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Cytotoxic and Apoptotic Effects of C-Myc Inhibition by 10058-F4 on Acute Promyelocytic Leukemia Cells

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ABSTRACT

BACKGROUND AND OBJECTIVE: C-Myc plays a very important role in controlling cell proliferation, apoptosis and differentiation. Due to the involvement of c-Myc in the regulation of a wide range of intracellular functions and based on its role in the pathogenesis of acute promyelocytic leukemia (APL), the present study was conducted to investigate the anti-leukemia effect of 10058-F4, as a known c-Myc inhibitor, on APL-derived HL-60 cells.

METHODS: In this experimental study, to evaluate the cytotoxic effects of 10058-F4 in acute promyelocytic leukemia, HL-60 cell line (prepared from Pasteur Institute of Iran) was treated with different doses of inhibitor (50, 100, 150 and 200 µM) and then in 24 -and 36- hours intervals, survival rate, cell count, metabolic activity and induction of apoptosis were respectively evaluated. In addition, transcriptional changes of apoptosis-related genes were studied by real-time PCR to investigate the molecular mechanisms of function of 10058-F4.

FINDINGS: The results showed that inhibition of c-Myc by 10058-F4 at doses of 150 and 200 µM in 24 hours reduced the growth of HL-60 cells by 38±4% and 49±3.2%, respectively, compared to the control group ($p<0.05$). In addition, the cytotoxic effects of the drug are due to the arrest of cells in the G1 phase and the induction of apoptosis; Because the percentage of cells stained with Annexin V/PI in cells treated at a dose of 100 µM after 24 hours increased by 31% compared to the control group ($p<0.05$).

CONCLUSION: In this study, the efficacy of 10058-F4 in HL-60 cells was fully established.

KEY WORDS: *Acute Promyelocytic Leukemia, C-Myc, 10058-F4, HL-60 Cell Line.*

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of blood malignancies in which we see the unbridled proliferation of neoplastic cells and the accumulation of blasts stopped at a stage of maturity and resistant to cell death in the bone marrow (1). Acute promyelocytic leukemia (APL) accounts for 5-15% of acute myeloblastic leukemias and can cause a high mortality rate for patients with this disease in case of therapy resistance or relapse. One of the current treatments for this disease is the use of arsenic trioxide (ATO), which largely covers the shortcomings of all-trans retinoic acid (ATRA) in inducing apoptosis and preventing the recurrence of the disease (2). At the same time, ATO has significant side effects in high doses and these doses are necessary for its effectiveness (3).

Efforts to untangle the molecular mechanisms involved in chemotherapy and cancer recurrence are advancing rapidly, and recent findings suggest that one of the most important mechanisms involved in immortality of cancer cells and resistance to apoptosis is the activity of the c-Myc protein (4). C-Myc plays a very important role in controlling cell proliferation, apoptosis and differentiation, and its misplaced expression is seen in many human cancers, including APL. (5). C-Myc is dimerized with another protein called Max to bind to a specific sequence on DNA (E-box with CACGTG central motif) in the promoter of its target genes. Therefore, by inhibiting c-Myc/Max dimerization, c-Myc binding to DNA can be prevented and thus its function inhibited (6).

Due to the important role of c-Myc in the survival of cancer cells, its inhibition can lead to the destruction of tumor cells. Due to the oncogenic properties of c-Myc, inhibition of this gene in various cancers has been considered today. Numerous inhibitors have been developed to target c-Myc. In an overview of these inhibitors, it can be said that Myc inhibitors are divided into two categories, direct and indirect, each of which has different types (7). Based on the above category, 10058-F4 is a direct Myc inhibitor (8).

This drug prevents the heterodimerization of c-Myc and Max and thus prevents its function. So far, various studies have been performed on the effect of this compound on various cancer cells and it has been shown that 10058-F4 inhibits the proliferation of liver, prostate and breast cancer cells (9, 10). Given the worrying side effects and deficiencies of current drugs used to treat APL as well as the expression of c-Myc protein in acute promyelocytic leukemia, these patients appear to be suitable candidates for treatment with c-Myc inhibitors. For this purpose, and to evaluate the efficacy of c-Myc inhibition strategy in APL disease, HL-60 cells were treated with different concentrations of 10058-F4 and the results were evaluated.

Methods

Cell culture and treatment: In this experimental study, after approval by the Research Ethics Committee of Shahid Beheshti University of Medical Sciences with the code IR.SBMU.RETECH.REC.1397.1012, HL-60 cells (derived from acute promyelocytic leukemia) (prepared from Pasteur Institute of Iran) were cultured in RPMI1640 medium (Gibco, USA) with 10% FBS, 100 U/ml penicillin and 100 g/ml streptomycin and stored in an incubator at 37 °C and under 5% carbon dioxide pressure. HL-60 cells were treated with different concentrations of 10058-F4 (50-200 µM) (Selleckchem, Germany) at 24 and 36 hours. In all experiments, HL-60 cells cultured in RPMI1640 medium without drug treatment were considered as the control group. All experiments were performed in triplicate to increase the accuracy of the experiments.

Trypan Blue Assay: To evaluate the effect of 10058-F4 drug on cell viability, HL-60 cells were incubated at 5×10^5 cells/ml in the presence of different doses of the drug for 24 hours. The treated cells were then mixed with 0.4% Trypan Blue dye at a ratio of 1:1, incubated for 1 to 2 minutes and then counted. Cells that absorb this dye are considered dead cells and cells that do not absorb any dye are considered live. Then, the cell survival was calculated using the following formula:

Survival rate (%)= number of live cells ÷ total number of cells × 100

Evaluation of cellular metabolic activity by MTT assay: To evaluate the effect of 10058-F4 on the metabolic activity of HL-60 cells, 5000 cells were added to each 96-well plate containing no drug and incubated for 24 hours in a CO_2 incubator. After the desired time, MTT solution (5 mg/ml) was added to the cells inside the plate and incubated at 37 °C for 3 hours. The plate was then centrifuged at 1000g for 10 minutes and after emptying the supernatant, 100 µl of DMSO was added to each well. The light absorption of each well was read by ELISA reader at 570 nm.

Evaluation of apoptosis index using flow cytometry: In order to evaluate the effect of drug on induction of programmed cell death (apoptosis), 5×10^5 cells were poured into each 12-well plate and treated with different

concentrations of 10058-F4 drug for 24 hours. After washing the cells with phosphate-saline buffer (PBS) and adding Annexin V-FITC/PI reagents (Roche, Germany), and incubation buffer, the samples were incubated for 15 minutes in the dark at room temperature. Staining of cells was performed using flow cytometry (PartecPasIII) with excitation wavelength of 488 nm and reflection of 518 nm. Data analysis was performed by FlowJo 7.6.1 software.

Evaluation of cell cycle activity: HL-60 cells were placed in 6-well plates. Then, they were treated with 10058-F4 for 24 hours and after incubation period, the cells were centrifuged at 2000 rpm for 5 minutes. Then, the cells were fixed in 70% ethanol and were kept at -20°C for 12 hours. Then, 0.5 µg/ml of RNase solution was added to PBS and after adding 50 µg/ml PI, the contents of cellular DNA were studied by flow cytometry and its analysis was performed through the obtained graphs.

Measurement of caspase-3 enzyme activity: To investigate whether 10058-F4-induced cell death in HL-60 cell line was caspase-dependent or not, caspase-3 enzyme activity was evaluated by caspase-3 kit (Sigma, USA). This test is based on the spectrophotometric detection of p-nitroaniline (pNA) molecule attached to the end of a specific caspase substrate. The cells were treated with the desired concentrations of 10058-F4 for 24 hours. After the desired time and centrifugation at 600 rpm for 5 minutes, to lyse the cell pellet, lysate buffer was added. It was centrifuged again at 20,000 rpm for 10 minutes. In a total volume of 100 µl, 5 µg of supernatant was incubated with 85 µl of assay buffer along with 10 µl of caspase 3 substrate for 2 hours in 96-well plate. Breakdown of the peptide by caspase-3 results in the release of pNA dye, which can be measured with a spectrophotometer at 405 nm.

RNA extraction and cDNA synthesis: After treating the cells with 50 and 100 µM 10058-F4, RNA extraction was performed after 24 hours using TRIzol. The quantity and purity of the extracted RNA were evaluated by spectrophotometry using 2000 Nanodrop ND apparatus. A cDNA synthesis kit (Takara, Japan) was used to reverse the transcription reaction. For cDNA synthesis according to the brochure, the samples were incubated for 15 minutes at 37°C and 15 seconds at 85°C.

Quantitative analysis of c-Myc, Bax and Bcl-2 genes: Quantitative Real Time PCR was performed for quantitative analysis of the expression of c-Myc, Bax and Bcl-2 genes. ABL gene was used as housekeeping gene in the assay process. For each reaction, 10 µl of SYBR green master mix (Amplicon), 2 µl of cDNA, 0.5 µl of each primer (10 picomolar) and 7 µl of nuclease-free distilled water were used. The temperature conditions used include an initial activation step at 95°C for 30 seconds followed by 40 cycles for denaturation (5 seconds, 95°C) and a combined connection/rearrangement step (20 seconds, 60°C). In order to investigate the specificity of the amplified product, the melting curve was evaluated. Furthermore, the relative number of amplified mRNA copies was calculated using the formula $2^{-\Delta\Delta ct}$. The sequence of primers used to perform the test is shown in Table 1.

Statistical analysis: All experiments were performed in the form of three independent tests and the reported values were recorded as Mean±SD. After ensuring the normal distribution of data using Shapiro-Wilk test and equality of variances using Leven test, in order to examine the intergroup differences, the one-way analysis of variance (One-Way ANOVA) test and Tukey post-hoc test were used. Calculations were performed using SPSS 23 software and $p<0.5$ was considered significant.

Table 1. Sequence of primers used in Quantitative Real Time PCR

Gene	Accession number	Reverse Primer (5' to 3')	Forward Primer (5' to 3')	Size (bp)
ABL	NM_005157	TCCTCGTCCTCCAGCTGTTA	ACCCGGAGCTTTTCACCTTT	218
c-Myc	NM_002467	CCACAGCAAACCTCCTCACAG	GCAGGATAGTCCTTCCGAGTG	105
Bax	NM_138761	GTGGGCGTCCCAAAGTAGG	CGAGAGGTCTTTTTCCGAGTG	242
Bcl-2	NM_000633	CGGTTCAGGTACTCAGTCATCC	CGGTGGGGTCATGTGTGTG	249

Results

10058-F4 reduces the survival rate of HL-60 cells in a dose- and time-dependent manner: The results showed that with increasing dose and over time, the survival rate of drug-treated HL-60 cells decreased. Although a dose of 50 µM of 10058-F4 did not significantly affect the survival rate of HL-60 cells, 24-hour treatment of cells with higher doses of this inhibitor (150 and 200 µM) resulted in a decrease in survival rate by 38±4 and 49±3.2% (Figure 1). In

general, this result indicates the cytotoxic effect of this drug in a dose- and time-dependent manner in HL-60 cells compared to the control group ($p<0.05$).

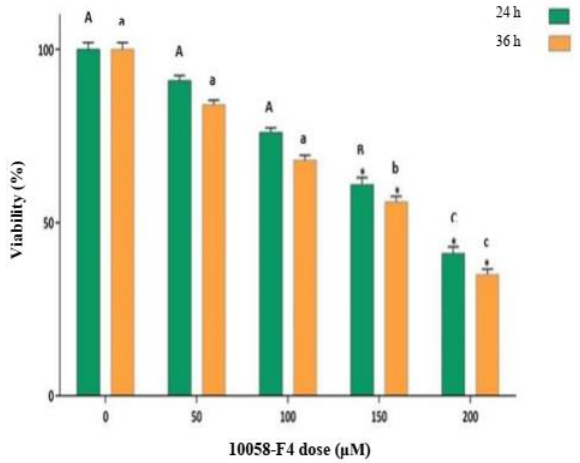

Figure 1. Effect of 10058-F4 on HL-60 cell viability. **Cells were treated with different doses of 10058-F4 for 24 and 36 hours, then cell viability was assessed by Trypan Blue Assay. (Capital letters are for 24 hours and lowercase letters are for 36 hours).**

Treatment of HL-60 cells with 10058-F4 drug reduces the rate of cell proliferation in a dose- and time-dependent manner: Results of live cell count after exposure to 10058-F4 drug by incubation for 24 hours and 36 hours showed that this inhibitor has anti-proliferative effects on cells derived from acute promyelocytic leukemia. Compared to the control group, treatment of cells with different doses of 10058-F4 reduced the number of living cells (Figure 2).

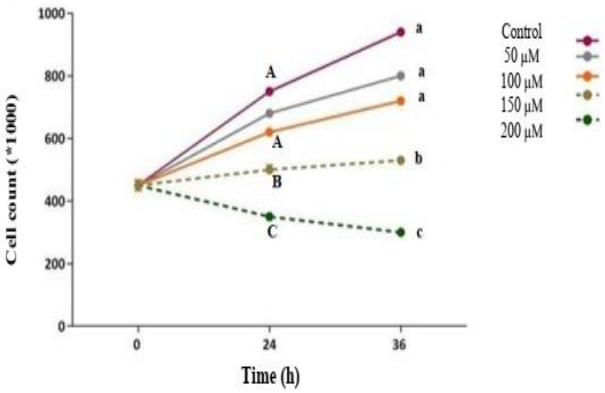

Figure 2. Dose-dependent effect of 10058-F4 on cell count in HL-60 cells. **HL-60 cells were treated at 105±5 with concentrations of 50 to 200 µM of 10058-F4. As shown in the figure, treatment with 10058-F4 decreased cell count in a dose- and time-dependent manner (Capital letters are for 24 hours and lowercase letters are for 36 hours).**

It should be noted that in confirmation of the results obtained from the analysis of cell viability, the number of cells reached 370,000±14,000 after exposure to 10058-F4 at a dose of 200 µM within 36 hours, which is significant compared to the control group ($p<0.05$) and among the doses used, it shows the greatest effect in reducing the number of cells.

10058-F4 reduces the metabolic activity of HL-60 cells: The metabolic activity of promyelocyte cells decreased dose- and time- dependently after drug exposure based on MTT assay. Treatment of cells with the highest dose of the drug (200 µM) reduced metabolic activity by approximately 63±3% after 24 hours ($p<0.05$) (Figure 3). In addition, the inhibitory effect increases over time; After 36 hours of cell treatment with a dose of 200 µM, the metabolic activity of HL-60 cells decreased by 76±4% ($p<0.05$) (Figure 3).

10058-F4 increases the population of HL-60 cells in the G1 phase of the cell cycle: In this study, it was shown that inhibition of c-Myc in HL-60 tumor cells stops the progression of the cell cycle and thus the accumulation of cell populations in Phase G1 (Figure 4). Examination of the cellular DNA content also revealed that 10058-F4 could increase the number of cells present in the Sub-G1 phase of the cell cycle (indicating dying cells). The percentage of this cell population at the 150 µm dose of the drug increased from 5.9±2.2% to about 25±3.1% compared to the control group ($p<0.05$).

10058-F4 induces apoptosis in HL-60 cells: Examination of the rate of phosphatidylserine externalization on the surface of drug-treated cells and comparison with the control group showed that the drug was able to induce apoptosis in HL-60 cell line. The c-Myc inhibitor was able to increase apoptosis five-fold at a dose of 150 µM compared to the control group (Figure 5). The results show that 10058-F4 reduces the survival of these cells by inducing apoptosis in HL-60 cells and this way, exerts its cytotoxic effect. To investigate whether apoptosis induced by the c-Myc inhibitor occurs through activation of the enzyme caspase-3, HL-60 cells were treated with the same doses used for the Annexin V/PI assay, and after 24 hours, the enzyme activity of caspase-3 was evaluated by caspase-3 assay kit. The results of this test also showed that inhibition of c-Myc in HL-60 cells is associated with dose-dependent activation of the enzyme caspase-3 (Figure 4) and probably, the c-Myc inhibitor induces apoptosis in this cell line through active caspase-dependent pathways.

Increased Bax expression and decreased Bcl-2 expression following treatment of Hl-60 cell line with 10058-F4: The results of Quantitative Real Time PCR test indicate that treatment of HL-60 cells with 10058-F4 is associated with increased expression of pro-apoptotic Bax gene and decreased expression of the anti-apoptotic Bcl-2 and c-Myc genes. Due to the increase in Bax gene expression and decreased Bcl-2 expression, the Bax/Bcl-2 ratio in cells exposed to doses of 100 and 150 µM was associated with an increase compared to control cells, which showed itself with an increase in apoptosis (Figure 6).

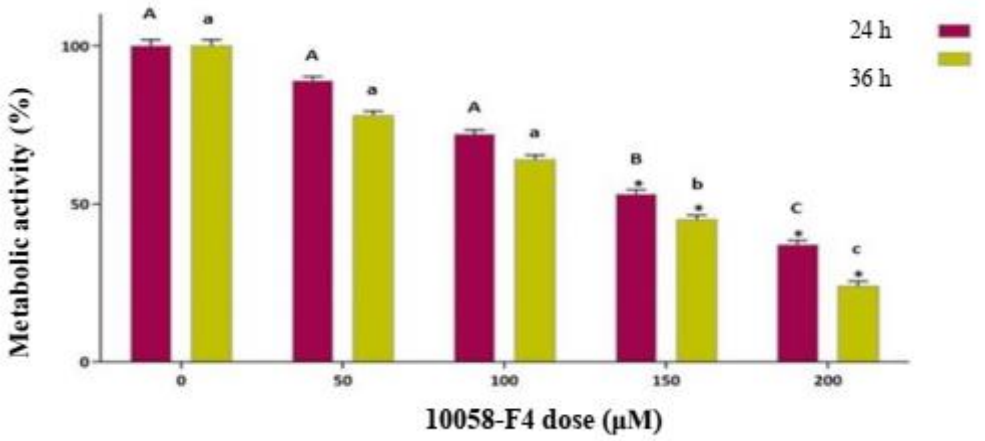

Figure 3. Evaluation of the effect of 10058-F4 on cellular metabolic activity of HL-60. **Cells were incubated in medium containing different doses of 10058-F4 for 24 and 36 hours and then MTT assay was performed for them. The IC50 in the study is estimated to be about 150 µM over 36 hours. (Capital letters are for 24 hours and lowercase letters are for 36 hours).**

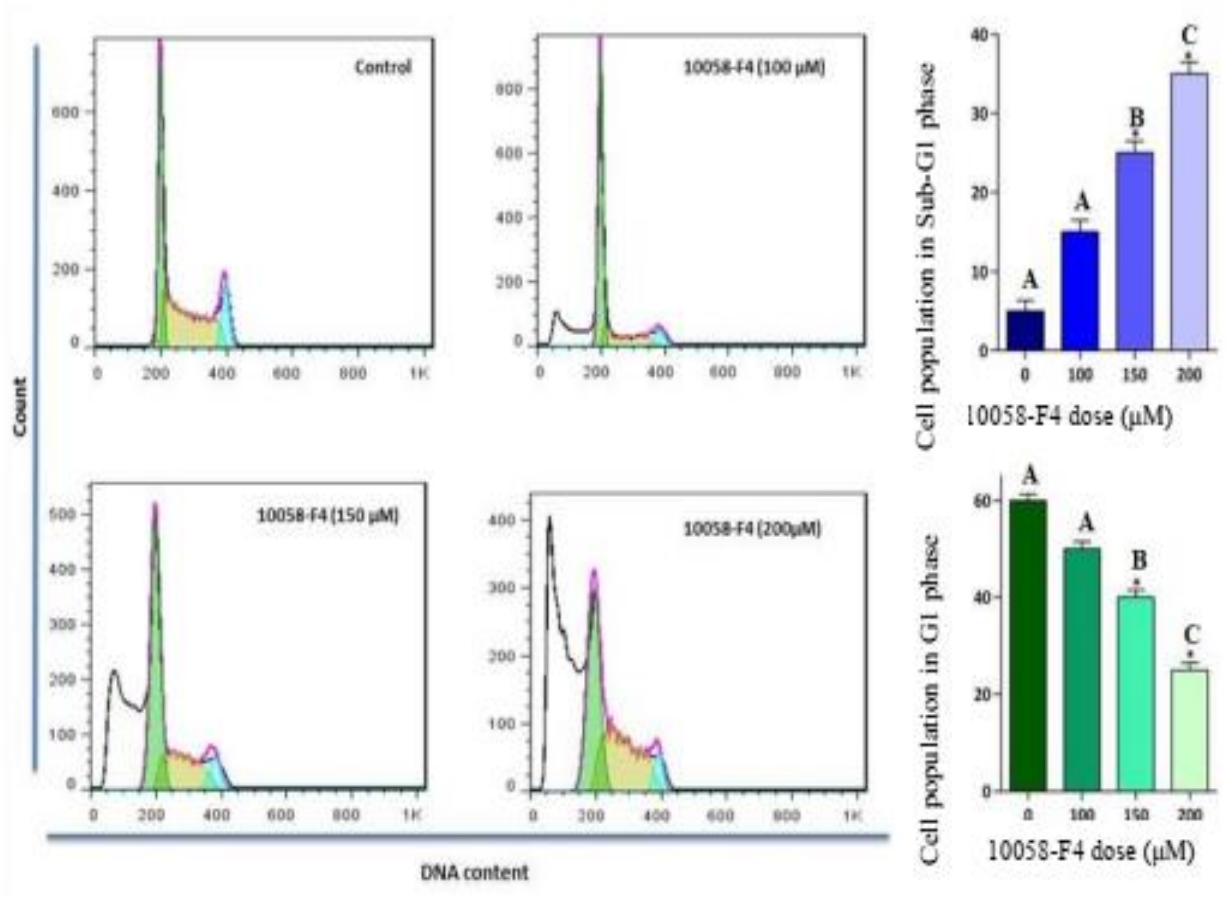

Figure 4. Effect of 10058-F4 on the distribution of HL-60 cells at different stages of the cell cycle. **Flow cytometric results of the distribution of inhibitor-treated cells showed that 10058-F4 increased cell growth in the G1 and Sub-G1 phases of the cell cycle.**

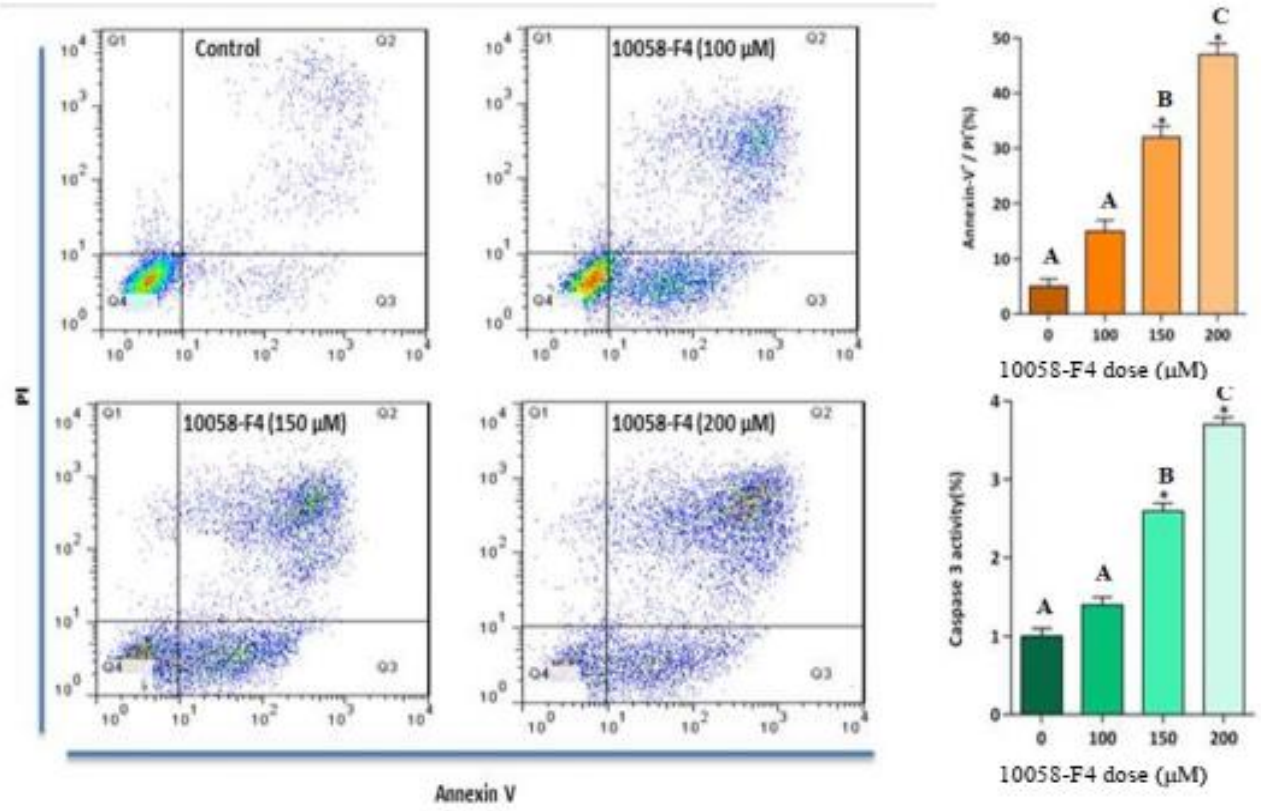

Figure 5. Evaluation of the percentage of apoptotic cell population after treatment with different doses of 10058-F4. **Treatment of HL-60 cells with c-Myc inhibitor increases the population percentage of both Ann-V$^{\pm}$ and Ann-V/PI$^{\pm}$ cells. Induction of apoptosis and enzyme activity of caspase-3 in HL-60 cell line were evaluated after treatment with 10058-F4.**

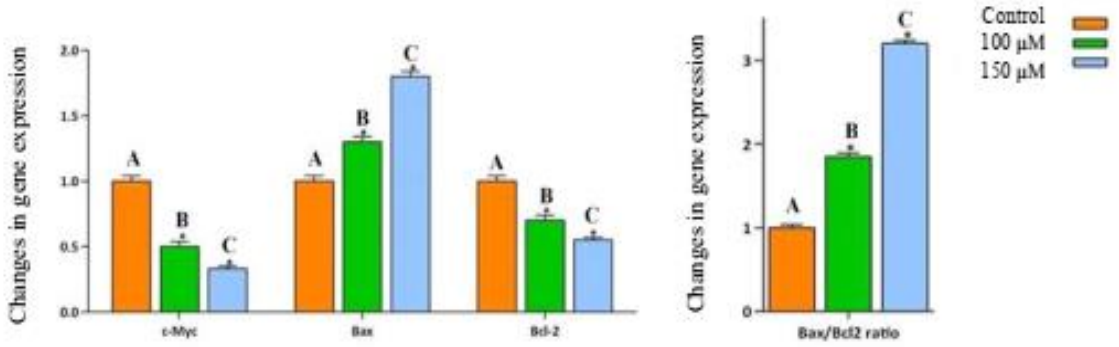

Figure 6. Increase in transcription of pro-apoptotic Bax gene and decrease in mRNA level of anti-apoptotic Bcl-2 and c-Myc genes during 24-hour treatment of HL-60 cells. **Cells were treated with 100 and 150 µM doses of 10058-F4 for 24 hours and after RNA extraction and cDNA synthesis, they were subjected to real-time PCR to evaluate the expression of the desired genes.**

Discussion

The results of this study confirm the effectiveness of 10058-F4 (which is a type of c-Myc inhibitor) as a cytotoxic and antiproliferative drug for HL-60 cells derived from acute promyelocytic leukemia. As an important transcription factor, the c-Myc oncogene is involved in many intracellular physiological activities such as cell cycle regulation, proliferation, proliferation, survival, apoptosis, transcription, translation, and cellular metabolism. For example, c-Myc is one of the major regulators of the hTERT gene, a catalytic unit of the human reverse transcriptase enzyme, which increases the expression and activity of hTERT, resulting in the proliferation and survival of cancer cells (11). The results of Trypan Blue Assay and MTT test

showed that cell proliferation and metabolic activity decreased significantly with increase in the dose and time of drug treatment of cells; a dose of 200 µM 10058-F4 has reduced metabolic activity and the number of live cells by 2 to 3 times compared to the dose of 50 µM of the same drug. Moreover, the 150 µM dose of c-Myc inhibitor reduced metabolic activity from 53% to 44% over a period of 24 hours to 36 hours. In a study by Lin et al., in hepatocellular carcinoma cell lines, 10058-F4 induced apoptosis by activating the mitochondrial pathway (12).

Furthermore, another study showed that inhibition of c-Myc function by reducing ROS levels significantly reduced the proliferation of ovarian cancer cells (13, 16). Although the anti-cancer properties of c-Myc have been demonstrated in many studies, the number of studies focusing precisely on the molecular mechanism of this inhibitor is limited (13). Escape from programmed cell death is one way for tumor cells to survive and develop drug resistance in patients. Symptoms of apoptotic cells include membrane germination, cell shrinkage, nuclear fragmentation, chromosome compression, DNA fragmentation, and increased phosphatidylserine expression on the outer surface of the cell (14).

To evaluate the induction of apoptosis after treatment with 10058-F4, the surface expression of phosphatidylserine was investigated by flow cytometry, which was confirmed by the results. The rate of apoptotic cells at a dose of 200 µM of the drug reached 63%. Due to the relevance of the c-Myc pathway in promoting proliferation and cell cycle, its inhibition is expected to cause changes in these processes. The results showed that 10058-F4 drug can activate the mechanisms of apoptosis in this cell line by inhibiting the metabolic activity of cells and keeping them in the Sub-G1 cell cycle, in addition to reducing cell proliferation. Many proteins are involved in the apoptotic process, the two most important of which are Bax and Bcl-2 proteins, and are directly or indirectly regulated by c-Myc (15).

Unlike Bax, which is a pro-apoptotic protein, Bcl-2 is anti-apoptotic and its reduction triggers the apoptotic pathway. As a result, the imbalance between the two proteins, that is, the increase in Bax relative to Bcl-2, is expected to lead the cell to apoptosis (16). Considering that in this study, after treating HL-60 tumor cells with a dose of 150 µM, the expression level of Bax was 3.21 times more than the expression level of Bcl-2, it is possible that 10058-F4 may have induced acute promyelocytic leukemia cells to apoptosis by altering the expression of these genes.

Based on all the information obtained from this study, it can be concluded that inhibition of c-Myc using 10058-F4 drug can lead to induction of apoptosis in HL-60 tumor cells, followed by reduction in the rate of proliferation and survival of these cells. It is hoped that 10058-F4 can be used as a new drug in the treatment of patients with acute promyelocytic leukemia, despite very few side effects. Of course, there is still a need for further investigation into how this drug works and its clinical effects in the treatment of this malignancy.

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