

## Identification of Licocoumarone as an Apoptosis-Inducing Component in Licorice

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**The 70% methanol soluble fraction from a licorice acetone extract was found to inhibit cell proliferation in human monoblastic leukemia U937 cells by inducing apoptosis. Separation by the methods including preparative HPLC provided us with an active compound, which was identified as licocoumarone. Several lines of evidence indicated that licocoumarone induced apoptosis in U937 cells. Thus, licocoumarone is suggested to be potentially useful as a natural anti-cancer agent.**

**Key words** licorice; apoptosis; licocoumarone; *Glycyrrhiza glabra*; U937 cell

Several chemotherapeutic compounds have been reported to induce apoptosis and apoptosis may be a primary mechanism of their anti-cancer activity.<sup>1,2)</sup> We have reported that several polyphenolic compounds including tea catechins and galloyl monosaccharides present in conventional medicinal plants induce apoptosis in human monoblastic leukemia U937 cells.<sup>3-5)</sup>

During the course of search for naturally occurring compounds with apoptosis-inducing activity, we have found that licorice (*Glycyrrhiza glabra*) contains active compounds. In the present paper, we report identification of one of them as licocoumarone.

### MATERIALS AND METHODS

**Materials and Chemicals** U937 cells were obtained from Health Service Research Resources Bank, Osaka, Japan, and cultured in 10% fetal bovine serum in RPMI 1640 medium (Iwaki Glass Co. Ltd., Chiba, Japan) with 50 U/ml penicillin, 50 µg/ml streptomycin, 2.5 µg/ml amphotericin B, and 50 µg/ml gentamycin at 37 °C under 5% CO<sub>2</sub>. A caspase inhibitor Z-Asp-CH<sub>2</sub>-DCB was obtained from Peptide Institute, Inc., Osaka, Japan. Hoechst 33342 (bisbenzimidazole H 33342 Fluorochrome) was obtained from Calbiochem-Novabiochem. Co., CA, U.S.A. SYBR Green I was obtained from Molecular Probes, Inc., OR, U.S.A.

**Fractionation of Acetone Extract of Licorice** Licorice roots were homogenized in water and insoluble materials were collected by centrifugation. They were then extracted with acetone and the acetone-soluble fraction was dissolved in 70% methanol. The soluble fraction was termed GL-1. GL-1 was separated by HPLC using a column of YMC ODS-A302 (4.6×150 mm). Isocratic elution with 70% methanol at a flow rate of 1 ml/min yielded 8 fractions. After elution of Fraction 8 a linear gradient to 99.5% methanol was started to give Fraction 9. For isolation of an active compound, HPLC using a column of Shiseido Capcell Pak C18 (20×250 mm) and elution with 70% methanol in H<sub>2</sub>O was repeatedly performed.

**Chemical Structure** The chemical structure of a purified compound was determined on the basis of: <sup>1</sup>H- and <sup>13</sup>C-NMR spectra by a JEOL α-400 spectrometer with tetra-

methylsilane as an internal standard; FAB mass spectra by a JEOL JMS-700 spectrometer; IR spectra by a Jasco FT/IR-550 Fourier transform infrared spectrometer; UV spectra by a Hitachi U-2000 spectrometer.

**Cell Proliferation** U937 cells (2×10<sup>4</sup>) in 200 µl of the culture medium were seeded in a 48 well microculture plate and incubated with or without a test sample solution in the culture medium. After 16 h, the numbers of viable cells were determined by the Alamar blue assay and the values were compared with control as described previously.<sup>3-5)</sup>

**DNA Fragmentation** U937 cells (1-2×10<sup>5</sup>) were incubated with a test sample solution for 16 h and the cells were pelleted by centrifugation. DNAs were isolated from the cell pellets according to the method described by Sellins and Cohen.<sup>6)</sup> They were electrophoresed in 2% agarose gel, stained with SYBR Green I, and then imaged with a FluoroImager (Molecular Dynamics Japan, Inc., Tokyo, Japan) as described previously.<sup>3-5)</sup> For confirmation of caspase-dependent DNA ladder formation, the caspase inhibitor Z-Asp-CH<sub>2</sub>-DCB<sup>7)</sup> was included in the culture medium and incubated as described above.

**Chromatin Condensation** U937 cells incubated in the presence or absence of a test sample solution at 37 °C for 16 h were pelleted by centrifugation and washed with phosphate buffered saline. After centrifugation, the cells were fixed with 1%-glutaraldehyde (Kanto Chemical, Co. Inc., Tokyo, Japan) at 4 °C for 2 h, and stained with Hoechst 33342 to examine chromatin condensation under a fluorescence microscope with excitation at 330-380 nm as described previously.<sup>5)</sup>

### RESULTS

**Effects of GL-1 on U937 Cell Proliferation** GL1 was dissolved in ethanol to give a concentration of 10 mg/ml and used after at least 200-fold dilution in the cell culture medium. As shown in Fig. 1, GL-1 inhibited cell growth of U937 cells. The observed morphological changes in the treated cells (not shown) suggested that this growth inhibition might be due to its apoptosis-inducing activity. To test this possibility, induction of DNA fragmentation and its inhibition by the caspase inhibitor<sup>7)</sup> were examined, since DNA

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ladder formation is one of the characteristics of apoptosis<sup>6</sup> and since caspases are essential proteases in execution of apoptosis.<sup>7-9</sup> The results of agarose gel electrophoresis of DNA extracted from the treated cells revealed that GL-1 induced DNA fragmentation in a nucleosome unit (Fig. 2a) and that this DNA fragmentation was inhibited by the general caspase inhibitor Z-Asp-CH<sub>2</sub>-DCB (Fig. 2b). These data indicated that GL-1 caused apoptosis in U937 cells.

**Fractionation of GL-1** To examine an active component

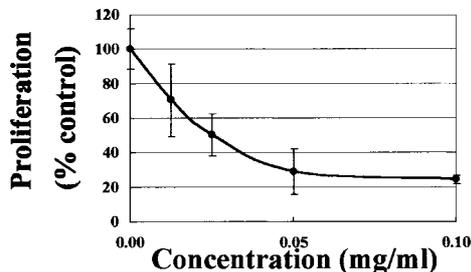


Fig. 1. Effects of GL-1 on Proliferation of U937 Cells

GL-1 was dissolved in ethanol, and an aliquot (2 μl) of the solution was added to 200 μl of culture medium containing 2 × 10<sup>4</sup> cells to give final concentration as indicated. After incubation for 16 h, Alamar blue was added. Each point represents the average of three determinations.

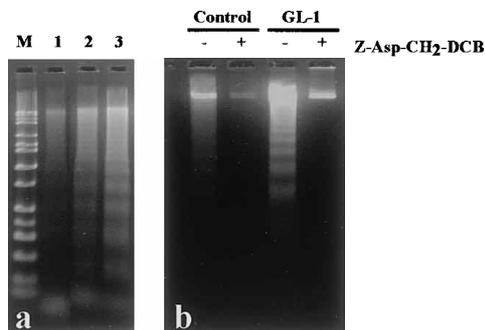


Fig. 2. (a) Fragmentation of DNA from U937 Cells Treated with GL-1

U937 cells were incubated at 37 °C for 16 h in the absence (lane 1) or presence of GL-1 at 0.025 mg/ml (lane 2) and 0.050 mg/ml (lane 3). M, size marker DNA.

(b) Effects of the Caspase Inhibitor on Fragmentation of DNA Induced by GL-1

U937 cells were treated with GL-1 at 0.05 mg/ml in the presence (+) or absence (-) of the caspase inhibitor Z-Asp-CH<sub>2</sub>-DCB (200 μM) for 16 h.

in GL-1, it was fractionated into 9 fractions as shown in Fig. 3. The yields were 8.7, 7.7, 5.3, 7.2, 4.8, 5.8, 6.3, 11.1, and 43.3% for Fractions 1, 2, 3, 4, 5, 6, 7, 8, and 9, respectively, on the weight basis. Each fraction was dried and dissolved in ethanol to evaluate the inhibitory activity against cell proliferation. The relative values of the cell number treated with Fractions 1, 2, 3, 4, 5, 6, 7, 8, and 9 were 107, 83, 67, 34, 44, 76, 51, 45, and 32%, respectively, as compared with the untreated cells (100%) after incubation for 16 h. Thus, Fraction 4 came second in the specific activity after Fraction 9.

**Isolation and Identification of an Active Compound**

Repeated HPLC of Fraction 4 provided us with a pure compound in a total yield of about 10 mg from 100 g of licorice roots. The spectrometric data were as follows: Yellow amorphous solid; UV (methanol) λ<sub>max</sub> (log ε) 321 (4.91), 336 (4.85) nm; IR (KBr) ν<sub>max</sub> 3520, 3380, 2980, 2900, 1620, 1600, 1510, 1480, 1450, 1320, 1290, 1180 cm<sup>-1</sup>; <sup>1</sup>H-NMR (acetone-*d*<sub>6</sub>, 400 MHz) δ 9.11 (1H, brs, 2'-OH), 8.54 (1H, brs, 4'-OH), 8.41 (1H, brs, 6-OH), 7.69 (1H, d, *J*=8.5 Hz, H-6'), 7.30 (1H, s, H-3), 6.77 (1H, s, H-7), 6.56 (1H, d, *J*=2.4 Hz, H-3'), 6.49 (1H, dd, *J*=8.5, 2.4 Hz, H-5'), 5.27 (1H, m, H-2''), 4.03 (3H, s, OCH<sub>3</sub>), 3.42 (2H, d, *J*=6.8 Hz, H-1''), 1.79 (3H, s, H-4''), 1.64 (3H, s, H-5''); <sup>13</sup>C-NMR (acetone-*d*<sub>6</sub>, 100 MHz) δ 159.13 (C-4'), 156.12 (C-2'), 154.69 (C-8), 154.23 (C-6), 151.99 (C-4), 151.45 (C-2), 130.39 (C-3''), 127.95 (C-6'), 125.02 (C-2''), 115.56 (C-5), 114.47 (C-9), 110.95 (C-1'), 108.25 (C-5'), 103.96 (C-3'), 101.89 (C-3), 93.25 (C-7), 60.37 (OCH<sub>3</sub>), 25.88 (C-5''), 23.38 (C-1''), 17.85 (C-4''); FAB-MS *m/z* 341 [M+H]<sup>+</sup>.

These data identified the isolated compound as licocoumarone (Fig. 4) on the basis of reported data.<sup>10,11</sup>

**Effects of Licocoumarone on U937 Cell Proliferation**

Licocoumarone was dissolved in ethanol to give a concentration of 10 mg/ml and used after at least 200-fold dilution in the cell culture medium. As shown in Fig. 5, licocoumarone inhibited cell growth of U937 cells dose-dependently.

**Apoptosis-inducing Activity of Licocoumarone**

As shown in Fig. 6, licocoumarone caused DNA ladder formation in a dose-dependent manner. This DNA fragmentation was inhibited by the presence of caspase inhibitor Z-Asp-CH<sub>2</sub>-DCB. When licocoumarone-treated cells were stained with Hoechst 33342, chromatin condensation was observed (Fig. 7). Chromatin condensation is also one of the features

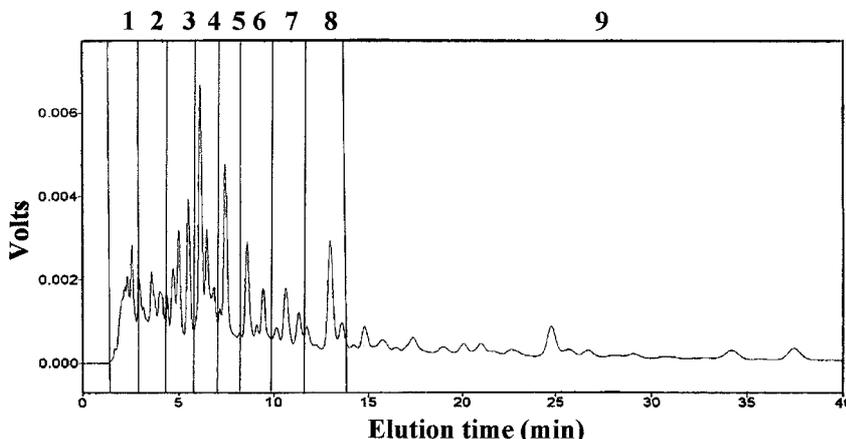


Fig. 3. Fractionation of GL-1 by HPLC Using a Column of YMC ODS-A302 (4.6 × 150 mm)

Isocratic elution with 70% methanol yielded 8 fractions. After elution of Fraction 8 a linear gradient to 99.5% methanol was started to give Fraction 9. Flow rate was 1 ml/min.

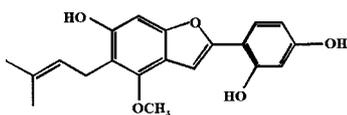


Fig. 4. Chemical Structure of Licocoumarone

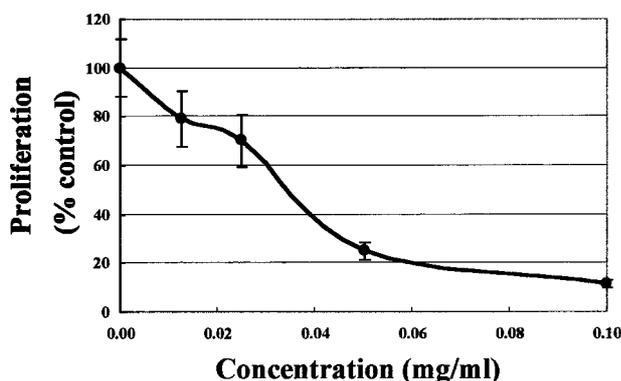


Fig. 5. Effects of Licocoumarone on Proliferation of U937 Cells  
Each point represents the average of three determinations.

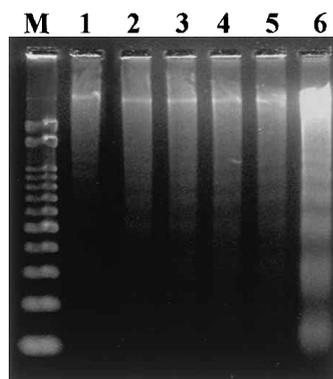


Fig. 6. Dose-Dependent DNA Fragmentation Induced by Licocoumarone  
U937 cells were incubated at 37 °C for 16 h in the absence (control, lane 1) or presence of licocoumarone at 0.003 mg/ml (lane 2), 0.006 mg/ml (lane 3), 0.013 mg/ml (lane 4), 0.025 mg/ml (lane 5), and 0.05 mg/ml (lane 6). M, size marker DNA.

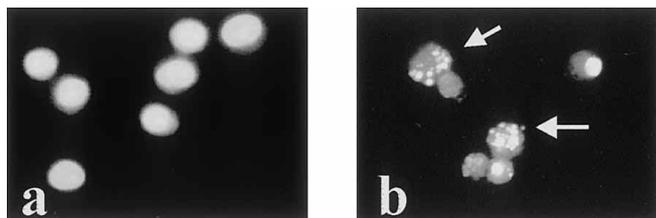


Fig. 7. Hoechst 33342 Staining of U937 Cells

Chromatin condensation as indicated by arrows was observed for the cells treated with licocoumarone at 0.025 mg/ml for 16 h (b). Untreated control cells are shown in (a).

characteristic of apoptosis.<sup>12)</sup> These results indicate that licocoumarone is an apoptosis inducer.

## DISCUSSION

In the present study, we found that the 70% methanol-soluble fraction from a licorice root extract exhibited inhibitory

activity for cell proliferation of human monoblastic leukemia U937 cells. Several purification steps provided us with a pure active compound and we identified it as licocoumarone. Several compounds derived from licorice are known to induce apoptosis. These include glycyrrhetic acid,<sup>13,14)</sup> isoliquiritigenin,<sup>15)</sup> and 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4'-hydroxy-phenyl)-1-propanone.<sup>16)</sup> Although antibacterial activity of licocoumarone has been reported,<sup>17)</sup> its apoptosis-inducing activity has not been described. Thus, present study adds a new member of the apoptosis-inducing components in licorice and suggests that it is potentially useful as a natural anti-cancer agent.

Licorice is known to have anti-tumor activities.<sup>18)</sup> Recently, glabridin from licorice has been reported to have antiproliferative properties in breast cancer cells.<sup>19)</sup> This activity has been claimed to be ascribable to its estrogenic activity,<sup>19)</sup> but it seems possible that apoptosis is also involved, since our preliminary experiments have shown that commercially available glabridin causes apoptosis in U937 cells. The findings that 8 fractions out of 9 fractions separated by HPLC exhibited more or less inhibitory activity against U937 cell proliferation suggest the possibility that licorice contains several unidentified compounds with apoptosis-inducing activity. The specific activity of licocoumarone was not so different from that of GL-1, suggesting that a certain combination of licocoumarone and the other compounds manifests a synergistic effect. The identification of the active compounds awaits future investigation.

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