

## Mesalazine induces apoptosis via mitochondrial pathway in K562 cell line

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### Abstract

Inflammation is an initial response of the body to infection and relationship between inflammation and cancer has been established. Nuclear factor kappa B (NF- $\kappa$ B) is a central factor in inflammation and its activity contributes to tumor progression and apoptosis prevention consequently leading to cancer promotion. As a result, NF- $\kappa$ B inhibitors can cause apoptosis. In this study, the effect of mesalazine as a NF- $\kappa$ B inhibitor on growth and apoptosis of K562 cells has been investigated. The K562 cells were first cultured in RPMI-1640 medium containing 10.00% fetal bovine serum. After that, they were treated for 72 hr with different concentrations of mesalazine (20.00, 40.00, 60.00 and 80.00  $\mu$ M mL<sup>-1</sup>). The MTT assay was used to evaluate cell viability. Hoechst staining and RT-PCR of apoptosis related genes (Bcl-2 and Bax) were carried out to illustrate apoptosis induction and immunocytochemistry was performed to investigate changes in c-Myc protein level. According to the results of MTT assay, all of applied mesalazine concentrations decreased K562 cells viability. Hoechst staining showed that the fragmented nuclei increased indicating apoptosis induction. Immunocytochemical results showed that mesalazine decreased c-Myc in treated cells. The RT-PCR results also showed an increase in Bax and a decrease in Bcl-2 expressions in mesalazine-treated cells. As the results suggest, mesalazine reduces cell viability by inducing apoptosis in K562 cell line; therefore, it can be used as a candidate for the leukemia treatment.

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### Introduction

Cancer is the second leading cause of death in world; thus, massive researches are performing for cancer treatment. Recently, multiple therapeutic approaches have been developed for cancer treatment including chemotherapy, immunotherapy, radiotherapy and surgery; however, the rate of cancer has been raised due to cancer complexity.<sup>1</sup>

One of the cancer types is leukemia which is a malignant clonal disorder of blood stem cells that increases not only myeloid cells but also peripheral erythroid cells, myeloid hyperplasia in the bone marrow and platelets in the blood. The average age of the disease is 35 years; but all age groups including children are affected. Chronic myelogenous leukemia diagnosis is usually based on the Philadelphia chromosome presence. Common symptoms of the disease include weakness and loss of appetite and weight; however, 5.00% of people are asymptomatic.<sup>2</sup>

Due to imbalance between oncoproteins and tumor suppressors in cancer, cell proliferation increases and

apoptosis decreases. Apoptosis is a programmed cell death that regulates cell population and its dysfunction correlates with some physiological and pathological disorders. As a result, apoptosis induction is the best approach in cancer cells elimination.<sup>3</sup> The process is induced by two pathways including extra-cellular and intra-cellular pathways. The Bcl-2 protein family has an important role at intra-cellular apoptosis pathway being initiated from mitochondria.<sup>4</sup> Multiple factors contribute to cancer promotion and tumor development such as genetic mutations, carcinogen exposure, inflammation, radiation and oncogenic viruses. Inflammation is defined as the response of body to infections being classified as acute and chronic inflammations. In acute status, the body responds rapidly to infections; while, in the chronic inflammation, the response occurs in a long time. According to stimulatory role of inflammatory factors on cell proliferation, in the chronic inflammation, unwanted cell proliferation increases which can lead to cancer initiation and tumor progression.<sup>5</sup>

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One of the most important intra-cellular factors being activated in inflammation is a transcription factor called nuclear factor kappa B (NF- $\kappa$ B). This factor is an essential factor for inflammatory response causing cell proliferation and apoptosis inhibition. The NF- $\kappa$ B over-expression and activation have been observed not only in immune cells but also in many cancers cell lines. Based on NF- $\kappa$ B role in cancer initiation, its inhibition could be a strategy in cancer treatment.<sup>6</sup>

Mesalazine, known as 2-aminosalicylate, is a member of non-steroidal anti-inflammatory drugs which can inhibit NF- $\kappa$ B activation and decrease cell proliferation.<sup>7</sup> This drug has been used to treat inflammatory bowel disease as well as for colon cancer prevention.<sup>8</sup> The cytotoxic effect of mesalazine on cancer cells has only been studied on colon cancer cell line.<sup>8</sup> Based on inhibitory effect of mesalazine on NF- $\kappa$ B, it may also have cytotoxic effects on other cancer cell lines. Because of this, for the first time in present work, the effect of mesalazine has been investigated on K562 cell line (erythromyeloid leukemia cell line) viability and apoptosis.

## Materials and Methods

### Cell culture, treatment and cell viability assay.

K562 (chronic myeloid leukemia) cell line was purchased from Iranian Pasteur Institute, Tehran, Iran. Cell culture medium (RPMI-1640; Gibco, Carlsbad, USA), penicillin streptomycin (Sigma, St. Louis, USA) and fetal bovine serum (FBS; Gibco) were also used. Cells were cultured in RPMI-1640 medium containing 10.00% FBS and 1.00% penicillin streptomycin and incubated at 37.00 °C, 95.00% humidity and 5.00% CO<sub>2</sub>. Mesalazine (Sigma) was dissolved in dimethyl sulfoxide (DMSO) as a solvent to yield different concentrations.

**Evaluation of cell viability by MTT assay.** Six thousand cells per well of 96-well plates were cultured and treated with different concentrations of mesalazine (20.00, 40.00, 60.00 and 80.00  $\mu$ M mL<sup>-1</sup>). The cells were cultured in three 96-well plates and 3 different times (24, 48 and 72 hr), respectively. After incubation, 10.00  $\mu$ L of MTT solution at a concentration of 5.00 mg mL<sup>-1</sup> was added into each well and the plate was shaken for a few sec to spread evenly. After 3 hr, 100  $\mu$ L of DMSO was poured into the wells. The plate was shaken for 15 min on a low-speed shaker until the formazan crystals were completely dissolved. The absorption of each well was read using the ELISA reader (BioTek, Winooski, USA) at 540 nm.<sup>9</sup> Cell viability was calculated as a percent of each well absorbance compared to control cell wells. Then, based on MTT data, mesalazine IC<sub>50</sub> was calculated by CompuSyn Software (version 1.0; ComboSyn Inc., Paramus, USA). The software has been designed by Chou and Talalay.<sup>10</sup>

**Illumination of the morphological effects of mesalazine on K562 cells.** 5.00  $\times$  10<sup>5</sup> cells were seeded in

each well of 24-well plates, then the cells were treated with different concentrations of mesalazine (20.00, 40.00, 60.00 and 80.00  $\mu$ M mL<sup>-1</sup>) for 72 hr at 37.00 °C and 5.00% CO<sub>2</sub>. The cells photos have been taken by inverted microscope (Olympus, Tokyo, Japan) after 72 hr.

**Evaluation of apoptosis by Hoechst staining.** 2.00  $\times$  10<sup>5</sup> cells per well were cultured in 24-well plates and treated with IC<sub>50</sub> concentration (54.00  $\mu$ M) of mesalazine for 72 hr. After incubation, the cells were centrifuged and washed with phosphate-buffered saline (PBS) three times and then fixed with cold methanol in - 20.00 °C for 20 min. After fixation, the cells were washed with PBS and incubated in Hoechst 33342 solution (Sigma) dissolved in PBS in concentration of 1.00 mg mL<sup>-1</sup> for 30 min at room temperature. After staining, the cells were washed with PBS, transferred on slides, observed using fluorescent microscope (Micros, Gewerbezone, Austria) and the cells photos were taken.<sup>11</sup>

**Analysis of Bcl-2 and Bax expressions by RT-PCR.** For RNA extraction, approximately 5.00  $\times$  10<sup>6</sup> K562 cells were treated with IC<sub>50</sub> concentration (54.00  $\mu$ M) of mesalazine for 72 hr. Next, cellular RNA was extracted based on the kit manufacturer's instructions (Takara, Tokyo, Japan). After determination of the extracted RNA concentration by Nanodrop, the cDNA was synthesized by the cDNA synthesis kit (Thermo Fisher Scientific, Bremen, Germany). The Bax, Bcl-2 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers were designed by Gene Runner Software with the sequences listed in Table 1.

**Table 1.** Primers sequences used in this study.

Genes	Sequences of primers
Bcl-2	F: 5'-GATGGGATCGTTGCCTTATGC -3'
	R: 5'-TTCTGGTGTTCCTCCCTTGG -3'
BAX	F: 5'-GCTTCAGGGTTTCATCCAGG -3'
	R: 5'-GCTGCCACTCGGAAAAAGAC -3'
GAPDH	F: 5'-AAGGGTCATCATCTCTGCC -3'
	R: 5'-ATGATGTTCTGGAGAGCCC -3'

The PCR reaction was performed with the following steps using Master Mix (Thermo Fisher Scientific, Waltham, USA): Pre-denaturation for 5 min at 95.00 °C followed by 35 cycles of 30 sec denaturation at 94.00 °C, annealing/extension for 30 sec at 57.00 °C and final extension for 30 sec at 72.00 °C. Afterwards, DNA concentration was determined at 260 nm. For electrophoresis, 50.00 ng DNA was loaded on a 2.00% agarose gel at 85.00 V for 90 min. Finally, the gel photo was captured by gel-documentation. Densitometric analysis of the bands was done using ImageJ software (National Health Institute, Bethesda, USA) and the software data were graphed by Excel software Excel (version 16.0; Microsoft Corporation, Redmond, USA).<sup>12</sup>

**c-Myc protein level analysis by immunocytochemistry.** One million cells were cultured in each well of 6-well plate and then treated with IC<sub>50</sub> concentration

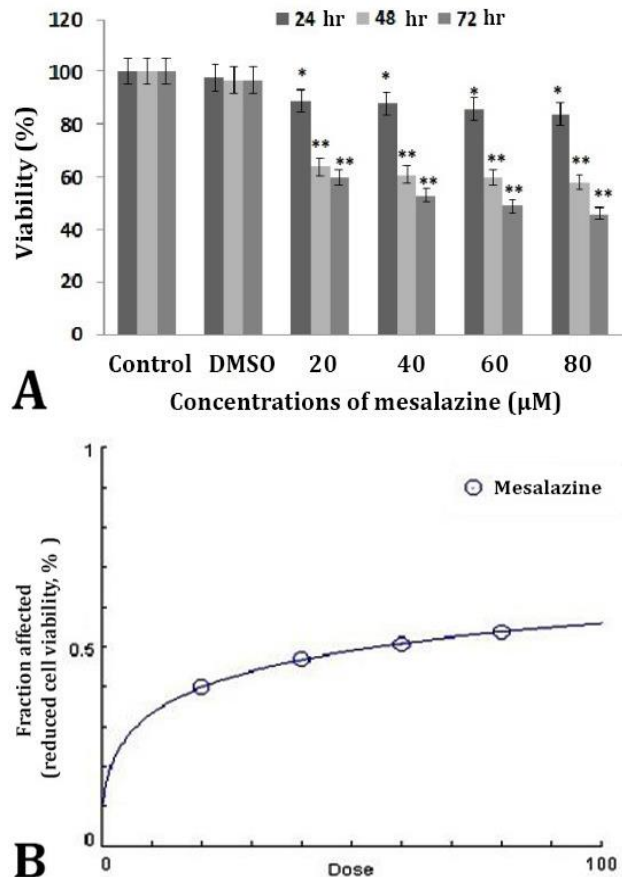
(54.00  $\mu\text{M}$ ) of mesalazine for 72 hr. After incubation, the cells were centrifuged and then washed with PBS followed by centrifugation. Cell pellets were fixed with methanol in  $-20.00\text{ }^{\circ}\text{C}$  for 20 min. Then, the cells were centrifuged and washed three times with anti-body buffer solutions (PBS, BSA, TX-100 and sodium azide) followed by incubation in c-Myc antibody solution (c-Myc anti-body, sc-42; Santa Cruz Biotechnology, Dallas, USA) and diluted by 1/100 in anti-body buffer solution for 2 hr at room temperature. After incubation, the cells were centrifuged and washed three times with anti-body buffer and then incubated in the second anti-body buffer (rabbit anti-mouse FITC, Fluorescein isothiocyanate, conjugated IgG, sc-516140; Santa Cruz Biotechnology and diluted by 1/100 in anti-body buffer solution). Thereafter, the cells were centrifuged and washed in PBS. The cell pellets were transferred on glass slides and finally photographed by a fluorescent microscope (Micros) with FITC filter.<sup>13</sup>

**Statistical analysis.** Charts were plotted in Excel software and data were analyzed by SPSS software (version 16.0; SPSS Inc., Chicago, USA) and ANOVA test to investigate the correlation between cell viability with treated drug concentration and treatment time compared to the control sample at  $p \leq 0.05$ .

## Results

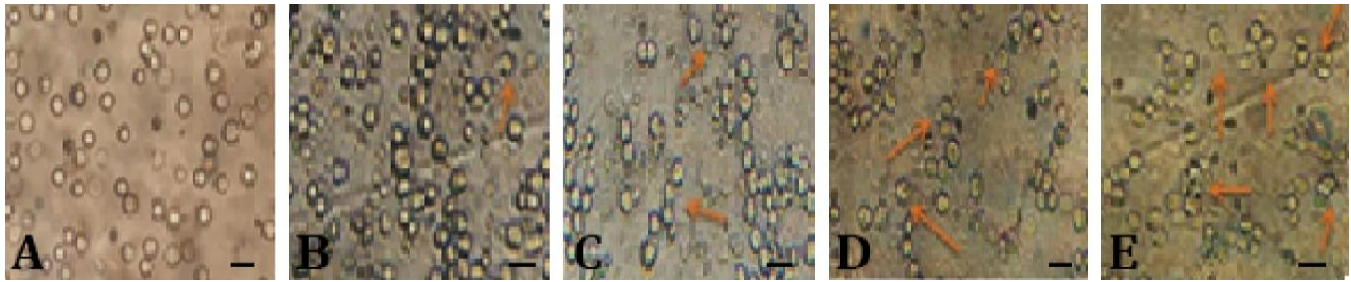
**Mesalazine reduced the cell viability of K562 cell line.** The cell viability was assessed after 24, 48 and 72 hr. Mesalazine in all administered concentrations reduced cell viability. Along with elevation of its dose and treatment time, mesalazine cytotoxic effect was raised on K562 cell line (Fig. 1A). So, it can be concluded that mesalazine cytotoxic effect is time- and dose-dependent. The most cytotoxic effect was observed at 72 hr and 80.00  $\mu\text{M}$  with 54.00% cytotoxicity. The percentages of cell viability reduction at 48 and 24 hr at 80.00  $\mu\text{M}$  concentration were respectively 42.00% and 16.00% being significant compared to the control group. Based on data obtained from the CompuSyn Software, the  $\text{IC}_{50}$  of drug was 54.00  $\mu\text{M}$  (Fig. 1B).

**Morphological effects of mesalazine on K562 cells.** The morphology of the treated cells was observed and photographed by inverted microscope after 72 hr treatment with 20.00, 40.00, 60.00 and 80.00  $\mu\text{M}$  of mesalazine as well as control cells. Figure 2 shows that the treated cells are undergone altered morphology and disrupted membrane. The control cells had intact membranes; whereas, most of the treated cells represented disintegrated membranes indicating cell death occurrence. In the treated cells, bleb structures were found on cell membrane indicating apoptosis induction; the structures increased with mesalazine dose elevation (Fig. 2).

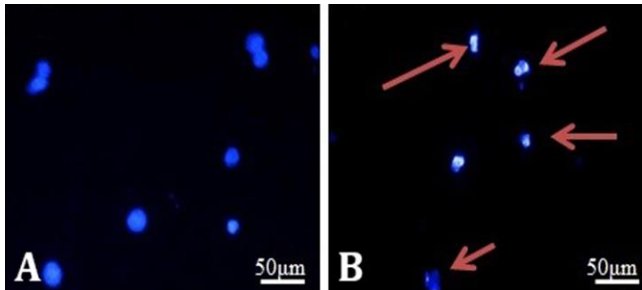


**Fig. 1.** Cytotoxic effect of mesalazine on K562 cell line. **A)** Effect of different concentrations of mesalazine on viability of K562 cancer cells after 24, 48 and 72 hr of treatment. Cell viability decreased after treatment with all concentrations. The highest cytotoxic effect was observed at 80.00  $\mu\text{M}$  of mesalazine 72 hr after treatment. \*  $p \leq 0.05$ ; and \*\*  $p \leq 0.01$ . **B)** Designed plot of the mesalazine different dose effect by the CompuSyn software.

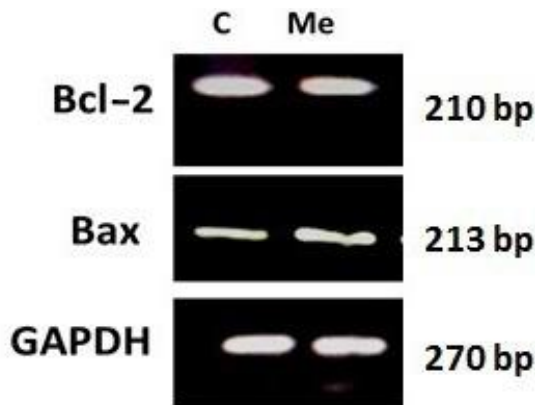
**Mesalazine induces apoptosis in K562 cell line.** In apoptosis, cell nucleus is fragmented as a sign of apoptosis occurrence. For observation of the cell nucleus fragmentation, the cells were stained with Hoechst stain. For this purpose, K562 cells were treated with  $\text{IC}_{50}$  concentration of mesalazine for 72 hr followed by Hoechst staining. As Figure 3 shows, in control cells, almost all of the cell nuclei are intact; whereas, in treated cells, the fragmented nuclei are apparent. For analysis of apoptotic gene expression level alterations in treated cells compared to control ones, the levels of Bax and Bcl-2 expressions in control and treated cells were assessed by RT-PCR. In treated cells, Bcl-2 expression is decreased and Bax expression is increased compared to control cells (Figs. 4 and 5). As Figure 5 shows, the level of Bax/Bcl-2 ratio in treated cells was raised about 70.00% compared to control ones being representative of apoptosis occurrence in treated cells.



**Fig. 2.** Morphological effects of mesalazine on K562 cells. **A)** Control cells; **B, C, D,** and **E)** 20.00, 40.00, 60.00, and 80.00 μM mesalazine concentration treated cells, respectively. As the dose of mesalazine increases, the cell morphology alterations elevate. Arrows indicate membrane changes and bleb structures (Scale bars = 50.00 μM).

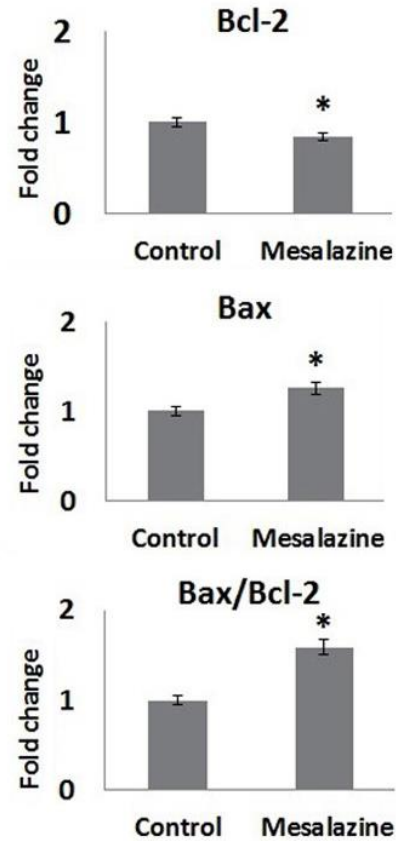


**Fig. 3.** Induction of apoptosis by mesalazine. K562 cells stained with Hoechst dye under fluorescence microscope. **A)** Untreated cells, and **B)** cells treated with the IC<sub>50</sub> concentration of the mesalazine drug. The arrows represent apoptotic cells with fragmented nuclei in comparison with the nuclei of control cells.

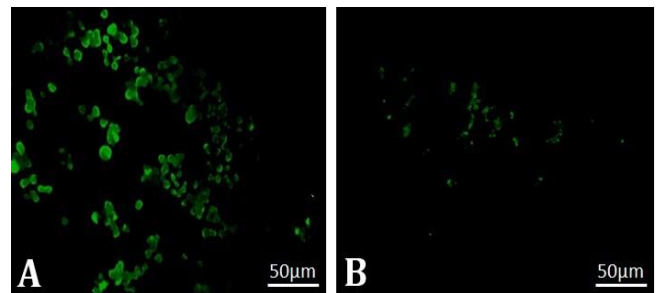


**Fig. 4.** Alterations of Bcl-2 and Bax mRNA levels in control and treated cells. The Bcl-2 mRNA level decreased after treatment and Bax mRNA level increased after treatment. C: Control; Me: Mesalazine. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

**c-Myc proto-oncogene changes in K562 cells treated with mesalazine.** The immunocytochemistry was performed to evaluate c-Myc proto-oncoprotein changes. In cancer cells, c-Myc protein is often expressed continuously. As seen in Figure 6, in control cells, c-Myc staining is high indicating that in untreated cells, c-Myc protein is highly expressed. Figure 6 shows also that in treated cells, c-Myc protein staining is lesser than it in control cells indicating that in treated cells, the level of c-Myc protein is decreased. Therefore, mesalazine has reduced c-Myc protein level in the cells.



**Fig. 5.** Compact bands analysis using ImageJ and Excel software. The Bax/Bcl-2 ratio increased after treatment with mesalazine compared to control cells. \*  $p \leq 0.05$ .



**Fig. 6.** Examination of c-Myc changes in K562 cells. **A)** Untreated cells; the cells were stained sharply as green color due to FITC green fluorescence. **B)** Treated cells; c-Myc staining (green stain) was decreased indicating c-Myc protein level reduction.

## Discussion

Chronic inflammation is a risk factor in cancer promotion and tumor progression.<sup>14</sup> Nuclear factor kappa B is a transcription factor playing a key role in several cellular processes including fetal growth, cell proliferation, apoptosis and immune response to infection and inflammation. It is an important intra-cellular factor being essential for inflammatory responses.<sup>15</sup> The NF- $\kappa$ B pathway activation has physiological roles in nerve cells, B lymphocytes and thymocytes functions; however, the pathway is inhibited in other cells except for cancer cells including breast, colon, pancreas, ovary, lymphoma and melanoma. The NF- $\kappa$ B activity contributes to tumor progression by inducing the secretion of cytokines and growth factors and activating some anti-apoptotic proteins such as survivin. Due to the roles of NF- $\kappa$ B, the inhibitors of the NF- $\kappa$ B pathway can reduce cancer cell viability.<sup>16</sup> Some of the NF- $\kappa$ B inhibitors include emetine, fluorosalan, metformin, mesalazine and aspirin, which are approved by the United States Food and Drug Administration.<sup>17</sup> Metformin is used to reduce blood glucose in the treatment of type 2 diabetes. It decreases the viability of all ESCC cell lines dose dependently by NF- $\kappa$ B inhibition and causes growth suppression and invasion inhibition in the cell lines.<sup>18</sup> Metformin also alters the expression of NRF-2 and NF- $\kappa$ B in HT29 cells in a dose- and time-dependent manner. It significantly decreases cell viability after 48 hr of treatment compared to 24 hr treatment.<sup>19</sup> Celastrol is a NF- $\kappa$ B inhibitor, also reduces LP-1 myeloma cells proliferation in a dose-dependent manner.<sup>20</sup> It also suppresses the invasion of ovarian cancer cells and inhibits the NF- $\kappa$ B/MMP-9 pathway.<sup>21</sup> Previous studies have shown that sulindac and its metabolites slow down the proliferation of the colon cancer cell lines by inhibiting the NF- $\kappa$ B pathway.<sup>22</sup> In the present study, mesalazine reduced the growth of K562 leukemia cells. Based on the results, it was observed that the viability of the treated cells decreased compared to the controls in a dose- and time-dependent manner being confirmed by morphological changes. Mesalazine induces apoptosis in colon cancer cells by caspase-3 activation without Bcl-2 family proteins levels alteration.<sup>23</sup> Morphological changes observed in cell phenotype of HT29 cells treated with metformin suggest that metformin not only can inhibit cell growth of HT29 cells but also can induce apoptosis in cancer cells through inner and outer apoptosis pathways.<sup>24</sup> It has been shown that the level of Bcl-2 significantly decreases after exposure to celastrol; while, a slight change in Bax level is occurred.<sup>20</sup> In this work, mesalazine-treated cells showed apoptotic nuclei indicating that mesalazine induces apoptosis. In treated cells, Bax expression elevation and Bcl-2 expression reduction led to Bax/Bcl-2 ratio increase indicating that mesalazine induces apoptosis via mitochondrial pathway.

For further investigation of the mesalazine apoptosis inducing effect, c-Myc protein level was analyzed. The c-Myc is a major transcription factor for cell proliferation and it has been implicated in many hematological and solid cancers.<sup>25</sup> In the present study, the results of immunofluorescence assay showed that mesalazine-treated cells had less staining than control cells indicating c-Myc protein level reduction in the treated cells. Mesalazine inhibits the Wnt/B-catenin pathway in CRC cells and increases B-catenin phosphorylation; thereby, reduces c-Myc expression playing an important role in tumorigenesis.<sup>26</sup> In the other hand, c-Myc continuously enhances B cell proliferation by activating NF- $\kappa$ B *in vitro* and *in vivo* leading to tumorigenesis.<sup>27</sup> As observed in this study, mesalazine induces apoptosis probably by NF- $\kappa$ B suppression via c-Myc protein reduction.

In conclusion, the results suggest that mesalazine dose and time dependently decreases cell viability and induces apoptosis via apoptotic internal pathway. Mesalazine may inhibit NF- $\kappa$ B by c-Myc protein reduction. Therefore, based on the results, mesalazine could be a suitable agent for treatment of erythromyeloid leukemia.

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## Conflict of interest

The authors declare no conflict of interest.

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