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5-Acetyl-6,7,8,4′-tetramethylnortangeretin induces apoptosis in multiple myeloma U266 cells

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Abstract

Multiple myeloma (MM) is one of the most common hematological malignancies and to date, it remains an incurable disease. In this study, we evaluated the inhibitory effect of 5-acetyloxy-6,7,8,4′-tetramethoxyflavone (5-ATMF), a compound from aged citrus peel extracts, on the MM U266 cell line. We found that the compound inhibited cell growth and induced cell apoptosis in multiple apoptotic assays. The apoptotic proteins caspase-3, caspase-9, and PARP (poly ADP-ribose polymerase) were cleaved when cells were treated with 5-ATMF. Along with the apoptosis process, the anti-apoptotic protein, Bcl-2 (B-cell lymphoma-2), was significantly downregulated and the pro-apoptotic protein, Bax (Bcl-2 associated X protein), upregulated along with the release of cytochrome C (Cyt c) and the reduction of mitochondrial membrane potential (MMP). Notably, we found that the phosphorylation of Bad (Bcl-2/Bcl-X_L-associated death promoter) was decreased but Bad remained unchanged in this process. On adding 5-ATMF to U266 cells, we observed that 5-ATMF repressed the phosphorylation of Akt (PKB Protein Kinase B PKB), thereby increasing the release of Cyt c and inhibiting the phosphorylation of Bad. These effects were enhanced by Ly294002, an inhibitor of Akt. These results suggested that 5-ATMF exerts a pro-apoptotic effect on U266 cells, possibly associated with the mitochondrial apoptotic pathway induced by the PI3K/Akt/Bad pathway.

Keywords: Citrus peel extract; Polymethoxylated flavones; 5-Acetyl-6,7,8,4′-tetramethylnortangeretin; Multiple myeloma; Apoptosis

1. Introduction

Multiple myeloma (MM) is a terminally differentiated B-cell malignant tumor characterized by malignant plasma infiltrated into the bone marrow combined with high levels of monoclonal protein in the blood or urine [1]. The majority of patients with multiple myeloma are older than 50, with most diagnosed in their mid-60s. Application of proteasome inhibitors and/or hematopoietic stem cell transplantation has prolonged, to some extent, the overall survival and the progression-free survival of patients. However, the disease is still thought to be incurable because of multidrug resistance and disease progression [2].

Concerning pathogenesis, constitutive activation of Akt has been found to occur frequently in both primary MM specimens and cell lines [3]. The Akt signaling pathway acts as an important regulator of cell apoptosis by modulating downstream factor Bad [4]. Furthermore, unlike the genomes of most hematological malignancies, MM genomes are similar to those of solid-tissue neoplasms and typified by mutations in oncogenes and

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tumor-suppressor genes as well as by chromosomal aberrations, some of which have been closely linked to disease pathogenesis [5].

It is well documented that flavonoid chemicals from fruits and vegetables can decrease the risk of various solid tumors [6]. Citrus is one of the most common sources of dietary flavonoids [7]. Polymethoxylated flavones (PMFs) are found specifically in citrus peels and have been reported to have a preventive effect against solid tumors [8]. Among PMFs, tangeretin is most often reported to modulate inflammation and enhance the chemosensitivity of cancer cells. The potential effects of tangeretin on some types of cancer cells are uncertain, although tangeretin can induce G1 cell cycle arrest [9,10]. Recently, compounds with modified groups, such as hydroxylation, glycosylation, or acetylation at the C5 position of tangeretin, have shown strong activity against solid cancers [11]. In nature, this modification takes place in aged citrus repeated heating and drying. In the present study, we used a novel PMF compound derived from acetylation of tangeretin, 5-acetyloxy-6,7,8,4′-tetramethoxyflavone (5-ATMF) to investigate whether this chemical has an inhibitory effect on a multiple-myeloma U266 cell line.

2. Materials and methods

2.1. Preparation and identification of 5-ATAN

5-ATAN was prepared as previously described [11]. Briefly, TAN was isolated from the peel of sweet orange (*Citrus sinensis*). 5-ATAN was obtained by selecting the 5-demethylation of tangeretin. The fractions of 5-ATAN solution were combined and the solvent removed to dryness to yield 5-ATAN as a pale yellow solid. The structure of 5-ATAN was confirmed by MS and proton nuclear magnetic resonance (^{1}H NMR).

2.2. Cell culture

The U266 cell line was provided by Dr. Zhibo Han, Institute of Hematology, Peking Union Medical College. Cells were incubated in 75 cm^2 flasks containing RPMI-1640 media (Thermo Fisher, Beijing, China) supplemented with 15% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified incubator containing 5% CO_2 at 37 °C. 5-ATAN and TAN were dissolved in dimethyl sulfoxide (DMSO, Solarbio Science and Technology, Beijing, China).

2.3. Detection of growth of U266 with MTT assay

Cell viability was measured with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT, Sigma, M5655, USA) according to Yang's method [12]. Briefly, 100 μL of cell suspension (5×10^3 cells/well) was seeded into 96-well plates and pretreated with a series of concentrations of 5-ATAN and TAN for 24 h. At the end of the treatment, 20 μL of MTT (5 mg/mL) was added to each well and the cells were incubated in a humidified incubator containing 5% CO_2 at 37 °C for 4 h. Cell growth was determined by scanning with an enzyme-linked immunosorbent assay reader with a 495-nm filter (Synergy HT; Bio-Tek Instruments). Inhibition rate = 1 − (experimental value − blank value)/(negative control value − blank value).

2.4. Apoptosis analysis by flow cytometry and cellular DNA flow and flow cytometry assay for Annexin V/PI and mitochondrial membrane potential (MMP)

Apoptosis detection was performed according to the manufacturer BD Annexin V-FITC/PI step (BD, New Jersey, USA). The cells were seeded in 6-wells plate (1×10^6 cells/well) and treated with different concentrations (2.5 μmol/L, 12.5 μmol/L, 25 μmol/L) of 5-ATAN at 37 °C for 24 h. Then, cells were harvested, washed twice with ice-cold PBS, and resuspended in 1 mL binding buffer. Resuspended cells (100 μL) were transferred to a 1.5 mL EP tube and 5 μL Annexin V-FITC plus 5 μL PI were added. The tube was gently vortexed and incubated for 15 min at room temperature in the dark. Binding buffer (400 μL) was then added and the cells were analyzed immediately by flow cytometry.

2.5. Western blotting analysis

Mitochondria and cytosolic fractions were separated using the Mitochondria/Cytosol fractionation kit according to the manufacturer's protocol (#89874, Pierce, Rockford, USA). Protein was extracted from U266 cells prepared with ice-cold lysis buffer (P1016, Solarbio Science and Technology, Beijing, China). Protein concentration in cell lysates was determined with a BCA protein kit (Beyotime, Shanghai, China). Samples containing equal amounts of protein (20 μg) were mixed with loading buffer with 5% 2-mercaptoethanol, heated for 5 min at 95 °C, and loaded onto a 10% SDS-PAGE gel and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% milk and 0.1% Tween 20 in Tris-buffered saline (TBS), membranes were incubated overnight at 4 °C with primary antibody (Bcl-2 (BM0200) was purchased from Boster (Wuhan, China); Akt (CS9272), P-Akt (CS9271), caspase-3 (CS9662), PARP (CS9542), caspase-8 (CS4790), caspase-9 (CS9502), Bad (CS9239) and p-Bad (CS4366) were purchased from Cell Signaling Technology (Boston, USA); cytochrome C (ab90529) and Bax (ab7977) were purchased from Abcam (Cambridge, UK); Cox IV (AC610) was purchased from Beyotime (Shanghai, China); β-Tubulin (CW0098) and GAPDH (CW0100) were purchased from CWBIO (Beijing, China)), and then incubated with the appropriate horseradish peroxide-conjugated secondary antibody at room temperature. Protein bands were detected using ECL reagents (WBKLS0100, Millipore, Billerica, MA, USA) according to the manufacturer's protocols. Intensity of the desired band was evaluated with Gel-Pro software. Experiments were repeated three times.

2.6. Mitochondrial membrane potential assay

Mitochondrial membrane potential was determined according to the specific JC-1 fluorescence dye kit's manufacturer instructions (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; JC-1; C2006,

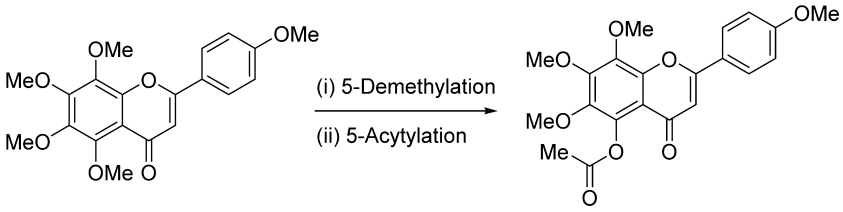

Fig. 1. Structure of 5-ATAN or TAN. 5-acetyl-6,7,8,4′-tetramethylnortangeretin (5-ATAN), which is replacing the methyl groups of tangeretin with acetyl groups at the C5 position of tangeretin.

Beyotime, Shanghai, China). Briefly, U266 cells were grown in 6-well plates and treated with a series of concentrations of 5-ATAN. After 24 h of treatment, the cells were stained with 1 mL JC-1 and gently pipetted, then placed in a CO_2 incubator for 20 min. After centrifuging at 600 × *g* at 4 °C for 3–4 min, the supernatant was discarded, and the residue washed twice with JC-1 staining buffer. Cells were resuspended immediately and subjected to flow cytometry. Mitochondrial stability was assessed by fluorescence microscopy. The green JC-1 signals were measured at 470/22 nm excitation and 510/42 nm emission and the red signals at 531/40 nm excitation and 593/40 nm emission. The experiment was repeated three times.

2.7. Statistical analysis

Data were expressed as mean ± SEM and analyzed by one-way analysis of variance. $P < 0.05$ was considered statistically significant. Data were means ± SD of three to five independent experiments.

3. Results and analysis

3.1. Effect of 5-ATAN and TAN on cell viability of U266 cells

The chemical structures of both tangeretin and 5-ATAN and TAN are shown in Fig. 1. An HPLC fingerprint chromatogram was established for the quality control of 5-ATAN, and the purity of 5-ATAN was 99.3%. To investigate the *in vitro* cytotoxity of these compounds against U266 cells, a MTT assay was conducted. The extent of cell viability was determined after

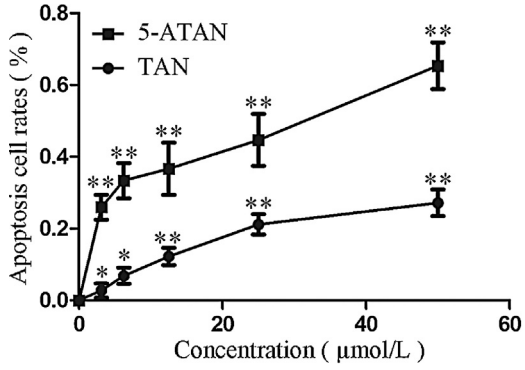

Fig. 2. Cell proliferation was analyzed by using MTT assay. Cell was treated with different dose of TAN (0, 3.125, 6.25, 12.5, 25, 50 μmol/L) or 5-ATAN (0, 3.125, 6.25, 12.5, 6.25, 25, 50 μmol/L). Results represent the mean ± SD from three independent experiments (* means compared with the control group, $P < 0.05$, ** means compared with the control group, $P < 0.0001$, $n = 5$).

treatment with different concentration of compounds for 24 h. As shown in Fig. 2, the 50% inhibitory concentration (IC50) values of 5-ATAN and TAN were 20.9 μmol/L and 46.9 μmol/L, respectively. The 5-ATAN treatment led to more potent inhibition of growth than that of TAN. Accordingly, in most of the subsequent experiments, we used 5-ATAN at a concentration of 25 μmol/L.

3.2. Apoptotic effect of 5-ATAN on U266

The previous assay suggested that 5-ATAN can inhibit the growth of U266 cells. Next we investigated whether apoptosis was associated with this growth inhibition. After treating U266 cells with 5-ATAN and TAN at 0 μm, 2.5 μm, 12.5 μm,

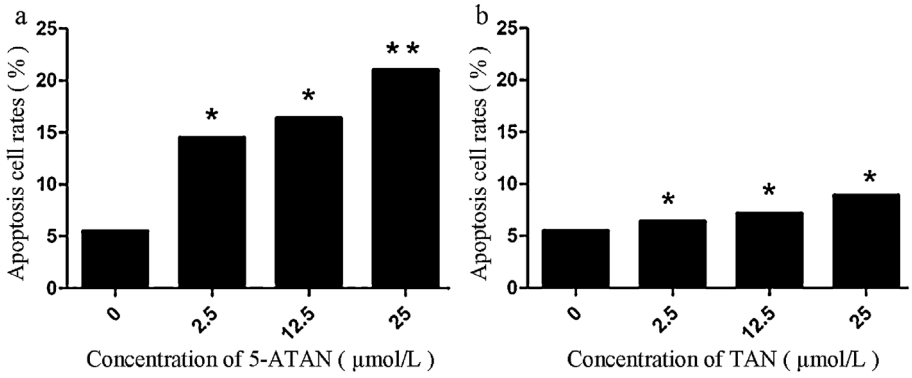

Fig. 3. Cell pro-apoptosis was analyzed by using flow (Annexin V-FITC/PI). Cell was treated with different dose of TAN (0, 2.5, 12.5, 25 μmol/L) or 5-ATAN (0, 2.5, 12.5, 25 μmol/L). Results represent the mean ± SD from three independent experiments (* means compared with the control group, $P < 0.05$, ** means compared with the control group, $P < 0.001$, $n = 3$).

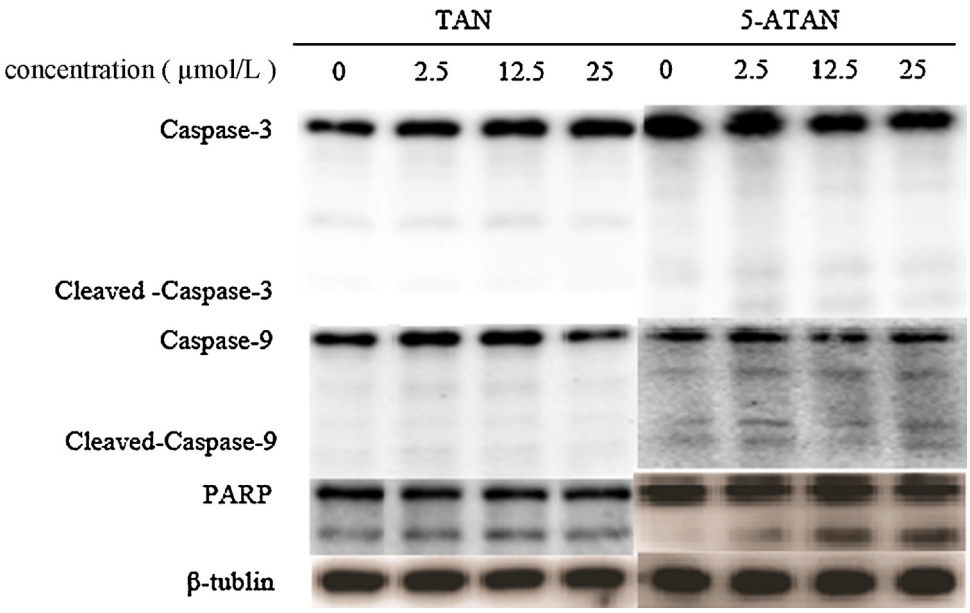

Fig. 4. Caspase-3 and caspase-9 were activated by 5-ATAN in a concentration dependent manner, 5-ATAN induced PARP cleavage in a concentration dependent manner; but did not occur by TAN.

or 25 μm for 24 h, we stained the cells with Annexin V-FITC/PI and subjected them to flow cytometry for apoptosis analysis. The apoptotic effects of U266 treated with 5-ATAN were 5.5%, 14.5%, 16.4%, and 21.0% (Fig. 3a) and those of the TAN group were 5.5%, 6.4%, 7.6%, and 8.9% (Fig. 3b). We found a clear dose-dependent increase of apoptotic rate in U266 cells. To corroborate this apoptotic effect, we analyzed the activation of caspase-3, caspase-9, and cleaved PARP. 5-ATAN showed a greater effect on U266 cells than TAN (Fig. 4). Taken together, these results indicated that growth inhibition of U266 cells by 5-ATAN is associated with apoptosis.

3.3. Effect of 5-ATAN on mitochondrial membrane potential

Mitochondrial involvement in apoptosis includes two crucial events: change in mitochondrial membrane potential (MMP) and release of cytochrome C (Cyt c), which is normally stored in the intramembrane space [13,14]. Given that 5-ATAN induced apoptosis in U266 cells, we hypothesized that mitochondria were affected. We evaluated the effect of 5-ATAN on mitochondrial membrane potential by JC-1 dye staining. The reductions of depolarized mitochondrial membranes in U266 at 0 μmol/L, 2.5 μmol/L, 12.5 μmol/L, and 25 μmol/L were 5%, 11.8%, 17%, and 27.1% (Fig. 5), showing that mitochondrial membrane potential increased as 5-ATAN concentration gradually decreased. Moreover, Western blotting assays showed that 5-ATAN dramatically increased the level of Cyt c in both dose- and time-dependent manners (Fig. 6). These results revealed that 5-ATAN-induced cell apoptosis involved mitochondria.

3.4. Mitochondrial-signaling downstream proteins induced by 5-ATAN

Based on the previous results, we further investigated the association of 5-ATAN in U266 cells with mitochondrial signaling pathway downstream proteins (Bcl-2, Bax, Bad) [15,16] by Western blotting. With the increasing concentration and prolonging effect time, anti-apoptotic Bcl-2 protein expression decreased and that of pro-apoptotic protein Bax increased gradually with concentration of 5-ATAN and time. Moreover, we further analyzed Bad and its phosphorylation [17]. The expression of P-Bad decreased, whereas there was no change with Bad in U266 cells (Fig. 7). Taken together, these results suggested that 5-ATAN-induced apoptosis of U266 cells involved the mitochondria-associated apoptotic pathway.

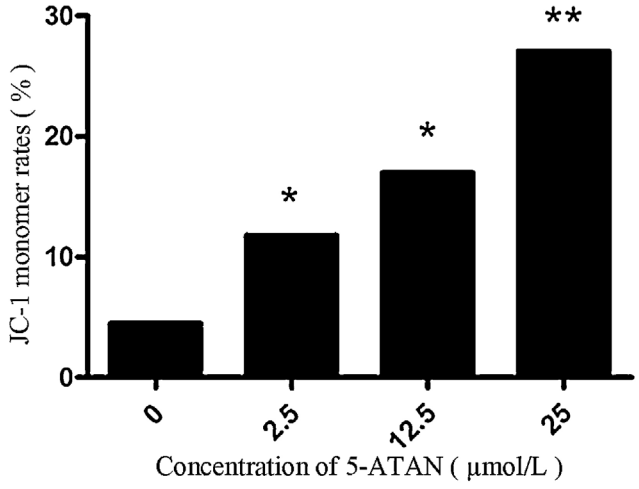

Fig. 5. Mitochondrial membrane potential detection indicates cell apoptosis. Treatment with 5-ATAN resulted in reduction of the aggregation of JC-1 dye in U266 cells [JC-1 dye was analyzed by using flow (Annexin V-FITC/PI). Cell was treated with different dose of 5-ATAN (0, 2.5, 12.5, 25 μmol/L). Results represent the mean ± SD from three independent experiments] (* means compared with the control group, $P < 0.05$, ** means compared with the control group, $P < 0.001$, $n = 3$).

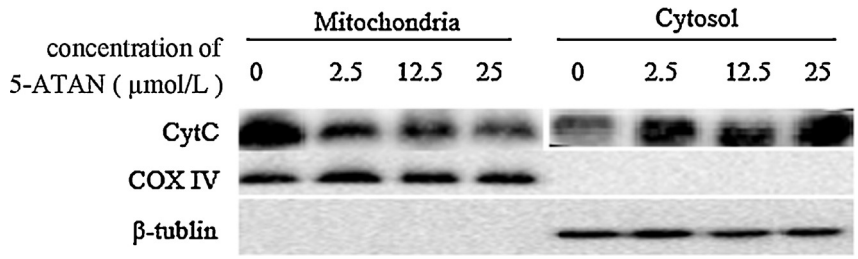

Fig. 6. 5-ATAN increased the level of cytochrome C in the cytoplasm. Cell was treated with different dose of 5-ATAN (0, 2.5, 12.5, 25 μmol/L).

3.5. PI3K/Akt in 5-ATAN-induced apoptosis of U266 cells

Based on the observation that the PI3K/Akt/Bad pathway is frequently overactivated in multiple myeloma and plays critical roles in disease progression and resistance to apoptosis, we assessed the effect of 5-ATAN on the PI3K/Akt signal pathway in U266 cells. As shown in Fig. 8, phosphorylation of Akt was significantly reduced, whereas total Akt was little affected when 5-ATAN alone was added to plates. To further investigate the role of the PI3K/Akt pathway in 5-ATAN induced apoptosis, we pretreated U266 cells with an Akt inhibitor, LY294002. LY294002 markedly enhanced the apoptotic ratio of U266 cells, with a 16.1% increase relative to TAN (Fig. 9) and inhibited the phosphorylation of both Akt and Bad (Fig. 10). These results suggested that 5-ATAN inhibited the activity of PI3K/Akt/Bad, which are involved in cellular apoptosis.

4. Discussion

Tangeretin is the best-studied compound among the polymethoxyflavones (PMFs), which are present exclusively in citrus peels and exhibit a broad spectrum of biological activities, such as anti-proliferative and anti-invasive activities [18,19]. However, the therapy response to tangeretin is unsatisfactory in some types of cancer cells. We prepared 5-ATMF with an acetyloxy group at the C5 position of the original tangeretin skeleton and then investigated whether 5-ATMF has impacts on MM. As expected, we observed that 5-ATMF has potent ability to induce

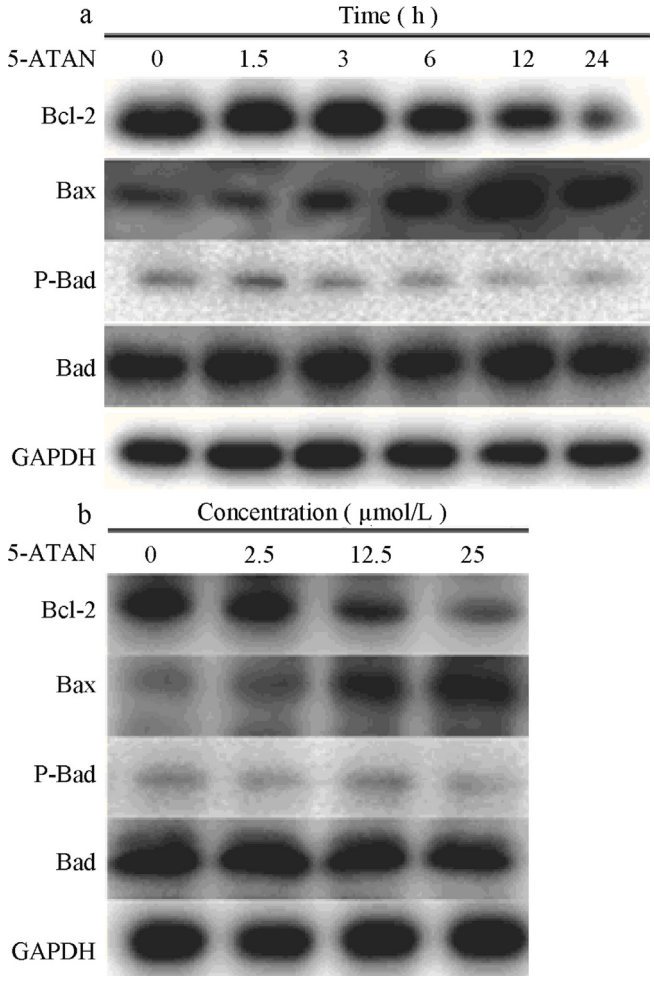

Fig. 7. Effect of 5-ATAN on expression of the mitochondrial signaling pathway downstream of related proteins of U266 cells. The protein levels of Bcl-2, Bax, P-Bad and Bad were analyzed by Western blotting. (a) Cell was treated with dose of 5-ATAN (25 μmol/L) in different times (0, 1.5, 3, 6, 12, 24 h). (b) Cell was treated with different dose of 5-ATAN (0, 2.5, 12.5, 25 μmol/L).

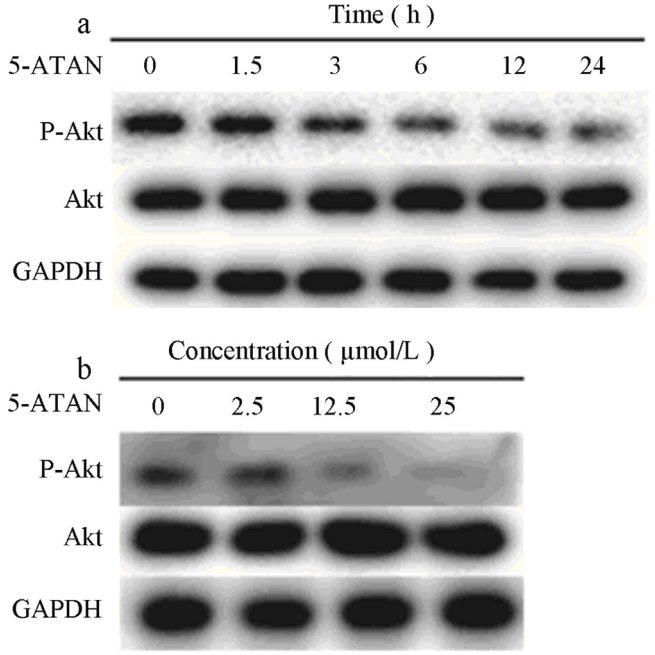

Fig. 8. The protein levels of P-Akt and Akt were analyzed by Western blotting. (a) Cell was treated with dose of 5-ATAN (25 μmol/L) in different times (0, 1.5, 3, 6, 12, 24 h). (b) Cell was treated with different dose of 5-ATAN (0, 2.5, 12.5, 25) μmol/L).

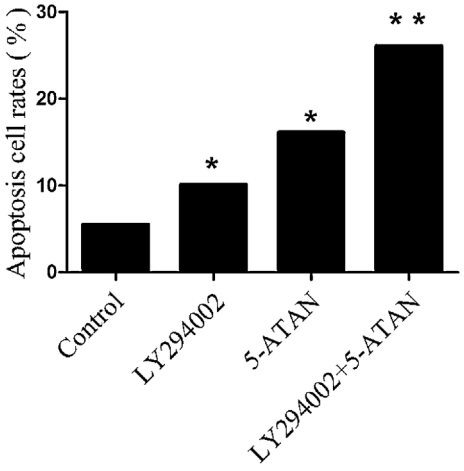

Fig. 9. Effect of 5-ATAN plus LY294002 or not on the pro-apoptosis of U266 cell lines [cell pro-apoptosis was analyzed by using flow (Annexin V-FITC/PI). Cell was treated with dose of 5-ATAN (25 μmol/L). Results represent the mean ± SD from three independent experiments] (* means compared with the control group, $P < 0.05$, ** means compared with the control group, $P < 0.001$, $n = 3$).

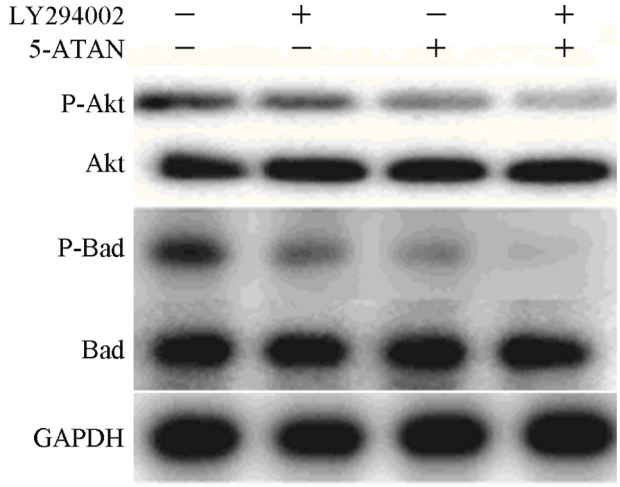

Fig. 10. Effect of 5-ATAN plus LY294002 or not on the protein levels of P-Akt, Akt, P-Bad and Bad were analyzed by Western blotting. Cell was treated with dose of 5-ATAN (25 μmol/L).

apoptosis of multiple myeloma U266 cells, and this apoptotic effect was correlated with the mitochondrial effect.

The Akt signal pathway is believed to be a pivotal signaling pathway regulating cell growth and apoptosis by interfering with mitochondria [20,21]. Activating Akt can lead to phosphorylation and inactivation of components of the apoptotic machinery, including Bad and caspase-9 [22]. Changes in Bad phosphorylation occur rapidly in response to apoptotic or survival stimuli and may further influence subcellular localization of Bad, causing its shift from cytosol to mitochondria [2]. Once reaching the mitochondrial, Bad interacts with the anti-apoptotic proteins Bcl-2 and/or Bcl-X_L [20]. This impact not only abrogates the function of the anti-apoptotic proteins but also activates the pro-apoptotic protein Bax [23]. Once activated, Bax may form pores in the mitochondrial membrane that directly or indirectly allow the release of Cyt c to drive cell death pathways [13]. The overactivated PI3K/Akt pathway is functionally linked with apoptosis regulation in MM cells [24]. Our data showed that application of 5-ATMF alone reduced the phosphorylation of Akt and altered its downstream factors. When LY294002 was combined with 5-ATMF, we observed a significant increase in both the release of Cyt c and the apoptotic ratio of U266 cells. In contrast, 5-ATMF treatment of U266 cells resulted in a decrease of phosphorylated Bad and phosphorylated Akt, which are both upstream regulators of the mitochondrial apoptotic pathway. Thus, our current data are consistent with a theoretical PI3K/AKT pathway that triggers changes in phosphorylated Bad and mitochondria, driving cell death when the pathway is influenced by apoptotic signaling components.

In summary, our results show for the first time that 5-ATMF has the ability of inducing apoptosis in the U266 multiple-myeloma cell line *in vitro*. Although the precise mechanism of 5-ATMF-induced cell death awaits further investigation, 5-ATMF seems at least partially to involve targeting Akt inhibition, by which 5-ATMF initiates mitochondrial driven cell death. Our data suggest the future preclinical investigation of natural citrus peel extract derivatives in multiple myeloma.

Acknowledgments

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