Kayeassamin A Isolated from the Flower of *Mammea siamensis* Triggers Apoptosis by Activating Caspase-3/-8 in HL-60 Human Leukemia Cells

Takuhiro Uto[1], Nguyen Huu Tung[1,2], Pinjutha Thongjankaew[3], Sorasak Lhieochaiphant[4], Yukihiro Shoyama[1]

[1]Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nagasaki International University, Sasebo, Nagasaki 859-3298, Japan, [2]School of Medicine and Pharmacy, Vietnam National University, Hanoi, Vietnam, [3]Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, [4]Faculty of Pharmacy, Payap University, Muang, Chiang Mai 50000, Thailand

ABSTRACT

Background: *Mammea siamensis* (Miq.) T. Anders. is used as a medicinal plant in Thailand and has several traditional therapeutic properties. In a previous study, we isolated eight compounds from the flower of *M. siamensis* and demonstrated that kayeassamin A (KA) exhibited potent antiproliferative activity against human leukemia and stomach cancer cell lines. **Objective:** In this study, we investigated the effect of KA on cell viability and apoptotic mechanisms in HL-60 human leukemia cells. **Materials and Methods:** Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Nuclear morphology and DNA fragmentation were observed using Hoechst 33258 staining and agarose gel electrophoresis, respectively. The sub-G1 phase of cells was analyzed by flow cytometry after the cellular DNA had been stained with propidium iodide. The protein levels of poly (ADP-ribose) polymerase (PARP) and caspases were determined by Western blotting. **Results:** KA exhibited a significant cytotoxic effect in a dose- and time-dependent manner, and induced chromatin condensation, DNA fragmentation, and sub-G1 phase DNA content, known as molecular events associated with the induction of apoptosis. In addition, KA strongly induced the activation of PARP and caspase-3 and -8, with weak caspase-9 activation. Furthermore, KA-induced DNA fragmentation was abolished by pretreatment with z-VAD-FMK (a broad caspase inhibitor), z-DEVD-FMK (a caspase-3 inhibitor), and z-IETD-FMK (a caspase-8 inhibitor), but not by z-LEHD-FMK (a caspase-9 inhibitor) pretreatment. **Conclusion:** These results indicate that KA triggers apoptotic cell death by activation of caspase-3 and -8 in HL-60 cells.

Key words: Apoptosis, caspase, kayeassamin A, leukemia, *Mammea siamensis*

SUMMARY

- Kayeassamin A (KA) isolated from the flower of *Mammea siamensis* exhibited a significant cytotoxic effect in HL-60 human leukemia cells. KA triggers apoptotic cell death by activating caspase-3/-8.
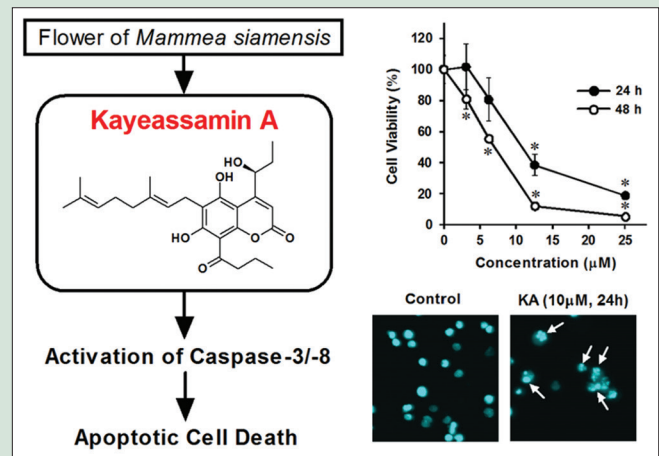
Abbreviations Used: KA: Kayeassamin A; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP: Poly (ADP-ribose) polymerase; PI: Propidium iodide; CA: Corosolic acid

Correspondence:

Prof. Yukihiro Shoyama,
Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki 859-3298, Japan.
E-mail: shoyama@niu.ac.jp
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INTRODUCTION

Mammea siamensis (Miq.) T. Anders., distributed throughout Thailand where it is known as "Sarapee," is a small evergreen tree with fragrant yellow or white flowers that belongs to the family *Calophyllaceae*.[1,2] In traditional medicine, its flowers have been used as an analgesic and a heart tonic, and for boosting appetite.[3] Previous phytochemical studies demonstrated that the flower of *M. siamensis* is a rich source of various coumarins.[4,5] Mahidol *et al.* isolated the series of typical coumarins from *M. siamensis*.[5] Laphookhieo *et al.*[6] and Morikawa *et al.*[7] also isolated several coumarins from *M. siamensis* and confirmed the cytotoxic activity and the inhibition activity against nitric oxide production in lipopolysaccharide-activated RAW264.7 cells, respectively. Win *et al.* isolated the structurally-related coumarins including typical derivatives such as kayeassammins from *Kayea assamica*.[8,9] In our previous study, we also isolated eight coumarin constituents from *M. siamensis* flowers including mammeanoyl, a

novel geranylated coumarin, and confirmed antiproliferative effects using several cell lines.[10]

Apoptosis is the process of programmed cell death that occurs in multicellular organisms and is characterized by unique cellular mitochondrial fragmentation and dysfunction, nuclear condensation,

cytoplasmic shrinkage, blebbing of the plasma membrane, and phagocytosis by neighboring cells.[11] Apoptosis is regulated by two major pathways, the mitochondrial (or intrinsic) pathway and membrane death receptor (or extrinsic) pathway.[12] The mitochondrial mediated pathway, which activates caspase-9, and the death receptor-activated pathway, which activates caspase-8, converge at caspase-3, and finally induce cell death.[13] Thus, the induction of apoptosis is a key target in chemotherapy and the development of novel anticancer agents.[14,15]

Our previous study demonstrated that three structurally-related coumarins, isolated from the flower of *M. siamensis*, showed statistically significant antiproliferative activity in human leukemia and stomach cancer cell lines. Of these, kayeassamin A (KA) [Figure 1] was the most potent antiproliferative compound.[10] Structure and activity relations in a naturally occurring coumarin antibiotics, novobiocin have been reviewed well resulted that a free hydroxyl group in coumarin nucleus was found to be important for its antifungal and antibacterial activity and substituent at C-4 or C-7 on coumarin nucleus contributed to high activity.[16] From the above evidences we selected KA as a candidate of anticancer agent through apoptosis phenomena. In this study, we aimed to investigate the antiproliferative and apoptosis-inducing mechanisms of KA in the human promyelocytic leukemia HL-60 cell line.

MATERIALS AND METHODS

Reagents

Antibodies against caspase-3, -8, -9, and poly (ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibody against β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fetal bovine serum (FBS) was from GIBCO (Gaithersburg, MD, USA). Caspase inhibitors were from Calciochem (San Diego, CA, USA). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of kayeassamin A

KA used for this study was isolated form the flower of *M. siamensis* as previously described.[10] Briefly, the air-dried sample of 100 g was extracted with MeOH. After the removal of solvent, the obtained residue (24.5 g) was suspended in water and successively partitioned with hexane, CH_2Cl_2, EtOAC, and n-BuOH to obtain soluble fractions of hexane (6.14 g), CH_2Cl_2 (1.31 g), EtOAC (1.31 g), and n-BuOH (6.42 g). The hexane and CH_2Cl_2 fractions were combined and then subjected to a silica gel column chromatography and a reverse-phase column chromatography for the purification of KA (150 mg).
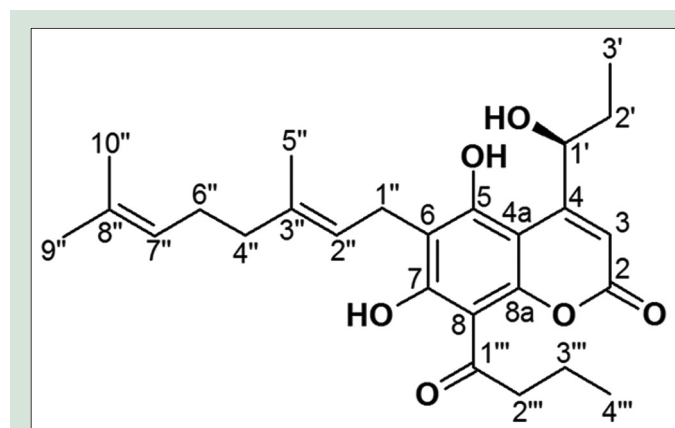

Figure 1: Chemical structure of kayeassamin A

Cell culture and treatment

HL-60 cell line was obtained from the RIKEN BioResource Center Cell Bank and cultured at 37°C in a 5% CO_2 atmosphere in RPMI1640 medium containing 10% FBS. For cell treatment, KA and caspase inhibitors were dissolved in dimethyl sulfoxide (DMSO) and were stored at −20°C before use. DMSO concentrations in the cell culture medium did not exceed 0.2% (v/v), and the controls were always treated with the same amount of DMSO as used in the corresponding experiments.

Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, the cells (1×10^4 cells/well) were treated with KA at various concentrations for 24 h or 48 h. At the end of treatment, MTT solution was added to each well, and the cells were incubated for another 4 h. The precipitated MTT-formazan was dissolved with 0.04 N HCl-isopropanol, and the amount of formazan was measured at 595 nm using a microplate reader (iMark, BioRad, Tokyo, Japan). Cell viability was expressed as a percentage of the control culture.

Nuclear staining by Hoechst 33258

Cells (1×10^6 cells) were treated with KA for 24 h, and then the cells were harvested, washed with PBS, and fixed with 1% glutaraldehyde for 30 min. After washing with PBS, the cells were stained with Hoechst 33258 for 10 min. The cells were washed with PBS, and nuclear morphology was observed by a fluorescent microscopy (Eclipse E600, Nikon, Tokyo, Japan).

DNA fragmentation analysis

Cells (1×10^6 cells) were treated with KA, and then cells were washed with ice-cold PBS and resuspended in lysis buffer (50 mM Tris-HCL, pH 8.0, 10 mM ethylenediaminetetraacetic acid, and 0.5% sodium dodecyl sulfate) with 0.2 mg/ml RNase A for 30 min at 50°C. Proteinase K was then added and incubated for overnight. The DNA was separated on 2% agarose gel and visualized under ultraviolet illumination after staining with ethidium bromide.

Flow cytometric detection of apoptosis cell by propidium iodide staining

Cell cycle analysis to detect the sub-G_1 phase was performed using a cell cycle phase determination kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, cells (1×10^6 cells) were treated with KA for 24 h, and then the cells were washed and fixed with fixative and suspended with staining solution containing propidium iodide (PI) and RNase A. The sub-G_1 peak was measured and analyzed in the FL2 channel of a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with a 488 nm excitation laser. A total of 10,000 cells were analyzed per sample.

Western blot analysis

Cells (1×10^6 cells) were plated in 6 cm dish. After treatment with KA for various periods, the harvested cells were lysed, and the supernatants were boiled for 5 min. Protein concentration was determined by using a dye-binding protein assay kit according to the manufacturer's manual (Bio-Rad, Richmond, CA, USA). Equal amounts of lysate protein were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred to polyvinylidene fluoride membranes and were detected as described previously.[17,18]

Statistical analyses

The experimental results are presented as mean ± standard error of the mean. Each experiment was repeated at least three times. Data were analyzed by ANOVA followed by Dunnett's test using GraphPad Prism 6 (GraphPad software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of kayeassamin A on cell proliferation

To determine the cytotoxicity of KA in HL-60 cells, we first examined cell viability after treatment with various concentrations of KA for 24 h and 48 h by using the MTT assay. A dose- and time-dependent decrease in viability was observed in the presence of KA [Figure 2]. The IC$_{50}$ values at 24 h and 48 h were 11.9 µM and 6.2 µM, respectively.
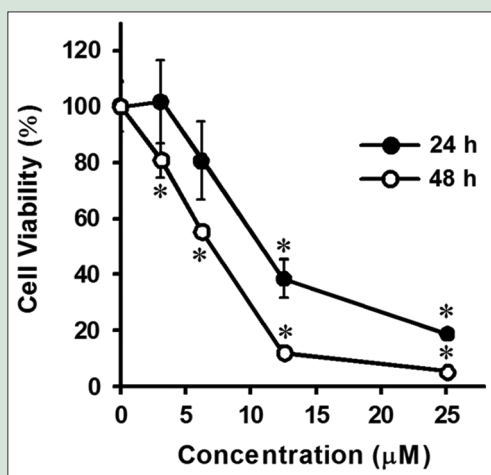
Effect of kayeassamin A on the induction of apoptosis

To explore whether the antiproliferative activity of KA is related to apoptosis, we evaluated the presence of markers of apoptosis, including nuclear morphological changes and DNA fragmentation, in HL-60 cells. Following treatment with 10 µM KA for 24 h, nuclear morphology was determined using Hoechst 33258 staining. As shown in Figure 3a, control cells exhibited normal nuclear morphology whereas cells treated with KA showed chromatin condensation. Furthermore, DNA fragmentation was examined based on the classical DNA laddering using agarose gel electrophoresis. Figure 3b shows that KA treatment (10 µM) led to the appearance of a DNA ladder in a time-dependent manner. Furthermore, we analyzed the sub-G1 phase of cells by flow cytometry after the cellular DNA had been stained with PI. As shown in Figure 3c, KA increased the accumulation of cells in the sub-G1 phase from 9.8% (control) to 68.1% (KA, 10 µM). In summary, these results clearly indicate that KA exerted its antiproliferative effect via the induction of apoptotic cell death.
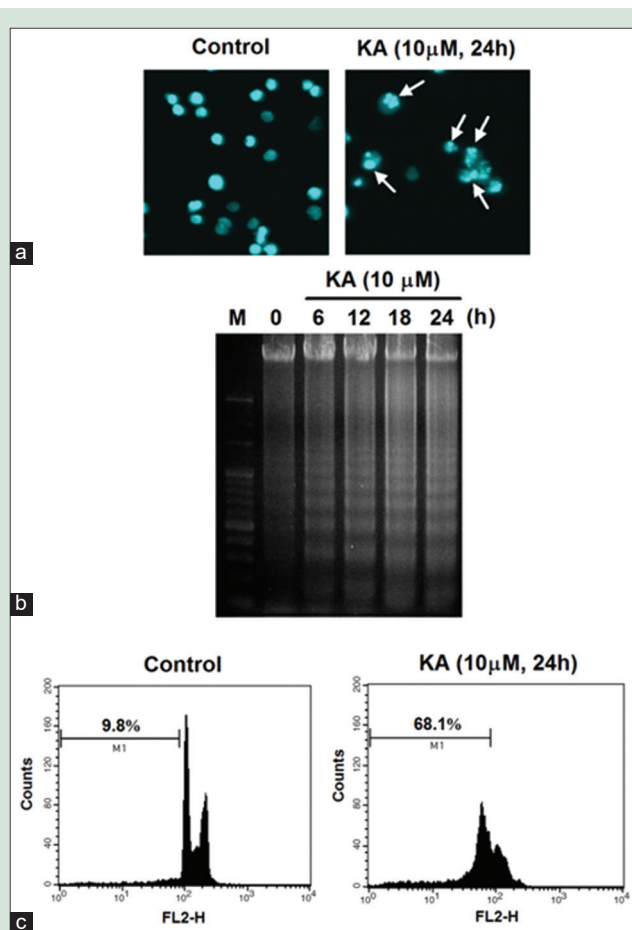
Effect of kayeassamin A on the caspase cascade

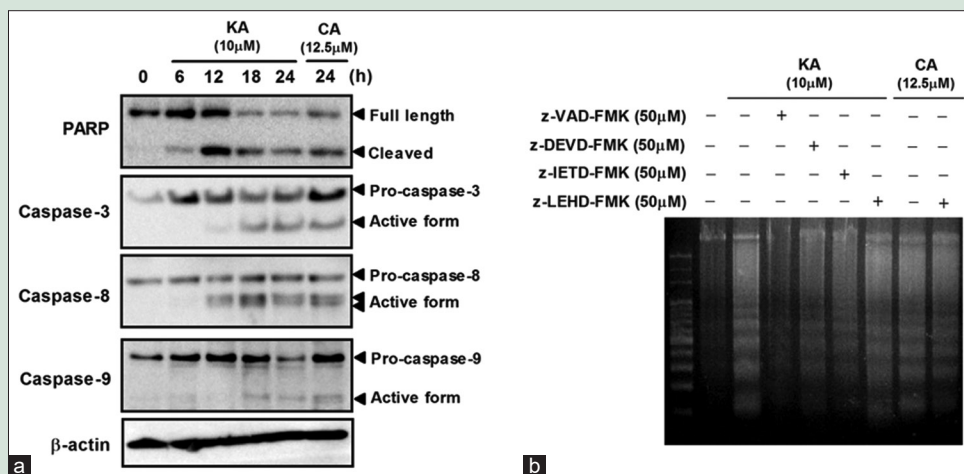In order to confirm the role of caspases in the induction of apoptosis by KA, we determined the protein levels of PARP and caspase-3, -8, and -9. Caspase-3 is the main downstream effector in the induction of apoptotic cell death, and PARP is the endogenous substrate protein of caspase-3.[19] As a positive control, we used corosolic acid (CA), which induces apoptosis through the activation of caspase-3, -8, and -9.[20] KA caused the cleavage of PARP and activated caspase-3 in a time-dependent manner [Figure 4a]. The activation of caspase-3 requires the activation of initiator caspases such as caspase-8 and -9. Our data demonstrated that KA strongly activated caspase-8, although it weakly induced the activation of caspase-9. To further confirm the involvement of specific caspases in KA-induced apoptosis, cells were treated with KA in the absence or presence of caspase inhibitors. As shown in Figure 4b, KA-induced DNA fragmentation was abolished by pretreatment with z-VAD-FMK (a broad caspase inhibitor), z-DEVD-FMK (a caspase-3 inhibitor), and z-IETD-FMK (a caspase-8 inhibitor). However, z-LEHD-FMK (a caspase-9 inhibitor) did not suppress KA-induced DNA fragmentation although it did inhibit DNA fragmentation induced by CA as a positive control. These results indicate that KA-induced apoptosis involves a caspase-3/8-dependent pathway in HL-60 cells.

Figure 3: Effect of kayeassamin A on apoptosis induction. (a) Induction of chromatin condensation by kayeassamin A. HL-60 cells were treated with kayeassamin A (10 µM) for 24 h and were stained with Hoechst 33258. (b) Induction of DNA fragmentation by kayeassamin A. HL-60 cells were treated with kayeassamin A (10 µM) for indicated times and the DNA fragmentation was analyzed by agarose gel electrophoresis. M is the 100-bp DNA marker. (c) Increase in the sub-G1 phase cells by kayeassamin A. HL-60 cells were treated with the indicated concentration of kayeassamin A (10 µM) for 24 h and were then analyzed by flow cytometry after staining with propidium iodide. The data shown are representative of 3 independent experiments that had similar results

Figure 2: Effects of kayeassamin A on cell proliferation in HL-60 cells. Cells were treated with kayeassamin A at various concentrations for 24 h or 48 h, and the cell viability was determined using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The data represent the mean ± standard error of the mean for three individual experiments. *$P < 0.05$ compared with the control group

Figure 4: Involvement of the caspase cascade in kayeassamin A-induced apoptosis. (a) The effect of kayeassamin A on poly (ADP-ribose) polymerase cleavage and the activation of caspase-3, -8, -9. HL-60 cells were treated with kayeassamin A (10 μM) for the times indicated. The cells were lysed, and poly (ADP-ribose) polymerase, caspase-3, -8, -9, and β-actin protein levels were determined by Western blotting. (b) The effect of caspase inhibitors on kayeassamin A-induced DNA fragmentation. After pretreatment with 50 μM caspase inhibitors for 1 h, HL-60 cells were treated with kayeassamin A (10 μM) for 18 h. DNA fragmentation was analyzed by agarose gel electrophoresis. The data shown are representative of three independent experiments with similar results. Corosolic acid is a positive control

DISCUSSION

Apoptosis is a highly-regulated programmed cell death process in which cells undergo inducible nonnecrotic cellular suicide, and therefore plays an important role in anticarcinogenesis. The induction of apoptosis involves the activation of caspases.[12] There are two major pathways in the caspase cascade; the intrinsic pathway or mitochondrial pathway involves spase-9 activation, while the extrinsic pathway involves death receptor-mediated activation via caspase-8.[12,13] Initiator caspases, such as caspase-8 and -9, self-activate in response to apoptotic stimuli, whereas the activation of effector caspases, such as caspase-3, requires the activation of initiator caspases.[12,13] The induction of apoptosis is the most frequently observed mechanism of anticancer agents. The strategy to selectively induce apoptosis in cancer cells is therefore important for cancer chemoprevention and chemotherapy.[14,15]

In our previous anticancer phytotherapeutic research, we reported the potent anticancer activity of alkannin derivatives from *Alkanna tinctoria*[21] and of crocin from saffron (*Crocus sativus*).[22,23] As part of an ongoing study, our preliminary screening of Thai medicinal plants showed promising antiproliferative effects of a methanol extract from *M. siamensis* flowers on several human cancer cell lines. In our previous study, bioassay-guided fractionation and a chemical investigation of the methanol extract of *M. siamensis* flower resulted in the isolation and identification of eight compounds, including a novel geranylated coumarin (mammeanoyl) and seven known compounds. Among the isolated compounds, three structurally-related coumarins showed statistically significant antiproliferative activities against human leukemia and stomach cancer cell lines. Of these, KA exerted the strongest activity.[10] Moreover, these compounds did not affect cell viability in colon cancer, hepatoma, and normal skin fibroblast cell lines.[10]

In this study, we investigated the mechanism of action of KA in HL-60 human leukemia cells. Our results indicated that KA strongly suppressed cell growth, with 24 h and 48 h IC_{50} values of 11.9 μM and 6.2 μM, respectively. Moreover, KA-induced apoptosis was shown to implicate a caspase-dependent pathway involving caspase-3 and its initiator caspases-8, but not caspase-9. The extrinsic pathway via caspase-8 involved binding of a ligand to one of the tumor necrosis factor families of death receptors, followed by activation of caspase-8 and -3.[24] To

examine the effect of KA on the Fas/FasL system, we investigated the expression of Fas and FasL. However, KA had no effect on either Fas or FasL protein expression (data not shown). To elucidate the mechanism by which KA triggers the activation of caspase-8, further investigation of the molecular mechanism of KA-induced apoptosis is required. Our findings suggest that KA is a potential anticancer candidate for use in the treatment of leukemia as we previously selected KA as a candidate of anticancer drug depending on the structure and activity relations.[16] Moreover, KA has three hydroxyl groups in a molecule. A phenolic hydroxyl group at C-5 can function chelating with some donor between a β-hydroxyl group at C-1′. Other phenolic hydroxyl group at C-7 makes chelation junction with carboxyl group at C-1‴. These structural functions may further promote activity. It would be interesting to determine whether KA-induced apoptosis contributes to anticancer activity *in vivo*.

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Conflicts of interest

There are no conflicts of interest.

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ABOUT AUTHOR

Yukihiro Shoyama, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nagasaki International University, Japan.

Yukihiro Shoyama