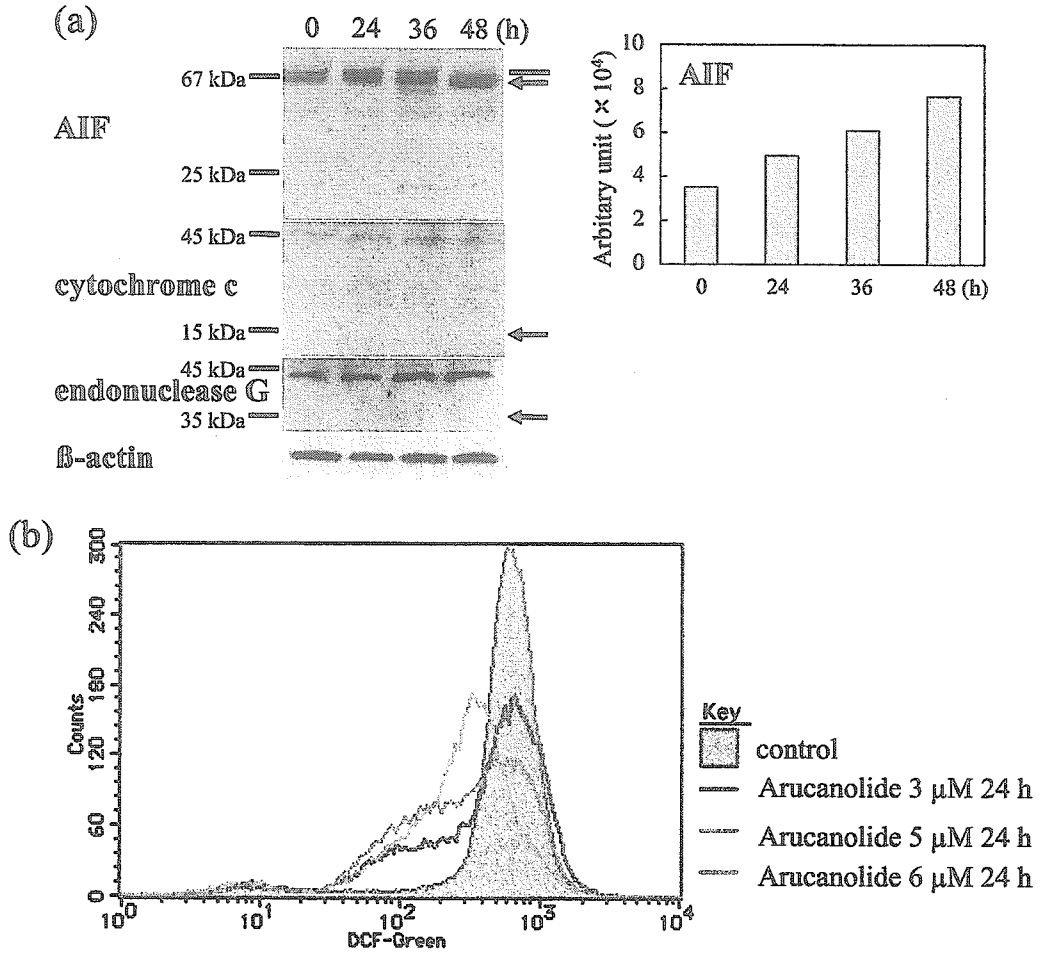

Fig. 4. Arucanolide-induced cell death in HL60. a: Activation of AIF, cytochrome c, and endonuclease G after the treatment with 6 μM arucanolide examined by Western blot analysis. b: Mitochondrial membrane potential after treatment with 3, 5, or 6 μM arucanolide. The relative fluorescence intensity of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

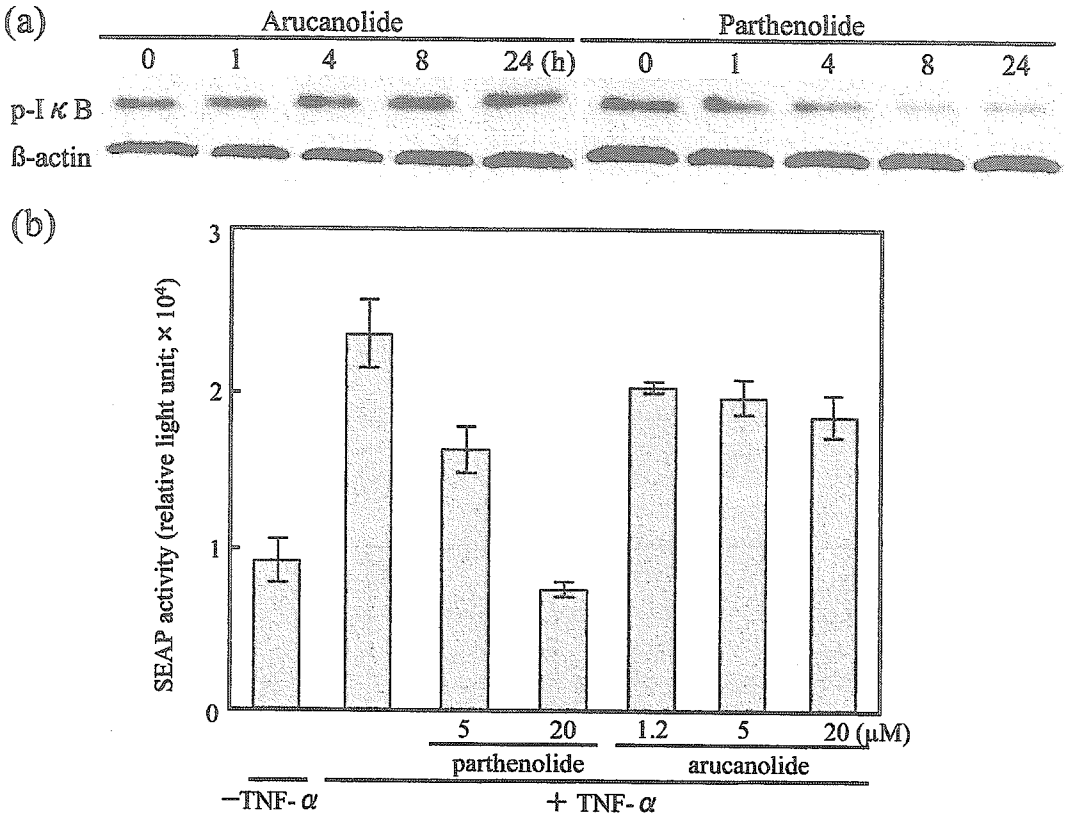

Fig. 5. NF-κB signaling in arucanolide-induced cell death. a: Phosphorylated form of IκB in HL60 cells examined by Western blot analysis compared with those in parthnolide-treated cells. b: Secreted alkaline phosphatase (SEAP) activety in parthenolide- and arucanolide-treated HeLa cells. The method is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

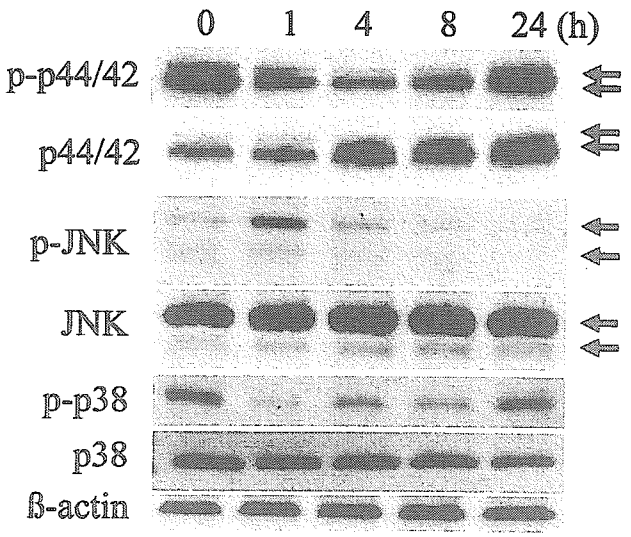

Fig. 6. Death-associated MAP kinase signaling of arucanolide-induced cell death in HL60 cells. p44/42 MAP kinase, p-p44/42 MAP kinase, JNK, p-JNK, p38, and p-p38 were examined by Western blot analysis.

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