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Secoaggregatalactone-A from *Lindera aggregata* Induces Apoptosis in Human Hepatoma Hep G2 Cells

Original Paper

Abstract

A new secobutanolide, secoaggregatalactone A (**1**) was isolated from the leaves of *Lindera aggregata*. Results obtained from the cytotoxicity assay revealed that secoaggregatalactone A exhibited a noticeable cytotoxicity (EC_{50} = 6.61 μg/mL; 22.1 μM) against the human hepatoma cell line (Hep G2 cell line). According to morphological observations, flow cytometric analysis, and DNA fragmentation analysis, it was proven that the cytotoxicity of secoaggregatalactone A on human cells was due to apoptosis. Moreover, based on the results from the protein expression assay and confocal laser scanning microscope observations, it is assumed that secoaggregatalactone A induced apoptosis through the mitochondria pathway by way of cleavage of Bit to release cytochrome *C* and activate caspases-9 and -3, and then degradation of PARP.

Key words
Secoaggregatalactone A · apoptosis · cytotoxicity · *Lindera aggregata* · Lauraceae · mitochondrial pathway

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Introduction

Lindera aggregata (Sims) Kosterm (*L. strychnifolia* Vill) (Lauraceae) is an endemic evergreen tree grown in Taiwan. It has a strong fragrance and is a traditional Chinese medicine used for treating several symptoms including chest and abdominal pain, indigestion, regurgitation, cold hernia, and frequent urination [1]. It has been proved that extracts from roots and leaves of *L. aggregata* have significant superoxide dismutase (SOD)-like activity [2]. Ohno et al. evaluated the possible preventive activity of *L. aggregata* on the progression of diabetic nephropathy. They suggested that the water extract of *L. aggregate* could be a novel therapeutic agent against diabetic nephropathy [3]. Wang and his co-workers demonstrated that treatment with alkaloids from the root of *L. aggregata* could reduce inflammation and joint destruction in a type II collagen-induced model of rheumatoid arthritis [4]. Several sesquiterpenes, proanthocynidin trimer, and alkaloids were isolated from *L. aggregata*. [5], [6], [7], [8], [9]. Recently, two new sesquiterpene lactones were isolated from the water extract of *L. aggregata* root, which showed strong cytotoxicity against human small cell lung cancer SBC-3, and lesser cytotoxicity against mouse fibroblast cells 3T3-L1 [10]. However, the mechanism of suppression of cell growth is not clear until now. In our systematic study on the bioactivities of Lauraceae trees grown in Taiwan, extracts of *L. aggregate* were potently cytotoxic [11]. To investigate the active principle of *L. aggregata* against human hepatoma Hep G2 Cells, the methanolic extract was isolated by bioactivity-guided fractionation. A new secobutanolide, namely secoaggregatalactone A (**1**) was identified from the EtOAc fraction of the methanolic extract. Secoaggregatalac-

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tone A (**1**) induces apoptosis-related proteins in human hepatoma cells. The results of our current study are reported herein.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, tryspin-EDTA and fetal bovine serum (FBS) were obtained from GIBCO BRL (Gaithersburg, MD, USA). Sodium dodecyl sulfate (SDS) and Tris–HCl were purchased from Bio Basic Inc. (Markham, Canada). Dimethyl sulfoxide (DMSO), propidium iodide, ribonuclease A (RNase A), NP-40, sodium deoxycholate, z-LEHD-fmk and β-actin antibody were bought from Sigma Chemical (St. Louis, MO, USA). Poly (ADP-ribose) polymerase (PARP), Bid, procaspase-3 and procaspase-8 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). All other chemicals and solvents used in this study were of the reagent grade and HPLC grade.

Plant material

The leaves of *L. aggregata* were collected from the Experimental Forest of National Chung-Hsing University, in July, 2005 and were identified by Dr. Yen-Hsueh Tseng (NCHU). The voucher specimen [*LCT001* (TCF)] was deposited in the herbarium of the same university.

Extraction and isolation

Air-dried leaves of *L. aggregata* (6.0 kg) were extracted with MeOH (60 L) at ambient temperature, and concentrated under vacuum to yield the MeOH extract (351.2 g). The MeOH extract was partitioned between EtOAc-H_2O to give the EtOAc-soluble fraction (60.1 g) and the H2O-soluble fraction. The EtOAc-soluble fraction displayed potent cytotoxicity (EC_{50} = 44.83 μg/mL), and was further chromatographed over silica gel (8 × 120 cm; 60 – 80 mesh; Merck; Darmstadt, Germany) eluted with *n*-hexane and a gradient of *n*-hexane-EtOAc (95 : 5; 90 : 10; 85 : 15; 80 : 20; 70 : 30; 60 : 40; 50 : 50; 40 : 60, 0 : 100, each 2 L) to produce 27 fractions. Part of fraction 7 (5.3 g) was subjected to silica gel chromatography by elution with *n*-hexane-EtOAc (85 : 15), and then eluted completely with EtOAc to give 15 fractions (7 – 1 to 7 – 15). Fraction 7 – 4 (2.8 g) displayed the strongest cytotoxicity (EC_{50} = 7.12 μg/mL), and was further separated by HPLC using a reversed-phase column (Prep Nova-Pak HR C_{18}, 300 × 7.8 mm, 6 μm column, Waters; Milford, MA, USA) with an MeOH/H_2O gradient (0 – 3 min, MeOH/H_2O = 80/20; 3 – 7 min, MeOH/H_2O = 80/20 linear gradient to 100/0; 10 – 20 min, MeOH/20 = 100/0) as eluant at a flow rate of 2.5 mL/min to obtain compound **1** (R.t. = 10.5 min) (1.68 g).

Secoaggregatalactone A (**1**): colorless oil; $[\alpha]_D^{25}$: + 73.75 (*c* 0.295, $CHCl_3$); UV (MeOH, log ε): λ_{max} = 215 nm (4.32); IR: ν_{max} = 3450 (br, OH), 1735 (ester), 1710 (ketone) cm^{-1}; ^{1}H-NMR (600 MHz, $CDCl_3$): δ = 0.86 (3H, t, *J* = 6.8 Hz, H-13), 1.28 (14H, br s, H-6 to H-12), 1.45 (2H, m, H-5), 2.13 (3H, s, H-3′), 2.33 (2H, q, *J* = 7.4 Hz, H-4), 3.71 (3H, s, OMe-1), 4.00 (1H, br d, *J* = 4.8 Hz, OH-1′, D_2O exchangeable), 4.88 (1H, br d, *J* = 4.8 Hz, H-1′), 7.06 (1H, t, *J* = 7.6 Hz, H-3); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 14.1 (C-13), 22.6 (C-12), 24.8 (C-3′), 28.6 (C-4), 28.7 (C-5), 29.0 – 30.0 (C-6 – 10), 31.9 (C-11), 52.1 (OMe-1), 73.3 (C-1′), 129.6 (C-2), 149.2 (C-3), 166.5 (C-1), 206.3 (C-2′); FAB-MS: *m*/*z* (rel. int.) = 299 ($[M + H]^+$), HR/FAB-MS: *m*/*z* = 299.4298 $[M + H]^+$, calcd. for $C_{17}H_{31}O_4$: 299.4295.

Cell culture and cytotoxicity assay

Hep G2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin, and were maintained at 37 °C and 5% CO_2. All cells (1×10^4) per well were seeded in 96-well plates and incubated for 24 h, and different dosages (4, 7, 10 μg/mL; 13.8, 23.4 and 33.4 μM) of secoaggregatalactone A (**1**) were added to each well in triplicate for 24 h. The cell viability was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Meanwhile, morphology features of cells and nuclear chromatin were observed by using a phase-contrast microscope and a confocal laser scanning microscope (CLSM) (Leica TCS Sp2 AOBS MP; Wetzlar, Germany).

Flow cytometric analysis

Hep G2 cells (1×10^6 cells/dish) seeded in a 60-mm dish were treated with 4 μg/mL (13.8 μM), 7 μg/mL (33.4 μM), and 10 μg/mL 33.4 (μM) of **1** for 24 h or treated with 10 μg/mL (33.4 μM) **1** for 0, 4, 10, 16, and 24 h, then cells were trypsinized and collected with ice cold PBS. The cells were resuspended in 200 μL PBS, and fixed in 800 μL of iced 95% ethanol at –20 °C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 mg/mL RNase A), and incubated at 37 °C for 30 min. Then, 1 mL of PI solution (50 μg/mL) was added, and the mixture was allowed to stand at 4 °C for 30 min. Fluorescence emitted from the PI–DNA complex was quantitated after excitation of the fluorescent dye by flow cytometry (FACScan, Becton Dickinson; Franklin Lakes, NJ, USA).

Caspase-3 activity and Western blotting assays

The activity of caspase-3 was determined using Chemicon's Caspase-3 Colorimetric Activity Assay Kits (Chemicon International; Billerica, MA, USA). The detailed analysis procedure is described in the manufacturer's protocol. The protein expression after treatment with **1** was determined by a Western blotting assay. Briefly, Hep G2 cells incubated in 60-mm culture dishes (1×10^6 cells per dish) were treated with **1** at the dosages of 4 (13.8), 7 (23.4), and 10 (33.4) μg/mL (μM) for 24 h or treated with **1** at dosages of 10 (33.4 μM) for 0, 4, 10, and 16 h. 20 μg of total cell proteins were separated by electrophoresis on 12% SDS–PAGE and transferred to PVDF membrane. Detection was performed by immunostaining using specific primary antibodies and horseradish peroxidase-conjugated anti-IgG antibody. The proteins were detected by chemiluminescence (ECL, Pierce Biotechnology, Inc.; Rockford, IL, USA). The following antibodies and working dilutions were used for the Western blots, i. e., rabbit polyclonal antibodies to human PARP (1 : 1000), procaspase-3 (1 : 1000), Bid (1 : 1000), procaspase-8 (1 : 1000), β-actin (1 : 500), Goat anti-rabbit immunoglobulin G (IgG)- horseradish peroxidase-conjugate (1 : 5000).

Statistical analysis

Data are expressed means ± SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences (* $p < 0.05$ and ** $p < 0.01$) between the control (untreated) and treated cells were analyzed by Dunnett's test.

Results and Discussion

Secoaggregatalactone A (**1**) (Fig. **1**) was obtained as a colorless oil. The molecular formula was determined to be $C_{17}H_{30}O_4$ by FAB-MS ([M +H]$^+$, *m/z* = 299). The UV spectrum showed absorption maxima at 215 nm and the IR spectrum showed characteristic absorption bands due to the presence of hydroxyl (3450 cm^{-1}), ester (1735 cm^{-1}), and ketone (1710 cm^{-1}) groups. In the ^{1}H-NMR spectra, **1** showed the signals for an olefinic proton at δ = 7.06 (1H, t, *J* = 7.6 Hz, H-3), a hydroxymethine proton at 4.88 (1H, br d, *J* = 4.8 Hz, H-1′), a methoxy group at 3.71 (3H, s, OMe-1), an acetyl group at 2.13 (3H, s, H-3′), and a terminal methyl group of a side chain at 0.86 (3H, t, *J* = 6.8 Hz, H-13). According to MS, UV, and ^{1}H NMR analysis, **1** was similar to secolincomolide A [12], except for the fact that secolincomolide A showed one more methylene units than **1** in the alkyl side-chain. The dextrorotatory optical activity ($[\alpha]_D^{25}$: + 73.75) of **1** indicated that C-1′ possesses the *S* configuration, which is similar to secoisolancifolide ($[\alpha]_D^{25}$: + 102.7) [13], but different from that of secokotomolide ($[\alpha]_D^{25}$: –11.5) [14]. Thus, compound **1** was defined structurally as (2*E*)-2-[(1*S*)-1-hydroxy-2-oxopropyl]-2-tridecenoic acid methyl ester, and the structure was further corroborated by ^{13}C-NMR, DEPT, HSQC, HMBC, and NOESY experiments. Fig. **1** shows the main HMBC and NOESY correlations of secoaggregatalactone A (**1**).

The cytotoxicity of **1** against Hep G2 cells was evaluated by using the standard MTT method. As shown in Fig. **2**, when Hep G2 cells were treated with **1** at dosages of 1 – 20 μg/mL, a dose-dependent decrease of cell viability was observed. Comparison with the anti-tumor compound curcumin [15], [16] revealed that it exhibited a stronger cytotoxicity. The EC_{50} value of **1** against Hep G2 cells was 6.61 μg/mL (22.1 μM). The growth-inhibiting activity of **1** was potent. With a dosage above 10 μg/mL (33.4 μM), the Hep G2 cells showed chromatin condensation, and apoptotic bodies were widespread throughout the entire population (see Supporting Information Fig. **1S**). Due to the observation of morphological changes in Hep G2 cells, it is believed that **1** might cause apoptotic cell death in the Hep G2 cells. To verify the induction of apoptosis and sub-G1 (sub-2N, which has been suggested to be the apoptotic DNA) cell population of **1** furthermore, we determined the cell cycle progression of Hep G2 cells after treatment with **1**. Flow cytometric analysis revealed a dose-dependent increased proportion of apoptotic cells in the sub-G_1 phase. When Hep G2 cells were treated with 0, 4 (13.8), 7 (23.4), and 10 (33.4) μg/mL (μM) of **1** for 24 h, the percentage of sub-G_1 cells was 7.1, 16.9, 25.1, and 40.7 %, respectively (Fig. **3**). On the other hand, in Hep G2 cells treated with 10 μg/mL (33.4 μM) **1** for different incubation periods, the percentage of cells in the sub-G_1 fractions was increased to 15.4 % (4 h), 17.6 % (10 h), and 25.7 % (16 h) (data not shown). The induction of apoptosis was further confirmed by flow cytometry of Hep G2 cells stained with annexin V–FITC and propidium iodide after treatment with 10 μg/mL (33.4 μM) **1** for 24 h. The annexin V assay measures phospholipid turnover from the inner to the outer lipid layer of the plasma membrane, an event typically associated with apoptosis. As indicated by flow cytometric analysis, the proportion of annexin V-staining cells increased with time in the cells treated with **1**; the percentage of annexin V-staining cells was 6.6 % at 0 h, and 50.6 % at 24 h, respectively (see Supporting Information Fig. **2S**). In addition to morphological observations and flow cytometric analyses, we assessed the DNA fragmentation in Hep G2 cells. Fig. **4** shows the DNA fragmentation induced by various concentrations of **1.** Based on the results obtained from morphological observations, flow cytometric and DNA fragmentation analysis, **1** at 7 μg/mL (23.4 μM) for 24 h or 10 μg/mL (33.4 μM) for 16 h efficiently induced apoptosis in Hep G2 cells.

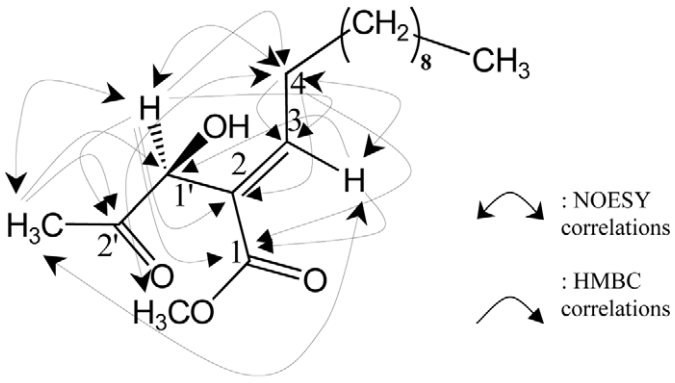

Fig. **1** Main HMBC and NOESY correlations of secoaggregatalactone A (**1**).

It is known that initiator caspases (e. g., caspases-8, -9, -10, and -12) are closely linked to proapoptotic signals [17]. When they are activated, these caspases are cleaved and activate downstream effectors caspases (e. g., caspases-3, -6, and -7), which sequentially cleave nuclear proteins, such as PARP and lamin A and then induce apoptosis [18]. As caspase-3 followed by PARP [Poly (ADP-ribose) polymerase] cleavage is the key event in the process of apoptosis, and as these events are also employed as markers of apoptosis induction [19], the effect of **1** on caspase-3 ac-

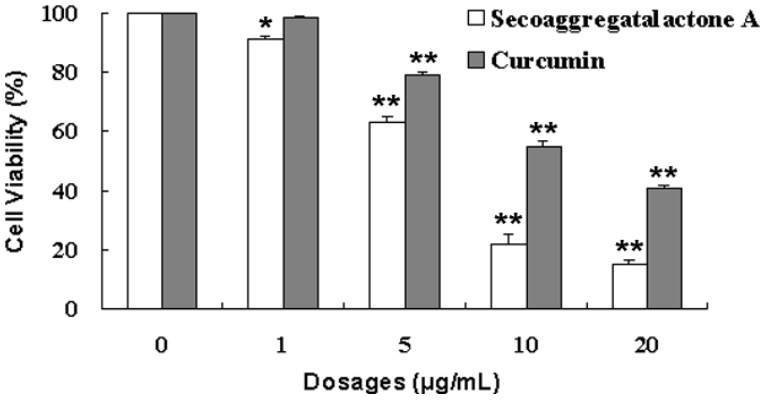

Fig. **2** Cytotoxic activity of secoaggregatalactone A and curcumin in Hep G2 cell lines. The cells were treated with various concentrations (1 – 20 μg/mL (3.3 – 66.9 μM)) of secoaggregatalactone A for 24 h. The data are presented in terms of proportional viability (%) by comparing the secoaggregatalactone A- and curcumin -treated groups with the untreated cells, the viability of which was assumed to be 100 %. Results represent the mean ± SE of three independent experiments. Statistical differences from control (untreated cells) as analyzed by Dunnett's test (* $p < 0.05$ and ** $p < 0.01$).

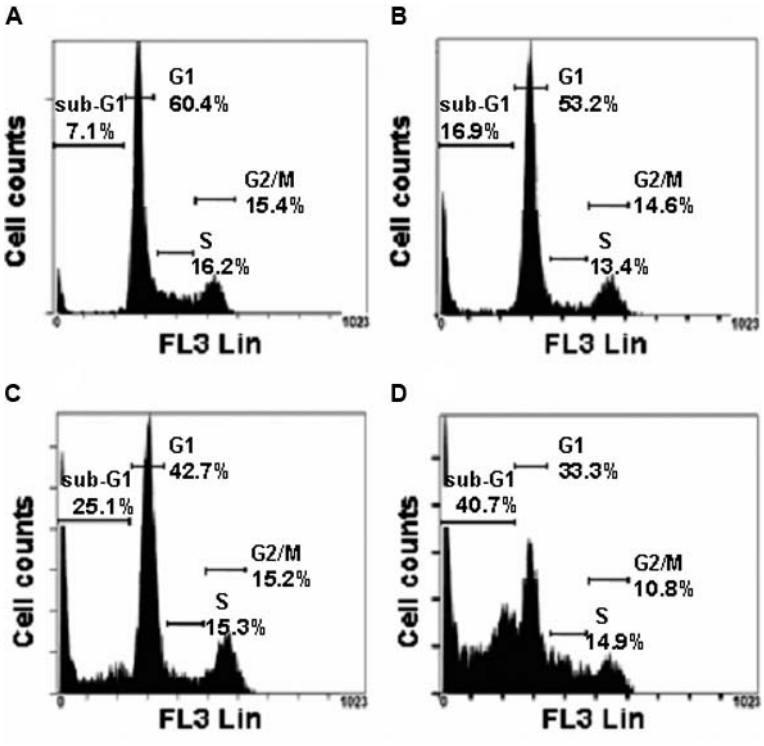

Fig. **3** Flow cytometric analysis of untreated Hep G2 cells and various dosages of secoaggregatalactone A treated cells after 24 h treatment. **A**: 0 μg/mL; **B**: 4 μg/mL (13.8 μM); **C**: 7 μg/mL (23.4 μM); **D**: 10 μg/mL (33.4 μM). Data represent the percentage of cell counts and display a sub-G1. Cell cycle distribution as assessed by flow cytometry.

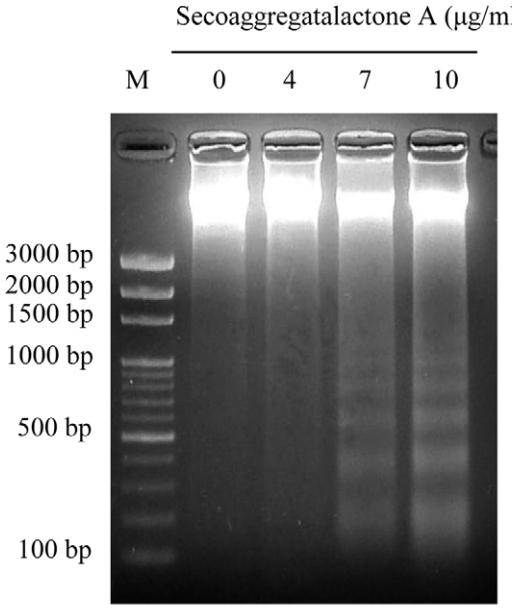

Fig. **4** Secoaggregatalactone A-induced DNA fragmentation in Hep G2 cells. Cells were treated with various concentrations (4 – 10 μg/mL (13.8 – 33.4 μM)) for 24 h. Genomic DNA was isolated and analyzed by 1.8% agarose gel electrophoresis.

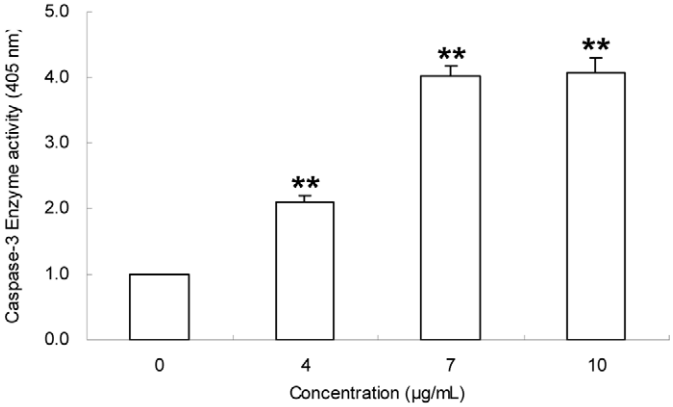

Fig. **5** Analysis of the caspase-3-like activity induced by secoaggregatalactone A. Hep G2 cells were treated with 4, 7, and 10 μg/mL (13.8, 23.4, and 33.4 μM) secoaggregatalactone A for 24 h and caspase-3-like activity in cell extracts was measured with a specific colorimetric substrate. Statistical differences from control (untreated cells) as analyzed by Dunnett's test (* $p < 0.05$ and ** $p < 0.01$).

tivity was evaluated first. Fig. **5** shows the activity of caspase-3 in Hep G2 cells, which was significantly enhanced after treatment with 4 (13.8), 7 (23.4), and 10 (33.4) μg/mL (μM) **1** for 24 h. Moreover, cleaved downstream procaspase-3 could activate the dimeric form of caspase-3 as an executioner of apoptosis. Fig. **6A** shows the results of procaspase-3, cleaved caspase-3, PARP, procaspase-8, and Bid expressions after treatment with 4 – 10 μg/mL (13.8 – 33.4 μM) **1** for 24 h or after treatment with 10 μg/mL (33.4 μM) for 4 – 24 h. It is obvious that **1** induced cleavage of procaspase-3 (32 kDa) into active caspase-3 (19 and 17 kDa), revealing that the expression of procaspase-3 was suppressed and caspase-3 activity was increased. Active caspase-3 could cleave PARP protein (116 kDa) into a 85 kDa fragment. In Fig. **6A**, **1** caused a dose- and time-dependent proteolytic cleavage of PARP, with accumulation of the 85-kDa fragment. Additionally, procaspase-8 and Bid protein expressions were markedly decreased by treatment with 10 μg/mL (33.4 μM) of **1** for different time periods (0, 4, 10, 16, and 24 h) (Fig. **6A**). On the other hand, when Hep G2 cells were incubated with 25 μM z-VAD-fmk (a general caspase-9 inhibitor) or 0.1% DMSO (control) for 1 h before treatment with 10 μg /mL (33.4 μM) **1** for 24 h, it was obvious that caspase-9 activity was inhibited (Fig. **6B**). Meanwhile, cytochrome *C* was released from mitochondria after treatment with 10 μg/mL (33.4 μM) secoaggregatalactone A for 10 h by

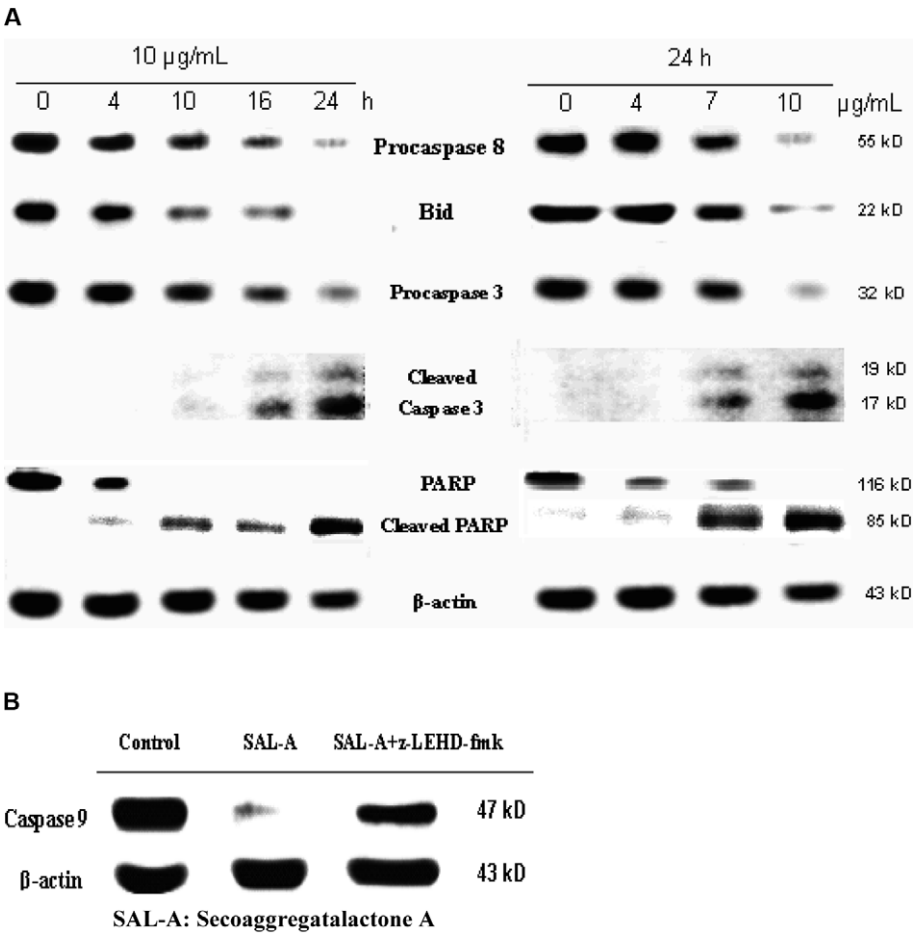

Fig. **6** **A** Hep G2 cells were incubated with 4, 7, and 10 μg/mL (13.8, 23.4, and 33.4 μM) secoaggregatalactone A for 24 h or treated with secoaggregatalactone A 10 μg/mL (33.4 μM) for 0, 4, 10, 16, and 24 h. Procaspase-3, cleaved caspase-3, PARP, procaspase-8 and Bid were determined by immunoblotting using specific antibodies. **B** Effect of z-LEHD-fmk on caspase-9 activity. Hep G2 cells were incubated with 25 μM z-VAD-fmk or 0.1% DMSO (control) for 1 h before treatment with 10 μg /mL (33.4 μM) **1** for 24 h.

using confocal laser scanning microscope (CLSM) observation (see Supporting Information Fig. **3S**). It has been reported that active caspase-8 triggers events downstream of the mitochondrial pathway of apoptosis and amplifies caspase-9 activation through the cleavage of Bid, which in turn was associated with the release of cytochrome *C* [20]. It is reported that **1** induced apoptosis through the mitochondrial pathway.

In summary, secoaggregatalactone A (**1**) was found to induce significant apoptotic cell death in Hep G2 cells, through the activation of caspase-8, Bid, and caspase-3, leading to cleavage of PARP and causing DNA fragmentation resulting in apoptosis.

References

[1] Duke JA, Ayensu ES. Medical Plants of China, Vol. 4. Algonac: Reference Publications; 1985: 399.

[2] Noda Y, Mori A, Anzai K, Packer L. Superoxide anion radical scavenging activity of uyaku (Lindera strychnifolia), a natural extract used in traditional medicine. In: Packer L, Hiramatsu M, Yoshikawa T, editors. Antioxidant food supplementations in human health San Diego: Academic Press; 1999: 471 – 9.

[3] Ohno T, Takemura G, Murata I, Kagawa T, Akao S, Minatoguchi S et al. Water extract of the root of *Lindera strychnifolia* shows down the progression of diabetic nephropathy in *db/db* mice. Life Sci 2005; 77: 1391 – 403.

[4] Wang C, Dai Y, Yang J, Chou G, Wang C, Wang Z. Treatment with total alkaloids from Radix Linderae reduces inflammation and joint destruction in type II collagen-induced model rheumatoid arthritis. J Ethnopharmacol 2007; 111: 322 – 8.

[5] Takeda K, Ishii H, Tozyo T, Minato H. Components of the root of Lindera strychnifolia Vill. 16, Isolation of lindenene showing a new fundamental sesquiterpene skeleton, and its correlation with linderene. J Chem Soc C; 1969: 1920 – 1

[6] Kouno I, Hirai A, Fukushige A, Jiang ZH, Tanaka T. New eudesmane sesquiterpenes from the root of *Lindera strychnifolia*. J Nat Prod 2001; 64: 286 – 8.

[7] Chou GX, Norio N, Ma CM, Wang ZT, Masao H. Isoquinoline alkaloids from *Lindera aggregata*. Chin J Nat Med 2005; 3: 272 – 5.

[8] Zheng CF, Sun QS, Wang ZT, Chou GX. Studies on constituents of the leaves of *Lindera aggregate* (Sims) Kosterm. Chin J Chin Mater Med 2001; 26: 765 – 7.

[9] Zhang CF, Sun QS, Wang ZT, Hattori M. One new A-type proanthocynidin trimer from *Lindera aggregate* (Sims) Kosterm. Chin Chem Lett 2003; 14: 1033 – 6.

[10] Ohno T, Nagatsu A, Nakagawa M, Inoue M, Li YM, Minatoguchi S et al. New sesquiterpene lactones from water extract of the root of *Lindera strychnifolia* with cytotoxicity against the human small cell lung cancer cell, SBC-3. Tetrahedron Lett 2005; 46: 8657 – 60.

[11] Lin CT, Chu FH, Tseng YH, Tsai JB, Chang ST, Wang SY. Bioactivity investigation of Lauraceae trees grown in Taiwan. Pharm Biol 2007; 45: 638 – 44.

[12] Tsai IL, Hung CH, Duh CY, Chen IS. Cytotoxic butanolides and secobutaolides from the stem wood of Formosan *Lindera communis*. Planta Med 2002; 68: 142 – 5.

[13] Tanaka H, Nakamura T, Ichino K, Ito K. Two lactonic compounds, lancifolide and isolancifolide, from *Actinodaphne lancifolia*. Phytochemistry 1989; 28: 1905 – 7.

[14] Chen FC, Peng CF, Tsai IL, Chen IS. Antitubercular constituents from the stem wood of *Cinnamomum kotoense*. J Nat Prod 2005; 68: 1318 – 23.

[15] Cao J, Jia L, Zhou HM, Liu Y, Zhong LF. Mitochondrial and nuclear DNA damage induced by curcumin in human hepatoma G2 cells. Toxicol Sci 2006; 91: 476 – 83.

[16] Zhao J, Zhao Y, Zhang Y, Chen W. Anti-tumor effect of curcumin on human cervical carcinoma HeLa cells *in vitro* and *in vivo*. Chin J Cancer Res 2007; 19: 32 – 6.
[17] Chen CN, Wu CL, Lin JK. Propolin C from propolis induces apoptosis through activating caspases, Bid and cytochrome c release in human melanoma cells. Biochem Pharmacol 2004; 67: 53 – 66.
[18] Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh J, Schlesinger PH. Pro-apoptotic cascade activates BID, which oligomerized BAK or BAX into pores that result in the release of cytochrome *C*. Cell Death Differ 2000; 7: 1166 – 73.
[19] Kaufmann S, Desnoyers S, Ottaviano Y, Davidson N, Poirier G. Specific proteolytic cleavage of poly (ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res 1993; 53: 3976 – 85.
[20] Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome *C* release from mitochondria in response to activation of cell death receptors. Cell 1998; 94: 481 – 90.