Reversal of multidrug resistance by magnetic Fe$_3$O$_4$ nanoparticle copolymerizing daunorubicin and MDR1 shRNA expression vector in leukemia cells

Bao-an Chen$^1$
Pei-pei Mao$^1$
Jian Cheng$^1$
Feng Gao$^1$
Guo-hua Xia$^1$
Wen-lin Xu$^2$
Hui-lin Shen$^2$
Jia-hua Ding$^1$
Chong Gao$^1$
Qian Sun$^1$
Wen-ji Chen$^1$
Ning-na Chen$^1$
Li-jie Liu$^1$
Xiao-mao Li$^4$
Xue-mei Wang$^5$

$^1$Department of Hematology, The Affiliated Zhongda Hospital, Clinical Medical School, Southeast University, Nanjing, People’s Republic of China; $^2$Department of Hematology, The Affiliated People’s Hospital, Jiangsu University, Zhenjiang, People’s Republic of China; $^3$Institution of Physiology, Southeast University, Nanjing, People’s Republic of China; $^4$Department of Physics, University of Saarland, Saarbruecken, Germany; $^5$State Key Lab of Bioelectronics (Chien-Shiung Wu Laboratory), Southeast University, Nanjing, People’s Republic of China

Abstract: In many instances, multidrug resistance (MDR) is mediated by increasing the expression at the cell surface of the MDR1 gene product, P-glycoprotein (P-gp), a 170-kD energy-dependent efflux pump. The aim of this study was to investigate the potential benefit of combination therapy with magnetic Fe$_3$O$_4$ nanoparticle [MNP (Fe$_3$O$_4$)] and MDR1 shRNA expression vector in K562/A02 cells. For stable reversal of “classical” MDR by short hairpin RNA (shRNA) aiming directly at the target sequence (3491–3509, 1539–1557, and 3103–3121 nucleotide) of MDR1 mRNA. PGC silencer-U6-neo-GFP-shRNA/MDR1 called PGY1–1, PGY1–2, and PGY1–3 were constructed and transfected into K562/A02 cells by lipofectamine 2000. After transfected and incubated with or without MNP (Fe$_3$O$_4$) for 48 hours, the transcription of MDR1 mRNA and the expression of P-gp were detected by quantitative real-time PCR and Western-blot assay respectively. Meanwhile intracellular concentration of DNR in K562/A02 cells was detected by flow cytometry (FCM). PGC silencer-U6-neo-GFP-shRNA/MDR1 was successfully constructed, which was confirmed by sequencing and PGY1–2 had the greatest MDR1 gene inhibitory ratio. Analysis of the reversal ratio of MDR, the concentration of daunorubicin (DNR) and the transcription of MDR1 gene and expression of P-gp in K562/A02 showed that combination of DNR with either MNP (Fe$_3$O$_4$) or PGY1–2 exerted a potent cytotoxic effect on K562/A02 cells, while combination of MNP (Fe$_3$O$_4$) and PGY1–2 could synergistically reverse multidrug resistance. Thus our in vitro data strongly suggested that a combination of MNP (Fe$_3$O$_4$) and shRNA expression vector might be a more sufficient and less toxic anti-MDR method on leukemia.

Keywords: K562/A02 cell line, multidrug resistance, magnetic nanoparticle of Fe$_3$O$_4$, recombinant plasmid vector PGY1–2

Introduction

Multidrug resistance (MDR) is a well-defined phenomenon of cross-resistance of mammalian cells to a number of anticancer agents following exposure to one such drug. It is a major obstacle to successful chemotherapy in leukemia and more than 90% of patients with malignant tumor died of MDR. A diverse range of agents involved in MDR include alkaloid compounds, bacterial and fungal antibiotics such as anthracyclines and etoposide. An accepted mechanism of MDR is related to a reduced intracellular accumulation and the altered subcellular distribution of toxic drugs, which is mediated by over expression of P-glycoprotein (P-gp) at the cell surface in many instances. P-gp, which is encoded by the MDR1 gene, is a major organic action transporter in tissues responsible for the excretion of xenobiotics (both drugs and toxins) by the biliary tract and proximal tubule of the kidney, so as to decrease intracellular drug accumulation.
Materials and methods

Main reagents

Adriamycin® (ADM; Hisun Pharmaceutical Co, Zhejiang, China) and daunorubicin (DNR; Main Luck Pharmaceuticals Inc., Shenzhen, China) stock solution (2 mg/mL) were prepared with 0.01 mol/L phosphate buffer saline (PBS, pH 7.4). Nanoparticles Fe₃O₄ (State Key Lab of Bioelectronics, Chien-Shiung Wu Laboratory, Southeast University, Nanjing, China) were well distributed in RPMI 1640 medium (Gibco®/BRL, Carlsbad, CA, USA) containing 10% (v/v) heat-inactivated new-born calf serum (Sijiqing, Hangzhou, China) by using ultrasound treatment in order to obtain MNP (Fe₃O₄) colloidal suspension. 1.0 µg/mL DNR conjugated with 10 µg/mL MNP (Fe₃O₄) were prepared by mechanical absorption polymerization at 4°C for 48 hours as previously.8,14 T4 DNA ligase, BamHI, Hind III NEB. Opti-MEM® (Gibco®). Trizol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA); SYBR® Green I dye (Takara, Shiga, Japan); Rotor-Gene 3000 (Corbett Research, Sydney, Australia).

Cell lines and culture conditions

Human leukemia cell line K562 and its Adriamycin®-selected P-gp-over expressing subline K562/A02 were obtained from the Institute of Hematology, Chinese Academy of Medical ZSciences (Tianjin, China). Both cells were cultured in RPMI 1640 medium (Gibco®/BRL) containing 0.1% heat-inactivated new-born calf serum, 100 µL/mL streptomycin, and 100 µg/mL streptomycin at 37°C, 5% CO₂ incubator. K562/A02 was cultured in the medium containing 1 µg/mL ADM for maintaining MDR phenotype, and maintained in drug-free medium for at least seven days before use. Briefly, cells were incubated with MNP (Fe₃O₄) and/or PGY1–2 at 37°C for 48 hours.

Vector construction and identification

Three 19-nt siRNAs targeting different nucleotide sites of MDR1 mRNA (M14758.1) designed according to the Tuschl principles were 5'-GGCTAAATTGCGAACCAT-3' (3491–3509 nt); 5'-GATACCCGCCTTGCAGG-3' (1539–1557 nt) and 5'-GATCGCTAC TGAAGCAATA-3' (3103–3121 nt). BLAST research was performed to ensure that the siRNAs did not have significant sequence homology with other genes. The 66-nt oligonucleotides were annealed and cloned into the BamHI and HindIII sites of the PGC silencer-U6-neo-GFP vector. The shRNA expression vectors called PGY1–1, PGY1–2, and PGY1–3 were designated and confirmed by sequencing.
Transfection
The human leukemia cell line K562/A02 was transfected in Opti-MEM® I medium using Lipofectamine™ 2000 (Invitrogen) under serum-free conditions according to the manufacturer’s recommendations. The plasmid (µg) to Lipofectamine 2000 (µL) was used to prepare for complexes at the ratio of 2:5. Cells were incubated at 37°C in a 5% CO₂ incubator for 48 hours for transgene expression. After staining with SYBR® Green I, the cells were calculated and differentiated for 48 hours for transgene expression. After staining with SYBR® Green I, the cells were calculated and differentiated according to whether the cell could light green fluorescence or not under the fluorescence microscope. The transfected efficiency was calculated by the ratio of transfected cells in one hundred of K562/A02 cells.

Cytotoxicity assay
The in vitro chemosensitivity was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, USA) assay. Account for the results of transfection and our previous reports, 1.3 µg/mL PGY1–2 vector, and 10 µg/mL MNP (Fe₃O₄) were selected for our study. Briefly, 1.5 × 10⁵/mL cells were suspended in 100 µL culture medium on 96-well culture plate (Costar; Fisher Scientific, Hampton, NH, USA) per well. To determine the antiproliferative effect of PGY1–2, MNP (Fe₃O₄) or DNR, a different concentration of DNR with or without 10 µg/mL MNP (Fe₃O₄) and 1.3 µg/mL PGY1–2 vector were added into 100 µL dilution of the culture medium. Meanwhile, RPMI 1640 medium was regarded as the bank control and cells without reagents were the negative control. After incubation for 48 hours at 37°C, 20 µL MTT (0.5 mg/mL) were added to each well and cultured for another 4 hours. The formazan was dissolved with 150 µL dimethyl sulfoxide (Sigma Aldrich, USA) after blotting the culture medium. The plates were shaken lightly for 10 minutes, and the reduction of MTT was quantified by absorbance at a wavelength of 540 nm using a microplate reader (Model-550; Bio-Rad Laboratories, Hercules, CA, USA).

Quantitative real-time PCR analysis
After treatment with drugs as described in cytotoxicity assay, total RNA was isolated using Trizol reagent according to the manufacturer’s protocol. One microgram of RNA was used to generate cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen Life Technologies). Primers involved were the MDR1 (205 bp) primers (forwards 5’-CCATCATTGCAATA GCAGG-3’, reverse 5’-AGGAGCGAGAAGT TTGAAC-3’) and the GAPDH primers (forwards 5’-CGGATTTCGTCGTAT TG-3’, reverse 5’-GAAGATGGTGATGCGATT-3’). QPCR was performed by monitoring in real-time the increase of fluorescence of SYBR® Green I dye with Rotor-Gene 3000 (Corbett Research, Sydney Australia). The relative gene copy number was calculated by the concentration-CT standard curve method and normalized using the average expression of GAPDH.

Western blot analysis
In order to examine the expression of P-gp, Western-blot assay was performed to analyze the level of protein from cells treated for 48 hours as described previously. Briefly, total protein was isolated and subjected to 10% sodium dodecyl sulfate PAGE, and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The blots were stained with mouse anti-P-gp or mouse anti-β-actin (1:1000–1:1200) in 5% nonfat dry milk overnight at 4°C, and then with horseradish peroxidase conjugated goat anti-rabbit as a secondary antibody (1:5000) for 1 hour at room temperature. The signal was detected with the enhanced chemiluminescence (ECL) Western Blotting Detection Kit (Zhong Shan Co, Beijing, China). After normalization by the corresponding expression of β-actin, the levels of P-gp protein expression were determined by densitometry scans (ECL system, Amersham, UK).

Cellular accumulation of DNR
The intracellular concentration of DNR in K562/A02 cells was determined by flow cytometry (FCM) assay. Briefly, K562/A02 cells were treated as described in cytotoxicity assay, collected and then incubated with CD34-FITC for 15 minutes. After being washed with PBS three times, cells were suspended and determined by a FCM (Becton Dickinson, USA) assay at excitation and emission wavelengths of 488 nm and 575 nm, respectively.

Statistical analysis
All experiments were repeated at least three times, data were expressed as mean ± SD and analyzed with the Statistical Package for Social Science (SPSS Release 11.5; SPSS Inc., Chicago, IL, USA). Differences among various groups were evaluated using one-way ANOVA. A P value of 0.05 was considered to be statistically significant.

Results
Vector feature
According to the targeting sequences, three pairs of 66-nt oligonucleotides coding shRNAs containing 19-nt reverted repeat, 9-nt linker sequence for all hairpins, HindIII
and BamHI restriction enzyme sites were designed and synthesized (Figure 1).

**Sequencing results**
The shRNA expression vector called PGY1–1, PGY1–2 and PGY1–3 were designated by sequencing. Based on the sequencing of PGY1–1, PGY1–2 and PGY1–3, shRNA was cloned into the vector successfully, respectively. (Figures 2A, B, C).

**Transfected cell morphology**
When the recombinant plasmids having GFP gene were transfected into K562/AO2 cells successfully, green fluorescence could be observed in these cells under fluorescence microscope. The transfected efficiency was 19.9% ± 1.2%, while there were no green fluorescence observed in the cells of control group ($P < 0.05$) (Figure 3).

**Screening masculine clone**
The transcription of MDR1 was significantly lower in K562/A02 cells transfected with PGY1–1, PGY1–2 or PGY1–3 than that in control group ($P < 0.05$). Furthermore, the transcription of MDR1 in K562/A02 transfected with PGY1–2 was less than that in transfected cells with PGY1–1 or PGY1–3 ($P < 0.05$), suggesting the PGY1–2 was the masculine clone we wanted to choose, which was further demonstrated by our Western-blot assay. Lower expression of P-gp in K562/A02 transfected with PGY1–2 than that in cells transfected with PGY1–1 or PGY1–3 was detected by ECL system ($P < 0.05$) (Figure 4).

**Cell growth and inhibition**
The MTT assay revealed that the growth and inhibition of DNR, MNP ($\text{Fe}_3\text{O}_4$) with DNR, PGY1–2 with DNR, MNP ($\text{Fe}_3\text{O}_4$) and PGY1–2 with DNR to K562/A02 cells. MNP ($\text{Fe}_3\text{O}_4$) and PGY1–2 symphysially showed significant reversal effect on DNR resistance in K562/A02 cell line, and its potency was greater than using MNP ($\text{Fe}_3\text{O}_4$) and PGY1–2 alone. The inhibitory concentration at 50% ($IC_{50}$) of DNR decreased from (14.82 ± 0.96) $\mu$g/mL to (0.97 ± 0.41) $\mu$g/mL ($P < 0.05$) at the combination of 10 µg/mL MNP ($\text{Fe}_3\text{O}_4$) and 1.3 µg/mL PGY1–2 with DNR, while the values were down to (4.75 ± 0.59) µg/mL and (1.08 ± 0.37) µg/mL for MNP ($\text{Fe}_3\text{O}_4$) and PGY1–2, respectively ($P < 0.05$). The fold reversals were 15.3 of the synergia compared with the 3.15 of MNP ($\text{Fe}_3\text{O}_4$) and 13.7 of PGY1–2 alone (Table 1).

**Transcription of MDR1 by QPCR**
To determine whether the shRNA expression vectors targeting MDR1 gene combined with MNP ($\text{Fe}_3\text{O}_4$) could down-regulate gene expression, K562/A02 cells were transfected with PGY1–2. DNR in the absence or presence of MNP ($\text{Fe}_3\text{O}_4$) or vector PGY1–2 all down-regulated the transcription of MDR1 in K562/A02 cells, these were statistically significant when compared to parented sensitive K562 cells ($P < 0.05$). Interestingly, MNP ($\text{Fe}_3\text{O}_4$) and PGY1–2 with DNR together decreased the expression of MDR1 mRNA more effectively than MNP ($\text{Fe}_3\text{O}_4$) or PGY1–2 with DNR, respectively ($P < 0.05$) (Figure 5), suggesting the combination of MNP ($\text{Fe}_3\text{O}_4$) and PGY1–2 has a potential benefit to sensitize MDR cells for down-regulating the transcription of MDR1.

**Expression of P-gp protein by Western-blot assay**
The effect of the shRNA/MDR1 on P-gp expression was determined by measuring cell surface P-gp. The inhibitory effect of shRNA expression vector combined with MNP ($\text{Fe}_3\text{O}_4$) on cellular P-gp in K562/A02 cells was assayed by Western-blot assay (Figure 6). The expression of P-gp in K562/A02 cells incubated with combination of MNP ($\text{Fe}_3\text{O}_4$) and PGY1–2 with DNR was decreased than that with shRNA expression vector or MNP ($\text{Fe}_3\text{O}_4$), respectively ($P < 0.05$), suggesting that MNP ($\text{Fe}_3\text{O}_4$) and PGY1–2 together has a potential benefit to sensitize MDR cells down-regulating the expression of P-gp.
Figure 2 Sequence of shRNA/MDR1. 
Notes: A) PGY1–1; B) PGY1–2; C) PGY1–3.

Intracellular concentration of DNR

The intracellular concentration of DNR in K562/A02 cells was explored by FCM assay after incubated for 48 hours. The mean fluorescence intensity of K562/A02 cells incubated with 0 µg/mL DNR was 170 ± 12; with DNR, 2490 ± 19; with MNP (Fe₃O₄) and DNR, 3318 ± 22; with PGY1–2 and DNR, 5513 ± 20; and with combination of DNR, MNP (Fe₃O₄) and PGY1–2, 6930 ± 12, implicating that MNP (Fe₃O₄) and PGY1–2 with DNR together increased the intracellular DNR in K562/A02 cells more effectively than that of MNP (Fe₃O₄) or PGY1–2 with DNR respectively (Figure 7).

Discussion

It is generally accepted that any access which could increase the effective concentration of intracellular chemotherapeutic agent was available to enhance the agent therapy. P-gp, among other ATP-binding cassette transporters, has important clinical implications since it is responsible for excretion of drugs and exotoxins and for resistance to multiple chemotherapeutic agents. The over-expression of P-gp in human tumors appears either at the time of diagnosis or prognosis. Inhibition of the function or expression of P-gp may result in re-sensitization of tumor cells to treatment with antineoplastic.

To increase the intracellular effective concentration of chemotherapeutic agent, drugs packaged by liposome or multimer have been developed recently, as one of the most commonly used magnetic nanoparticles, MNP (Fe₃O₄) are...
more easily gained than others and they may aggregate in water or tissue fluid spontaneously with good biocompatibility and low toxicity.\(^7\) MNP (Fe\(_3\)O\(_4\)) are widely used for targeted-drug carriers with target-orientation and sustained-release properties.\(^8\) Previous studies have demonstrated the synergistic effect of MNP (Fe\(_3\)O\(_4\)) with anticancer drug on the drug accumulation of leukemia K562 cells may help it to inhibit the relative drug resistance.\(^2\) Similar results were observed in our study, it showed that after incubation with different agents for 48 hours, fluorescence intensity of K562/A02 was increased when compared to control group. Interestingly, fluorescence intensity of K562/A02 treated with DNR, MNP (Fe\(_3\)O\(_4\)) and PGY1–2 was more intensified than that of MNP (Fe\(_3\)O\(_4\)) or PGY1–2 with DNR respectively, implicating that MNP (Fe\(_3\)O\(_4\)) and PGY1–2 with DNR together could increase the intracellular concentration of DNR in K562/A02 cells more effectively.

To further evaluate MNP (Fe\(_3\)O\(_4\)) and shRNAs on reversal of multidrug resistance cells, the shRNA expression vectors called PGY1–1, PGY1–2 and PGY1–3 were designated and confirmed by sequencing. The introduction of short, double-stranded RNA complexes by transfection have been found to be a very efficient way to knock down gene expression in mammalian cells.\(^6\) Recently, a number of studies have shown that shRNAs transcribed under the control of RNA polymerase III (Pol III) promoters effectively degrade mRNAs in culture cells. siRNA technology is an efficient tool for the specific knockdown of a given target mRNA. Up to now, mainly

Table 1 The cytotoxicity of DNR, MNP (Fe\(_3\)O\(_4\)) with DNR, PGY1–2 with DNR, MNP (Fe\(_3\)O\(_4\)) and PGY1–2 with DNR on K562/A02 for 48 hours determined by MTT assay (mean ± SD)

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<th>Groups</th>
<th>IC(_{50}) of DNR (µg/mL)</th>
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<tr>
<td>DNR 14.82 ± 0.96</td>
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<tr>
<td>10 µg/mL MNP (Fe(_3)O(_4)) + DNR</td>
<td>4.75 ± 0.59 (3.15)*</td>
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<tr>
<td>1.3 µg/mL PGY1–2 + DNR</td>
<td>1.08 ± 0.37 (13.7)*</td>
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<tr>
<td>10 µg/mL MNP (Fe(_3)O(_4)) + 1.3 µg/mL PGY1–2 + DNR</td>
<td>0.97 ± 0.41 (15.3)*</td>
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Notes: The values in parentheses were fold reversal (FR) calculated as follows: FR = IC\(_{50}\) DNR + MNP/IC\(_{50}\) DNR alone. *P < 0.05, compared with DNR group.

Abbreviations: DNR, daunorubicin; FR, fold reversal; IC\(_{50}\), inhibitory concentration at 50%, MNP (Fe\(_3\)O\(_4\)), magnetic nanoparticles of Fe\(_3\)O\(_4\) rate; SD, standard deviation.
the use of shRNAs, which expressed from stable integrated plasmids, can reduce MDR1 mRNA in K562/A02 cells.\textsuperscript{20} Our present toxicity results clearly indicated that the inhibitory activity of MNP (Fe$_3$O$_4$) and PGY1–2 was significantly greater than that of PGY1–2 or MNP (Fe$_3$O$_4$) alone at the same dose in the K562/A02 cells. The fold reversals were 15.3 of the synergy compared with the 3.15 of MNP (Fe$_3$O$_4$) and 13.7 of PGY1–2 alone. Furthermore, the transcription of MDR1 in K562/A02 cells preincubated with DNR in the absence or presence of MNP (Fe$_3$O$_4$) or vector PGY1–2 was higher when compared to parented sensitive K562 cells, furthermore, DNR with MNP (Fe$_3$O$_4$) and PGY1–2 decreased the transcription of MDR1 mRNA in K562/A02 cells more effectively than with MNP (Fe$_3$O$_4$) or with PGY1–2 alone, suggesting that combination of MNP (Fe$_3$O$_4$) and PGY1–2 has a potential benefit to down-regulate the transcription of MDR1 mRNA, we also found that MNP (Fe$_3$O$_4$) and PGY1–2 with DNR together decreased the expression of P-gp more effectively than MNP (Fe$_3$O$_4$) or PGY1–2 with DNR alone ($P < 0.05$), suggesting that a combination of MNP (Fe$_3$O$_4$) and PGY1–2 also has a potential benefit to down-regulate the expression of P-gp. Interestingly, the expression levels of P-gp were in accordance with the transcription MDR1 levels of mRNA. Therefore, MNP (Fe$_3$O$_4$) and PGY1–2 could synergistically reverse MDR. To obtain more data supporting this theory, further \textit{in vitro} and \textit{in vivo} studies are required.

**Conclusion**

Our study demonstrates for the first time that MNP (Fe$_3$O$_4$) and shRNA expression vector significantly reversed MDR, and the distinct synergistic effect of which owes to the down-regulation of expression of mdr1 gene, suggesting that a combination of MNP (Fe$_3$O$_4$) and shRNA expression vector may be a more sufficient and less toxic anti-MDR method.

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**Disclosure**

The authors report no conflicts of interest relevant to this study.

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