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Ethanol Extract of *Tripterygium wilfordii* Hook. f. Induces G0/G1 Phase Arrest and Apoptosis in Human Leukemia HL-60 Cells Through c-Myc and Mitochondria-Dependent Caspase Signaling Pathways

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1. Introduction

Tripterygium wilfordii Hook. f. is a traditional Chinese herb (Murphy, 2006; Qiu et al., 2003). The extract of *Tripterygium wilfordii* Hook. f. has been widely applied to the treatment of immune-related diseases, such as rheumatoid arthritis (RA), nephritis, and systemic lupus erythematosus (SLE) (Chang et al., 1999; Wang et al., 2000). Extracts of *Tripterygium wilfordii* Hook. f. have been shown to inhibit lymphocyte proliferation induced by mitogentic stimulation *in-vitro* (Wu et al., 2003). Triptolide (PG490, one of the most active components in *Tripterygium wilfordii* Hook. f. extract, possesses immunosuppressive, anti-inflammatory and anti-fertility actions *in vivo* and *in vitro* (Zhao et al., 2005; Leuenroth et al., 2005). Many reports have demonstrated that triptolide has anti-proliferate activity against L1210, U937, K562, HL60, and P388 leukemia cells (Lou et al., 2004; Chan et al., 2001; Wei et al., 1991). However, the cellular and molecular mechanisms underlying mediating *Tripterygium wilfordii* Hook. f.-induced differentiation and/or apoptosis in leukemia cells have not been well studied.

Leukemia is a malignant disease characterized by uncontrolled cellular growth and disrupted differentiation of hematopoietic stem cells (Lichtman et al., 2005; O'Hare et al., 2006). Chemotherapy can be effective in certain types of leukemia, but in cases in which it

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is not effective additional therapeutic strategies are needed. (Faderl et al., 2005; Frankfurt et al., 2006; ter Bals et al., 2005). Several compounds are capable of inducing the differentiation of leukemia cells into mature cells *in vitro*, and differentiation therapy has been shown to be an effective approach for treating leukemia (Altucci et al., 2004; Altucci et al., 2005; Takahashi et al., 2002). Human promyelocytic leukemia HL-60 cells and mouse monocytic leukemia WEHI-3 cells are commonly used to study various properties of leukemia cell proliferation and differentiation *in vitro* (Lin et al., 2006; Abe et al., 1987). Differentiation of HL-60 is induced into granulocytes by dimethyl sulfoxide (DMSO) and all-trans retinoic acid (ATRA), and into monocytic-like cells by phorbol ester (TPA) and 1,25-dihydroxy-vitamin D3 (Tsiftsoglou et al., 2003). In HL-60 cells, differentiation is induce-specific and is characterized by agents to differentiated is a marked increase in the proportion of G0/G1 cells (Yen et al., 2006), and the modulation of cyclin/CDK (Horie et al., 2004; Wang et al., 1996; Barrera et al., 2004; Pizzimenti et al., 1999; Kumakura et al., 1996).

In hematopoietic cells, apoptosis can be coupled to terminal differentiation of myeloid progenitor (Yazdanparast et al., 2005; Samudio et al., 2005). Cells undergoing apoptosis have observable morphology changes expressed as nuclear condensation, DNA fragmentation, and compact packaging of the cellular debris into apoptotic bodies (Fleischer et al., 2006; Bohm et al., 2006). The delivery and performance of apoptotic signals requires a coordinated cascade of caspase activation and action. The initiator caspases include caspase-8 in Fas-induced apoptosis, and caspase-9, the activation of which is triggered by cytochrome *c* release from mitochondria in response to various stimuli. Those caspases can directly activate downstream effectors of caspase-3, -6, and -7, which cleave death substrates, such as poly(ADP-ribose) polymerase (PARP) (Christophe et al., 2006; Lucken et al., 2005; Lockshin et al., 2005).

In this study, we investigated the cytotoxic effects of ethanol extract of *Tripterygium wilfordii* Hook. f. (ETW) on the promotion of cell cycle arrest and apoptosis in HL-60 cells. Our results indicated that ETW effectively induces both G0/G1 phase arrest and apoptosis of HL-60 cells *in vitro*. The mechanisms governing ETW-induced G0/G1 phase arrest included down regulation of cyclin E, Bcl-2 and Bax, and -triggered apoptosis through caspase-9, caspase-8 and caspase-3-dependent pathways.

2. Materials and methods

2.1 Chemicals and reagents

EDTA, Propidium iodide (PI), RNase A, Tris-HCl, Tritox X-100, Tween 20 and Proteinase K were obtained from Sigma Chemical Co. (St.Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), and L-glutamine, penicillin/streptomycin were obtained from Gibco BRL Co. (Grand Island, NY, USA). The caspase-3, caspase-8 and caspase-9 activity assay kits were bought from R&D Systems, Inc. (Minneapolis, MN, USA)

2.2 Ethanol *Tripterygium wilfordii* Hook. f. (ETW) extraction

Dried and powdered plant materials were subjected to continuous ethanol extraction in a Soxhlet extractor with absolute ethanol for 72 h. The ethanol extract was collected and concentrated by vacuum distillation. The extract was evaporated to dryness and reconstituted in ethanol before experiment.

2.3 Cell culture and viability assay

The human promyelocytic leukemia cell line (HL-60) was obtained from the Culture Collection and Research Center (CCRC, Taiwan, R.O.C.), originally from the American Type Culture Collection (ATCC, USA). Cells were cultured in RPMI-1640 culture medium (Gibco/Life Technologies, Taipei, Taiwan) supplemented with 10% heated-inactive fetal bovine serum (Gibco/Life Technologies), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Gibco/Life Technologies) and incubated at 37°C in humidified 5% CO2 atmosphere.

For viability analysis, 2.5 X 10^5 cells/well were seeded in 24-well culture plates. ETW was added to each well and the plates were incubated at 37°C for 24, 48 and 72 h. Cell viability was estimated by a propidium iodide (PI) incorporation assay and flow cytometry (FACS CaliburTM, Becton Dickinson) analysis (Aouacheria et al., 2002).

2.4 Cell cycle analysis

Cells were incubated with 50, 100 or 200 μg/mL of ETW for 0, 24 or 48 h. After treatment, cells were washed with phosphate-buffered saline (PBS) twice. The cells were re-suspended in hypotonic PI solution (0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml propidium iodide), and then cellular DNA content was determined by flow cytometry (Kamikubo et al., 2003).

2.5 Western blotting analysis

Total protein was prepared with protein lysising buffer (PRO-PREP™ protein extraction solution, iNtRON Biotechnology, Seongnam, Gyeonggi-Do, Korea). The concentration of protein was determined by the Bradford method using the Bio-Rad protein assay dye reagent. The lysates containing 40 μg of protein were separated by SDS-PAGE and transferred onto PVDF membrane. Nonspecific binding sites were blocked with 5% non-fat milk in PBST buffer (0.05 % Triton X-100 in PBS) for 1 h. The PVDF membrane was incubated overnight at 4°C with specific primary antibodies against cyclin D1, cyclin E, Bcl-2, and α-tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After being washed with PBST buffer, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz). Immunoreactive proteins were detected using a Western Blotting Chemiluminescence Reagent Plus kit (NENTM Life Science) and exposed to Chemiluminescence films (Choi et al., 2003).

2.6 Caspase activities assays

Cells were collected in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM DTT) and placed on ice for 10 min. The lysates were centrifuged at 15,800g at 4°C for 10 min. Cell lysates (50 μg of protein) were incubated with caspase -3, -9, and -8 specific substrates (Ac-DEVD-pNA, Ac-LEHD-pNA, and Ac-IETD-pNA) with reaction buffer in a 96-well plate at 37°C for 1 h. The caspase activity was determined by measuring OD405 of the released pNA (An et al., 2004).

2.7 Statistical analysis

Results are presented as mean ± S.D. Differences between the different treatment groups, which consisted of matched samples, were assessed by the Student's *t*-test. A p value of less than 0.05 was considered to be significant.

3. Results

3.1 Effects of ETW on cell viability in HL-60 and WEHI-3 cells

We treated HL-60 cells with ETW at the concentrations of 0, 50, 100 and 200 μg/ml. The number of viable cells was counted by a PI exclusion method 0, 24 and 48 h later. As shown in Fig. 1, ETW exerted a dose- and time-dependent loss of cell membrane integrity and viability in HL-60 cells.

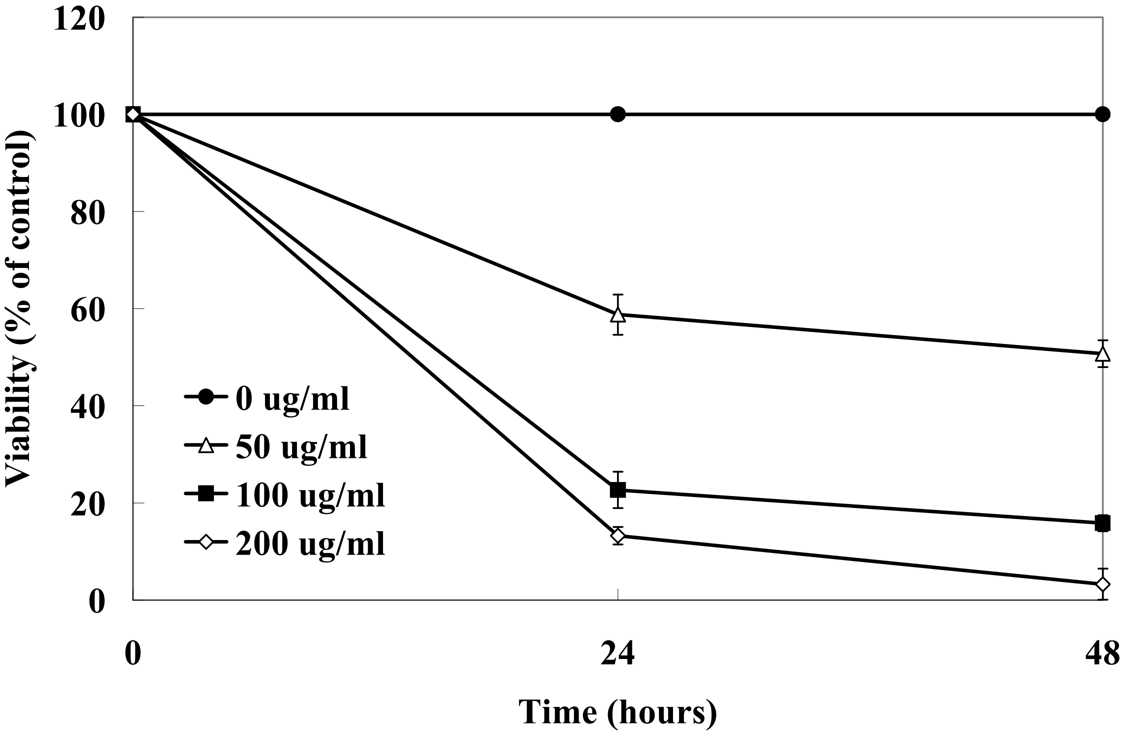
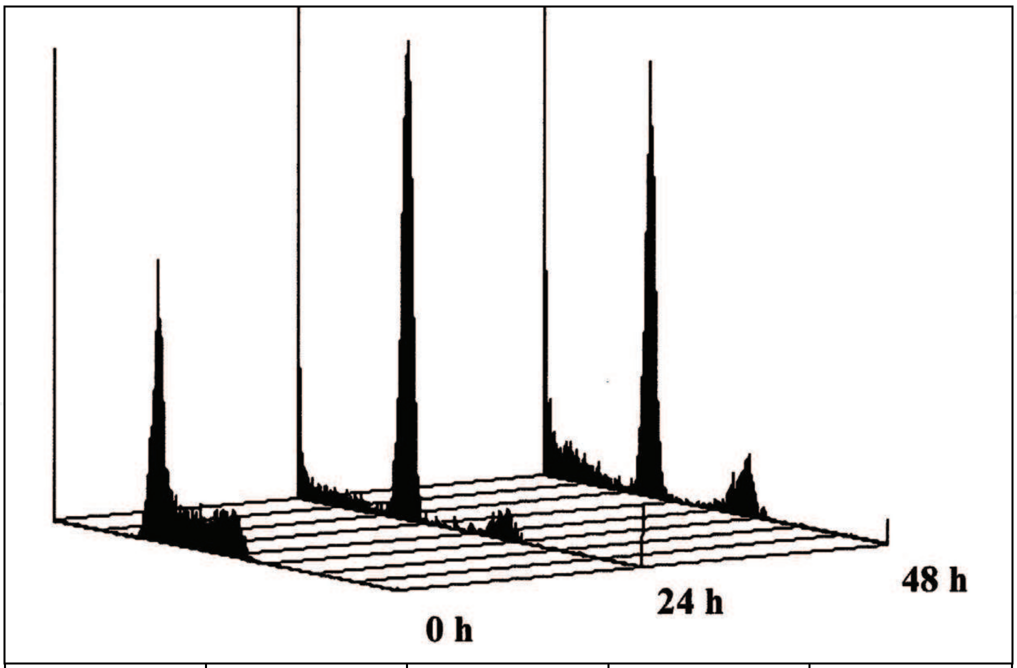


Fig. 1. Effects of cell viability in ETW treated HL-60 cells. Cells were treated with various concentrations of ETW for indicated duration. Viable cells were measured by PI exclusion and immediately analyzed by flow cytometry. The percentage of cell viability was calculated as a ratio between drug-treated cells and control cells. Each value represents mean±S.D. from three independent experiments

3.2 Effects of ETW on cell cycle progression in HL-60 cells

To investigate the mechanisms by which ETW induced cytotoxicity effect in HL-60 cells, we cultured cells for various time periods with 100 μg/ml ETW and analyzed DNA content by flow cytometry. Cell cycle analysis showed that ETW induced a prominent G0/G1 population arrest in HL-60 cells (Fig. 2.). In addition, 100 μg/ml of ETW increased the sub-G0/G1 nuclei population in HL-60 cells in a time-dependent manner (Fig. 2.).



Time	Sub-G1	G0/G1	S	G2/M
0 h	1.19 ± 0.05	52.80 ± 2.64	30.08 ± 1.97	17.12 ± 0.14
24 h	4.58 ± 3.25	77.74 ± 2.59	12.21 ± 2.96	10.05 ± 1.82
48 h	16.68 ± 4.66	81.96 ± 1.35	6.04 ± 1.68	12.00 ± 2.66

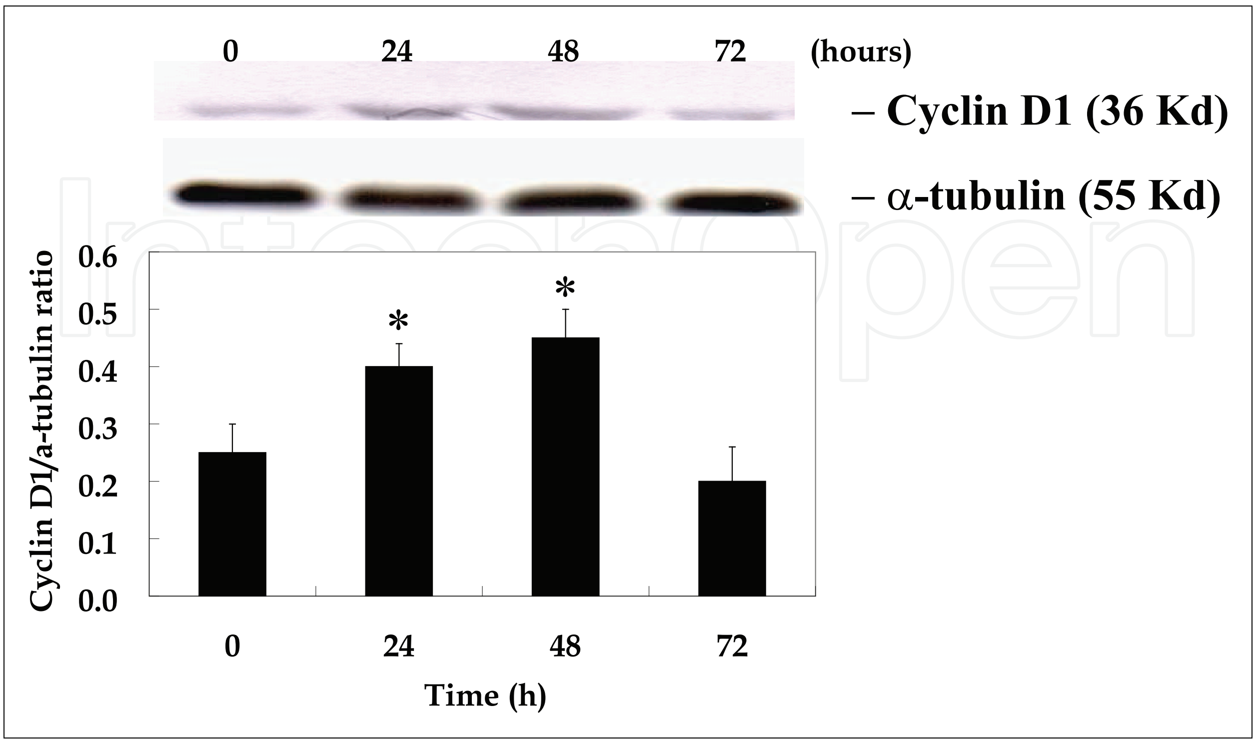
Fig. 2. Cell cycle progression on HL-60 cells after treated with ETW. Cells were treated with ETW for the indicated incubation times, then stained for DNA with PI, and analyzed for cell cycle progression or apoptosis by flow cytometry. Cell cycle analysis showed that ETW induced a prominent G0/G1 population arrest and apoptosis in HL-60 cells. Each value represents mean±S.D. from three independent experiments

3.3 Effects of ETW on cyclin D1, cyclin E, Bcl-2 and c-Myc proteins expression in HL-60 cells

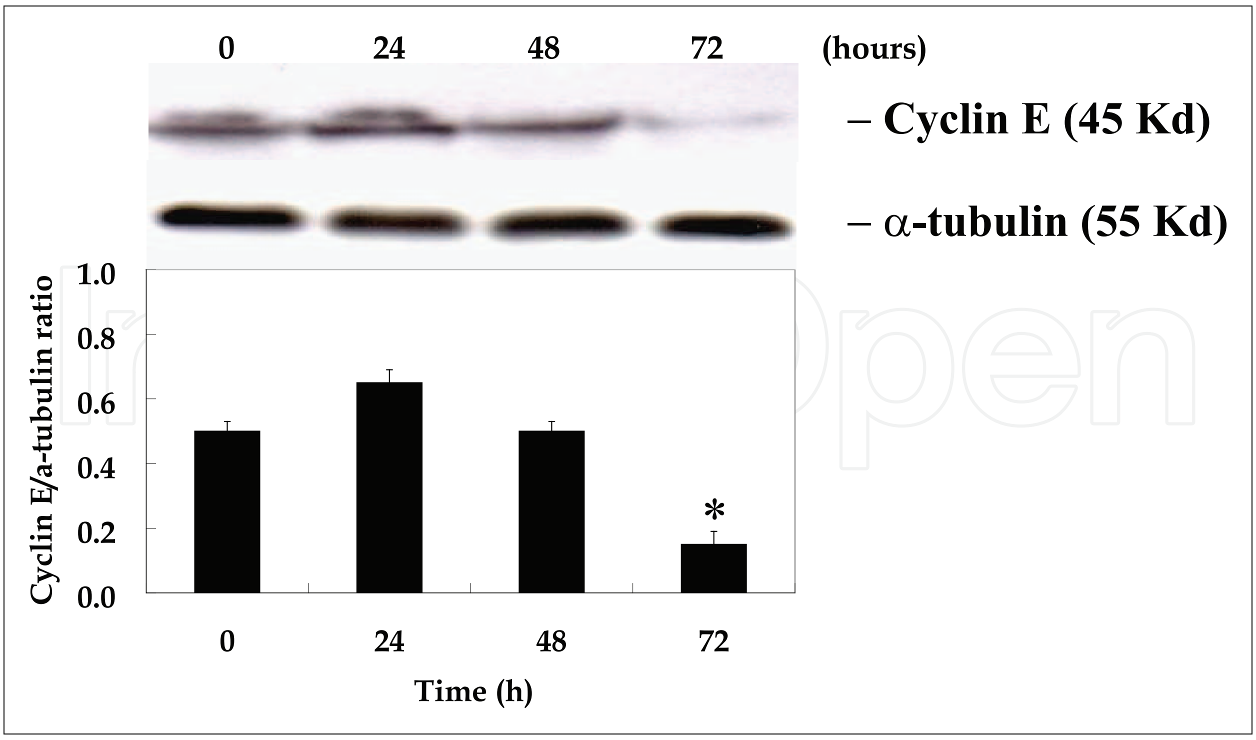
To better understand how ETW induces G0/G1 arrest, we investigated the protein expressions of cyclin D1 and cyclin E. After treatment with 100 μg/ml of ETW, there was a marked increase in protein levels of cyclin D1 and a marked decrease in cyclin E (Fig. 3A and 3B.) We also examined the expression levels of Bcl-2 and c-Myc protein. As shown in Fig. 3C and 3D, Bcl-2 and c-Myc protein levels decreased in HL-60 cells relative to controls. Our results suggest that ETW induces G0/G1 arrest and apoptosis in HL-60 cells by regulating cyclin D1, cyclin E, Bcl-2 and c-Myc protein expression.

3.4 ETW induced apoptosis is mediated by the activations of caspase-9, caspase-8 and caspase-3

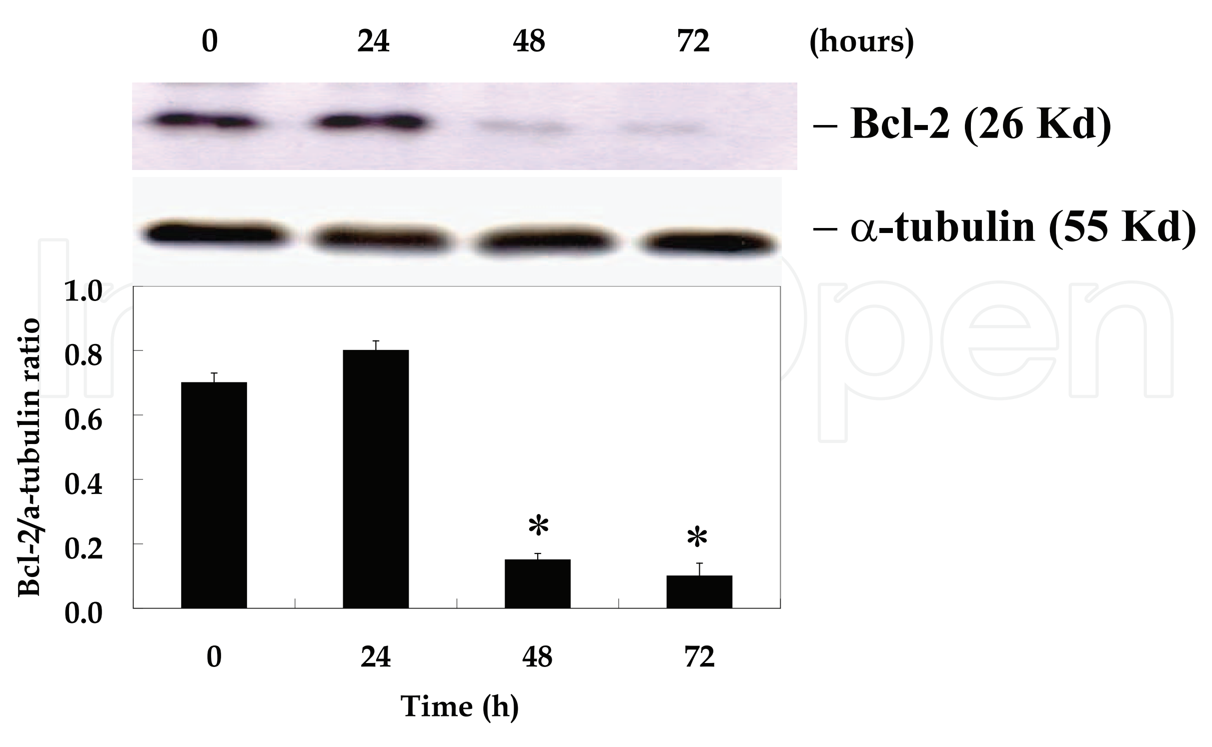
Activation of caspase plays a key role in the induction of apoptosis. We used a fluorogenic enzymatic assay to detect activated caspase-9, caspase-8 and caspase-3 in ETW-treated HL-60 cells. Both caspase-9 and caspase-3 activities increased 24 h after ETW treatment and caspase-8 activities increased 48 h after ETW treatment (Fig. 4). Our results suggest that ETW-induced apoptosis is mediated through the activation of caspase-9, caspase-3 and then caspase-8.


0
24
48
72
(hours)
– Cyclin D1 (36 Kd)
– α-tubulin (55 Kd)
Cyclin D1/a-tubulin ratio
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
0
24
48
72
Time (h)

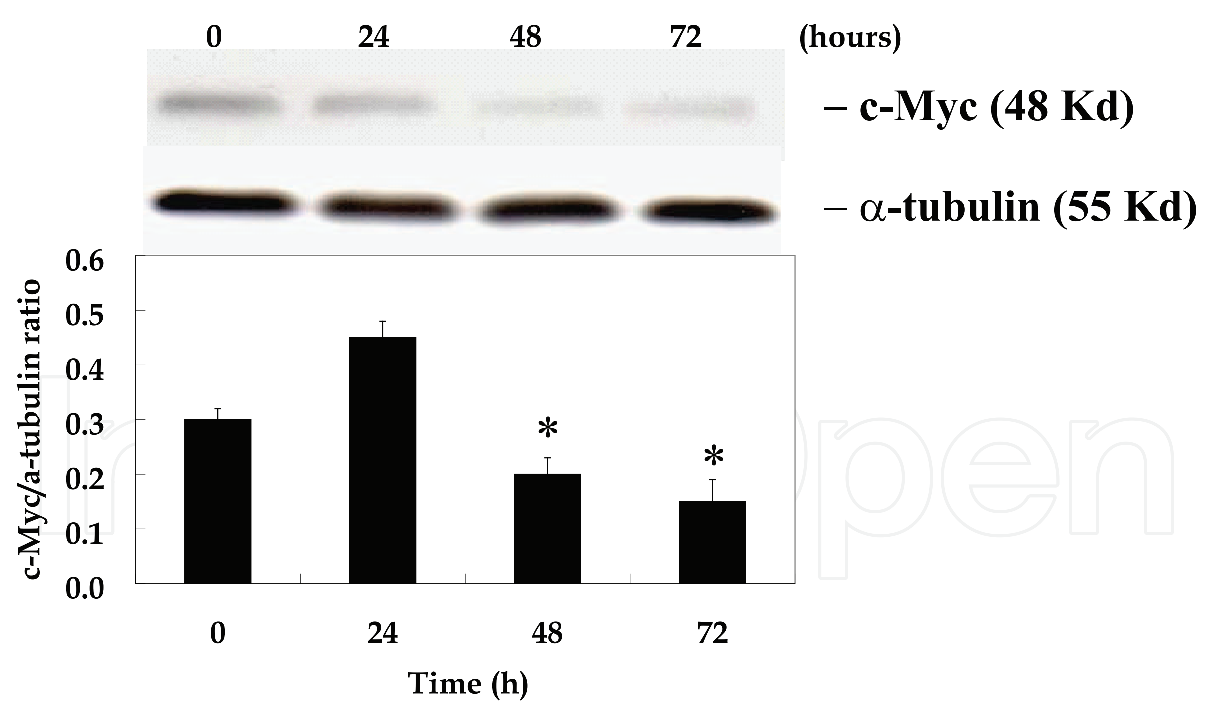
(a)


0
24
48
72
(hours)
– Cyclin E (45 Kd)
– α-tubulin (55 Kd)
Cyclin E/a-tubulin ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
0
24
48
72
Time (h)

(b)



(c)



(d)

Fig. 3. Representative Western blotting showing changes on the levels of (A) cyclin D1, (B) cyclin E, (C) Bcl-2 and (D) c-Myc in HL-60 cells after exposure to ETW (100 μg/ml). Cells were treated with ETW for the indicated incubation times then total protein were prepared and determined as described in Materials and Methods

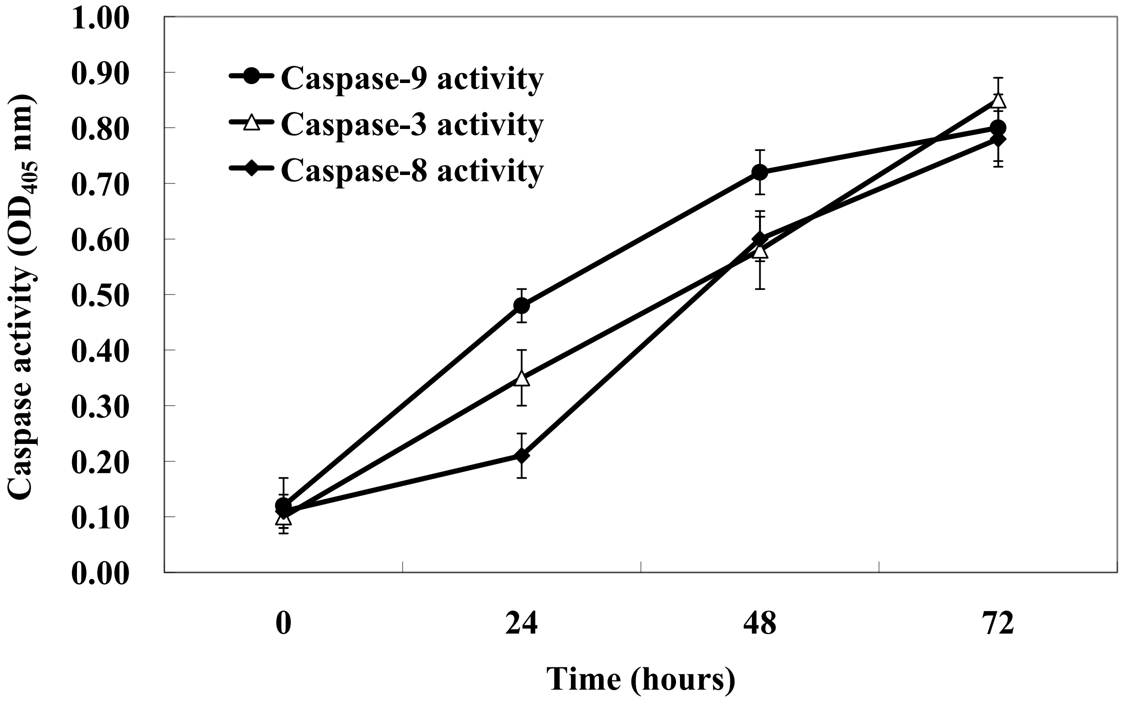


Fig. 4. Effects of ETW induced apoptosis on HL-60 cells by caspases-9, -8 and -3 activities. For caspase activity analysis, aliquots of total cell extracts were incubated with caspases-3, -9 and -8 specific substrates, respectively (Ac-DEVD-pNA, Ac-LEHD-pNA and Ac-IETD-pNA). The release of pNA was measured at 405 nm by a spectrophotometer

4. Discussion

Tripterygium wilfordii Hook. f. (TWHF) is used to treat inflammatory and immune-related diseases. Triptolide, a diterpenoid triepoxide extracted from the TWHF, exerts anti-tumorigenic actions against leukemia cells. In Differentiation -inducing activity study, some triterpene aglycones and Betulinic acid (pentacyclic triterpene) showed differentiation-inducing activity and against human acute promyelocytic leukemia HL-60 cells (Poon, 2004; Umehara et al., 1992). The preclinical laboratory work of identification and testing of potential anti-leukemia agents is designed for three categories: inhibition of cell proliferation, promotion of cell cycle arrest and induction of apoptosis. In the present study, we demonstrated that ETW induces cell cycle arrest and apoptosis in HL-60 cells. Hence, we suggest that ETW is a potent Chinese herb in HL-60 leukemia cells. However, it remains unclear whether ETW effectively induces the elimination of premalignant cells apoptosis *in vivo*.

The effects of ETW on HL-60 cells were associated with a specific disruption of cell cycle events and an induction of G0/G1 arrest. Our results show that ETW led to a loss of cell viability in a dose- and time-dependent manner (Fig. 1). Our study demonstrated that G1 cyclins (cyclin D1 and E) were regulated of HL-60 cells induced by ETW. A recent investigation of leukemia cell differentiation agent-induced differentiation of HL-60 leukemia cells has suggested that TPA to differentiate along the monocyte/macrophage lineage up-regulated of cyclin D1, and all-trans retinoic acid (ATRA) to differentiate along the Granulocyte lineage down-regulated cyclin E expression (Wang et al., 1996; Barrera et al., 2004; Pizzimenti et al., 1999). Thus, it could be suggested that the regulation of cyclin D1 and E as well as CDK2 might anticipate in part the early events in differentiation in ETW-treated HL-60 cells. Our studies found that ETW reduced the level of Bcl-2 and c-Myc in a time-dependent manner. Regulation of the relative levels of Bcl-2 and c-Myc may play an important role in modulating the susceptibility of cells to differentiation (Li et al., 2004; Wu et al., 2002). Previous studies have demonstrated that HL-60 cells exhibited an over-expression of Bcl-2 and c-Myc proto-oncogene and that alteration of cellular oncogenes occur during the differentiation of HL-60 cells (Kumakura et al., 1996). Within myeloid lineage, Bcl-2 is over-expressed in early myeloid precursors but under-expressed or absent in matured myeloid cells and neutrophils (Gazitt et al., 2001; Blagosklonny et al., 1996).

Apoptosis is an evolutionarily conserved process that regulates development and homeostasis, and defects in the mechanisms that regulate cell death are implicated in both tumor genesis and multidrug resistance. Two distinct pathways for apoptosis have been defined, namely the death-receptor pathway and mitochondria pathway (Bohm et al., 2006; Christophe et al., 2006; Lucken et al., 2005; Lockshin et al., 2005). The signal transmitted to the mitochondria pathway causes the release of cytochrome *c* into cytosol. We analyzed apoptosis induction in ETW-treated HL-60 cells by measuring the accumulation of sub-G1 nuclei overtime. We observed the induction of caspase-9 and caspase-8 at 24 h of treatment, and caspase-3 activities at 48 h of treatment before the onset of DNA fragmentation at 72 h treatment by at ETW (Fig. 4). Furthermore, we detected loss of mitochondria membrane potential ($\Delta\Psi$m) in ETW-treated HL-60 cells and release of mitochondrial cytochrome c to cytosol after 18 h of treatment (data not shown). Recent investigation of triptolide-induced apoptosis of U937 cells has suggested that induced caspase-3 activation and down-regulation of the caspase inhibitory protein, XIAP, are involved in this apoptotic process (Choi et al., 2003). Recent reports suggest that DNA damage results in onset of mitochondrial permeability transition, which plays a major role in the apoptotic processes (Choi et al., 2003). A common step in apoptosis involves the loss of mitochondrial membrane potential resulting in increased generation of reactive oxygen species (ROS) from the mitochondrial respiratory chain. Our results suggest that ETW-induced apoptosis is mediated through the loss of mitochondria membrane potential and activation of caspase cascades by activated caspase-9, -8 and caspase-3 in a cytochrome c-dependent manner.

In summary, our results show that ETW induced G0/G1 arrest of HL-60 leukemia cells by regulating the protein expression of cyclin D1, cyclin E, Bcl-2 and c-Myc, and that it induced apoptosis in HL-60 cells by activating caspase-9, caspase-8 and caspase-3. ETW might, therefore, be an alternative cancer therapy in treatment of leukemia patients.

5. References

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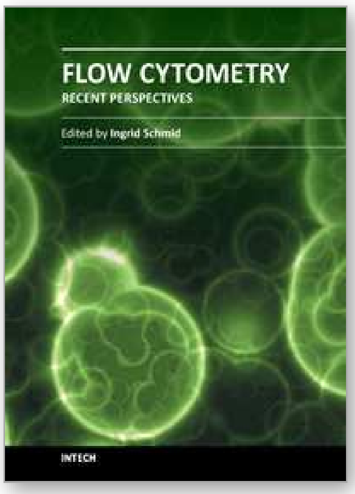

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