Modulating multidrug resistance gene in leukaemia cells by short interfering RNA

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ABSTRACT

Introduction: The multidrug resistance gene, MDR1, is one of the genes responsible for resistance to chemotherapy in the treatment of leukaemia and other cancers. The discovery of RNA interference in mammalian cells has provided a powerful tool to inhibit the expression of this gene. However, very little is known about the transfection of leukaemia cells with short interfering RNA (siRNA) targeted at MDR1. This study aims to evaluate the effectiveness of two chemically-synthesised siRNA in modulating MDR1 gene and inhibiting P-glycoprotein expression in leukaemic cells. We also evaluated two siRNA delivery methods in this study.

Methods: K562/Adr was transfected with two MDR1-targeted siRNA or negative control siRNA, by using cationic lipid-based transfection reagents or electroporator. Gene expression of MDR1 was quantified by real-time polymerase chain reaction and calculated as a percentage relative to the negative control siRNA. P-glycoprotein expression was evaluated via flow cytometry and drug sensitivity after treatment was assessed by cytotoxicity assays.

Results: The percentage of MDR1 gene knockdown from cells transfected with an electroporator was significantly higher (84.4 percent, p-value is 0.094) compared to cells transfected with cationic lipid-based transfection reagents (52.8 percent). Both siRNA significantly reduced the expression of MDR1 by 84.9 percent (p-value is 0.001) and 86.0 percent (p-value is 0.011), respectively. P-glycoprotein expression was down-regulated and drug sensitivity was increased after treatment with the siRNA.

Conclusion: This study shows that the two siRNA sequences are capable of modulating MDR1 and P-glycoprotein expressions and increased drug sensitivity. Transfection with an electroporator was superior to chemical transfection for leukaemia cells.

Keywords: leukaemia cells, multidrug resistance gene, P-glycoprotein, short interfering RNA transfection

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INTRODUCTION

Resistance to chemotherapy is a major obstacle in cancer treatment and has been associated with treatment failure. This phenomenon is termed multidrug resistance (MDR). In human malignancies, high MDR1 gene expression has been detected in adult acute myeloid leukaemia (AML), chronic lymphocytic leukaemia and myeloma.[1] In paediatric acute lymphoblastic leukaemia (ALL), the prognostic relevance of MDR has been confirmed in a large prospective study that shows an independent adverse effect of MDR on remission duration.[2,3] MDR1 protein expression has a similar adverse effect on remission duration in adult ALL.[4] In addition, the unfavourable significance of MDR1 expression has been previously demonstrated in AML.[5-9] Its expression, especially in older patients, has been significantly associated with a lower complete remission rate.[10]

MDR1 is located on chromosome 7q21 and its mRNA is 4,872 bp long.[11] The gene product, P-glycoprotein (P-gp) is a 170-kD transmembrane protein and a member of the ATP-binding cassette transporter superfamily.[12,13] P-gp confers cross-resistance to unrelated drugs that differ widely with respect to molecular structure and target specificity, including many natural product agents (e.g. paclitaxel, vincristine, and doxorubicin), as well as new targeted anticancer agents (e.g. Gleevec).[14] It has been shown that over production of P-gp causes not only an increase in drug secretion from cells, but also a decrease in intracellular level of drug accumulation, and subsequently reducing intracellular drug content.[15] Despite promising early

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studies showing that P-gp antagonists could reverse MDR,[16-19] the clinical goal of restoring therapeutic sensitivity in drug-resistant human cancer has been elusive. Research on successful reversal or prevention of drug resistance is still underway.

RNA interference mediated by short interfering RNA (siRNA) represents a recent breakthrough in the effective blocking of the target gene in mammalian cells.[20] A short double-stranded RNA (dsRNA) with 21–25 oligonucleotides in each strand, siRNA is capable of specific degradation of the target mRNA to effectively inhibit the subsequent protein expression, without incurring non-specific degradation of genes and cell death mediated by the use of long dsRNA for transfection. Compared with single-stranded antisense nucleotides, siRNA mediates more efficient and specific mRNA degradation, and is therefore widely applied in gene therapy research.[21,22] Studies on modulating MDR1 gene in cancer cells with siRNA have been published;[23-26] but a similar study for leukaemia has been lacking. Due to the severity of MDR in leukaemia treatments, effective siRNA sequences and delivery methods have to be determined for leukaemia cells. In this study, we report the effectiveness of two invalidated siRNA sequences in modulating the MDR1 gene and inhibiting P-gp in leukaemia cells. We also report the efficacy of two different siRNA delivery methods which will shed light to the better siRNA delivery method for leukaemia cells.

METHODS

K562/Adr cell line was purchased from RIKEN Cell Bank, Ibaraki, Japan. The cell line was cultivated with IMDM media (Invitrogen Corporation, Carlsbad, CA, USA) plus 10% foetal bovine serum (Invitrogen Corporation, Carlsbad, CA, USA), as described in the cell data sheet. Two different P-gp specific chemically-modified siRNA duplexes, Stealth siRNA (Invitrogen Corporation, Carlsbad, CA, USA) were designed to be homologous to the P-gp-encoding MDR1 consensus sequence (Gen Bank accession number NM-000927). The siRNA used were MDR1-a: 5'-AAUAUUAUCUGGUUUGUGCCCACUC-3' and MDR1-b: 5'-UCCCGUAGAAACCUUACAUUUAUGG-3'. Negative control siRNA were also obtained from the same manufacturer.

Transfections for MDR1-a were carried out with a cationic lipid-based reagent i.e. Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA) and also via electroporation using amaxa nucleofector and cell line nucleofector kit V (Amaxa GmbH, Nattermannallee, Cologne, Germany). MDR1-b was merely transfected with amaxa nucleofector. Transfections were carried out according to the manufacturers' instructions. Cell plating density in a 24-well plate for transfection with Lipofectamine 2000 was 1×10^5/ml and final concentration of MDR1-a was 40 pmol. For electroporation, cell number per nucleofection was 1×10^6 and 2 μg of siRNA was used. Transfections were carried out either in duplicates or triplicates. Uptake of siRNA was assessed by fluorescein-labelled ds RNA duplex, Block-it Fluorescent Oligo (Invitrogen Corporation, Carlsbad, CA, USA) for transfection with Lipofectamine 2000, and Cy3-labelled siRNA (Applied Biosystems, Foster City, CA, USA) for transfections with amaxa nucleofector.

Total RNA was extracted at 48 hours post-transfection. cDNA was synthesised with Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Nonnenwald, Penzberg, Germany). Real-time polymerase chain reaction (RT-PCR) was carried out using a LightCycler instrument (Roche Diagnostics, Nonnenwald, Penzberg, Germany). The oligodeoxynucleotide primers used for amplification were MDR1-fw: 5'-ATATCAGCAGCCCACATCAT-3', MDR1-rev: 5'- GAAGCACTGG ATGTCCGGT-3', GAPDH-fw: 5'-GCCAAAAGGGTCATCTC-3' and GAPDH-rev: 5'-GTAGAGGCAGGGATGATGTTC-3'. Amplication products were detected via intercalation of the fluorescent dye SYBR green from LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics, Nonnenwald, Penzberg, Germany). Cycling conditions for P-gp and GAPDH were as follows: initial enzyme activation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, 58°C for 5 s and 72°C for 10 s. All cycling reactions were performed in the presence of 4mM $MgCl_2$. Gene specific products were confirmed by melting curve analysis. Gene expression of MDR1 was normalised by the expression of GAPDH. Expression ratio of MDR1 gene from cells treated with MDR1-a or MDR1-b relative to cells treated with negative control siRNA were calculated by REST software[27] and converted to percentage of gene knockdown.

P-gp post-transfection was evaluated via flow cytometry. Cells at 48 hours post-transfection were harvested and stained with FITC-conjugated mouse anti-human P-gp monoclonal antibody (BD Biosciences, San Jose, CA, USA). Acquisition and analysis were performed by using FACScan (BD Biosciences, San Jose, CA, USA) with CellQuest software (BD Biosciences, San Jose, CA, USA). P-gp positive cells were gated by excluding negative cells that were stained with mouse IgG2b isotype negative control (BD Biosciences, San Jose, CA, USA). The percentage of P-gp protein expression was calculated based on the Mean Fluorescence Intensity values of transfected

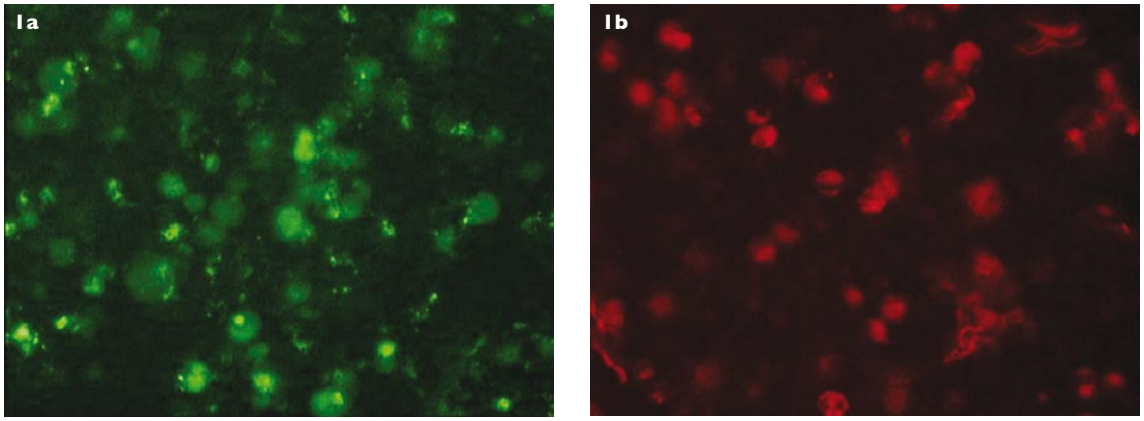

Fig. 1 Uptake of fluorescent-siRNA by K562/Adr at 48 hours post-transfection. K562/Adr was (a) transfected by fluorescein-labelled negative control siRNA using Lipofectamine 2000; or (b) transfected by Cy-3-labelled negative control siRNA using amaxa nucleofector. Images were observed under fluorescence phase contrast microscope (× 200).

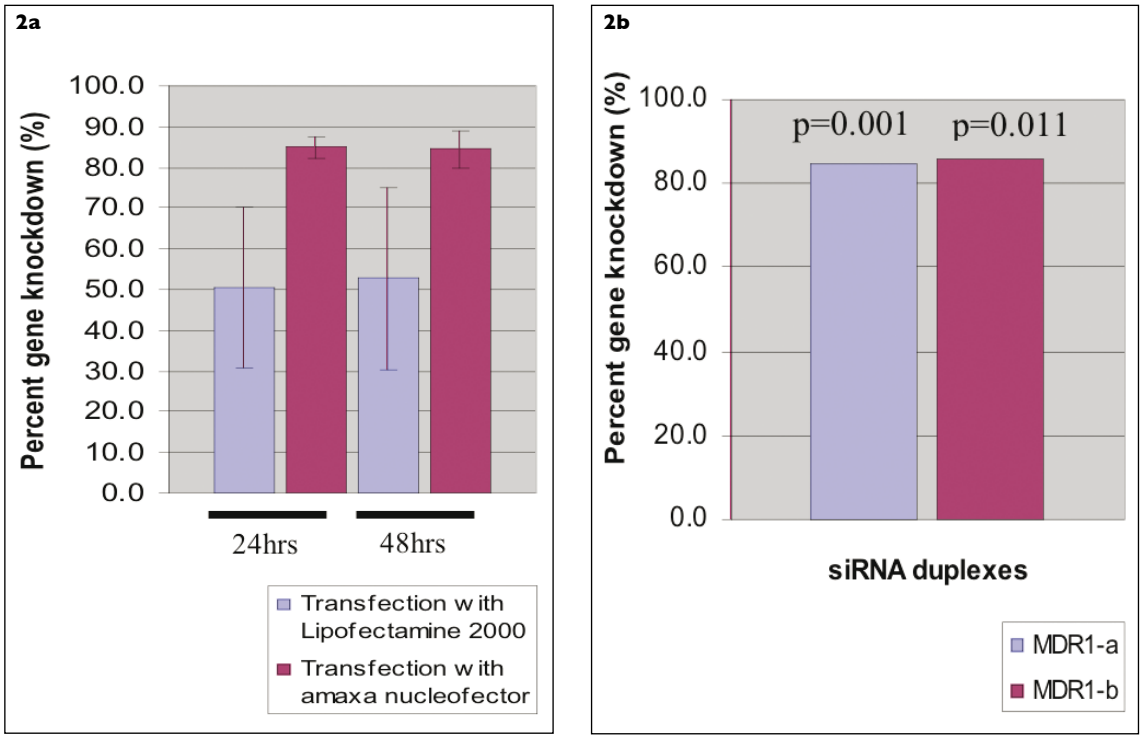

Fig. 2 Effect of siRNA on MDR1 expression in K562/Adr. (a) Histograms showing mean and standard deviation (error bars) of percent gene knockdown from three independent experiments. K562/Adr in duplicate wells were treated by MDR1-a and transfected either with Lipofectamine 2000 or amaxa nucleofector. Cells were harvested at 24 hours and 48 hours post-transfection and analysed by RT-PCR for MDR1 and GAPDH (normalisation control). Expression ratio of MDR1 in MDR1-treated cells relative to the negative controls were calculated with REST software and converted to percentage of gene knockdown. (b) K562/Adr were transfected with amaxa nucleofector in triplicates with either MDR1-a and MDR1-b. Cells were harvested at 48 hours post-transfection and analysed as described above.

cells with MDR1-a and MDR1-b as compared to the negative control.

The cytotoxicity assay and IC_{50} determination of doxorubicin on siRNA-treated and mock-treated cells without siRNA were analysed by the microculture tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) method.[29] Briefly, 3×10^4 cells per well were seeded in 96-well plates and culture medium supplemented with doxorubicin (Dabur Pharma Ltd, Baddi, Solan, India) in dilution series (0.00–3.04 μM) for 72 hours. Three repeated wells were

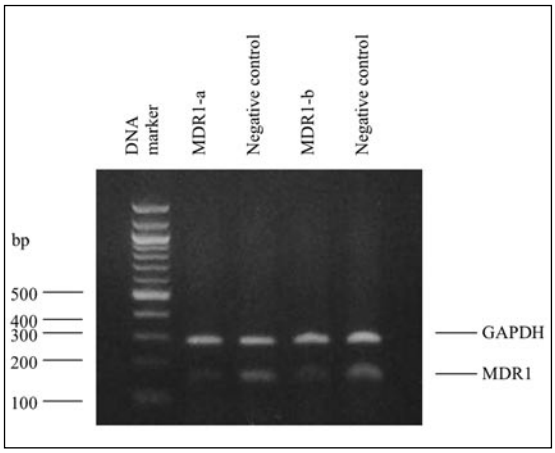

Fig. 3 Effect of siRNA on MDR1 mRNA expression in K562/Adr. Aliquots of RT-PCR products were electrophoresed on 1.5% agarose gel, and PCR fragments were visualised by ethidium bromide staining.

used for each concentration. After 72 hours of culture, the plate was centrifuged and the culture medium was aspirated from the plate. 100 μL of the medium and 20 μL of MTS (Promega Corporation, Madison, WI, USA) were added to each well. The plate was incubated for four hours before absorbance at 490 nm was measured with Dynatech MRX microplate reader (Dynex, Chantilly, VA, USA). The IC_{50} value was defined as the dosage of drug from which 50% of the cells died after 72 hours of treatment.

RESULTS

The uptake of fluorescent-labelled siRNA with Lipofectamine 2000 or amaxa nucleofector are shown in Figs. 1a and 1b. Transfection efficiencies for both methods, determined by manual counting, were

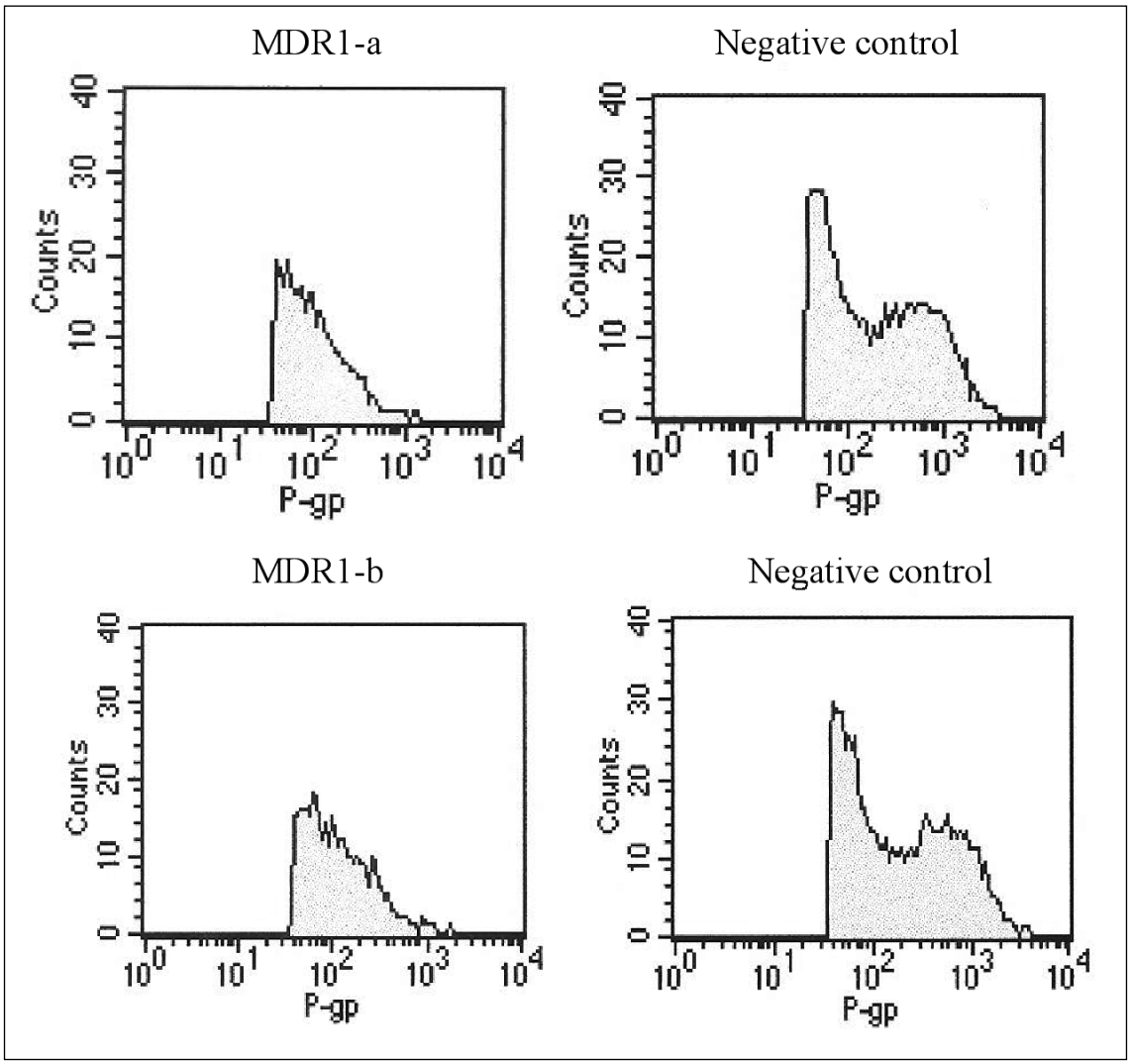

Fig. 4 P-gp protein analysis by flow cytometry. Cells were harvested at 48 hours post-transfection and stained with anti-P-gp monoclonal antibody. Acquisition and analysis were done with CellQuest software. Expressions of P-gp were decreased to 39.9% and 35.9% for cells treated with MDR1-a and MDR1-b, respectively, as compared to the negative control.

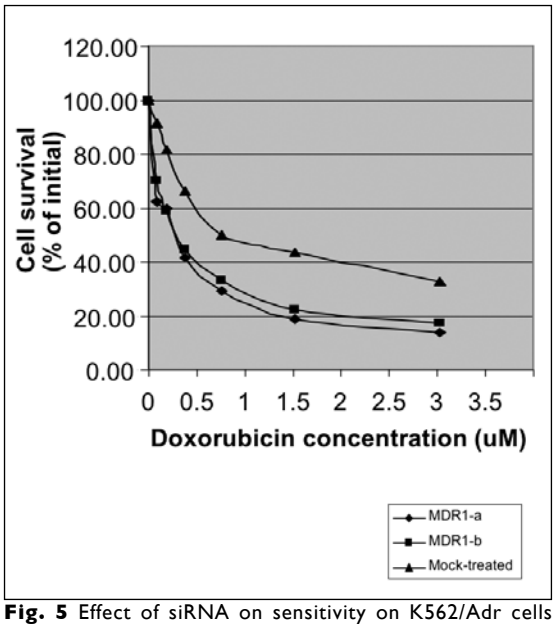

Fig. 5 Effect of siRNA on sensitivity on K562/Adr cells to doxorubicin. MDR1-a, MDR1-b or mock-treated cells plated in 96-well plate were incubated for 72 hours in the presence of series concentrations of doxorubicin. Cell numbers were determined and expressed as percentage of initial cell numbers by MTS assay. Each point represents the mean value from triplicate wells.

approximately 20%. We also found that both methods caused very little toxicity to K562/Adr, as cell viability determined by trypan blue dye exclusion was above 90%. Fig. 2a shows the results of gene knockdown by the transfection of MDR1-a with Lipofectamine 2000 and amaxa nucleofector. Transfection with amaxa nucleofector produced higher gene knockdown (84.4%, p = 0.094), as compared to Lipofectamine 2000 (52.8%) at 48 hours post-transfection. No significant difference of gene knockdown was observed for 24 hours and 48 hours post-transfection by both methods.

To test whether both designed methods of siRNA transfection worked efficiently, the cells were transfected with either MDR1-a or MDR1-b by amaxa nucleofector. Percent of gene knockdown mediated by MDR1-a and MDR1-b was 84.9% (p = 0.001) and 86.0% (p = 0.011), respectively (Fig. 2b). The gene products were determined by melting curve analysis (data not shown). Aliquots of RT-PCR product were electrophoresed on 1.5% agarose gel, and PCR fragments were visualised by ethidium bromide staining (Fig. 3). Lesser intensities of MDR1 gene products were observed for cells treated with MDR1-a and MDR1-b. The effect of siRNA on target protein expression was studied by flow cytometry. P-gp positive cells were gated and data was depicted in histogram plots. Expression of P-gp was decreased to 39.9% and 35.9% for cells treated with MDR1-a and MDR1-b respectively, as compared to the negative control (Fig. 4). To assess whether siRNA-directed suppression of P-gp sensitised K562/Adr to doxorubicin, we compared the drug sensitivity of siRNA-treated cells to the mock-treated cells using cytotoxicity assays (Fig. 5). The IC_{50} for cells treated with MDR1-a and MDR1-b were 0.335 μM and 0.299 μM, respectively, whereas the mock-treated cells had a higher IC_{50} value, i.e. 0.794 μM. Drug sensitivity of K562/Adr treated with MDR1-a and MDR1-b increased two-fold and four-fold, respectively, as compared to the controls.

DISCUSSION

Mammalian cells can be successfully loaded with exogenous siRNA, when the correct method and matrix of transfection conditions are employed. Chemical transfection (e.g. using lipid-based reagents) is used routinely to deliver siRNA into immortalised cells. Unfortunately, the transfer of siRNA into leukaemia cells by chemical transfection has not been reported extensively. Most of the transfections of these cells were carried out by electroporation.[28,29] To compare the efficiency of both methods, we choose Lipofectamine 2000 and amaxa nucleofector to transfect siRNA into K562/Adr cells. Our results showed that electroporation was more effective and reproducible in transfecting the cells compared to Lipofectamine 2000. However, many chemical transfection reagents are commercially available nowadays.[30,31] It is worthwhile to invest in with these reagents simply because one does not need an electroporator to transfect the cells.

We used fluorescence-labelled siRNA to determine the transfection efficiency of both methods as recommended by most manufacturers. However, we observed that there were no fluorescence signals after transfecting 2 μg of fluorescein-labelled siRNA by amaxa nucleofector. This might be caused by the bleaching of fluorescein during electroporation. Therefore, we switched to Cy3-labelled siRNA for electroporation and it emitted a strong fluorescence signal. Uptake of fluorescent siRNA did not reflect the degree of gene knockdown; rather, it merely showed that fluorescent siRNA had been transfected to the cells. Electroporation was more efficient for this cell line, because it involved applying an electric field pulse to induce the formation of microscopic pores in the cell membrane, and allow siRNA to traverse the membrane. Under a specific pulse condition, the pores reseal, and the electroporated cells recover and resume growth. Many factors can contribute to the degree of gene knockdown, such as cell type, design of siRNA, and delivery methods.[32,33]

Our results showed that both MDR1-a and MDR1-b were not only effective but also specific for depredating MDR1 mRNA and inhibiting P-gp expression, as compared to other reported siRNA duplexes and expression vectors.[23-26,34-36] Drug sensitivity to doxorubicin was also increased for siRNA-treated cells. However, we had not observed a dramatic increase of drug sensitivity as compared to similar studies conducted on other cancer cell lines. One of the possible reasons was because the majority of these studies applied more stably-transfected cell clones with short hairpin siRNA. Transfections with chemically synthesised siRNA used in this study were more transient, thus the expression of P-gp might have recovered during the treatment with doxorubicin.[23] Nevertheless, we used chemically-synthesised siRNA, as opposed to siRNA expressed from an expression vector system, because the reversal of clinical MDR by chemically-synthesised siRNA obviates the need to address technical and ethical problems raised by the use of expression vectors, especially potential retrovirus or adenovirus-based siRNA delivery systems.[34] There might be other underlying reasons such as the existence of unknown drug-resistant mechanisms involved, which deserves further studies.

In summary, the two siRNA presented here are capable of modulating MDR1 and P-gp expressions and increase drug sensitivity. Transfection via electroporation was superior to chemical transfection for leukaemia cells. Our findings support the idea of using siRNA as a molecularly-defined therapeutic approach for MDR in the treatment of leukaemia.

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