

# Inhibition of X-linked inhibitor of apoptosis protein enhances anti-tumor potency of pure total flavonoids on the growth of leukemic cells

LIQIANG WU<sup>1</sup>, XIUXIA ZHANG<sup>2</sup>, XIAOJIE LIN<sup>1</sup>, BO WANG<sup>1</sup>, CHANG HUANG<sup>1</sup>,  
YAO QIN<sup>1</sup> and SHENGYUN LIN<sup>1</sup>

<sup>1</sup>Department of Hematology, First Hospital Affiliated to Zhejiang University of Traditional Chinese Medicine, Hangzhou, Zhejiang 310006; <sup>2</sup>Department of General Surgery, Yuhang First People's Hospital, Hangzhou, Zhejiang 311100, P.R. China

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**Abstract.** Flavonoids, a vast group of polyphenols widely distributed in plants, are known to possess a range of biological activities and potential anti-tumor effects. X-linked inhibitor of apoptosis protein (XIAP) promotes the progression of leukemia by preventing tumor cells undergoing apoptosis. The present study investigated the potential effects and underlying mechanisms of pure total flavonoids from *Citrus paradisi* Macfad (PTFC) on human U937 cells, and explored the effects of short hairpin (sh)RNA-mediated XIAP knockdown on the anti-cancer effects of PTFC. Western blotting was used to determine level of apoptosis-associated effectors following PTFC treatment. A lentiviral vector of RNA interference of XIAP gene was constructed to downregulate XIAP expression. MTT assay and flow cytometry were used to determine the effects of PTFC separately or combined with XIAP-shRNA on inhibition and apoptosis of U937 cells, respectively. Treatment with PTFC effectively inhibited leukemic cell proliferation in a dose- and time-dependent manner. PTFC induced apoptosis of U937 cells in a dose-dependent manner, at a particular concentration range, by decreasing XIAP expression levels and activating caspases-3, -7 and -9. PTFC treatment combined with XIAP-shRNA additionally demonstrated a marked increase in cell apoptosis, compared with PTFC or XIAP-shRNA alone ( $P < 0.05$ ). Therefore, these findings suggest that PTFC inhibits growth and induces apoptosis in U937 cells *in vitro*. Furthermore, suppression of XIAP expression enhances these effects.

## Introduction

Leukemia represents a group of devastating hematopoietic malignancies that are seriously damaging to human health. Because of their diverse clinical and biological features, leukemia can be divided into a number of subtypes, which includes acute myeloid leukemia (AML) (1). AML is the most frequent leukemia affecting adults, its incidence increasing with the age, and continues to have the lowest survival rate among all types of leukemia (2,3). The current first-line treatment of AML is mainly dependent on chemotherapy to kill the malignant cells or induce leukemia cell differentiation (4,5). However, severe adverse side effects, drug resistance and relapse are the major failures in standard therapy (6,7). Thus, it would be much of benefit to research and develop new therapeutic reagents against AML with excellent sensitivity, selectivity and acceptable side-effects (8).

Some natural sources of chemicals or plant extracts often possess anti-tumor properties as well as relatively low toxicity, among them being ones that can control the biochemical function of tumor cells and induce apoptosis (8). Arsenic trioxide ( $As_2O_3$ ), for example, can greatly improve the prognosis of acute promyelocytic leukemia (APL) (9). Defined extracts from tea, such as theaflavin and epigallocatechin gallate (EGCG), have good potential as chemopreventive or anti-cancer agents causing growth inhibition *in vitro* (10,11). Although there are increasing reports on the anti-leukemic effects of plant extracts, alternatives are needed because of the complex nature of this disease as well as the urgent need of new therapeutic drugs. *Citrus paradisi* Macfad is one of the major *Citrus* species cultivated and used in the world (12). It is appreciated for its health-giving benefits and cosmetic properties, which make it popular in daily consumption. Specifically, *Citrus paradisi* Macfad has anti-cancer effects, which may be closely related with its plentiful flavonoid content (13,14). Flavonoids are naturally-occurring non-toxic material found widely in the plant kingdom, most of which have a wide range of biological activities. Exposure to flavonoids seems to reduce the risk for developing cancers (15-21). However, not much is known regarding the anti-cancer effects of pure total flavonoid compounds (PTFCs) extracted from the peel of

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**Correspondence to:** Dr Shengyun Lin, Department of Hematology, First Hospital Affiliated to Zhejiang University of Traditional Chinese Medicine, 54 Youdian Road, Hangzhou, Zhejiang 310006, P.R. China  
E-mail: linshengyun1113@163.com

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*Citrus paradisi* Macfad, and the molecular mechanisms of its action are poorly understood in human cancer cells.

X-linked inhibitor of apoptosis protein (XIAP) is the most important member of the inhibitor of apoptosis proteins (IAP) family in suppressing programmed cell death by physically interacting with and inhibiting the catalytic activity of caspases-3, -7, and -9 with the baculovirus IAP repeat (BIR) domains (22-24). Overexpression of XIAP is common in AML and other cancers (25-28); furthermore, overexpression in AML patients contributes to an unfavorable response to chemotherapy, increased rate of recurrence, and shorter overall survival (25,27), thus suggesting the prognostic and therapeutic potential of XIAP in AML. In this study, therefore, we isolated PTFC, tested its anti-leukemic activity on the AML cell line, U937, and investigated the effects of PTFC plus small hairpin RNA with specificity against XIAP (XIAP-shRNA) on the proliferation inhibition and apoptosis of U937 cells, the object being to get improve our understanding of the anti-tumor potential of PTFC and the unwelcome effects of XIAP.

## Materials and methods

**Preparation of PTFC.** An ultrasonic-assisted enzymatic method was used to extract PTFC from *Citrus paradisi* Macfad peel, as previously described (29,30). Briefly, *Citrus paradisi* Macfad peel was washed with water, dried and powdered. About 50 g was suspended in 500 ml phosphate-buffered saline (PBS) containing 5 g cellulose. After sonication, a part of the suspension was mixed with 500 ml 100% ethanol and filtered through a 0.45- $\mu$ m membrane. The soluble filtrates were dried by vacuum centrifugation before 7.5 g being was dissolved in water and extracted with 200 ml ethylacetate. The top layer was collected and the bottom layer was extracted again with ethylacetate, and the next top layer collected, both being dried by vacuum to recover the PTFC which was stored at 4°C with desiccants.

**Cell culture.** Leukemia cell lines U937 (CRL-3253™), HL60 and K562 were purchased from the American Type Culture Collection (ATCC) and propagated in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA), 1 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. 293T cells were also obtained from the ATCC and cultured in DMEM containing 10% (v/v) FBS, 1 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were incubated at 37°C in an incubator in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

**MTT assay.** The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to determine the effects of PTFC on the proliferation of U937 human leukemic cells. In brief, the U937 cells in logarithmic growth phase were seeded onto 96-well plates at 1x10<sup>4</sup> cells per well with 100  $\mu$ l RPMI 1640 medium containing 10% FBS. After overnight culture, the cells were treated with PTFC at 0.5, 1, 2, 4 and 8  $\mu$ M, before incubation for 24 or 48 h, with controls being given medium without PTFC. Human lymphocytes (1x10<sup>5</sup> cells/well) were similarly treated with PTFC for 24 h. The cells were

collected and treated with 20  $\mu$ l of MTT (0.5 mg/ml, Sigma, St. Louis, USA) and the resulting formazan dissolved in 200  $\mu$ l DMSO. The optical density at 570 nm (A570) was measured using a microplate reader (Berkeley, BIO-RAD, USA). The percentage of viable cells was calculated using the following formula:

$$\text{Survival (\%)} = \text{A570 (sample)} / \text{A570 (control)} \times 100\%.$$

**Western blotting.** After treatment with PTFC for 24 h, suspended cells were harvested and lysed in lysis buffer (Cell Signaling Technology, USA) for estimation of protein, quantified by the bicinchoninic acid (BCA) assay (Sangon Biotech, Shanghai, China). Protein samples (50  $\mu$ g/lane) were resolved on 12% polyacrylamide gel in SDS and transferred electrophoretically onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skimmed milk in Tris buffered saline Tween-20 for 2 h at room temperature and incubated with specific antibodies (1:1,000 dilutions) overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated (HRP) conjugated secondary antibody (1:5,000 dilutions) at room temperature for 2 h. Protein bands were visualized using the Western blotting luminol reagent (Biological Industries, Beit Haemek, Israel).

**Flow cytometric analysis.** The effect of PTFC on apoptosis in U937 cells was measured by flow cytometric analyses. Briefly, the cells were seeded in 6-well plates at 1x10<sup>5</sup> cells per well for 24 h. After treatment in duplicate with different concentrations (0.5-8  $\mu$ M) PTFC for 48 h, the cells were collected, washed with ice-cold PBS and centrifuged. The pellet was fixed in 75% (v/v) ethanol at 4°C for 1 h. Subsequently, the cells were washed once with PBS (pH 7.4) before being stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) using a commercially available apoptosis detection kit (Biouniquer, USA). The stained cells were analyzed in a FACSArca, (BD Sciences, San Jose, USA) apparatus to estimate the percentage of apoptotic cells, data being analyzed with Cellquest 1.2 software (BD Sciences).

**Lentiviral vector construction.** Small interference RNA (siRNA) sequences targeting XIAP and one scramble sequence (5'-TTCTCCGAACGTGTACAGT-3') serving as a negative control were designed, synthesized and inserted into a GV115 vector using the *AgeI/EcoRI* to generate short hairpin RNA (shRNA) expression cassettes-shXIAP and shCon. They were identified after the constructed plasmid DNA had been transformed into competent *Escherichia coli* cells by PCR.

For construction of the lentiviruses, 293T cells (5x10<sup>6</sup> cells/well) were seeded into 10 cm plastic dishes 24 h before DNA transfection. Lentivirus vectors shXIAP and shCon, combined with packaging plasmids (pHelper 1.0 and pHelper 2.0), were co-transfected into sub-confluent cells to produce shRNA lentiviral particles by the calcium phosphate co-precipitation method. At 48 h post-transfection, the supernatants containing retrovirus were collected, concentrated and frozen at -70°C until use. Lentivirus titer was determined by serial dilution with a gradient and the virus transfection to target U937 cells at different multiplicities of infection (MOI). The post-infection efficiency at 72 h was determined

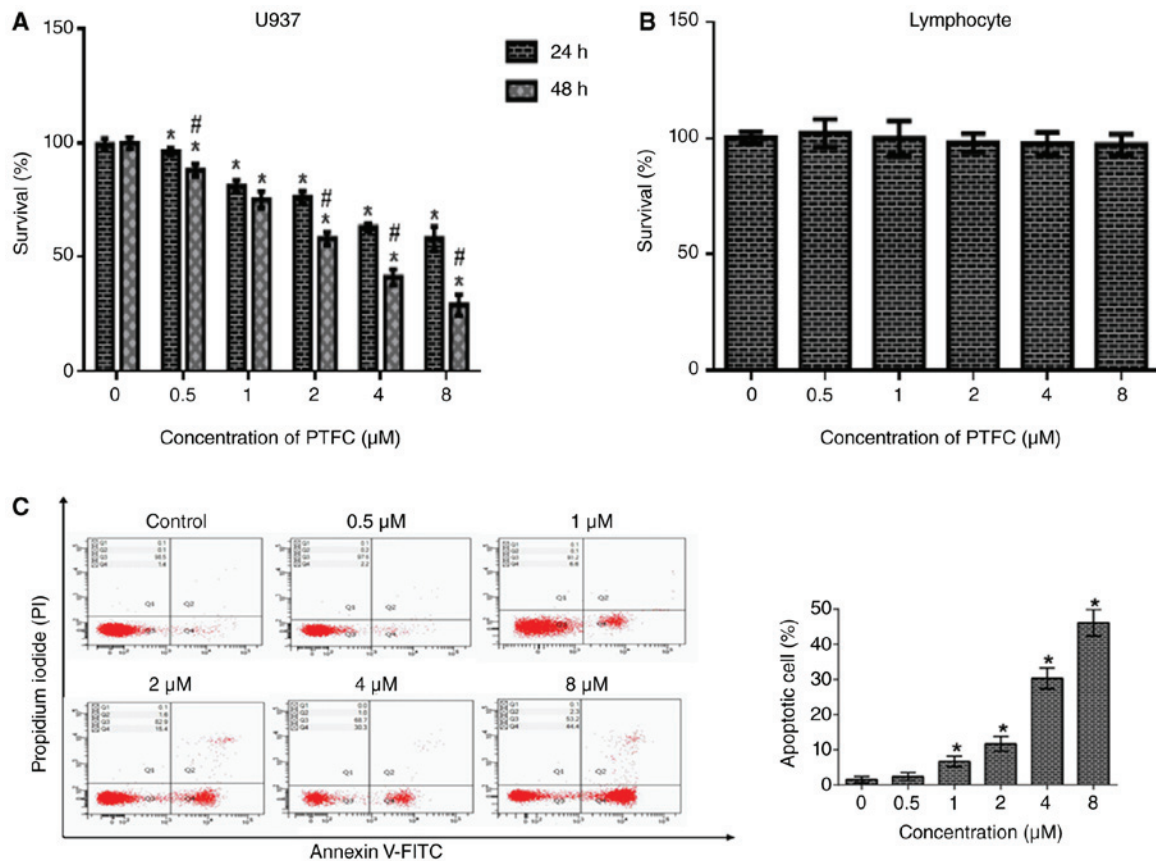


Figure 1. Effect of PTFC on proliferation of U937 cells *in vitro* by MTT. (A) Effect of PTFC treatment on proliferation of U937 cells at 24 or 48 h. (B) Effect of PTFC treatment on proliferation of human lymphocytes at 48 h. \*P<0.05 vs. vehicle-treated control cells; #P<0.05 vs. treatment of PTFC for 24 h. (C) Flow cytometry was used to analyses cell apoptotic treated with PTFC for 48 h in U937 cells. Note: \*P<0.05 vs. vehicle-treated control cells.

by fluorescence microscopy. Quantitative polymerase chain reaction (qPCR) was used to determine the inhibitory efficacy of XIAP mRNA expression in U937 cells. Total RNA was extracted using TRIzol reagent (Invitrogen), 1 μg total RNA being reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen).

**Statistical analysis.** Values are expressed as the mean ± standard deviation (x±s). Statistical significance was determined by the one-way analysis of variance (ANOVA) with post-test Neuman-Keuls for more than 3 groups and Student's t test for 2 groups. Moreover, we used CompuSyn Software (ComboSyn, Inc, Paramus, NJ, USA) to calculated combination index values (CI; CI<1 (synergism); CI=0 (additive effect); CI>1 (antagonism)) according to Chou and Talalay method (31). A P<0.5 was considered statistically significant.

## Results

**The effect of PTFC on the proliferation and apoptosis of U937 cells.** To determine the potential anti-leukemic activity of PTFCs, concentrations ranging from 0.5 to 8 μM of PTFC were tested on the viability of U937 cells. Fig. 1A gave survival rates of U937 cells, which decreased with exposure time to treatment and the dose. Treatment with 0.5 μM PTFC for 24 h significantly inhibited the proliferation of U937 cells compared with the controls (P<0.05). PTFCs in the 48 h treatment group were significant inhibitory at all concentrations

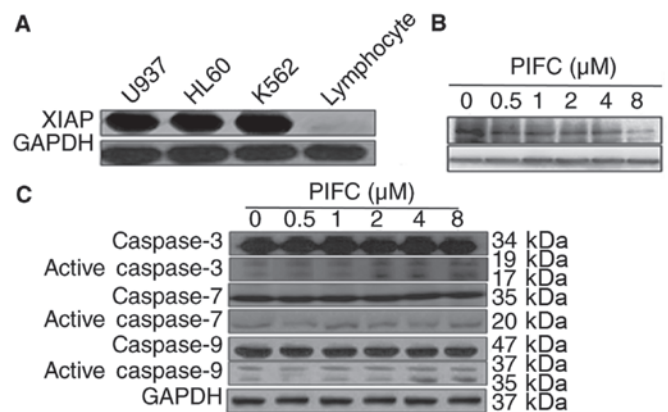


Figure 2. Effects of PTFC on expression levels of apoptosis-related regulators in U937 cells. (A) Expression level of XIAP in different kinds of leukemic cell lines. (B) Effect of PTFC treatment on expression level of XIAP on U937 cells. (C) Effect of PTFC treatment on expression level of caspases on U937 cells.

except 1 μM than in the 24 h treated group (P<0.05). The same PTFC concentrations did not reduce the viability of human lymphocytes *in vitro* (Fig. 1B). These data support the evidence that PTFC can inhibit the proliferation of U937 cell within a specific range of concentrations in a time-dependent manner. Almost immediately, we seeded U937 cells in triplicate into 96-well plates we treated with medium (control group) or PTFC at from 0.5 to 8 μM. After 48 h, apoptotic cells were counted



using flow cytometry, and at all concentrations PTFC induced apoptosis of U937 cells (Fig. 1C). PTFC treatment equal or  $>1 \mu\text{M}$  caused significantly more apoptosis than in the control group ( $P<0.05$ ). The ratio of apoptotic cells increased with the dose of PTFC, particularly in the higher range, therefore U937 apoptosis occurs in a dose-dependent manner.

*PTFC alters protein expression related to apoptosis.* To investigate its effect on the different concentration of XIAP was mediated by the changes of apoptosis proteins in leukemia cells, the expression of XIAP was measured by western blotting in 3 leukemic cell lines, U937, HL60 and K562. XIAP was highly expressed in all 3 tested leukemia cell lines, whereas there was little expression in normal human lymphocytes (Fig. 2A). PTFC treatment decreased XIAP expression in U937 cells in a dose-dependent manner (Fig. 2B). Treatment with PTFC strongly activated caspases-3, -7 and -9 (Fig. 2C). PTFC caused U937 apoptosis seemingly by downregulating the expression of XIAP and activating caspase-3/-7/-9 pathways.

*shXIAP decreases XIAP expression and treatment with PTFC inhibit proliferation and induce apoptosis in U937 cells.* To inhibit XIAP expression in U937 human leukemia cells, shRNA was designed and lentiviruses shXIAP targeting the expression of XIAP and the control lentiviruses shCon were collected finally to determine the titer of lentivirus. Purified recombinant shCon lentivirus gave a value of  $1.5 \times 10^7$  TU/ml, close to that of the shXIAP lentivirus, which was  $1.6 \times 10^7$  TU/ml. U937 cells were infected with the supernatant containing shXIAP lentivirus and XIAP mRNA expression was used to measure the effects of shRNAs on gene silencing after 48 h incubation. A portion of U937 cells was GFP-positive, indicating a successful transfection of shXIAP lentivirus (Fig. 3A). shXIAP transfection efficiency when MOI is 10 or 1 was also determined to find the optimum infection dosage. GFP fluorescence was seen in  $>80\%$  of U937 cells when MOI=10, which was suitable for subsequent experimental groups (Fig. 3B). qPCR and Western Blot showed that shXIAP significantly downregulated XIAP transcripts in U937 cells 48 h after lentivirus transfection (Fig. 3C and D;  $P<0.05$ ).

PTFC, as shown above, can inhibit the proliferation of U937 cells (Fig. 1). Treatment with  $4 \mu\text{M}$  PTFC combined with XIAP-shRNA for 24 h was significantly more inhibitory than either treatment on its own ( $P<0.05$ ; Fig. 3E). Furthermore, according to Chou and Talalay method, we used CompuSyn Software to calculate the synergistically effect of XIAP knockdown and PTFC treatment, the results showed that  $\text{CI}=0.86809<1$ . Similarly, following incubation of U937 cells with XIAP-shRNA plus PTFC for 24 h, the percentage of apoptotic cells was 67.2%, i.e. significantly higher than either PTFC or XIAP-shRNA induced apoptosis ( $P<0.05$ ) (Fig. 3F). Furthermore, western Blot showed that transfected with XIAP shRNA following treatment with PTFC activated caspases-3, -7 and -9 compared with PTFC treatment. Thus, PTFCs and XIAP-shRNA acted synergistically in inhibiting U937 cell proliferation and inducing apoptosis.

## Discussion

There is now evidence of the safety or enhancing anti-cancer activity of extracts or phytochemicals, which is has drawing

extensive interests in the possibility of controlling cancer with maximal efficiency and minimal toxicity. *Citrus paradisi* Macfad, grapefruit, is one of the most common fruit to contain several bioactive compounds, including flavonoids, carotenoids and organic acids, known to be greatly beneficial to human health (32-34). Our study, therefore, was conducted to explore anti-leukemia activity of pure total flavonoid compounds (PTFCs) extracted from the peel of *Citrus paradisi* Macfad on U937 cells and compare the joint effects of PTFC plus XIAP knockdown.

PTFC significantly inhibits the growth of U937 human leukemic cells in a dose- and time-dependent manner. It is known that PTFC is a functional inhibitor of growth in Kasumi-1, HL-60 and K562 cells (29,30). the cell lines derived from AML1/ETO fusion gene-positive AML M2 subtype, AML M2 subtype, and chronic myeloid leukemia (CML), respectively. Collectively, PTFC extracted from *Citrus paradisi* Macfad could have huge potential in inhibiting proliferation of all types of leukemic cell lines and possibly a other tumor cells, with broadly activity *in vivo* and *in vitro*. However, more studies are needed to draw firmer conclusions and verify its broad-spectrum anti-cancer activity. Of note, PTFC even at high dose had no inhibitory effect on human lymphocytes, and therefore seems to be specific in its action as a potential remedy for patients with leukemia.

To understand the mechanisms underlying the function of PTFC in inhibiting U937 cell proliferation, we found that treatment with different doses of PTFC for 24 h induced U937 cell apoptosis in a dose-dependent manner. PTFC treatment also decreased XIAP expression, but increased the expression of cleaved caspases-3, -7 and -9. The caspases are essential for programmed cell death including apoptosis, pyroptosis and necroptosis. Caspase-3 is formed from a 32 kDa zymogen that is cleaved into 17 kDa and 19 kDa subunits when activated in the apoptotic cell by both extrinsic (death ligand) and intrinsic (mitochondrial) pathways. Caspase-7 has been known as an executioner protein of apoptosis. Caspase-9 is an initiator caspase, identified in all mammals for which complete genome data are available. Activated caspase-9 goes on to cleave procaspase-3 and procaspase-7, which subsequently cleave several cellular targets, including poly ADP ribose polymerase (PARP). It is now widely accepted that XIAP is the important inhibitor of apoptosis regulator caspases, especially caspase-3/-7/-9 (23,24). Their activation is often the signal that ensures cellular components are degraded in a controlled manner, leading to cell death with minimal effect on surrounding tissues. The elevated of activated caspases-3, -7 and -9 and the reduced levels of XIAP indicate that PTFC induces caspase-dependent apoptosis of U937 cells by suppressing XIAP. In previous studies, Icariside II sensitizes U937 cells to apoptosis by targeting STAT3-related signaling (35). and flavonoids extracted from *Orostachys japonicus* A. Berger (FEOJ) triggered caspase-dependent apoptosis of U937 cells through p38 MAPK signaling pathway (21). Further investigation focusing on the precise molecular mechanisms by which PTFC trigger U937 cell apoptosis should yield interesting results.

Several cell lines that we used had high expression of XIAP, consistent with previous studies (25,27,28). XIAP belongs to a family of proteins that suppresses apoptosis by inhibiting the

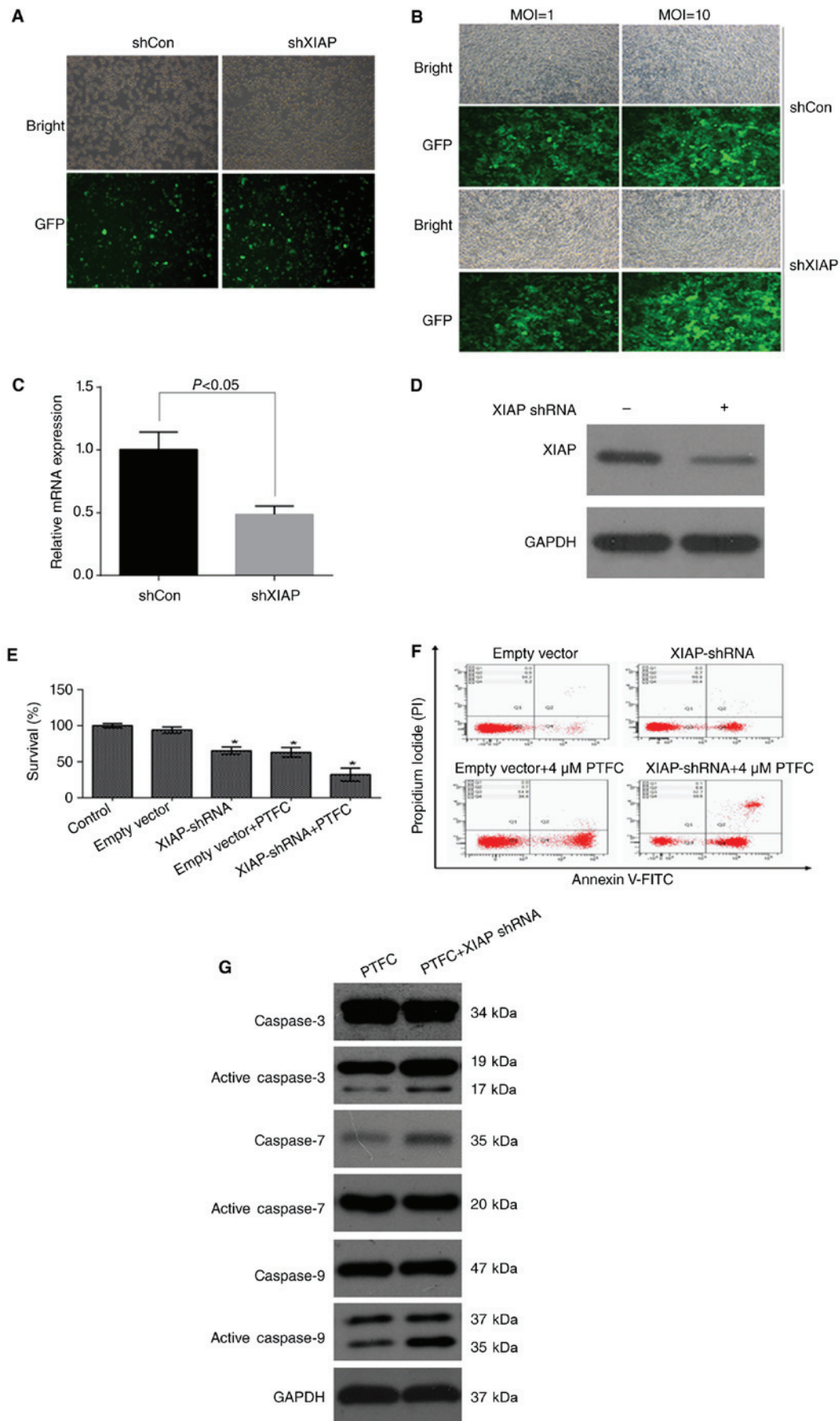


Figure 3. Effect of 4  $\mu$ M PTFC plus XIAP-shRNA on proliferation and apoptosis of U937 cells. (A) GFP was detected in cells to assess the transfection efficiency (magnification, x100). (B) shXIAP transfection efficiency when MOI is 10 or 1 (magnification, x100). (C) Relative mRNA expression of XIAP detected by qPCR. (D) Knockdown efficiency of XIAP was assessed by Western Blot. (E) MTT analyses. (F) Flow Cytometry analyses. \* $P<0.05$  vs. vehicle-treated control cells. (G) Western Blot was used to detect the expression level of caspases treatment with PTFC or XIAP shRNA plus PTFC in U937 cells.

activity of caspases in some cancers, conferring resistance to apoptosis induction by chemotherapeutic agents and leading to shorter survival of cancer patient (25,27,28). This study further indicates that combined treatment with PTFCs plus XIAP-shRNA to knockdown XIAP expression significantly increased apoptosis at several PTFC concentrations, suggesting that XIAP downregulation enhances the susceptibility of U937 cells to PTFC. Downregulation of XIAP by RNA interference (RNAi) is known to sensitize MCF-7 cells to etoposide and doxorubicin (36). XIAP-directed siRNA may also exert a strong sensitizing effect on TRAIL-reduced cell-viability (37). Coincidentally, our findings are consistent with other reports on the increased efficacy of chemical agents on tumor cells in which XIAP is depleted. Overall, XIAP seems to provoke certain injurious effects in U937 cells and other leukemias, and thus may have therapeutic potential clinically.

In brief, treatment with PTFC can inhibit U937 leukemic cell proliferation and induce caspase-dependent apoptosis *in vitro*. XIAP gene knockdown significantly enhances this effect, and therefore, may be intimately involved in PTFC-induced apoptosis of U937 cells. Given their potent anti-leukemic activity, PTFCs may be valuable for the development of new regimens for patients with leukemia, and XIAP knockdown might potentiate chemotherapies of human leukemia as well as other cancers.

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