

## RESEARCH ARTICLE

# BCR/ABL mRNA Targeting Small Interfering RNA Effects on Proliferation and Apoptosis in Chronic Myeloid Leukemia

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### Abstract

**Background:** To investigate the effects of small interference RNA (siRNA) targeting BCR/ABL mRNA on proliferation and apoptosis in the K562 human chronic myeloid leukemia (CML) cell line and to provide a theoretical rationale and experimental evidence for its potential clinical application for anti-CML treatment. **Materials and Methods:** The gene sequence for BCR/ABL mRNA was found from the GeneBank. The target gene site on the BCR/ABL mRNA were selected according to Max-Planck-Institute (MPI) and rational siRNA design rules, the secondary structure of the candidate targeted mRNA was predicted, the relevant thermodynamic parameters were analyzed, and the targeted gene sequences were compared with BLAST to eliminate any sequences with significant homology. Inhibition of proliferation was evaluated by MTT assay and colony-formation inhibiting test. Apoptosis was determined by flow cytometry (FCM) and the morphology of apoptotic cells was identified by Giemsa-Wright staining. Western blotting was used to analyze the expression of BCR/ABL fusion protein in K562 cells after siRNA treatment. **Results:** The mRNA local secondary structure calculated by RNA structure software, and the optimal design of specific siRNA were contributed by bioinformatics rules. Five sequences of BCR/ABL siRNAs were designed and synthesized *in vitro*. Three sequences, siRNA1384, siRNA1276 and siRNA1786, which showed the most effective inhibition of K562 cell growth, were identified among the five candidate siRNAs, with a cell proliferative inhibitory rate nearly 50% after exposure to 12.5nmol/L~50nmol/L siRNA1384 for 24,48 and 72 hours. The 50% inhibitory concentrations ( $IC_{50}$ ) of siRNA1384, siRNA1276 and siRNA1786 for 24hours were 46.6 nmol/L, 59.3 nmol/L and 62.6 nmol/L, respectively, and 65.668 nmol/L, 76.6 nmol/L, 74.4 nmol/L for 72 hours. The colony-formation inhibiting test also indicated that, compared with control, cell growth of siRNA treated group was inhibited. FCM results showed that the rate of cell apoptosis increased 24 hours after transfecting siRNA. The results of annexinV/PI staining indicated that the rate of apoptosis imcreased (1.53%, 15.3%, 64.5%, 57.5% and 21.5%) following treamtne with siRNAs (siRNA34, siRNA372, siRNA1384, siRNA1276 and siRNA1786). Morphological analysis showed td typical morphologic changes of apoptosis such as shrunken, fragmentation nucleus as well as “apoptotic bodies” after K562 cell exposure to siRNA. Western blot analysis showed that BCR/ABL protein was reduced sharply after a single dose of 50nmol/L siRNA transfection. **Conclusions:** Proliferation of K562 cells was remarkbly inhibited by siRNAs (siRNA1384, siRNA1276 and siRNA1786) in a concentration-dependent manner *in vitro*, with effective induction of apoptosis at a concentration of 50 nmol/L. One anti-leukemia mechanism in K562 cells appeared that BCR/ABL targeted protein was highly down-regulated. The siRNAs (siRNA1384, siRNA1276 and siRNA1786) may prove valuable in the treatment of CML.

**Keywords:** siRNAs - chronic myeloid leukemia (CML) - BCR/ABL - proliferation - apoptosis - K562

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### Introduction

Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20-25 base pairs in length. siRNA plays many roles, but it is most notable in the RNA interference (RNAi) pathway, where it interferes with the expression of specific genes with complementary nucleotide sequence (Seyed-Gogani et al., 2013). siRNA

also acts in RNAi-related pathways, e.g., as an antiviral mechanism or in shaping the chromatin structure of a genome (Shehata et al., 2010). Leukaemias are often characterised by nonrandom chromosomal translocations that, at the molecular level, induce the activation of specific oncogenes or create novel chimeric genes (Isakson et al., 2010; Lennon et al., 2014). They have frequently been regarded as optimal targets for gene silencing approaches, as these single abnormalities may directly initiate or

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maintain the malignant process. Since the ground-breaking discovery that double-stranded RNA molecules 21-23 nucleotides in length, named small interfering RNAs (siRNAs), are able to elicit gene-specific inhibition also in mammalian cells, the interest of the scientific community has rapidly been drawn to the potential of these siRNAs for targeting oncogenic fusion genes in leukaemic cells (Ramakrishnan et al., 2014). There has been a flurry of reports describing overexpressed or mutated genes that may also serve as attractive targets for therapeutic intervention by RNA silencing methods (Woyach et al., 2013; Zhang et al., 2014; Zhang et al., 2014).

With respect to cancer, the potential use of short RNAs in combination with conventional therapies such as chemotherapy or radiotherapy-either to complement or to augment the treatment modalities-has been proposed (Misumi et al., 2010). For example, it is well known that approximately one-third of patients acquire resistance to chemotherapeutic agents. This phenomenon has been termed multi-drug resistance (MDR) and in many cases is the result of over-expression of the MDR1 gene, which encodes P-glycoprotein (PGP), a member of the super-family of ABC transporters (Gao, et al., 2010). Although chemo-sensitizers such as verapamil may be used to circumvent MDR by inhibiting the drug efflux activity of the PGP pump, they are limited by their toxicity at pharmacologically active doses. Therefore, RNAi technology offers an alternative method for restoring chemo-sensitivity in drug-resistant cancer cells. Experiments have been conducted in cell culture systems and the findings have indicated successful modulation of the MDR phenotype using RNAi in various cell lines (Kamata et al., 2010; Snyder et al., 2010). Additionally, there is intense interest in the use of combinations of apoptosis inducing agents, for example tumor necrosis factor-related apoptosis-inducing ligand, with siRNAs that silence genes which inhibit apoptosis such as Bcl-2, FLICE-like inhibitory protein or inhibition of apoptosis proteins. Another approach that has been used to augment the effects of radiation and chemotherapy in cancer cells is RNAi-mediated silencing of DNA repair factors. Short RNAs targeting the pivotal proteins, ataxia telangiectasia-mutated protein (ATM), ataxia telangiectasia-related (ATR) protein and DNA-dependent protein kinase catalytic subunit (DNA-PKCs), in DNA damage signaling and repair pathways, have been used to enhance radiation and chemotherapy-mediated cell killing in human cancer cells (Hirose et al., 2010). These findings imply that short RNA molecules can potentially be developed for use as radio- or chemo-sensitizing agents (Inayoshi et al., 2010).

Chronic myeloid leukemia (CML) arises from the reciprocal translocation t(9;22) forming the highly stable, constitutively active tyrosine kinase BCR/ABL (Lee et al., 2010). This kinase activity is assumed to be sufficient and necessary to initiate CML. Imatinib, a potent Abl-specific tyrosine kinase inhibitor recently approved by the United States Food and Drug Administration (FDA), represents a highly effective therapy for this disease. However, clinical resistance occurs frequently, in particular during the late stages of CML. As molecular mechanisms leading to resistance point mutations in the Abl kinase domain of

BCR/ABL, and BCR/ABL overexpression have been described. Consequently, the investigation of alternative and supplementary therapies is still of great clinical importance.

We thereby in this article aimed to investigate the effects of small interference RNA (siRNA) targeting BCR/ABL mRNA on proliferation inhibition and induction of apoptosis in human chronic myeloid leukemia (CML) cell line K562 and to provide the theoretical rationale and experimental evidence for its potential clinical application for anti-CML.

## 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 (100 µg/ml) and penicillin (100 U/ml) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### 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](http://www.ambion.com/techlib/misc/siRNA_finder.html)). The NS-siRNA and irrelevant scrambled 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.

### K562 cells transfection

The day before transfection, cells were diluted at a density of 3×10<sup>5</sup>/ml in culture medium containing FBS (10%) and antibiotics in cell culture flask. After 24h, 2×10<sup>5</sup> 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 6h, 400 µl culture medium containing FBS (12.5%) and antibiotics were added to the cells.

### Growth inhibition and viability

To study proliferation and viability, the transfected and untransfected cells were seeded at a density of 2×10<sup>4</sup> 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 cytotoxicity were determined as mentioned previously. 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).

### RNA-i experiments

The si-RNA sequence targeting human BCR/ABL (from mRNA sequence; Invitrogen online) corresponds

to the coding region 377-403 relative to the first nucleotide of the start codon (target =5'-AAC ATC ACC TAT TGG ATC CAA ACT AC-3'). Computer analysis using the software developed by Ambion Inc. confirmed this sequence to be a good target. si-RNAs were 21 nucleotides long with symmetric 2-nucleotide 3' overhangs composed of 2'-deoxythymidine to enhance nuclease resistance. The si-RNAs were synthesized chemically and high pressure liquid chromatography purified (Genset, Paris, France). Sense si-RNA sequence was 5'-CAUCACCUAUUGGAUCCAAdTdT-3'. Antisense si-RNA was 5'-UUGGAUCCAUAAGGUGAUGdTdT-3'. For annealing of si-RNAs, mixture of complementary single stranded RNAs (at equimolar concentration) was incubated in annealing buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, and 10 mM MgCl<sub>2</sub>) for 2 minutes at 95°C followed by a slow cooling to room temperature (at least 25°C) and then proceeded to storage temperature of 4°C. Before transfection, cells cultured at 50% confluence in 6-well plates (10 cm<sup>2</sup>) were washed two times with OPTIMEM 1 (Invitrogen) without FCS and incubated in 1.5 ml of this medium without FCS for 1 hour. Then, cells were transfected with BCR/ABL-RNA duplex formulated into Mirus TRANSIT-TKO transfection reagent (Mirus Corp, Interchim, France) according to the manufacturer's instructions. Unless otherwise described, transfection used 20 nM RNA duplex in 0.5 ml of transfection medium OPTIMEM 1 without FCS per 5×10<sup>5</sup> cells for 6 hours and then the medium volume was adjusted to 1.5 ml per well with RPMI 2% FCS. Silencer<sup>TM</sup> negative control 1 si-RNA (Ambion Inc.) was used as negative control under similar conditions (20 nM). The efficiency of silencing is 80% in our assay.

#### Statistical analysis

Results are expressed as mean±standard deviation. Data were analysed using the unpaired two-tailed student's t test and the log rank test. *p* values of *p*<0.05 were considered significant.

## Results

### Screening of siRNA sequences

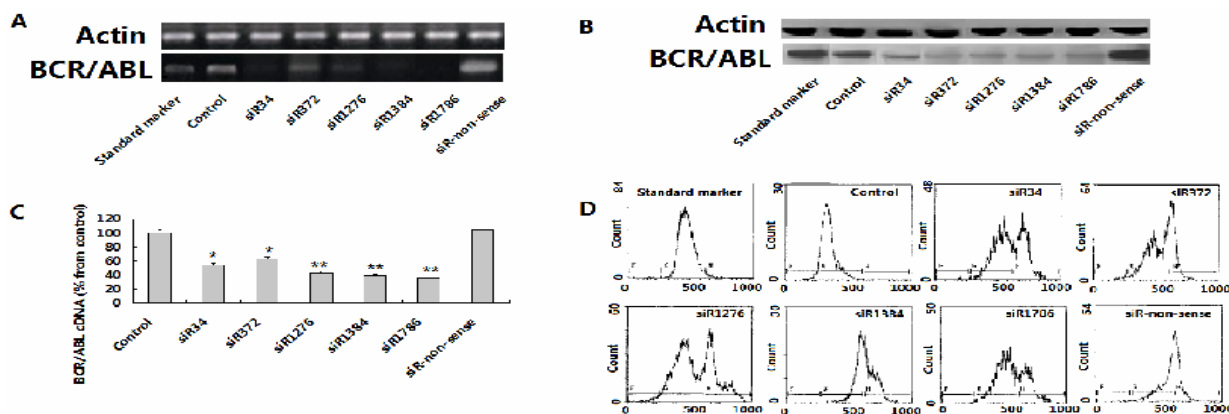
The specific double-stranded small interfering RNA was designed by siRNA target finder program at the Ambion website: ([http://www.ambion.com/techlib/misc/siRNA\\_finder.html](http://www.ambion.com/techlib/misc/siRNA_finder.html)). The siRNA and irrelevant scrambled siRNA (IR-siRNA) oligonucleotides 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. We finally chosen siRNA34, siRNA372, siRNA1276, siRNA1384 and siRNA1786 as the targeting gene according to the computer software design.

### Thermodynamic characteristics of target siRNA

Overall G37, Duplex G37, Break trag G37, Oligo-oligo G37 and Oligo-self G37 were drawn of each BCR/ABL mRNA siRNAs according to the RNA structure 5.2 analysis software. The Break trag G37 value of 4 of the total 5 siRNA pairs were 0 (siRNA372, siRNA1276, siRNA1384 and siRNA1786). The Oligo-self G37 of siRNA1786 was 0 and of the other 4 siRNA pairs were negative value and the order of their absolute order was siRNA34<siRNA372<siRNA1384, <siRNA1276. The order of the absolute value of Oligo-oligo G37 was siRNA1786<siRNA372<siRNA34<siRNA1384<siRNA1276. The order of the absolute value of Oligo-oligo G37 was siRNA1786<siRNA372<siRNA34<siRNA1384<siRNA1276.

### Effect of siRNAs on the level of BCR/ABL expression in K562 cells

The capacity of the designed siRNAs to reduce the amount of BCR/ABL mRNA in K562 cells was estimated by RT-PCR analysis (Figure 1A and B). The treatment of K562 with siRNAs reduced the level of BCR/ABL cDNA markedly. A reduction of approximately 60% was detected after treatment with a single dose of siRNA in K562 cells (Figure 1A). Besides, the treatment of K562 with siRNAs



**Figure 1. Effect of siRNAs on the level of BCR/ABL expression in K562 cells.** **A)** The capacity of the designed siRNAs to reduce the amount of BCR/ABL mRNA in K562 cells was estimated by RT-PCR analysis. The treatment of K562 with siRNAs reduced the level of BCR/ABL cDNA markedly. A reduction of approximately 60% was detected after treatment with a single dose of siRNA in K562 cells. **B)** The treatment of K562 with siRNAs also provoked more impressive decreases in BCR/ABL mRNA expression. An approximately 90% reduction in target mRNA was detected after treatment with siRNAs. **C)** Western blot analysis proved that the treatment of K562 with siRNAs reduced the level of BCR/ABL protein significantly. **D)** The BCR/ABL proteins were detected by flow cytometric registration of antigen-antibody complexes. The level of target oncoproteins in siRNA-treated K562 cells were significantly decreased (Figure 1D)

**Table 1. siRNAs Suppresses the Proliferation in a Number of K562 Cells Inhibiting Rates (%)**

Sequence	0nmol/L	12.5nmol/L	25nmol/L	50nmol/L
NC	0	0.43±9.36	6.32±8.73	14.25±3.34
siRNA372	0	29.38±14.78	32.42±7.61	30.13±10.08
siRNA1276	0	32.54±5.49	24.54±7.19	54.58±5.34
siRNA1384	0	19.13±6.72	24.69±6.93	43.92±6.63
siRNA1786	0	33.02±3.07	49.78±3.13	31.22±1.32

\**p*<0.05 \*\**p*<0.01, compared to control

**Table 2. Colony Formation Efficiency of K562 Cells in Soft Agar**

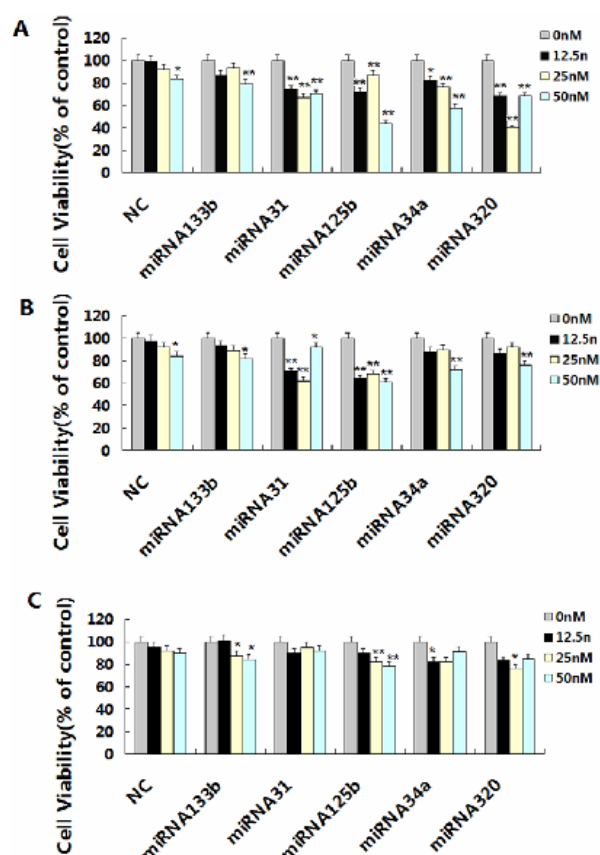
Sequence	Amount of colony		Colony forming rate (%)	
	control group	transfected group	control group	transfected group
siRNA1276	66.72±21.78	11.21±3.36	7.12±2.23	1.18±0.43*
siRNA1384	88.72±17.82	12.34±8.17	9.20±1.89	1.18±0.86*
siRNA1786	46.27±5.63	23.48±5.67	5.13±0.62	2.51±0.76*

\**p*<0.05, Compared to control

also provoked more impressive decreases in BCR/ABL mRNA expression (Figure 1B). An approximately 90% reduction in target mRNA was detected after treatment with siRNAs. Western blot analysis proved that the treatment of K562 with siRNAs reduced the level of BCR/ABL protein significantly. Similar effects were obtained on the level of target oncoproteins in siRNA-treated K562 cells (Figure 1D). The BCR/ABL proteins were detected by flow cytometric registration of antigen-antibody complexes.

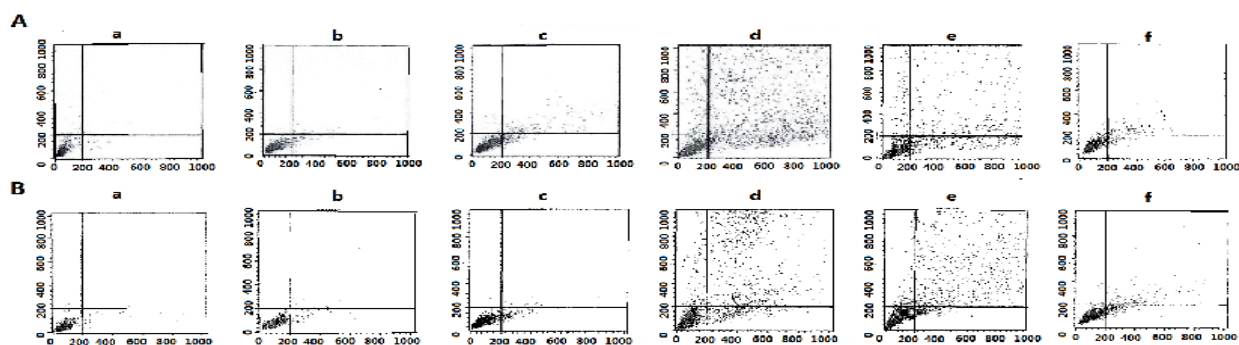
*The effects of different concentrations of siRNAs on inhibition and apoptosis of K562 cells*

The results indicated that siRNA372, siRNA1276, siRNA1384 and siRNA1786 all had the inhibitory effects on the proliferation of K562 cells under different concentration and the higher the concentration, the higher the proliferation inhibition efficiency within 0-50nmol/L (Table 1). siRNA1296 had the most obvious inhibitory effect on K562 inhibition and when the transfection concentration was 50nmol/L, the inhibitory rate of K562 was (54.76±5.32)% (*p*<0.01) 24 hours later. siRNA1276, siRNA1384 and siRNA1786 all had significant inhibitory effects on K562 with the concentration of 12.5nmol/L,



**Figure 2. siRNAs Significantly Suppressed the Proliferation of K562 Cells.** K562 cells were transfected with small interfering RNAs (siRNAs) at the concentrations of 0nM, 12.5nM, 25nM and 50nM respectively. The cells were harvested 24hours **A**), 48hours **B**) and 72hours **C**) after transfection and cell viabilities were measured. The value were shown as percentages of the siRNA negative control. \**p*<0.05 \*\**p*<0.01, compared to control

25nmol/L and 50nmol/L under 24 hours, 48 hours and 72 hours respectively as shown by Figure 2. Bivariate correlation analysis was done to the siRNA transfection concentration and the proliferation inhibition rate when the siRNA372, siRNA1276, siRNA1384 and siRNA1786 transfected with K562 24 hours later and the correlation value R were 0.667, 0.834, 0.841 and 0.572 respectively. Linear regression analysis showed that the correlation value were 0.667, 0.834, 0.841 and 0.572 respectively.



**Figure 3. siRNA induced apoptosis of K562 cells by Annexin V/PI staining.** **A**) K562 cells were treated with siRNA (NC: Negative Control, A: siRNA34, B: siRNA372, C: siRNA1276, D: siRNA1384, E: siRNA1786) at 50 nmol/L for 24 hours. Apoptotic cells were measured by Annexin V/PI staining. **B**) K562 cells were treated with siRNA (a: NC, Negative Control, b: siRNA34, c: siRNA372, d: siRNA1276, e: siRNA1384, f: siRNA1786) at 50 nmol/L for 48 hours. Apoptotic cells were measured by Annexin V/PI staining

**Table 3. The Apoptosis Rates of K562 Cell Which Cultures after Exposure to siRNA for 24 hours**

siRNAs	Cell apoptosis rate (%)			
	Normal Cells	Early stage Apoptosis	Late stage Apoptosis	Necrosis Cells
NC	96.23±1.67	0.89±0.65	2.34±0.97	1.26±0.08
siRNA34	95.71±2.34	4.72±0.85	0.89±0.31	0.17±0.07
siRNA372	80.21±5.78	9.57±3.72	8.78±1.69	0.82±0.23
siRNA1276	34.93±9.17	4.83±1.89	60.26±9.78	1.78±0.18
siRNA1384	41.25±11.52	5.12±1.63	53.72±11.23	1.89±0.46
siRNA1786	76.36±8.67	8.12±3.21	14.28±2.12	3.42±1.23

**Table 4. The Apoptosis Rates of K562 Cell which Cultures after Exposure to siRNA for 48 hours**

siRNAs	Cell apoptosis rate (%)			
	Normal Cells	Early stage Apoptosis	Late stage Apoptosis	Necrosis Cells
NC	95.12±1.08	2.81±0.33	3.32±0.56	1.31±0.35
siRNA34	92.27±1.45	7.68±0.96	1.16±0.23	0.09±0.02
siRNA372	89.87±1.29	10.56±1.14	1.47±0.29	0.27±0.06
siRNA1276	38.92±12.28	3.08±0.19	57.26±9.34	1.82±0.43
siRNA1384	41.68±11.17	3.15±0.07	54.12±10.23	1.67±0.38
siRNA1786	75.78±7.63	14.27±3.25	9.38±2.17	1.44±0.21

#### Decreased colony amount in the transfected siRNAs

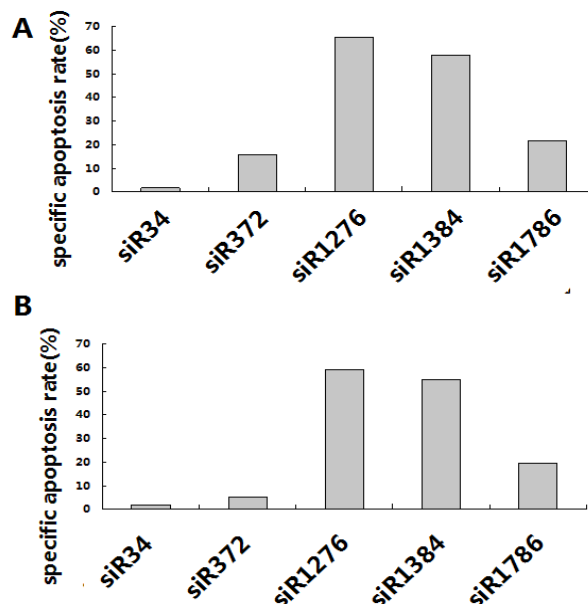
Colony formation assay showed that the number of amount of colony in the experimental group (siRNA1276, siRNA1384 and siRNA1786) were significantly reduced compared with the control group ( $p < 0.05$ ) (Table 2).

#### Effects of siRNAs transfection on the apoptosis of K562 cells

We used flow cytometry to analyze the effects of siRNAs on the apoptosis of K562 cells after Annexin V/PI staining and the results showed that the apoptosis rate of siRNAs (NC, siR34, siRNA372, siRNA1276, siRNA1384 and siRNA1786) in K562 after transfection were 3.12%, 5.76%, 19.73%, 65.32%, 58.16% and 22.69% respectively 24 hours later (Table 3) and 6.71%, 9.65%, 12.77%, 60.15%, 57.83% and 25.61% respectively 48 hours later (Table 4). There existed early stage apoptosis at both 24 and 48 hours (Figure 3). The apoptosis condition of different groups in the later stage varied significantly. There existed markedly differences in the siRNA1276, and siRNA1384 transfection group 24 hours later and the apoptosis rate were 65.37% and 58.78% respectively (Figure 4A). The specific apoptosis rate in the siRNA1276, and siRNA1384 transfection group 48 hours later were 59.41% and 56.14% respectively (Figure 4B).

## Discussion

In recent years, preliminary reports indicate that silencing of BCR-ABL using RNAi consistently reduces its expression (Koide et al., 2013; Tao et al., 2013). However, so far the molecular consequence of silencing of BCR-ABL has not been studied systematically. The previous studies showed that chemically synthesized siRNA specifically silenced the target gene (Woyach et al., 2013; Woyach et al., 2013). This study was performed on BCR-ABL in K562 cell. Chemically synthesized siRNA has a short half-life and therefore is needed a high and



**Figure 4. The Specific Apoptosis rates of K562 Cells Which Cultures after Exposure to siRNA. A)** There existed markedly differences in the siRNA1276 and siRNA1384 transfection group 24 hours later and the apoptosis rate were 65.37% and 58.78% respectively. **B)** The specific apoptosis rate in the siRNA1276 and siRNA1384 transfection group 48 hours later were 59.41% and 56.14% respectively

constant concentration of siRNA transfected to target cells. Fusion transcripts encoding oncogenic proteins may represent potential targets for a tumor-specific RNAi approach (Suresh et al., 2013; Hands et al., 2014). The Philadelphia (Ph) translocation t (9;22) (q34;q11) generates the BCR/ABL fusion gene characteristic for CML and Ph-acute lymphoblastic leukemia (ALL) (Omedes et al., 2013). BCR/ABL encodes a constitutively active cytoplasmic tyrosine kinase that is necessary and sufficient to induce and maintain leukemic transformation (Song et al., 2013).

Although success has already been achieved in clinical studies with Imatinib mesylate (STI571) in patients with Philadelphia chromosome-positive leukemias, these are only the first steps on the way to rationally designed target-directed therapies (Qiao et al., 2013). It has to be shown that RNAi-based therapies are as efficacious as small molecule drugs or monoclonal antibodies in targeting kinase enzymes (Zhang et al., 2013). Also, the main challenge to any gene therapy must also be overcome with RNAi: the double-stranded RNA or the expression vector encoding such molecules needs to reach the tumor cells efficiently. The neutralization of small interfering RNAs by the immune system may also be a foreseeable problem (Okuhashi et al., 2013). Besides, the advantageous extreme sequence specificity of RNAi may, in turn, form the basis for cancer cells to escape the RNAi-mediated attack. A single point mutation in the targeted region abolishes mRNA degradation and may cause RNAi-resistance in tumors (Markman et al., 2013). Recently we had to learn a similar lesson from patients who became resistant to the tyrosine kinase inhibitor STI571 by a variety of mechanisms including point mutations in the kinase domain. Thus, to some extent RNAi-based approaches

compete with the kinase inhibitors in the field of molecular therapy. But as is often the case with competitors in the same field: it may be that only their combined efforts will help to overcome the numerous problems associated with drug resistance in cancer treatment. The promising power of RNAi for therapeutic purposes and gives new hope that a potent tool for the reversion of an oncogenic phenotype is now in our hands (Humphries et al., 2013).

BCR/ABL protein is considered relatively stable to the antisense attack (half-life, 48 hours), and it is difficult to influence its level by a single application of anti- BCR/ABL substances (Kanduri et al., 2013). The effects of siRNAs seem to be transient and have a poor influence on cell viability despite the well expressed, siRNA-mediated reduction of BCR/ABL mRNAs and oncoproteins described in the current study (Almalik et al., 2013). This transient nature of BCR/ABL RNA interference also has been confirmed by other authors; and it may explain, at least in part, the relatively stable cell proliferation activity of K-562 cells during short-term treatment (24 hours) with anti- BCR/ABL siRNAs (Hsiao et al., 2013). Despite the transience of the effect of BCR/ABL RNA interference, there is a possibility of using long-term treatment with siRNAs to overcome this transient nature (Wang et al., 2013). Anti- BCR/ABL siRNAs have the potential to influence markedly the expression of respective target mRNAs and proteins after long-term treatment, as demonstrated in the current study and also by other authors (Tao et al., 2013). In addition, there is no risk of point mutations during siRNA treatment, unlike conventional anti-BCR/ABL drugs, confirming the possibility of a combined application for both substances (Xu et al., 2013).

The capacity of the designed siRNAs to reduce the amount of BCR/ABL mRNA in K-562 cells was estimated by RT-PCR analysis. The treatment of K562 cell lines with siRNA reduced the levels of BCR/ABL cDNA markedly, even 24 hours after a single transfection: A reduction of approximately 60% was detected after treatment with a single dose of siRNA in K-562 cells. It is interesting to note that the combined application of the 5 siRNA constructs (siR34, siRNA372, siRNA1276, siRNA1384 and siRNA1786) manifested a slightly stronger effect on the expression of BCR/ABL mRNA; it provoked an approximately 70-75% reduction in BCR/ABL mRNA expression after a single transfection, even though each sequence was applied at 3 times lower concentration.

The siRNAs we designed in this assay could significantly inhibited the proliferation of K562 cells. The three sequences, siRNA1384, siRNA1276 and siRNA1786 which showed the most effective inhibition of K562 cell growth, were identified among the five candidate siRNAs, with cell proliferative inhibitory rate nearly 50% after K562 cell exposed to 12.5nmol/L~50nmol/L siRNA1384 for 24, 48 and 72 hours. The 50% inhibitory concentrations ( $IC_{50}$ ) of siRNA1384, siRNA1276 and siRNA1786 for 24hours were 46.578 nmol/L, 59.324 nmol/L and 62.620 nmol/L, respectively; and 65.668 nmol/L, 76.549 nmol/L, 74.430 nmol/L for 72hours, respectively. Interestingly, siRNA1276 and siRNA1384 whose targeted sequences were located in the fusion sites had the most significant

proliferative inhibition while siRNA34 whose targeted sequences were located in the 3'-termini of the siRNA duplex, did not had obvious inhibition effects. So, the delivery of siRNAs to the proper sites of therapy remains problematic (Choi et al., 2013). This is especially true for their delivery to primary cells, because such cells often do not tolerate treatment with liposome transfection reagents (Hou et al., 2013). Chemical modification of siRNAs, such as changing the lipophilicity of the molecule may be considered-for example, phosphorothioate modifications present in antisense oligodeoxynucleotides, or the attachment of lipophilic residues at the 3'-termini of the siRNA duplex (Zhao et al., 2013). Delivery of siRNAs into organisms might be achieved with methods previously developed for the application of antisense oligonucleotides or nuclease-resistant ribozymes (Li et al., 2013; Wang et al., 2013).

The results of annexinV/PI staining detected cells apoptosis indicated that the rate of apoptosis improved (1.53%, 15.26%, 64.50%, 57.53% and 21.46%) following the augmentation of the different siRNA (siRNA34, siRNA372, siRNA1384, siRNA1276 and siRNA1786 for 24hours). Morphological analysis through microscopy showed the shrinking of cell volume and typical morphologic changes of apoptosis such as shrunken, fragmentation nucleus as well as "apoptotic body" after K562 cells exposure to siRNA. Western blot analysis showed that BCR/ABL proteins reduced sharply after a single dose of 50nmol/L siRNA transfection. Because all the siRNAs were targeted BCR/ABL mRNA and the siRNAs in different sites might had different effects which were correlated with the sequence structure of target sites. So, we suspected that the corresponding siRNAs might down-regulated the BCR/ABL expression and activated certain kinds of signaling pathways which inhibited the abnormal proliferation and prompted cell apoptosis. On the other hand, the activated BCR/ABL oncogene might changed its normal nuclear site and led to cell apoptosis. From our results, no matter apoptosis induction or proliferation inhibition, the effects were all alleviated 72 hours later indicated that the transfection efficiency and siRNAs stability were the key points in siRNA application. The major significant influence of siRNA was on the aspect of cancer stem cells cell cycle both in solid tumors and leukemia, but the mechanism understanding of it was still unclear. Karami found that the siRNA-mediated silencing of survivin inhibited proliferation and enhanced etoposide chemosensitivity in acute myeloid leukemia cells (Karami et al., 2013). Li found that the targeted silencing of inhibitors of apoptosis proteins with siRNAs was a potential anti-cancer strategy for hepatocellular carcinoma (Li et al., 2013).

Generally speaking, we here in this assay proved that the proliferation of K562 cells were remarkably inhibited by siRNAs (siRNA1384, siRNA1276 and siRNA1786) in a concentration-dependent manner *in vitro*. It is likely one of anti-leukemia mechanism in K562 cells treated with siRNA that BCR/ABL targeted protein was highly down-regulated. The siRNAs (siRNA1384, siRNA1276 and siRNA1786) were proved valuable in the treatment of CML.

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