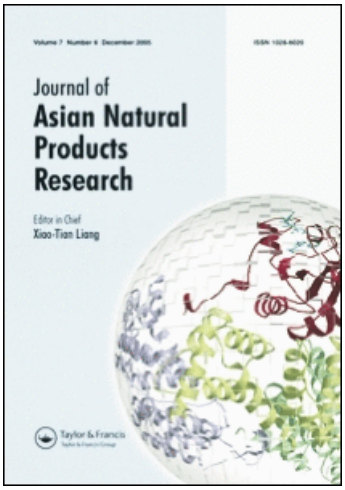

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Silymarin enhanced cytotoxic effect of anti-Fas agonistic antibody CH11 on A375-S2 cells

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Silymarin is a polyphenolic flavonoid from milk thistle *(Silybum marianum),* which has anti-inflammatory, cytoprotective as well as antioxidant effects. Our previous study demonstrated that silymarin has anti-apoptotic effect against UV irradiation. In this study, we assessed the effect of silymarin on anti-Fas agonistic antibody CH11-treated human malignant melanoma, A375-S2 cells. Pretreatment with silymarin (3×10^{-4} mol/L) significantly induced cell apoptosis in CH11-treated A375-S2 cells. Mitochondrial transmembrane potential ($\Delta\Psi_m$) was also down-regulated by silymarin pretreatment. Caspase-8, -9, -3 and pan-caspase inhibitors partially reversed silymarin-induced apoptosis of CH11-treated cells. The expression of Fas-associated proteins with death domain (FADD), a downstream molecule of the death receptor pathway, was increased by silymarin pretreatment, followed by cleavage of procaspase-8, whose activation induced cell apoptosis. Moreover, cleavage of procaspase-3 and digestion of its substrate, the inhibitor of caspase-activated DNase (ICAD), were also increased by silymarin pretreatment. These results suggested that silymarin could also exaggerate the apoptotic effect of anti-Fas agonistic antibody CH11 on A375-S2 cells.

Keywords: Silymarin; CH11; A375-S2 cell; Caspase; Mitochondrial transmembrane potential

1. Introduction

Extensive research within the last few years has shown that silymarin, a polyphenolic flavanoid, is clinically used as an antihepatotoxic agent for many liver diseases [1–4]. Pharmacological studies have indicated that silymarin is not toxic for cirrhosis patients even at high doses [3].

Apoptosis, or programmed cell death, is a genetically regulated, self-destructive cellular death process that is important in development, tissue remodeling, immune regulation and many diseases [5–8]. A variety of proteins and genes are involved in apoptosis. Caspases are a family of cysteine proteases that mediate apoptosis. Binding of death ligand such

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as FasL, or agonistic antibodies to their cognate receptors trigger the extrinsic pathway [9]. The trimerised cytoplasmic region then transduces the signal by recruiting FADD, which is responsible for downstream signal transduction by recruitment of caspase-8 and -10 [10]. Cellular stresses such as UV irradiation result in engagement of the intrinsic cell death pathway leading to procaspase-9 activation after mitochondrial damage. Both pathways lead to a cascade of executioner caspases (-3, -6, -7), which results in the cleavage of a number of caspase substrates responsible for apoptotic demise of the cells by cleaving intracellular proteins, altering or negating protein functions [11–14].

Our previous study demonstrated that silymarin was found to have an anti-apoptotic effect against UV irradiation in human malignant melanoma, A375-S2 cells [15]. In the present study, we report that anti-Fas agonistic antibody CH11 significantly induces apoptosis in A375-S2 cells after silymarin pretreatment.

2. Results and discussion

2.1 The cytotoxicity of anti-Fas agonistic antibody CH11 on A375-S2 cells was enhanced bypretreatment of silymarin

Table 1 showed the viability ratio of A375-S2 cells that were treated with various concentrations of silymarin for 1 h, and then further incubated with or without CH11 0.1 μg/ml for 12 h. It was found that silymarin could enhance cytotoxicity of CH11 on A375-S2 cells in a dose-dependent manner, while treatment with silymarin alone did not show obvious cytotoxic effect on the cells.

Figure 1 shows that silymarin could also time-dependently enhance the cytotoxicity of CH11. The viability ratio was decreased to 38.10% after silymarin pretreatment (3×10^{-4} mol/L) compared with 91.59% (silymarin-untreated) in CH11 (0.5 μg/ml)-treated A375-S2 cells at 24 h.

To determine whether silymarin-enhanced A375-S2 cell death was due to apoptosis, the morphologic changes in cell nuclei and DNA fragmentation were examined. When silymarin (3×10^{-4} mol/L)-pretreated cells continued to be incubated with CH11 0.1 μg/ml for 12 h, marked morphologic changes in cell nuclei were observed (figure 2d) compared with the cells which were treated by CH11 alone (figure 2c). The majority of cells became round, and many of these cells showed membrane blebbing (figure 2d), which was a hallmark of

Table 1. Effect of silymarin on cell viability of CH11-treated A375-S2 cells at 12 h: $\bar{x} \pm s$, $n = 3$.

Condition	*Cell viability (%)*
Medium	100 ± 0.67
CH11 0.1 μg/ml	96.82 ± 2.41
Silymarin (1×10^{-5} mol/L) + CH11 0.1 μg/ml	95.93 ± 1.06
Silymarin (5×10^{-5} mol/L) + CH11 0.1 μg/ml	89.47 ± 1.23†
Silymarin (1×10^{-4} mol/L) + CH11 0.1 μg/ml	85.91 ± 2.11†
Silymarin (3×10^{-4} mol/L) + CH11 0.1 μg/ml	68.68 ± 5.68†
Silymarin (1×10^{-4} mol/L) + CH11 0.1 μg/ml	60.32 ± 3.59†
Silymarin (1×10^{-5} mol/L)	100.2 ± 1.19
Silymarin (5×10^{-5} mol/L)	102.8 ± 3.01
Silymarin (1×10^{-4} mol/L)	101.9 ± 2.18
Silymarin (3×10^{-4} mol/L)	98.4 ± 1.38
Silymarin (1×10^{-4} mol/L)	98.7 ± 1.94

† $p < 0.01$ *vs* CH11 0.1 μg/ml group.

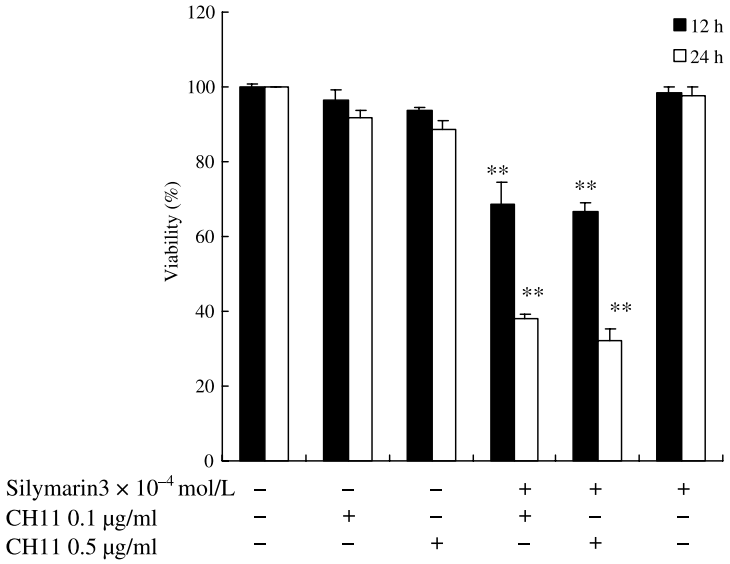

Figure 1. Effect of silymarin on the viability of CH11-treated A375-S2 cells. A375-S2 cells were pretreated with silymarin (3×10^{-4} mol/L) for 1 h, then incubated with CH11 0.1 or 0.5 μg/ml for 12 (■) or 24 h (□). Cell viability was evaluated using the MTT method. $\bar{x} \pm s$, $n = 3$, $^{**}p < 0.01$.

apoptosis, while morphologic changes in CH11-treated cells (silymarin untreated) were not obvious (figure 2c). DNA fragmentation, another hallmark of apoptosis, was also observed in this study (figure 3). It was also found that DNA fragmentation was effectively enhanced by silymarin in CH11-treated cells and the effect of (anti-Fas antagonistic antibody UB2 + CH11) group on DNA fragmentation was a negative control (figure 3). Therefore,

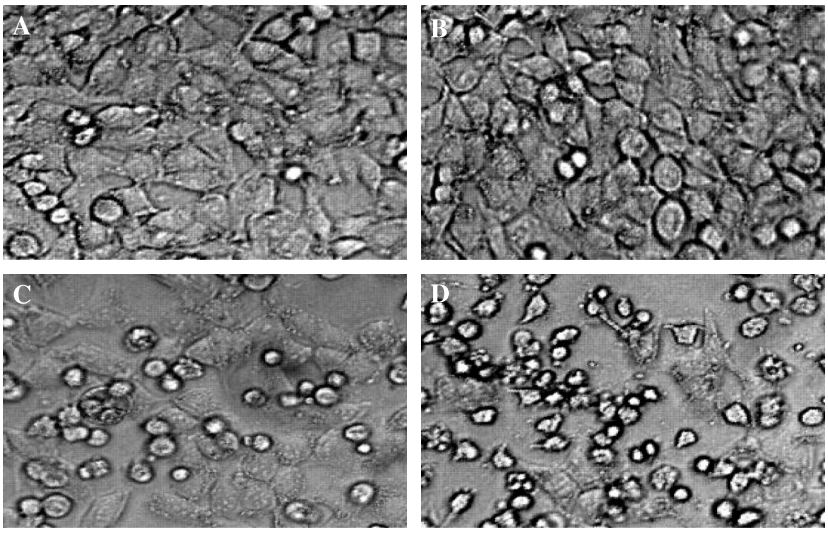

Figure 2. Silymarin-induced significant morphologic changes in CH11-treated A375-S2 cells. (a) Medium control; (b) the cells were incubated with silymarin (3×10^{-4} mol/L) for 12 h; (c) the cells were incubated with CH11 0.1 μg/ml for 12 h; (d) the cells were pretreated with silymarin (3×10^{-4} mol/L) for 1 h, then incubated with CH11 0.1 μg/ml for 12 h. Morphologic changes were observed by phase-contrast microscopy (original magnification × 200).

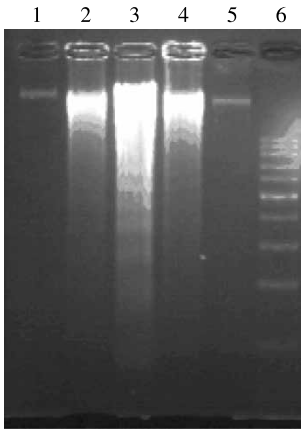

Figure 3. DNA fragmentation induced by silymarin pretreatment in CH11-treated A375-S2 cells. Lane 6: Hae-III-digested ΦX-174 DNA fragments were used as a molecular marker. Lane 1: medium control. Lanes 2, 5: the cells were incubated with CH11 0.1 μg/ml or silymarin (3×10^{-4} mol/L) for 12 h. Lanes 3, 4: the cells were pretreated with silymarin (3×10^{-4} mol/L) or UB2 0.05 μg/ml for 1 h, then incubated with CH11 0.1 μg/ml for 12 h. DNA was isolated by agarose gel electrophoresis and analysed by ethidium bromide staining.

these results demonstrated that the cause of CH11-treated cell death induced by silymarin pretreatment was related to apoptosis.

2.2 Mitochondrial transmembrane potential ($\Delta\Psi_m$) was greatly reduced by silymarin in CH11-treated A375-S2 cells

Kinetic data indicate that mitochondria undergo major changes in membrane integrity before classical signs of apoptosis become manifest [16]. These changes concern both the inner and the outer mitochondrial membranes, leading to a disruption of the inner transmembrane potential ($\Delta\Psi_m$) and the release of intermembrane proteins through the outer membrane. In this study, mitochondrial transmembrane potential ($\Delta\Psi_m$) was further assessed using rhodamine 123, a specific fluorescent probe for analysis of mitochondrial transmembrane potential. It was found that the fluorescent intensity was significantly decreased in silymarin and CH11 treated cells (figure 4d) compared with the other three groups (figures 4a,b,c). The percentage of stained cells by rhodamine 123 was also decreased to 32.84% using a FACScan flow cytometer (figure 4h). Moreover, caspase-9 was found to be activated in our study, which is activated by post-mitochondrial pathway [17]. These results suggested that down-regulated mitochondrial transmembrane potential by silymarin pretreatment in CH11 treated A375-S2 cells might trigger the opening of the mitochondrial megachannel (also called permeability transition pore), followed by release of cytochrome *c*, from the mitochondrial intermembrane, resulted in activation of caspase-9 apoptotic pathway.

2.3 Caspases activated during silymarin-enhanced apoptosis in CH11-treated A375-S2 cells

20 μM z-IETD-fmk, z-LEHD-fmk, z-DEVD-fmk, and z-VAD-fmk (inhibitors for caspase-8, -9, -3, and pan-caspase, respectively) partially blocked silymarin-enhanced apoptosis in

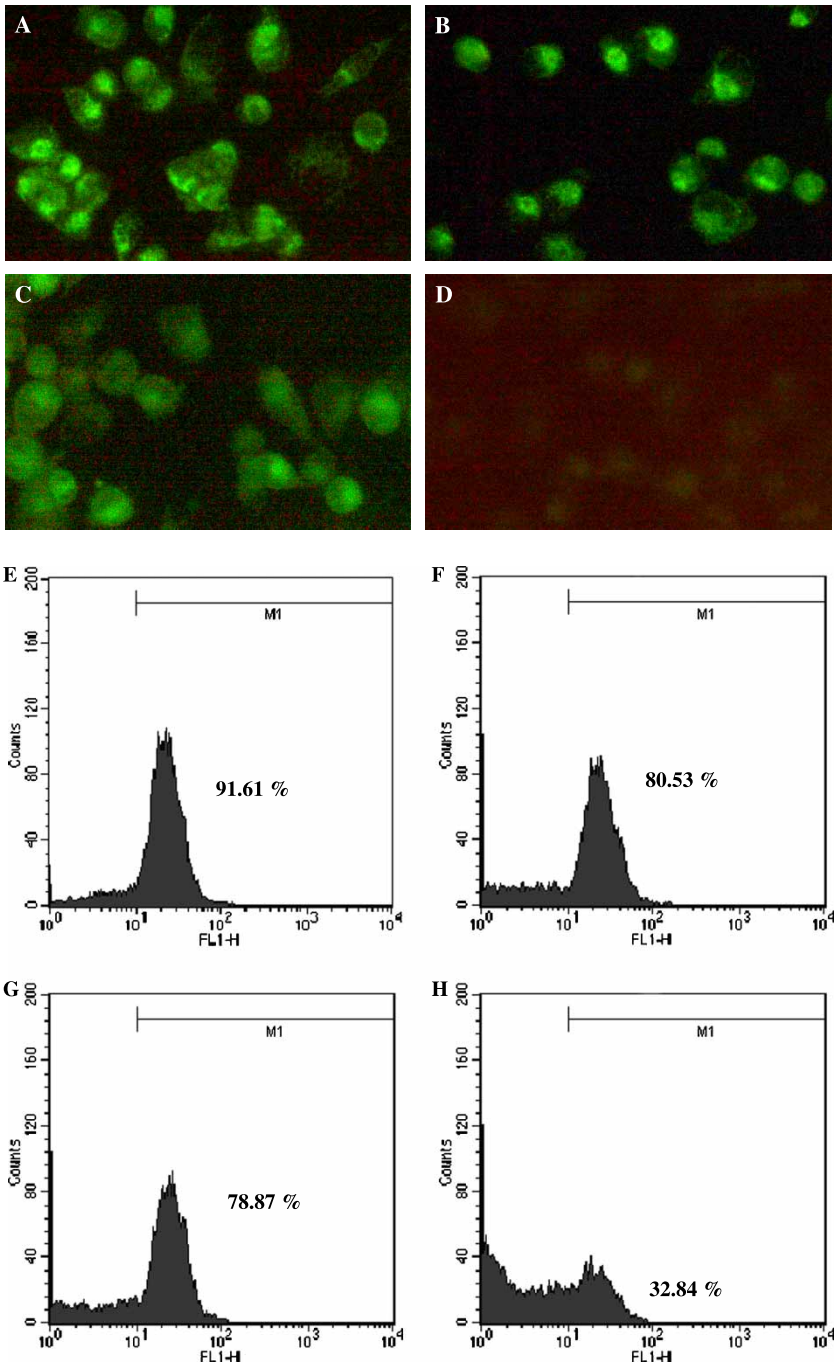

Figure 4. Mitochondrial transmembrane potential ($\Delta\Psi_m$) was reduced by silymarin in CH11-treated A375-S2 cells. (a, e) Medium control; (b, f) the cells were incubated with silymarin (3×10^{-4} mol/L) for 12 h; (c, g) the cells were incubated with CH11 0.1 μg/ml for 12 h; (d, h) the cells were pretreated with silymarin (3×10^{-4} mol/L) for 1 h, then incubated with CH11 0.1 μg/ml for 12 h. The cells were collected and washed with PBS, followed by incubation with PBS containing 1 μg/ml rhodamine 123 at room temperature for 30 min. The fluorescence of cells was observed using fluorescence microscopy (a–d) and measured by a FACScan flow cytometer (e, f).

CH11-treated A375-S2 cells. The viability ratio was increased to 67.84%, 62.55%, 73.13%, and 70.28%, respectively, compared with 55.36% in silymarin and CH11-treated cells. In addition, it was found that caspase-10 inhibitor (z-AEVD-fmk) did not have anti-apoptotic effect (the viability of the cells was 53.87%) (figure 5).

Western blot analysis was carried out to further confirm the participation of caspase-8 and -3. The 56 kDa band of procaspase-8 and the 30 kDa band of procaspase-3 were effectively degraded after 12 h treatment with silymarin 300 μM and CH11 0.1 μg/ml, indicating the activation of these two caspases (figures 6, 7).

The inhibitor of a caspase-dependent Dnase (ICAD) referred to as CAD (DEF40/CPAN) is a caspase substrate that is cleaved to be inactivated and allow CAD to execute the characteristic fragmentation of DNA. ICAD is expressed as two isoforms, one of 45 kDa (ICAD-L/DFF45) and other one of 35 kDa (ICAD-S/DFF35). Although both ICAD-L/DFF45 and ICAD-S/DFF35 bind to and inhibit CAD, only ICAD-L/DFF45 was reported to be functional [18,19]. Thus the ICAD-L/DFF45 expression in A375-S2 cells was examined to confirm the activation of caspase-3. After exposure to silymarin 300 μM and CH11 0.1 μg/ml for 12 h, ICAD-L was significantly degraded compared with exposure only to CH11 0.1 μg/ml (figure 7).

All these results suggest that the caspase cascade plays a critical role in silymarin-mediated apoptosis in CH11-treated A375-S2 cells.

2.4 Silymarin increased the expression of FADD in CH11-treated A375-S2 cells

It has been reported that activation of CD95 (Fas/APO-1) requires trimerisation of the receptor, which results in recruitment of the FADD [20,21]. Our result showed that silymarin

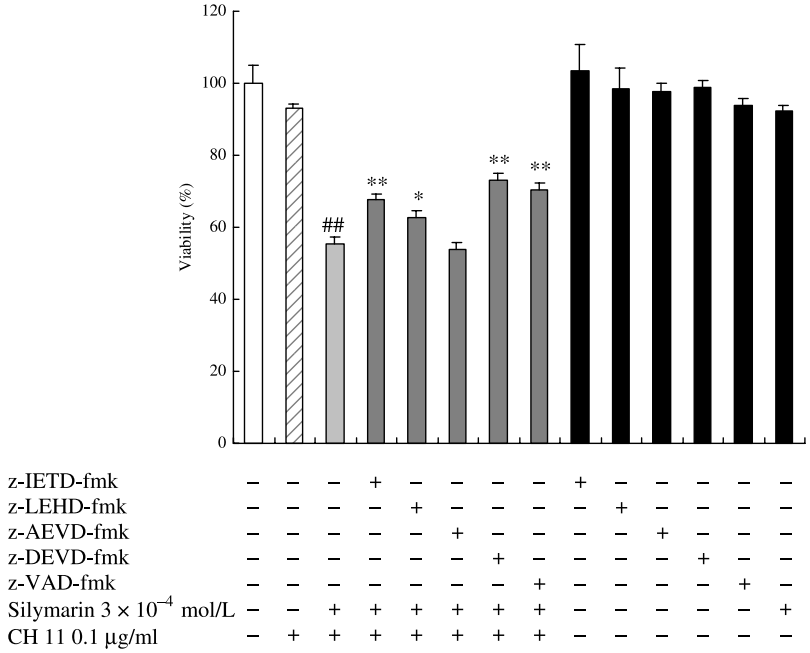

Figure 5. Effect of caspase inhibitors on silymarin-enhanced apoptosis in CH11-treated A375-S2 cells. The cells were cultured in the presence or absence of caspase inhibitors 1 h prior to the addition of silymarin and then incubated with CH11 for 20 h ($n = 3$). *$p < 0.05$, **$p < 0.01$ *vs* the silymarn and CH11-treated group, ##$p < 0.01$ *vs* medial control group or CH11-treated group.

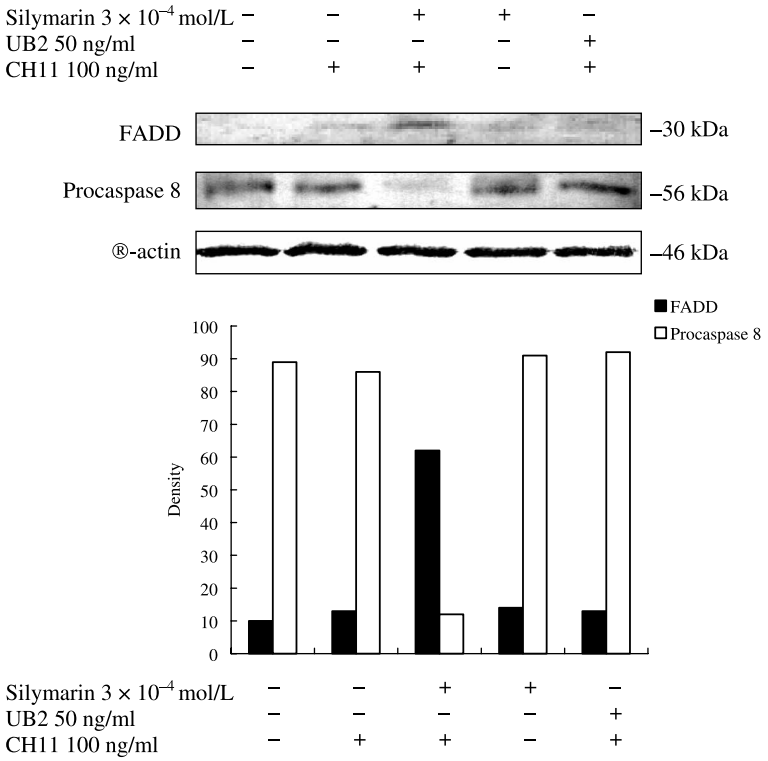

Figure 6. Expressions of FADD and procaspase-8 in A375-S2 cells. The cells were pretreated with silymarin (3×10^{-4} mol/L) or UB2 0.05 μg/ml for 1 h, then incubated with CH11 0.1 μg/ml for 12 h. Cell lysates were separated by 12% SDS-PAGE, and expressions of FADD and procaspase-8 were detected by Western blot analysis. β-Actin was used as an equal loading control.

pre-incubation up-regulated the expression of FADD in CH11-treated cells (figure 6), which might increase the recruitment of FADD to CD95, resulting in activation of anti-Fas agonistic antibody CH11-triggered CD95 pathway. CH11 treatment alone did not increase the expression of FADD.

In summary, the data presented here indicate that silymarin enhanced the cytotoxicity of CH11 on A375-S2 cells through activation of caspases, CD95 and post-mitochondrial pathway.

3. Experimental

3.1 Chemicals

Silymarin (m.w. = 482.4) was obtained from the Beijing Institute of Biologic Products (Beijing, China), the purity of which is determined to be about 98% by HPLC. Rhodamine 123, RNase A, proteinase K, 3,3-diaminobenzidine tetrahydrochloride (DAB) and 3-(4,5-dimetrylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). z-IETD-fmk, z-LEHD-fmk, z-AEVD-fmk, z-DEVD-fmk, and z-VAD-fmk for inhibitors of caspase-8, -9, -10, -3, and pan-caspase, respectively, were obtained from Enzyme Systems (Livermore, CA, USA). Anti-Fas agonistic antibody CH11 and anti-Fas antagonistic antibody UB2 were from Medical & Biological Laboratories

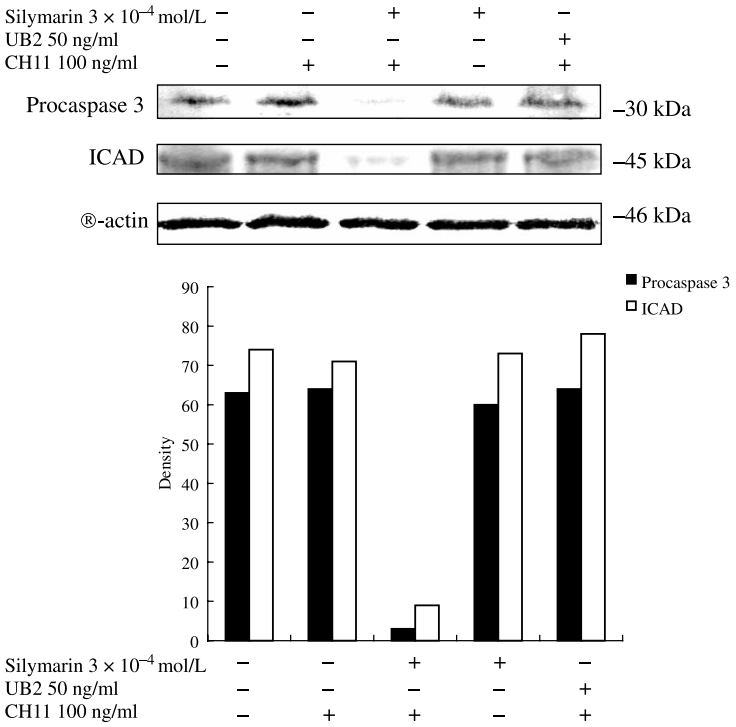

Figure 7. Expressions of procaspase-3 and its death substrates ICAD in A375-S2 cells. The cells were pretreated with silymarin (3×10^{-4} mol/L) or UB2 0.05 μg/ml for 1 h, then incubated with CH11 0.1 μg/ml for 12 h. Cell lysates were separated by 12% SDS-PAGE, and expressions of procaspase-3 and ICAD were detected by Western blot analysis. β-Actin was used as an equal loading control.

(Nagoya, Japan). Rabbit polyclonal antibodies against FADD, procaspase 8, procaspase 3, ICAD and horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

3.2 Cell culture

The cells were cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Dalian Biological Reagent Factory, Dalian, China), L-glutamine (2 mM, GIBCO, Grand Island, NY, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) and maintained at 37°C with 5% CO_2 in a humidified atmosphere.

3.3 Cell growth assay

A375-S2 cells were dispensed in 96-well flat-bottomed microtiter plates (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells/well. (1) After preincubation with or without various concentrations of silymarin for 1 h, the cells were incubated with CH11 0.1 or 0.5 μg/ml for 12 or 24 h. (2) The cells were incubated with the caspase inhibitors z-IETD-fmk, z-LEHD-fmk, z-AEVD-fmk, z-DEVD-fmk and z-VAD-fmk at given concentrations for 1 h, then treated with silymarin (3×10^{-4} mol/L) for another 1 h. The cells were further incubated with CH11 0.1 μg/ml for 20 h. Cell growth was measured using the MTT assay as described [22] with an ELISA reader (TECAN SPECTRA, Wetzlar, Germany).

The percentage of cell viability was calculated as follows:

$$\text{Cell viability}(\%) = (A_{490} - A_{490,\text{blank}})/(A_{490,\text{control}} - A_{490,\text{blank}}) \times 100$$

3.4 Observation of morphologic changes

A375-S2 cells in RPMI-1640 containing 10% FBS were seeded into 25-ml culture bottles and incubated overnight. The cells were incubated with CH11 0.1 μg/ml for 12 h after pretreatment with silymarin (3×10^{-4} mol/L) for 1 h, and the cellular morphology was observed using phase-contrast microscopy (Leica, Wetzlar, Germany).

3.5 DNA fragmentation assay

A375-S2 cells (1×10^6 cells) were harvested and centrifuged at 1,500 × *g* for 5 min, and then washed with PBS. The cells were pelleted and suspended in 10 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0) and 0.5% Triton X-100, and maintained at 4°C for 10 min. The supernatant was incubated with 20 mg/ml RNase A (2 μl) and 20 mg/ml proteinase K (2 μl) at 37°C for 1 h, then stored in 0.5 M NaCl (20 μl) and ispropanol (120 μl) at −20°C overnight, and centrifuged at 15,000 × *g* for 15 min. DNA was dissolved in TE buffer [10 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0)] and subjected to 2% agarose gel electrophoresis at 50 V for 40 min and stained with ethidium bromide.

3.6 Measurement of mitochondrial transmembrane potential

To measure the mitochondrial transmembrane potential ($\Delta\Psi_m$), the cells were washed with PBS and then incubated for 30 min at room temperature with PBS containing 1 μg/ml rhodamine 123. After two times of washes and final resuspension in PBS, the fluorescence of cells was observed using fluorescence microscopy (Leica, Wetzlar, Germany) and measured by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

3.7 Western blot analysis

After incubation for 12 h, both adherent and floating A375-S2 cells were collected. Western blot analysis was carried out as previously described [23] with some modification. The cells were lysed on ice in lysis buffer [50 mM HEPES (pH 7.4), 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride (PMSF)], supplemented with the proteinase inhibitors, aprotinin 100 μg/ml, leupeptin 10 μg/ml, and pepstatin 100 μg/ml for 1 h. The protein concentration was determined using the folin assay. The lysate was centrifugated at 16,000 × *g* at 4°C for 10 min, equal amounts of total proteins were mixed in 2 × loading buffer [50 mM Tris–HCl (pH 6.8), 2% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue], boiled for 5 min, and subjected to a 12% SDS–polyacryamide gel eletrophoresis. Proteins were electrotransferred onto nitrocellulose membranes and detected with antibodies against FADD, procaspase 8, procaspase 3, and ICAD, followed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibody and 3,3-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate.

3.8 Statistical analysis

All data represent at least three independent experiments and are expressed as mean ± SD, unless otherwise indicated. Statistical comparisons were made using Students' *t*-test. *p*-Values of less than 0.05 were considered to represent a statistically significant difference.

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