Nucleostemin Depletion Induces Post-G1 Arrest Apoptosis in Chronic Myelogenous Leukemia K562 Cells

Negin Seyed-Gogani[1], Marveh Rahmati[2], Nosratollah Zarghami[2,3], Iraj Asvadi-Kermani[3], Mohammad Ali Hoseinpour-Feyzi[1], Mohammad Amin Moosavi[1,3,4]*

[1] *Department of Zoology, Faculty of Natural Science, University of Tabriz, Tabriz, Iran.*

[2] *Department of Biochemistry, Faculty of Medicine, Tabriz University of Medical Science, Tabriz, Iran.*

[3] *Hematology and Oncology Research Center, Tabriz University of Medical Science, Tabriz, Iran.*

[4] *National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.*

A R T I C L E I N F O

A B S T R A C T

Purpose: Despite significant improvements in treatment of chronic myelogenous leukemia (CML), the emergence of leukemic stem cell (LSC) concept questioned efficacy of current therapeutical protocols. Remaining issue on CML includes finding and targeting of the key genes responsible for self-renewal and proliferation of LSCs. Nucleostemin (NS) is a new protein localized in the nucleolus of most stem cells and tumor cells which regulates their self-renewal and cell cycle progression. The aim of this study was to investigate effects of *NS* knocking down in K562 cell line as an in vitro model of CML.
Methods: *NS* gene silencing was performed using a specific small interfering RNA (NS-siRNA). The gene expression level of *NS* was evaluated by RT-PCR. The viability and growth rate of K562 cells were determined by trypan blue exclusion test. Cell cycle distribution of the cells was analyzed by flow cytometry.
Results: Our results showed that *NS* knocking down inhibited proliferation and viability of K562 cells in a time-dependent manner. Cell cycle studies revealed that *NS* depletion resulted in G_1 cell cycle arrest at short times of transfection (24 h) followed with apoptosis at longer times (48 and 72 h), suggest that post-G1 arrest apoptosis is occurred in K562 cells.
Conclusion: Overall, these results point to essential role of *NS* in K562 cells, thus, this gene might be considered as a promising target for treatment of CML.

Introduction

Chronic myelogenous leukemia (CML) is a clonal pluripotent hematopoietic stem cell disorder caused by indefinite proliferation of leukemic stem cells (LSCs).[1] Reciprocal translocation between the *abl* gene (on chromosome 9) and the *bcr* gene (on chromosome 22) causes formation of *Bcr-Abl* oncogene.[1,2] The fusion product of *Bcr-Abl* is an oncogenic protein displays up-regulated tyrosine kinase activity.[2] At present, CML therapies mostly included chemotherapy, differentiation therapy, α-interferon treatment, Bcr-Abl tyrosine kinase inhibitors and bone marrow transplantation.[3,4] Although, recent tyrosine kinase inhibitors improved therapeutical options in CML patients, some adverse effects such as drug resistance and late relapse were observe in clinical trials.[5] It has been suggested that current therapeutic approaches would not completely eliminate all LSC in CML patients and relapse of disease was observed. In fact, unlimited self-renewal capacity and impaired

differentiation property of LSCs allow continuously proliferation and prevent terminal differentiation and apoptosis that normally occur in blood cells.[6,7] Obviously, elucidation of the mechanisms involved in LSC proliferation, differentiation and apoptosis enumerates first-line investigations for improving CML therapeutic strategies.

In 2002, Tsai and McKay discovered that a novel gene called *Nucleostemin* (*NS*), apparently expressed in rat embryonic and adult central nervous system stem cells.[8] The protein coded by *NS* gene was found in the nucleoli of undifferentiated cells, such as adult and embryonic stem cells, neural stem cells and human bone marrow stem cells but not in differentiated counterpart cells, indicating that *NS* is silenced during normal cells differentiation.[9,10] Interestingly, recent reports suggest that *NS* gene is also abundantly expressed in several human cancer cell lines such as SGC-7901 (gastric), Hela (cervical), 5637

*Corresponding author: Mohammad Amin Moosavi, Nanobiomaterial and Tissue Engineering Research Center, National Institute of Genetic Engineering and Biotechnology, P.O Box: 14965/161, Tehran, Iran. Tel.: +98 21 44580335, Fax: +98 21 44580399, Email: a-moosavi@nigeb.ac.ir

(bladder), PC-3 (prostate), and HL-60 (acute myelocytic leukemia).[11-15] In parallel with significant of this gene in cancer, several knocking down experiments using RNA interference (RNAi) showed that inhibition of *NS* gene expression markedly inhibited proliferation and cell cycle progression of cancerous cells followed with induction of differentiation and/or apoptosis.[11-15] Recently, a high expression level of NS has been reported in leukemia patients, particularly CML.[15] Consistent with this, RNAi-mediated *NS* knocking down inhibited proliferation and induced differentiation and apoptosis in HL-60 human acute myeloblastic leukemia.[16] However, importance of *NS* in other types of leukemia, especially CML, needs to be addressed.

This study was designed to investigate functional importance and therapeutic potential of *NS* gene expression and effects of *NS* knockdown on cell cycle and apoptosis in K562 cells. Our result showed that RNA interference (RNAi)-mediated *NS* silencing induced G_1 cell cycle arrest followed with apoptosis in K562 leukemia cells.

Materials and Methods
Cell culture
The human K562 cell line was cultured in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (10% v/v), streptomycin (100μg/ml) and penicillin (100 U/ml) at 37 °C in a humidified atmosphere of 5% CO_2.

siRNA design and synthesis
NS specific double-stranded small interfering RNA (NS-siRNA) was designed by siRNA target finder program at the Ambion website: (http://www.ambion.com/techlib/misc/ siRNA_finder.html). The NS-siRNA and irrelevant scrambeled siRNA (IR-siRNA) oligonucleotides were synthesized by Eurofin MWG Operon (Germany). A siRNA labeled by fluorescein at 3' end of antisense strand was used to determine efficiency of cellular transfection. The sense and antisense sequence of NS-siRNA and IR-siRNA were as followed:
 NS-siRNA Sense: 5'-GAACUAAAACAGCAGCAGAdTdT-3' and Antisense: 5'-UCUGCUGCUGUUUUAGUUCdTdT-3'
IR-siRNA (Sense: 5'-CACCGCCTCTCATCGTCGTC-3', Antisense: AAUCAGACGUGGACCAGAAGAdTdT)

K562 cells transfection
The day before transfection, cells were diluted at a density of 3×10^5/ml in culture medium containing FBS (10%) and antibiotics in cell culture flask. After 24 h, 2×10^5 cells/ well were seeded in 24-well plates (SpL Life sciences, South Korea) with 100 μl culture medium containing FBS (10%) and antibiotics. For cell transfection, 200 nM siRNA associated with 6 μl HiPerfect (Qiagen, USA) transfection reagent and 100 μl serum free medium mixed and vortexed. This mixture was incubated for 10 min at room temperature and then added

to cells. After 6 h, 400 μl culture medium containing FBS (12.5%) and antibiotics were added to the cells.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)
Total RNA was isolated from transfected cells as well as untransfected cells 12-72 h after transfection, using RNX plus™ (Cinagen, Tehran) according to the manufacturer's protocol. Total RNA (1 μg) converted to cDNA in a final volume of 20 μl by oligo dT and RevertAid™ M-MuLV reverse transcriptase (Fermentase, UK). PCR reactions were carried out using Taq DNA polymerase. The sequences of primers for all isoforms of NS (NM014366, NM206825, NM206826) were: Reverse: 5 '-AAAGCCATTCGGGTTGGAGT-3', Forward: 5'-ACCACAGCAGTTTGGCAGCAC-3'. *β2microglobulin (β2m)* gene was used as a control for adjusting the relative amounts of total RNA between the samples. *β2m* forward primer and reverse primer were 5'- CTA CTC TCT CTT TCT GGC CTG-3' and 5'- GAC AAG TCT GAA TGC TCC AC-3', respectively. PCR for *NS* gene included an initial denaturation step at 94 °C for 2 min, followed by 35 amplification cycles consisting of denaturation at 94 °C for 30 s, annealing at 60 °C for 40 s, extension at 72 °C for 1 min and final extension at 72 °C for 5 min (418 bp). PCR for *β2m* gene included an initial denaturation step at 94 °C for 5 min, followed by 30 amplification cycles consisting of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 5 min (191 bp). The amplified product was identified by electrophoresis on 1.5% agarose gel. *NS* and *β2m* primers were synthesized by Eurofin MWG Operon (Germany).

Growth inhibition and viability
To study proliferation and viability, the transfected and untransfected cells were seeded at a density of 2×10^4 cells/well in 24-well plates. After different times of transfection, viable and dead cells were counted by trypan blue exclusion assay and percent of growth inhibition and cytotoxixicty were determined as mentioned previously.[17] The transfected cells were stained with 0.4% trypan blue at a dilution of 1:1, and counted using a neubauer hemocytometer slide under an inverted light microscopy (Olympus, Japan). Morphological studies of cells were also performed by inverted light microscopy (Olympus, Japan).

Cell cycle analyses
DNA contents of cells were analyzed using flow cytometry as described previously.[18] Control and transfected cells were harvested and washed twice with PBS (Phosphate Buffer Saline), fixed in 70% ethanol and kept at −20 °C until analysis. Then the cells were stained with 20 μg/ml PI containing 20 μg/ml RNase (DNase free) for 2 h. The stained cells were analyzed by flow cytometry (Partec Pas, Germany). The population of G_0/G_1, S, G_2/M and sub-G_1 cells was determined using Mulicycle Cell

Cycle Software. The results are expressed as percentage of the cells in each phase.

Fluorescent microscopic study of apoptosis

Control and transfected cells were washed in cold PBS and adjusted to a cell density of 5×10^4 cell/20μl of PBS and gently mixed with a mixture of AO (1 μg/ml) and EtBr (1 μg/ml) solution (1 : 1, v/v). The suspension was placed on a microscopic slide and viewed under a fluorescent microscopy (Nikon E-1000, Japan).

Statistical analyses

All data represent the mean ±SEM of three independent experiments. Significant differences between groups were evaluated by multiple mean comparisons via one-way ANOVA test, SPSS 14.0 and Microsoft Excel 210. $P<0.05$ were considered statistically significant.

Results

Expression of NS was efficiently inhibited by NS-siRNA in K562 cells

Based on our preliminary data about high expression level of *NS* in leukemia cell lines, we examined different RNAi techniques for silencing of this gene in K562 cells.[19] One of the designed siRNAs, called NS-siRNA, could efficiently inhibit NS expression in K562 cells (Figure 1). As depicted in Figure 1, NS-siRNA at 200 nM was efficiently delivered into K562 cells (Figure 1A) and significantly inhibited *NS* expression in a time-dependent manner (Figure 1B). In fact, no significant reduction in *NS* expression was observed after 6-12 h NS-siRNA transfection of K562 cells, whereas *NS* mRNA level were significantly inhibited between 16 h and 48 h of transfection (Figure 1B and C). The inhibition rate of *NS* expression in comparison with corresponding *β2m* internal control after 16 h, 24 h and 48 h were about 20%, 24% and 55%, respectively (Figure 1C).
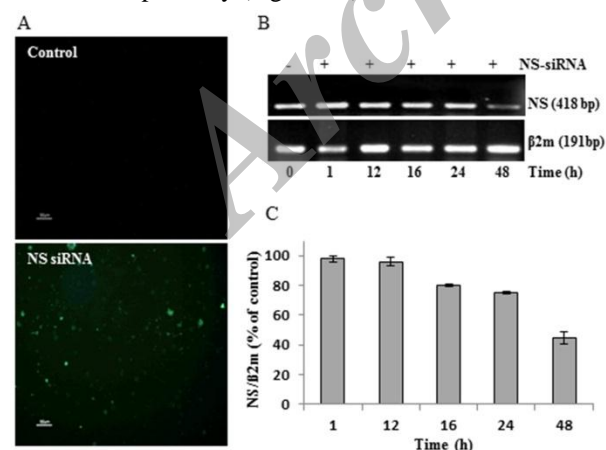
Figure 1. Knockdown of *NS* expression by siRNA in K562 cell line. (A) NS-siRNA delivery into K562 cells. After 24 h of transfection with fluorescein-labeled NS-siRNA, K562 cells were harvested and analyzed by fluorescent microscopy. (B) RT-PCR study of *NS* gene expression level in K562 cells. Following transfection with 200 nM IR- and NS-siRNAs, K562 cells were collected and mRNA levels of NS were dtermined by semi quantitative RT-PCR. (C) Analyzing of NS mRNA level in K562 cells. The densitometry analysis of *NS* mRNA over *β2m* mRNA data was studied by UVltec software. Each value represents the mean±SEM of three independent experiments and *P*<0.05 (*) were considered statistically significant.

Knockdown of NS inhibits growth and viability of K562 cells

To evaluate biological consequence of *NS* silencing, the growth rate and viability of K562 cells were studied for various time intervals (Figure 2). No significant growth inhibitory effects were observed 12 h after transfection, while growth was inhibited by 28.8%, 33.7% and 36.4% after 24 h, 48 h and 72 h, respectively (Figure 2A). In comparison with control or IR-siRNA transfected cells, the viability of NS depleted cells was also reduced but with different kinetics (Figure 2B). Indeed, no significant cell death was observed after 12-24 h of NS depletion, while viability of K562 cells was significantly reduced by 17.6% and 34.9% after 48 h and 72h, respectively. These findings together with the growth results (Figure2A) suggest that growth inhibition is prominent effects of NS-siRNA at short times while cell death is appeared at long times (48-72 h).
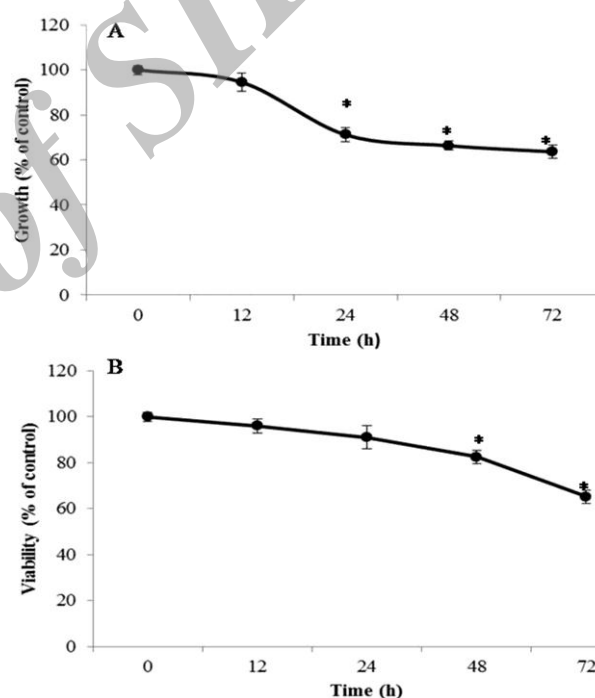
Figure 2. Effects of NS-siRNA on growth and viability of K562 cells. Following transfection with 200 nM IR- and NS-siRNAs, K562 cells were collected and number of viable cells (white cells), and dead cells (blue cells) were estimated . Growth inhibition (a) and viability (b) were studied using trypan blue test , respectively. Each value represents the mean±SEM of three independent experiments. *P*<0.05 (*) were considered statistically significant.

### Knockdown of NS leads to profound morphological changes in K562 cells

The morphology of K562 cells after NS-siRNA transfection was shown in Figure 3. Aggregation of K562 cells and decrees in cell confluency was typically observed in *NS* depleted K562 cells. However, some cell death criteria such as cell shrinking and cell debris were observed after 48-72 h of NS-siRNA transfection.
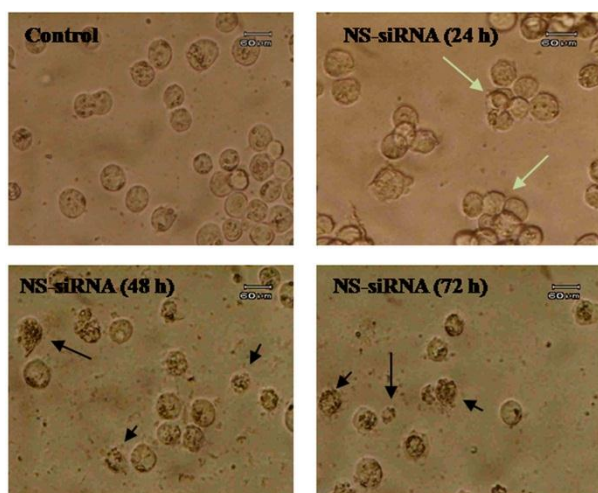
Figure 3. Morphological changes of K562 leukemia cells after transfection with NS- siRNA. The cells were transfected by 200 nM NS-siRNA for 24-72 h, and then morphological changes were studied using light microscopy (magnification 40x). After 24 h, cell aggregation (white arrow) were observed in NS-siRNA transfected K562 cells whereas after longer times (48-72 h) cell shrinking (long black arrows) and apoptotic bodies (short black arrows) were clearly observed.

Knockdown of NS induces apoptosis in K562 cells

To determine mode of cell death in NS-siRNA transfection cells, we studied apoptosis and necrosis by AO/EtBr double staining of the cells (Figure 4). The results clearly showed that *NS* siRNA transfected cells underwent apoptosis after 48 h. The apoptotic criteria, including nuclear fragmentation chromatin condensation, and apoptotic bodies were clearly observed. In these figure, viable cells were equally green whereas early apoptotic cells had bright green blots in their nuclei indicating chromatin condensation and nuclear fragmentation. Late apoptotic cells, however, stained orange and showed condense and fragmented nuclei. Necrotic cells were uniformly orange.
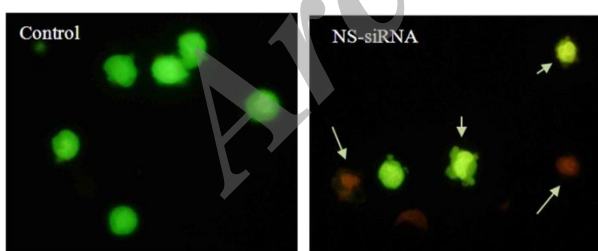
Figure 4. Apoptotic effects of NS-siRNA in K562 cells.
Frothy eight hours after transfection of K562 cells with 200 nM IR- and NS-siRNAs, the cells were collected.
Control (IR-siRNA) and NS-siRNA transfected K562 cells were double stained with AO/EtBr and studied by fluorescent microscopy (magnification, 40x).Viable cells are equally green, early apoptotic cells are green and contained bright green dots in their nuclei (short arrows) and late apoptotic cells are orange (long arrows).

Knockdown of NS induces G_0/G_1 cell cycle arrest in K562 cells

Evidence suggests that the cell fate decision is made within G1 phase of cell cycle. Therefore, the cell cycle distribution of NS-siRNA transfected K562 cells was also studied in this work (Figure 5). When compared with control cells, NS-siRNA transfected cells showed a significant increase in G_0/G_1 phase of cell cycle population with concurrent decrease in S and G_2M phase after 24 h of transfection. As might be expect, a sub-G_1 peak (apoptotic cells) was apparent after longer times of transfection. For example, After 24 h, the G_0/G_1 cell cycle population of NS-siRNA transfected cells (59%) was higher than control cells (45%). Moreover, the sub- G_1cell population (apoptotic cells) was increased from from 18-36% 48 -72 h of transfection, respectively.

Discussion

Several reports have suggested that *NS* is a marker of stem cells that is involved in controlling self-renewal, cell cycle progression and proliferation in both stem cells and cancerous cells.[10,20] As we took this matter into consideration that *NS* plays a critical role in cell proliferation, consequently, we examined *NS* expression and its function in K562 cell line as a model of CML stem cells. K562 cell line has been established from the pleural extravasation of a patient with CML in blast crisis which behaves as pluripotent hematopoietic stem cells. In addition to abnormal *Bcr-Abl* gene, K562 cells have also mutated p53 gene.[16] These combined mutations make the cells a suitable and worldwide *in vitro* model to study effects of new chemotherapy drugs and CML stem cells targeted therapies.[16] With our knowledge, functional importance of NS in CML has not been studied until now. Our results indicated that *NS* mRNA was highly expressed in K562 cells. This finding is aligned with previous studies based on *NS* over-expression in several human cancer cell lines.[11-15] In our study, role of NS in cell cycle progress and apoptosis of K562 cells was determined by a *NS* specific siRNA as a genomic nanoparticle. These oligos led to a significant decrease in the *NS* mRNA expression (Figure 1). The results showed that *NS* knocking down inhibited growth of K562 cells 24 h after transfection. Apoptosis began after 48 h and increased to its highest level after 72 h. Therefore, NS depletion in K562 cells resulted in growth inhibition at short times and apoptosis at longer times. These results are in full agreement with cell cycle results where an accumulation in G_1 phase population was observed after 24 h of NS-siRNA transfection. After this time point, however, the cells population at G_1 phase decreased and a sub-G_1 peak was appeared, suggest that post-G_1 arrest apoptosis is exact mode of action of NS-siRNA in K562 cells. Most literature reports suggest that NS depletion inhibited proliferation and induced cell cycle arrest in cancer cell lines.[13,14,21,22] For instance, *NS* specific siRNA in bladder cancer cells led to G_1 cell cycle arrest in prostate PC-3 cells and bladder cancer 5637cells.[13,14] However, NS may also induce G_2/M cell cycle arrest as the case of bladder cancer SW1710 cells.[13] Apparently, the role of NS in regulation of G_1 phase of cell cycle in K562 cells are in full agreement with most of these literature reports.
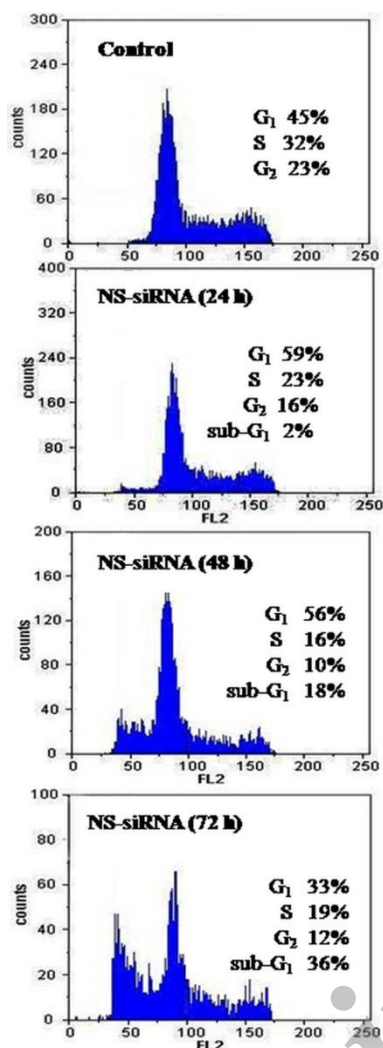
Figure 5. Effects of NS-siRNA on cell cycle distribution of K562 cells. Following NS –siRNA transfection, the K562 cells were collected at different time intervals (24-72 h) and their DNA contents were analyzed by flow cytometry as mentioned in materials and methods. The results are from a typical experiment.

Although, several reports point to apoptotic effects of NS depletion in different cancerous cells, induction of apoptosis following G_1 cell cycle arrest is a novel finding of this paper. In fact, it has been previously reported that *NS* depletion induced a rapid apoptosis response in HeLa cells, PC-3 cells, human bladder (5637) cells and HL-60 cells.[12-14,16] In our experiments, however, we observed a delayed apoptosis response in K562 cells. This may be related to different levels of NS depletion and protein contents of the cells used in distinct experiments.

Several studies have provided evidence that the p53 signaling pathway is involved in cell-cycle arrest induced by the NS depletion. The knockdown of NS enhanced the interaction between the p53-binding protein MDM2 and the ribosomal protein L5 or L11, preventing MDM2 from inducing ubiquitylation-based p53 degradation.[8,21] However, NS depletion induced cell-cycle arrest and decreased cell proliferation in rat bone marrow stromal stem cells and several cancerous cells in a p53-independent manner.[22] Considering this fact that K562 cells and three other cancerous cell lines HL-60, PC-3 and 5637 cells have no functional p53 protein, it can be concluded other proteins might be involved in NS effects.[14] It is possible that NS induces apoptosis via interaction with other molecules than p53. Consistent with this conclusion, it has been reported that NS depletion inhibited ribosome biogenesis in a p53-depndent manner.[23] Further works are in progress to address this question.

Conclusion

Attain to potent growth inhibitory and apoptotic effects of NS-siRNA in human myeloid leukemia K562 cells, the silencing of this gene can be a considered as a therapiutic target for treatment of leukemia.

Acknowledgments

Conflict of Interest

The authors declare that they have no conflict of interest.

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