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Cytotoxic and pro-apoptotic activities of cynaropicrin, a sesquiterpene lactone, on the viability of leukocyte cancer cell lines

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Abstract

Cynaropicrin, a sesquiterpene lactone from *Saussurea lappa*, has been reported to possess immunomodulatory effects on cytokine release, nitric oxide production and immunosuppressive effects. In this study, we have examined cytotoxic effect of cynaropicrin against several types of cell lines such as macrophages, eosinophils, fibroblasts and lymphocytes. Cynaropicrin potently inhibited the proliferation of leukocyte cancer cell lines, such as U937, Eol-1 and Jurkat T cells, but some other cells such as Chang liver cells and human fibroblast cell lines were not strongly suppressed by cynaropicrin treatment. The cytotoxic effect of cynaropicrin was due to inducing apoptosis and cell cycle arrest at G1/S phase, according to flow-cytometric, DNA fragmentation and morphological analyses using U937 cells. Evidence that combination treatment with L-cysteine and *N*-acetyl-L-cysteine, reactive oxygen species scavengers, or rottlerin (1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2, 2-dimethyl-2*H*-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one), a specific protein kinase (PK) Cδ inhibitor, abolished cynaropicrin-mediated cytotoxicity and morphological change, and that cynaropicrin-induced proteolytic cleavage of PKCδ suggests that reactive oxygen species and PKCδ may play an important role in mediating pro-apoptotic activity by cynaropicrin. Taken together, these results indicate that cynaropicrin may be a potential anticancer agent against some leukocyte cancer cells such as lymphoma or leukemia, through pro-apoptotic activity.

Keywords: Cynaropicrin; Sesquiterpene lactone; Cytotoxicity; Apoptosis; Cell cycle arrest; PKCδ

1. Introduction

Sesquiterpene lactones, with flavonoids and lignans, are regarded as major classes of natural products with a wide spectrum of biological activities including antitumor, antiulcer, antiinflammatory, neurocytotoxic and cardiotonic activities (Robles et al., 1995). For example, costunolide and dehydrocostus lactone exhibited inhibitory effects on killing function of cytotoxic T lymphocytes (Taniguchi et al., 1995), nitric oxide (NO) production (Lee et al., 1999) and hepatitis B virus surface antigen expression (Chen et al., 1995). Helenalin alleviated carrageen-induced edema of rat hindfeet (Hall et al., 1979) and suppressed cancer cell growth (Hall et al., 1977). Parthenolide and encelin showed strong inhibitory effects on the expression of cyclooxygenase and tumor necrosis factor (TNF)-α (Hwang et al., 1996). These wide range of biological activities are known to be due to inactivation of nuclear factor (NF)-κB via a α-methylene γ-butyrolactone group which is chemically reactive (Hwang et al., 1996; Bork et al., 1997; Hehner et al., 1998).

We have previously reported that at non-cytotoxic concentrations, cynaropicrin (Fig. 1) from *Saussurea lappa* suppresses cytokine production such as TNF-α and cytokine-induced neutrophil chemoattractant-1 (interleukin-8) (Cho et al., 1998; Jung et al., 1998) and NO release (Cho et al., 2000a). Moreover, this compound was reported to potently attenuate mitogenic stimulation for

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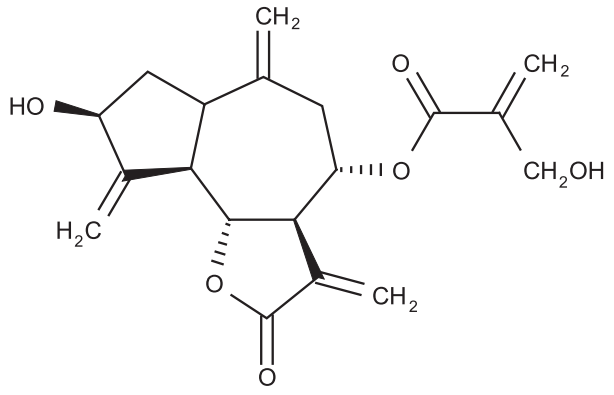

Fig. 1. Chemical structure of cynaropicrin.

CD4+ and CD8+ lymphocyte proliferation (Cho et al., 2000a), suggesting that cynaropicrin may be a useful agent for treatment of acute and chronic inflammatory diseases.

In this study, we have investigated the cytotoxic and pro-apoptotic effects of cynaropicrin, because long-term treatment of cynaropicrin more sensitively affected cell viability of some cancer cells. Indeed, cynaropicrin potently suppressed the proliferation of human leukocyte cancer cell lines such as U937, Eol-1 and Jurkat T cells, via the induction of apoptosis. Pharmacological and biochemical evidence suggested that reactive oxygen species and protein kinase (PK) Cδ may play an important role in mediating the pro-apoptotic activity of cynaropicrin. Thus, L-cysteine and *N*-acetyl-L-cysteine, reactive oxygen species scavengers (Ballarin et al., 1998; Li et al., 2000), and rottlerin (1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)-methyl]-5,7-dihydroxy-2, 2-dimethyl-2*H*-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one), a selective PKCδ inhibitor, abolished cynaropicrin-mediated cytotoxicity and morphological changes, and cynaropicrin also induced proteolytic cleavage of PKCδ. These results suggest that cynaropicrin may be a potential anticancer agent against some leukocyte cancer cells such as lymphoma or leukemia, through pro-apoptotic activity.

2. Materials and methods

2.1. Materials

Cynaropicrin (Fig. 1, molecular weight: 346.38) was purified from roots of *S. lappa* as described previously (Cho et al., 1998). Structural identity of cynaropicrin was determined spectroscopically (^{1}H and ^{13}C NMR, IR, MS). Purity (>97.5%) was analyzed by high-performance liquid chromatography. L-Cysteine, *N*-acetyl-L-cysteine, RNase A, 5-fluorouracil, propidium iodide (PI), staurosporin, cyclosporin A and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Z-VAD-fmk (*Z*-Val–Ala–Asp–fluoromethylketone: a broad-spectrum caspase inhibitor), Z-IEDT-fmk (*Z*-Ile–Glu–[*O*-ME]–Thr–Asp[*O*-ME] fluoromethylketone: a caspase 8 inhibitor), PD98059 (2-[2-amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one), SB203580 (4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]-1*H*-imidazole), SP600125 (anthra(1,9-cd)pyrazol-6(2H)-one 1,9-pyrazoloanthrone), rottlerin and GF109203X (2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide) were obtained from Calbiochem (La Jolla, CA). Cell Death Detection ELISA Plus assay kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN), and anti-PKCδ antibody was obtained from Transduction Laboratories (Lexington, KY). Fetal bovine serum, penicillin, streptomycin and RPMI1640 were obtained from Gibco (Grand Island, NY). Eol-1 (eosinophil cell line), Chang liver cells (primary hepatocytes), Detroit 551 (fibroblast cell line) and U937 (promonocytic cell line) cells were purchased from American Type Culture Collection (Rockville, MD). Explanted fibroblast cells were prepared by methods reported previously (Park et al., 2000). All other chemicals were of reagent grade.

2.2. Cell culture

All tested cell lines were maintained in RPMI1640 supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin and 10% fetal bovine serum and were grown at 37 °C and 5% CO_2 in humidified air.

2.3. Cytotoxicity assay

Cytotoxicity of cynaropicrin was evaluated by MTT assay with minor modification as reported previously (Cho et al., 1998). Cells ($0.1-1\times10^6$) were cultured in 96-well plate in the presence of testing compounds for indicated times. Finally, 10 μl of MTT solution (10 mg/ml in phosphate-buffered saline [PBS], pH 7.4) was added to each well 4 h before stopping. OD was measured with a microplate reader at 570 nm ($OD_{570-630}$). The mean value of OD content of four wells was used for calculating the viability (% of control).

2.4. Lactate dehydrogenase assay

Lactate dehydrogenase activity in the cell-free extracellular supernatant was quantified as an index of cell death. Therefore, we tested lactate dehydrogenase activity from culture supernatant as described previously (Cho et al., 1998).

2.5. Phase contrast microscope

Images of cells in culture at indicated time point were obtained using an inverted phase contrast microscope, attached to a video camera, and captured using NIH image software.

2.6. Flow-cytometric analysis

Flow-cytometric analysis for PI staining was performed as described previously (Lee et al., 2000). In brief, U937 (10^6) cells were washed with PBS, fixed in ethanol, suspended in PI solution (1 mg/ml RNase A, 50 μg/ml PI and 0.1% Triton X-100 in 3.8 mM sodium citrate) and incubated on ice for 30 min in the dark. After washing three times with FACS buffer, PI fluorescent intensity was analyzed on a FACScan (Becton Dickinson).

2.7. DNA fragmentation assay

Quantitative DNA fragmentation assay was performed using a recently developed Cell Death Detection Enzyme Linked Immunosorbent Assay (ELISA) Plus assay kit, as reported previously (Anantharam et al., 2002). This is a fast, highly sensitive and reliable assay for the detection of early changes in apoptotic cell death and measures the appearance and amount of histone-associated low-molecular-weight DNA in the cytoplasm of cells. Briefly, U937 (0.5×10^6) cells were exposed to 20 μM cynaropicrin for 3 h. After cynaropicrin treatment, cells were spun down at $200 \times g$ for 5 min and washed once with 1 × PBS. Cells were then incubated with a lysis buffer (supplied with the kit) at room temperature. After 30 min, samples were centrifuged, and 20-μl aliquots of the supernatant were then dispensed into streptavidin-coated 96-well microtiter plates followed by addition of 80 μl of antibody cocktail and incubated for 2 h at room temperature with mild shaking. The antibody cocktail consisted of a mixture of anti-histone biotin and anti-DNA-horseradish peroxidase (HRP) directed against various histones and antibodies to both single-stranded DNA and double-stranded DNA, which are major constituents of the nucleosomes. After incubation, unbound components were removed by washing with the incubation buffer supplied with the kit. Quantitative determination of the amount of nucleosomes retained by anti-DNA-HRP in the immunocomplex was determined spectrophotometrically with 2,2′ -azino-di-(3-ethylbenzthiazoline sulfonate(6)) diammonium salt (ABTS) as an HRP substrate (supplied with the kit). Measurements were made at 405 nm against an ABTS solution as a blank (reference wavelength ~ 490 nm) using a Molecular Devices Spectramax Microplate Reader (Sunnyvale, CA).

2.8. Western blot analysis for PKCδ

One milliliter of U937 (5×10^6 cells/ml) cells was plated in 24-well plates under serum-free conditions (Cho et al., 2001a). After 3 h recuperation, cynaropicrin was added to the cells as appropriate for a further 1 h, and the cells were then transferred onto ice and washed three times with cold PBS containing 1 mM sodium orthovanadate and lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzimide and 2 mM hydrogen peroxide) for 30 min with rotation at 4 °C. The lysates were clarified by centrifugation at $16{,}000 \times g$ for 10 min at 4 °C. Samples containing equal amount (40 μg/lane) of protein were loaded in each lane and separated on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel. Proteins were transferred by electroblotting to nitrocellulose membrane. Membranes were blocked for 60 min in 3% BSA TTBS (Tris-buffered saline containing 20 mM NaF, 2 mM EDTA, 0.2% Tween 20) at room temperature. Immunoblot for PKCδ was carried out using specific monoclonal antibody and secondary horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins. Peroxidase activity was visualised by chemiluminescence detection (ECL reagents, Amersham, Little Chalfont, Buckinghamshire, UK).

2.9. Statistical analysis

All values expressed as mean ± S.E.M. were obtained from indicated observations. The Student's *t*-test for unpaired observation between control and experimental samples was carried out for statistical evaluation of a difference; *P* values of 0.05 or less were considered as statistically significant.

3. Results

3.1. Cynaropicrin strongly suppressed the viability of leukocyte cancer cell lines

The inhibitory effect of cynaropicrin on cancer cell viability was examined first. Fig. 2 shows that cynaropicrin was more cytotoxic to leukocyte-derived cancer cells than fibroblast or Chang liver cells tested under our conditions. Thus, cynaropicrin dose-dependently decreased viability of U937, Eol-1 and Jurkat T cells with IC_{50} values of 3.11, 10.9 and 2.36 μM, respectively (Table 1), in 48 h incubation, while it was not cytotoxic against U937 cell viability at 6 h incubation up to 30 μM (Cho et al., 2000a). In contrast, cynaropicrin only weakly blocked the proliferation of other cells such as fibroblasts (Detroit 551 and explanted primary cells) and Chang liver cells (data not shown) with IC_{50} values of more than 25 μM in 48 h incubation. The inhibitory potency of cynaropicrin on the viability of leukocyte cancer cell lines (U937, Jurkat and Eol-1 cells) was increased in a time-dependent manner (Fig. 2B). Cynaropicrin inhibition of cell viability was also confirmed by determination of lactate dehydrogenase released. Namely, cynaropicrin dose-dependently increased the release of lactate dehydrogenase.

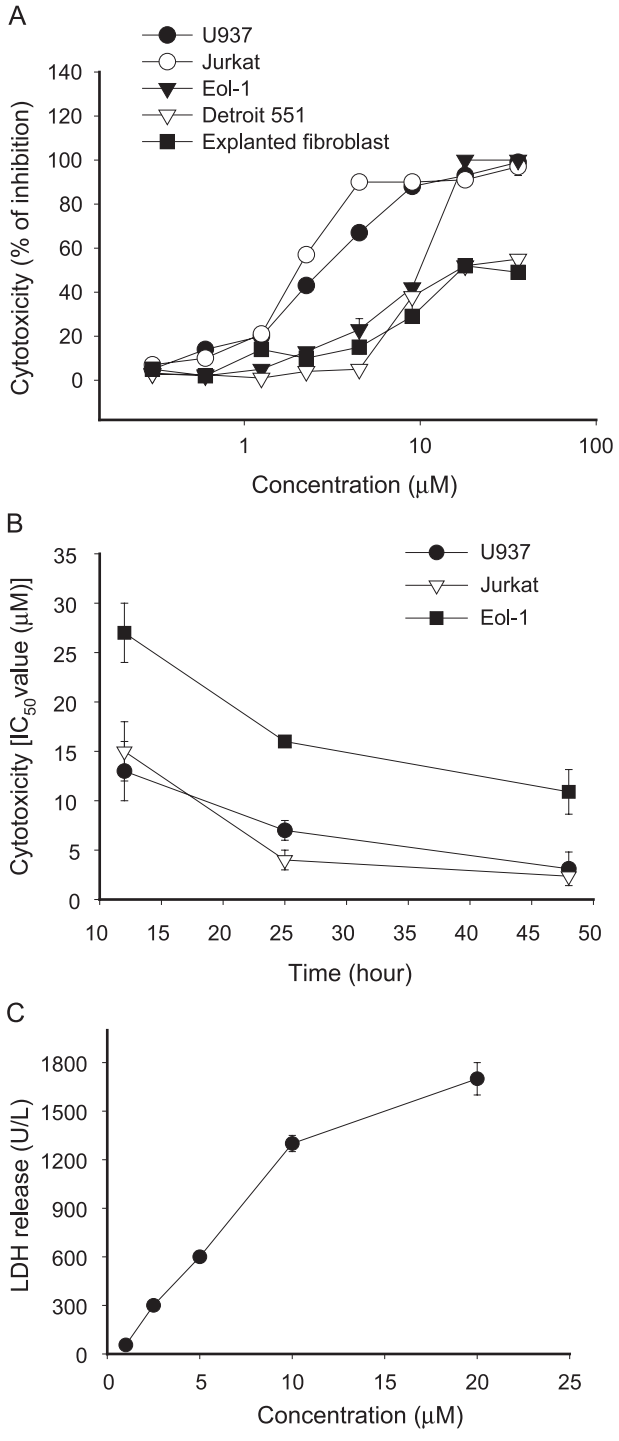

Fig. 2. Cytotoxic effect of cynaropicrin on the viability of primary and cancerous cell lines. (A) Cytotoxicity of cells was assayed by conventional MTT method as described in Materials and methods. Cells (0.1×10^6) were cultured in 96-well plate in the presence of testing compounds for 48 h. Data represent mean ± S.E.M. of three separate observations performed in triplicate. (B) IC_{50} profiles at incubation time indicated were obtained by cytotoxicity assay as described in Materials and methods. (C) Lactate dehydrogenase activity assay was performed by the method described in Materials and methods using U937 cells under the same condition with cytotoxicity assay.

Table 1
Inhibitory effect of cynaropicrin on viability of primary and cancerous cell lines

Cell line	IC_{50} (μM)
U937	3.11 ± 0.21
Jurkat	2.36 ± 0.03
Eol-1	10.9 ± 2.26
Detroit 551	26.3 ± 2.11
Explanted fibroblast	29.4 ± 3.88

Cells (10^5) were incubated with cynaropicrin for 2 days, and cell viability was assayed by conventional MTT method as described in Materials and methods. Data represent mean ± S.E.M. of three separate observations performed in triplicate.

3.2. Cynaropicrin-induced cytotoxic effect is mediated by apoptosis

To test whether cynaropicrin-induced cytotoxic effect is mediated by apoptotic cell death, several experiments such as determination of DNA fragmentation, PI staining and flow-cytometric cell cycle analysis were performed. Fig. 3 showed that cynaropicrin induced U937 cell apoptosis. Thus, DNA flow-cytometric analysis after 24 h incubation (Fig. 3A) indicated that 67% of cells contained small DNA fragments which were generally found in apoptotic cells, like staurosporin (51.8%). In contrast, normal cultures of U937 showed DNA staining corresponding to cells with a full content of intact DNA (G1, S and G2). DNA fragmentation was also confirmed in cynaropicrin-treated cells by a quantitative rapid ELISA method (Fig. 3B). DNA fragmentation was increased by cynaropicrin exposure, but attenuated by cotreatment with a broad-spectrum caspase inhibitor, Z-VAD-fmk, but not the caspase 8 inhibitor, Z-IEDT-fmk, as shown in the case of 5-fluorouracil, a positive control. Continuously, we investigated the influence of cynaropicrin on cell cycle process using hydroxyurea (1.5 mM) which blocks the cell at the G1/S boundary. After washing into fresh medium, the population proceeded synchronously through the cell cycle (data not shown) and at 12 h incubation in fresh medium, the cells displayed almost similar level of G1/S and G2/M phases (Fig. 3C). However, cynaropicrin arrested hydroxyurea-synchronised cells at the G1/S transition stage throughout one cell cycle and even during an additional 12 h incubation as shown in the case of typical apoptosis-inducing agents such as ginsenoside-Rg5 (Lee et al., 1997). Cynaropicrin also induced morphological change after 6 h incubation. This compound induced clearly visible formation of blebs and apoptotic bodies (arrows seen in Fig. 4A).

3.3. Cynaropicrin-induced apoptotic pathway is mediated by reactive oxygen species generation and PKCδ cleavage

To dissect the signaling pathways involved in the induction of apoptosis by cynaropicrin, the effects of a number of enzyme inhibitors were tested. Reactive oxygen species-generating pathway was examined first, based on

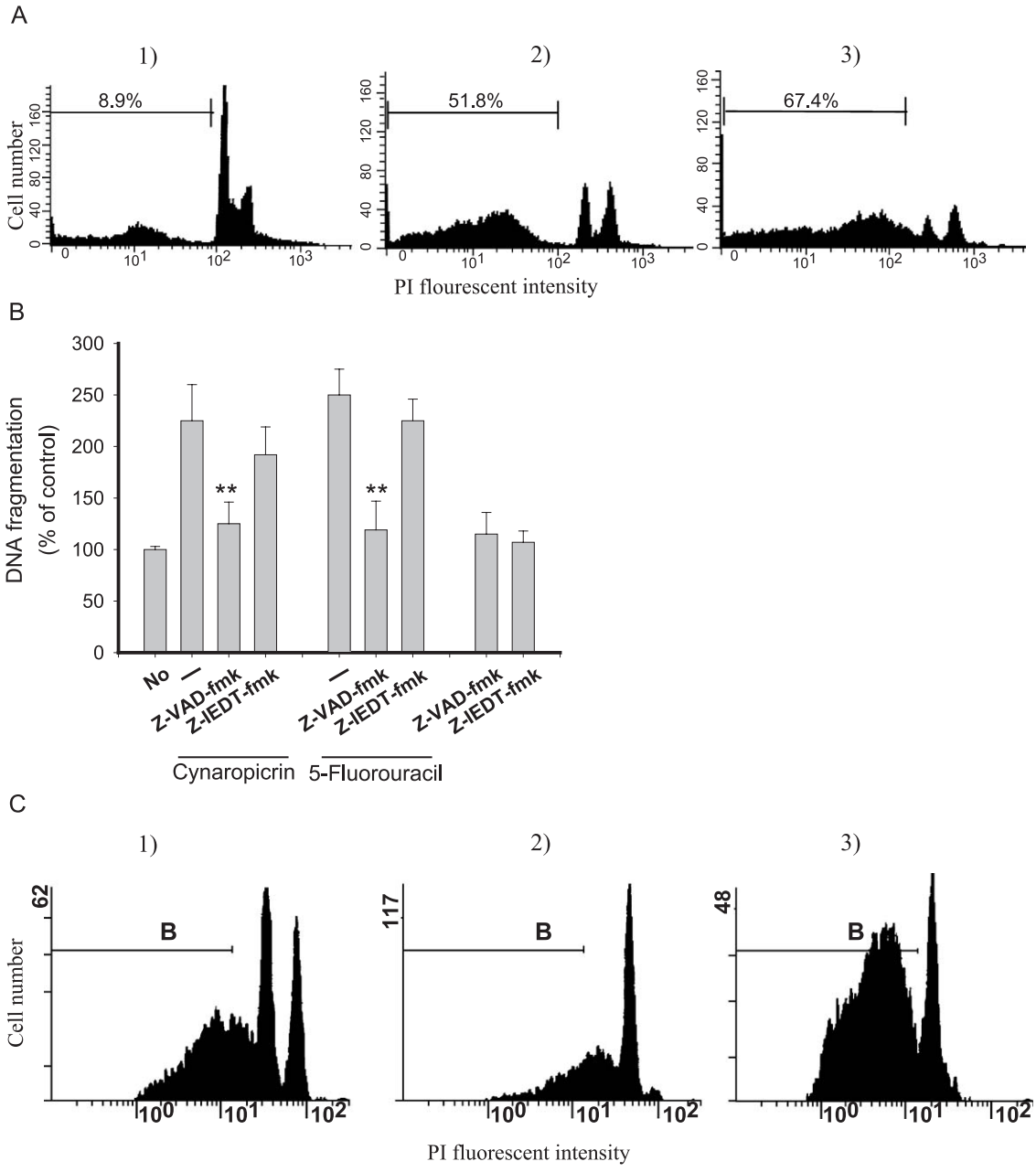

Fig. 3. Evidence for cynaropicrin-induced apoptosis. (A) Propidium iodide (PI) florescent intensity was measured by flow cytometry as described in Materials and methods. Each histogram indicates the number of untreated (1), staurosporin (2.5 μM)-treated (2) and cynaropicrin (10 μM)-treated (3) cells for 24 h as a function of propidium iodide fluorescence. (B) DNA fragmentation was measured by DNA fragmentation assay as described in Materials and methods. U937 (10^6) cells were pretreated with caspase inhibitors (Z-VAD-fmk, 50 μM and Z-IEDT-fmk, 50 μM) for 2 h, and then the cells were further incubated in the presence or absence of cynaropicrin (20 μM) or 5-fluorouracil (1 mg/ml) for 3 h. Data represent mean ± S.E.M. of three separate observations performed in triplicate (**$P<0.01$ compared to cynaropicrin or 5-fluorouracil alone). (C) Flow-cytometric cell cycle analysis of untreated and cynaropicrin-treated cells were performed by PI staining. U937 (10^6) cells were exposed to 1.5 mM hydroxyurea for 14 h and then treated with cynaropicrin (10 μM) for 12 and 24 h. Cell samples were analyzed for DNA content by flow cytometry. Cells in G1 are represented by the first peak (2N); cells in G2M are represented by the second peak (4N), and cells in S are represented by the area between in peaks: (1) 12 h after, normal, (2) 12 h after, cynaropicrin and (3) 24 h after, cynaropicrin.

previous reports (Hwang et al., 1996; Bork et al., 1997; Robles et al., 1997; Wen et al., 2002). As expected, two scavengers, L-cysteine (150 μM) and *N*-acetyl-L-cysteine (800 μM) (Ballarin et al., 1998; Li et al., 2000) abolished the cynaropicrin-induced morphological changes (Fig. 4A) and DNA fragmentation (Fig 4B), suggesting that the apoptotic phenomena may be due to reactive oxygen species generation.

In the next study to confirm the effect of reactive oxygen species, we investigated the involvement of PKCδ which is a key signaling molecule undergoing proteolytic cleavage in the reactive oxygen species-induced apoptotic pathway

(Anantharam et al., 2002; Lee et al., 2002; Emoto et al., 1995; Ghayur et al., 1996). To test this possibility, rottlerin, a selective PKCδ inhibitor, was employed. Interestingly, Fig. 4 demonstrated that the postulation is reliable. Thus, rottlerin blocked morphological change, DNA fragmentation and cytotoxicity induced by cynaropicrin, whereas GF109203X (a broad-spectrum PKC inhibitor) at concentration displaying limited inhibitory effect to conventional PKC (Cho et al., 2003b) did not. Furthermore, cynaropicrin exposure induced the appearance of the 42-kDa catalytic domain of PKCδ (Fig. 4D), according to biochemical analysis, suggesting that the effect of reactive oxygen species may be relevant to PKCδ cleavage.

Because PKCδ-mediated apoptosis has also been linked to the activation of extracellular signal-regulated kinase

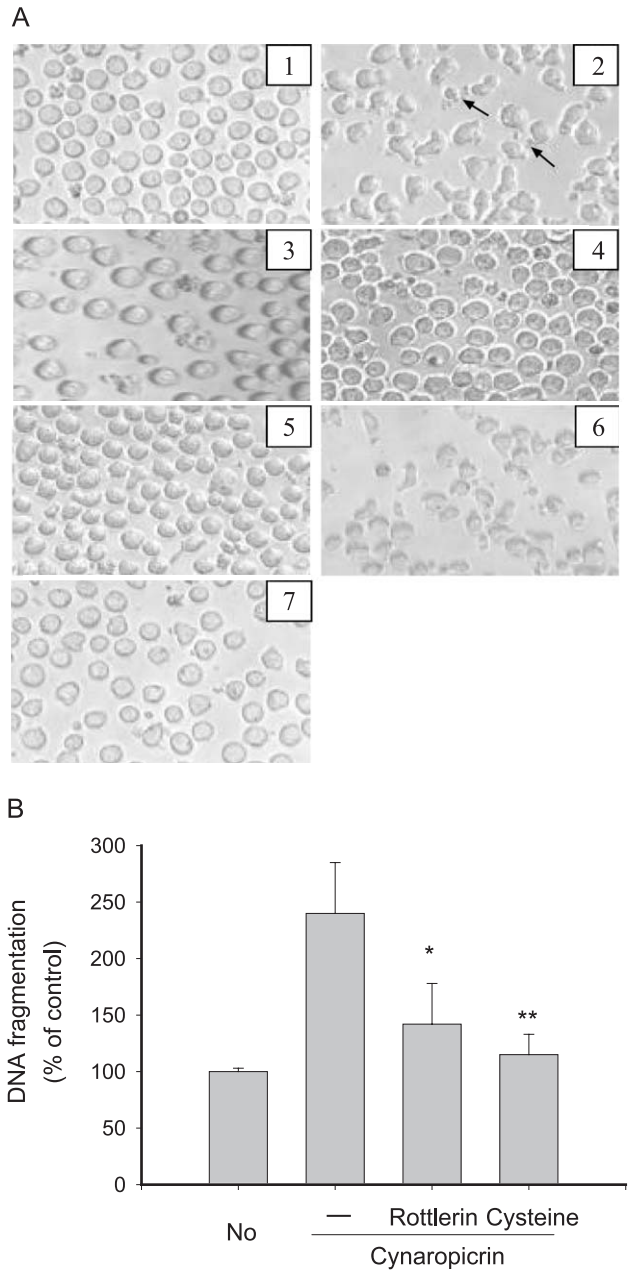

Fig. 4. ROS and PKCδ involvement in cynaropicrin-mediated morphological change and cytotoxicity. (A) Images of cells in culture at 6 h were obtained using an inverted phase contrast microscope, attached to a video camera, and captured using NIH image software. U937 (10^5) cells were pretreated with rottlerin (10 μM), L-cysteine (150 μM), *N*-acetyl-L-cysteine (800 μM) or GF109203X (5 μM) for 30 min and further incubated in the presence or absence of cynaropicrin (10 μM). (1) Normal, (2) cynaropicrin, (3) cynaropicrin + L-cysteine, (4) cynaropicrin + *N*-acetyl-L-cysteine, (5) cynaropicrin + rottlerin, (6) cynaropicrin + GF109203X and (7) rottlerin alone. (B) DNA fragmentation was measured by DNA fragmentation assay as described in Materials and methods. U937 (10^6) cells were pretreated with rottlerin (10 μM) and L-cysteine (150 μM) for 2 h, and then the cells were further incubated in the presence or absence of cynaropicrin (20 μM) for 3 h. Data represent mean ± S.E.M. of three separate observations performed in triplicate (**$P < 0.01$ and *$P < 0.05$ compared to cynaropicrin alone). (C) Cell cytotoxicity induced by cynaropicrin was evaluated after pretreatment of pharmacological inhibitors. Cells (0.5×10^6) 45 min pretreated with rottlerin (10 μM), L-cysteine (150 μM), *N*-acetyl-L-cysteine (800 μM) and GF109203X (5 μM) were cultured in 96-well plate in the presence of cynaropicrin for 12 h. Cell viability was assayed by conventional MTT method as described in Materials and methods. Data represent mean ± S.E.M. of three separate observations performed in triplicate (**$P < 0.01$ compared to cynaropicrin alone). (D) Proteolytic cleavage of PKCδ was assay by Western blotting as described in Materials and methods. U937 (5×10^6) cells were incubated with cynaropicrin for 30 min, and lysates were prepared as described in Materials and methods. PKCδ was analyzed by immunoblotting using an antibody (αPKCδ) that recognizes the PKCδ. Lanes 1: no treatment; lane 2: cynaropicrin (20 μM).

(ERK) or p38 (Lee et al., 2002), the effects of a combination of three different mitogen-activated protein kinase (MAPK) inhibitors [PD98059 (a specific ERK inhibitor), SB20380 (a specific p38 inhibitor) and SP60089 [a c-*Jun* N-terminal

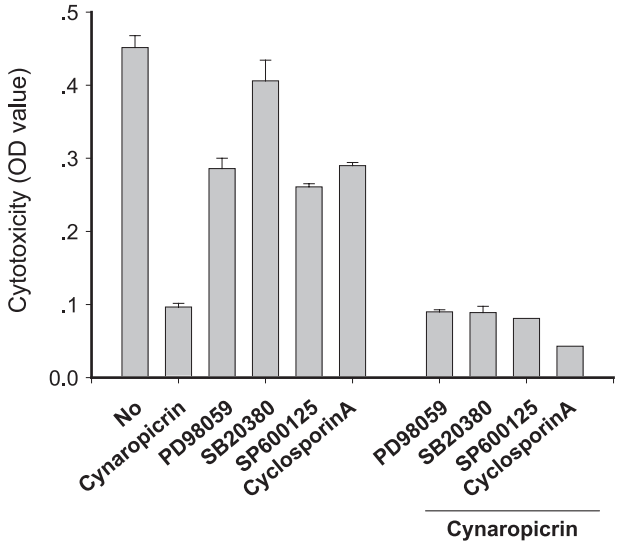

Fig. 5. Pharmacological characterization of cynaropicrin-induced cytotoxicity. U937 (10^6) cells were pretreated with pharmacological inhibitors (PD98059: 25 μM, SB203580: 1 μM, SP600125: 25 μM and cylcosporin A: 10 μg/ml) for 30 min, and then the cells were further incubated in the presence or absence of cynaropicrin (15 μM) for 24 h. Cell viability was assayed by conventional MTT method as described in Materials and methods. Data represent mean ± S.E.M. of two separate observations performed in triplicate.

kinase (JNK) inhibitor] at concentrations previously shown to block phorbol 12-myristate 13-acetate-induced interleukin-2 promoter activity (Cho et al., 2003a) were explored. As Fig. 5 shows, however, these inhibitors did not alter cynaropicrin-induced morphological change (data not shown) or cytotoxicity (Fig. 5), suggesting that additional MAPK pathway may not be required for cynaropicrin-induced apoptosis and cytotoxicity.

4. Discussion

In the course of screening of natural products to find novel anti-inflammatory drugs as TNF-α inhibitors (Cho et al., 1999a,b, 2000b, 2001b), we have discovered that sesquiterpene lactones including cynaropicrin, reynosin and satamarine from *S. lappa* have potent suppressive effects on cytokine production (Lee et al., 1999; Cho et al., 1998; Jung et al., 1998). In particular, cynaropicrin strongly inhibited TNF-α release and NO production from murine macrophage stimulated by lipopolysaccharide (Cho et al., 1998, 2000a) at non-cytotoxic concentrations and potently blocked mitogen-stimulated lymphocyte proliferation (Cho et al., 2000a). Although the pharmacological targets of sesquiterpene lactones are not fully identified, effects on tyrosine phosphorylation, mitogen activation of MAPKs and NF-κB have been reported (Hwang et al., 1996; Bork et al., 1997; Hehner et al., 1998). Nevertheless, the biological activities of sesquiterpene lactones (including cynaropicrin) seem to be specific, regardless of structural feature having extra cyclic methylene group as a potent alkylating agent. Thus, cynaropicrin only suppressed U937 cell homotypic aggregation induced by aggregative antibodies to CD29 (β1 intgerins) and CD98, but not CD43 (Cho et al., 2004). Moreover, these compounds did not block DNA binding of pro-inflammatory transcription factors such as activator protein-1 (AP)-1 and octamer transcription factor (OCT)-1 (Bork et al., 1997). Therefore, we believed that exploring molecular mechanism of sesquiterpene lactone-mediated pharmacological activity could expand our knowledge to apply these compounds to numerous human diseases. In this study, because cynaropicrin was shown to strongly decrease viability of some cancer cells after 2 days incubation, we examined the cytotoxic and pro-apoptotic effects of cynaropicrin on the viability of leukocyte cancer cell lines.

Cynaropicrin was found to be more cytotoxic to leukocyte-derived cancer cells than fibroblast or Chang liver cells tested under our conditions, though cynaropicrin potently blocked the mitogenic proliferation of normal splenocytes (Cho et al., 2000a). Thus, cynaropicrin decreased viability of U937, Eol-1 and Jurkat T cells with IC_{50} values of 3.11, 10.9 and 2.36 μM, respectively (Fig. 2 and Table 1), in 48 h incubation, while it was not cytotoxic against U937 cell viability at 6 h incubation up to 30 μM (Cho et al., 2000a). In contrast, cynaropicrin only weakly blocked the proliferation of other cells such as fibroblasts (Detroit 551 and explanted primary cells) and Chang liver cells (data not shown) with IC_{50} values of more than 25 μM. Wen et al. (2002) also reported that parthenolide did block proliferation of hepatoma SH-J1, but not Chang live cells. The inhibitory activity of cynaropicrin on the viability of leukocyte cancer cell lines (U937, Jurkat and Eol-1 cells) was also time dependent, as shown in Fig. 2B. The increased level of lactate dehydrogenase, an index of cell death, in the cell-free extracellular supernatant confirms that cynaropicrin is cytotoxic in a dose-dependent manner (Fig. 2C). Although we could not test the cytotoxic effect of cynaropicrin on the viability of solid tumor cells, our data obtained using leukocyte cancer cells were higher than those against other solid tumor cells such as SK-MEL, KB, BT-549 and SK-OV-3 (Muhammad et al., 2001). At present, we are unable to clearly explain why cynaropicrin exhibited high in vitro activity against leukocyte cancer cells and low activity against other cells. It may be due to different either bioavailability of the drug or sensitivity toward some biological target in the cells. It has been known that sesquiterpene lactone as a potent alkylating agent can produce adduct compound (although the adducts have not been identified yet from drug-treated cell fraction) with intracellular thiol molecules (such as glutathione and L-cysteine), which also act as physiological antioxidants (Heilmann et al., 2001). Furthermore, these adducts showed lower biological activities (Heilmann et al., 2001). Therefore, the level of these thiol molecules in

some cells may affect in part the cytotoxic activity of cynaropicin, rather than the extent of bioavailability.

Sesquiterpene lactones, such as costunolide (Park et al., 2001), helenalin (Dirsch et al., 2001a,b) and parthenolide (Wen et al., 2002), are known to be strong inducers of cytotoxicity by triggering apoptosis (programmed cell death) through reactive oxygen species-mediated oxidative stress. Therefore, we next addressed whether cynaropicrin-induced cytotoxicity is linked to apoptosis. Figs. 3 and 4 clearly indicate that cynaropicrin induces apoptosis. The first evidence to demonstrate this point was obtained by DNA flow-cytometric analysis after 24 h incubation (Fig. 3A), as reported previously with other sesquiterpene lactones such as parthenolide, helenalin and thapsigargin (Furuya et al., 1994; Dirsch et al., 2001b; Wen et al., 2002). Normal cultures of U937 showed DNA staining corresponding to cells with a full content of intact DNA (G1, S and G2). However, cynaropicrin (10 μM) and staurosporin (2.5 μM, a good control for a cellular apoptotic model) induced the appearance of a broad sub-G1 peak suggestive of DNA fragmentation, a hallmark of apoptosis, up to 52% (staurosporin) and 67% (cynaropicrin). DNA fragmentation was confirmed in cynaropicrin-treated cells by a quantitative rapid ELISA method (Fig. 3B), which is a fast, highly sensitive and reliable assay (Anantharam et al., 2002), as shown in the cases of helenalin (Dirsch et al., 2001b) and parthenolide (Wen et al., 2002). The DNA fragmentation was blocked by a broad-spectrum caspase inhibitor, Z-VAD-fmk, but not the caspase 8 inhibitor, Z-IEDT-fmk-like 5-fluorouracil as a positive control in this study. The inhibitory pattern was also seen in parthenolide (Dirsch et al., 2001b; Wen et al., 2002) but not helenalin which induced caspase 8 activity (Gertsch et al., 2003). The influence of cynaropicrin on cell cycle was also investigated. Hydroxyurea (1.5 mM) was used to block the cell at the G1/S boundary, and most cells indeed accumulated at the G1 phase as reported previously (Lee et al., 1997). After washing into fresh medium, the population proceeded synchronously through the cell cycle (data not shown), and at 12 h incubation in fresh medium, the cells displayed almost similar level of G1/S and G2/M phases (Fig. 3C). In contrast, cynaropicrin [as reported previously for parthenolide and thapsigargin (Wen et al., 2002; Furuya et al., 1994)] arrested hydroxyurea-synchronised cells at the G1/S transition stage throughout one cell cycle and even during an additional 12 h incubation. As further evidence of apoptosis, morphological change after cynaropicrin treatment was investigated. Cynaropicrin induced clearly visible formation of blebs and apoptotic bodies (arrows seen in Fig. 4A), consistent with the induction of apoptosis, as reported previously with thapsigargin (Furuya et al., 1994; Rao et al., 2002; Song et al., 2002). Taken together, these results suggest that cynaropicrin may cause cell death by inducing caspase-dependent apoptosis, resulting in cell cycle arrest and DNA fragmentation.

To dissect the signaling pathways involved in the induction of apoptosis by cynaropicrin, the effects of a number of enzyme inhibitors were tested. First, based on the previous reports that sesquiterpene lactones (parthenolide and repin) generate reactive oxygen species which triggers cellular apoptotic pathways (Hwang et al., 1996; Bork et al., 1997; Robles et al., 1997; Wen et al., 2002), we tested L-cysteine and *N*-acetyl-L-cysteine, efficient reactive oxygen species scavengers (Ballarin et al., 1998; Li et al., 2000). As predicted, the pretreated L-cysteine (150 μM) and *N*-acetyl-L-cysteine (800 μM) abolished the cynaropicrin-induced morphological changes (Fig. 4A) and DNA fragmentation (Fig 4B) via probably removing reactive oxygen species generation, even though we cannot exclude a possibility that those compounds directly reacted with cynaropicrin to form an adduct product (not identified yet) which shows a lower biological activity (Heilmann et al., 2001; Cho et al., 2000a). However, recent evidence that parthenolide generated cellular reactive oxygen species and depleted intracellular glutathione and these can be restored by treatment with several antioxidants such as *N*-acetyl-L-cysteine, pyrrolidine dithiocarbamate and nordihydroauaiaretic acid (Wen et al., 2002) strongly supported the role of those compounds as effective antioxidants. The other evidence to prove reactive oxygen species generation by cynaropicrin is that PKCδ was involved in cynaropicrin-mediated apoptosis. PKCδ has also been reported as a key signaling molecule in the reactive oxygen species-induced apoptotic pathway through generation of an active catalytic fragment by proteolytic cleavage (Anantharam et al., 2002; Lee et al., 2002; Emoto et al., 1995; Ghayur et al., 1996). The activated PKCδ catalytic fragment via interaction with p73β is associated with chromatic condensation, nuclear fragmentation, appearance of sub-G1 DNA and lethality (Ghayur et al., 1996; Ren et al., 2002; Bharti et al., 1998; Robins and Cobb, 2000). Rottlerin, a selective PKCδ inhibitor which blocks PKCδ-induced neuronal cell cytotoxicity and apoptosis (Anantharam et al., 2002) and CD98-mediated PKCδ activation in U937 cells (Cho et al., 2003b), was therefore tested in this model. To distinguish PKCδ involvement from conventional PKC isoforms (α, β and γ) in this cellular event, we simultaneously tested GF109203X (a broad-spectrum PKC inhibitor) at concentration displaying limited inhibitory effect to conventional PKC (Cho et al., 2003b). As shown in Fig. 4A–C, rottlerin (10 μM, selective inhibitory concentration for PKCδ) but not GF109203X (5 μM, selective inhibitory concentration for conventional PKC) blocked cynaropicrin-induced morphological changes, DNA fragmentation and cytotoxicity, suggesting selective involvement of PKCδ. This pharmacological data was supported by biochemical analysis showing that cynaropicrin exposure induced the appearance of the 42-kDa catalytic domain of PKCδ (Fig. 4D). Proteolytic cleavage of PKCδ may therefore be essential for mediating cynaropicrin-mediated apoptosis.

PKCδ-mediated apoptosis has also been linked to the activation of ERK or p38 (Lee et al., 2002) [although ERK is generally considered to promote cell survival (Robins and Cobb, 2000)]. We therefore tested the effects of a combination of three different MAPK inhibitors [PD98059 (a specific ERK inhibitor), SB20380 (a specific p38 inhibitor) and SP60089 (a JNK inhibitor)] at concentrations previously shown to block phorbol 12-myristate 13-acetate-induced interleukin-2 promoter activity (Cho et al., 2003a). However, these inhibitors did not alter cynaropicrin-induced morphological change (data not shown) or cytotoxicity (Fig. 5), suggesting that additional MAPK pathway may not be required for apoptosis and cytotoxicity.

In conclusion, we have shown that cynaropicrin dose-dependently induces cytotoxicity of leukocyte cancer cell lines such as U937 and Jurkat T cells, and the effect may be due to activation of apoptosis. Thus, pro-apoptotic effect by cynaropicrin may be explained by pharmacological and cell biological analyses including DNA fragmentation, cell cycle arrest, morphological change, caspase activation and proteolytic cleavage of PKCδ. As recently, thapsigargin has been reported to induce apoptosis in an Apaf-1 (also cytochrom *c*)-independent manner (Rao et al., 2002), including the activation of glycogen synthase kinase-3β (Song et al., 2002), the next study will be further examined as to whether cynaropicirn-induced apoptosis involves these pathways. Taken in conjunction with the fact that sesquiterpene lactones can also stimulate cellular differentiation of myeloid cell lines (Lee et al., 2000; Gu et al., 2002; Choi et al., 2002), these results suggest that cynaropicrin and perhaps also other sesquiterpene lactones may be useful drugs for the treatment of leukocyte cancers such as lymphoma or leukemia.

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