

# Strategies for Inhibition of MDR1 Gene Expression

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## ABSTRACT

Several distinct strategies have been used to modulate the expression of cancer-associated genes, including antisense oligonucleotides, small interfering RNAs (siRNAs), and artificial transcriptional factors. One major cause for chemotherapeutic treatment failure in cancer is the overexpression of P-glycoprotein, the product of the multidrug resistance gene *MDR1*. In this study, we tested the ability of siRNAs to inhibit *MDR1* gene expression. We evaluated the efficiency of chemically synthe-

sized dsRNAs as well as vector-based hairpin siRNAs and investigated the behavior of clones of multidrug-resistant NCI/ADR-RES breast carcinoma cells stably transfected with hairpin siRNA vectors. The effects of siRNA on the MDR phenotype were compared with those elicited by antisense oligonucleotides or by designed transcription factors targeting the *MDR1* promoter. These studies suggest that there are several comparably effective strategies for inhibiting *MDR1* expression.

Selective regulation of the expression of cancer-associated genes represents an important emerging approach in cancer therapeutics. Over the last decade, several technologies have been developed that enable the targeted regulation of specific genes. Thus, antisense oligonucleotides (Bennett, 1998) and ribozymes (Freelove and Zheng, 2002) cause enzymatic degradation of specific mRNAs and pre-mRNAs. Designed transcription factors, usually based on zinc finger libraries, can allow either activation or repression of the transcription of specific genes (Beerli and Barbas, 2002; Falke and Juliano, 2003). Most recently, RNA interference has emerged as a powerful technique for the selective suppression of gene expression in mammalian cells through catalytic degradation of mRNAs triggered by short double-stranded RNAs and mediated by a multiprotein cytoplasmic complex (McManus and Sharp, 2002). All of these strategies have been applied to influence the expression of cancer-associated genes, but there is little information on their relative effectiveness. In this study, we have used three distinct approaches to reduce the expression of the *MDR1* multidrug resistance gene, thus allowing an evaluation of the merits and liabilities of these approaches within a consistent experimental model.

Resistance to multiple anticancer drugs is a serious obstacle to chemotherapy. A common form of multidrug resistance (MDR) is caused by the overexpression of P-glycoprotein, the product of the *MDR1* gene. P-glycoprotein is a 170-kDa transmembrane ATPase that transports a variety of structurally and functionally diverse chemotherapeutic drugs from cells (Ambudkar et al., 1999; Gottesman et al., 2002).

Many small-molecule organic compounds have been shown to reverse the MDR phenotype (Leyland-Jones et al., 1993; Yang et al., 1994); however, these compounds often display nonspecific toxicities that limit their use in the clinic (Dalton et al., 1995). Because of this, investigators have sought to use various types of large molecules to reverse MDR by suppressing P-glycoprotein expression. Thus, several types of antisense oligonucleotides have been used (Alahari et al., 1996, 1998; Fisher et al., 2002; Ramachandran and Wellham, 2003), as have hammerhead ribozymes (Kobayashi et al., 1994; Nagata et al., 2002). Our laboratory has also successfully used designed zinc-finger transcription factors that selectively regulate *MDR1* expression (Xu et al., 2002). *MDR1* transcription is increasingly well understood and may be a favorable locus of regulation (Scotto, 2003).

Small interfering RNAs (siRNAs) of approximately 19–22 bp mediate sequence specific mRNA degradation in mammalian cells (Elbashir et al., 2001a,b). Although transfection of chemically synthesized siRNA can be partially effective, to achieve long-term target gene suppression in cells and whole organisms, RNA polymerase III promoter-driven hairpin siRNA-producing cassettes have been developed and are commonly used in vector-based systems (Lee et al., 2002; Paul et al., 2002; Sui et al., 2002). Recent studies have shown that transient transfection of chemically synthesized siRNA can modulate the MDR phenotype (Nieth et al., 2003; Wu et al., 2003). In this report, we describe several additional aspects of the regulation of *MDR1* by siRNA. Thus, we designed and evaluated siRNAs targeting different sites in *MDR1* transcript, including the 5' and 3' untranslated regions as well as the coding region. We also constructed vectors con-

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**ABBREVIATIONS:** siRNA, small interfering RNA; MDR, multidrug resistance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

taining a U6 promoter-siRNA cassette that expressed various lengths of hairpin MDR1 siRNAs. We examined the effects of the various forms of siRNA in the multidrug resistant NCI/ADR-RES breast cancer cell line. This included the selection of sublines stably expressing anti-MDR1 siRNA hairpins. We then investigated the level of P-glycoprotein expression in both transiently transfected and stably transfected cell lines and the degree of reversal of the MDR phenotype in the stable transfectants. We further compared the effects of siRNA on the MDR phenotype with those evoked by anti-MDR1 antisense oligonucleotides and by a designed transcriptional repressor targeted to the MDR1 promoter.

## Materials and Methods

**Cell Culture and Antibodies.** NCI/ADR-RES human breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in minimal essential medium with L-glutamine (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum. MRK-16 is an anti-cell surface P-glycoprotein antibody (Kamiya Biochemicals, Thousand Oaks, CA), whereas C219 is useful for Western-blotting P-glycoprotein (Signet Labs, Dedham MA). An anti-HLA1 antibody was from Accurate Scientific (Westbury NY), whereas (R)-phycoerythrin-conjugated goat anti-mouse IgG was from Sigma-Aldrich (St. Louis, MO).

**siRNA Design and Sequences.** The siRNA duplexes were designed to target the following regions of MDR1 mRNA: 5' untranslated region at nt 225–245 (5'UTR); coding region at nt 1545–1565 (ORF1); nt 1939–1959 (ORF2); nt 3109–3129 (ORF3); 3'UTR at nt 4438–4458. The sequences are indicated in Table 1. The siRNA duplexes were synthesized by Dharmacon Research, Inc. (Lafayette, CO). A phosphorothioate antisense oligonucleotide (ISIS 5995) bracketing the start codon of the MDR1 message was also used; its sequence was 5'-CCATCCCGACCTCGCGCTCC-3'.

TABLE 1  
siRNA sequences

siRNA		
MDR1	Target Region	Sequence
5' UTR	225–245	5'-GCAGAGGCCGCGUUCGUUdTdT-3' 3'-dTdTTCGUCUCCGGCGACAAGCAA-5'
ORF1	1545–1565	5'-GUAUUGACAGCUAUUCGAAdTdT-3' 3'-dTdTCAUAAACUGUCGAUAGCUU-5'
ORF2	1939–1959	5'-GGAAGCCAAUGCCUUAUGACdTdT-3' 3'-dTdTCCUUCGGUUAACGGAUACUG-5'
ORF3	3109–3129	5'-GAUCGCUACUGAAGCAAUAdTdT-3' 3'-dTdTTCUAGCGAUGACUUCGUUUAU-5'
3'UTR	4438–4458	5'-CAUCAUCAAGUGGAGAGAAdTdT-3' 3'-dTdTGUAGUAGUACACCUCUCUU-5'
EGFP		5'-GGUUUAUGUACAGGAACGCAdTdT-3' 3'-dTdTCCAUAUACAUGUCCUUGCGU-5'
Nischarin		5'-CCUCGUGCACCUCUAGCCUGdTdT-3' 3'-dTdTGGAGCAGCUGGAACUGGAC-5'
Scrambled		5'-GCAUCGCGCACUCACCAAGdTdT-3' 3'-dTdTTCGUAGCGCGUGAGUGGUUC-5'

TABLE 2  
Hairpin MDR1 siRNA inserts based on ORF1

	Sequence	Length of hairpin siRNA
19-mer	5'-GTATTGACAGCTATTTCGAAttcaagagaTTCGAATAGCTGTCAATACTtttttt-3'	19
24-mer	5'-GCCAAGTATTGACAGCTATTTCGAAttcaagagaTTCGAATAGCTGTCAATACTTGGCtttttt-3'	24
25-mer	5'-GTATTGACAGCTATTTCGAAGAGTGGttcaagagaCCACTCTTCGAATAGCTGTCAATACTtttttt-3'	25
28-mer	5'-GTATTGACAGCTATTTCGAAGAGTGGGCAttcaagagaTGCCCACTCTTCGAATAGCTGTCAATACTtttttt-3'	28
28 mismatch	5'-GTATTGACAGCTATTTCGAAGAGTGGGCAttcaagagaTGCCCACTCTTCGAATAGCTGTCAATACTtttttt-3'	

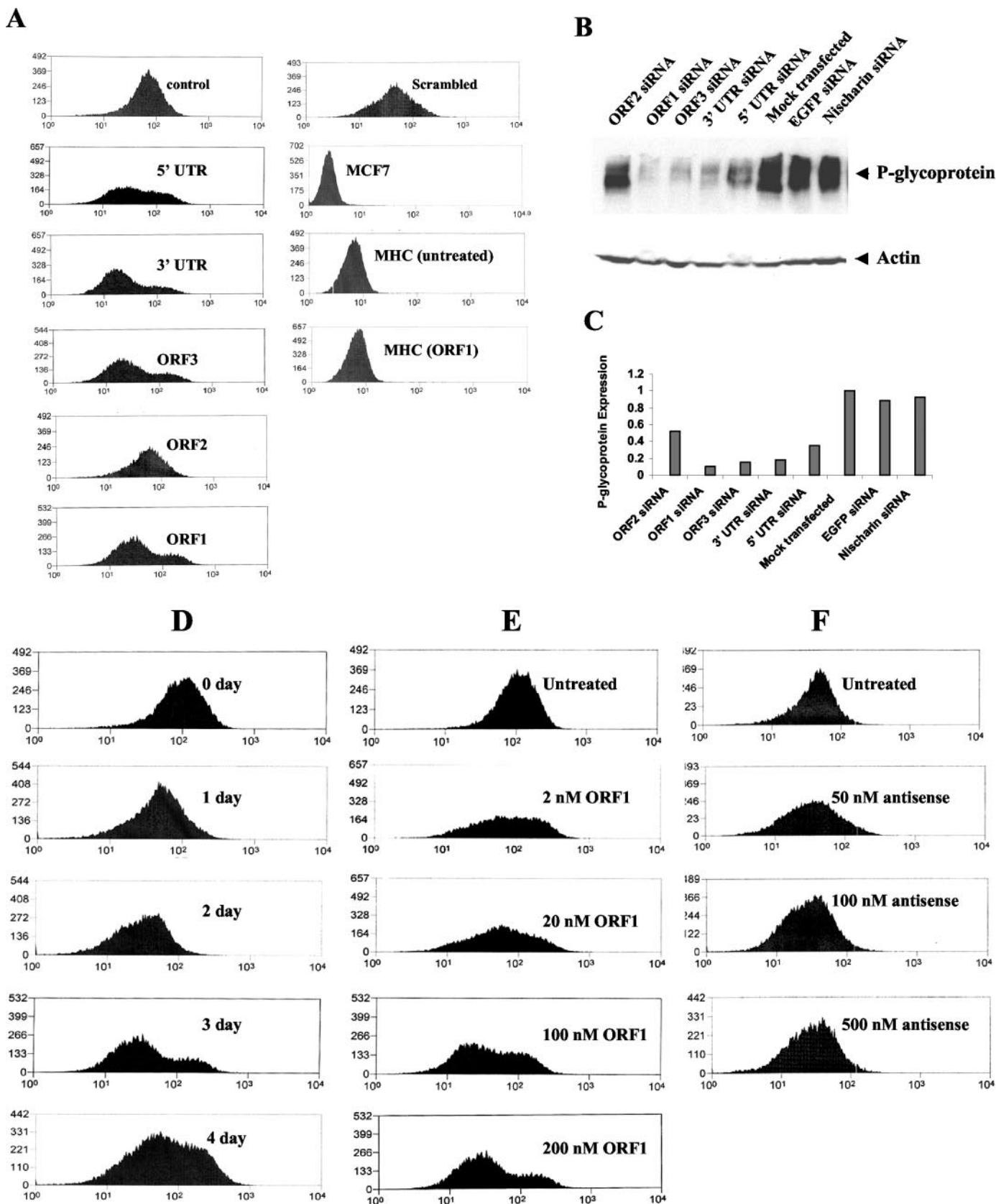
**Plasmid Construction.** DNA oligonucleotides targeting MDR1 mRNA at nt 1545–1565 or its extensions were synthesized and inserted into BamHI-HindIII linearized pSilencer 2.1-U6 hygro vector (Ambion, Austin, TX) according to manufacturer's instructions. These insertions expressed various lengths of hairpin siRNAs based on siRNA ORF1. The sequences of the hairpin siRNAs are indicated in Table 2. All constructs were sequenced to ensure that no mutations were introduced during the cloning.

**Transfection.** Transfection was carried out using LipofectAMINE2000 (Invitrogen) or Superfect (QIAGEN, Valencia, CA) per the manufacturer's instructions. Cells were transfected with the indicated amount of siRNA or siRNA plus plasmid (using LipofectAMINE2000), or with plasmid DNA alone (using Superfect). For cotransfection, the ratio between hairpin siRNA-producing vector and EGFP-expressing or CD4-expressing vector was 10:1 or 1:1.

**Stable Cell Line Production.** NCI/ADR-RES cells were transfected with the indicated pSilencer-siMDR vector or with pSilencer vector as a control. Forty-eight hours after transfection, resistant cells were selected in medium containing 500 µg/ml of hygromycin B. Clones were tested for the presence of integrated U6 promoter-siMDR cassette by PCR with primers flanking the cassette. Ten positive clones were selected for each construct and maintained in the presence of hygromycin B (0.25 µg/ml).

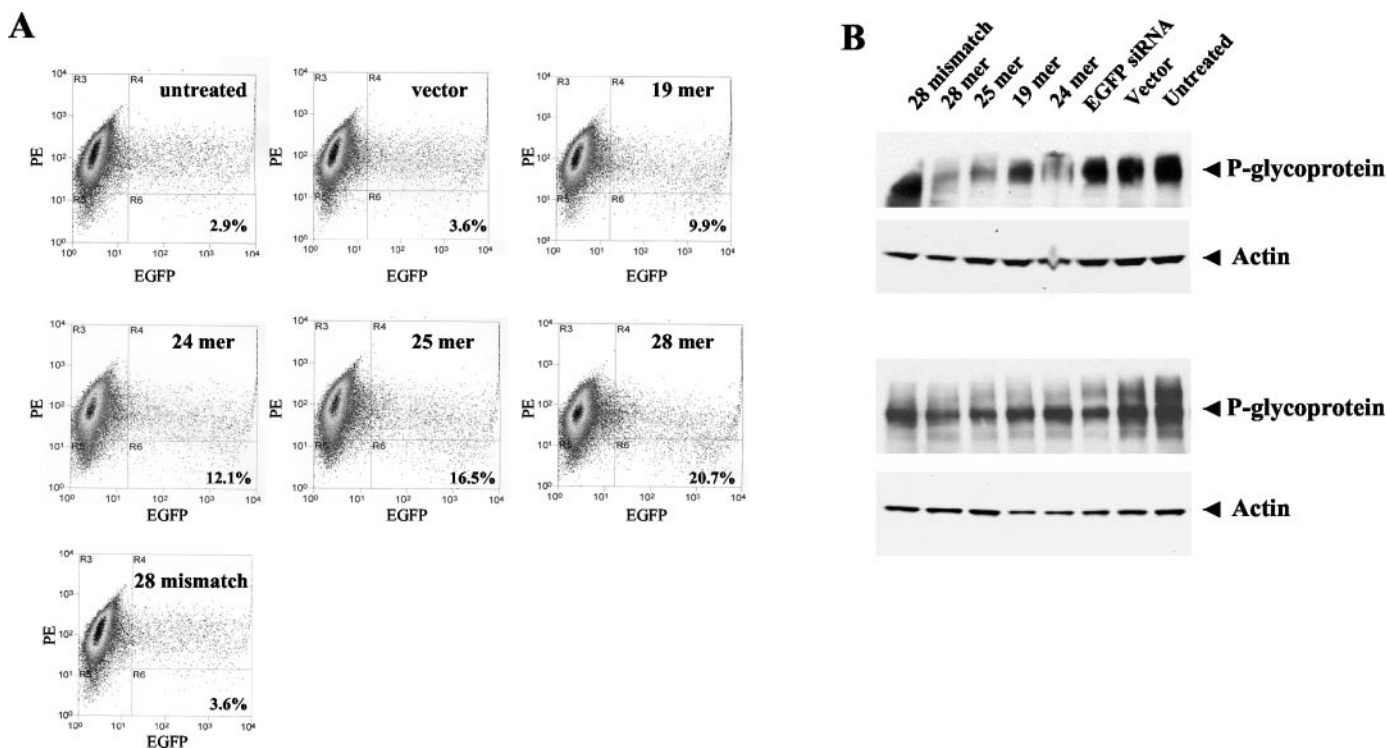
**RNA Extraction and Real-Time RT-PCR.** Total RNA was isolated using an RNeasy kit (QIAGEN), and cDNA was synthesized from total RNA using an oligo-dT primer. Primers (Oligonucleotide Synthesis Core Facility, University of North Carolina) and probes (Integrated DNA Technologies, Santa Clara, CA) were designed using Primer 4 software and were designed to span exon-intron junctions. MDR1 probes were labeled at the 5' end with the reporter dye 5-carboxyfluorescein and at the 3' end with the quencher dye 5-carboxytetramethylrhodamine. The human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was labeled at the 5' end with the reporter dye tetrachloro-6-carboxy-fluorescein and at the 3' end with the quencher dye 5-carboxytetramethylrhodamine. The sequences are as follows: for MDR1: probe, 5'-TCAGTAGCGATCTTCCAGCACCT-3'; sense primer, 5'-GTCTGGACAAGCACTGAAA-3'; antisense primer, 5'-AACACGCTTCGGAAGTTT-3'. For human GAPDH, probe, 5'-CAAGCTTCCCCTTCTCAGCC-3'; sense primer, 5'-ACCTCAACTACATGGTTTAC-3'; antisense primer, 5'-GAAGATGGTGATGGGATTTC-3'. PCR reactions of cDNA samples and standards were performed with the use of Platinum Quantitative PCR SuperMix-UDG (Invitrogen) in a total reaction volume of 15 µl. Real-time PCR was performed using the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA). The PCR conditions were 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 56°C for 1.5 min. Standard curves were constructed with PCR-II TOPO cloning vector (Invitrogen) containing the same fragment as amplified by the Taqman system. The expression in each sample was calculated based on standard curves generated for MDR1 or GAPDH. Samples were normalized by dividing the copies of MDR1 by the copies of human GAPDH.

**CD4 Enrichment.** Seventy-two hours after siRNA cotransfection with CD4-expressing vector, cells were harvested and selected for transfected cells with magnetic anti-CD4 Dynabeads M-450 (DynaL ASA, Oslo, Norway) following the manufacturer's instructions. Transfected cells on Dynabeads were lysed in modified radioimmu-



**Fig. 1.** Inhibition of MDR1 expression by synthesized siRNA. NCI/ADR-RES cells were treated with 200 nM concentrations of siRNAs targeting various positions of the *MDR1* message as indicated, or with one of the following siRNAs as control: scrambled siRNA, EGFP siRNA, Nischarin siRNA. Seventy-two hours later, P-glycoprotein expression was estimated by immunostaining with MRK-16 antibody, (R)-phycoerythrin second antibody, and flow cytometry (A), and by Western blot (B). In A, the abscissa represents the relative fluorescence from the fluorophore-conjugated secondary antibody. The ordinate indicates the number of cells at each level of fluorescence. MCF7 cells are a closely related, non-drug-resistant cell line. The





**Fig. 2.** Inhibition of P-glycoprotein expression by vector-based siRNA. NCI/ADR-RES cells were cotransfected with plasmids coding for the various length of hairpin *MDR1* siRNA based on ORF1 or “empty” vector along with an EGFP-expressing vector (A) or a CD4-expressing vector (B). A. Seventy-two hours after transfection, cells were immunostained, followed by flow cytometry for P-glycoprotein expression and EGFP. The abscissa represents the relative fluorescence from EGFP. The ordinate represents the relative fluorescence from an R-PE-conjugated secondary antibody for the detection of P-glycoprotein. B. Seventy-two hours after transfection, cells were harvested and selected with magnetic anti-CD4 Dynabeads. Both selected (upper) and unselected cells (lower) were lysed in modified RIPA buffer and used in western blots. P-glycoprotein was detected with C219 antibody, and the same membrane was re-probed with anti-actin antibody.

noprecipitation assay buffer, followed by Western blot. Unselected cells were lysed and used as controls.

**Flow Cytometry Measurement of Cell Surface Proteins.** Measurement of cell surface expression of P-glycoprotein or of other membrane proteins was carried out by immunostaining and flow cytometry, as we have described in detail elsewhere (Fisher et al., 2002; Xu et al., 2002).

**Western Blotting.** Detection of P-glycoprotein and of other proteins by Western blotting was carried out as we have described previously (Alahari et al., 1998; Xu et al., 2002).

**Rhodamine 123 Uptake and Cytotoxicity.** Studies of the uptake of the P-glycoprotein substrate Rhodamine 123 were conducted as we have described previously (Xu et al., 2002). Cytotoxicity studies were initiated by plating cells into 24-well plates (Nalge Nunc International, Naperville, IL) at  $5 \times 10^4$  cells/well. Cells were incubated overnight followed by the addition of different amounts (0,

0.1–10  $\mu$ M) of doxorubicin for 24 h. Drug and control medium were then removed and replaced with fresh, drug-free medium and incubated for an additional 48 h. The surviving fraction was determined by the MTT dye assay as described previously (Carmichael et al., 1987).

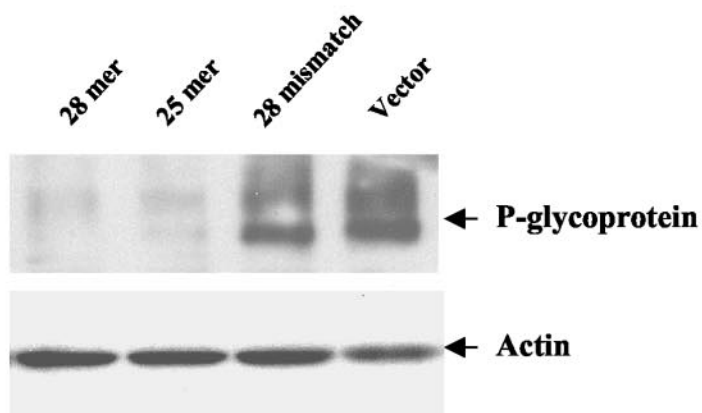
## Results

**Efficiency of Synthesized siRNAs in Reducing *MDR1* Expression.** To assess the efficacy of siRNA targeting different locations of *MDR1* mRNA, we designed siRNA duplexes homologous to loci in the 5'UTR, ORF1, ORF2, ORF3, and 3'UTR regions. Scrambled siRNA and irrelevant siRNAs directed toward EGFP or the protein Nischarin (Alahari et al., 2000) were used as controls. Drug-resistant human breast cancer cells NCI/ADR-RES were treated for 72 h with 200 nM of the above-mentioned siRNAs. Changes in P-glycoprotein expression levels were then detected by flow cytometry and Western blotting. A similar but non-drug-resistant cell line (MCF 7) was used as a negative control for P-glycoprotein. Figure 1, A–C, shows that all the *MDR1* siRNAs partially inhibited *MDR1* gene expression and, consequently, the level of cell surface P-glycoprotein in NCI/ADR-RES cells, whereas the scrambled siRNA and siRNA targeting EGFP or Nischarin did not. The ORF2 siRNA was much less effective than the other *MDR1* siRNAs tested and was not studied further. HLA1, an MHC antigen displayed on the cell surface, was not affected by the *MDR1*-targeted siRNAs; this indicated the specificity of the siRNA effect. There are functional siRNAs from both the untranslated

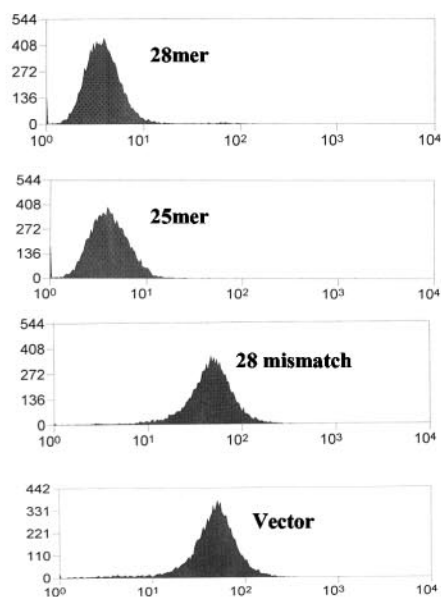
lack of effect of siRNA ORF1 on levels of cell surface MHC antigen (HLA1) is seen in the last two panels on the right. In B, top, P-glycoprotein was detected with C219 antibody. Bottom, the same membrane was re-probed with anti-actin antibody. In C, the ratio of P-glycoprotein to actin is depicted graphically. The Western blots of (B) were scanned with a densitometer [Fluor-S scanner and Quantity One software (Bio-Rad, Hercules, CA)]. The ratios of the actin and P-glycoprotein peaks were calculated and normalized based on the ratio in the mock-transfected control being set equal to 1. NCI/ADR-RES cells were treated with *MDR1* siRNA duplex ORF1 at 200 nM for various times (D), or at different dosages for 3 days (E). P-glycoprotein expression was estimated by immunostaining and flow cytometry. The dose-dependent effect of a phosphorothioate antisense oligonucleotide (5995) on P-glycoprotein expression is seen in F (note that F represents a separate experiment; in this case the immunostaining of P-glycoprotein in the untreated controls was less than that in D or E). The abscissa represents the relative fluorescence from an (R)-phycoerythrin-conjugated secondary antibody. The ordinate represents the number of cells at each level of fluorescence.

## MDR1 Message and Protein Expression

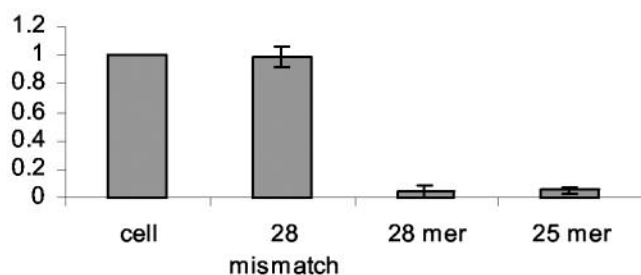
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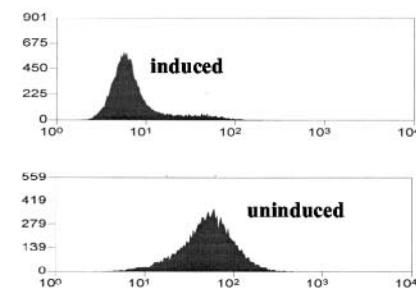
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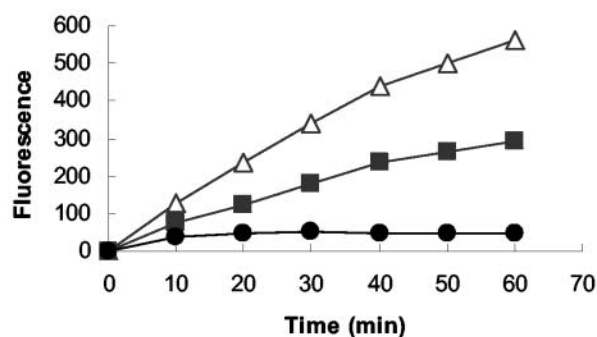


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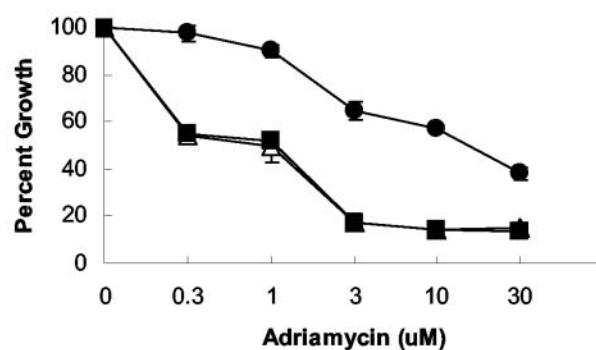


## Drug Uptake and Cytotoxicity

E



F



## Rhodamine 123 Accumulation

## Doxorubicin Toxicity

**Fig. 3.** Inhibition of MDR1 expression by stably transfected hairpin siRNA. Subclones of NCI/ADR-RES were selected to resist hygromycin and contain stably incorporated vectors producing the indicated hairpin siRNA. A, Western blot of P-glycoprotein in stable cell lines. Top, P-glycoprotein was detected with C219 antibody. Bottom, the same membrane was reprobbed with anti-actin antibody. B, flow cytometry of cell surface P-glycoprotein

region and the coding region, although one coding region siRNA (ORF2) was ineffective, which suggests that siRNA effects are dependent on sequence and local structure, as proposed in previous reports (Harborth et al., 2003).

NCI/ADR-RES cells were treated with siRNA duplex ORF1 at different dosages and for various times. The maximum inhibition using 200 nM siRNA was observed after 3 days of treatment (Fig. 1D), consistent with the high expression level of P-glycoprotein in NCI/ADR-RES cells and the long turnover time of P-glycoprotein (Richert et al., 1988). Four days after the treatment, cells began to re-express P-glycoprotein, probably because transiently transfected siRNAs were diluted as cells divided. Figure 1E shows that 200 nM ORF1 siRNA provided the greatest inhibition of P-glycoprotein expression during 72 h of exposure; higher concentrations began to display toxicity. We also compared the potency of the *MDR1* siRNAs to that of a phosphorothioate antisense oligonucleotide (ISIS 5995). This compound had been previously selected from among approximately 20 anti-*MDR1* oligonucleotides for being able to significantly inhibit P-glycoprotein expression (Alahari et al., 1996). As seen in Fig. 1F, the phosphorothioate antisense compound was considerably less potent than the ORF1 siRNA (for example, compare the leftward shifts of the distribution at 100 nM concentrations of each type of oligonucleotide).

**Sustained Inhibition of *MDR1* Expression by Stably Transfected Hairpin siRNAs.** To overcome the limitations of transient transfection, we decided to make stable cell lines expressing siRNAs directed toward *MDR1*. Because the ORF1 siRNA seemed reasonably potent, we chose this as a basis for vector construction. We initially constructed a plasmid that generated a hairpin siRNA version of ORF1 with 19 bases. Considering the possibility of variations in the efficiency of silencing caused by the length of hairpin siRNA (Harborth et al., 2003), we also constructed plasmids producing different lengths of hairpin siRNA with 5- to 9-base pair extensions of ORF1: 24-mer, 25-mer, and 28-mer. We then cotransfected cells with each plasmid, along with an EGFP-expressing vector (Fig. 2A) or a CD4-expressing vector (Fig. 2B), to monitor or select the transfected cells. In Fig. 2A, at 72 h after transfection, the cells were analyzed for EGFP ( $\alpha$ -axis) and cell surface P-glycoprotein ( $\gamma$ -axis) expression levels. Cell populations in the upper right quadrant repre-

sent the cells with a high level of both P-glycoprotein and EGFP, whereas the ones in the lower right quadrant represent the cells with a high level of EGFP but low level of P-glycoprotein. As shown in Fig. 2A, more cells were shifted into the lower right quadrant using the longer hairpin. The percentage of the total population of the two right quadrants present in the lower quadrant was 9.9% (19-mer), 12.1% (24-mer), 16.5% (25-mer), and 20.7% (28-mer). This suggested that the 28-mer hairpin siRNA was the most favorable substrate for intracellular processing and production of siRNAs. An 'empty' vector control as well as a hairpin 28 mismatch (18 G was missing in the antisense strand) had no effect on the expression of *MDR1*.

Western blot of anti-CD4 selected transfected cells showed the same trend (Fig. 2B). At the top, less P-glycoprotein was observed with the cells transfected with longer hairpin siRNA producing plasmids, whereas the  $\beta$ -actin level remained the same. On the bottom, the cells not selected by anti-CD4 had a level of P-glycoprotein similar to that of untreated control cells, indicating that the siRNA-producing vectors largely coexpressed with the CD4 marker vector. It is also worth noting that a much smaller fraction of the total cell population was affected by transient transfection of the siRNA vectors compared with transfection of siRNA oligonucleotides.

We then selected stable cell lines expressing 28-mer, 25-mer, or 28-mer mismatch hairpin siRNAs. Flow cytometry and Western blot analyses showed a dramatic reduction of P-glycoprotein expression in the 28-mer and 25-mer cell lines. Thus, as shown in Fig. 3, A and B, there was approximately a 1-log reduction in P-glycoprotein expression. Real-time RT-PCR (Fig. 3C) showed a comparable degree of reduction in the *MDR1* mRNA level. Stable cell lines expressing pSilencer vector or 28-mer mismatch hairpin siRNA expressed levels of P-glycoprotein similar to untreated drug-resistant cells.

Because we had previously studied the ability of a designed transcription factor to repress *MDR1* gene expression (Xu et al., 2002), we decided to compare the siRNA hairpin vectors to the designed repressor approach. Thus, we used a stable subline of NCI/ADR-RES that displayed ponasterone-regulated expression of a repressor (K25F) targeted to the *MDR1* promoter. As seen in Fig. 3D, induction of the K25F-designed repressor also caused a reduction of approximately 1 log in cell surface P-glycoprotein levels. Thus, in our hands, the designed repressor reduced *MDR1* gene expression to a degree similar to that of the best hairpin siRNA constructs that we have devised.

The strong inhibition of P-glycoprotein expression in pSilencer 28-mer and pSilencer 25-mer stable cell lines led to dramatic increases in drug uptake as assayed by flow cytometry using Rhodamine 123 as a P-glycoprotein substrate (Fig. 3E); by contrast, the drug uptake in the pSilencer 28 mismatch stable cell line remained the same as in the parental cell line (data not shown). Cytotoxicity assays of these cell lines (Fig. 3F) showed that doxorubicin dose-response profiles of pSilencer 28-mer and pSilencer 25-mer clones were significantly shifted leftward, with  $IC_{50}$  values of 1.1 and 1.7  $\mu$ M, respectively, compared with 17  $\mu$ M in the parental cells. Therefore, 28-mer and 25-mer hairpin siRNA led to a substantial reversal of doxorubicin resistance in these cells. Interestingly, although the 28-mer seemed somewhat more

in stable cell lines. The abscissa represents the relative fluorescence from an R-PE-conjugated secondary antibody. The ordinate is the number of cells at each level of fluorescence. C, real-time RT-PCR analysis of *MDR1* mRNA level in stable cell lines. *MDR1* mRNAs from the indicated cell lines were quantified by real-time PCR. Values were normalized with those of GAPDH as described in Methods and expressed as fold change over parental cells. Each experiment was repeated three times. D, inhibition of *MDR1* expression by a designed transcription factor. Shown are the inhibition of P-glycoprotein expression in a NCI/ADR-RES subclone stably transfected with the ponasterone-inducible designed transcriptional factor, K2-5F. E, effects of anti-*MDR1* siRNA on drug accumulation. Selected clones stably transfected with vectors producing 28-mer (open triangles) or 25-mer (solid squares) hairpin *MDR1* siRNA, or parental cells (solid circles) were exposed to 1  $\mu$ g of Rh123 for various times and cell levels of this compound were quantitated by flow cytometry. Repeat experiments gave similar results. F, effects of anti-*MDR1* siRNA on drug toxicity. Selected clones stably transfected with vectors producing 28-mer (open triangles) or 25-mer (solid squares) hairpin *MDR1* siRNA, or parental cells (solid circles) were exposed for 24 h to various concentrations of Adriamycin (doxorubicin). After a further 48 h in complete, drug free, growth medium, cell numbers were determined and expressed as percentage of control.



effective than the 25-mer in the Rhodamine 123 uptake assay, both hairpins seemed approximately equally effective in the doxorubicin toxicity assay; the reason for this is unclear at present.

## Discussion

The advent of RNA interference technology for mammalian cells has elicited a great deal of excitement about its potential use in therapeutic gene regulation. However, the relative merits of siRNA, compared with earlier approaches of gene regulation, remain to be defined. In the experiments presented here, transient transfection of anti-MDR1 siRNA oligomers into multidrug resistant cells provided a moderate degree of inhibition of P-glycoprotein expression using oligomer concentrations in the 100 to 200 nM range; this effect was transient with a duration of about 3 to 4 days. These observations are similar to other recent reports using chemically synthesized anti-MDR1 siRNA oligonucleotides in which similar, relatively modest, effects were observed (Nietz et al., 2003; Wu et al., 2003). Transient transfection using vectors expressing hairpin oligonucleotides with the same siRNA sequence as the chemically synthesized oligomers resulted in even more modest P-glycoprotein suppression in the bulk cell population. Presumably, this is because transfection of a large vector is less efficient than for small oligonucleotides and because the chemically synthesized oligomers begin to act immediately, whereas the hairpin oligonucleotides are gradually synthesized within the cell.

It has been widely assumed that siRNAs are more effective than conventional antisense oligonucleotides, although there are reports that dispute this assumption (Vickers et al., 2003). In the current study, several of the siRNAs tested were clearly more potent than a phosphorothioate anti-MDR1 oligonucleotide that we had used previously (Alahari et al., 1996). However, although phosphorothioates are the most widely used form of antisense, newer chemical modifications provide more stable and potent compounds. Using methoxyethoxy-based (Alahari et al., 1998) or hexitol-based antisense compounds (H. Kang, unpublished observations) we have attained substantial inhibition of MDR1 expression using concentrations of oligonucleotides in the 100 nM range, thus paralleling the siRNA effects seen here. Antisense oligonucleotides depend on the action of RNaseH (Bennett, 1998), whereas siRNAs depend on the RISC complex (McManus and Sharp, 2002). Thus, the relative abundance and activity of these distinct protein complexes is likely to be an important determinant of the relative effectiveness of antisense and siRNA oligonucleotides. However, in many cases, the limiting factor for both antisense and siRNA effects may be the efficiency of intracellular delivery.

The most dramatic inhibition of *MDR1* gene expression was attained in the cell lines stably expressing the hairpin oligonucleotides. Thus, greater than 10-fold reductions in MDR1 message and P-glycoprotein levels were attained, with parallel increases in drug uptake and susceptibility to drug-mediated cell killing. Thus, as might be expected, continuous intracellular production of siRNA produces a degree of gene inhibition not readily attained by transient delivery of identical chemically synthesized siRNA. It is interesting to note that this contrasts with the case of vector-expressed single-strand antisense RNAs, which have largely proven disap-

pointing in terms of their ability to block gene expression (Denhardt, 1992). It also seems worth comparing the effectiveness of the siRNA vectors to previously described vectors that produce a designed repressor of the *MDR1* promoter (Xu et al., 2002). Interestingly expression of the repressor protein was approximately equally effective as stable expression of the siRNA hairpins. Furthermore, the repressor is under tight control of an inducible promoter, whereas effective induction of siRNA expression is still problematic.

The MDR1/P-glycoprotein system is a challenging target for selective gene regulation because the gene is highly amplified, the message is stable and abundant, and the protein is stable. These characteristics provide a stringent testing ground for gene regulation technologies. Although it is impossible to know whether each reagent tested here was fully optimized, they were all designed based on the best available current information. It seems clear that transient transfection of siRNA suffers from some of the same limitations as antisense in that only a portion of the population is transfected and thus only moderate inhibition of expression is seen in the bulk population. The generation of stable siRNA cell lines obviates this problem and allows very effective inhibition of *MDR1* gene expression, similar to that attained in stable cell lines expressing a designed repressor. It is interesting to note, however, that even in the favorable case of stable hairpin siRNA expression, there is not total ablation of *MDR1* gene expression; thus, use of mammalian RNA interference does not equate with the generation of truly null cells, at least in this case. It also remains to be determined whether the strong inhibitory effects seen here in stable hairpin-expressing cell lines can be mimicked by using viral vectors to efficiently deliver the hairpin expression cassette to cells.

The results presented suggest that vectors expressing siRNA and those expressing a designed transcriptional repressor may be comparably effective in inhibiting gene expression. Likewise, although siRNA oligonucleotides are more potent than standard phosphorothioate antisense compounds, newer antisense derivatives may offer effectiveness comparable with that of siRNA.

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