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Small Interfering RNA-induced Suppression of *MDR1* (P-Glycoprotein) Restores Sensitivity to Multidrug-resistant Cancer Cells[1]

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ABSTRACT

Overexpression of P-glycoprotein (P-gp), the *MDR1* gene product, confers multidrug resistance (MDR) to cancer cells. Clinically, MDR is one of the major causes for chemotherapeutic treatment failure in cancer patients. To explore a new approach to circumventing MDR, we adopted RNA interference to target *MDR1* gene expression. RNA interference is a conserved biological response to double-stranded RNA, which results in sequence-specific gene silencing [G. J. Hannon, Nature (Lond.), *418:* 244–251, 2002]. We report that introduction of an *MDR1*-targeted small interfering RNA duplex into drug-resistant cancer cells markedly inhibited the expression of *MDR1* mRNA and P-gp, as determined by reverse transcription-PCR and Western blot. Inhibition of P-gp expression by small interfering RNA enhanced the intracellular accumulation of and selectively restored sensitivity to drugs transported by P-gp. These studies indicate that RNA interference can modulate MDR in preclinical models.

INTRODUCTION

Drug resistance hampers successful chemotherapy in cancer patients. One form of MDR[3] is caused by overexpression of P-gp, the *MDR1* gene product (1, 2). P-gp is a transmembrane phosphoglycoprotein capable of transporting a variety of structurally and functionally diverse chemotherapeutic drugs such as vinblastine, doxorubicin, and paclitaxel, leading to reduced intracellular drug concentration and decreased cytotoxicity (3). Despite promising early studies showing that P-gp antagonists could reverse MDR (4–7), the clinical goal of restoring drug sensitivity to drug-resistant human cancer has been elusive. Successful reversal or prevention of drug resistance is still awaiting new therapeutic strategies or pharmaceuticals. In this study, we tested the feasibility of using siRNA to inhibit P-gp expression and reverse MDR. siRNA generated from double-stranded RNA can trigger silencing of homologous gene expression by inducing degradation of the complementary mRNA, an evolutionarily conserved mechanism termed RNA interference or post-transcriptional gene silencing (8, 9). Using the RNA interference approach, we show that the expression of the endogenous as well as transfected *MDR1* gene can be effectively inhibited, and that the sensitivity to P-gp-transportable drugs can be restored. Our study demonstrates the utility of siRNA for therapeutically modulating P-gp-mediated drug resistance.

MATERIALS AND METHODS

Cell Lines and Culture. The MDR human breast cancer cell lines, MCF-7/AdrR and MCF-7/BC-19, and their parental, sensitive line, MCF-7, were kindly supplied by Dr. Kenneth Cowan of the Eppley Institute for Research in Cancer (Omaha, NE). They were maintained in RPMI 1640 containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2/95% air. MCF-7/AdrR was developed by step-wise selection (10), and MCF-7/BC-19 is a *MDR*1 transfectant (11). Human ovarian carcinoma cell lines, A2780 and A2780Dx5, were provided by Dr. Youcef Rustum (Roswell Park Cancer Institute, Buffalo, NY) and were grown in DMEM containing 10% fetal bovine serum under the identical condition as described above except that for A2780Dx5, 2 μM of doxorubicin was added to the medium for the maintenance of the MDR phenotype (12). Cells were checked routinely and found to be free of contamination by *Mycoplasma* or fungi. All of the cell lines were discarded after 3 months and new lines obtained from frozen stocks.

siRNA Preparation and Transfection. The siRNA sequence targeting *MDR1* corresponded to the coding region 79–99 (5′-AAGGAAAAGAAACCAACTGTC-3′) relative to the start codon. The siRNA duplex with the following sense and antisense sequences was used: 5′-GGAAAAGAAACCAACUGUCdTdT (sense) and dTdTCCUUUUCUUUGGUUGACAG-5′ (antisense). Lamin A/C siRNA duplex has the following sequences: 5′-CUGGACUUCCAGAAGAACAdTdT (sense) and dTdTGACCUGAAGGUCUUCUUGU-5′ (antisense).

All of the siRNA duplexes were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2′-ACE protection chemistry.

Cells in exponential phase of growth were plated in six-well plates at 5×10^5 cells/well, grown for 24 h then transfected with siRNA (P-gp siRNA: 200 nM; lamin A/C siRNA: 100 nM) using oligofectamine and OPTI-MEM I reduced serum medium (Invitrogen Life Technologies, Inc., Carlsbad, CA), according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose-response studies. Silencing was examined 24–48 h after transfection. Control cells were treated with oligofectamine and serum-reduced medium (mock).

Reverse Transcription-PCR. Total RNA was extracted from cells with TRIzol reagent (Invitrogen Life Technologies, Inc.) and quantified by UV absorbance spectroscopy. The reverse transcription reaction was performed using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies, Inc.) in a final volume of 20 μl containing 5 μg of total RNA, 200 ng of random hexamers, 1× reverse transcription buffer, 2.5 mM $MgCl_2$, 1 mM deoxynucleotide triphosphate mixture, 10 mM DTT, RNaseOUT recombinant ribonuclease inhibitor, 50 units of Superscript reverse transcriptase, and diethylpyrocarbonate-treated water. After incubation at 42°C for 80 min, the reverse transcription reaction was terminated by heating at 70°C for 15 min. The newly synthesized cDNA was amplified by PCR. The reaction mixture contained 2 μl of cDNA template, 1.5 mM $MgCl_2$, 2.5 units of Tag polymerase, and 0.5 μM of *MDR1* primer (5′-ATATCAGCAGCCCACATCAT-3′; 5′-GAAGCACTGGGATGTCCGGT-3′; Ref. 13). GAPDH primer (5′-GCCAAAAGGGTCATCATCTC-3′; 5′-GTAGAGGCAGGGATGATGTTC-3′) was used as an internal control. Amplication cycles were: 94°C for 3 min, then 33 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1.5 min, followed by 72°C for 15 min. Aliquots of PCR product were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining.

Western Blot Analysis. Cells were washed twice with PBS containing 1 mM phenylmethylsulphonyl fluoride, scraped off the dishes, and pelleted at $500 \times g$ for 10 min. Cell pellets were then lysed in cold TNT buffer [20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, and 1% aprotinin) for 45 min with occasional rocking. The lysates were transferred to Eppendorf tubes and clarified by centrifugation at $12{,}000 \times g$ for 40 min at 4°C. Identical amounts (50 μg of protein) of cell lysates were resolved by 8% SDS-PAGE. Transfer of proteins to nitrocellulose was carried out by the method of Towbin *et al.* (14). The membranes were incubated in blocking solution consisting of 5% powered milk in TBST [10

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[3] The abbreviations used are: MDR, multidrug resistant or multidrug resistance; P-gp, P-glycoprotein; siRNA, small interfering RNA; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

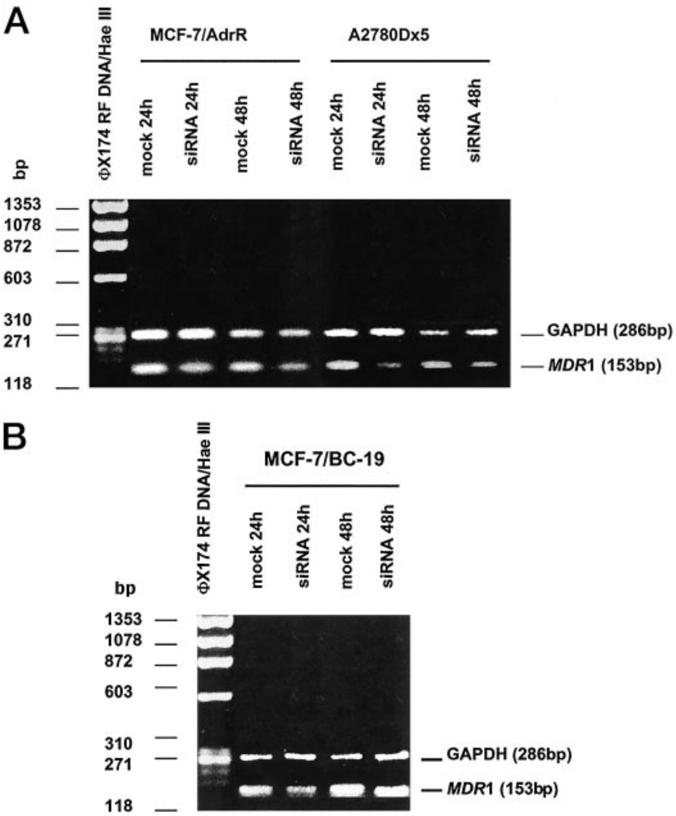

Fig. 1. Effect of siRNA on *MDR1* mRNA expression in MDR cancer cells. MDR MCF-7 and A2780 (*A*) or MCF-7/BC-19 (*B*) cells were treated with 200 nM of *MDR1* siRNA or mock, which only consisted of oligofectamine and OPTI-MEM I reduced serum medium. Twenty-four and 48 h later, total RNA was extracted from the cells and the reverse transcription reaction was performed using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies, Inc.). The newly synthesized *MDR1* cDNA was amplified by PCR using the *MDR1* primer (5′-ATATCAGCAGCCCACATCAT-3′; 5′-GAAGCACTGGGATGTCCGGT-3′). GAPDH primer (GCCAAAAGGGTCATCAT-CTC; GTAGAGGCAGGGATGATGTTC) was used as an internal control. Aliquots of PCR product were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining. Results are the representative of three similar experiments.

mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] at room temperature for 1 h, then immunoblotted with monoclonal anti-P-gp antibody C219 (Calbiochem, San Diego, CA), antilamin A/C antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or antitubulin antibody (Sigma-Aldrich, St. Louis, MO). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Protein expression was quantified by Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA).

Paclitaxel and Doxorubicin Accumulation. Steady-state paclitaxel accumulation was assayed by a method described previously by our laboratory (15). Briefly, sensitive and MDR MCF-7 cells transfected with siRNA or mock were seeded in 24-well plates and grown for 48 h. Then, the growth medium was aspirated and replaced with 0.25 ml of RPMI 1640 containing 25 mM HEPES (pH 7.4) and 50 nM of [^{3}H]paclitaxel (10.0 Ci/mmol; Moravek Biochemicals, Inc., Brea, CA). Cells were incubated with [^{3}H]paclitaxel for 2 h and were then cooled on ice, washed three times with ice-cold PBS, and solubilized with 0.25 ml of 1% SDS. The radioactivity in each sample was determined by scintillation counting.

To assess steady-state doxorubicin accumulation, sensitive and MDR MCF-7 cells transfected with siRNA or mock were incubated with 25 μM of doxorubicin for 1 (sensitive MCF-7) or 2 h (MCF-7/AdrR). At the end of incubation, cells were washed three times with PBS and observed under a fluorescence microscope with 100× lens (Nikon ECLIPSE TE200 microscope; Nikon Inc., Melville, NY).

Clonogenic Assay. siRNA-transfected cells were plated in 60-mm cell culture dishes (250 cells/dish) and incubated at 37°C in a humidified atmosphere containing 5% CO_2/95% air for 12 days in the presence of various concentrations of vinblastine, doxorubicin, or hydroxyurea. At the end of the incubation period, cells were stained with 1% methylene blue in 50% methanol for 30 min, washed with water, and colonies counted. IC_{50} was defined as the concentration of drug that inhibited colony formations by 50% as compared with that of vehicle-treated control. Student's *t* test was used to determine the degree of significance. Fold reversal was the IC_{50} for cytotoxic drug in mock-treated cells divided by the IC_{50} for drug in siRNA-treated cells.

RESULTS

To test whether siRNA could be used to modulate MDR, we treated MDR cells overexpressing P-gp with an siRNA duplex designed to target coding region 79–99 after the start codon of *MDR1*. Fig. 1 shows that as compared with mock transfection, the siRNA duplex reduced the levels of the endogenous (Fig. 1*A*) as well as the transfected (Fig. 1*B*) *MDR1* mRNA in drug-resistant cells at 24 and 48 h after treatment, as determined by reverse transcription-PCR. The siRNA had no effect on GAPDH RNA (Fig. 1).

To determine the effect of siRNA on target protein expression, cell lysates from the treated cells were analyzed by Western blot. Fig. 2*A* demonstrates that the expression of P-gp was decreased by 24 h in all three of the cell lines. The P-gp content remained lower in siRNA-treated A2780Dx5 and MCF-7/BC-19 cell lines at 48 h after treatment as compared with the controls. However, the protein level began to increase in MCF-7/AdrR cells 48 h after treatment (Fig. 2*A*). *MDR*1

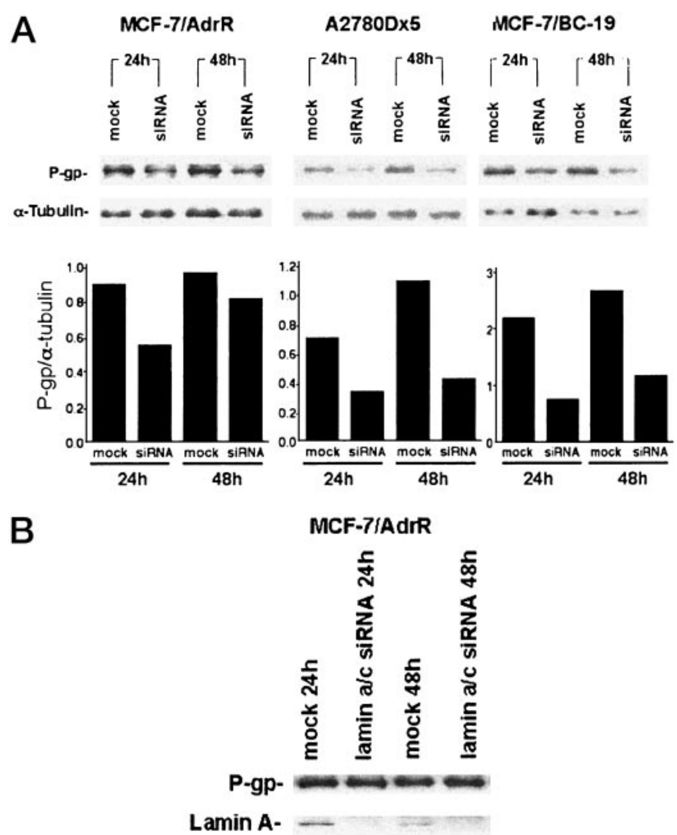

Fig. 2. Effect of siRNA on P-gp expression in MDR cancer cells. MDR MCF-7 and A2780 cells were treated with 200 nM of *MDR1* siRNA, 100 nM of lamin A/C siRNA, or mock. Twenty-four and 48 h later, cell lysates were prepared from the siRNA- or mock-treated cells. Equal amounts (50 μg proteins) of cell lysates were separated by 8% SDS-PAGE, then transferred onto nitrocellulose membrane. The membranes were immunoblotted with monoclonal anti-P-gp antibody C219, antilamin A/C antibody, or antitubulin antibody. Detection of P-gp was performed using enzyme-linked chemiluminescence. Protein expression was quantified by Molecular Analyst software. Results are the representative of three similar experiments.

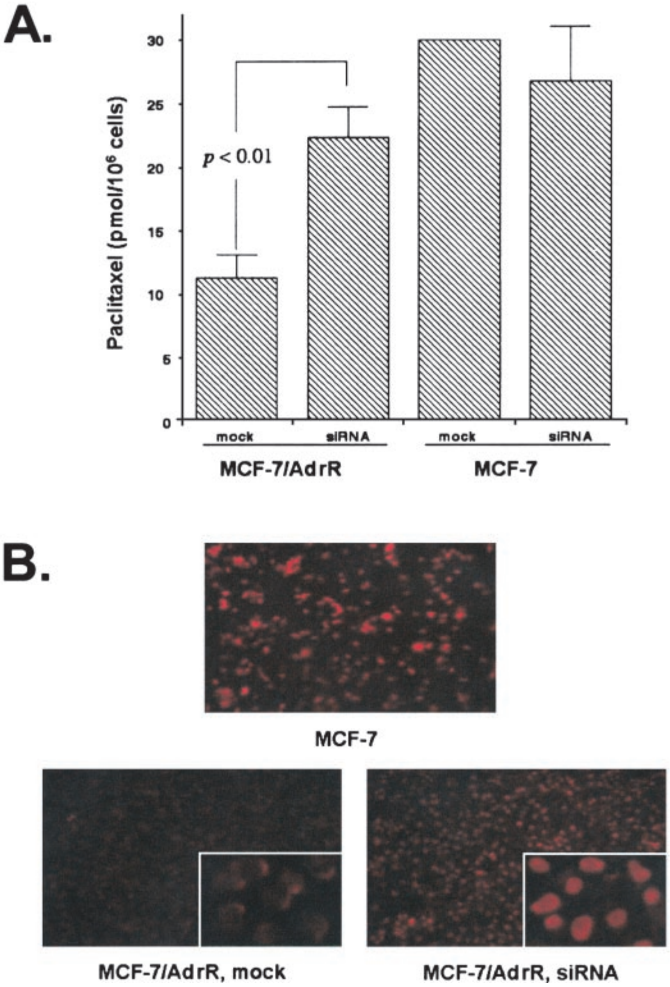

Fig. 3. Effect of siRNA on accumulation of paclitaxel and doxorubicin in MDR cancer cells. *A*, sensitive and MDR MCF-7 cells treated with *MDR1* siRNA or mock were seeded in 24-well plates and grown for 48 h. Cells were then incubated with 50 nM [^{3}H]paclitaxel for 2 h at 37°C. At the end of incubation, cells were cooled on ice, washed three times with ice-cold PBS, and solubilized with 0.25 ml of 1% SDS. The radioactivity in each sample was determined by scintillation counting. *Bar*, ±SD of triplicate determinations. *B*. siRNA- or mock-transfected MDR MCF-7 and sensitive MCF-7 cells were treated with 25 μM of doxorubin for 1 (sensitive MCF-7) or 2 h (MCF-7/AdrR). At the end of incubation, cells were washed three times with PBS, then observed under a fluorescence microscope with ×100 lens. Results are the representative of two similar experiments.

siRNA did not affect α-tubulin expression (Fig. 2*A*). To confirm the specificity of the siRNA-mediated silencing of P-gp expression, we tested an unrelated sequence, lamin A/C siRNA (16), on gene expression. Fig. 2*B* demonstrates that treatment of P-gp-overexpressing MDR cells with lamin A/C siRNA reduced the expression of lamin A protein but had no effect on the expression of P-gp.

Treatment of cells with the siRNA enhanced intracellular drug accumulation. Fig. 3*A* shows that in siRNA-treated MCF-7/AdrR cells, the accumulation of paclitaxel, a P-gp-transportable compound, was increased significantly as compared with that of the mock-transfected cells ($P < 0.01$). The accumulation of doxorubicin, another drug that is transported by P-gp, was also increased in the siRNA-treated MDR MCF-7 cells in comparison with the mock-transfected controls (Fig. 3*B*).

To assess whether siRNA-directed suppression of P-gp sensitized MDR cancer cells to cytotoxic agents, we compared the drug sensitivity of the siRNA-treated to that of the mock-treated MDR cells using clonogenic assay. As shown in Table 1 and Fig. 4, the sensitivity of the MDR cells to vinblastine, doxorubicin, and paclitaxel was increased significantly by the introduction of *MD*R1-targeted siRNA ($P < 0.05$). For example, in MCF-7/AdrR, MCF-7/BC-19, and A2780Dx5 cell lines, siRNA caused a 232-, 49-, and 4-fold reversal of cellular resistance to vinblastine, respectively. In MCF-7/AdrR cells, treatment with siRNA caused a 12-fold reversal of resistance to doxorubicin and 95-fold reversal of resistance to paclitaxel. The sensitivity to hydroxyurea, a drug that is not transported by P-gp, was not affected by silencing of P-gp expression (Table 1; Fig. 4).

DISCUSSION

Because Elbashir *et al.* (16) reported that RNA interference can be triggered in mammalian cells by introduction of 21-nucleotide siRNA, siRNA has been shown to be an effective approach for silencing gene expression that has been applied recently to inhibiting HIV-1 replication and infection in cell cultures (17–20). We now demonstrate that introduction of an siRNA duplex decreases the expression of P-gp (Fig. 2), increases intracellular drug accumulation (Fig. 3), and restores drug sensitivity (Fig. 4) in human MDR cancer cells. We also found that the modulation of MDR results from the siRNA-directed degradation of *MDR1* mRNA (Fig. 1).

P-gp is expressed in many human tumors either at the time of diagnosis or after treatment. Inhibition of the function or expression of P-gp can sensitize MDR cells to chemotherapeutic drugs (3, 21). Although modulations of MDR by pharmacological agents (3), antibodies (22), antisense oligonucleotides (23), and inhibitors of signal transduction (24) have been reported, the clinical benefit of these approaches has not been realized. Gene silencing induced by RNA interference was shown to be specific and potent (16, 25). siRNAs target the expression of the genes from which the siRNA sequences are derived without detectable effects on the expression of unrelated genes (16, 25). In *Caenorhabditis elegans*, only a few molecules of

Table 1 *Effect of siRNA on sensitivity of MDR cells to chemotherapeutic drugs*

Cells were plated in 60-mm cell culture dishes (200 cells/dish) and exposed to various concentrations of cytotoxic drugs for 12 days. At the end of incubation, cells were stained with 1% methylene blue and the colonies counted. IC_{50} is the concentration that produced 50% inhibition of colony formation compared to vehicle-treated controls. Results represent the mean of two or mean ± standard error of three separate experiments.

		IC_{50}			
		Vinblastine (nM)	Doxorubicin (μM)	Paclitaxel (nM)	Hydroxyurea (μM)
MCF-7		0.085	0.002	0.09	157
MCF-7/AdrR	Mock	293 ± 28	0.60 ± 0.06	1450 ± 278	165 ± 7.6
	siRNA	1.26 ± 0.40^a (232)	0.05 ± 0.01^a (12)	15.3 ± 1.1^a (95)	172 ± 3.0 (1)
MCF-7/BC-19	Mock	6.90 ± 0.80	0.03 ± 0.01	21 ± 4.3	
	siRNA	0.14 ± 0.05^a (49)	0.01 ± 0.00^b (3)	1.18 ± 0.42^b (18)	
A2780		0.16	0.01	1.15	
A2780Dx5	Mock	65.3 ± 10.8	0.61 ± 0.14	2233 ± 338	
	siRNA	15.0 ± 2.0^b (4)	0.12 ± 0.03^b (5)	247 ± 37^a (9)	

^{a} $P \leq 0.01$ *versus* mock treatment. Numbers in the parentheses represent fold-reversal.
^{b} $P < 0.05$ *versus* mock treatment.

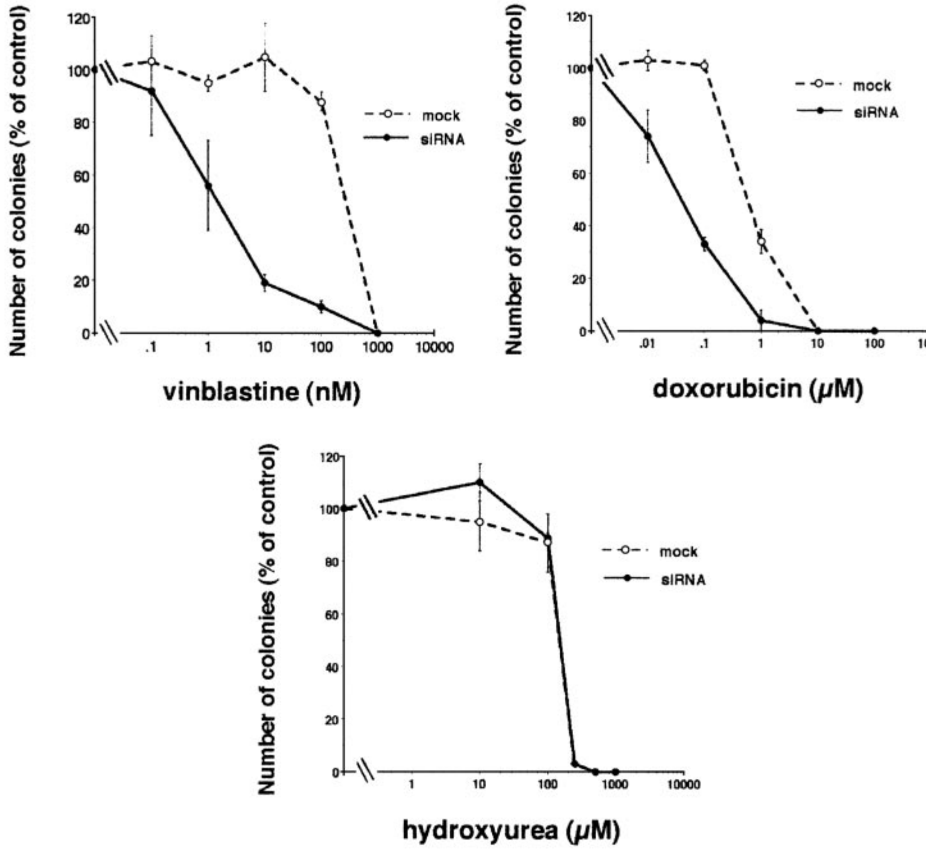

Fig. 4. Effect of siRNA on sensitivity of MDR cancer cells to vinblastine, doxorubicin, and hydroxyurea. *MDR1* siRNA- or mock-treated MCF-7/AdrR cells plated in 60-mm cell culture dishes were incubated at 37°C in a humidified atmosphere containing 5% CO_2/95% air for 12 days in the presence of a series of concentrations of vinblastine, doxorubicin, or hydroxyurea. At the end of incubation, cells were stained with 1% methylene blue in 50% methanol for 30 min, washed with water, and the colony number counted. Results are the representative of two similar experiments; *bars*, ±SD.

siRNA per cell are required for silencing and the spread of the silencing effect through a broad region of the organism (25); a greater number of molecules per cell may be required to acquire the desired result in mammalian cells. Nevertheless, siRNA-induced RNA interference may offer an alternative strategy for overcoming drug resistance.

This report demonstrates the feasibility of using siRNA to specifically and effectively modulate MDR. *MDR1*-targeted siRNA inhibits the expression of *MDR1* RNA and P-gp with minimum effect on GAPDH and tubulin expression in comparison with mock treatment (Fig. 1 and Fig. 2*A*); lamin A/C siRNA decreased lamin A expression but had no effect on the expression of P-gp (Fig. 2*B*). Furthermore, *MDR1*-targeted siRNA reversed resistance to P-gp-transportable drugs, but did not affect the sensitivity to hydroxyurea, a non-P-gp substrate (Table 1; Fig. 4). These data suggest that silencing of P-gp expression mediated by siRNA is specific. Despite using the optimum concentration (200 nM) of siRNA determined by dose-response studies, the maximum inhibition of P-gp expression was 65% (Fig. 2*A*). The lack of complete inhibition is likely because of the high content of P-gp (10–12), the relatively long half-life (14–17 h) of the protein (26), and transfection efficiency. The incomplete inhibition of P-gp expression may explain the incomplete restoration of drug sensitivity in MCF-/AdrR and A2780Dx5 (Table 1), two highly resistant MDR cell lines (10). Furthermore, because MCF-7/AdrR and A2780Dx5 are lines selected by prolonged exposure to doxorubicin, additional mechanisms of drug resistance are known to exist (12, 27). In contrast, siRNA was more effective against the *MDR1*-transfected MCF-7/BC-19 cell line, which is 10–50-fold less resistant than the MCF-7/AdrR line (11). The effect of siRNA on drug resistance was similar to that reported for chemical modulators (3–7).

Similar to several other studies using siRNA (17, 18), the silencing effect on P-gp expression is short-lived. The maximum decreases in *MDR1* mRNA are seen at 24 h, and begin to recover 24 h later. By 72 h, the message had returned to baseline values (data not shown). As anticipated, the recovery of P-gp expression tends to lag behind that of the RNA (Fig. 2*A*). Because the half-life of P-gp is 14–17 h (26), a greater decrease in P-gp expression may be attained through the use of a DNA vector-based siRNA expression system (28–31).

Treatment of MDR cells with P-gp siRNA increases the intracellular accumulation of paclitaxel and doxorubicin, two P-gp substrates (Fig. 3), enhances the sensitivity to doxorubicin, paclitaxel, and vinblastine, but has no effect on the non-P-gp transportable drug, hydroxyurea (Fig. 4; Table 1). Successful delivery of siRNA to postnatal and adult mice by high-pressure tail-vein injection has been reported recently (32, 33). Therefore, studies in animals harboring MDR tumors are warranted as precursors to testing this approach in humans.

In summary, our study demonstrates the effectiveness of siRNA in reversing MDR. Therefore, the RNA interference approach may hold promise for the treatment of drug-resistant cancer.

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