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Atromentin-Induced Apoptosis in Human Leukemia U937 Cells

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In the course of screening for apoptotic substances that induce apoptosis in human leukemia U937 cells, a fungal strain, F000487, which exhibits potent inducible activity, was selected. The active compound was purified from an ethyl acetate extract of the microorganism by Sep-pak C_{18} column chromatography and HPLC, and was identified as atromentin by spectroscopic methods. This compound induced caspase-3 processing in human leukemia U937 cells. The caspase-3 and poly (ADP-ribose) polymerase (PARP) were induced by atromentin in a dose-dependent manner. Furthermore, DNA fragmentation was also induced by this compound in a dose-dependent manner. These results show that atromentin potently induces apoptosis in U937 cells and that atromentin-induced apoptosis is related to the selective activation of caspases.

Keywords: Apoptosis, atromentin, U937 cells

Apoptosis is genetically programmed cell death that is essential for development, the maintenance of tissue homeostasis, and the elimination of unwanted or damaged cells from multicellular organisms [7]. When cells are dysregulated, apoptosis can contribute to various diseases such as cancer, autoimmune, and neurodegenerative diseases [1, 15]. Therefore, understanding the mechanism of apoptosis is important for preventing and treating many diseases [5]. Cell death resulting from apoptosis is characterized by cell shrinkage, chromatin condensation, nuclear collapse, and cellular fragmentation into apoptotic bodies. In most cases, these morphological changes are accompanied by internucleosomal DNA fragmentation [25]. Central components of the conserved apoptotic machinery include Bcl-2, Apaf-1 (apoptotic protease activating factor 1), and caspase family members [3]. Caspases are a conserved family of cysteine proteases that play pivotal roles in apoptosis [21]. The

caspases are synthesized as dormant proenzymes, which upon proteolytic activation, acquire the ability to cleave key intracellular substrates that result in the morphological and biochemical changes associated with apoptosis [3, 11]. A substrate for caspase-3 is poly (ADP-ribose) polymerase (PARP), a 116-kDa enzyme involved in DNA repair [27]. 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 [22]. In most cases, apoptosis involves the release of cytochrome c from the mitochondria [23, 26]. In the cytosol, cytochrome c activates caspase-9, which in turn activates effector caspases such as caspase-3 [6]. The induction of apoptosis in tumor cells has been shown to be the most common anti cancer mechanism targeted by therapy. Compounds used in cancer chemotherapy, such as etoposide, cisplatin, doxorubicin, and paclitaxel, have apoptosis-inducing activity [2, 8, 17]. Therefore, chemical agents exhibiting strong apoptosis-inducing activity but minimal toxicity would be expected to have potential utility as anticancer drugs.

In particular, the regulation of caspase-3 activity could be a promising way to control apoptosis. Based on this idea, we have set up a cell-based chemical screening system to discover new pro-apoptotic agents from microbial metabolites. Consequently, we isolated and identified atromentin from the culture broth of fungal strain F000487 as a potent apoptosis inducer. In the present study, the isolation and the new regulatory activity of atromentin on apoptosis are described.

MATERIALS AND METHODS

Materials
The silica gel (Merck Kieselgel 60, 70–230 mesh, 63–200 mm) and silica TLC plates (Silica gel 60F$_{254}$) 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

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purchased from Falcon and the media and additives were purchased from Gibco (BRL, U.S.A.).

Cells and Culture Conditions

Human promyeloid leukemia U937 cells obtained from the Korean Collection of Type Cultures KRIBB (KCTC, Daejeon, Korea) were grown in RPMI 1640 medium (Gibco BRL, U.S.A.) containing 10% FBS, 5 mM HEPES (pH 7.0), 1.2 mg/ml NaHCO$_3$, 100 units/ml penicillin, and 100 µg/ml streptomycin.

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

The activities of caspase-3 and -9 were measured in U937 cells, which were incubated 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% CO$_2$–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. Caspase-3 activity in the cell lysate was estimated using a substrate of caspases [18, 20]. The cleavage of the peptide substrate was monitored by 7-amino-4-trifluoro methylcoumarin (AFC) 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 [11]. In the MTS assay, the cell suspension was plated (100 µl) on a 96-well microculture plate (BD, Franklin Lakes, NJ, U.S.A.). 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 assay plates without additional processing.

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 µg/ml leupeptin, 1 µg/ml aprotinin). An aliquot of lysate was used to determine the protein concentration by the Bradford method. Fifty µg of 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, the size-separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.) at 250 mA for 2 h. The membranes were blocked with 5% skimmed milk for 1 h and washed with 0.05% TBST (TBS containing 0.05% Tween-20). The membranes were then incubated for 2 h with antibodies specific to caspase-3 (R&D System, Minneapolis, MN, U.S.A.), caspase-9 (Cell Signaling Technology, Beverly, MA, U.S.A.), and PARP (BD Pharmingen, San Diego, U.S.A.). After washing three times in 0.05% TBST, the 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 β-actin was used as a normalizing control.

DNA Fragmentation Assay

The DNA fragmentation assay was conducted as previously described [12]. Cells were lysed with a buffer (EGTA, Triton-X100, and Tris-HCl, pH 7.4) and incubated for 20 min on ice, and then centrifuged at 500 ×g for 10 min at 4°C. Cytosolic DNA in the supernatant was extracted with phenol:chloroform (1:1). The DNA was treated with 0.1 mg/ml RNase A for 30 min at 37°C, and fragments were separated by gel electrophoresis on 1% agarose and visualized with ethidium bromide staining.

RESULTS AND DISCUSSION

Isolation and Purification of Atromentin

The fungal strain F000487 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 F000487 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 C$_{18}$ cartridge (Waters; 5 g), and the column was eluted with increasing proportions of MeOH-H$_2$O (8:2). The active fraction was further purified by using a reversed-phase column (Capcell Pak C$_{18}$, 250×10 mm, S-5 µm, 120 Å) with an acetonitrile-H$_2$O gradient solvent system, resulting in pure compound 1 (3.7 mg). The structures of purified substances were determined by instrumental analyses, including ESI–MS, ^{1}H-NMR, and ^{13}C-NMR. From the observation of ESI–MS, the molecular weight of compound 1 could be assigned as 326. In the ^{1}H-NMR (CD$_3$OD, 300 MHz, ppm) spectrum of 1, 6.68 (2 H, d, J=8.5 Hz) and 7.46 (2 H, d, J=8.5 Hz) signals were detected. In the ^{13}C-NMR (CD$_3$OD, 75 MHz, ppm) spectrum of 1, 115.8 (CH×2), 125.1 (C×2), 127.8 (CH×2), 131.5 (C×2),
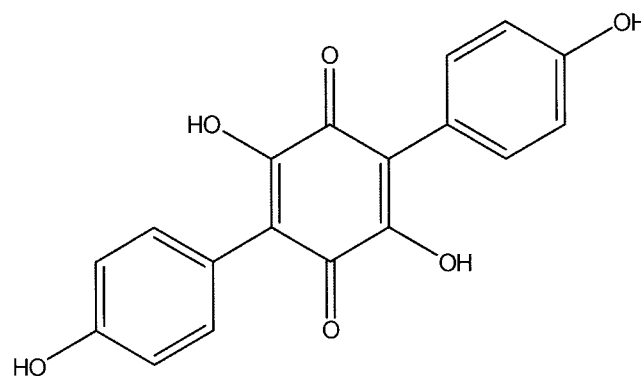

Fig. 1. Chemical structure of atromentin isolated from a fungal strain F000487.

157.7 (C×2), 168.0 (C×2), and 178.6 (C×2) signals were detected. Therefore, the chemical structure of compound 1 was identified as atromentin [9, 14] by comparison of the spectral results from a previous study to the literature values (Fig. 1). Atromentin, which belongs to the mushroom pigments and p-terphenyl compounds, was first described in 1878 by Thörner [24] and reviewed in 2006 by Liu [19]. The structure of atromentin isolated from a lignicolous mushroom, *Paxillus panuoides,* and other mushrooms has been spectroscopically elucidated [9, 14]. Atromentin has been reported to possess an anticoagulant activity and enoyl-[acyl carrier protein (ACP)] reductase (FabK)-specific inhibitors [28].

Atromentin Induces Apoptosis in Human Leukemia U937 Cells

We initiated our study by examining the cytotoxic effects of atromentin using the MTS assay in human leukemia U937 cells. The viability of U937 cells was inhibited in a dose-dependent manner (Fig. 2). The 50% inhibited concentration upon incubation with atromentin for 24 h was 18.4 μM.

Caspases play key roles in promoting the degradative changes in DNA that are associated with apoptosis. Caspase-9 plays a role mainly in the mitochondria-mediated pathway. On the other hand, caspase-3, appears to be essential for apoptosis [16]. Therefore, we examined whether the induction of apoptosis by atromentin resulted in activation of upstream caspase-9 and downstream caspase-3 (Fig. 3). The activation of caspases was measured using fluorogenic and Western blot analyses of U937 cells treated with atromentin. After treatment with atromentin, U937 cells showed increased caspase-9 activity (Fig. 3), whereas procaspase-9 clearly cleaved forms of caspase-9 (Fig. 4A). Atromentin showed an inducing activity for caspase-9 and caspase-3 production-inducing activity in a dose-
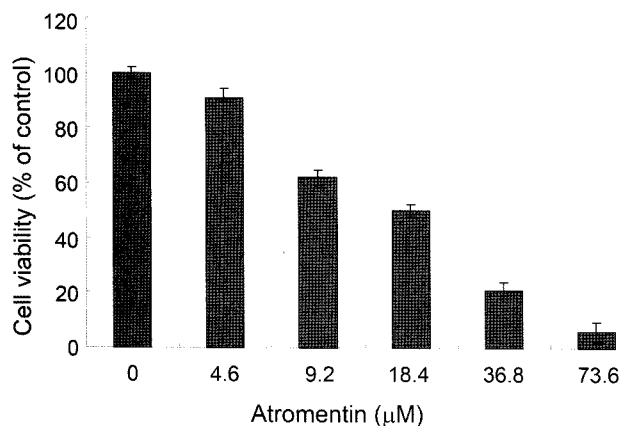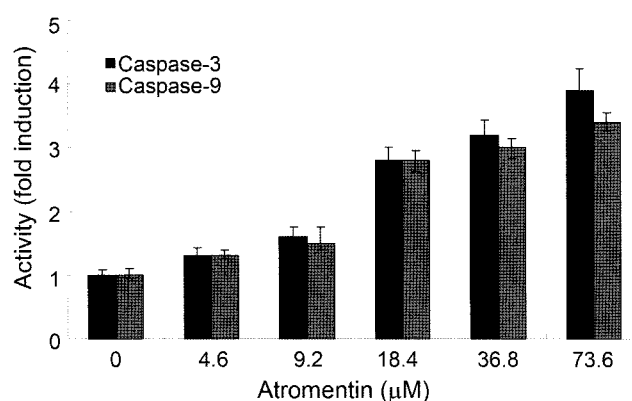

Fig. 3. Dose response effects of atromentin on caspase-9 and caspase-3 activities in human leukemia U937 cells.

The caspase-9 and -3 activities were measured as described in the Methods section. The results are averages of triplicate experiments, and the data are expressed as means±SD.

dependent manner (Fig. 3). Next, to determine the level of apoptosis-related protein expression induced in the U937 cells, we investigated caspase-9, caspase-3, and PARP by Western blot analysis. As shown in Fig. 4, the Western blot analysis revealed a dose-dependent decrease in procaspase-3 and its cleavage into an active form. In addition, the activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. Although PARP is not essential for cell death, the cleavage of PARP is another hallmark of apoptosis. It is known that PARP is characteristically processed during apoptosis from its native 116 kDa form into a truncated 85 kDa product [4]. The atromentin treatment of U937 cells induced PARP degradation, whereas β-actin, an internal control, was not affected (Fig. 4D).

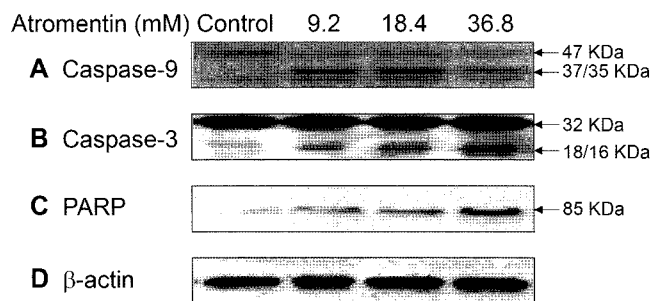Since internucleosomal DNA fragmentation is a biochemical feature of the apoptotic process, we also

Fig. 2. Inhibitory effects of atromentin on the growth of U937 cells.

Cytotoxicity was determined by an MTS assay, as described in the Methods section. The results are averages of triplicate experiments, and the data are expressed as means±SD.

Fig. 4. Western blot analysis of caspase-9, caspase-3, and PARP levels in atromentin-treated human leukemia U937 cells.

Cells were treated with 9.2, 18.4, and 36.8 μM of atromentin for 8 h. Blots were prepared and probed with rabbit anti-caspase-9 (A), goat polyclonal anti-caspase-3 antibody (B), rabbit polyclonal anti-PARP (C), or mouse monoclonal anti-β-actin (D) antibodies. Immunoreactivity was determined using anti-mouse (Amersham) or anti-rabbit (Amersham) peroxidase-conjugated secondary immunoglobulin G antibody, followed by enhanced chemiluminescence (ECL, Amersham). The figures are representative of one of three independent experiments.
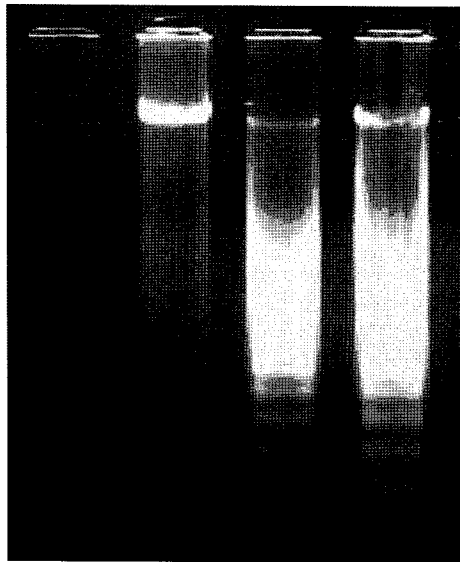
Atromentin (μM)   —   9.2   18.4   36.8

Fig. 5. Effect of atromentin on DNA fragmentation in human leukemia U937 cells.
U937 cells (1×10⁶) were collected after incubation for 8 h with the indicated concentration of atromentin, and then harvested in order to analyze apoptotic DNA fragmentation.

investigated the effect of atromentin on the induction of DNA fragmentation (Fig. 5). When U937 cells were treated with atromentin, DNA fragmentation occurred in a dose-dependent manner.

Carcinogens usually cause genomic damage in exposed cells. As a consequence, the damaged cells may be triggered either to undergo apoptosis or to proliferate, leading to the formation of cancerous cells that generally exhibit cell cycle abnormalities [2]. Thus, the ability of atromentin alone to induce apoptosis suggests its potential use as a chemopreventive agent because many anticancer drugs are known to exert their antitumor function by inducing apoptosis in the target cells. Compounds capable of inducing apoptosis of human cancer cells have recently attracted a great deal of attention owing to their potential utilization as anticancer agents, and many known chemopreventive agents exert their anticancer effects by inducing apoptosis in various cancer cells [13]. In conclusion, atromentin isolated from a fungal strain, F000487, was found to induce apoptosis in human leukemia U937 cells. We are currently investigating whether atromentin can be developed further as a chemopreventive agent.

Acknowledgments

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