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Combination Effect of *MicroRNA-15a* and *MicroRNA-16-1* on Chemosensitivity of the Leukemia Cells

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ABSTRACT

Background: Down-regulation of *miRNA-15a* and *miRNA-16-1* is associated with Bcl-2 and Mcl-1 expression and chemoresistance in tumor cells. In this study, the combined effect of *miRNA-15a* and *miRNA-16-1* on apoptosis and sensitivity of the chronic lymphocytic leukemia (CLL) cells to fludarabine and ABT-199 was investigated.

Materials and Methods: The experiment groups were as follows: ABT-199, negative control (NC) miRNA, *miRNA-15a*, miRNA-16, *miRNA-15a*+*miRNA-16*, NC miRNA+ABT-199, *miRNA-15a*+*miRNA-16*+ABT-199, erlotinib blank control, miRNA blank control and combination blank control. The expression levels of *Bcl-2* and *Mcl-1* were measured using quantitative real-time PCR (qRT-PCR). The effect of treatments on cell growth and survival was measured by trypan blue staining and MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assay, respectively. Apoptosis was measured using caspase-3 activity and ELISA cell death assays.

Results: Transfection of *miRNA-15a* or *miRNA-16-1* significantly suppressed the expression of *Bcl-2* and *Mcl-1* in a time-dependent manner ($P<0.05$ versus negative control miRNA or blank control). Other experiments showed that up-regulation of *miRNA-15a* or *miRNA-16-1* decreased cell growth, induced apoptosis, and synergistically lowered the IC_{50} value of fludarabine and ABT-199 in CLL cells. Moreover, co-transfection of two miRNAs showed a more significant effect on cell survival, apoptosis, and drug sensitivity than single transfection.

Conclusion: Our study shows that *miRNA-15a* and *miRNA-16-1* can effectively enhance the anticancer effects of fludarabine and ABT-199 in CLL cells and may offer a new promising therapeutic strategy for CLL-resistant patients.

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Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent form of leukemia, characterized by a gradual accumulation of mature B lymphocytes [1, 2]. Numerous signaling pathways contribute to the defective apoptotic response and increased survival in CLL [3]. Despite countless advances in the treatment of CLL, the disease finally relapses and therefore, additional therapy is needed [4]. Therefore, understanding the mechanisms leading to resistance and developing novel therapeutics are required.

The intrinsic pathway of apoptosis is under the control of Bcl-2 family proteins that comprise two classes of prosurvival proteins (Bcl-2, Mcl-1, Bcl-xL, Bcl-w, etc.) and proapoptotic proteins (Bad, Bid, Bax, Bak, etc.) [5, 6]. It has become clear that high expression levels of prosurvival proteins such as Mcl-1 and Bcl-2 correlate with resistance to treatment with fludarabine and shorter overall survival in CLL patients. Given their role in the leukemogenesis and survival of CLL cells, targeting prosurvival proteins is considered an attractive strategy for improving apoptosis-based therapies in CLL [7, 8].

ABT-199 (venetoclax) is an oral Bcl-2-specific inhibitor that binds to Bcl-2 but not to Mcl-1 and Bcl-xL. Although ABT-199 has shown high cytotoxic activity in CLL cells and improved clinical outcomes in CLL patients, the cells expressing high levels of Mcl-1 demonstrate resistance to this agent. Therefore, a rational combination therapeutic approach through Mcl-1 inhibitors could effectively overcome ABT-199 resistance in CLL patients [9-11].

MicroRNAs (miRNAs) are a group of evolutionarily conserved, 18-25 nucleotides RNAs that specifically bind to the 3' untranslated region (3'-UTR) of target messenger RNAs (mRNAs), leading to the translation inhibition or mRNA degradation [12]. Today, it is clear that miRNAs act as important biomarkers in numerous cancers, such as lung, pancreas, colorectum, and breast [13]. Moreover, miRNAs are involved at different levels of hematopoiesis and lymphopoiesis, suggesting the pivotal role of miRNAs in CLL [14]. Genetic abnormalities have been diagnosed in more than 80% of CLL patients. These aberrations include the low-risk 13q deletion, intermediate-risk 11q deletion, and high-risk 17p deletion [15]. The most common genetic change seen in more than 50% of CLL cases is 13q14 deletion. Moreover, miRNAs are correlated with chromosomal aberrations. Previous studies have demonstrated that the *miRNA-15a*, *miRNA-16-1* and *DLEU2* genes were absent or down-regulated in CLL patients with 13q14 deletion [16]. *MiRNA-15a* and *miRNA-16-1* act as tumor suppressor genes by inhibiting several molecules such as cyclin D1, cyclin D3, cyclin-dependent kinase 6 (CDK6) and Bcl-2, which are involved in cell growth and apoptosis [17]. In addition, bioinformatics and experimental data have shown a significant association between the expression of *miRNA-15a* and *miRNA-16-1* with Mcl-1 expression in CLL cell lines and patients' samples [14]. However, little is known about the role of *miRNA-15a* and *miRNA-16-1* in apoptosis and drug resistance of CLL cells.

We hypothesized that down-regulation of *miRNA-15a* and *miRNA-16-1* would inhibit cell apoptosis and confer resistance to fludarabine and ABT-199 via the suppression of Mcl-1 and Bcl-2 expression. Therefore, this study investigated the effect of *miRNA-15a* and *miRNA-16-1* on the sensitivity of the CLL cells to fludarabine and ABT-199.

Materials and Methods

Cell culture

The CLL-CII cell line (Pasteur Institute, Tehran, Iran) was propagated in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich), antibiotics (100 U/mL penicillin-streptomycin) (Sigma-Aldrich), 2 mM of glutamine and 1% sodium pyruvate at 37 ˚C in a humidified atmosphere containing 5% CO_2. The cells were subcultured every 48-72 h with an initial concentration of 1×10^5 cells/mL and used in exponentially growing cultures in whole experiments.

MicroRNA transfection

The miRNA used in transfection experiments as *miRNA-15a* mimic was 5'-UAG CAG CAC AUA AUG GUU UGU G-3', *miRNA-16-1* mimics: 5'-UAG CAG CAC GUA AAU AUU GGC G-3' and negative control (NC) miRNA: 5'-ACU ACU GAG UGA CAG UAG-3'. All of these were ordered from Dharmacon (Lafayette, Co., USA). The cells were transfected with miRNAs using Lipofectamine™2000 reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM I reduced serum medium (Invitrogen) according to the manufacturer's protocol. Optimal concentrations of miRNAs (10 nM) and lipofectamine (3 µL/mL) for transfection were determined empirically.

Quantitative real-time PCR (qRT-PCR)

At different time points after treatments, total cellular RNA was extracted by AccuZolTM reagent (Bioneer, Daedeok-gu, Daejeon, Korea) according to the manufacturer's protocol. cDNA was synthesized from 1 µg of purified total RNA using MMLV reverse transcriptase (Promega, Madison, WI, USA) and oligo-dT primer according to the manufacturer's instructions. qRT-PCR was performed on a LightCycler 96 System (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan). Each PCR reaction had the following components: 12 µL of SYBR green reagent, 1 µL of RT product, 0.2 µM of the forward and reverse primers, and 6 µL of nuclease-free distilled water. The specific primer sequences were as follows: Forward, 5'- TAA GGA CAA AAC GGG ACT GG-3' and reverse 5'-ACC AGC TCC TAC TCC AGC AA-3' for *Mcl-1*; forward, 5'-ATC GCC CTG TGG ATG ACT GAG -3' and reverse, 5'-GCC AGG AGA AAT CAA ACA GAG GC-3' for *Bcl-2*, and forward, 5'-TCC CTG GAG AAG AGC TAC G-3' and reverse, 5'-GTA GTT TCG TGG ATG CCA CA-3' for β–actin. The initial denaturation step at 95 ˚C for 5 min was followed by 40 cycles at 95 ˚C for 15 s and 60 ˚C for 1 min. The relative transcript abundance was measured with the $2^{-(\Delta\Delta Ct)}$ method [18, 19], using β-actin as an internal control.

3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay

The effect of *miRNA-15a* and *miRNA-16-1* on the response of CLL cells to ABT-199 and fludarabine was investigated using MTT assay. In brief, the cells were seeded in 96-well plates (6×10^4 cells per well) and then transfected with miRNAs for 6 h. Next, the cells were treated with various concentrations of fludarabine and ABT-199 (0-3.2 µM). Twenty-four and 48 hours after transfection, the cytotoxicity was determined using an MTT assay kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. The absorbance at 490 nm was measured spectrophotometrically with a multi-plate reader (Awareness Technology, Palm City, FL, USA). IC_{50} (half maximal inhibitory concentration) values were calculated using Prism software, version 6.01 (GraphPad Software Inc., San Diego, CA, USA).

Analysis of combined drug effects

To further explore the effect of combination therapy, the combination index (CI) analysis was performed based on the principles described by Chou and Talalay [20, 21]. The cell survival values of the MTT assay were converted to fraction affected (Fa; where Fa=1 is 0% cell survival and Fa=0 is 100% cell survival) and analyzed by CompuSyn software, version 1.0 (ComboSyn Inc., Paramus, NJ, USA). CI<1, CI>1, or CI=1 indicate synergy, antagonism or additivity, respectively.

Cell growth assay

The effect of treatments on cell growth was determined by trypan blue exclusion assay. CLL cells (1×10^5 cells/well) were treated with *miRNA-15a*, *miRNA-16-1*, fludarabine, and ABT-199 in 6-well cell culture plates for 24-120 h as described in the MTT assay. At different time points, the cells were collected and stained with 0.4% trypan blue (Merck KGaA, Darmstadt, Germany) for 3 min. Next, the number of viable cells (N, unstained cells) was quantified by an inverted microscope (Nikon Instrument Inc. Melville, NY, USA) and a hemocytometer. The cell viability was determined from the Equation 1:

1. Cell viability (%)=$(N_{Test}/N_{Control})\times100$.

The cell viability percentage for the blank control group was considered as 100%.

Apoptosis ELISA assay

The effect of *miRNA-15a*, *miRNA-16-1*, fludarabine, and ABT-199 on apoptosis was evaluated with a cell death detection ELISA plus kit (Roche Diagnostics GmbH) that measures the amount of cytosolic mono- and oligo-nucleosomes produced during apoptosis. The CLL-CII cells were plated at a density of 1×10^5 cells/well in 6-well plates. Twenty-four and 48 hours after treatment, cells were harvested and resuspended in lysis buffer. Following centrifugation, 20 µL of the cell supernatants and 80 µL of immunoreagent containing anti-histone-biotin and anti-DNA-peroxidase were transferred to each well of a streptavidin-coated plate. After 2 h of incubation at 25 ˚C, the wells were washed, and 100 µL of 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) solution was transferred to each well. The reactions were stopped with ABTS stop solution, and absorbance was determined at 405 nm using a microplate reader. Results were shown as the fold increase in the absorbance of treated cells compared to the control group.

Caspases-3 activity assay

Induction of caspase-3 activity was measured using a colorimetric caspase activity assay kit according to the manufacturer's protocol (Abnova, Taipei, Taiwan). In brief, cells were resuspended in 50 µL of chilled

cell lysis buffer and incubated on ice for 10 min. The cell suspension was centrifuged at 10000×g for 1 min. Next, 50 µL reaction buffer and 5 µL of 4 mM caspase-3 (DEVD-pNA) colorimetric substrate were added to each cell supernatant and incubated at 37 ˚C for 24 h. The absorbance was measured spectrophotometrically using a microplate reader (Awareness Technology, Palm City, FL, USA) at a wavelength of 405 nm.

Statistical analysis

Quantitative data are presented as the Mean±SD of at least three independent experiments. Statistical evaluation was performed using analysis of variance (ANOVA) and Bonferroni test to differentiate the means of different groups. A P<0.05 was considered statistically significant. GraphPad Prism software was used to analyze all data.

Results

MiRNA-15a and *MiRNA-16-1* effect on ABT-199 and increasing Mcl-1 expression

First, we examined the effect of treatments on Mcl-1 and Bcl-2 expression in CLL cells by qRT-PCR. Results of qRT-PCR demonstrated that 24 and 48 h transfection of *miRNA-15a* or *miRNA-16-1* significantly reduced the expression of both *Mcl-1* and *Bcl-2* mRNA relative to the blank control (Figure 1). After treatment of the cells with ABT-199, the relative *Mcl-1* mRNA expression was significantly increased, while the expression levels of Bcl-2 mRNA did not change. PCR results also showed that combination treatment with specific miRNAs and ABT-199 did not change the expression levels of *Bcl-2* mRNA compared to the *miRNA-15a* or *miRNA-16-1*. Moreover, the expression of Mcl-1 mRNA in this combination group was higher and lower than in the cells treated with only specific miRNAs or ABT-199, respectively. An insignificant effect on mRNA expression was observed in NC miRNA or fludarabine-treated cells (P>0.05). Together, these results indicate that ABT-199 increases the expression of *Mcl-1* in CLL cells and *miRNA-15a* and *miRNA-16-1* can counteract this effect.

MiRNA-15a and *miRNA-16-1* sensitization of CLL-CII cells to fludarabine and ABT-199

To investigate whether down-regulation of Bcl-2 and Mcl-1 by miRNAs can sensitize CLL-CII cells to fludarabine and ABT-199, a combination treatment with specific miRNAs, fludarabine and ABT-199 was applied. As measured by MTT assay, monotherapy with fludarabine or ABT-199 caused a dose- and time-dependent reduction of cell survival (Figures 2 and 3). The IC_{50} values of 24 and 48 h were respectively 0.35 and 0.26 µM for ABT-199 and 1.18 and 0.79 µM for fludarabine (Table 1). Twenty-four hours after transfection of the *miRNA-15a* and *miRNA-16-1*, the cell survival rates decreased to 90.5% and 87%, respectively, relative to the blank control (Figure 2; P<0.05). However, 48 h transfection lowered the cell survival rates to 86% and 83.5%, respectively (Figure 3; P<0.05). Furthermore, compared with a single treatment, combination therapy further reduced the cell survival of the leukemia cells. Single transfection of *miRNA-15a* or *miRNA-16-1* markedly reduced the IC_{50} values of fludarabine or ABT-199 at indicated time points. Moreover, the combination of *miRNA-15a* and *miRNA-16-1* further reduced the IC_{50} of treatments relative to the single transfection (Tables 1, 2 and 3; P<0.05). The effect of NC miRNA on the cell survival and chemosensitivity of the cells was minimal (P>0.05).

Combination effects of specific MiRNAs with fludarabine and ABT-199 on CLL cells

To explore whether the effects of miRNAs with fludarabine and ABT-199 on the survival of the CLL cells are responsible for their synergistic interaction, we performed the CI analysis using CompuSyn software. The CI–Fa plots indicated that the combination effects of *miRNA-15a* (50 nM) and *miRNA-16-1* (50 nM) with fludarabine or ABT-199 were synergistic (CI<1) in all of the combinations. The results of the CI analysis are shown in full in Figures 4 and 5.

MiRNA-15a and *miRNA-16-1* and the growth inhibitory effects of fludarabine and ABT-199 in CLL Cells

As up-regulation of Bcl-2 and Mcl-1 is associated with the growth and survival of cancer cells, we sought to examine the effect of *miRNA-15a* and *miRNA-16-1*, alone and in combination with fludarabine and ABT-199, on CLL cells. Results showed that monotreatment with *miRNA-15a*, *miRNA-16-1*, fludarabine, or ABT-199 significantly decreased the cell viability time-dependently (Figure 6). *MiRNA-15a* and *miRNA-16-1* enhanced the growth inhibition effects of fludarabine and ABT-199. Furthermore, fludarabine, in combination with ABT-199, showed a stronger effect on inhibiting cell growth than fludarabine or ABT-199 alone (P<0.05). NC miRNA had no significant effect on cell growth compared to the control group.

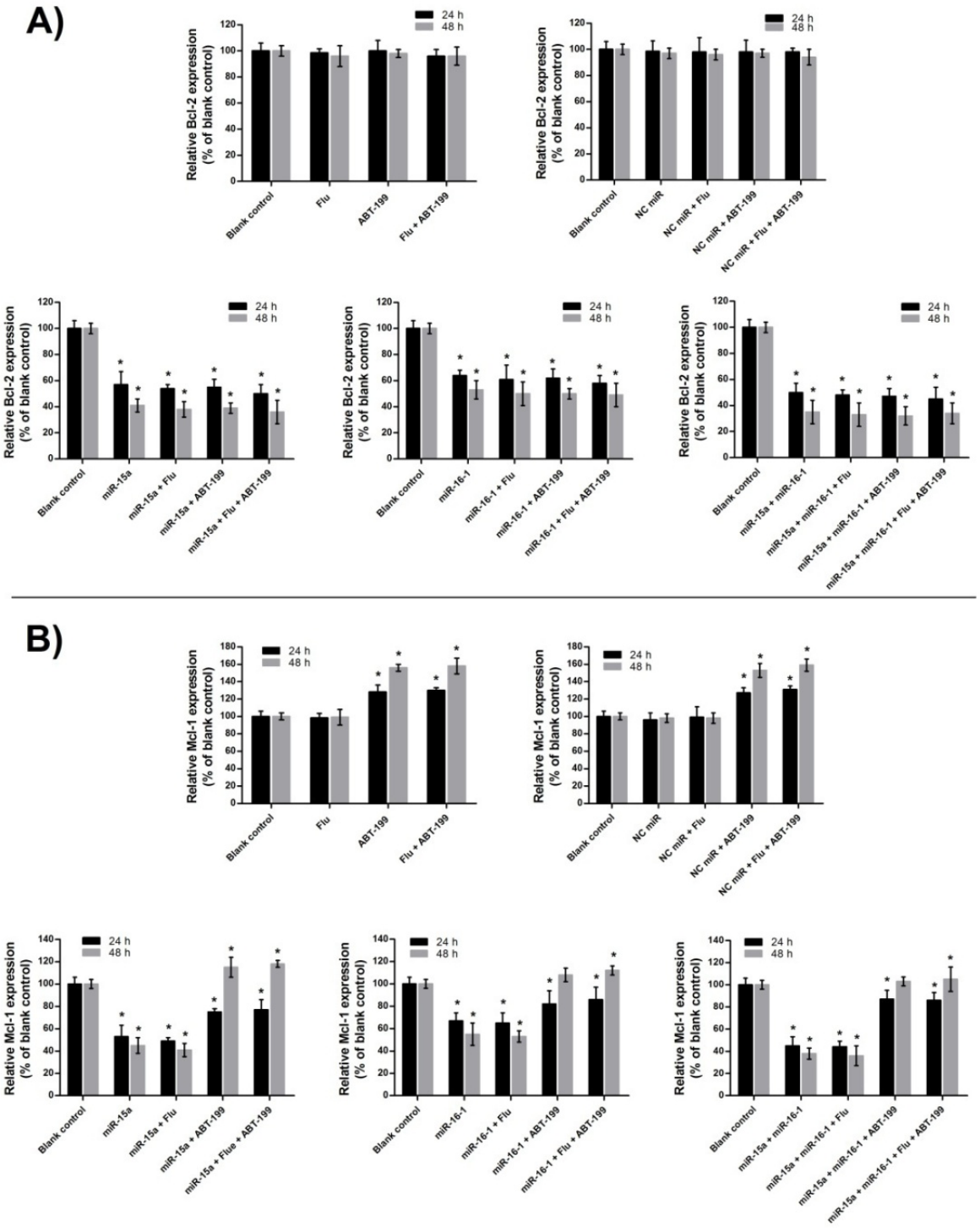

Figure 1. The *Bcl-2* and *Mcl-1* mRNA expression analysis in CLL-CII cells

Abbreviations: CLL: Chronic lymphocytic leukemia; NC: Negative control; miR: miRNA; Flu: Fludarabine.

*P<0.05 versus corresponding blank control.

Note: The cells were treated with *miRNA-15a*, *miRNA-16-1*, fludarabine and ABT-199, alone and in combination, for 24 and 48 h. Then, relative *Bcl-2* (A) and *Mcl-1* (B) mRNA expression were measured using RT-qPCR. The data are presented as Mean±SD of the results of three experiments.

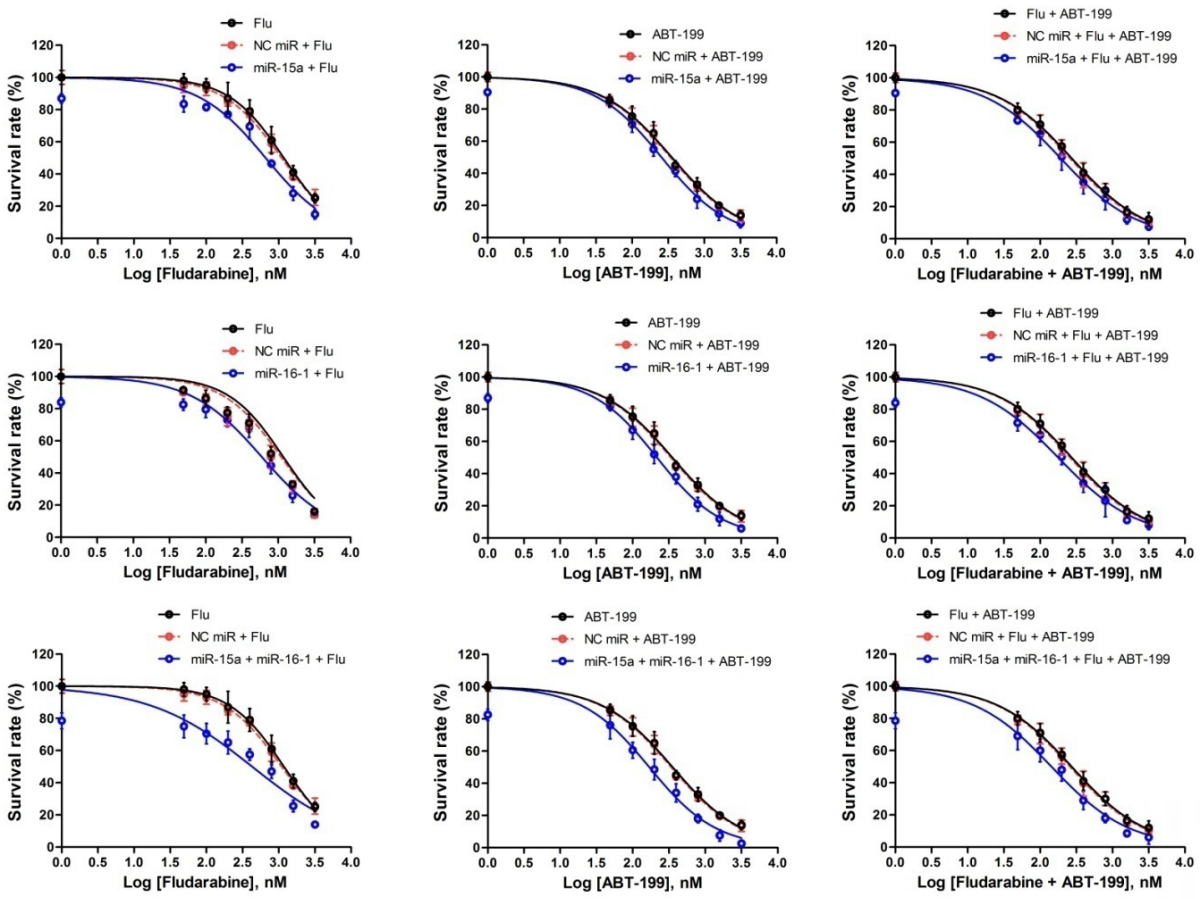

Figure 2. The effect of MiRNAs on chemosensitivity of the CLL-CII cells

Abbreviations: CLL: Chronic lymphocytic leukemia; NC: Negative control; miR: miRNA; Flu: Fludarabine.

Note: The cells were transfected with *miRNA-15a* and *miRNA-16-1* and then exposed to various concentrations of ABT-199 and fludarabine. After 24 h, the cell survival rate was measured using MTT assay. Cell survival curves were plotted using GraphPad Prism software. The data are expressed as Mean±SD (n=3).

Up-regulation of *MiRNA-15a* and *MiRNA-16-1* and the apoptotic effects of ABT-199 and fludarabine

To explore whether the sensitizing effect of the *miRNA-15a* and *miRNA-16-1* was associated with enhancing apoptosis, the combination effects of *miRNA-15a*, *miRNA-16-1*, ABT-199 and fludarabine on apoptosis were assessed by ELISA cell death assay. Results demonstrated that 24 and 48 h exposure of the cells with *miRNA-15a*, *miRNA-16-1*, ABT-199 and fludarabine significantly enhanced apoptosis compared to the blank control (Figure 7A). Pretreatment of the cells with *miRNA-15a* or *miRNA-16-1* increased the apoptotic effects of ABT-199 and fludarabine ($P<0.05$, compared with a single treatment). In addition, ABT-199 enhanced the effect of fludarabine on the induction of apoptosis. Furthermore, co-transfection of *miRNA-15a* and *miRNA-16-1* had a greater effect on inducing apoptosis than single transfection ($P<0.05$). However, NC miRNA showed no significant effect on the extent of apoptosis. These results indicate that *miRNA-15a* and *miRNA-16-1* enhance the chemosensitization effect of ABT-199 in CLL cells through the induction of apoptosis.

MiRNA-15a and *MiRNA-16-1* and the caspase-3 activity in CLL cells

To investigate the induction mechanism of apoptosis in CLL cells, caspase-3 activity was measured after 24 and 48 h of treatment. Results showed that compared with the blank control group, caspase-3 activity was significantly enhanced in the cells treated with the *miRNA-15a*, *miRNA-16-1*, ABT-199, or fludarabine (Figure 7B). Moreover, combining *miRNA-15a* or *miRNA-16-1* with ABT-199 or fludarabine markedly increased caspase-3 activity compared to the mono treatment ($P<0.05$). The combination effects of *miRNA-15a* with *miRNA-16-1* as well as ABT-199 with fludarabine were also greater than their single effects. However, compared to the blank control, caspase-3 activity did not change in cells treated with NC miRNA ($P>0.05$).

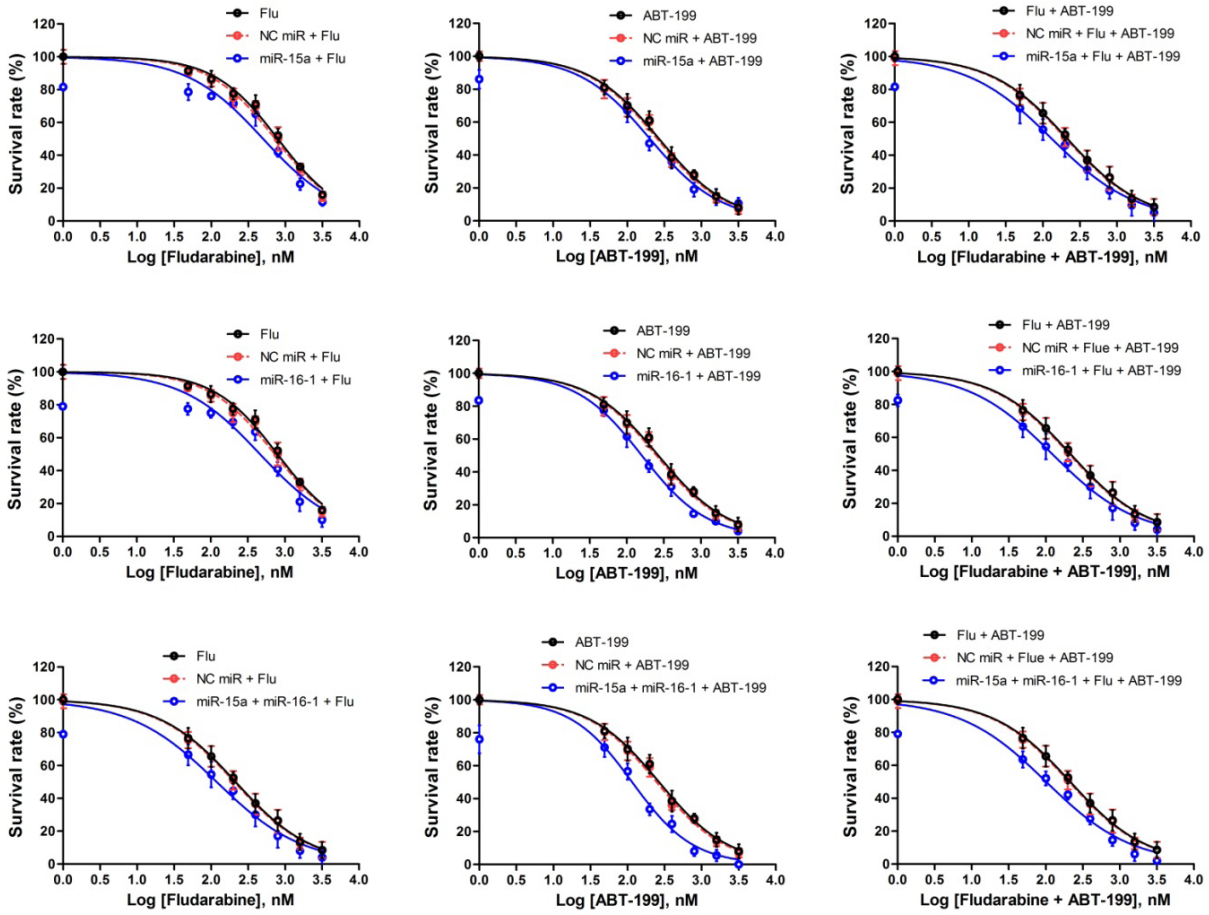

Figure 3. The effect of MiRNAs on chemosensitivity of the CLL-CII cells after 48 h

Abbreviations: CLL: Chronic lymphocytic leukemia; NC: Negative control; miR: miRNA; Flu: Fludarabine.

Note: The cells were transfected with *miRNA-15a* and *miRNA-16-1* and then exposed to various concentrations of ABT-199 and fludarabine. The cell survival rate was measured using an MTT assay. Cell survival curves were plotted using GraphPad Prism software. The data are expressed as Mean±SD (n=3).

Table 1. IC_{50} values of ABT-199, alone and in combination with miRNA, in CLL cells

Treatment	Mean±SD	
	IC_{50} (μM)	
	24 h	48 h
ABT-199	0.35±2.09	0.26±0.84
NC miR+ABT-199	0.34±1.77	0.25±1.41
miR-15a+ABT-199	0.25±0.28*	0.2±1.24*
miR-16-1+ABT-199	0.21±2.51*	0.15±2.58*
miR-15a + miR-16-1+ABT-199	0.16±1.03*	0.11±2.09*

CLL: Chronic lymphocytic leukemia; NC: Negative control; miR: miRNA.

*P<0.05 relative to the corresponding ABT-199.

Note: IC_{50} values were calculated using a sigmoidal dose-response model using GraphPad Prism software, version 6.01.

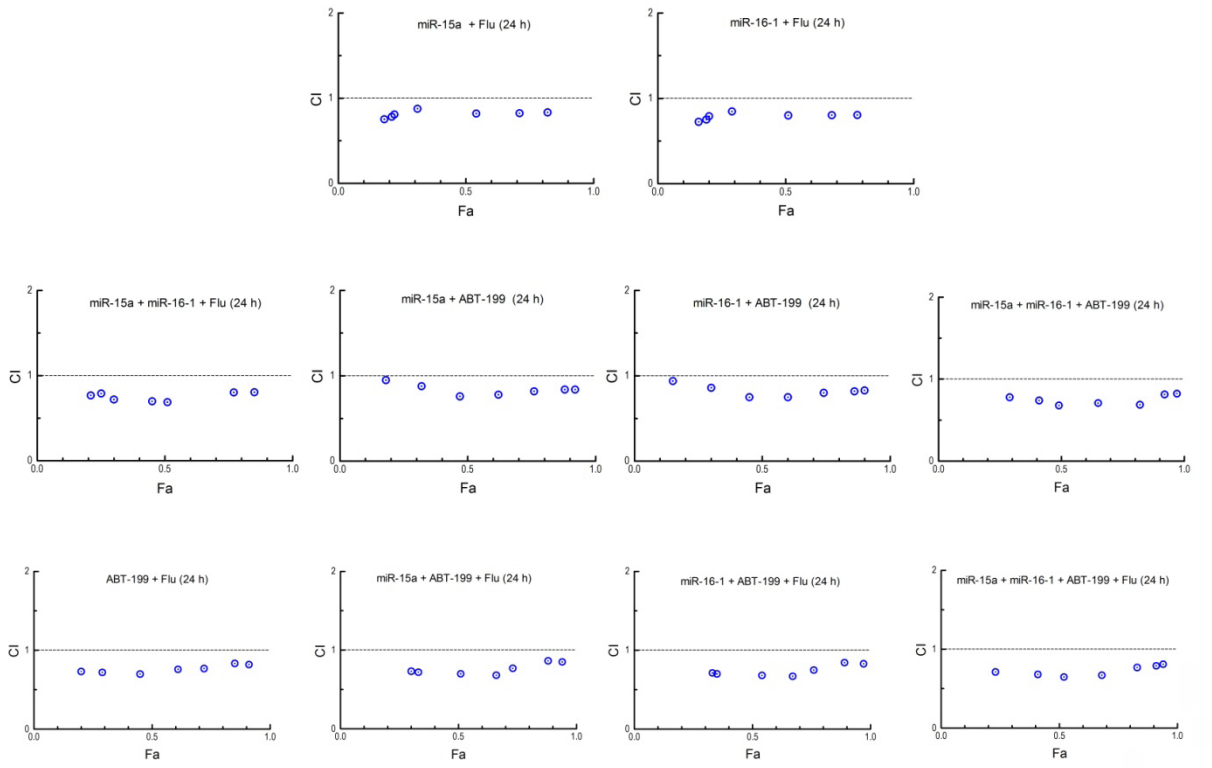

Figure 4. Combination effects of MiRNAs with fludarabine and ABT-199 on CLL cells after 24 h

Abbreviations: CI: Combination index; Fa: Fractional effect; CLL: Chronic lymphocytic leukemia; NC: Negative control; miR: miRNA; Flu: Fludarabine.

Note: Results of the MTT assay were used to plot the CI versus Fa using the Chou and Talalay method and CalcuSyn software. Dashed lines represent CI=1.

Table 2. IC_{50} values of fludarabine, alone and in combination with MiRNA in CLL cells

Treatment	Mean±SD	
	IC_{50} (μM)	
	24 h	48 h
Flu	1.18±2.50	0.79±1.11
NC miR+Flu	1.10±2.61	0.72±1.95
miR-15a+Flu	0.66±1.83*	0.50±2.61*
miR-16-1+Flu	0.59±0.60*	0.46±0.75*
miR-15a+miR-16-1+Flu	0.42±0.55*	0.36±1.38*

Abbreviations: CI: Combination index; Fa: Fractional effect; CLL: Chronic lymphocytic leukemia; NC: Negative control; miR: miRNA; Flu: Fludarabine.

*P<0.05 relative to the corresponding fludarabine.

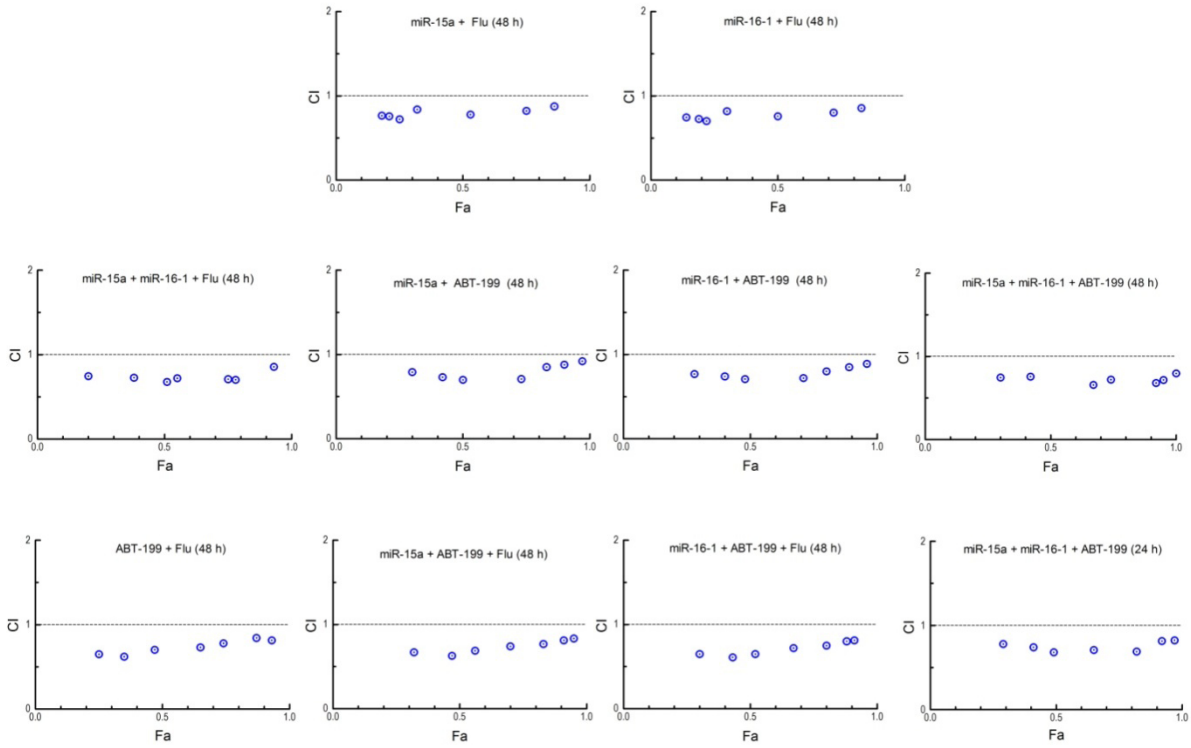

Figure 5. CI analysis of CLL cells after 48 h treatment with MiRNAs, fludarabine and ABT-199

Note: Chou and Talalay method and CalcuSyn software were used to plot the CI versus Fa. Dashed lines represent C=1.

Discussion

Although most patients with CLL respond to fludarabine, a significant fraction do not respond or become resistant to this agent over time [2]. As the deregulation of apoptotic machinery is involved in CLL's resistance to therapy, the reactivation of apoptotic processes for overcoming this resistance has attracted much attention. It has been reported that up-regulation of Mcl-1 and Bcl-xL levels has been associated with ABT-199 resistance [10, 22, 23]. Therefore, pretreatment of CLL cells with Mcl-1 inhibitors could improve the apoptotic effect of ABT-199 in CLL resistance cells.

Table 3. IC_{50} values of ABT-199 and fludarabine in combination with MiRNA in CLL-CII cells

Treatment	Mean±SD	
	IC_{50} (μM)	
	24 h	48 h
ABT-199+Flu	0.27±1.22	0.21±1
NC miR+ABT-199+Flu	0.25±0.13	0.2±2.56
miR-15a+ABT-199+Flu	0.19±0.45	0.13±2.93
miR-16-1+ABT-199+Flu	0.17±2.67	0.12±2.49
miR-15a+miR-16-1+ABT-199+Flu	0.14±1.02	0.1±1.38

Abbreviations: CLL: Chronic lymphocytic leukemia; NC: Negative control; miR: miRNA; Flu: Fludarabine.

Note: P<0.05 relative to the corresponding ABT-199 plus fludarabine treated cells.

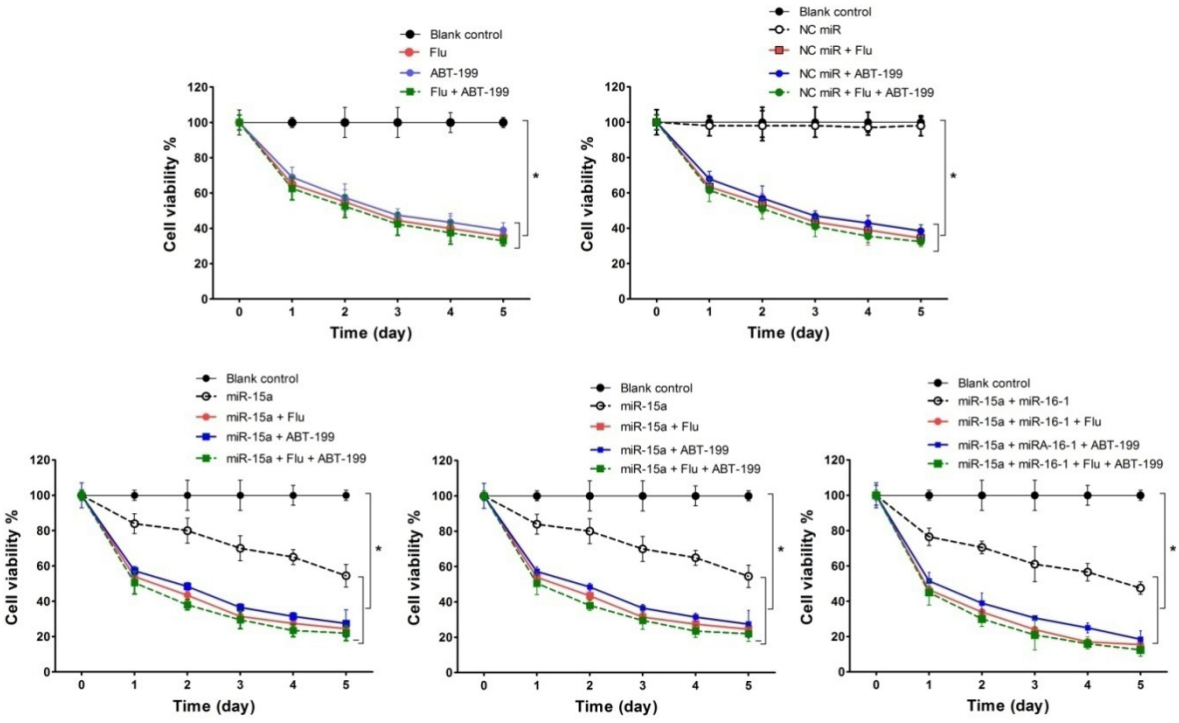

Figure 6. Growth curve of CLL-CII cells

Abbreviations: CLL: Chronic lymphocytic leukemia; NC; Negative control; miR, miRNA; Flu: Fludarabine.

*P<0.05 versus blank control.

Note: The cells were treated with ABT-199 and fludarabine alone and combined with miRNAs. Cell growth was measured using trypan blue staining for 24-120 h. Results are expressed as Mean±SD (n=3).

Our study demonstrated that suppression of Mcl-1 and Bcl-2 by *miRNA-16-1* and *miRNA-15a* was associated with inhibition of cell proliferation and synergistically enhanced the sensitivity of CLL-CII cells to fludarabine and ABT-199. Following the results of our study, several studies indicate that high levels of Mcl-1 in the CLL were correlated with resistance to fludarabine both in vitro and in vivo [24-26]. Moreover, Robertson et al. [27] showed that high expression levels of Bcl-2 in CLL patients were associated with shorter overall survival and resistance to fludarabine. Zhu et al. [28] targeted down-regulation of Bcl-1 and Mcl-1 expression that sensitized the CLL cells to fludarabine-dependent apoptosis. Also, our data confirm the results of the above reports and suggest that *miRNA-15a* and *miRNA-16-1* can enhance the sensitivity of CLL cells to fludarabine by inhibition of Bcl-1 and Mcl-1 expression.

The cytotoxicity assay demonstrates that transfection of *miRNA-15a* and *miRNA-16-1* significantly reduced the IC_{50} value and enhanced the sensitivity of the CLL cell to ABT-199. So far, several studies have explored the role of apoptotic proteins in the sensitivity of tumor cells to ABT-199. One study shows that treatment with A-1210477, a Mcl-1-selective inhibitor, counteracts the resistance to ABT-199 in Mcl-1-dependent acute myeloid leukemia cells, in vitro and in vivo [11]. In addition, Choudhary et al. [22] reported that substantial AKT activation and up-regulation of Bcl-xL and Mcl-1 were associated with the inherent and acquired resistance to ABT-199 in CLL and non-Hodgkin lymphoma cell lines. Their study demonstrated that treatment with a specific inhibitor of the AKT pathway resulted in the loss of Mcl-1 expression and sensitized the tumor cells to ABT-199. Our study further confirms the results of the above reports and suggests that inhibition of Mcl-1 by *miRNA-15a* and *miRNA-16-1* can enhance the ABT-199 sensitivity in CLL cells that depend on Mcl-1 for survival.

MiRNA-15a and *miRNA-16-1* act as tumor suppressor genes by targeting critical molecules in CLL cells. However, the role of these miRNAs in drug resistance of CLL has not been thoroughly investigated [7, 29]. So far, several studies have been performed to show the relationship of miRNA with drug resistance. For example, Zhu et al. [28] reported that miRNA-34, miRNA-181a/b,

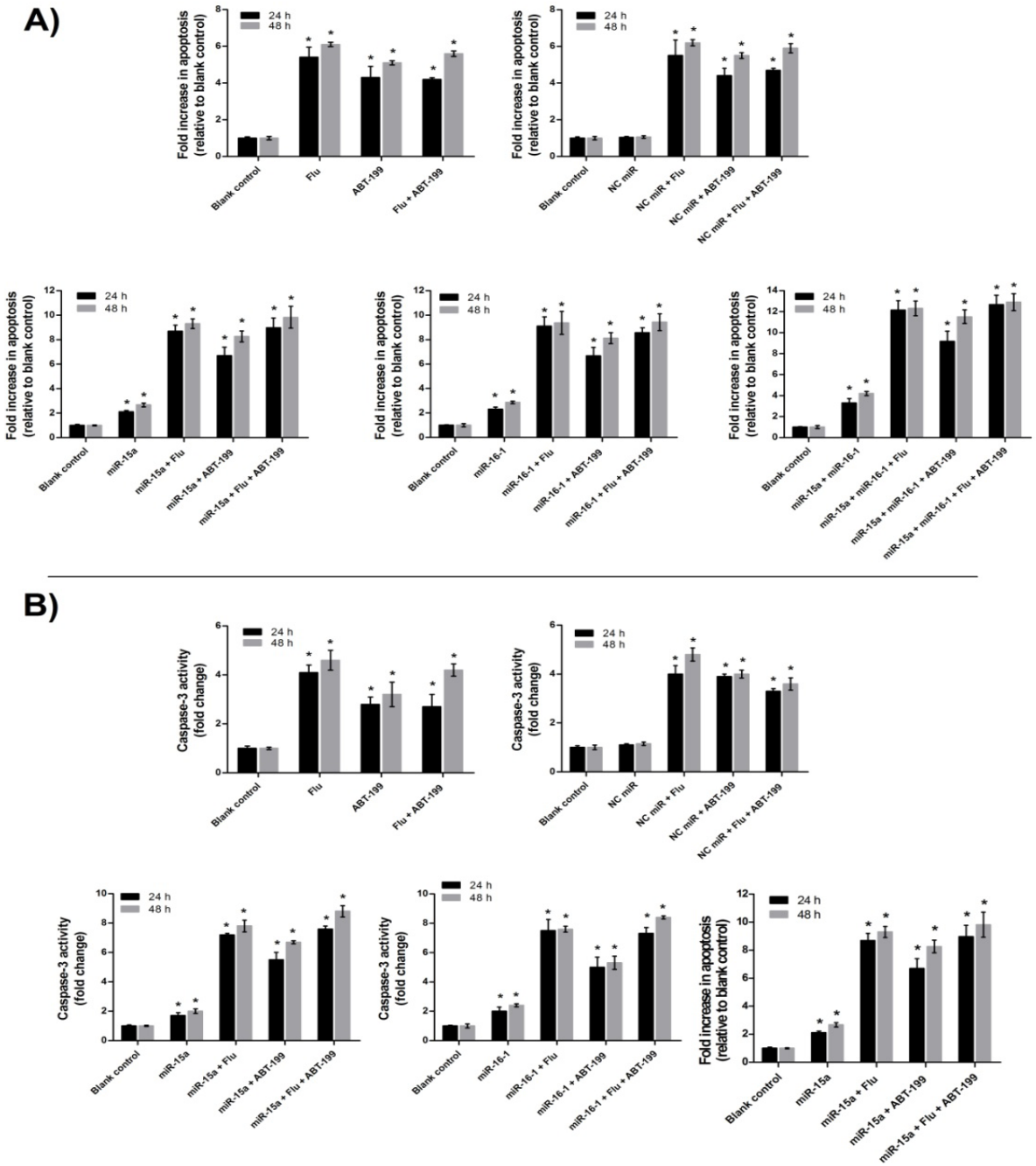

Figure 7. The effect of MiRNA-15a and MiRNA-16-1 in combination with ABT-199 and fludarabine on apoptosis of CLL cells

Abbreviations: CLL: Chronic lymphocytic leukemia; NC: Negative control; miR: miRNA; Flu: Fludarabine.

*P<0.05 compared with control.

Note: Cells were treated with miRNAs (50 nM), fludarabine, and ABT-199 (IC_{50} doses of 24 and 48 h), alone and in combination. Next, the cell death was assessed using ELISA apoptosis assay (A). Caspase-3 activity of CLL-CII cells was measured using a Caspase-3 activity assay Kit (B). Results are presented as Mean±SD of three independent experiments.

miRNA-15a, and *miRNA-16-1* sensitized the CLL cells to fludarabine-induced killing by inhibition of Mcl-1 and Bcl-2 expression. Since the over-expression of Mcl-1 is associated with resistance of cancer cells to Bcl-2 inhibitors, other studies have investigated the effect of miRNAs on Mcl-1 expression and the sensitivity of tumor cells to Bcl-2-specific inhibitors. It has been demonstrated that miRNA-193b is reduced in resistant melanoma cells, and treatment with miRNA-193b can restore the ABT-737 sensitivity of the melanoma cells by targeting Mcl-1 [30, 31]. Also, Lam et al. [30] also recognized a panel of 12 miRNAs linked to reduced Mcl-1 expression and sensitivity of melanoma cells to ABT-263. However, the relationship between miRNAs expression and ABT-199 has not been investigated. Here, we showed that *miRNA-15a* and *miRNA-16-1* increased the sensitivity of the CLL cells to fludarabine and ABT-199 via inhibition of Mcl-1 and Bcl-2 expression. Our findings further confirm the role of miRNAs in drug resistance and suggest that *miRNA-15a* and *miRNA-16-1* can be helpful in future therapeutic approaches to restore the drug sensitivity of the CLL cells.

We next examined the effect of treatments on cellular apoptosis. Our results demonstrate that in the presence of ABT-199, the apoptotic effect of fludarabine increases, and caspase-3 activity is enhanced. The inhibition of Mcl-1 and Bcl-2 by *miRNA-15a* or *miRNA-16-1* is associated with the induction of apoptosis and enhancement of the fludarabine or ABT-199-mediated apoptosis. Moreover, co-transfection of *miRNA-15a* and *miRNA-16-1* has a stronger effect on cellular apoptosis than single transfection.

The intrinsic pathway of cell death is induced by different stimuli, such as DNA damage, cytotoxic drugs, radiation, and oxidative stress. The Bcl-2 family of pro- and anti-apoptotic proteins regulates this pathway. In apoptotic conditions, the proapoptotic effectors proteins such as Bax and Bak are activated, leading to the change in the mitochondrial outer membrane permeability, cytochrome c release, and the downstream activation of the caspases 3, 6 and 7. The antiapoptotic proteins such as Mcl-1 and Bcl-2 prevent apoptosis by sequestering with Bak and Bax [5, 7]. Previous studies have shown that fludarabine triggers apoptosis in CLL cells by restraining several enzymes involved in RNA and DNA synthesis. However, the effect of fludarabine on the activation of the intrinsic pathway of apoptosis is not well known [32, 33]. It is also demonstrated that ABT-199 induces an intrinsic apoptosis pathway by inhibiting Bcl-2 [11]. Up-regulation of the antiapoptotic proteins Bcl-xL, Bcl-1, and Mcl-1 was associated with resistance to fludarabine and ABT-199 in CLL cells [7, 11, 34]. Moreover, caspase-3 activation has been observed in tumor cells treated with either fludarabine or ABT-199 [32, 34]. Following the above reports, we have shown that targeting Mcl-1 and Bcl-1 by *miRNA-16-1* and *miRNA-15a* enhances the apoptotic effects of ABT-199 and fludarabine in the CLL cells. *MiRNA-15a* and *miRNA-16-1* act with fludarabine and ABT-199 to exert synergistic anticancer efficacy against CLL cells.

Conclusion

In conclusion, the data presented here reveal that *miRNA-15a* and *miRNA-16-1* act in concert with fludarabine and ABT-199 to exert synergistic anticancer efficacy against CLL cells, which is attributed to the suppression of Mcl-1 and Bcl-2. In addition, *miRNA-15a* and miRNA-16-1 could augment the execution of apoptosis induced by fludarabine and ABT-199. The intrinsic pathway of apoptosis and caspase activation may be a part of the underlying mechanisms involved in this process. Our study shows that the *miRNA-15a* and miRNA-16-1 can efficaciously enhance the anticancer effects of fludarabine and ABT-199 in CLL cells and may offer a new promising therapeutic strategy for patients with CLL.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Arak University of Medical Sciences, Arak, Iran (Code: ARAKMU.REC.1395.113).

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Authors contribution's

Conceptualization, study design and critical revision: Hadi Karami and Alireza Amani; Data collection and writing the original draft: Nooshin Ashofteh, Razieh Amini and Alireza Amani; Data analysis and interpretation: Hadi Karami, Alireza Amani and Razieh Amini; Funding acquisition: Hadi Karami.

Conflict of interest

The authors declared no conflict of interest.

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