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Gentisyl Alcohol Inhibits Apoptosis by Suppressing Caspase Activity Induced by Etoposide

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Abstract In the course of our screening for small molecules to inhibit apoptosis of U937 human leukemia cells induced by etoposide (10 μg/ml), *Penicillium* sp. F020150 with potent inhibitory activity was selected. The active compound was purified from ethyl acetate extract of the microorganism by Sephadex LH-20 column chromatography and HPLC, and was identified as gentisyl alcohol (2,5-dihydroxybenzyl alcohol) by spectroscopic methods. The compound inhibited caspase-3 induction with IC_{50} value of 3.0 μg/ml after 8 h of etoposide treatment. The expression levels of caspase-3 and PARP were dose-dependently inhibited by the compound, suggesting that gentisyl alcohol inhibits etoposide-induced apoptosis via downregulation of caspases.

Key words: Apoptosis, U937 cells, gentisyl alcohol

Apoptosis is a key physiological event responsible for the elimination of unwanted cells during the process of development and the removal of self-reactive lymphocytes [21]. Efficient removal of these cells is important to maintain morphogenesis and homeostasis, and is achieved by an intrinsic mechanism, resulting in cellular suicide [7]. Indeed, it is well documented that AIDS, stroke, Alzheimer's diseases, 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 of specific cells [2, 6, 13, 14, 15, 24].

The distinct morphological and biochemical features of apoptosis include the collapse of the nucleus due to chromatin condensation, the formation of globular apoptotic bodies, increased transglutaminase activity, and genomic DNA fragmentation. Among several players responsible for these features, proteolytic cleavage and activation of caspase-3 may be functionally important in the induction of apoptosis [20]. Caspase (CPP32), one of the members in the CED-3/ICE family, encodes structurally related cysteine proteases which have the unusual substrate specificity for cleavage of Asp-X peptide bonds [7]. Because caspase-3 shows the highest homology to CED-3, and its tetrapeptide inhibitor (DEVD-CHO) often blocks apoptosis induced by a variety of apoptosis inducers, caspase-3 is now thought to be a key regulator in the signaling pathway to control mammalian apoptosis [19]. Notably, caspase-3 is known to be upregulated by the treatment of xenobiotic agents such as etoposide, leading to the apoptotic cell death [22]. As such, the regulation of caspase-3 activity could be a promising way for the control of 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 gentisyl alcohol from the culture broth of *Penicillium* sp. F020150 as a potent apoptosis inhibitor. *Penicillium* is one of the microorganisms that produces metabolites with an important anti-oxidant activity. Moreover, gentisyl alcohol, a phenolic compound, is responsible for DPPH-scavenging activity and oxidative mutagenicity [1]. However, the anti-apoptotic effect of gentisyl alcohol has not previously been reported.

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In the present study, the isolation and the new inhibitory activity of gentisyl alcohol on etoposide-induced apoptosis are described.

MATERIALS AND METHODS

Materials

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 from Bio-Rad (Hercules, CA, U.S.A.). The tissue culture plastics were from Falcon (Becton Dickinson, Franklin Lakes, U.S.A.), and the media and additives were from Gibco (Grand Island, U.S.A.).

Cell and Culture Conditions

Human promyeloid leukemia U937 cells were used for the cell lines of apoptosis induction. The U937 cells obtained from the Korean Collection of Type Cultures KRIBB (KCTC, Daejeon, Korea) were grown in RPMI 1640 (Gibco, Grand Island, U.S.A.) containing 10% FBS, 5 mM HEPES (pH 7.0), 1.2 mg/ml $NaHCO_3$, 100 units/ml penicillin, and 100 mg/ml streptomycin.

Fermentation

A slant culture of the F020150 grown on PDA (Difco, Becton Dickinson, U.S.A.) was used to inoculate a 500-ml baffled flask containing 100 ml of the medium which consisted of yeast extract 0.2%, polypeptone 0.5%, magnesium sulfate 0.05%, potassium dihydrogenphosphate 0.1%, and glucose 2%. The seed culture was cultivated on a rotary shaker at 200 rpm at 25°C for 3 days and then inoculated to a 15-l jar fermenter containing 10 l of the same medium. The fermentation was continued for 6 days at 25°C with agitation rate of 200 rpm and aeration of 1.0 vvm.

Instrumental Analysis

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

Cytotoxicity Assay

Cytotoxicity of the compounds was evaluated by MTS assay (microculture tetrazolium assay) [8]. In the MTS assay, the cell suspension was plated (100 µl) in a 96-well microculture plates. After seeding, various concentrations of test compounds were added to the plate and incubated for 24 h. MTS/PMS solution was prepared by mixing 25 µl of phenazinemethosulfate (PMS) (1.53 mg/ml in PBS) for every 975 µl of 3-(4,5-dimethylthiazol-1)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTS) (1.71 mg/ml in PBS). Finally, 50 µl of MTS/PMS solution were added to each well and incubated for 1 to 3 h. Absorbance of formazan at 490 nm was measured directly from the 96-well assay plates without additional processing.

Measurement of Caspase Activity

The activities of etoposide-induced caspase-3, -8, and -9 were measured in U937 leukemia cells. Etoposide (10 µg/ml) was added to the U937 cells in the presence or absence of various concentrations of the compound to be tested. The cells were incubated for 7 h at 37°C in a 5% CO_2-95% air atmosphere. After observing apoptotic cells under a microscope, the activity of caspase-3, -8, -9 activity was estimated from the cell lysate, using each substrate of caspases [10, 12, 17]. 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 cleavage of the peptide substrate was spectrofluorimetrically (Perkin-Elmer LS-50B, U.K.) monitored by measuring AFC (7-amino-4-trifluoro methylcoumarin) liberation at 400 nm excitation and 505 nm emission wavelengths.

Western Blot Analysis

The cells were washed three times with ice-cold PBS, lysed, and homogenized in 0.2 ml of ice-cold lysis buffer (0.1 M Tris-HCl, pH7.2, 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 protein concentration by the Bradford method [4]. Fifty µg proteins per lane were loaded onto 15% and 8% SDS-polyacrylamide gels to detect caspase-3 and PARP, respectively [9]. After running at 100 V for 2 h, the size-separated proteins were transferred to a PVDF membrane (Millipore, Bedford, MA, U.S.A.) at 250 mA for 2 h. The membranes were blocked with 5% skim 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 antibody for caspase-3 (R&D System, Minneapolis, U.K.) and PARP (BD Pharmingen, San Diego, U.S.A.), respectively. After washing three times in 0.05% TBST, the membranes were incubated with the secondary antibodies conjugated with horseradish peroxidase (Amersham, Buckinghamshire, U.K.) and detected by using the Amersham ECL system. The expression of β-actin was used as a normalizing control.

DNA Fragmentation Assay

The DNA fragmentation assay was done as described [3, 5, 23]. Cells were lysed with buffer (EGTA, Triton-X100 and Tris-HCl, pH 7.4), incubated for 20 min on ice, and then centrifuged at 500 ×*g* for 10 min at 4°C. Cytosolic DNA was extracted by phenol:chloroform (1:1) extraction

of the supernatants. DNA was treated with 0.1 mg/ml RNase A for 30 min at 37°C, and the digest was separated by 1% agarose gel electrophoresis and visualized with ethidium bromide staining.

RESULTS AND DISCUSSION

Isolation and Purification

After 6 days of cultivation, cultured broth (10 l) of *Penicillium* sp. F020150 was filtered with 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 Sep-pak C18 cartridge (Waters, 5 g), and the column was eluted with increasing proportion of MeOH. The active fractions were concentrated and subjected to Sephadex LH-20 column chromatography with MeOH-H_2O (4:6) elution. The active fraction was further purified by using a reversed phase HPLC column (Capcell Pak C18, 250×10 mm, S-5 μm, 120 Å) with the solvent system of acetonitrile-H_2O (1:3), resulting in pure compound **1** (5.0 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 the compound **1** was found to be 140. In the ^{1}H-NMR (CD_3OD, 300 MHz, ppm) spectrum of **1**, 6.74 (1H, d, *J*=2.7), 6.62 (1H, d, *J*=8.7), 6.49 (1H, dd, *J*=8.7, 3.0), 4.79 (2H, d, *J*=5.4) signals were detected. In the ^{13}C-NMR (CD_3OD, 75 MHz, ppm) spectrum of **1,** 150.02 (C), 149.54 (C), 130.02 (C), 117.34 (CH), 116.02 (CH), 115.90 (CH), 60.02 (CH_2) signals were detected. Compound **1** was, therefore, identified as 2,5-dihydroxybenzyl alcohol (gentisyl alcohol) by comparison of its spectral data with the literature values (Fig. 1) [11]. Alfaro *et al.* [1] recently reported the isolation and identification of gentisyl alcohol from *Penicillium novae-zeelandiae* and showed that gentisyl alcohol, which displays both radical-scavenging activity and oxidative mutagenicity, was able to inhibit mutagenesis induced by an organic hydroperoxide.

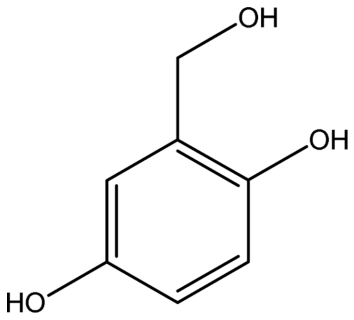

Fig. 1. Chemical structure of gentisyl alcohol isolated from *Penicillium* sp. F020150.

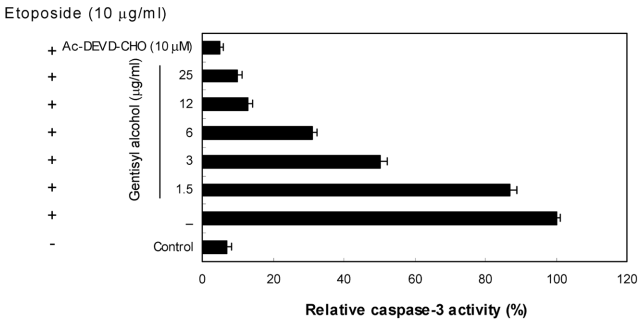

Fig. 2. Effects of various concentrations of gentisyl alcohol on the etoposide-induced caspase-3 activity in U937 human leukemia cells.

Ten μg/ml etoposide was used. The caspase-3 activity was measured after 8 h of etoposide treatment.

Gentisyl Alcohol Suppresses the Etoposide-Induced Apoptosis in U937 Cells

To date, several caspases, including caspase-3, caspase-8, and caspase-9, have been implicated in the apoptotic process [19]. In particular, caspase-3 is the final executioner enzyme associated with cell death during stimuli-induced apoptosis [18, 19]. Once activated, caspase-3 is free to initiate various processes involved in apoptosis. Therefore, we investigated the effect of gentisyl alcohol on the enzymatic activity of caspase-3, caspase-8, and caspase-9 in human leukemia U937 cells previously treated with etoposide. Gentisyl alcohol showed an inhibitory activity on caspase-3 production in etoposide-induced U937 cells with IC_{50} value of 3.0 μg/ml (Fig. 2). As shown in Fig. 3, caspase-8 and caspase-9 activities in etoposide-treated cells were also inhibited by gentisyl alcohol. Nevertheless, gentisyl alcohol was not cytotoxic to U937 cells at the concentrations ranging from 3 to 50 μg/ml (Fig. 4).

The inhibition of caspase-3 activity in the *in vitro* caspase activation system could be due to either direct inhibition of the caspases involved or to inhibition of the

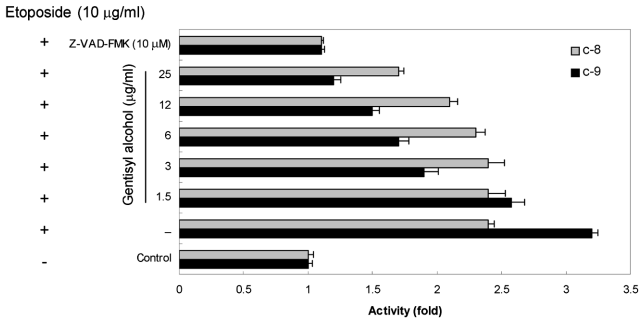

Fig. 3. Effects of various concentrations of gentisyl alcohol on the etoposide-induced caspase-8 and caspase-9 activities in U937 human leukemia cells.

Ten μg/ml etoposide was used. The caspase-8 and caspase-9 activities were measured after 8 h of etoposide treatment.

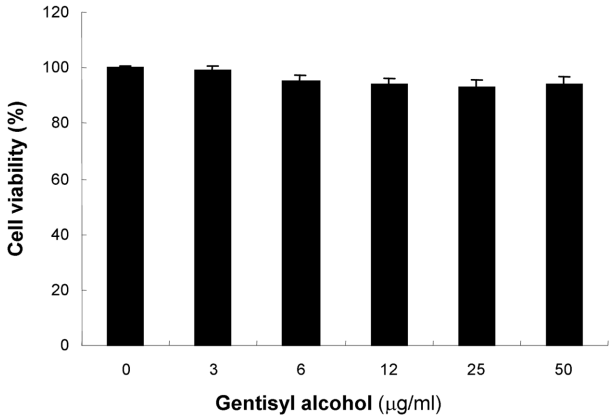

Fig. 4. Cytotoxic effects of gentisyl alcohol on U937 human leukemia cells after 24 h of gentisyl alcohol treatment, measured by MTS assay.

formation of the apoptosome complex [16]. To test whether gentisyl alcohol was a specific caspase inhibitor, it was incubated with recombinant caspase-3, caspase-8, and caspase-9, and caspase activities were measured using specific substrates for caspase-3, caspase-8, and caspase-9, respectively. Gentisyl alcohol *in vitro* failed to inhibit any of the recombinant caspase-3, caspase-8, and caspase-9 at concentrations ranging from 1.5 to 50.0 μg/ml, whereas the specific tetrapeptide caspase inhibitor zVAD-FMK practically completely inhibited the activities (Table 1). Therefore, gentisyl alcohol does not appear to inhibit caspases directly.

We next investigated the effects of gentisyl alcohol on caspase-3-like specific substrate degradation. As shown in Figs. 5A and 5B, procaspase-3, a classical substrate for active caspase-3, and PARP degradation were inhibited by gentisyl alcohol, while β-actin, an internal control, was not affected (Fig. 5C), suggesting that the inhibitory activity of the compound is specific for caspases. Since internucleosomal DNA fragmentation is one of the biochemical features in the apoptotic process, we also investigated the effect of

Table 1. Direct effects of gentisyl alcohol on the recombinant caspase-3, caspase-8, and caspase-9 enzymatic activities.

Gentisyl alcohol (μg/ml)	Activity (%)[a]		
	Caspase-3	Caspase-8	Caspase-9
50	99±0.8	94±0.5	98±0.6
25	97±0.5	95±0.6	94±0.6
12	98±0.3	95±0.8	95±0.9
6	97±0.6	96±1.0	93±0.7
3	99±0.8	95±0.8	95±1.1
1.5	96±0.7	94±0.7	97±0.4
z-VAD-FMK (10 μM)	12±0.3	10±0.4	17±0.3

[a]Activity (%) was measured using specific substrates for caspase-3, caspase-8, and caspase-9, respectively. Each value represents the mean SD of three experiments.

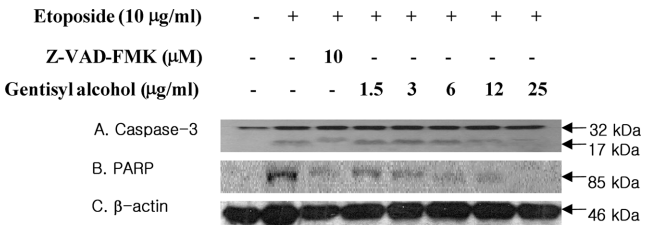

Fig. 5. Western blot analysis of caspase-3 and PARP in etoposide-induced U937 human leukemia cells.
Cells were treated with 1.5, 3, 6, 12, and 25 μg/ml of gentisyl alcohol for 8 h and analyzed by Western blotting, as described in Materials and Methods. Experiments were repeated at least three times.

gentisyl alcohol on the inhibition of DNA fragmentation (Fig. 6). When U937 cells were treated with etoposide in the presence or absence of various concentrations of gentisyl alcohol, the compound dose-dependently inhibited the DNA fragmentation in U937 cells induced by etoposide. These results suggest that gentisyl alcohol inhibits etoposide-induced apoptosis via the suppression of caspase activation.

In summary, we report herein that gentisyl alcohol exhibits the inhibitory activity of etoposide-induced caspase-3-like protease activation. This interesting biological activity of gentisyl alcohol is expected to encourage 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 gentisyl alcohol as an apoptosis inhibitor provides new possibility in the discovery of therapeutic candidates for the treatment of such diseases. Our further studies will be focused on the *in vivo* application of gentisyl alcohol for the curative possibility of apoptosis-related diseases.

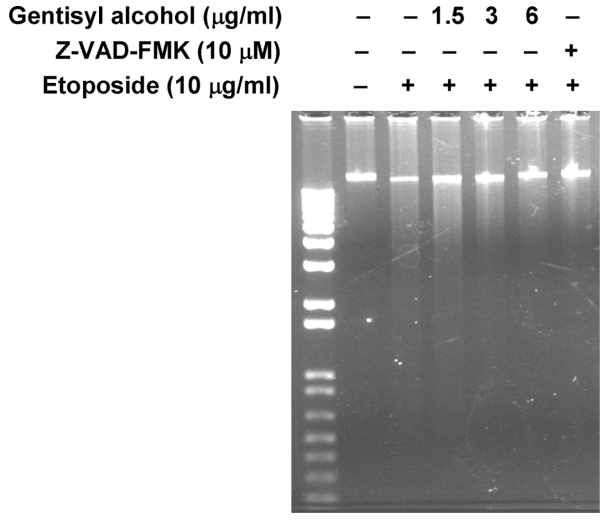

Fig. 6. Effects of gentisyl alcohol on apoptotic DNA fragmentation in the etoposide-induced U937 human leukemia cells.
U937 cells (1×10^6) were collected after incubation for 8 h at the indicated concentration of gentisyl alcohol, and the cells were then harvested to analyze the apoptotic DNA fragmentation.

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