

## RESEARCH ARTICLE

# Down-Regulation of Mcl-1 by Small Interference RNA Induces Apoptosis and Sensitizes HL-60 Leukemia Cells to Etoposide

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### Abstract

**Background:** Acute myeloid leukemia (AML) is a fatal hematological malignancy which is resistant to a variety of chemotherapy drugs. Myeloid cell leukemia-1 (Mcl-1), a death-inhibiting protein that regulates apoptosis, has been shown to be overexpressed in numerous malignancies. In addition, it has been demonstrated that the expression level of the Mcl-1 gene increases at the time of leukemic relapse following chemotherapy. The aim of this study was to target Mcl-1 by small interference RNA (siRNA) and analyze its effects on survival and chemosensitivity of acute myeloid leukemia cell line HL-60. **Materials and Methods:** siRNA transfection was performed with a liposome approach. The expression levels of mRNA and protein were measured by real-time quantitative PCR and Western blot analysis, respectively. Trypan blue assays were performed to evaluate tumor cell growth after siRNA transfection. The cytotoxic effects of Mcl-1 siRNA (siMcl-1) and etoposide were determined using MTT assay on their own and in combination. Apoptosis was quantified using a DNA-histone ELISA assay. **Results:** Transfection with siMcl-1 significantly suppressed the expression of Mcl-1 mRNA and protein in a time-dependent manner, resulting in strong growth inhibition and spontaneous apoptosis. Surprisingly, pretreatment with siMcl-1 synergistically enhanced the cytotoxic effect of etoposide. Furthermore, Mcl-1 down-regulation significantly increased apoptosis sensitivity to etoposide. No significant biological effects were observed with negative control siRNA treatment. **Conclusions:** Our results suggest that specific suppression of Mcl-1 by siRNA can effectively induce apoptosis and overcome chemoresistance of leukemic cells. Therefore, siMcl-1 may be a potent adjuvant in leukemia chemotherapy.

**Keywords:** Mcl-1 - apoptosis - siRNA - etoposide - leukemia - HL-60

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### Introduction

Acute myeloid leukemia (AML) is a genetic disorder characterized by accumulation of abnormal myeloid progenitor cells in the bone marrow, resulting in hematopoietic failure (Robak and Wierzbowska, 2009). AML is commonly associated as a disease of the elderly, with the majority of patients dying with the condition. (Kupsa et al., 2012). Standard treatment options for AML are chemotherapy and hematopoietic stem cell transplantation (Robak and Wierzbowska, 2009; Smits et al., 2009). The majority of patients with AML does not achieve complete remission (CR) or are expected to relapse even with intensive chemotherapy. This is mainly due to the development of drug resistance in tumor cells (Xiao et al., 2009; Kupsa et al., 2012; Szer, 2012). In addition, owing to the occurrence of relapse and development of graft-versus-host-disease (GVHD) in stem

cell transplantation, the treatment of these patients is still controversial (Robak and Wierzbowska, 2009; Smits et al., 2009). Therefore, the design of new therapeutic strategies using less toxic and more specific agents is necessary.

Dysregulation of the apoptosis (Programmed cell death) machinery contributes to the formation of neoplasia and subsequent resistance to chemotherapy (Zhang et al., 2012; Li et al., 2013). As most of the chemotherapy agents exert their anti-tumor effects by triggering apoptosis, new approaches for cancer treatment have focused on targeting mediators of this pathway (High et al., 2010; Akagi et al., 2013).

Myeloid cell leukaemia-1 (Mcl-1), a highly regulated member of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) family of proteins, was initially founded in the ML-1 human myeloid leukemia cell line during differentiation (Kozopas et al., 1993). This protein is expressed in different tissue and tumor cells and possesses an important

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role in the control of apoptosis and cell cycle program. Moreover, some reports have indicated that Mcl-1 is needed for the function of hematopoietic cell systems and survival of tumor cells (Brunelle et al., 2009; Yecies et al., 2010; Glaser et al., 2012). Studies have shown that Mcl-1 is overexpressed in various malignancies and down-regulation of this protein by antisense oligonucleotide (ASO) or RNA interference (RNAi) technology induced apoptosis and sensitized tumor cells to anti-cancer agents (Chetoui et al., 2008; Skoda et al., 2008; Warr and Shore, 2008; Quinn et al., 2011; Akagi et al., 2013). Moreover, elevated expression of Mcl-1 at the time of leukemic relapse, offers a possible explanation to the role of this protein in the survival of leukemia cells after chemotherapy (Kaufmann et al., 1998).

In this study, we investigated whether suppression of Mcl-1 by small interference RNA (siRNA) could sensitize HL-60 human AML cell line to chemotherapeutic agent etoposide. We therefore examined the effects of either Mcl-1 specific siRNA (siMcl-1) or etoposide alone, versus their combination treatment in promoting tumor cells apoptosis.

## Materials and Methods

### Cell culture conditions

The HL-60 leukemic cells (Pasteur Institute, Tehran, Iran) were maintained in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) (Sigma-Aldrich), 2 mM of glutamine, and 1% sodium pyruvate at 37°C in 95% humidified atmosphere containing 5% CO<sub>2</sub>. The cells were sub-cultured with an initial concentration of 5 × 10<sup>4</sup> cells/ml and used in the logarithmic growth phase in whole experiments.

### Transfection of siRNA

The Mcl-1 siGENOME siRNA and a negative control (NC) siRNA were ordered from Dharmacon (Lafayette, CO, USA). Just before transfection, HL-60 cells were cultured in RPMI-1640 medium without FBS and antibiotics. Transfection of siRNAs was performed using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. In brief, siRNAs (at a final concentration of 80 nM) and lipofectamine (4 µl/ml of transfection medium) were diluted in Opti-MEM I medium (Invitrogen) separately and mixed gently. After incubation for 15 min at ambient temperature, the diluted solutions were combined and incubated for another 15 min at ambient temperature. Following on, the complexes were then added to the culture medium. Treatment with only lipofectamine was considered as siRNA blank control. After 6 h incubation of the cells at 37°C in a humidified CO<sub>2</sub> incubator, RPMI medium plus FBS was added to a final FBS concentration of 10%, with cells being cultured under the same conditions. For assessment of effective gene silencing, transfection (5 × 10<sup>5</sup> cells/well) was performed in 6-well culture plates. After 24, 48 and 72 h, down-regulation of

Mcl-1 was monitored by real-time quantitative PCR (RT-qPCR) and Western blotting.

### Cytotoxicity assay

The effect of siMcl-1 on the sensitivity of HL-60 cell line to etoposide (Sigma- Aldrich) was evaluated using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. The experiment was divided into eight groups: etoposide, siMcl-1, NC siRNA, siMcl-1 and etoposide, NC siRNA and etoposide, siRNA blank control, etoposide blank control and combination blank control. Briefly, cells were cultivated at a density of 2 × 10<sup>4</sup> cells/well in 96-well tissue culture plates. Six hours after transfection, the cells were treated with various concentrations of etoposide (0.001, 0.2, 0.5, 1, 2 and 4 µM). Cells treated with only 1% DMSO, solvent of etoposide, were served as etoposide blank controls. Also, treatment with a mixture of 1% DMSO and lipofectamine without siRNA were considered as a combination blank control. After 18 h of incubation, the cytotoxicity of the treatments was determined using a cell proliferation MTT kit (Roche Diagnostics GmbH, Mannheim, Germany) as described by the manufacturer. The absorbance (A) of the formazan dye was quantified with an ELISA plate reader (Awareness Technology, Palm City, FL, USA) at 570 nm with a reference wavelength of 650 nm. The survival rate (SR) was calculated from the following formula: SR (%) = (A Test /A Control) ×100%. IC<sub>50</sub> (concentration that produced 50% cytotoxicity) values were determined using Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

### Combination effect analysis

The effect of the interaction between siMcl-1 and etoposide was determined using the value of the coefficient of drug interaction (CDI), based on the Chou-Talalay method (Chou and Talalay, 1984; Han et al., 2013). The CDI was calculated using the following equation: CDI = AB/ (A×B), where AB is the survival rate of the combination group relative to the control group, A and B are the survival rate of siMcl-1 and etoposide relative to the corresponding control group. CDI > 1, CDI = 1 and CDI < 1 indicate antagonistic, additive and synergistic effects, respectively.

### Cell viability assay

The effect of siMcl-1 on cell growth was measured by the trypan blue exclusion assay. HL-60 cells (4 × 10<sup>4</sup> cells/well) were transfected with siRNAs in 24-well culture plates and incubated for 24-120 h. At indicated time points, the cells were harvested and cell suspensions stained with equal volume of 0.4% trypan blue (Merck KGaA, Darmstadt, Germany). After 3 min of incubation, the number of viable cells (unstained blue) was counted under an inverted microscope (Nikon Instrument Inc., Melville, NY, USA) using a hemocytometer. The percentage of viable cells was determined by dividing the number of viable cells in test group by the number of viable cells in blank control group and multiplying by 100. Also, the viability of the blank control group in each time was considered as 100%.

### RT-qPCR

Following treatments, total RNA was isolated from the cells using AccuZol™ reagent (Bioneer, Daedeok-gu, Daejeon, Korea) according to the manufacturer's protocol. Then, 1  $\mu$ g of total RNA was reverse transcribed into complementary DNA (cDNA) by use of MMLV reverse transcriptase and oligo-dT primer following the manufacturer's instructions (Promega, Madison, WI, USA). Relative gene expression was quantified by RT-qPCR using SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan) and the Rotor-Gene™ 6000 system (Corbett Life Science, Mortlake, NSW, Australia). The PCR was performed in a 20  $\mu$ l reaction system containing 1  $\mu$ l of cDNA template, 12  $\mu$ l of SYBR green reagent, 0.2  $\mu$ M of each of the primers and 6  $\mu$ l of nuclease-free distilled water. The sequences of PCR primers were as follows: forward, 5'-TAAGGACAAAACGGGACTGG-3', reverse, 5'-ACCAGCTCCTACTCCAGCAA-3', for Mcl-1, and forward, 5'-TCCCTGGAGAAGAGCTACG-3', and reverse, 5'-GTAGTTTCGTGGATGCCACA-3', for  $\beta$ -actin. The PCR conditions for Mcl-1 and  $\beta$ -actin were 95°C for 10 min followed by 45 cycles at 95°C for 20 sec and 60°C for 1 min. Relative gene expression of Mcl-1 was calculated with the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001), using  $\beta$ -actin as an internal control.

### Western blot analysis

Cells were collected, washed twice with cold phosphate-buffered saline (PBS) and resuspended in lysis buffer (1% Triton X-100, 1% SDS, 150 nM NaCl, 50 mM Tris-HCl, pH 7.4 and 1mM EDTA, pH 8) containing protease inhibitor cocktail complete (Roche Diagnostics GmbH). After 30 min of incubation on ice, the suspensions were centrifuged at 14,000 rpm for 10 min at 4°C and the supernatants were collected. Protein concentrations were measured by Bradford reagent (Sigma-Aldrich). Equal amounts of each protein sample (50  $\mu$ g) were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF) (GE Healthcare, Amersham, Buckinghamshire, UK).

The membranes were incubated at room temperature for 45 min in blocking buffer (3% fat-free milk and 0.05% Tween-20 in PBS) and then probed overnight at 4°C with primary mouse monoclonal antibodies specific for Mcl-1 (1:500, Abcam, Cambridge, MA, UK) and  $\beta$ -actin (1:1,000, Abcam) diluted in 3% fat-free milk in PBS. Following three 5 min washes with PBS containing 0.05% Tween-20, membranes were incubated with corresponding horseradish peroxidase (HRP)-linked goat anti-mouse secondary antibody (1:3,000, Abcam) diluted in PBS for 2 h at room temperature. Next, the membranes were washed again and bound antibodies visualized using enhanced chemiluminescence plus western blotting detection Kit (GE Healthcare) and X-ray film (Estman Kodak, Rochester, NY, USA). Densitometry was performed by ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) and the signal intensity of each protein band was normalized for the respective actin loading control.

### Apoptosis ELISA assay

Cell death was quantified with a cell death detection ELISA plus kit (Roche Diagnostics GmbH) that measures mono- and oligonucleosomes released into the cytoplasm of apoptotic cells. The HL-60 cells were seeded at a density of  $4 \times 10^4$  cells/well in 24-well plates and treated with siMcl-1, NC siRNA, the IC<sub>50</sub> dose of etoposide or a combination of the two, as described previously. Twenty-four hours after transfection, cells were harvested and ELISA assay was performed according to the manufacturer's protocol. In brief, the cell lysates were obtained and centrifuged at 200 g for 10 min. Twenty microliters of the supernatants and 80  $\mu$ l of immunoreagent containing anti-histone-biotin and anti-DNA-peroxidase were then transferred to each well of a streptavidin-coated plate and the plate was incubated for 2 h in 25°C. The wells were washed with incubation buffer and 100  $\mu$ l of 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) solution was added. The reactions were stopped with ABTS stop solution and absorbance was measured using an ELISA plate reader at 405 nm (with a reference wavelength of 490 nm). Results were expressed as the fold increase in the absorbance of treatment groups relative to the blank control group.

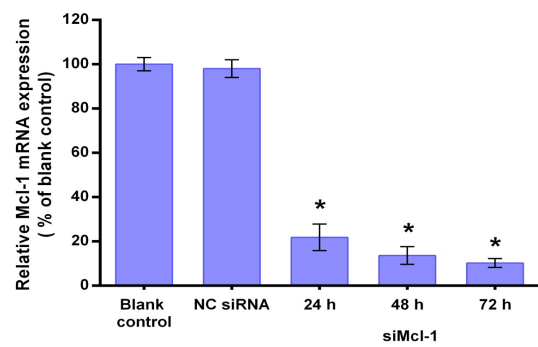
### Statistical analysis

Data were presented as mean  $\pm$  standard deviation (SD). Analysis of variance (ANOVA) followed by Bonferroni's test was used to determine the significant differences between groups. Values of P less than 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism software.

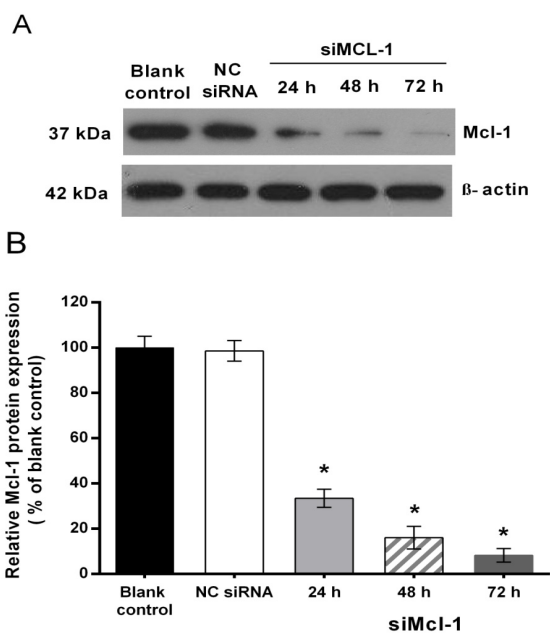
## Results

### BsiRNA down-regulated Mcl-1 mRNA and protein levels in HL-60 cells

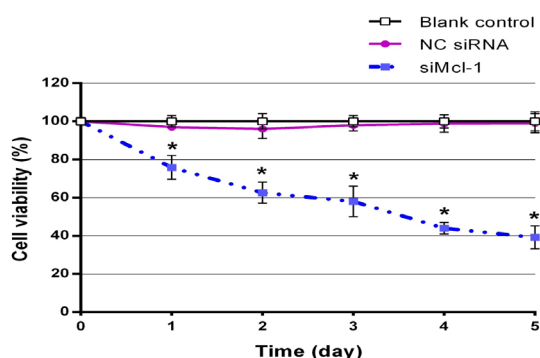
Firstly, we examined the effect of siRNA on Mcl-1 gene expression in tumor cells by RT-qPCR and Western blotting. Relative gene expression was calculated in relation to the blank control, which was considered as 100%. Compared with the blank control, treatment with siMcl-1 markedly reduced Mcl-1 mRNA and protein



**Figure 1. Down-regulation of Mcl-1 mRNA by siRNA in HL-60 Cells.** Cells were transfected with Mcl-1 siRNA (siMcl-1) or negative control (NC) siRNA for 24, 48 and 72 h. Mcl-1 mRNA levels were examined by RT-qPCR and relative mRNA expression levels were quantified by the  $2^{-\Delta\Delta Ct}$  method. The data represent mean  $\pm$  SD (n=3); \*p<0.05 versus blank control



**Figure 2. The Expression Levels of Mcl-1 Protein in HL-60 Cells Transfected with siRNA.** (A) Representative western blot of Mcl-1 and  $\beta$ -actin proteins from cells transfected with siRNAs. (B) The expression level of each band was measured by densitometry and normalized to the corresponding  $\beta$ -actin. The data represent mean $\pm$ SD (n=3); \*p<0.05 versus blank control

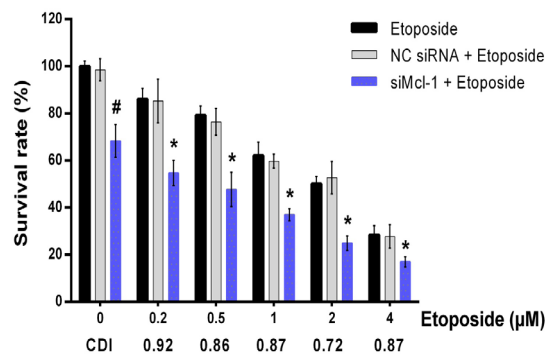


**Figure 3. Growth Curve of HL-60 Cells Transfected with siMcl-1 or NC siRNA.** Cell viability in each time was measured by trypan blue staining. The data represent mean $\pm$ SD (n=3); \*p<0.05 versus blank control

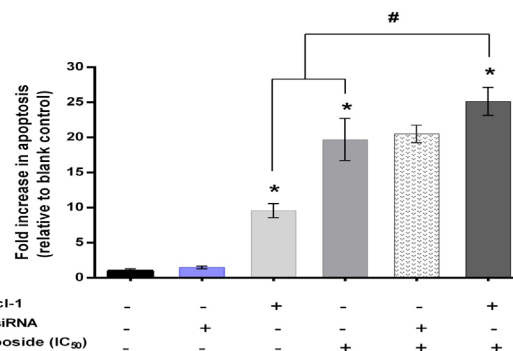
levels in a time-dependent manner (p<0.05; Figure 1 and 2). At 24, 48 and 72 h posttransfection, the relative expression of Mcl-1 mRNA were 21.87%, 13.60% and 10.26%, respectively (Figure 1), while relative expression of Mcl-1 protein were 33.45%, 16.01% and 8.28%, respectively (Figure 2B) (p<0.05). Notably, treatment with NC siRNA had minimal effect on mRNA and protein levels compared with the blank control (p>0.05).

*Down-regulation of Mcl-1 inhibited cell growth*

As overexpression of Mcl-1 is associated with survival of leukemic cells; we therefore sought to examine whether down-regulation of this protein could inhibit the growth of HL-60 cells. The cells were transfected with siMcl-1 and NC siRNA. Cell viability was then determined every 24 h for 5 days by trypan blue assay. In our study, the cell growth curve showed that compared with blank control



**Figure 4. Effect of siMcl-1 on the Sensitivity of HL-60 Cell Line to Etoposide.** The cytotoxic effects of siMcl-1, NC siRNA, etoposide and their combination were determined by MTT assay as described in methods section. The values of the coefficient of drug interaction (CDI) were calculated to determine the interaction effects between etoposide and siMcl-1. The data represent mean $\pm$ SD (n=4); \*p<0.05 versus etoposide or siMcl-1 alone; p<0.05 versus blank control



**Figure 5. Effect of Mcl-1 Down-regulation on Etoposide-induced Apoptosis.** HL-60 cells were treated with siRNAs and etoposide alone or in combination. Twenty-four hours after transfection, apoptosis was detected by cell death ELISA assay as described previously. The data represent mean $\pm$ SD (n=3); \*p<0.05 versus blank control; #p<0.05

group, siMcl-1 significantly reduced cell viability in a time dependent manner (p<0.05; Figure 3). Beginning at 24 h after transfection of Mcl-1 siRNA, the cell viability decreased rapidly to 76.68% and dropped to 39.23% on day 5. In contrast, no significant differences in cell viability were found between the NC siRNA transfected cells and the blank control group (p>0.05; Figure 3).

*siMcl-1 increased sensitivity to etoposide in a synergic manner*

To analyze whether reduced Mcl-1 expression could enhance the sensitivity of HL-60 cells to etoposide, a combination treatment with siMcl-1 and etoposide was performed. As shown in Figure 4, single treatment with etoposide induced cytotoxicity in a dose-dependent manner. The results showed that siMcl-1 alone significantly lowered the cell survival rate to 68.32%, relative to the blank control (p<0.05). Furthermore, combination therapy further reduced the cell survival rate compared with siMcl-1 or etoposide monotherapy (p<0.05). The IC<sub>50</sub> value of etoposide drastically decreased from 2.03  $\mu$ M to 1.01  $\mu$ M in the presence of siMcl-1. The combination effects of siMcl-1 and etoposide were also synergistic

with the CDI values of less than 1 in all concentrations of etoposide (Figure 4). Meanwhile, NC siRNA had an insignificant effect on the chemosensitivity of the cells compared with etoposide alone ( $p>0.05$ ; Figure 4).

#### *Down-regulation of Mcl-1 enhanced etoposide-induced apoptotic cell death*

To confirm whether the observed synergistic cytotoxic effects between siMcl-1 and etoposide were related to the increase in the extent of apoptosis, the effects of specific siRNA and etoposide alone and in combination on apoptosis, were examined using an ELISA-based cell death detection system. Figure 5, demonstrates that treatment of leukemia cells with siMcl-1 or etoposide alone significantly increased apoptosis by 9.58 and 19.70 fold, respectively, compared to the blank control. Moreover, combination treatment further enhanced apoptosis to 25.16 fold ( $p<0.05$ ). On the other hand, treatment with NC siRNA alone or in combination with etoposide displayed no distinct alterations in the extents of apoptosis relative to the blank control or etoposide monotreatment, respectively ( $p>0.05$ ). Therefore, we concluded that the chemosensitization effect of Mcl-1 down-regulation is partially attributed to the enhancement of apoptosis.

## Discussion

Despite intensive efforts in the treatment of AML, it is unfortunately still deemed as an incurable disease with a high mortality rate. Owing to the occurrence of chemoresistance in leukemia cells, the majority of patients does not achieve CR or show relapse after first CR, following the standard therapy (Smits et al., 2009; Xiao et al., 2009; Szer, 2012). Therefore, development of new strategies for improved therapy is required. It has been shown that Mcl-1 up-regulates in a variety of malignancies, such as leukemia (Warr and Shore, 2008). On the contrary, others have demonstrated that down-regulation of Mcl-1 can sensitize cancer cells to anti-tumor agents (Chetoui et al., 2008; Skoda et al., 2008). The above reports indicate that Mcl-1 is linked to drug resistance and may be an appropriate target for chemosensitization of tumor cells. However, the effect of Mcl-1 depletion on sensitivity of AML cells to the chemotherapeutic agent etoposide has yet to be investigated. Thus, we examined a gene therapy strategy using siMcl-1 in combination with etoposide on HL-60 leukemia cell line.

RT-qPCR and Western blot revealed that treatment with siMcl-1 led to steady decrease in the expression of Mcl-1 at the mRNA and protein levels over a 3-day period. These results suggest that siMcl-1 effectively blocked the translation of the Mcl-1 protein through cleavage of the corresponding mRNA. The results of the cell viability assay indicated that the down-regulation of Mcl-1 significantly decreased the viability of cells compared with the blank control group during a 5-day period. These results show that Mcl-1 may play a critical role in the survival and growth of leukemia cells. The MTT assay findings demonstrated that pretreatment with siMcl-1 synergistically enhanced the cytotoxicity of etoposide

in HL-60 cells, and the  $IC_{50}$  value was subsequently decreased substantially. Therefore, it is proposed that down-regulation of Mcl-1 could sensitize HL-60 cells to etoposide.

To further investigate the role of Mcl-1 in the chemoresistance of tumor cells, we examined the effect of Mcl-1 down-regulation on etoposide-induced apoptosis. ELISA cell death assay showed that the chemotherapeutic agent etoposide, alone, caused remarkable apoptosis in leukemia cells. Of note, we found that the inhibition of Mcl-1 using siRNA also resulted in significant apoptosis in the absence of etoposide, which is in contrast to the finding of others on solid tumors (Thallinger et al., 2003, Thallinger et al., 2004). However, this observation is similar to that found on other studies on hematological malignancies (Hussain et al., 2007; Quinn et al., 2011), illustrating the important biological role of Mcl-1 in leukemic cells. In addition, siMcl-1, in combination with etoposide dramatically enhanced apoptosis compared with siRNA alone or etoposide alone. In contrast, neither NC siRNA nor lipofectamine treatment changed the impact of chemotherapy, which supports the specific effect of siMcl-1. These results are in agreement with previous reports using other tumor cells (Kano et al., 2009, Akagi et al., 2013). The above-mentioned results confirm that the presence of anti-apoptotic Mcl-1 protein is required for the survival and development of chemoresistance in the HL-60 cell line. Thus, targeting of this protein could induce apoptosis and sensitize tumor cells to chemotherapy agents.

Apoptosis can be initiated through two major signaling pathways: the intrinsic, mitochondrial pathway and the extrinsic, death receptor pathway (Warr and Shore, 2008). Mcl-1 mainly inhibits the intrinsic pathway by the sequestering and neutralization of pro-apoptotic Bcl-2 family members such as Bak, Bax and Bim, thereby preserving mitochondria integrity. This action blocks the release of cytochrome c from the inner mitochondrial membrane space that is required for caspase activation and next apoptotic events (Warr and Shore, 2008; Zhang et al., 2012; Akagi et al., 2013). Recent studies on melanoma cells have indicated that up-regulation of Mcl-1 inhibited the death receptor pathway of apoptosis (Chetoui et al., 2008; Boisvert-Adamo et al., 2009). However, the exact role of Mcl-1 in the regulation of the extrinsic pathway of leukemic cells is not clear. Etoposide is a potent cytotoxic agent that induces apoptosis by stabilizing a covalent DNA topoisomerase II-DNA complex, causing DNA double-strands to break. In addition, it was shown that treatment of HL-60 cells with etoposide triggers both intrinsic and extrinsic pathways of apoptosis by activation of different caspases (Martins et al., 1997; Montecucco and Biamonti, 2007). Our study demonstrates that suppression of Mcl-1 protein induces apoptosis and augments the effect caused by etoposide. However, the precise molecular mechanisms of the sensitization remain unclear and further investigations are needed.

RNAi or post-transcriptional gene silencing is a powerful strategy for the knockdown of a specific gene in which double-stranded RNA (called siRNA) is introduced to the cells and suppresses gene expression through

cleavage of the related messenger RNA (mRNA). Due to advantages of RNAi such as its efficacy, specificity, and low cytotoxicity, this phenomenon is extensively used in gene therapy studies (Yang and Mattes, 2008; David et al., 2010; Shan, 2010; Ramachandran and Ignacimuthu, 2012). In comparison of the antisense oligonucleotide technology, siRNA has several advantages such as a greater resistance to nuclease-induced degradation (Reischl and Zimmer, 2009; Kanasty et al., 2012). In contrast, transient nature of chemically synthesized siRNA is one of the most important limitations of siRNA-based therapeutics. The use of constitutive siRNA-based vector systems with higher efficacy may overcome this drawback, especially for long term in vivo studies (Yang and Mattes, 2008; David et al., 2010; Shan, 2010).

Together, we have demonstrated that Mcl-1 plays a pivotal role in the survival and resistance of HL-60 cells to etoposide. Specific down-regulation of Mcl-1 by siRNA induced significant apoptosis in leukemia cells in vitro. Most importantly, the combination of etoposide with siMcl-1 showed synergistic anti-tumor effect. Our data underline the potential of siMcl-1 for apoptosis induction and sensitization of leukemia cells to anti-tumor drugs. This phenomenon appears to decrease the serious side-effects related to high-dose chemotherapy. Thus, our study suggests that the Mcl-1 gene can be considered as an attractive target in gene therapy for AML patients and may be a novel strategy to overcome chemoresistance in the future.

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## References

Akagi H, Higuchi H, Sumimoto H, et al (2013). Suppression of myeloid cell leukemia-1 (Mcl-1) enhances chemotherapy-associated apoptosis in gastric cancer cells. *Gastric Cancer*, **16**, 100-10.

Boisvert-Adamo K, Longmate W, Abel EV, Aplin AE (2009). Mcl-1 is required for melanoma cell resistance to anoikis. *Mol Cancer Res*, **7**, 549-56.

Brunelle JK, Ryan J, Yecies D, Opferman JT, Letai A (2009). MCL-1-dependent leukemia cells are more sensitive to chemotherapy than BCL-2-dependent counterparts. *J Cell Biol*, **187**, 429-42.

Chetoui N, Syla K, Gagnon-Houde JV, et al (2008). Down-regulation of mcl-1 by small interfering RNA sensitizes resistant melanoma cells to fas-mediated apoptosis. *Mol Cancer Res*, **6**, 42-52.

Chou TC, Talaly P (1984). Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*, **22**, 27-55.

David S, Pitard B, Benoit JP, Passirani C (2010). Non-viral nanosystems for systemic siRNA delivery. *Pharmacol Res*, **62**, 100-14.

Glaser SP, Lee EF, Trounson E, et al (2012). Anti-apoptotic Mcl-

1 is essential for the development and sustained growth of acute myeloid leukemia. *Genes Dev*, **26**, 120-5.

Han P, Luan Y, Lin Y, et al (2013). Small interfering RNA targeting Rac1 sensitizes colon cancer to dihydroartemisinin-induced cell cycle arrest and inhibited cell migration by suppressing NFkappaB activity. *Mol Cell Biochem*, **379**, 171-80.

High LM, Szymanska B, Wilczynska-Kalak U, et al (2010). The Bcl-2 homology domain 3 mimetic ABT-737 targets the apoptotic machinery in acute lymphoblastic leukemia resulting in synergistic in vitro and in vivo interactions with established drugs. *Mol Pharmacol*, **77**, 483-94.

Hussain SR, Cheney CM, Johnson AJ, et al (2007). Mcl-1 is a relevant therapeutic target in acute and chronic lymphoid malignancies: down-regulation enhances rituximab-mediated apoptosis and complement-dependent cytotoxicity. *Clin Cancer Res*, **13**, 2144-50.

Kanasty RL, Whitehead KA, Vegas AJ, Anderson DG (2012). Action and reaction: the biological response to siRNA and its delivery vehicles. *Mol Ther*, **20**, 513-24.

Kano R, Yano T, Nagamatsu K, et al (2009). Effectiveness of small interfering RNA (siRNA) against the Mcl-1 gene in a canine mammary gland tumor cell line. *Res Vet Sci*, **87**, 64-6.

Kaufmann SH, Karp JE, Svingen PA, et al (1998). Elevated expression of the apoptotic regulator Mcl-1 at the time of leukemic relapse. *Blood*, **91**, 991-1000.

Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW (1993). MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc Natl Acad Sci U S A*, **90**, 3516-20.

Kupsa T, Horacek JM, Jebavy L (2012). The role of cytokines in acute myeloid leukemia: a systematic review. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*, **156**, 291-301.

Li G, Chang H, Zhai YP, Xu W (2013). Targeted silencing of inhibitors of apoptosis proteins with siRNAs: a potential anti-cancer strategy for hepatocellular carcinoma. *Asian Pac J Cancer Prev*, **14**, 4943-52.

Livaki KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-ΔΔCT</sup> Method. *Methods*, **25**, 402-8.

Martins LM, Mesner PW, Kottke TJ, et al (1997). Comparison of caspase activation and subcellular localization in HL-60 and K562 cells undergoing etoposide-induced apoptosis. *Blood*, **90**, 4283-96.

Montecucco A, Biamonti G (2007). Cellular response to etoposide treatment. *Cancer Lett*, **252**, 9-18.

Quinn BA, Dash R, Azab B, et al (2011). Targeting Mcl-1 for the therapy of cancer. *Expert Opin Investig Drugs*, **20**, 1397-411.

Ramachandran PV, Ignacimuthu S (2012). RNA interference as a plausible anticancer therapeutic tool. *Asian Pac J Cancer Prev*, **13**, 2445-52.

Reischl D, Zimmer A (2009). Drug delivery of siRNA therapeutics: potentials and limits of nanosystems. *Nanomedicine*, **5**, 8-20.

Robak T, Wierzbowska A (2009). Current and emerging therapies for acute myeloid leukemia. *Clin Ther*, **31**, 2349-70.

Shan G (2010). RNA interference as a gene knockdown technique. *Int J Biochem Cell Biol*, **42**, 1243-51.

Skoda C, Erovcic BM, Wachek V, et al (2008). Down-regulation of Mcl-1 with antisense technology alters the effect of various cytotoxic agents used in treatment of squamous cell carcinoma of the head and neck. *Oncol Rep*, **19**, 1499-503.

Smits EL, Berneman ZN, Van Tendeloo VF (2009). Immunotherapy of acute myeloid leukemia: current approaches. *Oncologist*, **14**, 240-52.

- Szer J (2012). The prevalent predicament of relapsed acute myeloid leukemia. *Hematol Am Soc Hematol Educ Program*, **2012**, 43-8.
- Thallinger C, Wolschek MF, Maierhofer H, et al (2004). Mcl-1 is a novel therapeutic target for human sarcoma: synergistic inhibition of human sarcoma xenotransplants by a combination of mcl-1 antisense oligonucleotides with low-dose cyclophosphamide. *Clin Cancer Res*, **10**, 4185-91.
- Thallinger C, Wolschek MF, Wacheck V, et al (2003). Mcl-1 antisense therapy chemosensitizes human melanoma in a SCID mouse xenotransplantation model. *J Invest Dermatol*, **120**, 1081-6.
- Warr MR, Shore GC (2008). Unique biology of Mcl-1: therapeutic opportunities in cancer. *Curr Mol Med*, **8**, 138-47.
- Xiao J, Yin S, Li Y, et al (2009). SKP2 siRNA inhibits the degradation of P27kip1 and down-regulates the expression of MRP in HL-60/A cells. *Acta Biochim Biophys Sin*, **41**, 699-708.
- Yang M, Mattes J (2008). Discovery, biology and therapeutic potential of RNA interference, microRNA and antagomirs. *Pharmacol Ther*, **117**, 94-104.
- Yecies D, Carlson NE, Deng J, Letai A (2010). Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. *Blood*, **115**, 3304-13.
- Zhang S, Li G, Ma X, et al (2012). Norcantharidin enhances ABT-737-induced apoptosis in hepatocellular carcinoma cells by transcriptional repression of Mcl-1. *Cell Signal*, **24**, 1803-9.