

Heptelidic Acid, a Sesquiterpene Lactone, Inhibits Etoposide-Induced Apoptosis in Human Leukemia U937 Cells

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In the course of screening for substances that inhibit etoposide (10 µg/ml)-induced apoptosis in human leukemia U937 cells, fungal strain F000120, which exhibits potent inhibitory activity, was selected. The active compound was purified from an ethyl acetate extract of the microorganism by Sep-pak C₁₈ column chromatography and HPLC, and was identified as heptelidic acid (koningic acid) by spectroscopic methods. This compound inhibited caspase-3 induction in U937 cells with an IC₅₀ value of 40 µM after 8 h of etoposide treatment. Fluorescent dye staining with acridine orange and ethidium bromide showed that heptelidic acid inhibited apoptosis. Furthermore, it was found that DNA fragmentation and caspase-3 activation, the biological hallmarks of apoptosis, were inhibited by the compound in a dose-dependent manner, suggesting that heptelidic acid inhibits etoposide-induced apoptosis via downregulation of caspases.

Keywords: Apoptosis, heptelidic acid, U937 cells

Apoptosis is a physiological event important in a diverse array of biological processes ranging from embryonic development [23] to bacterial infection [24]. Indeed, it is well documented that AIDS, stroke, Alzheimer's, Huntington's diseases, Parkinson's diseases, amyotrophic lateral sclerosis, traumatic brain injury, and spinal cord injury are associated with excess apoptosis, whereas cancers, systematic lupus erythematosus, multiple sclerosis, diabetic mellitus, and rheumatoid arthritis are due to inadequate apoptosis in specific cells [4, 10, 11].

Cell death from apoptosis is characterized by microvilli, cell shrinkage, chromatin condensation, nuclear collapse, and cellular fragmentation into apoptotic bodies. In most cases, these morphological changes are accompanied by internucleosomal DNA fragmentation [19]. Caspases play

an integral role in the signal transduction pathway leading to apoptosis. Caspases are cysteine proteolytic enzymes that mediate apoptosis by cleaving aspartic acid residues in order to activate downstream effectors and substrates [2]. A substrate for caspase-3 is poly(ADP-ribose) polymerase (PARP), a 116-kDa enzyme involved in DNA repair [21]. Activated caspase-3 cleaves PARP between amino acids 216 and 217, generating 89- and 24-kDa inactive fragments. The loss of PARP function precludes DNA repair, which contributes to the apoptotic phenotype [17].

Therefore, regulating caspase-3 activity could be a promising means of controlling apoptosis. Based on this idea, we have set a cell-based chemical screening system to discover new anti-apoptotic agents from microbial metabolites. Consequently, we isolated and identified heptelidic acid from the culture broth of *Trichoderma* sp. F000120 as a potent apoptosis regulator. The anti-apoptotic effect of heptelidic acid has not previously been reported. In the present study, the isolation of heptelidic acid and its inhibitory effect on etoposide-induced apoptosis are described.

MATERIALS AND METHODS

Materials

The silica gel (Merck Kieselgel 60, 70–230 mesh, 63–200 µm) and silica TLC plates (Silica gel 60F₂₅₄) were purchased from Merck (Darmstadt, Germany). Etoposide was purchased from Sigma (St. Louis, U.S.A.), and electrophoresis chemicals were purchased from Bio-Rad (Hercules, CA, U.S.A.). The tissue culture plastics were purchased from Falcon, and the media and additives were purchased from Gibco (BRL, U.S.A.).

Cell and Culture Conditions

Human promyeloid leukemia U937 cells were used for apoptosis induction. Cells were obtained from the Korean Collection of Type Cultures KRIBB (KCTC, Daejeon, Korea) and grown in RPMI 1640 (Gibco BRL, U.S.A.) containing 10% FBS, 5 mM HEPES (pH 7.0), 1.2 mg/ml NaHCO₃, 100 units/ml penicillin, and 100 mg/ml streptomycin.

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Instrumental Analysis

Mass spectra were obtained on ESI-MS (electrospray ionization mass spectrometry; Fisons VG Quattro 400 mass spectrometer, U.S.A.). NMR spectra were recorded on a Bruker AMX-500 (U.S.A.).

Measurement of Caspase Activity

Etoposide-induced caspase-3, -8, and -9 activities were measured in U937 cells. Etoposide (10 $\mu\text{g/ml}$) was added to cells in the presence or absence of various concentrations of the compound to be tested. The cells were incubated for 8 h at 37°C in a 5% CO₂-95% air atmosphere. After observing apoptotic cells under a microscope, the cells were lysed with a TTE buffer (10 mM Tris-HCl, 0.5% Triton X100, 10 mM EDTA, pH 8.0), kept on ice for 30 min, and then centrifuged. The activities of caspase-3, -8, -9 were estimated using each substrate of the caspases [12, 13]. The cleavage of the peptide substrate was monitored by AFC (7-amino-4-trifluoro methylcoumarin) liberation using 400 nm excitation and 505 nm emission wavelengths. The released fluorescence was measured on a spectrofluorimeter (Perkin-Elmer LS-50B).

Cell Viability Assay

Cell viability was evaluated by a 3-(4,5-dimethylthiazol-1)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTS) assay [6]. In the MTS assay, the cell suspension was plated (100 μl) in a 96-well microculture plate. After seeding, various concentrations of the compound were added to the plate and incubated for 24 h. The MTS/PMS solution was prepared by mixing 25 μl of phenazinemethosulfate (PMS) (1.53 mg/ml in PBS) for every 975 μl of MTS (1.71 mg/ml in PBS). Finally, 50 μl of MTS/PMS solution was added to each well and incubated for 1 to 3 h. The absorbance of formazan at 490 nm was measured directly from the 96-well plates without additional processing.

Acridine Orange and Ethidium Bromide Morphological Fluorescence Dye Staining

Acridine orange (AO) stains DNA bright green, allowing visualization of the nuclear chromatin pattern. Apoptotic cells have condensed chromatin that is uniformly stained. Ethidium bromide (EB) stains DNA orange but is excluded by viable cells. Dual staining allows separate enumeration of populations of viable non-apoptotic, viable (early) apoptotic, nonviable (late) apoptotic, and necrotic cells. The assay was performed as previously described [1, 3]. Briefly, cells were pelleted, resuspended in 50 μl of complete media, and stained with 4 μl of AO-EB working solution (100 $\mu\text{g/ml}$ of AO and 100 $\mu\text{g/ml}$ of EB in 0.9% phosphate-buffered saline). Samples were immediately analyzed by fluorescence microscopy utilizing a fluorescein filter (excitation at 488 nm, emission at 520 nm) [22].

Western Blot Analysis

The cells were washed with ice-cold PBS three times, lysed, and homogenized in 0.2 ml of ice-cold lysis buffer (0.1 M Tris-HCl, pH 7.2, 1% NP-40, 0.01% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g/ml}$ leupeptin, and 1 $\mu\text{g/ml}$ aprotinin). An aliquot of lysate was used to determine the protein concentration by the Bradford method. Fifty μg per proteins per lane was loaded onto 15% and 8% SDS-polyacrylamide gels to detect caspase-3 and PARP, respectively [7]. After running at 100 V for 2 h, size-separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.) at 250 mA for 2 h. Membranes were blocked with 5% skim milk for 1 h and washed with 0.05% TBST (TBS containing

0.05% Tween 20). Membranes were then incubated for 2 h with antibodies specific for caspase-3 (R&D System, Minneapolis, MN, U.S.A.) and PARP (BD Pharmingen, San Diego, U.S.A.), respectively. After washing three times in 0.05% TBST, membranes were incubated with anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Amersham, Buckinghamshire, U.K.) and detected with the Amersham ECL system. The expression of b-actin was used as a normalizing control.

DNA Fragmentation Assay

The DNA fragmentation assay was conducted as previously described [8, 20]. Cells were lysed with buffer (EGTA, Triton-X100, and Tris-HCl, pH 7.4) and incubated for 20 min on ice, and then centrifuged at 500 $\times g$ for 10 min at 4°C. Cytosolic DNA in the supernatant was extracted with phenol:chloroform (1:1). DNA was treated with 0.1 mg/ml RNase A for 30 min at 37°C, and then separated by 1% agarose gel electrophoresis and visualized with ethidium bromide staining.

RESULTS AND DISCUSSION

Isolation and Purification of Heptelidic Acid

The fungal strain F000120 was isolated from a soil sample that was collected around Odae mountain, Kangwon-do, Korea. After 6 days of cultivation, a cultured broth (3 l) of fungal strain F000120 was filtered through Whatman No. 2 filter paper. The filtrate was concentrated by evaporation and extracted with ethyl acetate. The ethyl acetate extract on concentration left a residue of a dark syrup, which was loaded onto a Sep-pak C₁₈ cartridge (Waters; 5 g), and the column was eluted with increasing proportions of MeOH-H₂O (8:2). The active fraction was further purified by using a reversed-phase column (Capcell Pak C₁₈, 250 \times 10 mm, S-5 μm , 120 Å) with an acetonitrile-H₂O gradient solvent system, resulting in pure compound **1** (3.8 mg). The structures of purified substances were determined by instrumental analyses, including ESI-MS, ¹H-NMR, and ¹³C-NMR. Based on ESI-MS results, the molecular weight of compound **1** was determined to be 280. In the ¹H-NMR (CD₃OD, 300 MHz, ppm) spectrum of **1**, 0.8–1.6 (6 H, m), 1.02 (3H \times 2, d, J =14.5 Hz), 1.80 (1 H, m), 1.82 (1 H, m), 2.59, 2.36 (2 H, d, J =5.0 Hz), 4.74, 4.76 (2 H, d, J =5.0 Hz), and 7.18 (1 H, dd, J =4.5, 1.5 Hz) signals were detected. In the ¹³C-NMR (CD₃OD, 75 MHz, ppm) spectrum of **1**, 21.2 (CH₃ \times 2), 22.2 (CH₂), 30.0 (CH), 30.2 (CH), 33.0 (CH₂), 45.4 (CH), 50.7 (CH), 52.0 (CH₂), 56.0 (C), 61.6 (CH₂), 128.6 (C), 156.2 (CH), 170.0 (C), and 177.0 (C) signals were detected. Therefore, the chemical structure of this compound **1** was identified as heptelidic acid [5, 9] by comparison of the spectral results from a previous study with the literature values (Fig. 1). Heptelidic acid (koningic acid) was reported to be a fungal metabolite that is active against anaerobic bacteria and showed antimalarial activity [9, 18]. The inhibition of glyceraldehyde-3-phosphate dehydrogenase was also reported [5]. Nakazawa *et al.* [16] have reported

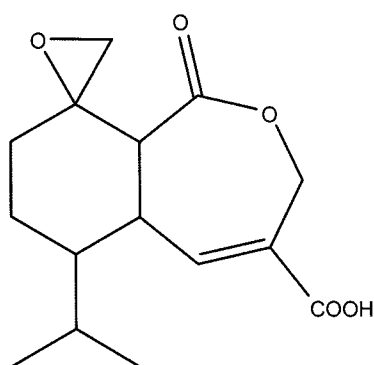


Fig. 1. Chemical structure of heptelidic acid isolated from *Trichoderma* sp. F000120.

that heptelidic acid induces nucleosomal DNA fragmentation in NG108-15 cells. However, the anti-apoptotic effect of heptelidic acid has not previously been reported.

Heptelidic Acid Suppresses Etoposide-Induced Apoptosis in U937 Cells

A family of caspases plays a pivotal role in the execution of apoptosis. Caspases, including caspase-3, caspase-8, and caspase-9, have been reported to be involved in the apoptotic process [15]. Caspase-3 is the final executioner enzyme associated with cell death during stimuli-induced apoptosis [14]. Therefore, we investigated the effect of heptelidic acid on caspase-3, caspase-8, and caspase-9 activity in human leukemia U937 cells. Heptelidic acid showed an inhibitory effect on caspase-3 production in etoposide-induced U937 cells, with an IC_{50} value of 40 μ M (Fig. 2). As shown in Fig. 3, caspase-8 and caspase-9 activity in etoposide-induced cells was inhibited by heptelidic acid. Nevertheless, heptelidic acid was not cytotoxic to U937 cells at concentrations ranging from 10 to 160 μ M (Fig. 4). The occurrence of apoptosis was also assessed by the complementary, semi-

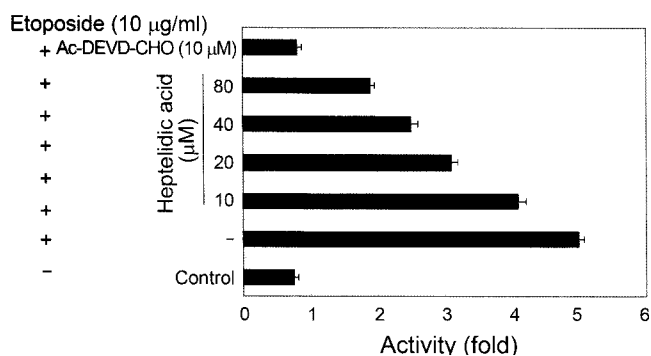


Fig. 2. Effects of various concentrations of heptelidic acid on the etoposide-induced caspase-3 activity in human leukemia U937 cells.

The caspase-3 activity was measured in U937 cells, as described in Materials and Methods. The results are averages of triplicate experiments, and the data are expressed as means \pm SD.

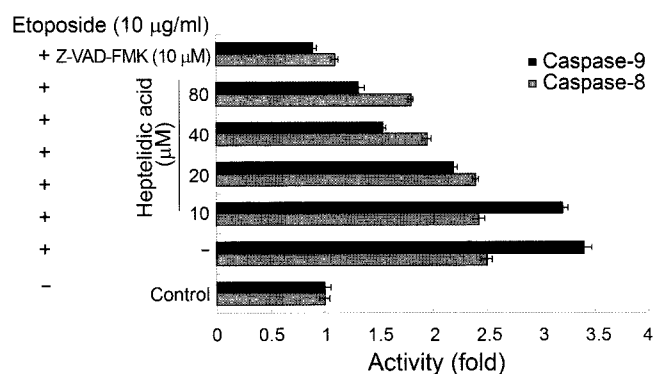


Fig. 3. Effects of various concentrations of heptelidic acid on etoposide-induced caspase-8 and caspase-9 activity in human leukemia U937 cells.

The caspase-8 and caspase-9 activities were measured in U937 cells, as described in Materials and Methods. The results are averages of triplicate experiments, and the data are expressed as means \pm SD.

quantitative fluorescent dye technique using acridine orange and ethidium bromide. As shown in Fig. 5, viable cells (A) and heptelidic acid (B) were uniformly stained green, whereas cells treated with etoposide showed nuclear chromatin condensation (Fig. 5C). However, late apoptotic cells were not observed following treatment with both etoposide and heptelidic acid for 24 h (Fig. 5D).

Next, we investigated the effects of heptelidic acid on caspase-3-like-specific substrate degradation. As shown in Figs. 6A and 6B, degradation of procaspase-3, a classical substrate for active caspase-3, and PARP were inhibited by heptelidic acid, whereas β -actin, an internal control, was not affected (Fig. 6C). Since internucleosomal DNA fragmentation is a biochemical feature of the apoptotic process, we also investigated whether heptelidic acid inhibits DNA fragmentation (Fig. 7). When U937 cells were treated with etoposide in the presence or absence of various concentrations of heptelidic acid, DNA fragmentation was inhibited in a dose-dependent manner. These results suggest that heptelidic

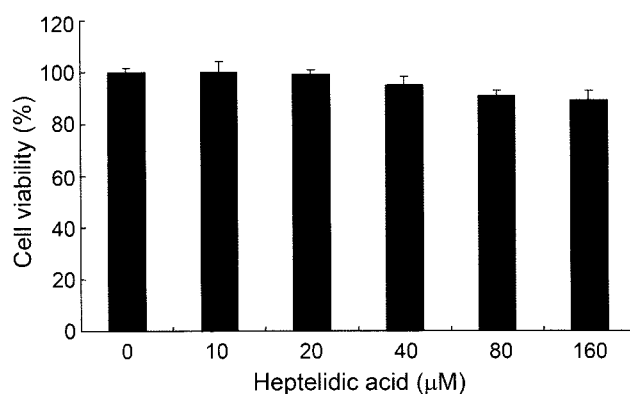


Fig. 4. Cytotoxic effects of heptelidic acid on human leukemia U937 cells after 24 h of heptelidic acid treatment, as measured by MTS assay (described in Materials and Methods).

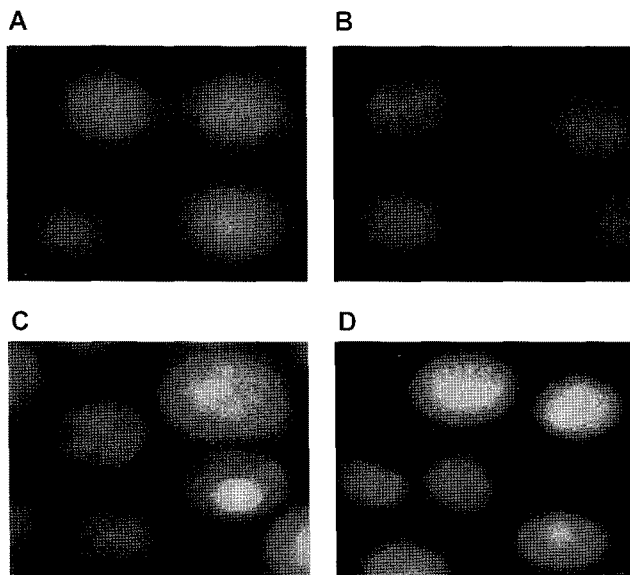


Fig. 5. Effect of heptelidic acid on nuclear morphology of etoposide-treated cells as revealed by fluorescence dye staining. A representative nuclear morphology of normal (viable), early (viable) apoptotic, late (nonviable) apoptotic, and necrotic (dead) U937 cells obtained by fluorescence dye staining procedure at 400× magnification. **A**, untreated cells; **B**, cells treated with heptelidic acid only (40 μM); **C**, cells treated with etoposide only (10 μg/ml); **D**, cells treated with etoposide (10 μg/ml) and heptelidic acid (40 μM).

acid inhibits etoposide-induced apoptosis *via* caspase activation.

In summary, we conclude that heptelidic acid plays a role in the inhibition of etoposide-induced caspase-3-like protease activation. This specific molecular function of heptelidic acid encourages its development as an apoptosis inhibitor in certain cells. Given the present lack of appropriate drugs for treating apoptosis-related diseases, such as neurodegenerative disorders, the identification of heptelidic acid may be important in the discovery of improved therapeutic candidates for the treatment of these diseases. Further studies will focus on the *in vivo* molecular

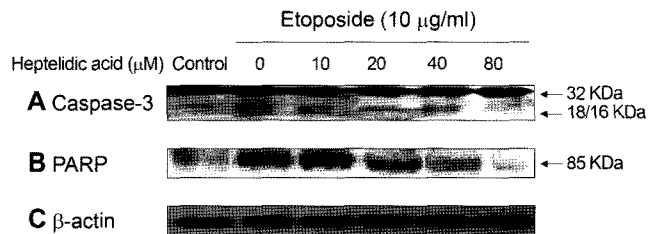


Fig. 6. Western blot analysis of caspase-3 and PARP in etoposide-induced human leukemia U937 cells. Cells were treated with 10, 20, 40, and 80 μM of heptelidic acid for 8 h. Blots were prepared and probed with goat polyclonal anti-caspase-3 antibody (**A**), rabbit polyclonal anti-PARP (**B**), or mouse monoclonal anti-β-actin (**C**) antibodies. Immunoreactivity was determined using anti-mouse or anti-rabbit peroxidase-conjugated secondary immunoglobulin G antibody, followed by enhanced chemiluminescence. Experiments were repeated at least three times.

Etoposide (10 μg/ml)	-	+	+	+	+
Z-VAD-FMK (10 μM)	-	-	-	-	+
Heptelidic acid (μM)	-	-	20	40	-

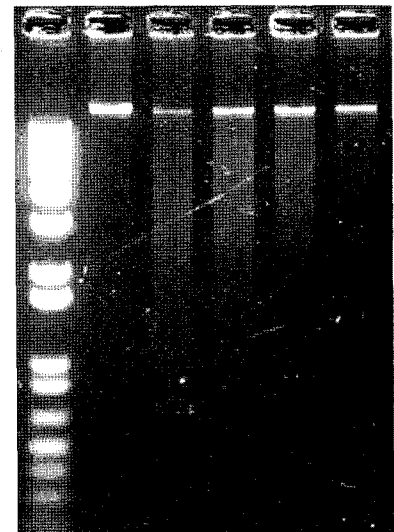


Fig. 7. Effect of heptelidic acid on apoptotic DNA fragmentation in etoposide-induced human leukemia U937 cells. U937 cells (1×10⁶) were incubated for 8 h in the indicated concentration of heptelidic acid, and then collected and harvested to analyze DNA fragmentation.

mechanism approach for the curative possibility of such apoptosis-related diseases by heptelidic acid.

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