Pure total flavonoids from *Citrus paradisi* Macfadyen act synergistically with arsenic trioxide in inducing apoptosis of Kasumi-1 leukemia cells *in vitro**

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Abstract: To investigate the potential effects of pure total flavonoid compounds (PTFCs) from *Citrus paradisi* Macfadyen separately or combined with arsenic trioxide on the proliferation of human myeloid leukemia cells and the mechanisms underlying the action of PTFCs. The effects of PTFCs separately or combined with arsenic trioxide on the proliferation and apoptosis of leukemia cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluorescence microscopy, and flow cytometry. Their effects on the expression levels of apoptosis-related regulators were determined by Western blot assay. PTFCs combined with arsenic trioxide significantly inhibited the growth of Kasumi-1 cells, and apoptosis was confirmed by flow cytometry analysis. Hoechst 33258 staining showed more significant morphological changes and more apoptosis following the combined treatment. Western blots showed changes in the expression of genes for poly ADP-ribose polymerase (PARP), caspase 3/9, and P65. The results indicated that PTFCs separately or combined with arsenic trioxide inhibited proliferation of leukemia cells *in vitro* and induced their apoptosis by modulating the expression of apoptosis-related regulator genes.

Key words: Pure total flavonoid compounds, Human myeloid leukemia cells, Growth inhibition, Synergistic effect, Apoptosis

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1 Introduction

Leukemia is a hematological malignancy that seriously harms human health. Currently, treatment of leukemia is dependent on chemotherapies to kill malignant cells or to induce leukemia cell differentiation (Piccaluga *et al.*, 2006; Krause and Crispino, 2013). However, the available chemotherapies usually have severe adverse side effects and are difficult to tolerate

(Goldman and Melo, 2003; Schlenk *et al.*, 2008). Therefore, the discovery and development of new therapeutic reagents with a safe profile would be of great significance (Parekh *et al.*, 2009).

Previous studies have demonstrated that the dietary intake of flavonoids is closely associated with a reduced risk for the development of cancer (Middleton *et al.*, 2000; Havsteen, 2002). Flavonoids extracted from different plants have several kinds of active components and there have been many studies of their anti-cancer effects (Ranelletti *et al.*, 2000; Salucci *et al.*, 2002; Pan *et al.*, 2005; Leung *et al.*, 2007). We found that the pure total flavonoid compounds (PTFCs) extracted from the peel of *Citrus paradisi* Macfadyen can inhibit the growth of

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leukemia cells *in vitro*, but the mechanisms of this effect are unclear.

Arsenic trioxide is used in the treatment of leukemia and other cancers. It is a promising drug for patients with relapsed acute promyelocytic leukemia (APL), but its clinical efficacy is burdened by its serious cardiac toxicity (Mathews *et al.*, 2013). In this study, we tested the separate and combined effects of PTFCs and arsenic trioxide on leukemia cell proliferation activity and the potential mechanisms underlying their anti-leukemia activity *in vitro*. We found that combining the two kinds of drugs produced a synergistic effect. Both inhibited leukemia cell proliferation and triggered leukemia cell apoptosis. These effects were associated with activation of the caspase-apoptotic pathway. Our findings may provide new insights into understanding the regulation of leukemia cell proliferation by flavonoids and help in the design of new therapies for combined chemotherapeutic regimens in the treatment of leukemia.

2 Materials and methods

2.1 Materials

2.1.1 Preparation of PTFC

The *C. paradisi* Macfad. peel was washed with water, dried, and powdered. The *C. paradisi* Macfad. peel powder (50 g) was re-suspended in 500 ml of phosphate-buffered saline (PBS) containing 5 g cellulose, and sonicated. Subsequently, one portion of the *C. paradisi* Macfad. peel suspension was mixed with 500 ml of 100% ethanol and filtrated through a 0.45- μ m filter. The soluble filtrates were dried by vacuum centrifugation. The filtrates (7.50 g) were dissolved in water and extracted with 200 ml of ethyl acetate. The top layer was collected and the bottom layer was further extracted with ethyl acetate, followed by collecting the top layer. The collected top layers were dried by vacuum to yield the PTFC.

2.1.2 Cell culture

Human acute myeloid leukemia cells (Kasumi-1) were purchased from the American Type Culture Collection (ATCC). The cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS; Invitrogen, Carlsbad, USA) at 37 °C in a humidified of 5% CO₂ atmosphere.

2.2 Methods

2.2.1 MTT

The impact of PTFCs on the proliferation of leukemia cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, Kasumi-1 cells (2×10^4 cells/well) were cultured in 10% FCS RPMI 1640 medium on 96-well plates overnight, then treated with different PTFC concentrations (0.125–2.000 mg/ml) for 24 or 48 h. Kasumi-1 cells (2×10^4 cells/well) were then treated with 0.250–2.000 mg/ml PTFC and/or 1–8 μ mol/L arsenic trioxide (SL Pharm, Beijing, China) for 24 h. Human lymphocytes (1×10^5 cells/well) were treated with different concentrations (0.125–2.000 mg/ml) of PTFC for 24 h as a control. During the last 4-h incubation, the cells were exposed to 20 μ l of MTT (0.5 mg/ml, Sigma, St. Louis, USA) and the resulting formazan was dissolved in 200 μ l DMSO. Absorbance at 570 nm (A_{570}) was recorded using a microplate reader (Berkeley, BIO-RAD, USA). The percentage of viable cells was calculated using the following formula: survival (%) = A_{570} (sample)/ A_{570} (control) $\times 100\%$.

2.2.2 Hoechst 33258 staining

Kasumi-1 cells treated with PTFCs and/or arsenic trioxide were harvested and washed twice with PBS. The cells were fixed in 4% (0.04 g/ml) para-formaldehyde at 37 °C for 30 min and treated with 0.5% (0.005 g/ml) Triton X-100 in PBS for 30 min at room temperature. After being washed, the cells were stained with 1 μ g/ml of Hoechst 33258 (Sigma-Aldrich, St. Louis, USA) for 20 min. A total of 2000 cells from each group were counted and the percentages of apoptotic cells determined using a fluorescence microscope (BX51T- 32H01, Olympus, Japan).

2.2.3 Flow cytometry analysis

The impact of PTFCs on leukemia cell apoptosis was determined by flow cytometry analysis. Briefly, Kasumi-1 cells (1×10^5 cells/ml) were cultured in 10% FCS RPMI 1640 medium on 6-well plates overnight and treated in duplicate with 1 mg/ml of PTFCs and/or 4 μ mol/L arsenic trioxide for 24 h. The cells were washed with ice-cooled PBS and stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) using an apoptosis detection kit,

according to the manufacturer's instructions (Biouniquer, USA). The percentages of apoptotic cells were determined using a flow cytometer (FACSArica, BD Biosciences, San Jose, CA, USA) and the data were analyzed using CellQuest 1.2 software (BD Biosciences).

2.2.4 Western blotting analysis

After treatment with PTFCs and/or arsenic trioxide for 24 h, the cells were harvested and lysed in lysis buffer (Cell Signaling, MA, USA). The concentrations of proteins in cell lysates were determined by bicinchoninic acid (BCA) assay (Sangon Biotech, Shanghai, China). The cell lysates (50 μ g/lane) were separated by 12% (0.12 g/ml) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST) for 2 h and incubated with primary antibodies (1:1000 dilutions, Cell Signaling Technology, USA) overnight at 4 °C. After being washed, the bound antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:5000 dilutions) at room temperature for 2 h and visualized using the Western Blotting Luminol

Reagent (Biological Industries, Beit Haemek, Israel). The relative levels of target protein to the control β -actin were determined by densitometric analysis.

3 Results

3.1 PTFCs separately or combined with arsenic trioxide inhibit the proliferation of human leukemia cells *in vitro*

To determine the potential anti-leukemia activity of PTFCs, we tested the impact of different concentrations on the viability of Kasumi-1 cells. We found that treatment with PTFCs at 0.125–2.000 mg/ml significantly inhibited the proliferation of Kasumi-1 cells *in vitro* (Fig. 1a). Treatment with 0.250–2.000 mg/ml PTFCs and/or 1–8 μ mol/L arsenic trioxide for 24 h, showed that the inhibitory effects of PTFCs combined with arsenic trioxide were significantly greater than the effects of either treatment on its own. Thus, PTFCs and arsenic trioxide had a synergistic effect on inhibiting Kasumi-1 proliferation *in vitro* (Figs. 1b and 1c). We found that the same concentration of PTFCs has no inhibitory effect on human lymphocytes *in vitro* (Fig. 1d).

Fig. 1 Effect of PTFC and/or arsenic trioxide on leukemia cell proliferation *in vitro*

(a) Effect of 0.125–2.000 mg/ml of PTFC on Kasumi-1 cells after 24 or 48 h. (b) Effect of PTFC and/or arsenic trioxide on Kasumi-1 cell proliferation after 24 h. (c) Synergistic killing effect of PTFCs and arsenic trioxide was analyzed using CalcuSyn 2.0 software and expressed as lgCI (combine index) versus fraction affected. When CI<1 was calculated, PTFC and arsenic trioxide then had synergistic effect. (d) Effect of PTFCs on human lymphocytes after 24 h

3.2 PTFCs separately or combined with arsenic trioxide trigger Kasumi-1 cell apoptosis

To understand the mechanism underlying the action of PTFCs separately or combined with arsenic trioxide in inhibiting leukemia cell proliferation, we examined whether treatment with PTFCs and/or arsenic trioxide could trigger leukemia cell apoptosis *in vitro*. Kasumi-1 cells were treated with 1.000 mg/ml PTFCs and/or 4 μ mol/L arsenic trioxide for 24 h and stained with Hoechst 33258, followed by examination under a fluorescence microscope. The numbers of apoptotic cells following the combined treatment were significantly greater than those following the separate treatments (Fig. 2a). In parallel, Kasumi-1 cells were treated with 1.000 mg/ml PTFCs and/or 4 μ mol/L arsenic trioxide for 24 h, and the apoptotic cells were characterized by flow cytometry analysis (Fig. 2b).

Fig. 2 Effect of PTFCs and/or arsenic trioxide on the Kasumi-1 cell apoptosis

PTFCs and/or arsenic trioxide induce Kasumi-1 cell apoptosis *in vitro*. Kasumi-1 cells were treated with the indicated concentrations of PTFCs and/or arsenic trioxide for 24 h and the cells were stained with Hoechst 33258 or FITC-Annexin V/PI. The apoptotic cells were characterized by fluorescence microscopy or flow cytometry analysis. (a) Fluorescent analysis of apoptotic cells; (b) Flow cytometry analysis of apoptotic cells

3.3 PTFCs separately or combined with arsenic trioxide induce apoptosis in leukemia cells and alter protein expression level related to apoptosis

Further analysis revealed that treatment with PTFCs and/or arsenic trioxide greatly activated poly ADP-ribose polymerase (PARP), caspase 3, and caspase 9 in Kasumi-1 cells (Fig. 3). In addition, the treatments inhibited P65 expression in Kasumi-1 cells. Apparently, PTFCs separately or combined with arsenic trioxide triggered Kasumi-1 cell apoptosis by activating the PARP and caspase 3/9 pathways.

Fig. 3 Effects of PTFCs and/or arsenic trioxide on the expression levels of apoptosis-related regulators in Kasumi-1 cells

After treatment with PTFCs and/or arsenic trioxide for 24 h, the cell lysates were subjected to Western blotting analysis

4 Discussion

C. paradisi Macfadyen, also known as grapefruits, contains several phytochemicals such as flavonoids, carotenoids, limonoids, organic acids, pectin, and folate, which have been known to benefit human health (Girennavar *et al.*, 2008; Patil *et al.*, 2009). We pioneered the extraction of the new compounds (PTFCs) from the peel of *C. paradisi* through high performance liquid chromatography (HPLC), and found that PTFCs can inhibit the growth of leukemia cells *in vitro*.

We studied the biological function of PTFCs and found that treatment with PTFCs inhibited the proliferation of Kasumi-1 cells in a dose- and time-dependent manner. There was also a synergistic effect between PTFCs and arsenic trioxide in inhibiting leukemia cell proliferation. Moreover, PTFCs had no inhibitory effect on human lymphocytes at high dose. Conceivably, combination medication may be a valuable strategy for the development of new therapies for patients with leukemia.

To understand the potential mechanisms underlying the action of PTFCs separately or combined with arsenic trioxide in inhibiting leukemia cell proliferation, we tested whether treatment with PTFCs and/or arsenic trioxide could trigger leukemia cell apoptosis. Further characterization indicated that treatment with PTFCs and/or arsenic trioxide increased the relative levels of activated PARP, cleaved caspase-3, and cleaved caspase-9 expression in Kasumi-1 cells, and the effect of the combined treatment was significantly greater than that of each separate treatment alone. The PARPs participate in DNA repair and respond to cell stress by activation. The activated PARP can bind to single strand DNA breaks and form poly(ADP-ribose) (PAR), which can stimulate mitochondria to release apoptosis-inducing factor (AIF) and activate caspase 9 and caspase 3, leading to cell apoptosis (Crowder and El-Deiry, 2012). Activation of transcription factor nuclear factor- κ B (NF- κ B) has been shown to play a role in cell proliferation, apoptosis, cytokine production, and oncogenesis (Yu *et al.*, 2004), and it acts as an anti-apoptotic protein (Ghosh *et al.*, 1998). P65 is a member of the NF- κ B/Rel protein family, and is over-expressed in a variety of cancers (Balcerczak *et al.*, 2002; Yu *et al.*, 2004). P65/P50 is one of the most important dimers commonly found in cytoplasm and takes part in regulating the expression of a series of anti-apoptosis genes. It is considered a key component of the regulation of NF- κ B activity. P65 is also the co-enzyme substrate of NF- κ B transcriptional regulation kinases, such as protein kinase A (PKA), mitogen- and stress-activated protein kinase 1 (MSK1), and MSK2. Its phosphorylation and acetylation are associated with transcriptional activity. P65 is up-regulated in malignant tumors like breast cancer, stomach cancer as well as leukaemia. With the progress of leukemia, the expression of P65 will increase and *vice versa* (Guzman *et al.*, 2001). P65 is an anti-apoptosis protein (Ni *et al.*, 2001) that can promote cell proliferation to a certain extent. It is involved in the inhibitor of NF- κ B (I κ B)-dependent serine (Ser) phosphorylation pathway. The various stimuli that activate NF- κ B cause phosphorylation of I κ B, which is followed by its ubiquitination and subsequent degradation (Hatta *et al.*, 2003). P65/P50 enters into the nucleus by binding to specific DNA sequences, thus regulating gene transcription (Bueso-Ramos *et al.*,

2004). In our study, PTFCs and arsenic trioxide each down-regulated the expression of P65, and in combination this effect was significantly increased. The elevated levels of activated PARP, caspase 3, and caspase 9 and the reduced levels of P65 in the PTFCs and/or arsenic trioxide-treated leukemia cells suggest that PTFCs separately or combined with arsenic trioxide may induce cell stress and leukemia cell apoptosis by activating the PARP and caspase pathways, together with down-regulation of P65 expression. We are interested in further investigating the precise molecular mechanisms by which PTFCs combined with arsenic trioxide trigger leukemia cell apoptosis.

In summary, our study indicated that treatment with PTFCs separately or combined with arsenic trioxide inhibited leukemia cell proliferation, and the effect of the combined treatment was significantly greater than that of each treatment on its own. The effect was associated with activation of the PARP and caspase pathways and down-regulation of P65 expression in leukemia cells. The results indicated that PTFCs and arsenic trioxide have a synergistic effect in inhibiting Kasumi-1 cell proliferation *in vitro*. Considering their potent anti-leukemic activity, PTFCs may be valuable for the development of new therapies for patients with leukemia.

Compliance with ethics guidelines

Bo WANG, Sheng-yun LIN, Ying-ying SHEN, Li-qiang WU, Zhen-zhen CHEN, Jing LI, Zhi CHEN, Wen-bin QIAN, and Jian-ping JIANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题 目：葡萄柚黄酮协同三氧化二砷诱导白血病细胞 Kasumi-1 凋亡的体外实验研究

目 的：初步探索葡萄柚黄酮 (PTFCs) 联合三氧化二砷 (As_2O_3) 协同诱导白血病 Kasumi-1 细胞株凋亡及凋亡相关机制。

创新点：采用生物酶法与超声波辅助提取技术结合的方法从天然植物葡萄柚中提取和纯化出黄酮组分已取得国家发明型专利，发现其具有明显的抑制白血病细胞增殖作用，并且与 As_2O_3 存在协同作用。

方 法：通过 MTT 法验证其抑制 Kasumi-1 细胞增殖作用，通过流式细胞术、Hoechst 33258 染色、蛋白质印迹 (Western blot) 等技术探索其相关作用机制。

结 论：发现 PTFCs 可以协同 As_2O_3 诱导 Kasumi-1 细胞凋亡。

关键词：葡萄柚黄酮；白血病细胞；抑制增殖；协同作用；凋亡