

RESEARCH ARTICLE

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# The siRNA targeted to *mdr1b* and *mdr1a* mRNAs *in vivo* sensitizes murine lymphosarcoma to chemotherapy

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## Abstract

**Background:** One of the main obstacles for successful cancer polychemotherapy is multiple drug resistance phenotype (MDR) acquired by tumor cells. Currently, RNA interference represents a perspective strategy to overcome MDR via silencing the genes involved in development of this deleterious phenotype (genes of ABC transporters, antiapoptotic genes, etc.).

**Methods:** In this study, we used the siRNAs targeted to *mdr1b*, *mdr1a*, and *bcl-2* mRNAs to reverse the MDR of tumors and increase tumor sensitivity to chemotherapeutics. The therapy consisting in *ex vivo* or *in vivo* application of *mdr1b/1a* siRNA followed by cyclophosphamide administration was studied in the mice bearing RLS<sub>40</sub> lymphosarcoma, displaying high resistance to a wide range of cytostatics.

**Results:** Our data show that a single application of *mdr1b/1a* siRNA followed by treatment with conventionally used cytostatics results in more than threefold decrease in tumor size as compared with the control animals receiving only cytostatics.

**Conclusions:** In perspective, *mdr1b/1a* siRNA may become a well-reasoned adjuvant tool in the therapy of MDR malignancies.

## Background

A substantial progress in the study of cancer development was achieved during last decades. However, mortality from these diseases still remains high despite the appearance of new highly efficient therapeutics and new polychemotherapy programs. One of the main obstacles for successful polychemotherapy of cancer diseases is the development of multiple drug resistance (MDR) phenotype, the resistance to a wide range of chemotherapeutic agents, acquired by cancer cells [1].

The primary cause of MDR phenotype is attributed to an overexpression of some members of a highly conserved gene family (from bacteria and barley to man) of transmembrane ATP-binding cassette proteins, referred

to as the ABC transporters superfamily [2-4]. The human *MDR1* (*ABCB1*) gene and two mouse genes, *mdr1b* and *mdr1a* (*Abcb1b* and *Abcb1a*), that belong to subfamily B of ABC-transporters encode P-glycoprotein (Pgp), involved in active ATP-dependent transport of cytotoxic agents out of the cells [5-7]. Being a complex process, MDR arises also from the expression abnormalities of other genes, namely, the genes encoding pro- and antiapoptotic factors p53, Bax, Bad, and Bcl-2 [5-10]; genes of the glutathione pathway [2,4]; and genes encoding topoisomerases [3,4,11]. However, the most frequent cause of the MDR phenotype development in many human and rodent tumors is an increased level of Pgp [12-14]. One of the strategies to overcome MDR is to increase the intracellular concentration of chemotherapeutics using P-glycoprotein inhibitors [15]. Another strategy is to silence the genes involved in MDR using small interfering RNA (siRNA), which seems to be a promising tool.

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In our previous work [16], RLS<sub>40</sub> cell line, exhibiting MDR, was obtained by cultivation of murine ascitic lymphosarcoma RLS cells, resistant to apoptosis-inducing agents, in the presence of vinblastine. The MDR of RLS<sub>40</sub> cells was provided by a high expression of *mdr1b* and *mdr1a* genes and a moderate expression of *bcl-2* and appeared as a 20-fold decrease in the cell sensitivity to a wide range of cytostatics (cyclophosphamide, cisplatin, vinblastine, and rubomycin) [16]. The obtained RLS<sub>40</sub> cell line generates tumors in mice and mimics the tumor status observed in patients after several chemotherapy courses [17]. Thus, we had at our disposal two lymphosarcoma models displaying MDR: the MDR of RLS<sub>40</sub> is *mdr1b/1a*-associated and the MDR of RLS tumor is predominantly *bcl-2*-associated.

In this study, we applied the *mdr1b/1a* siRNA targeted to *mdr1b* and *mdr1a* mRNAs and *bcl-2* siRNA addressed to *bcl-2* mRNA aiming to reverse the MDR phenotype of tumor cells and increase their sensitivity to chemotherapeutics. The potential of siRNAs was studied *in vitro*: we showed that *mdr1b/1a* and *bcl-2* siRNAs caused five- and two-fold decrease in the corresponding mRNA levels, respectively, and *mdr1b/1a* siRNA exerted an increase in cell sensitivity to cytostatics. The application of siRNA followed by cyclophosphamide administration was studied *ex vivo* and *in vivo* using RLS<sub>40</sub> and the related RLS lymphosarcomas developed in mice. Our data showed that single application of *mdr1b/1a* siRNA and cyclophosphamide resulted in a decrease in the MDR and a threefold decrease in the tumor size as compared with the control animals treated only with cyclophosphamide, while *bcl-2* siRNA was ineffective *in vivo*.

## Methods

### Mice

Male 10-12 week-old CBA/LacSto (hereinafter, CBA) mice obtained from the animal breeding facility with the Institute of Cytology and Genetics (Siberian Branch, Russian Academy of Sciences, Novosibirsk, Russia) were used in this study. All animal procedures were carried out in accordance with approved protocol and recommendations for proper use and care of laboratory animals [European Communities Council Directive 86/609/CEE].

### Tumor generation

Ascites RLS and RLS<sub>40</sub> lymphosarcomas are permanent and maintained in CBA mice by regular passaging of tumor cells. For generating ascites tumors, 2 × 10<sup>5</sup> tumor cells in 0.2 ml of buffered saline were intraperitoneally injected into the abdominal cavities of CBA mice. For generating solid tumors, intramuscular injections of 2 × 10<sup>5</sup> tumor cells in 0.1 ml of solution were made into the right lower limbs of CBA mice.

### Isolation of primary tumor culture from ascites

On days 10-12 of RLS or RLS<sub>40</sub> tumor development, 0.5 ml of phosphate buffered saline (PBS) was injected into the abdominal cavity. Then mice were sacrificed by cervical dislocation to collect the ascites fluid with syringe. To eliminate the erythrocytes, the collected cell suspension was diluted with 5 ml of PBS, layered over 3 ml of LSM (lymphocyte separation medium, MP Biomedicals, United States), and centrifuged at 1500 rpm for 15 min. The tumor cells, localized to the mononuclear fraction, were collected, washed with PBS, and centrifuged at 1000 rpm for 5 min. The collected cells were suspended in the Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic antimycotic solution (10 000 µg/ml streptomycin, 10 000 IU/ml penicillin, and 25 µg/ml amphotericin; ICN, Germany). The cells were cultivated at 37°C in a humidified incubator with 5% CO<sub>2</sub> until a complete adhesion of macrophages to the flask bottom. Then the cell suspension containing lymphocytes was transferred to a new flask and cultivated under the same conditions. In the *in vitro* experiments on analysis of siRNA silencing activity and sensitivity of siRNA-treated tumor cells to cytostatics, the cells were cultivated not more than 1 month. To perform *ex vivo* experiments, the isolated cells were inoculated in mice immediately after transfection with the corresponding siRNA.

### Design of siRNAs

The sequence for *mdr1b/1a* siRNA was selected using the data from GenBank [GenBank: [M14757](#)]; the sequence for *bcl-2* siRNA was chosen according to Wacheck *et al.* [18]. The siRNAs had the following sequences: *mdr1b/1a* siRNA, sense 5'-GGCUGGACAAGCUGUGCAUGG-3' and antisense 5'-AUGCACAGCUUGUCCAGCCAA-3' and *bcl-2* siRNA, sense 5'-GCCUUUGUGGAAC-UUAUUGGdTdT-3' and antisense 5'-CCAUAUAGUUC-CACAAAGGCdTdT-3'; luciferase siRNA was used as a control (sense 5'-CGUUAUUUAUCGGAGUUGCAG-3' and antisense 5'-GCAACUCCGAUAAAUAACGCG-3') [19]. The siRNAs were chemically synthesized at the Laboratory of RNA Chemistry (Institute of Chemical Biology and Fundamental Medicine).

### Duplex formation of siRNAs

The sense and antisense strands of siRNAs at a concentration of 20 µM were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2 mM magnesium acetate) at 90°C for 1 min and then at 37°C for 1 h. The formed duplexes were kept at -20°C.

### Cell transfection with siRNAs

Tumor cells were placed in a serum-free medium into six-well plates immediately before transfection. The cells

were incubated with siRNA precomplexed with Lipofectamine™ 2000 (Invitrogen, United States) in an Opti-MEM medium (Invitrogen, United States) at 37°C for 4 h according to the manufacturer's protocol. Then the medium was replaced with a supplemental medium containing 10% FBS, and the cells were cultivated for 24-96 h under standard conditions. In the *ex vivo* experiments, the cells after transfection were twice washed with PBS and diluted with PBS to a concentration of  $2 \times 10^6$  cells/ml.

#### RNA isolation

Total RNA was isolated from cells according to published protocol [20]. RNA concentration in the samples was measured by absorbance at 260 and 280 nm using a Bio-Mate 3 (Thermo Electron Corporation) spectrophotometer.

#### Semiquantitative RT-PCR analysis

The primers and RT-PCR conditions were earlier described in detail [16].  $\beta$ -Actin product was used as an internal control. Amplification was performed in the following mode: the initial step at 95°C for 5 min; 24, 25, and 30 cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min for *mdr1b*, *mdr1a*, and *bcl-2*, respectively; and the final elongation step at 72°C for 5 min. The PCR products were analyzed by 8% PAGE, visualized with ethidium bromide staining, photographed in UV light, and densitometrically quantified using a Gel-Pro Analyzer 4.0. The data were presented as the ratio of specific gene expression level to  $\beta$ -actin expression level.

#### Cell viability in the presence of cytostatics (MTT test)

The change in sensitivity of RLS<sub>40</sub> cells to vinblastine after transfection with siRNA was tested by MTT assay [21]. Vinblastine (Sigma, United States) was added to cells at concentrations of 10 to 700 nM 48 h after transfection, and the cells were incubated at 37°C for 48 h in a humidified incubator with 5% CO<sub>2</sub>. Then MTT solution was added to cells at a final concentration of 0.5 mg/ml, and the cells were incubated for 3-4 h under the same conditions. The medium was removed; the crystallized formazan was dissolved in 100  $\mu$ l of DMSO; and optical density was measured at a wavelength of 570 nm (background, at 620 nm) in a Multiscan RS (TermoLabsystems) multi-channel spectrophotometer.

#### Rhodamine 123 efflux assay

The intracellular accumulation of the substrate of Pgp, Rhodamine 123 (Rh123), was measured to evaluate the Pgp activity in RLS<sub>40</sub> cells after the transfection with *mdr1b/1a* siRNA according to [22]. Cells ( $5 \times 10^5$ ) were washed with PBS, resuspended in 0.5 ml of serum-free IMDM, and incubated with 10  $\mu$ M of the Pgp inhibitor

Verapamil for 45 min at 37°C and 5% CO<sub>2</sub>. To determine the influx, Rh123 was added to cells to a final concentration of 5  $\mu$ M. The cells were incubated for 15 min at 37°C and 5% CO<sub>2</sub> in the darkness. After the influx step, the cells were washed with serum-free medium, resuspended in 0.5 ml of this medium, and cultivated for 3 h at 37°C and 5% CO<sub>2</sub> to determine the efflux. Rh123 efflux was stopped by cooling the cells at 4°C, and the cells were washed with ice-cold PBS. Propidium iodide was added to the cells for assessing viability. The analysis was performed in a Cytomics FC 500 (Beckman Coulter) flow cytometer by accumulating events in the FL1 channel. The median fluorescence ( $\Delta F$ ) of samples was used to calculate the percentage of Rh123 intracellular accumulation (A%):

$$A\% = 100 - (\Delta F_{\text{Ver}} - \Delta F_{\text{mdr1b/1a siRNA}}) / (\Delta F_{\text{Ver}} - \Delta F_{\text{luc siRNA}}) \times 100,$$

where  $\Delta F_{\text{Ver}}$  is a median fluorescence of the cells incubated with Rh123 and Verapamil;  $\Delta F_{\text{mdr1b/1a siRNA}}$ , a median fluorescence of the cells transfected with *mdr1b/1a* siRNA and incubated with Rh123; and  $\Delta F_{\text{luc siRNA}}$ , a median fluorescence of the cells transfected with luciferase siRNA and incubated with Rh123.

#### Tumor development in the presence of siRNA and/or cytostatics

*Ex vivo*. RLS<sub>40</sub> or RLS ascites were taken from CBA mice. The RLS<sub>40</sub> cells isolated from ascites fluid by filtration through LSM were divided into four portions: (1) wild-type cells (hereinafter, wt cells), (2) the cells transfected with control luciferase siRNA, (3) the cells transfected with *mdr1b/1a* siRNA at a concentration of 100 nM, and (4) the cells transfected with *bcl-2* siRNA at a concentration of 200 nM. The RLS cells isolated from ascites fluid were divided into three portions: (1) wt cells; (2) the cells transfected with control luciferase siRNA; and (3) the cells transfected with *bcl-2* siRNA at a concentration of 200 nM. The cell suspensions (0.1 ml,  $2 \times 10^6$  cells/ml) 4 h after transfection were intramuscularly inoculated into the right lower limb of 14-16-week-old male CBA mice for solid tumor development. On days 2 and 4 after tumor cell inoculation, a half of the RLS<sub>40</sub>-bearing animals from each group was intraperitoneally injected with 100 mg/kg of cyclophosphamide (Biokhimik, Saransk, Russia) or with 1.5 mg/kg of vinblastine and a half of RLS-bearing animals from each group was once intraperitoneally injected with 200 mg/kg of cyclophosphamide on day 2 after transplantation. The other half of each group was injected with buffered saline. Each group included 40 mice.

*In vivo*. The CBA mice with generated RLS<sub>40</sub> ascites tumors were divided into two groups: the mice were

intraperitoneally injected with (1) 15 µg of control luciferase siRNA or (2) 15 µg of *mdr1b/1a* siRNA. Prior to injection, the siRNAs were precomplexed with Lipofectamine in Opti-MEM medium. The ascites 4 h after injection were collected from each group of animals, diluted with PBS to a concentration of  $2 \times 10^6$  cells/ml, and 0.1 ml of this suspension was intramuscularly inoculated into the right lower limb of 14-16-week-old male mice for solid tumor development. On day 2 after tumor inoculation, each group of animals was divided into three subgroups (20 mice per subgroup): subgroup 1 was intraperitoneally injected with 200 mg/kg of cyclophosphamide; subgroup 2 intravenously injected with 2 mg/kg of embichin (mechlorethamine hydrochloride, Aldrich, United States); and subgroup 3 (control subgroup) was injected with buffered saline.

As soon as tumors began to be palpable, the tumor volumes were measured every 2-3 days using calipers. When the experiment was finished, the animals were sacrificed by cervical dislocation. Tumor weight was determined as the difference between the weights of the amputated tumor-bearing stump and the tumor-free contralateral stump. The inhibition of tumor growth was estimated as follows:  $[\text{mean tumor weight}_{\text{control}} - \text{mean tumor weight}_{\text{experiment}}] / \text{mean tumor weight}_{\text{control}}$ . The liver was weighed for hepatic index calculation. Hepatic index was estimated as follows:  $(\text{liver weight}/\text{mouse weight}) \times 100\%$ . The liver index of healthy CBA mice was 5.0%.

### Statistics

The data were statistically processed using the Student's *t*-test (two-tailed, unpaired);  $p < 0.05$  was considered statistically significant.

## Results

### Design of siRNAs

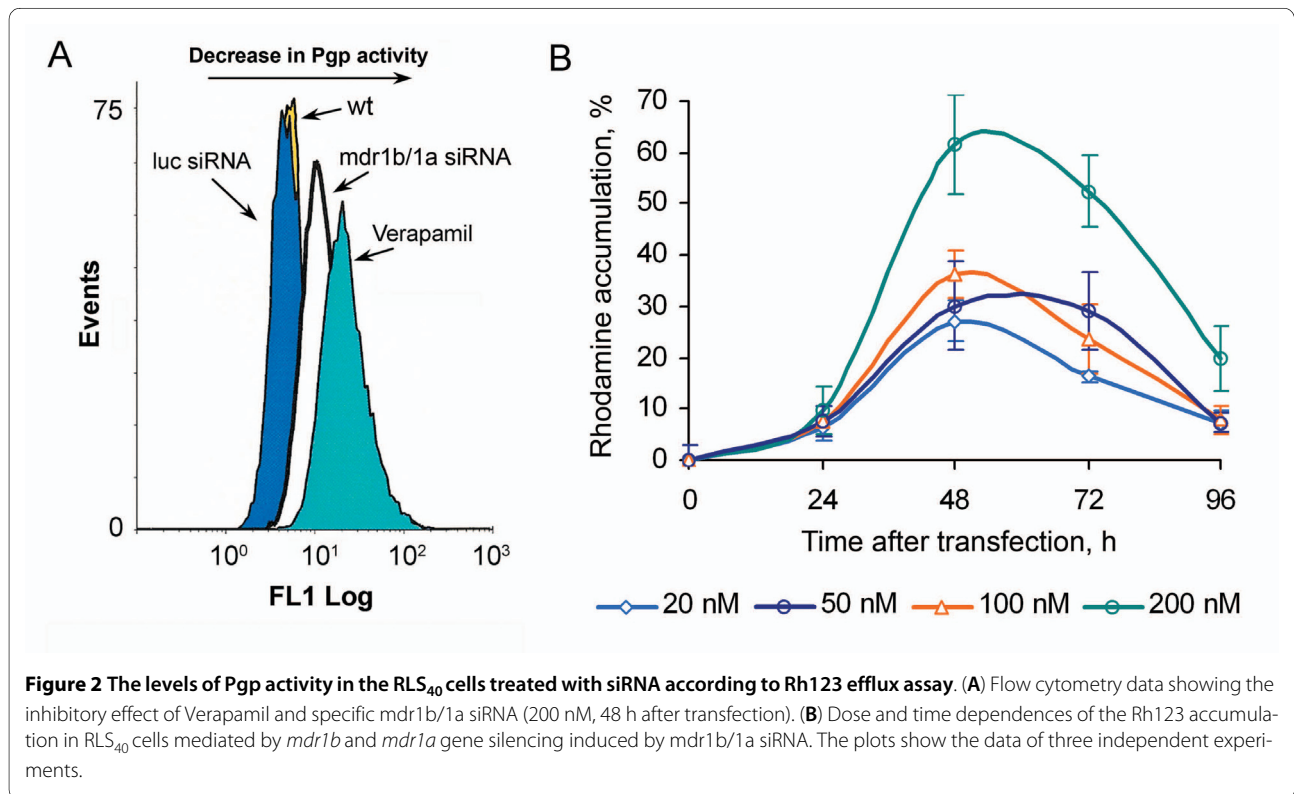
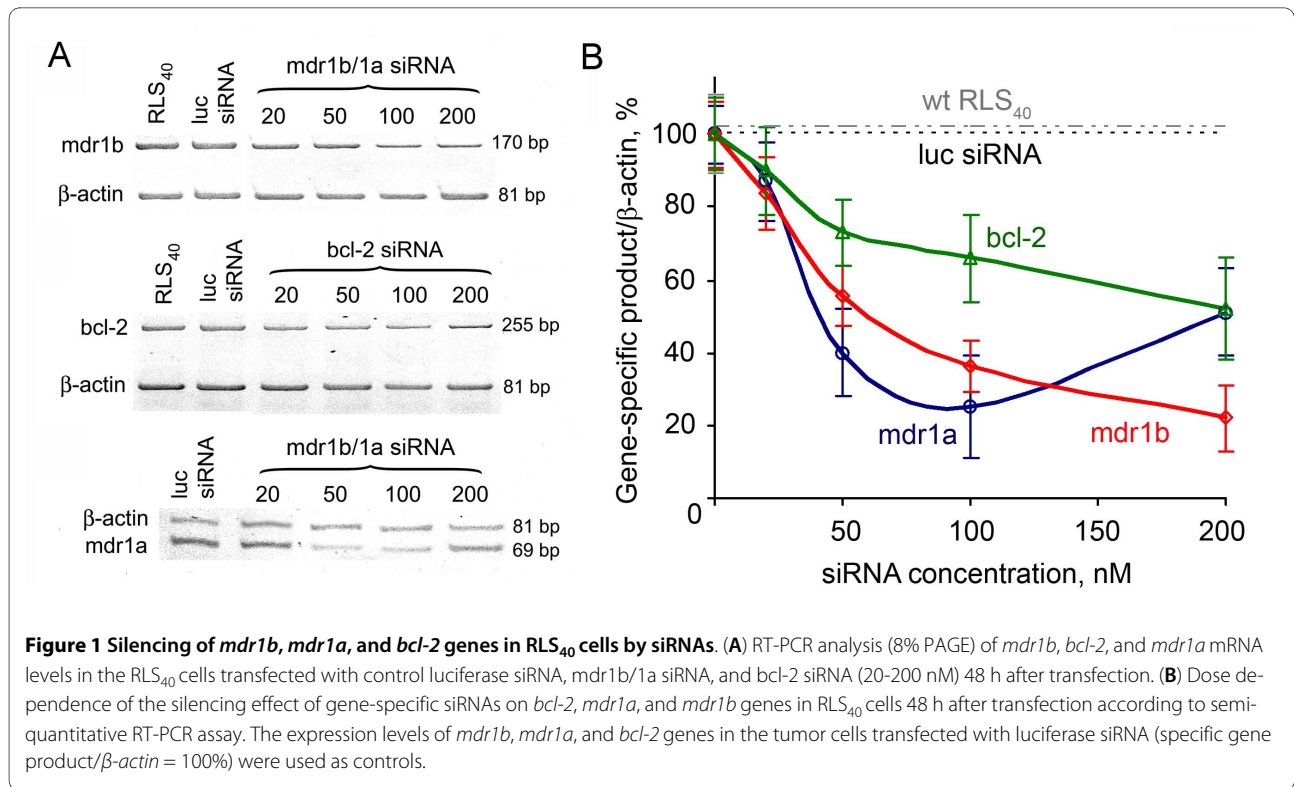
To reverse the MDR phenotype of tumor cells, we applied in our experiments two siRNAs: the *bcl-2* siRNA targeted to *bcl-2* mRNA and the *mdr1b/1a* siRNA targeted to *mdr1b* and *mdr1a* mRNAs. The *mdr1b/1a* siRNA was designed to target *mdr1b* and *mdr1a* genes simultaneously. The sequence of *mdr1b/1a* siRNA antisense strand is complementary to the *mdr1b* mRNA and is able to form a duplex with three mismatches with *mdr1a* mRNA. Note that the mismatches are located at the 5'-end of the antisense strand, which makes this strand favorable for RISC loading [23]. It has been shown earlier that an efficient silencing of *c-myc* and *N-myc* genes is feasible using single siRNA [24]. The sequences of *mdr1b* and *mdr1a* mRNAs for targeting with *mdr1b/1a* siRNA were chosen based on the results of the studies [25,26] where the siRNAs addressed to various regions of *MDR1* mRNA were screened: the designed siRNAs displayed silencing activity and no off-target effects.

### In vitro

The siRNAs targeted to the mRNA of *mdr1b*, *mdr1a* and *bcl-2* genes were tested for their ability to downregulate the synthesis of Pgp and Bcl-2 proteins in the RLS<sub>40</sub> cell line, displaying MDR phenotype. The siRNA targeted to the mRNA of firefly luciferase gene was used as a control. RLS<sub>40</sub> cells were transfected with specific siRNA at concentrations of 20 to 200 nM in the presence of Lipofectamine and 24-96 h post transfection, the expression levels of *mdr1a*, *mdr1b*, and *bcl-2* genes were measured by semiquantitative RT-PCR.

According to the kinetics of the gene silencing by specific siRNAs, the minimal levels of *mdr1a*, *mdr1b*, and *bcl-2* mRNAs in RLS<sub>40</sub> cell line were detected 48 h after transfection (primary data not shown). At that time point, the *bcl-2* and *mdr1b/1a* siRNAs caused a twofold, fivefold and fourfold reduction in the level of the *bcl-2*, *mdr1b* and *mdr1a* mRNAs, respectively (Figure 1A, B). However, 96 h after transfection, the levels of mRNAs restored. Analysis of the concentration dependences of gene silencing demonstrated that the *bcl-2* and *mdr1b/1a* siRNAs at a concentration of 200 nM caused a maximal decrease in *bcl-2* and *mdr1b* mRNA levels (Figure 1A, B). A further increase in *mdr1b/1a* siRNA concentration up to 200 nM failed to additionally decrease the *mdr1a* mRNA level: the observed change in mRNA levels was within the experimental error.

The *mdr1a* and *mdr1b* genes of mice encode Pgp, which exports chemotherapeutic agents out of cancer cells, thereby providing for a decrease in drug accumulation in tumors. The silencing effect of *mdr1b/1a* siRNA on *mdr1a* and *mdr1b* genes should be accompanied by a decrease in Pgp activity; therefore, we studied the effect of *mdr1b/1a* siRNA on the Pgp pump function. Rh123 is a well-known substrate for Pgp efflux and is commonly used for the measurement of Pgp bioactivity. In the Rh123 efflux assay, the Pgp activity was blocked by a commonly used Pgp inhibitor, Verapamil (as a positive control). Thus, the efficacy of *mdr1b/1a* siRNA was evaluated relative to the inhibitory effect of Verapamil. Accumulation of Rh123 in the tumor cells cultivated with Verapamil was considered 100%. As expected according to the Rh123 efflux assay, tumor cells intensively pumped out Rh123 in the absence of inhibitors of Pgp activity. The control luciferase siRNA did not influence the Pgp activity (Figure 2A), and Rh123 cellular accumulation was low (regarded as 0%), whereas the efflux of fluorescent dye was considerably decreased in the RLS<sub>40</sub> cells transfected with *mdr1b/1a* siRNA (Figure 2A, B). The highest level of Rh123 accumulation was observed 48 and 72 h after transfection with 100 and 200 nM of *mdr1b/1a* siRNA: the Rh123 accumulation reached 38 and 62%, respectively, as compared with the cells transfected with control siRNA (Figure 2B).



The downregulation of *mdr1a* and *mdr1b* gene expression by specific siRNA resulted in a decrease in the Pgp quantity in the cytoplasmic membrane, which contributes to accumulation of cytostatics in the cytoplasm leading to cell death. The sensitivity of RLS<sub>40</sub> cells to vinblastine was evaluated 48 h after transfection with *mdr1b/1a* siRNA, when the levels of *mdr1a* and *mdr1b* mRNAs were the lowest. The IC<sub>50</sub> of vinblastine (concentration of vinblastine at which 50% of the cells remained viable) for the RLS<sub>40</sub> cells transfected with luciferase siRNA was 691.4 ± 44.2 nM, being similar to the IC<sub>50</sub> for untransfected RLS<sub>40</sub> cells. The transfection of RLS<sub>40</sub> cells with *mdr1b/1a* siRNA resulted in an up to threefold increase in the cell sensitivity to vinblastine (Figure 3): the IC<sub>50</sub> of vinblastine was 315.4 ± 25.1, 278.3 ± 23.1, and 235.5 ± 20.1 nM for the cells transfected with 20, 50, and 100 nM of *mdr1b/1a* siRNA, respectively. Note that for the parental RLS cell line, which is more susceptible to chemotherapy, the IC<sub>50</sub> value for vinblastine was 6.2 nM.

#### Ex vivo

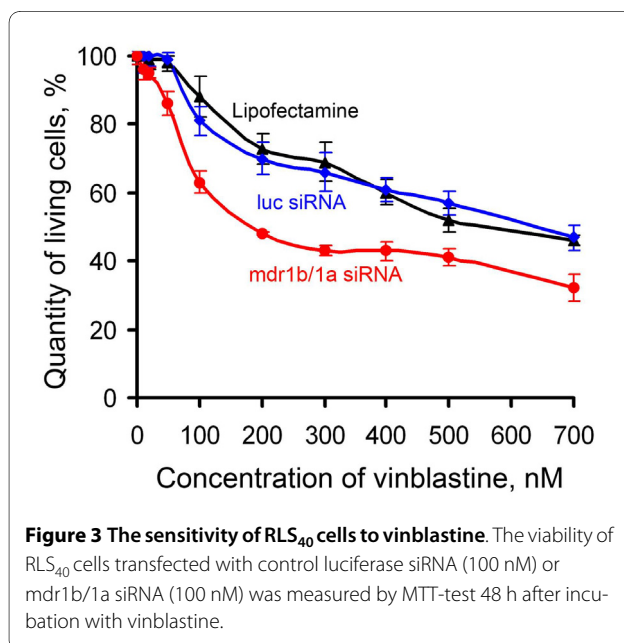
The ability of *mdr1b/1a* and *bcl-2* siRNAs to overcome the MDR phenotype was tested in *ex vivo* experiments. The ascites of RLS<sub>40</sub> or RLS tumors were collected; the cells were isolated and transfected with siRNAs as described in Materials and Methods. Then tumor cells were inoculated into the right thigh muscle of mice for solid tumor development. In the experiment with RLS<sub>40</sub> tumor, the mice were twice treated with cyclophosphamide (100 mg/kg), on days 2 and 4 after transplantation; in the experiment with parental RLS tumor, the mice were once treated with cyclophosphamide (200 mg/kg)

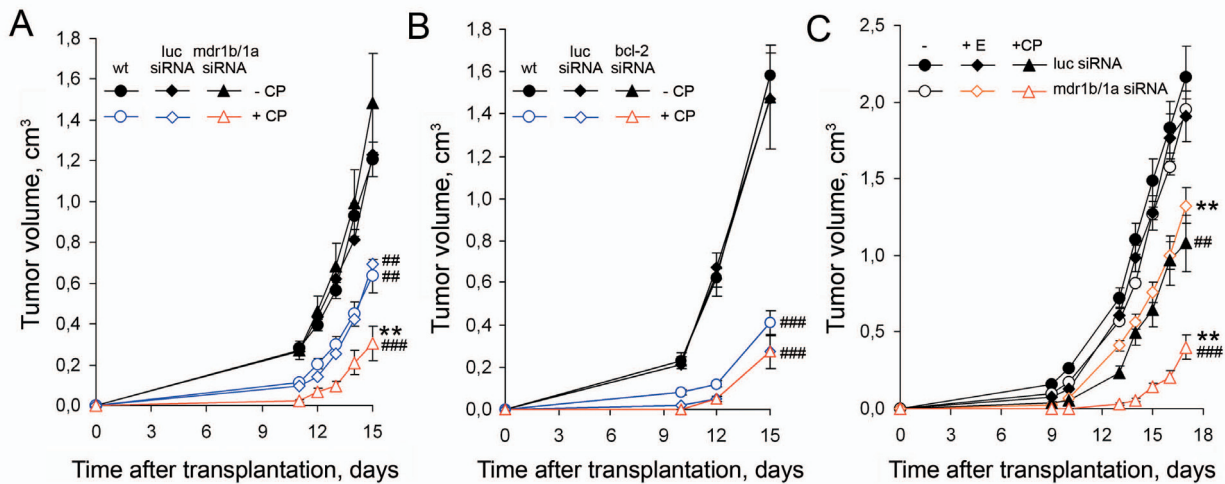
on day 2 after transplantation. After transfection, approximately 5 × 10<sup>5</sup> cells from each sample were left and incubated at 37°C and 5% CO<sub>2</sub> for 48 h. Then the cells were used for measuring of *mdr1a*, *mdr1b*, and *bcl-2* mRNA levels. RT-PCR analysis demonstrated that *mdr1b/1a* siRNA caused a fourfold downregulation of expression of *mdr1a* and *mdr1b* mRNAs and *bcl-2* siRNA - a twofold downregulation of expression of *bcl-2* gene as compared with the wt cells and luciferase siRNA-treated cells.

The dynamics of tumor growth is shown in Figure 4. The growth rates of the RLS<sub>40</sub> tumors either wt or transfected with luciferase or *mdr1b/1a* siRNA were similar (Figure 4A, black curves), while cyclophosphamide administration resulted in a twofold retardation of tumor growth in the mice with wt RLS<sub>40</sub> tumors and mice bearing the tumors transfected with luciferase siRNA (Figure 4A, blue curves), thus showing the absence of specific effect of the control siRNA on the tumor growth. The administration of cyclophosphamide in the case of the RLS<sub>40</sub> cells transfected with *mdr1b/1a* siRNA resulted in a twofold retardation of tumor growth as compared with only cyclophosphamide action and sixfold, as compared with the untreated tumors (Figure 4A, red curve). The treatment of RLS<sub>40</sub> cells with the *bcl-2* siRNA followed by cyclophosphamide administration had no effect on the RLS<sub>40</sub> tumor growth (data not shown), which is explainable by a major contribution of *mdr1a* and *mdr1b* gene products to MDR development (Pgp-mediated MDR phenotype) as compared with *bcl-2* gene.

The block of apoptosis is a distinctive feature of the parental RLS tumor: a sixfold increase in the expression level of *bcl-2* mRNA was observed for the parental RLS lymphosarcoma as compared with RLS<sub>40</sub> tumor. Therefore, an antitumor potential of *bcl-2* siRNA and cyclophosphamide was examined *ex vivo* using the RLS lymphosarcoma. Analysis of dynamics of RLS tumor growth failed to find any significant difference between the growth rates of wt tumor, the tumor transfected with control luciferase siRNA, and specific *bcl-2* siRNA after cyclophosphamide impact (Figure 4B).

The ability of *mdr1b/1a* siRNA to sensitize RLS<sub>40</sub> tumor to cytostatics was also studied in an *ex vivo* experiment using vinblastine. The dynamics of tumor growth showed that the double treatment including *mdr1b/1a* siRNA and vinblastine had no effect on tumor progress (data not shown). The reason could be a high resistance of RLS<sub>40</sub> tumor cells to this particular cytostatic (IC<sub>50</sub> = 680 ± 42 nM): the tolerable dose of vinblastine administered to animals was lower than the IC<sub>50</sub> values observed *in vitro* even after silencing of *mdr1b* gene with the corresponding siRNA (283 ± 4 nM).





**Figure 4** The dynamics of tumor development in mice after *ex vivo* and *in vivo* successive treatment with siRNA and cytostatics. (A)  $RLS_{40}^{-}$ -bearing animals: **wt**, control mice bearing wild type tumor; **luc siRNA**, the mice bearing tumor cells transfected *ex vivo* with control luciferase siRNA; and **mdr1b/1a siRNA**, the mice bearing tumor cells transfected *ex vivo* with mdr1b/1a siRNA; (**+CP**) the animals treated with cyclophosphamide (100 mg/kg) on days 2 and 4 after tumor transplantation and (**-CP**) the animals without chemotherapeutic treatment. (B) RLS-bearing animals: **wt**, the control animals bearing wild type tumor; **luc siRNA**, the mice bearing tumor cells transfected *ex vivo* with control luciferase siRNA; and **bcl-2 siRNA**, the mice bearing tumor cells transfected *ex vivo* with bcl-2 siRNA; (**+CP**) the animals treated with cyclophosphamide (200 mg/kg) on day 2 after tumor transplantation and (**-CP**) the animals without chemotherapeutic treatment. (C)  $RLS_{40}^{-}$ -bearing animals: **luc siRNA**, the mice with tumors transfected *in vivo* with luciferase siRNA and **mdr1b/1a siRNA**, the mice with tumors transfected *in vivo* with mdr1b/1a siRNA; (-) the animals without chemotherapeutic treatment, (**+E**) the animals treated with embichin (2 mg/kg) or (**+CP**) cyclophosphamide (200 mg/kg) on day 2 after tumor transplantation. \*\*  $p < 0.01$ , compared with cytostatic treatment; ## and ###,  $p < 0.01$  and  $p < 0.001$ , respectively, compared with wt (A) or -/luc siRNA and -/mdr1b/1a siRNA (C).

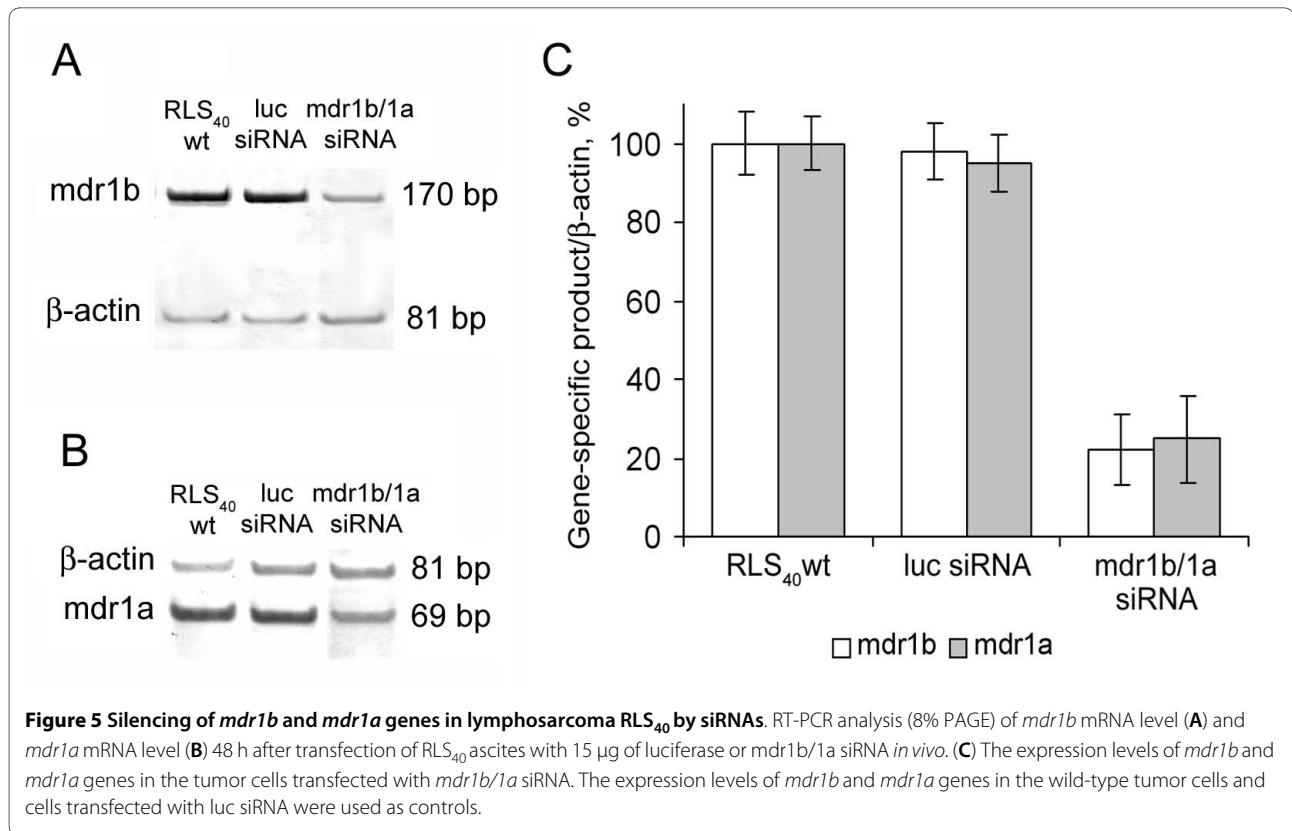
In all groups of mice, the hepatic index (HI), an indicator of animal health, was measured. The HI in healthy CBA mice is 5.0%. In the groups of the  $RLS_{40}$ -bearing animals treated with cyclophosphamide only or cyclophosphamide following the transfection with the luciferase siRNA, the HI was  $8.6 \pm 0.6\%$ . The HI was  $6.1 \pm 0.9\%$  in the mice bearing  $RLS_{40}$  tumors treated with cyclophosphamide after mdr1b/1a siRNA transfection. An average lifetime for mice of this group was  $25 \pm 1.8$  days versus  $21.4 \pm 1.5$  days in the groups treated with cyclophosphamide only or cyclophosphamide with the control siRNA.

#### In vivo

The efficacy of cytostatic treatment following the silencing of *mdr1b* and *mdr1a* genes with siRNA was tested *in vivo*.  $RLS_{40}$  ascites tumors were transfected *in vivo* with (1) luciferase siRNA or (2) mdr1b/1a siRNA. The  $RLS_{40}$  ascites were extracted 4 h post transfection, and the cells were intramuscularly transplanted into new groups of mice for solid tumor formation. After transfection, approximately  $10^6$  cells were isolated from the ascites fluid and incubated at  $37^{\circ}C$  and  $5\% CO_2$  for 48 h. Then the cells were used for measuring the *mdr1a* and *mdr1b* mRNA levels. RT-PCR analysis demonstrated that the mdr1b/1a siRNA caused a fourfold downregulation of

expression of *mdr1a* and *mdr1b* genes as compared with the luciferase siRNA-treated cells (Figure 5).

On day 2 of tumor development, the mice were treated with embichin (2 mg/kg) or cyclophosphamide (200 mg/kg). Analysis of the tumor growth dynamics showed that the tumors derived from the cells transfected with mdr1b/1a siRNA were more sensitive to chemotherapy as compared with the cells transfected with the control siRNA (Figure 4C). Both cytostatics, embichin and cyclophosphamide, caused a significant retardation of tumor growth only in the mice treated with mdr1b/1a siRNA: the tumors were 1.4-fold smaller in the case of embichin (Figure 4C, orange curve) and 3.3-fold smaller in the case of cyclophosphamide (Figure 4C, red curve) as compared with the corresponding control groups. On the other hand, administration of embichin only did not influence the tumor growth in the control group (the tumors transfected with luciferase siRNA) (Figure 4C, black curve, diamond points). Cyclophosphamide had a more pronounced effect on the tumor growth: the tumors transfected with luciferase siRNA were twofold smaller (Figure 4C, black curve, triangle points) than the tumors without treatment. The HI in the group received luciferase siRNA and cyclophosphamide was  $9.2 \pm 0.7\%$  versus  $7.7 \pm 0.8\%$  in the group received mdr1b/1a siRNA. An average lifetime for the mice of this group was  $20.6 \pm 0.7$  days versus



16.8 ± 0.5 days in the group treated with cyclophosphamide only or cyclophosphamide with the control siRNA.

## Discussion

The range of siRNA application continues to expand. Researchers attempt to explore the potential of RNA interference (RNAi) technology for the therapy of communicable, autoimmune, cancer, and inflammatory diseases; neural disorders; chronic pains; adiposis; and insulin-independent diabetes [27-34]. The potential of siRNA strategy to overcome multidrug resistance *in vitro* was demonstrated in a number of studies [25,26,35-39], and the success in *in vivo* experiments is retarded by unsettled challenges in the *in vivo* siRNA delivery technologies [40,41].

Development of MDR to a variety of conventional and novel chemotherapeutic agents is a significant obstacle in effective therapy of refractory cancer types. In our previous work, we established two murine lymphosarcoma models with a stable MDR, namely, RLS and RLS<sub>40</sub> [16]. These two related tumors differed in the origin of drug resistance: the MDR of RLS<sub>40</sub> tumor is *mdr1a/mdr1b*-associated and the MDR of RLS tumor is *bcl-2*-associated. These lymphosarcomas are the allografts, exhibiting the adequate response of the body.

In this work, we applied the siRNA targeted to the mRNAs of *bcl-2*, *mdr1b*, and *mdr1a* genes to overcome

the MDR phenotype of lymphosarcomas RLS<sub>40</sub> and RLS. It was shown that *mdr1b/1a* siRNA caused the decrease in both *mdr1b* and *mdr1a* mRNA levels. Although the *mdr1b/1a* siRNA has three mismatches in the binding site of *mdr1a* mRNA, we believe that this siRNA does not provide a miRNA effect and acts via the RNA interference mechanism. The key features that distinguish a miRNA from a siRNA are (1) a noncomplementarity between the center on the miRNA and the targeted mRNA [42,43] and (2) a decrease in the protein level rather than the mRNA level [44]. In our case, we observed a concentration-dependent reduction in the *mdr1a* mRNA level after treating the cells with *mdr1b/1a* siRNA. The mismatches between *mdr1b/1a* siRNA and *mdr1a* mRNA are localized to the 5'-end of the siRNA antisense strand rather than to its central part. Therefore, the total effect of MDR reversing is provided by the degradation of both *mdr1b* and *mdr1a* mRNAs via an RNA-interference pathway.

The effect of *mdr1b/1a* siRNA resulted in a substantial increase in the sensitivity of cancer cells to chemotherapeutics both *in vitro* and *in vivo* and led to a threefold retardation of tumor growth upon cyclophosphamide administration. Interestingly, we observed a more efficient inhibition of tumor growth in *in vivo* experiment as compared with *ex vivo* experiment. It could be explained by the difference in the treatment schemes, namely, a



double administration of cyclophosphamide on days 2 and 4 after tumor implantation at a dose of 100 mg/kg in the *ex vivo* experiment and a single administration of cyclophosphamide on day 2 at a doubled dose in the *in vivo* experiment.

Our data demonstrated that the *mdr1b/1a* siRNA *in vivo* sensitized RLS<sub>40</sub> tumor cells to cyclophosphamide and embichin. Cyclophosphamide itself is not cytotoxic but undergoes metabolic changes in the body predominantly in the liver with the formation of an active derivative, phosphoramidate mustard [45], which is an alkylating agent capable of forming cross-links in DNA molecules and initiating apoptosis. Phosphoramidate mustard has not been regarded as a substrate of Pgp [46]. The observed inhibition of RLS<sub>40</sub> tumor growth after an *ex vivo* treatment of tumor cells with *mdr1b/1a* siRNA followed by *in vivo* administration of cyclophosphamide suggests that this toxic agent is yet a substrate of Pgp. To confirm this assumption in *in vivo* experiment, mice were injected with embichin (nitrogen mustard), another active alkylating agent with similar cytotoxic properties. Although embichin was not as efficient as cyclophosphamide, the obtained results supported this hypothesis.

Our data showed that *bcl-2* siRNA specifically downregulated the *bcl-2* mRNA level by 50% *in vitro*; however, the *bcl-2* siRNA *in vivo* neither showed any sensitizing effect on RLS tumor nor influenced the tumor growth. In addition to the classical Pgp-based mechanism of multidrug resistance of cancer cells [47-50], there is an alternative mechanism associated with putative effector pathways of drug action, apoptosis, and senescence [51-59]. It was shown that Pgp could modulate caspase activation, e.g., caspase 8, and inhibit apoptosis [60]. Thus, the Pgp inhibitors, which reverse drug resistance, can also block its caspase-inhibitory function [60]. Although an MDR phenotype of RLS is considered as *bcl-2*-associated, the expression levels of *mdr1a* and *mdr1b* genes in these cells are also rather high [16]. In both RLS and RLS<sub>40</sub> cells, high levels of Pgp and Bcl-2 resulted in a "double" suppression of apoptosis. The *mdr1b/1a* siRNA-induced decrease in the Pgp level resulted in triggering of proapoptosis, while the decrease in Bcl-2 caused by *bcl-2* siRNA is not sufficient to overcome the MDR of tumor due to a high Pgp level. Another possible reason for the absence of tumor sensitivity to cyclophosphamide after *bcl-2* siRNA transfection is that a 50% downregulation of *bcl-2* mRNA expression can be insufficient.

The results demonstrating the sensitizing properties of *mdr1b/1a* siRNA cannot be immediately applied in the clinical practice; however, it is really encouraging that even a single administration of siRNA resulted in essential tumor growth retardation and increase in the average lifetime by approximately 20%. Thus, the examined ther-

apy has significant promise in the clinical management of refractory cancer diseases.

## Conclusions

In view of our results, the synthetic siRNA targeted to *mdr1b* and *mdr1a* genes overcame MDR phenotype *in vivo* and, in the successive treatment with conventionally used cytostatics, evoked more than threefold increase in the tumor cell sensitivity to chemotherapy after a single siRNA administration. In clinical practice, a twofold decrease in the cancer cell resistance to chemotherapy is sufficient to achieve a significant enhancement in the efficiency of antitumor therapy.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

OP performed most of the experiments and data analysis and drafted the manuscript. NM coordinated the study and participated in the experiments and manuscript preparation. NP participated in the design and coordination of the animal study. VK participated in the animal study and revision of the manuscript. VN participated in the animal study and data interpretation. WV conceived the study and revised the manuscript. MZ developed the experimental concept and design and supervised the study, data interpretation, and manuscript revision.

All authors read and approved the final manuscript.

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