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Enhancement of the Sensitivity of the Acute Lymphoblastic Leukemia Cells to ABT-737 by Formononetin

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ABSTRACT

Overexpression of (myeloid leukemia cell differentiation protein 1) Mcl-1 is associated with the reduction of ABT-737 toxicity and secondary resistance. In this study, the effect of formononetin (biochanin B) on Mcl-1 expression, cell growth, apoptosis, and ABT-737 sensitivity of the acute lymphoblastic leukemia (ALL) cells was investigated. In this experimental study, the cell proliferation and MTT assays were used to investigate the effect of formononetin on cell growth and survival. qRT-PCR was performed for the measurement of gene expression. Hoechst 33342 staining and caspase-3 activity assay were used for the determination of apoptosis. Our data showed that formononetin and ABT-737 both led to a significant reduction in the IC_{50} value and synergistically reduced the cell growth and survival relative to single treatment. Overexpression of Mcl-1 was found after the treatment with ABT-737. Formononetin decreased the expression of B-cell lymphoma 2 (Bcl-2) and Mcl-1 and increased the Bcl-2-associated protein x (Bax) and P21 expression. Moreover, formononetin enhanced the apoptotic effect of ABT-737 in ALL cells. In summary, formononetin showed anti-carcinogenic activities in human ALL cells *via* suppression of cell growth and survival. Formononetin enhanced the apoptotic effect of ABT-737, with contribution by inhibition of the Mcl-1 expression.

Keywords: ABT-737, ALL, apoptosis, Formononetin, Mcl-1

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Introduction

Acute lymphoblastic leukemia (ALL) is aggressive hematologic disorder arising from hematopoietic blasts (1).Despite great efforts over recent decades to improve the treatment of pediatric and Philadelphia-positive ALL patients, almost half of children and adult patients still present high rates of relapse and develop drug-resistance. Therefore, an expansion of new drugs or new treatment strategies is an urgent need for ALL patients (2, 3).

Anti-apoptotic Bcl2 family proteins (Bcl-2 and Mcl-1) by binding and inhibiting pro-apoptotic proteins such as Bax and Bak prevent their interaction with mitochondria and then inhibit apoptosis (4). Altered expression of pro-apoptotic and anti-apoptotic proteins has been proven in many hematological cancers such as ALL. Overexpression of anti-apoptotic proteins as well as the decrease in Bax/Bcl2 ratio has been observed in ALL patients, which these changes are associated with an increase in growth, drug resistance, decreased cell apoptosis and poor prognosis (5-7). Therefore, targeting Bcl-2 family anti-apoptotic proteins has been proposed as a potential therapeutic approach in many cancers, especially ALL.

ABT-737 is a synthetic BH3-mimic that inhibits B-cell lymphoma-extra Large (Bcl-xL) and Bcl-2, which has shown significant anticancer activity alone and in combination with chemotherapy drugs in several solid and hematological cancers (8, 9).This compound binds with high affinity to Bcl-2, Bcl-xL and Bcl-w proteins, but its affinity to Mcl-1 is low(10). Also, reports show that up-regulation of Mcl-1 is associated with the reduction of ABT-737 toxicity and secondary resistance.Inhibition of Mcl-1 protein has been proposed as a potential strategy to increase sensitivity to ABT-737 (11-13).

Natural products are a rich source of various chemical compounds that have shown great potential in the treatment of various diseases, including cancer (14). Formononetin(biochanin B) is a non-steroidal methoxylated isoflavonoid that is extracted from several plants such as Red clover (*Trifolium pratense*) and *Astragalus membranaceus* and has significant antioxidant, anti-inflammatory and anti-cancer effects(15-17). Formononetin has been shown to inhibit the cell cycle at several stages in ovarian, colon, prostate, and liver cancers(17-20).Also, the evidence shows that formononetin changes the expression of Bax, Bcl-2, matrix metalloproteinase-2 (MMP-2), tumor protein P53 (P53) and cyclin-dependent kinase inhibitor 1 (P21), and activates caspases through mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), janus kinase/signal transducers and activators of transcription (JAK/STAT) and phosphoinositide 3-kinase(PI3K) cellular signaling pathways and by which regulates the internal and external pathway of apoptosis (1, 6, 19-21). Therefore, formononetin can increase the sensitivity to chemical drugs such as ABT-737 by inhibiting anti-apoptotic proteins such as Mcl-1.In this study, the effect of formononetin on the growth, apoptosis and sensitivity of the ALL cells to ABT-737 has been investigated.

Materials and methods

Chemical reagents

Formononetin (purity ≥98.0%), ABT-737 (purity ≥98.0%), RPMI-1640 culture medium, antibiotics, MTT solution andHoechst 33342 (purity ≥98.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).Trypan blue was obtained fromMerck (KGaA, Darmstadt, Germany)

Cell culture

In this experimental study, the human T-ALL cell lines MOLT-4 and MOLT-17 were obtained from thePasteur Institute (Tehran, Iran). The cells were propagated in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) containing 1% antibiotics (100 µg/mL streptomycin, 100 IU/mL penicillin,) (Sigma-Aldrich), 1% Glutamax, 20% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Both cell lines were cultured at 37°C in 5% CO_2.

MTT assay

The methyl thiazolyl tetrazolium (MTT) assay was used to measurethe cell cytotoxicity of formononetin and ABT-737, alone and in combination on ALL cells. The assay was divided into five groups: blank control, solvent control, formononetin, ABT-737 and formononetin+ABT-737. In brief, the cells were cultivated at a density of 2×10^4 cells/well in 96 well platesfor 24 h. Then, the cells were exposed to different concentrations of formononetin (0, 12.5, 25, 50, 100, 200 and 400 µM) and ABT-737 (0.125, 0.5, 1, 2 and 4 µM) and continued to further incubate for 24 h. Next, 10 µL of MTT solution (Sigma-Aldrich) was added to the wellsand incubated for 4 h at 37 ºC. The supernatants were discarded and 150 µL of DMSO was added to the cells to dissolve the sediment. Absorbance at 490 nm was determined and used for the measurement of the ratio of surviving cells. The survival rate (SR) was determined according to the equation as follows: SR (%) = ($A_{Experiment}$ /$A_{Control}$) × 100%. The concentration that reduced 50% survival rate (IC_{50}) was measured by GraphPad software (GraphPad Software Inc., San Diego, CA, USA). In the next experiments, the IC_{50} doses of compounds were used. Figure 1 shows the schematic view of our study.

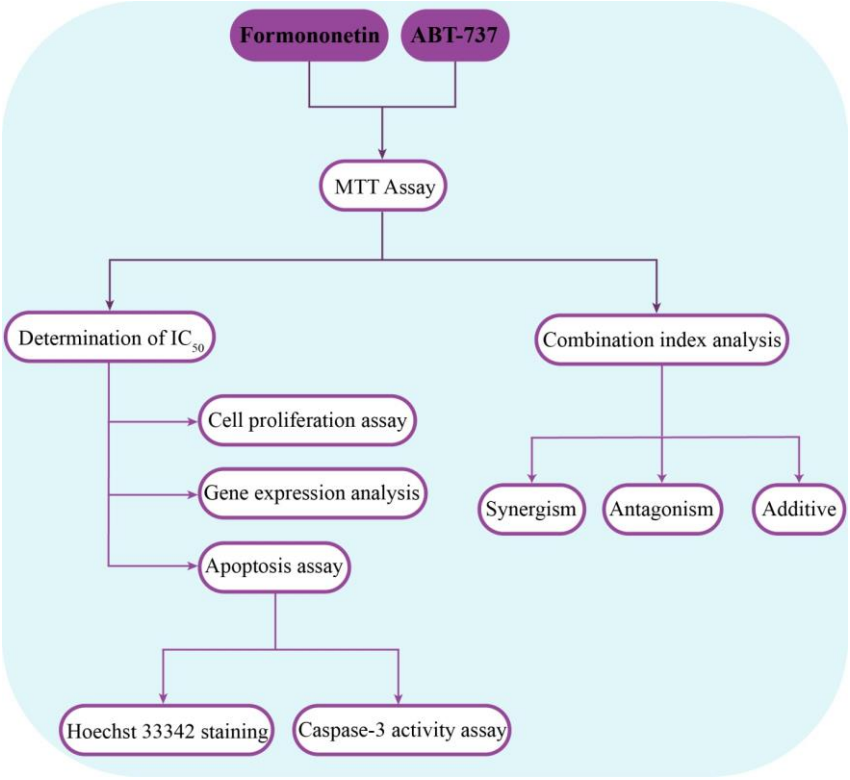

Fig.1. In this study, the toxicity of the different concentrations of formononetin and ABT-737 on MOLT-4 and MOLT-17 ALL cells were investigated. After determining the IC_{50}, this dose was used to investigate the effect of compounds on cell proliferation, gene expression and apoptosis.

Chou-Talalay combination-effect assay

The combination index (CI) theorem of Chou-Talalay was used to investigate the interaction between formononetin and ABT-737(22, 23). The all results obtained from the MTT were converted to Fraction affected (Fa; range 0-1; where Fa = 1 represents 0% cell survival and Fa = 0 represents 100% cell survival) and analyzed with the CompuSyn program (ComboSyn Inc., Paramus, NJ, USA). A CI=1, >1or <1, indicates additive, antagonistic and synergistic effects, respectively.

Cell proliferation assay

The effect of formononetin and ABT-737 on cancer cell proliferation was assessed by the trypan blue exclusion assay. MOLT-4 and MOLT-17 cell lines (5×10^4 cells per well) were treated with ABT-737 and formononetin, alone and in combination, in 6-well plates for 24-120 h as described above. At indicated time points, the leukemic cells were collected and then stained with 0.4% trypan blue (Merck KGaA, Darmstadt, Germany) and incubated for two minutes. Next, the number of unstained cells was counted under an inverted microscope (Nikon Instrument Inc., Melville, NY, USA) by using a hemocytometer. The cell viability was counted by dividing the number of viable cells in the treated group by the number of viable cells in the blank control group and multiplying by 100. The percentage of cell viability in the blank control group each time was considered as 100%.

RNA isolation and reverse transcription polymerase chain reaction

After 24 h of treatment, the total RNA was isolatedusingRNA extraction reagent (Parstous, Tehran, Iran) as described by the manufacturer's protocol. cDNA was synthesized from 1 µg of purified RNA by using of MMLV reverse transcriptase and oligo-dT primer according to the manufacturer's instructions (Parstous). qRT-PCR was performed by the LightCycler 96 system (Roche DiagnosticGmbH, Mannhein, Germany)using SYBR green reagent (Parstous). Each PCR reaction had the following components: 1 µL of cDNA template, 0.2 µM each of the forward and reverse primers, 12 µL of SYBR green and 6 µL of nuclease-free distilled water. The specific primers used for PCR analysis are listed in Table 1. The initial PCR step at 95˚C for 10 min was followed by 35 cycles at 95°C for 20 sec and 60°Cfor 1 min. Relative quantitation was determined with the $2^{-(\Delta\Delta Ct)}$ method(24, 25), by β-actin as the internal gene control.

Table 1. Sequence of primers used in real-time PCR.

Genes	Sequence (5'→3')	Primer length (base)	Tm (°C)
β-actin FW	GACATCCGCAAAGACCTGTA	20	57.62
β-actin RV	GGAGCAATGATCTTGATCTTCA	22	56.22
Bcl-2 FW	GGATGCCTTTGTGGAACTG	19	56.50
Bcl-2 RV	CAGCCAGGAGAAATCAAACAG	21	57.16
Bax FW	GCTTCAGGGTTTCATCCAG	19	55.88
Bax RV	TTACTGTCCAGTTCGTCCC	19	56.39
P21 FW	TGGAGACTCTCAGGGTCGAAA	19	58.32
P21 RV	CGGCGTTTGGAGTGGTAGAA	21	60.20
Mcl-1 FW	TAGTTAAACAAAGAGGCTGGGA	20	60.32
Mcl-1 RV	CCTTCTAGGTCCTCTACATGG	21	56.33

FW: Forward, RV: Reverse

Hoechst 33342 staining

The MOLT-4 and MOLT-17 cell lines were incubated with IC_{50} of formononetin and ABT-737 for 24 h. Subsequently, the cells were washed with PBS and fixed with 3.7% formaldehyde for 30 min. Next, 50 µg⁄mL of Hoechst 33342 (Sigma-Aldrich) was used to stain the cells for 30 min. Next, apoptotic morphological features were observed using a fluorescence microscope (Olympus, Tokyo, Japan). The fragmented or condensed nuclear of the cells were considered as apoptotic cells.

Caspase-3 activity assay

The induction of caspase-3 activity was investigated using a colorimetric caspase assay Kit (Abnova, Taipei, Taiwan). In brief, the cells were resuspended in 50 µL lysis buffer and centrifuged in 11,000 g for 2 minutes. Then, 5 µl of the DEVD-pNA substrate and 50 µL of reaction buffer (contents were added to each sample. After 2 h incubation at 37°C, the absorbance was measured using a microplate reader (Awareness Technology, Palm City, FL, USA) at 405 nm.

Statistical analysis

Quantitative data in this study were presented as mean ± standard deviation (SD) of three independent experiments. Statistical significance of differences between groups was evaluated by using the analysis of variance (ANOVA) and *t*-test using GraphPad Prism software. A value of p less than 0.05 was considered significant.

Results

Formononetin enhanced the sensitivity of T-ALL cells to ABT-737

To test whether formononetin could increase the sensitivity of the ALL cells to ABT-737, a combination treatment of formononetin and ABT-737 on MOLT-4 and MOLT-17 cells was performed. The results of MTT assay revealed that treatment with formononetin and ABT-737 alone, significantly decreased the cell survival rate in a dose-dependent manner (relative to the blank control) (Figure 2). Furthermore, combination treatment further reduced the cell survival rate relative to formononetin or ABT-737 alone ($p<0.05$). The IC_{50} values of formononetin and ABT-737 for 24 h treatment were155.8 and 1.59 µM in MOLT-4 cells, and183.2 and 2.27 µM in MOLT-17 cells, respectively (Table 2). The IC_{50} values of combination were also 1.05 and 1.36 µM in MOLT-4 and MOLT-17 cells, respectively. As illustrated in Table 2, the combination treatment with formononetin and ABT-737 led to a significant reduction in the IC_{50} value, relative to the monotreatment. These results suggest that formononetin can increase the sensitivity of T-ALL cells to the chemotherapeutic agent ABT-737.

The combination effect of formononetin and ABT-737 on ALL cells was synergistic

To determine whether the effect of formononetin and ABT-737 on cell survival is responsible for their synergistic effect, we determined the combination index based on the non-constant method of Chou–Talalay and CompuSyn software. The CI–Fa plot showed that the combination index of formononetin (0–4 μM) with ABT-737 (0–4 μM) on ALL cells was synergistic interaction (CI<1) in all of the combinations. Moreover, our data showed that the strongest synergistic effect of 24 h was obtained at 4 μM formononetin in combination with 4μM ABT-737 (CI=0.70), with Fa level of 0.81 in MOLT-4 cell line (Figure 2). Furthermore, in MOLT-17 cell line,the strongest synergistic effect was observed at 2 μM formononetin in combination with 2μM ABT-737 (CI=0.71), with Fa level of 0.58 (Figure 2).

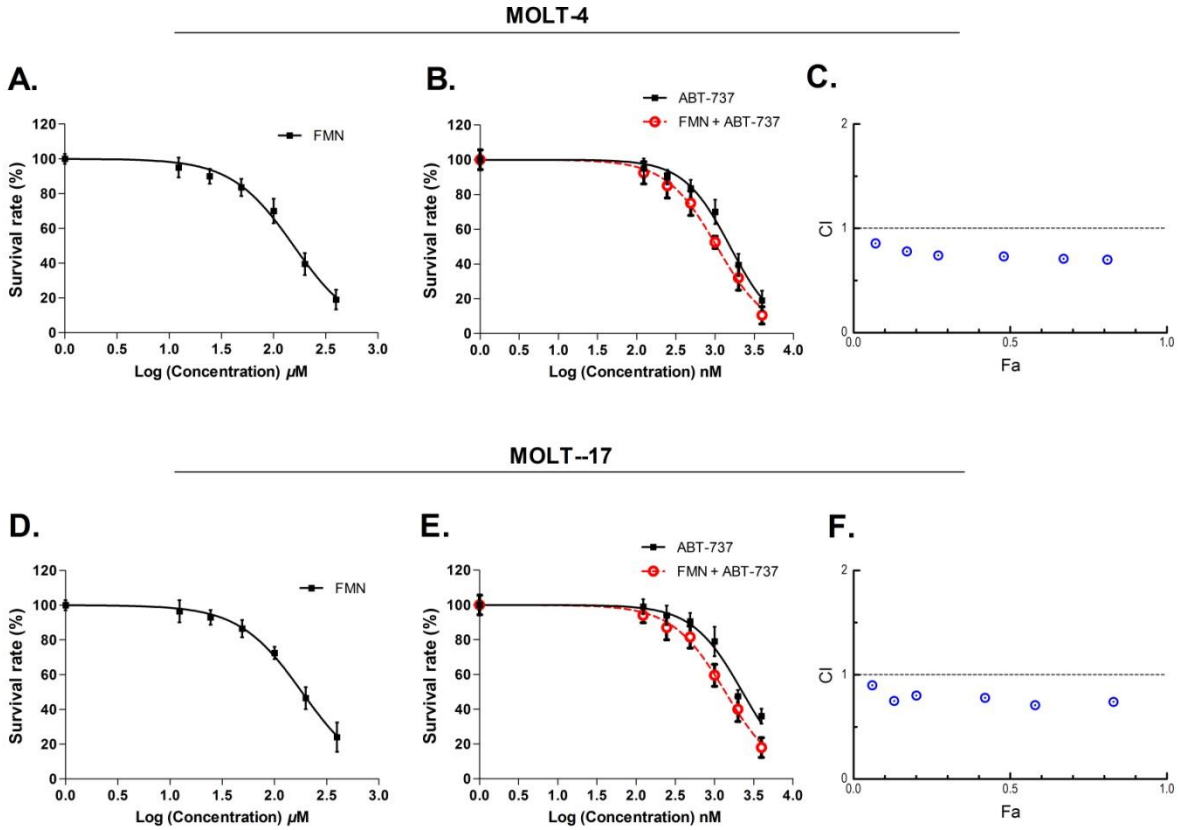

Fig. 2. Effect of formononetin in combination with ABT-737 on cell survival. The MOLT-4 and MOLT-17 ALL cells were treated with formononetin (FMN) and ABT-737 at indicated concentrations. Twenty-four hours after treatment, the cell survival was determined using MTT assay. The cell survival curves were plotted using GraphPad software (A, B, D and E). Data are showed as mean±SD of three experiments. The combination index (CI) values were measured using the fractional affected (Fa) values of MTT assay and CalcuSyn software (C and F).

Table 2. IC_{50} values of the ABT-737 and formononetin in ALL cells.

	IC_{50} (24 h, μM)	
	MOLT-4	**MOLT-17**
ABT-737	1.59	2.27
Formononetin	155.8	183.2
Combination	1.05*	1.36*

IC_{50} was calculated using sigmoidal dose-response model and Prism software. Data are expressed as the mean±SD (n=3). *p<0.001 versus single treatment

Formononetin increases the growth inhibitory effect of ABT-737

We then studied whether formononetin and ABT-737 could inhibit the proliferation of the ALL cells. The cells were treated with formononetin and ABT-737, alone and in combination. Cell viability was then evaluated by trypan blue assay during a period of 5 days, and the results were expressed as the percentage of viable cells in relation to the total number of cells. The cell proliferation assay showed that comparing with the control groups, the viability of the cells in formononetin, ABT-737 and combinatorial group significantly reduced in a time-dependent way. In MOLT-4 cells, the cell viability in formononetin, ABT-

737 and combinatorial group decreased to 74 %, 69 % and 63 % respectively, after 24 h of treatment, and then to a further 36 %, 29 % and 26 %, at the end of the experiment (p<0.05; Figure 3). Similar results were observed in the MOLT-17 cell line.

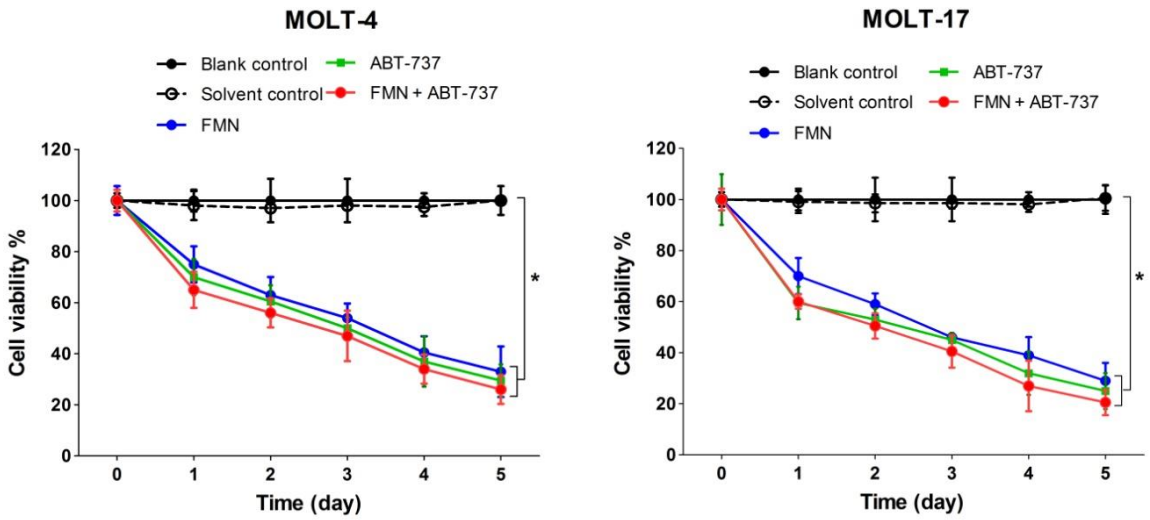

Fig.3. Proliferation inhibition of leukemic cells. The MOLT-4 and MOLT-17 cells were treated with ABT-737 and formononetin (FMN) for 1-5 day, and the cell viability was measured using trypan blue assay at the end of each day. The data are represented as mean±SD of three experiments. *p<0.05, versus blank control or solvent control.

Formononetin altered the expression of apoptotic genes

qRT-PCR method was used to investigate the effect of formononetin and ABT-37 on gene expression. The results showed that 24 h after the treatment of MOLT-17 and MOLT-4 cells with formononetin, the mRNA levels of Bcl-2 and Mcl-1 decreased compared to the blank control group, while, the mRNA expression of Bax and P21 increased (p<0.001). Moreover, treatment with ABT-737 did not cause a significant change in the mRNA expression of Bcl-2, Bax and P21, and increased the expression of Mcl-1 (Figure 4). In addition, combination therapy significantly decreased the expression of Bcl-2 and increased the expression of Bax, P21 compared to the blank control, and ABT-737 treated cells. These changes were significantly less than formononetin group. Mcl-1 gene expression in the combination group showed a clear difference with the single treatment and blank control groups (Figure4). In the solvent control group, there was no significant difference in gene expression compared to the blank control group (p>0.05).

Formononetin augmented the apoptotic effect ofABT-737 in ALL cells

To understand whether the cytotoxic effect of treatments was related to apoptosis, the MOLT-4 and MOLT-17 cells were treated with IC_{50} doses of formononetin, ABT-737and their combination for 24 h as described above. Next, we conducted Hoechst 33342and caspase-3 activity assays. Nuclear morphological changes are one of the most typical characteristics of apoptosis. So, Hoechst 33342 staining was used to detect apoptotic morphologic changes in ALL cells. As shown in Figure 5, apoptotic bodies containing nuclear fragments were observed in formononetin and ABT-737 treated cells but not in the control groups. Moreover, the number of apoptotic cells in the combination group did not significantly differ from the single treatment group.

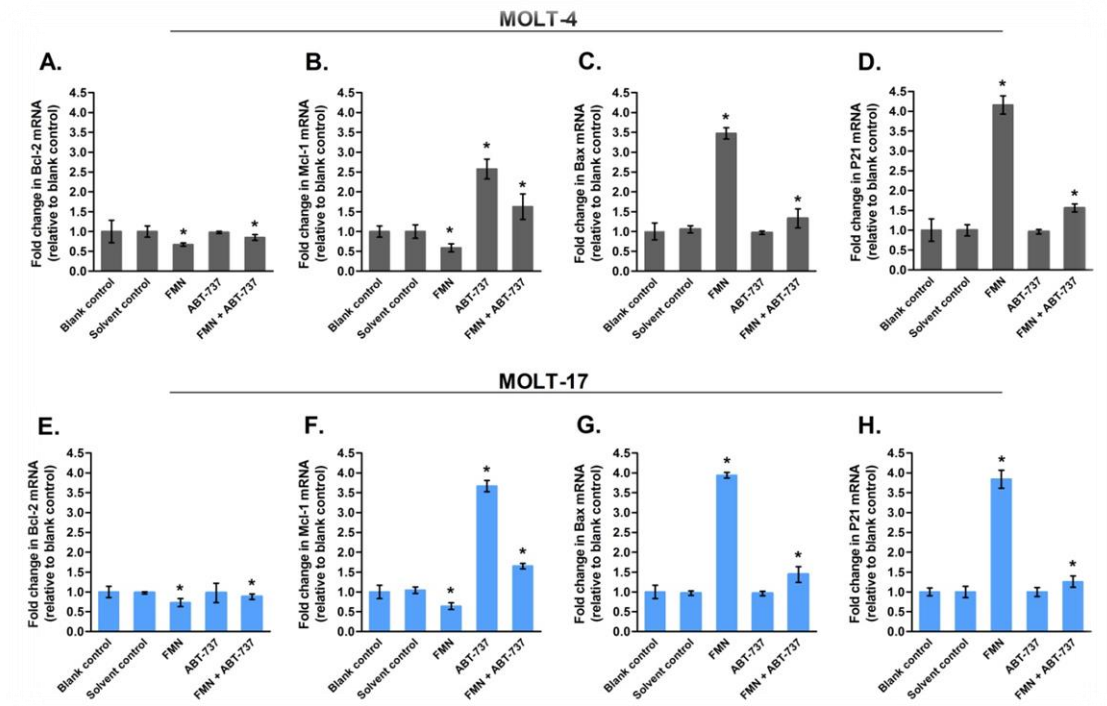

Fig.4. RT-qPCR analysis of leukemic cells. The MOLT-4 and MOLT-17 cells were treated with ABT-737 and formononetin (FMN) (IC_{50} doses) for 24 h. Relative expression levels of Bcl-2 (A and E), Mcl-1 (B and F), Bax (C and G) and P21 (D and H) mRNAs were measured by RT-qPCR using $2^{-(\Delta\Delta Ct)}$ method. Data are presented as mean±SD (n=3). *p<0.001, relative to blank control.

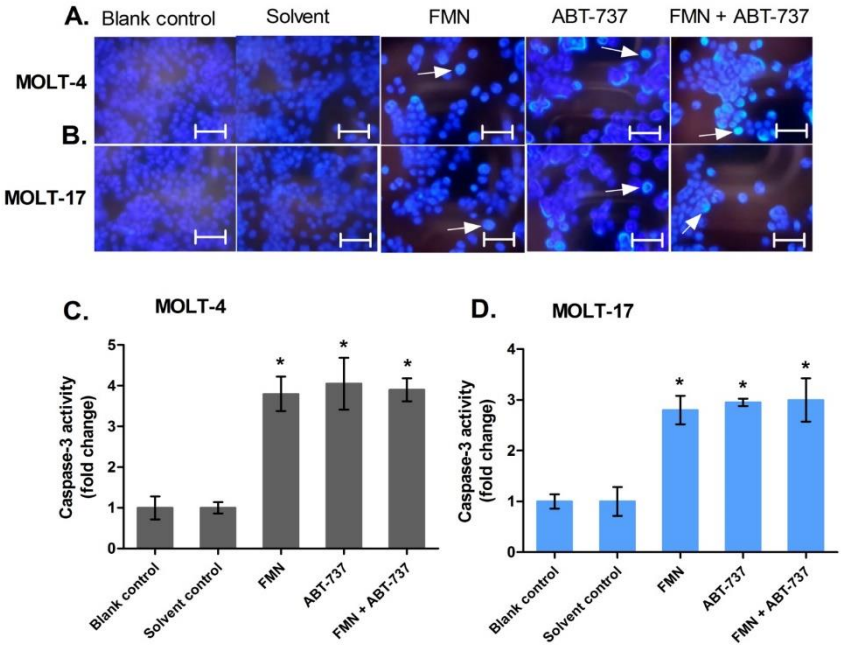

Fig.5. Combination effects of ABT-737 and formononetin on apoptosis of ALL cells. Cells were treated with ABT-737 and formononetin (FMN) (IC_{50} doses) for 24 h. Next, the apoptosis was determined using Hoechst 33342 staining (A and B) and caspase-3 activity assay (C and D). The data are presented as mean ± SD (n=3) of three independent experiments. *p<0.001 compared with blank control. Arrows show the apoptotic cells. Scale bar: 10 µM.

To understand the molecular basis of apoptosis induced by formononetin and ABT-737, caspase-3 activity assay was performed. The results indicated that there was a significant increase in caspase-3 activity in ALL cells treated with formononetin and ABT-737. The percentage of caspase-3 activity in the cells treated with the combination of formononetin and ABT-737 was not significantly different compared to caspase-3 activity in the cells treated with either formononetin or ABT-737 alone (Figure 5).

Because the IC_{50} dose of the combination treatment is lower than the IC_{50} dose of either compound alone, these results demonstrate that the combination of the two agents has a greater effect on inducing cell apoptosis compared to treatment with each single agent. The enhanced apoptotic effect with the combination is consistent with the results from the MTT assay.

Discussion

Despite significant efforts over the past few decades to improve the treatment of pediatric and Philadelphia-positive ALL patients, almost half of the children and the adult patients still present the high rates of relapse and develop drug-resistance (1-3). These highlight the need for new drugs or treatment strategies to address these challenges and improve outcomes for ALL patients. Overexpression of anti-apoptotic proteins is associated with increased growth, drug resistance, decreased cell apoptosis, and poor prognosis in ALL patients (5-7). Reports show that up-regulation of Mcl-1 is associated with the reduction of ABT-737 toxicity and secondary resistance. Inhibition of Mcl-1 protein has been proposed as a potential strategy to increase sensitivity to ABT-737 (11-13). In this study, the effect of formononetin on the growth, apoptosis and sensitivity of the MOLT-4 and MOLT-17 ALL cells to ABT-737 has been investigated.

The results of our study showed that treatment with either formononetin or ABT-737 alone significantly reduced the cell growth and survival and induced apoptosis. Combination treatment with formononetin and ABT-737 led to a significant reduction in the IC_{50} value and synergistically reduced the cell survival rate relative to formononetin or ABT-737 alone. The IC_{50} dose of the combination treatment was lower than the IC_{50} dose of either compound alone, therefore our data show that the combination of the two agents has a greater effect on cell growth and apoptosis compared to treatment with each single agent. So far, various studies have investigated the relationship between the expression level of Mcl-1 gene in cancer cells and resistance to ABT-737. For example, Mazumder *et al*. (13) showed that ABT-737 treatment led to Mcl-1 phosphorylation and resistance to ABT-737 in leukemic cells. Moreover, they found that increased NADPH oxidase activator 1 (NOXA) expression, a pro-apoptotic protein, could overcome ABT-737 resistance by enhancing Mcl-1 degradation. Yecies *et al*. (11) found that lymphoma cells that had acquired resistance to ABT-737 up-regulated the Mcl-1 and, combination of ABT-737 with Mcl-1 inhibitors could overcome this resistance.Konopleva *et al*. (26) investigated the mechanisms underlying the sensitivity and resistance of acute myeloid leukemia (AML) cells to the ABT-737. They found that AML cells with high levels of Bcl-2, Mcl-1 or Bcl-xL and low levels of Bim, a pro-apoptotic protein, are more resistant to ABT-737. Moreover, the activation of survival pathways, such as the PI3K/Akt pathway, confers resistance to ABT-737 in AML cells. In a study conducted by Tahir *et al*. (27), they investigated the contribution of Bcl-2 family members to the cellular response of several small-cell lung cancer (SCLC) cell lines to ABT-737. They found that the relatively higher levels of Bcl-2, Bcl-xL, Bim, and Noxa, and the lower levels of Mcl-1 are characterized

SCLC cell lines that were sensitive to ABT-737. Wang *et al*. (28) in another study showed thatA-1210477, a selective Mcl-1 inhibitor, could overcome resistance to ABT-737 in AML cells that up-regulated Mcl-1. The combination of A-1210477 and ABT-737 induced synergistic apoptosis in AML cells.In our study, we showed that formononetin decreases the expression of the Mcl-1 mRNA and increases the apoptosis caused by ABT-737 in MOLT-4 and MOLT-17 ALL cells. Our results are in agreement with the above reports and show that formononetin can increase the sensitivity of the ALL cells to ABT-737 by suppressing the Mcl-1expression.

We also investigated the effect of formononetin and ABT-737 on mRNA expression. The result of qPCR showed that ABT-737 increases the expression of Mcl-1 without affecting the expression of Bcl-2, Bax and P21. Formononetin also decreased the expression of Bcl-2 and Mcl-1 and increased the Bax and P21 expression. In combination treatment, formononetin lowered the increased Mcl-1 mRNA induced by ABT-737, which was associated with enhanced sensitivity to ABT-737. In accordance with our study, various studies have been conducted regarding the effect of formononetin on gene expression and drug resistance.A previous study showed that formononetin inhibits tumor proliferation and promotes mitochondrial apoptosis by suppression of EGFR-Akt-Mcl-1axis in non-small cell lung cancer (29).The results of another study revealed that formononetin inhibits the growth of HCT 116 colon cancer cells and promotes apoptosis. These processes were accompanied by caspase activation and down-regulation of the -Bcl-2 and Bcl-xL proteins (30). Yang *et al*. (31) showed that formononetin inhibits cell proliferation, induces G1-phase cell cycle arrest and promotes apoptosis in of A549 and NCI-H23 lung cancer cells. On the molecular level, they found that exposure to formononetin suppresses the expression levels of cell cycle arrest-associated proteins P21, cyclin A and cyclin D1. In addition, the expression level of Bcl-2 decreased after treatment with formononetin.In a study by Zhang *et al*. (32) investigated the effect of formononetin in combination with temozolomide on C6 glioma cells.Results showed that formononetin in combination with temozolomide have synergy effect on C6 cells. Moreover, drug combination enhanced the expression of Bax, decreased the expression of Bcl-2, and augmented tumor cells apoptosis.Xin *et al*. (33) in another study demonstrated that formononetin down-regulates Bcl-2 mRNA expression and triggers apoptosis in MCF-7 breast cancer cells. The combinational use of formononetin and metformin enhanced cell growth inhibition, and the induction of apoptosis.The result of above reports is in agreement with our data and show that treatment with formononetin can potentially sensitize the tumor cells to chemotherapeutic agents, such as ABT-737, through induction of apoptosis.

The mitochondrial or intrinsic pathway of cell death is activated by an array of stimuli such as toxins, radiation, hypoxia and free radicals. The regulation of this pathway is performed by the pro- and anti-apoptotic members of the Bcl-2 family proteins (34, 35). In apoptotic conditions, the pro-apoptotic members such as Bak and Bax are activated. Activated Bak and Bax cause the mitochondrial outer membrane permeability (MOMP), release of cytochrome c into the cytoplasm, and subsequently, the activation of caspases. The anti-apoptotic proteins such as the Bcl-2 and Mcl-1, when not sequestered by pro-apoptotic members, inhibit apoptosis (34-36). P21 functions as an anti-proliferative effecter and cell cycle inhibitor in normal cells, and is deregulated in some tumor cells (37). The role of P21 in the intrinsic pathway of apoptosis is not straightforward and depends on various factors, including the type of cells and the interplay

between pro- and anti-apoptotic proteins (38). ABT-737 is a synthetic BH3-mimic that binds with high affinity to Bcl-2, Bcl-Xl, and Bcl-w proteins, but its affinity to Mcl-1 is low. Studies show that up-regulation of Mcl-1 is associated with the reduction of ABT-737 toxicity and secondary resistance. Inhibition of Mcl-1 protein has been proposed as a potential strategy to increase sensitivity to ABT-737 (11-13). Formononetin has shown to cause the cell cycle arrest in various types of cancer cells(17-20).The evidence shows that formononetin changes the expression of Bax, P21, Mcl-1 and Bcl-2 through MAPK, NF-kB, JAK/STAT and PI3K cellular signaling pathways by which trigger the internal and external pathway of apoptosis (1, 6, 19, 20, 39). In this study, we showed that formononetin decreases the expression of Bcl-2 and Mcl-1, and increases the expression of Bax and P21 in ALL cells. These changes in gene expression were associated with the induction of cellular apoptosis. Moreover, suppression of Mcl-1 was associated with the increased sensitivity to ABT-737. These results suggest that formononetin does not only stimulate the intrinsic pathway of apoptosis, but also can enhance the sensitivity of the ALL cells to chemotherapeutic agents such as ABT-737 through changing the expression of Mcl-1.

In summary, we found that combination treatment with formononetin and ABT-737 leads to a significant reduction in the IC_{50} value and synergistically reduces the cell growth and survival relative to single treatment. Formononetin triggers the intrinsic pathway of apoptosis in MOLT-4 and MOLT-17 ALL cells by down-regulation of Bcl-2 and up-regulation of Bax and P21. Overexpression of Mcl-1 was found after treatment with ABT-737. Moreover, formononetin can enhance the apoptotic effect of ABT-737 in ALL cells by suppressing the Mcl-1. The role of P21 in the intrinsic pathway of apoptosis is not straightforward.

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