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Rengyolone Inhibits Apoptosis *via* Etoposide-Induced Caspase Downregulation

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In the course of screening for substances inhibiting apoptosis of U937 human leukemia cells induced by etoposide (10 μg/ml), *Forsythiae fructus*, which showed a high level of inhibition, was selected. The regulating compounds were purified from the ethyl acetate extract by silica gel column chromatography and HPLC. The active substance was purified and identified as rengyolone by spectroscopic methods. This compound showed inhibitory activity on caspase-3 induction, a major protease of the apoptosis cascade, with an IC_{50} value of 38.96 μM after 8 h of etoposide treatment in U937 cells. The expression level of caspase-3 and poly(ADP-ribose) polymerase (PARP) were dose-dependently inhibited by the compound, suggesting that rengyolone inhibits etoposide-induced apoptosis *via* downregulation of caspases.

Keywords: Apoptosis, U937 cells, rengyolone

Apoptosis is a form of cell death that is essential for the control of cell populations during normal development and in many diseases. The importance of cell death through apoptosis in the maintenance of normal tissue homeostasis is now widely recognized [26]. Indeed, it is well documented that AIDS, stroke, Alzheimer's, Huntington's disease, Parkinson's disease, 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 [2, 6, 15–17, 28].

Cell death is caused by various pathological as well as physiological stimuli, through either necrotic or apoptotic processes. It is recognized by distinct morphological changes, including cell shrinkage, nuclear condensation, and fragmentation.

One set of cell-death-regulating genes, the CED-3/ICE family, encodes structurally related cysteine proteases that have the unusual substrate specificity for cleavage at Asp-X peptide bonds [8]. Recent studies have suggested that proteolytic cleavage and activation of the caspase-3 may be functionally important in the induction of apoptosis [25]. Caspase-3 (CPP32) has been well characterized and seems to be placed in the central pathway of the apoptotic process. Because caspase-3 shows the highest homology to CED-3, and its tetrapeptide inhibitor (DEVD-CHO) often blocks apoptosis induced by a variety of inducers, it is now thought to be a human equivalent of CED-3. Caspase-3 plays important roles in the signaling pathway controlling mammalian apoptosis [24].

In the course of screening for antiapoptotic agents, we isolated and identified rengyolone from *Forsythiae fructus*, as a potent apoptosis inhibitor. The fruits of *F. koreana* are known to exhibit antibacterial, anti-inflammatory, and diuretic activities [19, 20]. Moreover, rengyolone is reported to be responsible for their antibacterial activity [13]. However, the antiapoptotic effect of rengyolone has not been reported so far.

In the present study, we investigated the modulating activity of rengyolone on etoposide-induced apoptosis.

Materials and Methods

Materials

The silica gel (Merck Kieselgel 60, 70–230 mesh, 63–200 μm) 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 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 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 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.

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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.).

Cell Viability Assay
Cell viability was evaluated by a 3-(4,5-dimethylthiazol-1)-5-(3-carboxymeth-oxyphenyl)-2H-tetrazolium (MTS) assay [9]. In the MTS assay, the cell suspension (100 μl) was plated in a 96-well microculture plate. After seeding, various concentrations of the compound 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 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.

Measurement of Caspase Activity
The etoposide-induced caspase-3, -8, -9 activity assay was measured in the U937 leukemia cells using the standard inhibitor. Etoposide (10 μg/ml) was added to the U937 cells in the presence or absence of various concentrations of the compound. The cells were incubated for 7 h at 37 °C in a 5% CO_2-95% air atmosphere. After observing apoptotic cells on a microscope, the activity of caspase-3, -8, -9 activity was estimated from the cell lysate using each substrate of caspases [11, 14, 21]. The cells were lysed with a TTE buffer (10 mM Tris-HCl, 0.5% Triton X100, 10 mM EDTA, pH 8.0) and kept on ice for 30 min, and then centrifuged. 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).

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 [4]. Fifty μg of proteins per lane was loaded onto 15% and 8% SDS-polyacrylamide gels to detect caspase-3 and PARP, respectively [10]. 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, MN, U.S.A.) and PARP (BD Pharmingen, San Diego, U.S.A.), respectively. After washing three times in 0.05% TBST, the membranes were incubated with the anti-rabbit 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 conducted as previously described [3, 5, 27]. 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 ×*g* for 10 min at 4°C. Cytosolic DNA was extracted by phenol:chloroform (1:1) extraction of the supernatants. The DNAs were treated with 0.1 mg/ml RNase A for 30 min at 37°C, separated by 1% agarose gel electrophoresis, and visualized with ethidium bromide staining.

Results and Discussion

Isolation and Purification of Rengyolone

Dried medicinal plants (2 kg) were percolated with MeOH at 25°C for 2 weeks. The residue obtained after removal of the solvent (130 g) was diluted with H_2O (1 l) and extracted with ethyl acetate (1 l, ×3). The EtOAc extract on concentration left a dark syrup (40 g), which was chromatographed on a silica gel column with $CHCl_3$ and MeOH mixtures of increasing polarity. The active fractions were further purified by Sephadex LH-20 column chromatography using a solvent system of MeOH-H_2O (4:6). Final purifications were conducted by high-performance liquid chromatography using a reversed-phase column (Capcell Pak C_{18}, 250×10 mm, S-5 μm, 120 Å) with an acetonitrile-H_2O gradient solvent system and resulted in pure compound **1** (12.3 mg). The structure of purified rengyolone was 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 154. In the ^{1}H-NMR (CD_3OD, 300 MHz, ppm) spectrum of **1**, 6.78 (1H, d, *J*=10.2), 5.95 (1H, d, *J*=10.2), 4.15 (1H, dd, *J*=1.8, 4.3), 3.95 (1H, dd, *J*=5.6, 8.5), 3.82 (1H, dd, *J*=7.2, 7.8), 3.6 (1H, d, *J*=6.6), 2.75 (1H, dd, *J*=4.8), 2.58 (1H, dd, *J*=7.2, 8.7), and 2.22 (1H, dd, *J*=5.6, 7.8) signals were detected. In the ^{13}C-NMR (CD_3OD, 75 MHz, ppm) spectrum of **1,** 199.1 (C), 150.7 (CH), 128.8 (CH), 82.3 (CH), 67.2 (CH_2), 40.54 (CH_2), and 40.38 (CH_2) signals were detected. Compound **1** was identified as rengyolone by comparison of its spectral data with the literature (Fig. 1) [7]. Kim and colleagues [19] have recently reported the inhibitory effects of a butanol fraction of the aqueous extract of *Forsythia koreana* on the nitric oxide production by murine macrophage-like RAW 264.7 cells. Moreover, we recently reported that rengyolone attenuates the

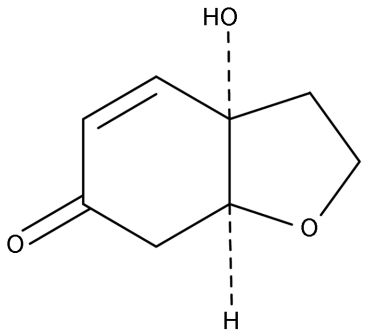

Fig. 1. Chemical structure of rengyolone isolated from *Forsythiae fructus*.

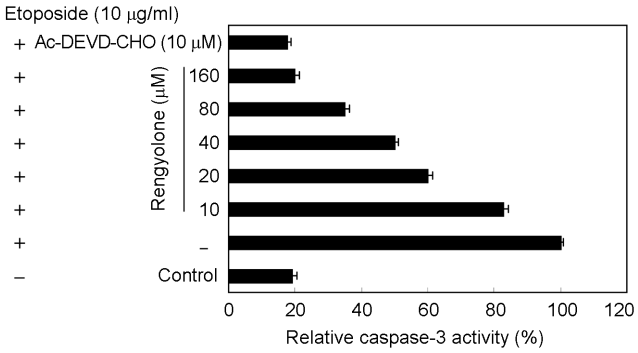

Fig. 2. Effects of various concentrations of rengyolone on the etoposide-induced caspase-3 activity in U937 human leukemia cells.
The caspase-3 activity was measured in U937 cells, as described in Materials and Methods. The results are averages of triplicate experiments, and the data are expressed as means ± SD.

inflammation through inhibition of NO production and iNOS expression by blockade of NF-κB and p38 MAPK activation in LPS-stimulated RAW 264.7 cells [18].

Rengyolone Suppresses Etoposide-Induced Apoptosis in U937 Cells

To date, numerous caspases have been reported to play pivotal roles in the cascade degradation of specific cellular and nuclear substrates. Caspases, including caspase-3, caspase-8, and caspase-9, have been reported to be involved in the apoptotic process [24]. Caspase-3 is the final executioner enzyme associated with cell death during stimuli-induced apoptosis [23, 24]. Hence, we investigated the effect of rengyolone on alterations of the enzymatic activitjies of caspase-3, caspase-8, and caspase-9 in human leukemia U937 cells. Rengyolone showed an inhibitory activity for caspase-3 production in etoposide-induced U937 cells with IC_{50} value of 6.0 μg/ml (Fig. 2). As shown in Fig. 3, the caspase-8 and caspase-9 activities in etoposide-induced

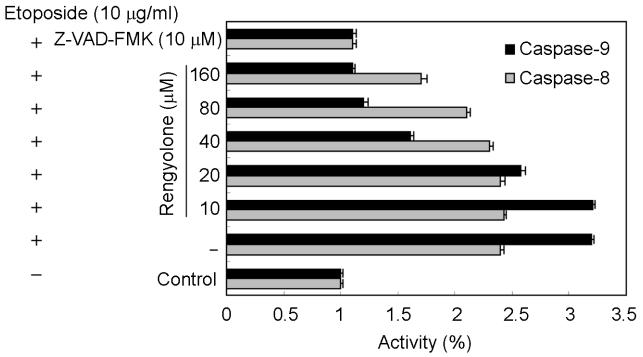

Fig. 3. Effects of various concentrations of rengyolone on the etoposide-induced caspase-8 and caspae-9 activity in U937 human leukemia cells.
The caspase-8 and caspase-9 activities were measured in U937 cells, as described in Materials and Methods. The results are averages of triplicate experiments, and the data are expressed as means ± SD.

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

Rengyolone (μg/ml)	Activity (%)[a]		
	Caspase-3	Caspase-8	Caspase-9
25	98 ± 0.6	96 ± 0.7	96 ± 0.6
12	96 ± 0.4	95 ± 0.5	94 ± 0.8
6	97 ± 0.7	96 ± 1.0	93 ± 0.7
3	96 ± 0.5	95 ± 0.8	95 ± 1.0
1.5	97 ± 0.7	97 ± 0.5	97 ± 0.4
z-VAD-FMK (10 μM)	13 ± 0.4	11 ± 0.5	18 ± 0.6

[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.

cells were inhibited by rengyolone. Examination of the cytotoxicity of rengyolone in U937 cells by MTS assay indicated that, even at 25 μg/ml, rengyolone did not affect the viability of U937 cells (data not shown).

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 formation of the apoptosome complex [21]. To test whether rengyolone was a specific caspase inhibitor or not, it was incubated with recombinant caspase-3, caspase-8, and caspase-9, and the caspase activities were measured using specific substrates for caspase-3, caspase-8, and caspase-9 at concentrations ranging from 1.5 to 25 μg/ml. The specific tetrapeptide caspase inhibitor z-VAD-FMK practically and completely inhibited the activities (Table 1). Therefore, rengyolone does not appear to inhibit caspases directly.

We next investigated the effects of rengyolone on caspase-3-like-specific substrate degradation. As shown in Fig. 4A and 4B, procaspase-3, a classical substrate for active caspase-3, and PARP degradation were inhibited by rengyolone, whereas β-actin, an internal control, was not affected (Fig. 4C). Since the internucleosomal DNA fragmentation

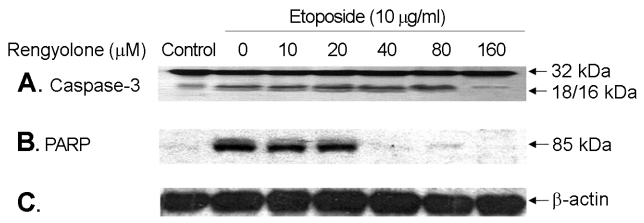

Fig. 4. Western blot analysis of caspase-3 and PARP in etoposide-induced U937 human leukemia cells.
Cells were treated with 1.5, 3, 6, 12, and 25 μg/ml of rengyolone for 8 h and analyzed using Western blotting. Blots were probed with goat polyclonal anti-caspase-3 antibody (**A**), rabbit polyclonal anti-PARP (**B**), or mouse monoclonal anti-β-actin (**C**) antibodies. Immunoreactivity was anti-mouse (Amersham) or anti-rabbit (Amersham) peroxidase-conjugated secondary immunoglobulin G antibody, followed by enhanced chemiluminescence (ECL, Amersham). Experiments were repeated at least three times.

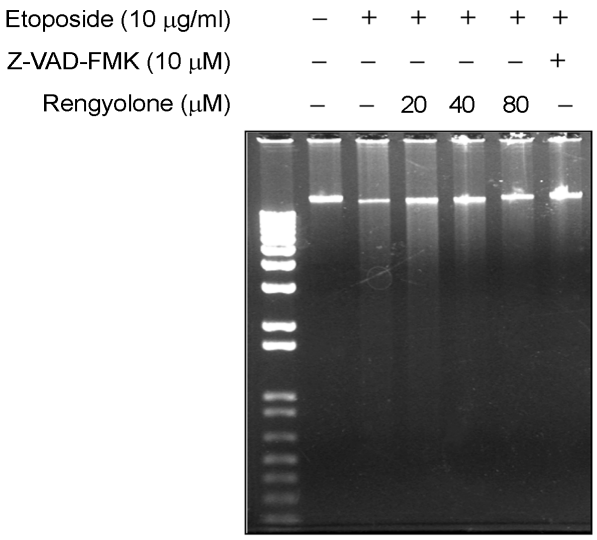

Fig. 5. Effects of rengyolone on apoptotic DNA fragmentation on etoposide-induced U937 human leukemia cells.
U937 cells (1×10^6) were collected after incubation for 8 h at the indicated concentration of rengyolone, and then the cells were harvested to analyze the apoptotic DNA fragmentation.

is a biochemical feature of the apoptotic process, we also investigated the effect of rengyolone on the inhibition of DNA fragmentation (Fig. 5). When U937 cells were treated with the etoposide in the presence or absence of its various concentrations, rengyolone inhibited the DNA fragmentation in U937 cells that was induced by etoposide in a dose-dependent manner. These results suggest that rengyolone inhibits etoposide-induced apoptosis *via* caspase activation.

In summary, we conclude that rengyolone plays a role in the inhibition of etoposide-induced caspase-3-like protease activation. This specific molecular function of rengyolone 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, ischemic injury, and toxin-induced liver diseases, the identification of rengyolone 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 mechanism approach for the curative possibility of such apoptosis-related diseases by rengyolone.

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