Advanced Pharmaceutical Bulletin, **2014**, 4(3), 243-248
doi: http://dx.doi.org/10.5681/apb.2014.035
http://apb.tbzmed.ac.ir/

Therapeutic Effects of Myeloid Cell Leukemia-1 siRNA on Human Acute Myeloid Leukemia Cells

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ARTICLEINFO

Article Type:
Research Article

Article History:
Received: 27 November 2013
Revised: 1 January 2014
Accepted: 3 January 2014
ePublished: 7 February 2014

Keywords:
Mcl-1
siRNA
Leukemia
HL-60
Apoptosis
Proliferation

ABSTRACT

***Purpose*:** Up-regulation of Mcl-1, a known anti-apoptotic protein, is associated with the survival and progression of various malignancies including leukemia. The aim of this study was to explore the effect of Mcl-1 small interference RNA (siRNA) on the proliferation and apoptosis of HL-60 acute myeloid leukemia (AML) cells.
***Methods*:** siRNA transfection was performed using Lipofectamine™2000 reagent. Relative mRNA and protein expressions were quantified by quantitative real-time PCR and Western blotting, respectively. Trypan blue assay was performed to assess tumor cell proliferation after siRNA transfection. The cytotoxic effect of Mcl-1 siRNA on leukemic cells was measured using MTT assay. Apoptosis was detected using ELISA cell death assay.
***Results*:** Mcl-1 siRNA clearly lowered both Mcl-1 mRNA and protein levels in a time-dependent manner, leading to marked inhibition of cell survival and proliferation. Furthermore, Mcl-1 down-regulation significantly enhanced the extent of HL-60 apoptotic cells.
***Conclusion*:** Our results suggest that the down-regulation of Mcl-1 by siRNA can effectively trigger apoptosis and inhibit the proliferation of leukemic cells. Therefore, Mcl-1 siRNA may be a potent adjuvant in AML therapy.

Introduction

Acute myeloid leukemia (AML) is a lethal disorder characterized by the accumulation of abnormal myeloid progenitor cells in the bone marrow, which results in hematopoietic failure. Despite various efforts in detection and treatment, many patients with AML continue to die of this cancer.[1-3] Therefore, understanding the cellular mechanisms linked to the AML formation and progression could be beneficial in designing of the new therapeutic strategies for AML.
Myeloid cell leukaemia-1 (Mcl-1) is a highly regulated member of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) family of proteins, was originally isolated from the ML-1 human myeloid leukemia cells during differentiation.[4] This protein was founded in various tissue and tumor cells and has a critical role in the regulation of cell cycle program and apoptosis. Some previous studies have revealed that Mcl-1 is needed for the survival of hematopoietic and tumor cells.[5-7] Furthermore, other studies have demonstrated that Mcl-1 is overexpressed in different malignancies including leukemia, and suppression of this protein by RNA interference (RNAi) or antisense oligonucleotide (ASO) technology triggered apoptosis and inhibited the growth of tumor cells.[8-13]
RNAi or post-transcriptional gene-silencing technology is a potent phenomenon in which a double stranded RNA (called siRNA) is transfered into the cells where it suppresses the expression of a specific gene by cleavage of the homologous mRNA. There have been many confirmed studies that RNAi-mediated gene silencing can successfully induce apoptosis and arrest the proliferation of the malignant cells in vivo and in vitro, and siRNA-based gene therapy has been turned into an effective approach for cancer therapy.[14-16]
In this study, Mcl-1 small interference RNA (siRNA) was transfected into AML cell line HL-60 in vitro, to

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investigate the impact of Mcl-1 gene suppression on the human AML cell apoptosis and proliferation.

Materials and Methods

Cell culture conditions

The HL-60 AML cell line (Pasteur Institute, Tehran, Iran) was maintained in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 15% fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM of glutamine, 1% sodium pyruvate and 1% antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) (Sigma-Aldrich) at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were sub-cultured 48-72 h later with an initial concentration of 5 × 10^4 cells/ml and used in the logarithmic growth phase in whole experiments.

siRNA transfection

The Mcl-1 specific and negative control (NC) siGENOME siRNA sequences were ordered from Dharmacon (Lafayette, CO, USA). Just before transfection, the cells were grown in RPMI-1640 medium free of antibiotics and FBS. siRNA transfection (at a final concentration of 50 nM in all experiments) was performed using Lipofectamine™2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, siRNAs and lipofectamine (4 µl/ml of transfection medium) were separately diluted in Opti-MEM I Reduced Serum Medium (Invitrogen) and incubated for 10 min at ambient temperature. The diluted solutions were then combined and incubated for 20 min at ambient temperature. Following on, the mixtures were added to each well containing medium and cells. Furthermore, the treated cells with only lipofectamine were considered as a siRNA blank control group. The cells were then incubated for 6 h at 37 °C in a humidified CO_2 incubator. Subsequently, complete cell culture medium (with final FBS concentration of 15%) was added and the cells were incubated under the same mentioned conditions. To monitor the effect of siRNA on gene silencing, transfection (5 × 10^5 cells/well) were done in 6-well plates for 24-48 h. Down-regulation of Mcl-1 expression was then measured by quantitative real-time PCR (qRT-PCR) and Western blot analysis.

Cytotoxicity assay

The cytotoxic effect of Mcl-1 siRNA on HL-60 was determined using 3-(4, 5 Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. The experiment was subdivided into three groups: Mcl-1 siRNA, NC siRNA and blank control. Briefly, leukemic cells were seeded at a density of 15 × 10^3 cells in 96-well cell culture plates. The cells were then transfected with siRNAs as described previously. After 24 and 48 h of incubation, the cytotoxicities of the treatments were measured using the MTT assay kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's recommendations. The amounts of formazan dyes were quantified by measuring their absorbance (A) at 570 nm with a reference wavelength of 650 nm using an ELISA plate reader (Awareness Technology, Palm City, FL, USA). The cell survival rate (SR) was measured from the following formula:

$$SR\ (\%) = (A_{\text{Experiment}} / A_{\text{Blank control}}) \times 100\%.$$

Cell proliferation assay

The antiproliferative effect of Mcl-1 siRNA was evaluated using trypan blue exclusion assay. The cells (5 × 10^4 cells/well) were transfected with Mcl-1 specific and NC siRNAs in 24-well cell culture plates and then incubated for 24-120 h. At different time points after transfections, the cells were collected and stained with equal volume of 0.4% trypan blue dye (Merck KGaA, Darmstadt, Germany) for 1 min. Following on, the number of viable cells (N, unstained cells) was quantified using a hemocytometer and an inverted microscope (Nikon Instrument Inc., Melville, NY, USA). The percentage of viable cells was then determined from the equation as follows: Cell viability (%) = ($N_{\text{Experiment}} / N_{\text{Blank control}}$) × 100. The percentage of viable cells in each time was also considered as 100% for blank control group.

qRT-PCR

Following transfections, total RNA was extracted by AccuZol™ reagent (Bioneer, Daedeok-gu, Daejeon, Korea) as described by the manufacturer. Complementary DNA (cDNA) was generated from 1 µg of total RNA by use of oligo-dT primer and MMLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's recommendations. qRT-PCR was then performed using SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan) in the Rotor-Gene™ 6000 system (Corbett Life Science, Mortlake, NSW, Australia). The PCR was done in a 20 µl reaction system containing 12 µl of SYBR green reagent, 0.2 µM of each primer, 1 µl of cDNA template and 6 µl of nuclease-free distilled water. The primer sequences were as follows: forward, 5'-TCCCTGGAGAAGAGCTACG-3', reverse, 5'-GTAGTTTCGTGGATGCCACA-3', for β–actin and forward, 5'-TAAGGACAAAACGGGACTGG-3', and reverse, 5'-ACCAGCTCCTACTCCAGCAA-3', for Mcl-1. The initial denaturation step at 95 °C for 10 min was followed by 45 cycles at 95 °C for 20 sec and 60 °C for 1 min. Relative Mcl-1 mRNA expression was calculated with the $2^{-(\Delta\Delta C_T)}$,[17] using β-actin as a reference gene.

Western blot analysis

At indicated time points after transfection, the cells were harvested, washed with cold PBS and resuspended in lysis buffer (1% SDS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA, pH 8) containing protease inhibitor cocktail

complete (Roche Diagnostics GmbH) for 30 min on ice. Cell suspensions were then centrifuged at 12,000 rpm for 10 min at 4 °C and cellular debrises were removed. Protein concentrations were measured using Bradford reagent (Sigma-Aldrich). Next, 50 µg of each protein sample was separated by 12% SDS-PAGE, transferred to PVDF membrane (GE Healthcare, Amersham, Buckinghamshire, UK), and then blocked with 3% skim milk in PBS/Tween-20 (0.05%, v/v) for 45 min at room temperature. Subsequently, the membrane was probed overnight at 4 °C with primary monoclonal antibodies against β-actin (1:1000, Abcam, Cambridge, MA, UK) and Mcl-1 (1:500, Abcam) diluted in 3% skim milk in PBS. After three 7 min washes with a buffer containing PBS and 0.05% Tween-20, membrane was incubated with appropriate horseradish peroxidase-linked secondary antibody (1:4,000, Abcam) diluted in PBS for 1.5 h at room temperature. Following on, the membrane was washed and protein bands visualized using enhanced chemiluminescence detection Kit (GE Healthcare) and X-ray film (Estman Kodak, Rochester, NY, USA). The protein bands were then analyzed by ImageJ 1.62 software (National Institues of Health, Bethesda, Maryland, USA) and signal intensity of each band was normalized to its corresponding β-actin loading control.

Apoptosis ELISA assay

The HL-60 leukemia cells were seeded at a density of 5 × 10^4 cells/well in 24-well cell culture plates and then transfected with Mcl-1 specific and NC siRNAs as described above. At 24 and 48 h after transfection of siRNAs, cells were collected and apoptosis was detected using an ELISA cell death detection kit (Roche Diagnostics GmbH) according to the supplier's recommendations. This assay determines the amount of cytosolic mono- and oligonucleosomes produced during apoptosis. In brief, the cells were lysed and centrifuged at 500 rpm for 10 min. Following the addition of 20 µl of the cell supernatant and 80 µl of immunoreagent containing anti-histone-biotin and anti-DNA-peroxidase to each well, streptavidin-coated plate was incubated for 2 h at ambient temperature. Following washing with incubation buffer, 100 µl of 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) solution was transfered to each well. The reactions were stopped and absorbance at 405 nm was quantified with a microplate reader (with a reference wavelength of 490 nm). The fold increase in apoptosis was calculated by dividing the absorbance of the experiment group by the absorbance of the control group.

Statistical analysis

Data in this study were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by two-tailed unpaired t-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 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

Results

siRNA suppressed Mcl-1 mRNA and protein levels in leukemia cells

First, we explored the effect of siRNA on Mcl-1 gene expression in HL-60 cells by qRT-PCR and Western blotting. Relative Mcl-1 gene expression was calculated in relation to the blank control group, which was considered as 100%. As shown in Figure 1 and 2, Mcl-1 siRNA led to a clear time-dependent reduction of both Mcl-1 mRNA and protein levels ($P<0.05$; compared with the blank control and NC siRNA groups). At 24, 48 and 72 h posttransfection, the relative Mcl-1 mRNA expression levels were 45.11%, 37.24% and 16.22%, respectively (Figure 1), while the relative Mcl-1 protein expression levels were 60.19%, 39.43% and 20.76%, respectively (Figure 2B) ($P<0.05$). Notably, transfection with NC siRNA had an insignificant effect on Mcl-1 gene expression compared to the blank control group.

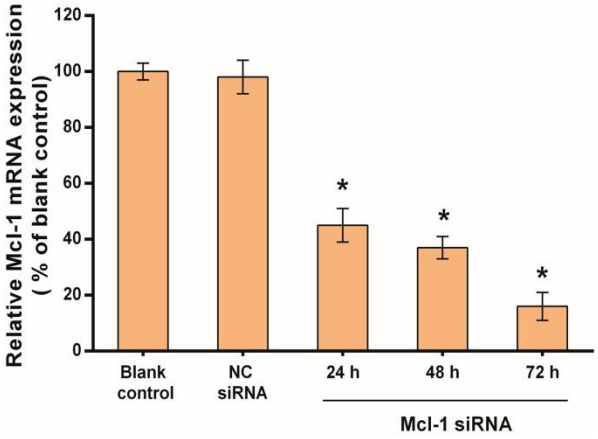

Figure 1. Down-regulation of Mcl-1 mRNA expression by siRNA in HL-60 cells. The cells were transfected with negative control (NC) siRNA or Mcl-1 siRNA for 24, 48 and 72 h. Relative mRNA expression was then measured by qRT-PCR using $2^{(-\Delta\Delta Ct)}$ method. The results are expressed as mean ± SD (n = 3); *P<0.05 versus blank control and NC siRNA.

Mcl-1 siRNA decreased the cell survival rate of the leukemic cells

To assess the cytotoxic effect of Mcl-1 down-regulation on HL-60 leukemia cells, the cells were treated with Mcl-1 siRNA for 24 and 48 h and then analyzed in MTT assay. As shown in Figure 3, the cytotoxicity significantly enhanced at 24 and 48 h in the Mcl-1 siRNA transfected cells relative to the blank control and NC siRNA transfected cells. The results showed that Mcl-1 siRNA significantly lowered the cell survival rate to 81.21% (24 h) and 65.15% (48 h) compared with the blank control group ($P<0.05$). In contrast, transfection with NC siRNA had a minimal cytotoxic effect on the leukemic cells relative to the blank control group ($P>0.05$; Figure 3).

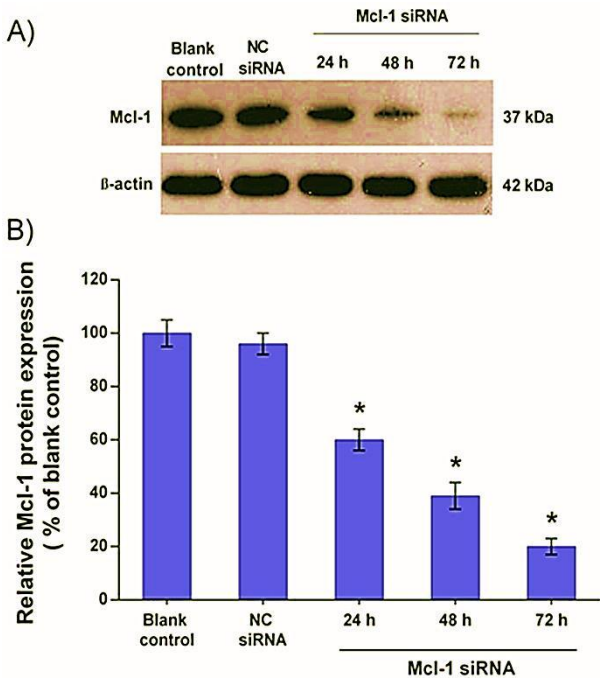

Figure 2. Mcl-1 protein expression levels in HL-60 cells transfected with siRNAs. (A) Representative western blot of β-actin and Mcl-1 proteins from cells transfected with NC siRNA or Mcl-1 siRNA. (B) The expression level of each band was quantified using densitometry and normalized to the respective β-actin. The results are expressed as mean ± SD (n = 3); *P<0.05 versus blank control and NC siRNA.

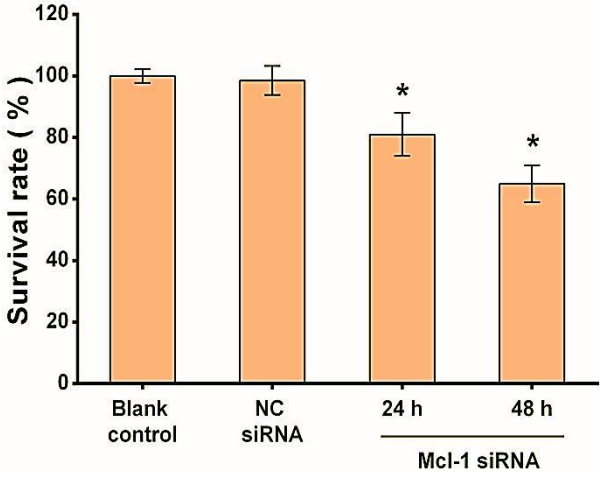

Figure 3. Effect of Mcl-1 siRNA on the survival rate of leukemia cells. The HL-60 cells were transfected with NC siRNA or Mcl-1 siRNA for 24 and 48 h and then the cytotoxicities of the treatments were measured by MTT assay. The results are expressed as mean ± SD (n = 4); *P<0.05 versus blank control and NC siRNA.

Down-regulation of Mcl-1 expression inhibited the proliferation of HL-60 cells

As up-regulation of Mcl-1 is involved in the survival of leukemia cells; we therefore sought to examine whether suppression of this protein could inhibit the proliferation of AML cells. The HL-60 cells were therefore transfected with Mcl-1 specific and NC siRNAs for 5 days and cell viability was measured every 24 h by trypan blue exclusion assay. Results showed that compared with the blank control or NC siRNA groups, Mcl-1 siRNA significantly inhibited the proliferation of HL-60 cells over a period of 5 days (P<0.05; Figure 4). At 24 h after transfection of Mcl-1 siRNA, the percentage of viable cells dropped to 84.40% and then to a further 53.37% at the end of the experiment (day 5). Meanwhile, no significant differences in cell proliferation was observed between the NC siRNA group and the blank control groups (P>0.05; Figure 4).

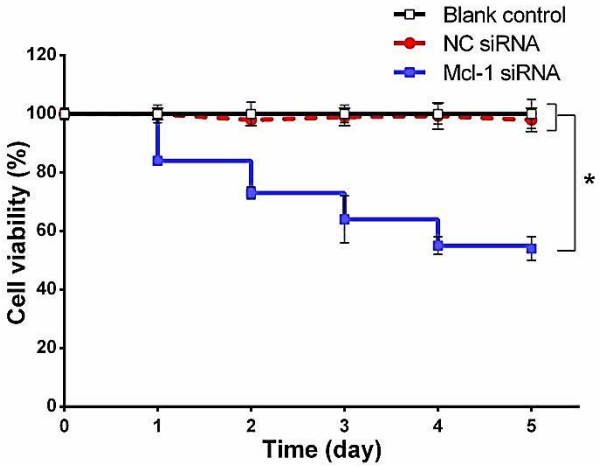

Figure 4. Proliferation inhibition of HL-60 cells transfected with NC siRNA or Mcl-1 siRNA. Cell viability was determined by trypan blue assay over a period of 5 days. The results are expressed as mean ± SD (n = 3); *P<0.05 versus blank control and NC siRNA.

Suppression of survivin expression enhanced apoptotic cell death

To analyze whether the observed cytotoxic effect of Mcl-1 Down-regulation was linked to the enhancement of apoptosis, the effect of Mcl-1 siRNA on apoptosis were examined using an ELISA cell death assay. Results showed that Mcl-1 siRNA significantly enhanced the extent of apoptosis at 24 and 48 h relative to the blank control group and the NC siRNA transfected group (Table 1; P<0.05). However, NC siRNA transfected cells displayed no distinct alteration in the extent of apoptosis relative to the blank control group (P>0.05). These results indicate that the cytotoxic effect of survivin suppression is partially due to the induction of apoptosis.

Table 1. Fold increase in apoptosis of HL-60 cells after transfection of siRNAs.

Groups	Fold increase in apoptosis
Blank control	1
NC siRNA	1.33
Mcl-1 siRNA (24 h)	7.01*
Mcl-1 siRNA (48 h)	11.38*

Results from the ELISA cell death assay showed that Mcl-1 siRNA significantly enhanced the extent of apoptosis at 48 and 72 h after transfection relative to the blank control and the NC siRNA groups. *P <0.05.

Discussion

Molecular targeted therapy is a new emerging technology for treatment of cancer. Gene therapy is a potent kind of targeted therapy, in which the target is a specific gene overexpressed in tumor cells. The therapeutic agents, including ASO, ribozyme and siRNA are used to interfere with the expression of the target gene. As a result, the formation, growth and metastasis of tumors are inhibited.[14-16] Overexpression of Mcl-1, a member of the anti-apoptotic Bcl-2 family of proteins, is attributed to the tumor formation, development and metastasis.[13] On the contrary, different reports have shown that suppression of Mcl-1 expression can induce apoptosis and inhibit the proliferation of tumor cells.[8,9] Thus, we used a siRNA-based gene therapy strategy to target Mcl-1 and evaluate its antileukemic effects.

Quantitative PCR and Western blotting findings showed that transfection with Mcl-1 siRNA led to steady decrease in the expression levels of both Mcl-1 mRNA and protein over a 3-day period. These data revealed that Mcl-1 siRNA effectively blocked the synthesis of the Mcl-1 protein by cleavage of its corresponding mRNA. The results of cytotoxicity assay exhibited that Mcl-1 siRNA distinctly lowered the cell survival rate. Most notably, the results of the cell proliferation assay demonstrated that the suppression of Mcl-1 expression significantly decreased the viability of HL-60 cells during a 5-day period, suggesting the critical role of Mcl-1 in the proliferation of leukemic cells. In contrast, treatment with NC siRNA or lipofectamine displayed no significant changes in the gene expression and cellular events, demonstrating the specific impact of Mcl-1 siRNA.

To further investigate the cellular role of Mcl-1 in the development of leukemic cells, we examined the effect of Mcl-1 suppression on induction of apoptosis. Results of ELISA cell death assay indicated that siRNA-mediated silencing of Mcl-1 led to a remarkable spontaneous apoptosis. These results are in contrast to the other studies on solid tumors.[18,19] Meanwhile, our observations are in agreement with the results of similar studies on hematological tumors,[11,20] illustrating the important biological role of Mcl-1 in the survival and growth of leukemia cells. The above-mentioned results confirm that the presence of Mcl-1 protein is required for the development and progression of HL-60 cells. Therefore, silencing of Mcl-1 expression could induce spontaneous apoptosis and inhibit the proliferation of AML cells.

Cellular apoptosis can be controlled by two major signaling pathways. The intrinsic pathway responds to toxic intracellular stimuli, resulting in release of cytochrome c from inner mitochondrial membrane space which leads to the activation of caspases-9. The extrinsic pathway triggers by ligands binding to extracellular death receptors and causes caspase-8 activation. Both pathways converge at caspase-3 that activates the other caspases and leading to a proteolytic cascade and the next apoptotic events. Mcl-1 mainly blocks the mitochondrial pathway by the sequestering and neutralization of pro-apoptotic Bcl-2 family members such as Bim, Bax and Bak, thereby preserving mitochondria integrity. This action inhibits the release of cytochrome c that is necessary for caspase-9 activation.[12,13,21]

Moreover, recent studies on melanoma cells have revealed that overexpression of Mcl-1 inhibited the death receptor pathway of apoptosis.[8,22] Our study showed that transfection of Mcl-1 siRNA induced apoptosis in leukemia cells. Therefore, we suggest that suppression of Mcl-1 expression by siRNA may trigger apoptosis through caspase-3-dependent mechanisms. However, the exact roles of Mcl-1 in the regulation of the apoptosis pathways remain unclear.

RNAi-mediated gene silencing is a powerful strategy for the knockdown of a particular gene in which siRNA is introduces to the target cells and suppresses the expression of specific gene by degradation of the corresponding complementary mRNA. Because of its unique characteristics such as specificity, high efficacy and low cytotoxicity, siRNA is extensively used in gene-based medicine investigations.[14-16] Moreover, owing to its advantages such as the greater resistance to cellular nucleases, siRNA is preferred to the ribozyme and ASO technologies.[23,24] On the other hand, transient nature of double-stranded siRNA is one of the major drawbacks of long term siRNA-based therapeutics which can be overcome by use of the siRNA-based vector systems.[14-16]

Conclusion

In summary, we have demonstrated that Mcl-1 has a critical role in the survival and growth of HL-60 cells. Specific knockdown of Mcl-1 by siRNA induced apoptosis and inhibited the proliferation of leukemia cells in vitro. We therefore suggest that the siRNA-mediated silencing of Mcl-1 may be considered as a novel treatment strategy for AML patients in the future. Future studies on animal models could further examine whether Mcl-1 can be efficiently suppressed by siRNA expressed from a vector-based system, such that siRNA can effectively silence Mcl-1 in AML cells in a long term period.

Acknowledgements

This work was supported by a grant from the Immunology Research Center (IRC), Tabriz University of Medical Sciences (No. 9032). We thank staff of the IRC and Biochemistry Department for their technical assistance.

Conflict of Interest

The authors declare that they have no conflict of interest.

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