Original Article

MiRNA-16-1 Suppresses Mcl-1 and Bcl-2 and Sensitizes Chronic Lymphocytic Leukemia Cells to BH3 Mimetic ABT-199

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Abstract

Objective: Chronic lymphoid leukemia (CLL) is the most common type of leukemia among adults. Increased levels of Mcl-1 and Bcl-xL is linked to resistance to Bcl-2 inhibitors including ABT-199. In this study, we investigated the effect of *miRNA-16-1* on apoptosis and sensitivity of the CLL cells to ABT-199.

Materials and Methods: In this experimental study, the Mcl-1 and Bcl-2 expression were measured using qualitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blotting. The effect of treatments on cell survival and growth were explored with MTT assay and Trypan blue assay, respectively. The drug interaction was evaluated using combination index analysis. Apoptosis was assessed by ELISA cell death and caspase-3 activity assays.J

Results: *MiRNA-16-1* markedly inhibited the expression of Mcl-1 and Bcl-2 in a time dependent manner ($P<0.05$, relative to blank control). Pretreatment with *miRNA-16-1* synergistically suppressed the cell growth and survival and reduced the half-maximal inhibitory concentration (IC_{50}) value of ABT-199. Moreover, *miRNA-16-1* markedly augmented the apoptotic effect of ABT-199 in CLL cells ($P<0.05$).

Conclusion: Our findings propose that *miRNA-16-1* act in concert with ABT-199 to exert synergistic anticancer efficacy against CLL, which is attributed to the inhibition of Bcl-2 and Mcl-1. This may propose a promising strategy for CLL resistant patients.

Keywords: ABT-199, Bcl-2, Chronic Lymphocytic Leukemia, Mcl-1

Introduction

Chronic lymphoid leukemia (CLL) is the most common type of leukemia among adults with a median age of diagnosis 72 years (1, 2). Despite recent advances in treatments over single-agent chemotherapy such as fludarabine or chemo-immunotherapy combinations, CLL remains an incurable disease (2). A variety of parameters such as deregulated production of survival signals or intrinsic defects in apoptotic machinery contribute to the therapy resistance in CLL patients (3-5). As a result, there remains a need for understanding the detailed molecular pathophysiology of CLL as well as the development of new drugs for clinical treatment of CLL (6).

Apoptosis is induced by the two extrinsic and intrinsic pathways. The intrinsic pathway cell death is controlled by the Bcl-2 family proteins members, including the proapoptotic and the antiapoptotic proteins (5, 7). Over-expression of some antiapoptotic proteins such as Mcl-1 and Bcl-2 is correlated with shorter overall survival and chemoresistance in CLL patients. Accordingly, many targeted strategies have been developed to the Bcl-2 and Mcl-1 to overcome drug-resistance of CLL patients (8, 9).

The Bcl-2-specific antagonist ABT-199 or venetoclax has showed improved clinical efficacy in patients with CLL (10, 11). ABT-199 is demonstrated high cytotoxicity against CLL cells *in vitro* but is much less effective against CLL cells that have expressed high levels of Mcl-1. Therefore, combination therapy of CLL cell with Mcl-1 inhibitors and ABT-199 have been suggested for improvement of apoptosis-based therapies in CLL resistance cells (12-14).

MicroRNAs (miRNAs) are a small family of endogenous, single-stranded, non-coding RNAs with 20-22 nucleotides in length that are involved in numerous cellular processes such as cell survival, cell death, differentiation and proliferation. They act by directly binding to the specific target mRNA, causing inhibition of the gene expression (15, 16). Several studies have demonstrated that miRNAs are recognized as important diagnostic and therapeutic biomarkers in numerous types of cancers, such as colon and breast cancer (17, 18). Furthermore, miRNAs are involved in almost all hematological processes, suggesting the important role of miRNAs in CLL (19-21). Genetic abnormalities have been observed in the majority of CLL cases. These aberrations include the 11q deletion with intermediate risk, the 13q deletion with low risk, and the 17p deletion with high risk (22). The 13q14 deletion is the most common genetic aberrations observed in more than

50% of CLL cases. The results of previous studies have clarified that the *miRNA-16-1* gene was absent or down-regulated in CLL cases with 13q14 deletion. *MiRNA-16-1* is a tumor suppressor gene that involved in the regulation of cell proliferation and cell death via targeting of several molecules (cyclin-dependent kinase 6, cyclin D1, cyclin D3, and Bcl-2) (23). In addition, the results of experimental studies show that there is a significant relationship between *miRNA-16-1* and Mcl-1 expression levels in samples of CLL patient (19, 24). However, the exact role of miRNA-16-1 in pathogenesis and drug resistance of CLL has not been fully investigated.

We hypothesized that reducing the expression of the *miRNA-16-1* gene could lead to increased expression of Bcl-2 and Mcl-1, and subsequently resistance to the ABT-199 in CLL cells. Therefore, we investigated the combination effect of *miRNA-16-1* and ABT-199 on survival and apoptosis of the CLL cells.

Materials and Methods
Cell culture conditions
The CLL-CII leukemia cells (Pasteur Institute, Iran) were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS, Sigma Aldrich, USA), streptomycin (100 mg/ml), penicillin (100 U/ml), 1% (v/v) Glutamax (Sigma Aldrich, USA), and 1% sodium pyruvate at 37°C in 5% CO_2 (25). The cells were seeded in suspension at a concentration of 1×10^5 cells/ml with the medium changed every two days.

This research was ethically wise approved from Deputy of Research and Technology, Arak University of Medical Sciences, Arak, Iran (IR.ARAKMU.REC.1395.185).

Transfection of miRNA
The *miRNA-16-1* mimics with the sense strand sequence 5´-UAG CAG CAC GUA AAU AUU GGC G-3´ and the negative control (NC) miRNA sense strand sequence 5´-ACU ACU GAG UGA CAG UAG A-3´ were 5´-ACU ACU GAG UGA CAG UAG A-3´ bought from Dharmacon (Lafayette, CO, USA) and used in transient transfection of CLL-II cells. Cell transfection was executed with using Lipofectamine™2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM I reduced serum medium (Invitrogen, USA) according to the manufacturer's recommendation. In brief, the cells were cultured at 40-50% confluence in culture medium without serum and antibiotics one day before transfection. To make the transfection complex, we diluted *miRNA-16-1* mimics or *NC miRNA* (50 nM) with Lipofectamine™2000 (4 µl/ml of transfection medium) in Opti-MEM I. The diluted solutions were thoroughly mixed and incubated for 15-20 minutes at room temperature. Next, the mixture was added to the culture medium. After 6 h of incubation, medium was replaced with a complete growth medium (10% FBS). At different time points after transfection, the

CLL-II cells were harvested and various experiments performed.

MTT assay
The cytotoxic effects of *miRNA-16-1* and ABT-199 (Sigma- Aldrich, USA) on CLL cells was evaluated using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay (25). The experiment was divided into eight groups: ABT-199, *miRNA-16-1* mimics, NC miRNA, *miRNA-16-1* mimics and ABT-199, NC miRNA and ABT-199, miRNA blank control, ABT-199 blank control and combination blank control. Briefly, the cells were cultivated in 96-well tissue plates at a density of 5×10^4 cells per well, and then transfected with miRNAs. Six hours later, the cells were treated with different concentrations of ABT-199 (0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 µM). After 24 and 48 hours of incubation, the cytotoxicity was determined using a MTT assay kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The absorbance (A) was measured spectrophotometrically at 570 nm with a microplate reader (Awareness Technology, Palm City, FL, USA). Half-maximal inhibitory concentration (IC_{50}) (drug concentration that reduced 50% survival rate) value of the ABT-199, alone or in combination with miRNA, was calculated with Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA). In the next experiments, the IC_{50} doses of fludarabine were used.

Combination effect analysis
The combination index (CI) analysis was performed to explore the interaction between ABT-199 and *miRNA-16-1* (25-27). The results obtained from the MTT experiment were converted to Fraction affected (Fa, where Fa=0 is 100% cell survival and Fa=1 is 0% cell survival) and analyzed by CompuSyn 1.0 software from Combosyn (Paramus, NJ, USA). Additive, synergistic and antagonistic effects are indicated by CI=1, CI<1 and CI>1, respectively.

Quantitative real time polymerase chain reaction
After treatments, total RNA was extracted by using AccuZolTM reagent (Bioneer, Daedeokgu, Daejeon, Korea) according to the manufacturer's instructions. Then, reverse transcription of 1 µg of purified total RNA was performed by use of PrimeScript RT reagent kit (Promega, Madison, WI, USA), following the manufacturer's protocol. Relative gene expression was measured by qualitative reverse transcription-polymerase chain reaction (qRT-PCR) using SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan) and the LightCycler 96 System (Roche Diagnostics GmbH). RT-PCR carried out in a final volume of 20 µl containing 1 µl of cDNA template, 10 µl of SYBR green reagent and 0.2 µM of each of the primers. The sequences of PCR primers were as follows:

β–actin-

F: 5′-TCC CTG GAG AAG AGC TAC G-3′
R: 5′-GTA GTT TCG TGG ATG CCA CA-3′

Mcl-1-

F: 5′-TAA GGA CAA AAC GGG ACT GG-3′
R: 5′-ACC AGC TCC TAC TCC AGC AA-3′

Bcl-2-

F: 5′-ATC GCC CTG TGG ATG ACT GAG T-3′
R: 5′-GCC AGG AGA AAT CAA ACA GAG GC-3′.

The protocol parameters were as follows: initial incubation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing and extension at 60°C for 50 seconds. The relative mRNA levels were determined using the comparative CT method, $2^{-(\Delta\Delta Ct)}$ (26, 27), and *β-actin* as an endogenous control.

Immunobloting analysis

After treatments, the cells were washed with phosphate-buffered saline (PBS, Sigma-Aldrich, USA) and cell lysates prepared by disrupting cells in lysis buffer (1% NP-40, 0.1% sodium dodecyl-sulfate (SDS, Sigma-Aldrich, USA), 0.5% sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, USA), 50 mM Tris-HCl pH=7.4, 150 mM NaCl) containing protease inhibitor cocktail (Roche Diagnostics GmbH). Protein samples (fifty micrograms) were separated on 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Sigma-Aldrich, USA) gels and transferred onto PVDF membrane (GE Healthcare, Amersham, Buckinghamshire, UK). Mouse primary monoclonal antibodies for Mcl-1 (Abcam, Cambridge, MA, UK), Bcl-2 (Abcam) and β-actin (Abcam) were used at 1:1000 dilutions. HRP-conjugated secondary antibodies (Abcam) were used at 1:4000 dilutions. The blot signals were detected using ECL plus western blotting detection Kit (GE Healthcare) and X-ray film (Estman Kodak, Rochester, NY, USA) and quantified via ImageJ 1.62 software (National Institutes' of Health, Bethesda, Maryland, USA).

Cell growth assay

The effect of *miRNA-16-1* and ABT-199 on tumor cell growth was assessed by the trypan blue staining. CLL-CII cells (1×10^5 cells/well) were treated with *miRNA-16-1* and ABT-199 in 6-well culture plates for 5 days as described previously. At the end of each day, the cells were collected and cell suspensions stained with 0.4% trypan blue dye (Merck KGaA, Darmstadt, Germany). After 2 minutes of incubation, the number of viable cells was measured using a hemocytometer and an inverted microscope (Nikon Instrument Inc., Melville, NY, USA). The percentage of cell viability in control group was considered as 100%.

Apoptosis ELISA assay

Cell death was determined with an ELISA apoptosis

kit (Roche Diagnostics GmbH) that determines mono- and oligonucleosomes released into the cytoplasm of apoptotic cells (25). The CLL-CII cells were cultivated at a density of 1×10^5 cells/well in 6-well culture plates and exposed to *miRNA-16-1* and ABT-199, as described previously. After 24-48 hours of incubation, the cells were lysed and ELISA assay was performed according to the manufacturer's instructions. Briefly, 20 µl of the supernatants and 80 µl of immunoreagent containing DNA-peroxidase and histone-biotin antibodies were added to each well of a streptavidin-coated plate and the plate was incubated for 2 hours at room temperature. After washing with incubation buffer, 100 µl of ABTS solution was added. Finally, the reactions were stopped with ABTS stop solution and absorbance was quantified immediately by an ELISA reader (Awareness Technology, Palm City, FL, USA) at 405 nm. Data were calculated as the fold increase in the absorbance of test groups relative to the control group.

Caspase-3 activity assay

The *in vitro* induction of caspase-3 activity was determined using a colorimetric caspase assay Kit (Abnova, Taipei, Taiwan) (25). Briefly, the treated cells were resuspended in 50 µl cooled lysis buffer and then centrifuged in 10,000 g for 1 minute. Then, 5 µl of the 4 mM DEVD-pNA substrate and 50 µl of 2X reaction buffer (containing 10 mM DTT) were added to each sample. After 2 hours incubation at 37°C the absorbance was quantified using a microplate plate reader (Awareness Technology, Palm City, FL, USA) at 405 nm.

Statistical analysis

All results in this study are demonstrated as mean ± standard deviation (SD) of three experiments. ANOVA followed by Bonferroni's test was used to determine the significant differences between groups. A P<0.05 was considered significant. All results were analyzed using Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

Results

MiRNA-16-1 inhibited the expression of Mcl-1 and Bcl-2 mRNA and protein in CLL-CII cells

First, we explored the effect of *miRNA-16-1* on Mcl-1 and Bcl-2 levels in CLL-CII leukemic cells by qRT-PCR and western blotting. As shown in Figure 1A and 1B, transfection of *miRNA-16-1* markedly reduced both *Mcl-1* and *Bcl-2* mRNA levels in a time-dependent way (P<0.05, relative to the blank control). At 24 and 48 hours after treatment with ABT-199, the relative Mcl-1 mRNA expression levels were significantly enhanced, while the expression levels of Bcl-2 mRNA did not change. In *miRNA-16-1* and ABT-199 combination group, the expression of Bcl-2 mRNA was similar to the cells transfected with only *miRNA-16-1*. In addition, the expression of Mcl-1 mRNA in the combination group was

higher and lower than the cells treated with only *miRNA-16-1* or ABT-199, respectively. However, NC miRNA had a negligible effect on mRNA expression compared to the blank control (P>0.05). The results of western blotting were in agreement with the PCR results (Fig.1C-F).
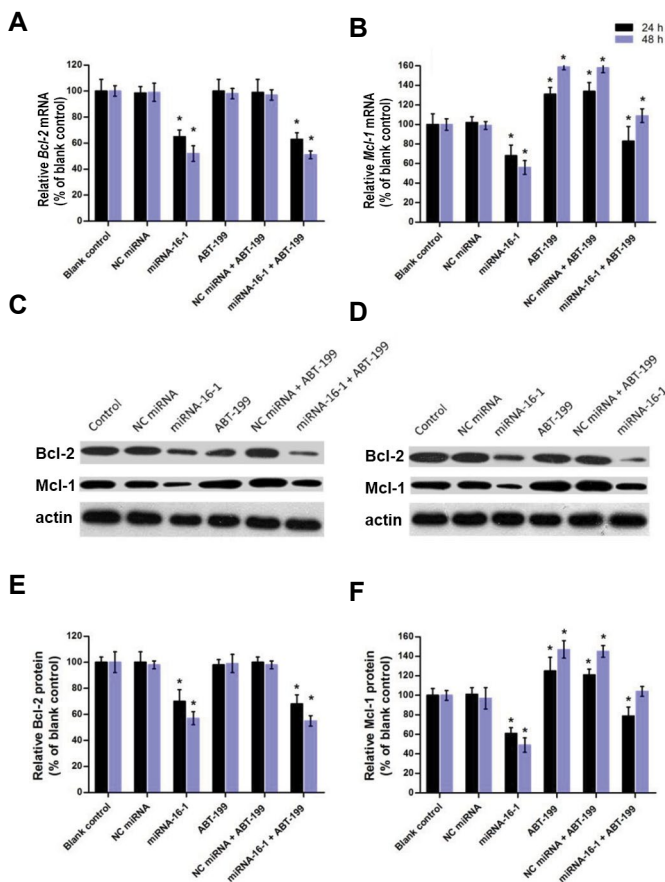
199 monotreatment (P<0.05). Furthermore, in the presence of *miRNA-16-1*, the IC_{50} values of ABT-199 dramatically decreased from 0.37 µM to 0.22 µM and 0.24 µM to 0.14 µM after 24 and 48 hours, respectively (Table 1). Notably, NC miRNA had a minimal effect on survival and chemosensitivity of the cells (P>0.05, Fig.2, Table 1).

Fig.1: Bcl-2 and Mcl-1 expression analysis in CLL-CII cells treated with *miRNA-16-1* and ABT-199. The cells were treated with *miRNA-16-1*, ABT-199 and combination of them for 24 and 48 hours, and then relative **A.** *Bcl-2* and **B.** *Mcl-1* mRNA expression was measured using RT-qPCR. Representative western blots of Mcl-1, Bcl-2 and β-actin after **C.** 24 and **D.** 48 hours. The density of **E.** Bcl-2 and **F.** Mcl-1 protein bands was measured and normalized to the corresponding β-actin. The data are presented as mean ± SD of the results of three experiments. *; P<0.05 versus corresponding blank control or NC miRNA transfected cells, RT-qPCR; Reverse transcription qualitative-polymerase chain reaction, and NC; Negative control.

Table 1: IC_{50} of ABT-199 in combination with miRNAs, in CLL cells

Treatment	IC_{50} (µM)	
	24 hours	**48 hours**
ABT-199	0.37 ± 1.33	0.24 ± 1.30
NC miRNA and ABT-199	0.33 ± 1.54[#]	0.22 ± 0.80[#]
miRNA-16-1 and ABT-199	0.22 ± 2.41[*]	0.14 ± 1.53[*]

IC_{50} of ABT-199 was calculated by GraphPad Prism 6.01 software and sigmoidal dose-response model. Data expressed as the mean ± SD of three independent experiments. CLL; Chronic lymphoid leukemia, [*]; P<0.05 relative to the corresponding ABT-199, [#]; P>0.05 relative to the corresponding ABT-199, and IC_{50}; Half-maximal inhibitory concentration.

MiRNA-16-1 synergistically enhanced the effect of ABT-199 on CLL-CII cells

To assess whether the combination of *miRNA-16-1* and ABT-199 on CLL-CII cells is synergistic, the combination analysis using the Chou-Talalay method was carried out. The results showed that the effects of *miRNA-16-1* (50 nM) and ABT-199 (0.05-3.2 µM) were synergistic with the CI values of >1 in all concentrations of ABT-199 (Fig.2B, D). CI-Fa curved demonstrated that the most synergistic effects of 24 hours (CI=0.77) and 48 hours (CI=0.72) of treatment were seen at 0.4 and 0.2 µM of ABT-199 with Fa values of 0.61 and 0.49, respectively (Table 2).
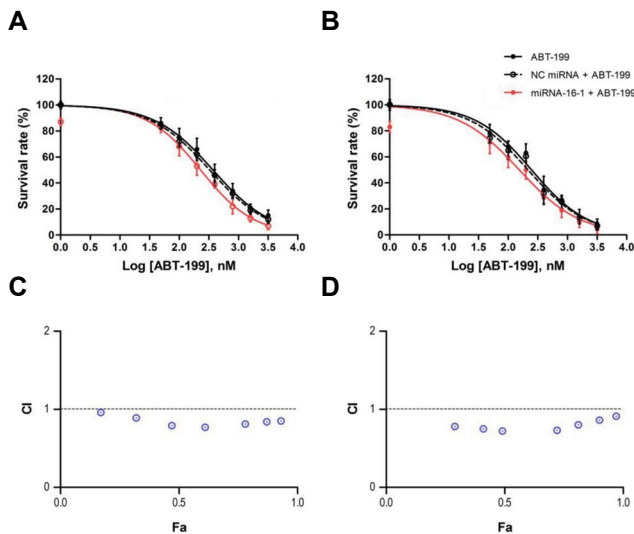
MiRNA-16-1 increased ABT-199 sensitivity in CLL-CII leukemia cells

To explore whether suppression of Mcl-1 and Bcl-2 by *miRNA-16-1* could increase the sensitivity of leukemia cells to ABT-199, a combination study with *miRNA-16-1* and ABT-199 was done. The results of MTT assay showed that single treatment with ABT-199 reduced the cell survival in a dose-dependent way. Moreover, transfection of *miRNA-16-1* at 24 and 48 h markedly reduced the cell survival to 87.10% and 83.162% respectively, relative to the control (Fig.2, P<0.05). Combination treatment with *miRNA-16-1* and ABT-199 further reduced the survival rate of the CLL cells compared with *miRNA-16-1* or ABT-

Fig.2: The effect of *miRNA-16-1* on ABT-199 sensitivity of the CLL-CII cells. The leukemia cells were exposed to *miRNA-16-1* (50 nM) and different concentrations of ABT-199 for **A, B.** 24 hours and **C, D.** 48 hours. Next, the cell survival rate was evaluated using MTT assay. Cell survival curves were plotted by GraphPad Prism software. The results are shown as mean ± SD (n=3). Chou and Talalay method and CalcuSyn software were used to plot the CI vs. Fa. Dashed lines represent CI value equal to 1. CI; Combination index and Fa; Fraction affected.

Table 2: CI analysis of *miRNA-16-1* and ABT-199 in CLL cells

ABT-199 concentration (μM)	24 hours			48 hours		
	Fa	**CI**	**Combined effect**	**Fa**	**CI**	**Combined effect**
0.05	0.17	0.96	S	0.29	0.78	S
0.1	0.32	0.89	S	0.41	0.75	S
0.2	0.47	0.79	S	0.49	0.72	S
0.4	0.61	0.77	S	0.72	0.73	S
0.8	0.78	0.81	S	0.81	0.80	S
1.6	0.86	0.84	S	0.90	0.86	S
3.2	0.93	0.85	S	0.97	0.91	S

The CI analysis was measured using CompuSyn software and combination index method of Chou-Talalay. CLL; Chronic lymphoid leukemia, CI value >1, =1 and <1 show antagonistic, additive and S effects, respectively, CI; Combination index, S; Synergistic, and Fa; Fraction affected.

MiRNA-16-1 enhanced the effect of ABT-199 on CLL cell growth

As over-expression of Mcl-1 and Bcl-2 is linked to the cell growth; we therefore explored whether *miRNA-16-1* could inhibit the proliferation of the CLL-CII cells. The CLL-CII cells were treated with *miRNA-16-1* (50 nM), ABT-199 (IC_{50} of 25 hours) and combination of them for 1-5 days and the percent of the viable cells was counted every day by trypan blue staining assay. Data showed that in comparison with the control group, treatment with *miRNA-16-1* or ABT-199 significantly suppressed the growth of CLL-CII cells over a period of 5 days. At 24 hours after treatment with *miRNA-16-1* or ABT-199, the cell viability dropped to 83.30% and 62.23% respectively, and then to a further 54.15% and 25.67% at the end of the experiment (day 5). Moreover, combination therapy with *miRNA-16-1* and ABT-199 had a stronger effect on inhibition of cell growth compared to single therapy (P<0.05). However, no significant difference in cell growth was seen between the NC miRNA and the blank control groups (Fig.3).

Increased levels of *miRNA-16-1* enhanced ABT-199-induced apoptotic

To explore whether the observed sensitizing effect of the *miRNA-16-1* was associated with the increased amount of apoptosis, the effects of *miRNA-16-1* and ABT-199 alone and in combination on apoptosis, were assessed using an ELISA apoptosis assay. Results demonstrate that 24 h treatment with *miRNA-16-1* or ABT-199 increased apoptosis by 1.93 fold and 4.30 fold, respectively, compared to the control group (Fig.4A, P<0.05). Furthermore, combination treatment enhances the extent of cell death to 7.44 fold (P<0.05, compared with either ABT-199 alone or *miRNA-16-1* alone). Moreover, 48 h exposure of the cells with *miRNA-16-1* or ABT-199 alone, increased apoptosis by 2.46 and 5.12 fold, respectively, relative to the control

group (P<0.05). Also, the combination of *miRNA-16-1* and ABT-199 further augmented the induction of apoptosis to 8.56 fold during same period of time (P<0.05, compared with the blank control or monotreatment). However, NC miRNA (alone or in combination with ABT-199) showed no significant effect on extents of apoptosis compared with the *miRNA-16-1* or ABT-199, respectively (Fig.4A, P>0.05). The results of ELISA apoptosis assay shows that miRNA-16-1 sensitizes the chronic lymphocytic leukemia cells to ABT-199 partially via enhancement of apoptosis.
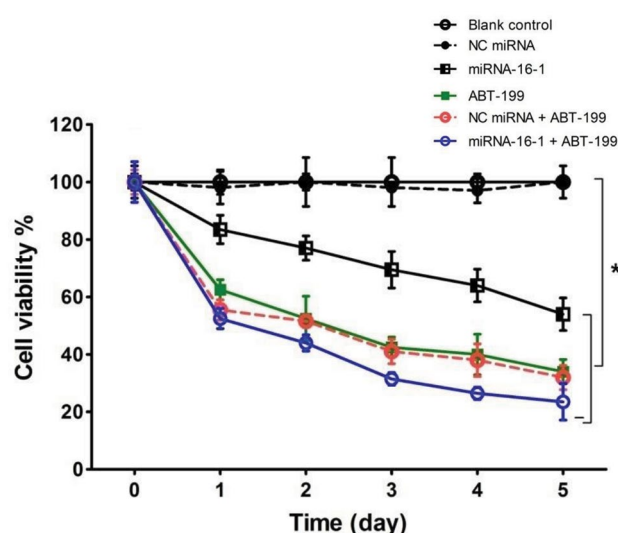

Fig.3: Cell growth curve of CLL-CII cells after treatment with *miRNA-16-1* and ABT-199. Cell viability was measured using trypan blue staining over a period of 24-120 hours. Results are expressed as mean ± SD (n=3). *; P<0.05 versus blank control or NC miRNA and NC; Negative control.

MiRNA-16-1 enhanced the effect of ABT-199 on caspase-3 activity in CLL-CII cells

To explore the mechanism by which apoptosis occurred

in the treated cells, changes in the activation of the caspases-3 were determined by using caspase-3 activity assay Kit. Figure 4B shows the changes in caspases-3 activity in the CLL cells treated with the *miRNA-16-1*, ABT-199 (IC$_{50}$) and their combination for 24 hours, that show the caspase-3 activity was enhanced by 1.65, 3.42, and 6.21 times, respectively, relative to the blank control group (P<0.05). As indicated in Figure 4B, *miRNA-16-1* alone and in combination with ABT-199 activated caspase-3 activity in a time dependent way. However, treatment with NC miRNA did not show a notable effect on caspase-3 activity relative to the blank control group (P>0.05).
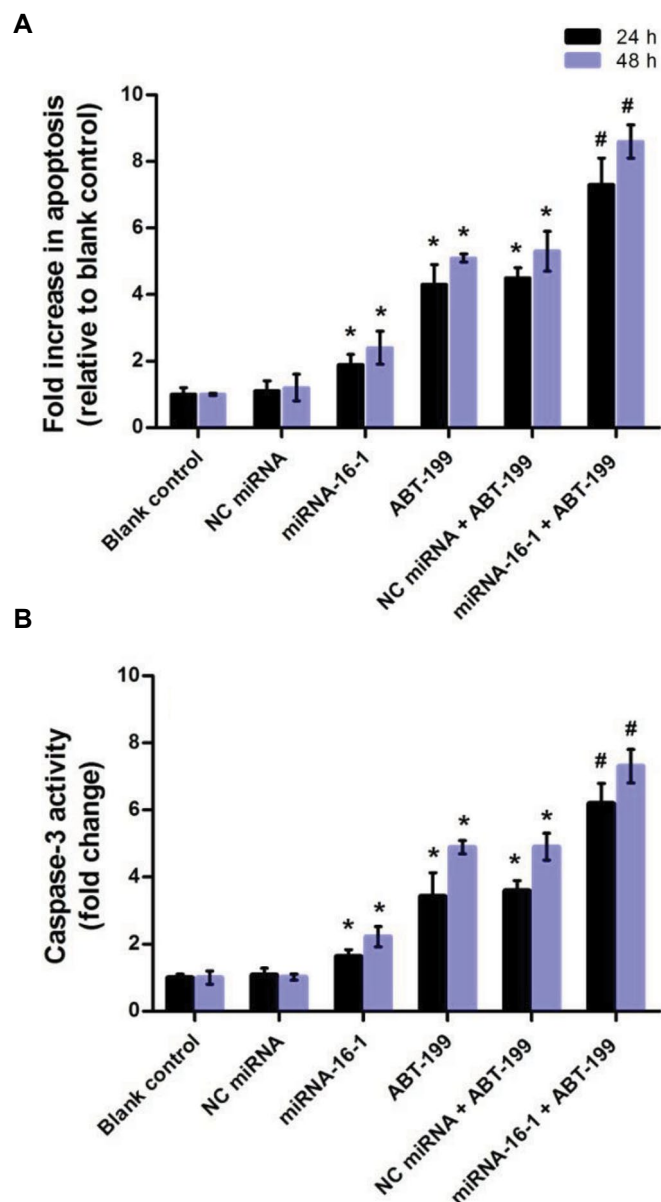

Fig.4: The effects of *miRNA-16-1* and ABT-199 on apoptosis of CLL cells. Cells were treated with *miRNA-16-1* (50 nM), NC miRNA (50 nM) and ABT-199 (IC$_{50}$ doses of 24 and 48 hours), alone and in combination. **A.** Next, the cell death was assessed using ELISA cell death assay. **B.** Caspase-3 activity of CLL cells was measured using a caspase-3 activity assay Kit. The data are showed as mean ± SD (n=3). CLL; Chronic lymphoid leukemia, IC$_{50}$; Half-maximal inhibitory concentration, NC; Negative control, *; P<0.05 relative to the control, and #; P<0.05 versus *miRNA-16-1* or ABT-199 monotreatment.

Discussion

Although ABT-199 has shown high clinical activity against CLL, some patients do not respond or become resistant to this Bcl-2 inhibitor. It has been reported that genetic factors such as Bcl-2 and Bax mutations have been associated with ABT-199 resistance. Sustained activation of B-cell receptor and AKT as well as up-regulation of Mcl-1 and Bcl-xL levels is also related to this process (12-14). However, the exact mechanisms of resistance are not fully known. Our findings propose that *miRNA-16-1* act in concert with ABT-199 to exert synergistic anticancer efficacy against CLL, which is attributed to the inhibition of Bcl-2 and Mcl-1.

Our study demonstrated that inhibition of Mcl-1 and Bcl-2 by *miRNA-16-1* was associated with inhibition of cell proliferation and increased the sensitivity of the CLL cells to ABT-199 in a synergistic way. So far, various studies have investigated the role of apoptotic proteins, especially Mcl-1, in the sensitivity of tumor cells to ABT-199. For example, Wang et al. (28) showed that Mcl-1-dependent AML cells were resistant to ABT-199 and Mcl-1-specific inhibitors such as A-1210477 that could counteract these resistance *in vitro* and *in vivo*. Other study indicated that treatment with A-1592668, a small-molecule inhibitor of CDK9, resulted in the loss of Mcl-1 expression and apoptosis in Mcl-1 dependent lymphoma and AML cell lines. Moreover, the A-1592668 plus ABT-199 combination showed efficacy superior to either agent alone with minimal toxicity in mouse models (29). In addition, Choudhary et al. (13) explored the mechanisms of resistance to ABT-199 in CLL and non-Hodgkin lymphoma cell lines. Their study demonstrated persistent activation of AKT as well as over-expression of Bcl-xL and Mcl-1 levels in the acquired and inherent ABT-199 resistant cells. Moreover, treatment with specific inhibitor of AKT pathway reduced Mcl-1 levels and sensitized the tumor cells to ABT-199. However, our data further confirms the results of the above studies and suggests that downregulation of Mcl-1 by *miRNA-16-1* can enhance the ABT-199 sensitivity in CLL cells that depend on Mcl-1 for survival.

MiRNA-16-1 acts as a tumor suppressor by targeting critical molecules in CLL cells. However, few studies have been performed on the role of this miRNA in the chemoresistance of CLL (30). In our study, transfection of *miRNA-16-1* increased the ABT-199 sensitivity of the CLL cells. So far, several investigations have been performed to show the relationship of miRNA with chemoresistance. Zhu et al. (31) reported that *miRNA-15a, miRNA-16-1, miRNA-34* and *miRNA-181a/b* sensitized the CLL cells to fludarabine-induced apoptosis through the inhibition of Mcl-1 and Bcl-2. Some other studies of miRNA expression reported that *miRNA-221* and *miRNA-181a* were significantly up-regulated and *miRNA-29a* strongly down-regulated in fludarabine-resistant cells *in vitro* (24, 32). Since the increased expression of Mcl-1 is associated with resistance of tumor cells to Bcl-2-specific inhibitors, other investigations have been performed to

explore the effect of miRNAs on Mcl-1 expression and the sensitivity of tumor cells to these inhibitors. *MiRNA-193b* is down-regulated in melanoma cells, and induced expression of this miRNA restores ABT-737 sensitivity of the resistant cells by targeting Mcl-1 (33, 34). Similarly Lam et al. (34) recognized a panel of 12 miRNAs that were linked to the reduced Mcl-1 protein levels that can sensitize melanoma cells to the apoptosis induced by ABT-263. In accordance with the above reports, our findings showed that *miRNA-16-1* increases ABT-199 sensitivity of the CLL cells. No other study has been done on the relationship between miRNAs and sensitivity to ABT-199 in cancer cells.

We also examined the effects of *miRNA-16-1* and ABT-199 on cellular apoptosis. Our results demonstrated that ABT-199 significantly triggered apoptosis and enhanced caspase-3 activity in CLL cells. Moreover, suppression of Mcl-1 and Bcl-2 by *miRNA-16-1* was associated with the induction of apoptosis and enhancement of the ABT-199-mediated apoptosis. The intrinsic pathway of apoptosis is induced with different stimuli such as DNA damage, oxidative stress, cytotoxic drugs and radiation. This pathway is under the control of Bcl-2 family of pro- and anti-apoptotic proteins (35, 37). The pro-apoptotic members Bcl-2 family such as Bak and Bax when activated lead to protein hemodimerization, change in the mitochondrial outer membrane permeability (MOMP), release of cytochrome c, and ultimately the downstream activation of the caspases 3, 6 and 7. The anti-apoptotic members such as Bcl-2 and Mcl-1 inhibit apoptosis by heterodimerising with Bax and Bak (5, 9). ABT-199 induces intrinsic pathway of apoptosis in CLL cells by inhibiting Bcl-2. It has been shown that caspase-3 activation is induced by the ABT-199. Moreover, it has been shown that the tumor cells which over-expressed Bcl-2, Mcl-1 and Bcl-xL were resistant to ABT-199 (9, 28, 29). The above reports are in accordance with our results and propose that targeting of anti-apoptotic family members would be a promising strategy to enhance the activity of ABT-199 in various malignancies including CLL.

Conclusion

The data presented here indicate that *miRNA-16-1* act in concert with ABT-199 to exert synergistic anticancer efficacy against CLL, attributed to the inhibition of Bcl-2 and Mcl-1. Moreover, our study demonstrated that *miRNA-16-1* could augment the execution of apoptosis induced by ABT-199. The intrinsic pathway of apoptosis and caspase activation may be a part of the underlying mechanisms involved in this process. Collectively, our findings show that the combination of *miRNA-16-1* and ABT-199 can efficaciously induce the apoptosis of CLL cells, and may offer a promising strategy for patients with CLL.

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Authors' Contributions

H.K.; Study concept and design. N.A., A.S.A.M., M.B.; Acquisition of data. N.A., H.K., M.B., A.S.A.M.; Analysis and interpretation of data. N.A., H.K., M.B.; Drafting of the manuscript. N.A., H.K., A.S.A.M.; Critical revision of the manuscript for important intellectual content. H.K., A.S.A.M.; Funding recipients. All authors read and approved the final manuscript.

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