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The Reduction of P-Glycoprotein Expression by Small Interfering RNAs Is Improved in Exponentially Growing Cells

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ABSTRACT

Small interfering RNAs (siRNAs) are powerful tools in specifically silencing gene expression. Nevertheless, their efficiency can be limited when targeting proteins with an unusually long half-life, such as P-glycoprotein (P-gp), which is involved in the multidrug resistance phenomenon. P-gp is characterized by a long half-life, which may vary depending on the cell line and, for some of them, on serum deprivation or high cell density. In the present paper, involvement of an exponential cell growth phase in the improvement of siRNA efficiency has been suggested. The doxorubicin-selected human line MCF7-R was shown to be a more adapted model than NIH-MDR-G185 cells stably transfected with human *mdr1*. Nonspecific effects occurring at moderate (100 nM) siRNA concentration have been shown. Two efficient siRNAs led to a very satisfactory P-gp extinction (only 20% P-gp expression remaining) with siRNA concentration as low as 20 nM.

INTRODUCTION

RNA INTERFERENCE (RNAi) is a sequence-specific posttranscriptional gene silencing (PTGS) process triggered by double-stranded RNAs (dsRNAs) homologous to the silenced genes (for review, see Hammond et al., 2001; Arenz and Schepers, 2003). dsRNAs introduced into cells are processed by the RNase III-like enzyme Dicer to approximately 21–23 nt duplex small interfering RNAs (siRNAs). These siRNAs are incorporated into the multiprotein complex RNA-induced silencing complex (RISC) that recognizes and cleaves the target mRNA. In mammals, dsRNAs longer than 30 bp activate the interferon (IFN) response, leading to nonspecific mRNA degradation. This problem was circumvented by Tuschl's group (Elbashir et al., 2001). They found that siRNAs designed to mimic the products of the Dicer enzyme bypass this nonspecific response when transfected into cultured mammalian cells and trigger RNAi. Silencing of many endogenous genes has been carried out with success (Elbashir et al., 2001; Harborth et al., 2001; Vickers et al., 2003; Holen et al., 2002).

Because major cellular proteins can be efficiently silenced, some authors assume that difficulties in the siRNA strategy are expected only when targeting proteins with unusually long half-lives (Harborth et al., 2001), such as P-glycoprotein (P-gp). P-gp is a transmembrane pump involved in the multidrug resistance (MDR) phenomenon and encoded by the *mdr1* gene (Fojo et al., 1987; Roninson, 1992). P-gp half-life varies from 16 hours (Cohen et al., 1990) to about 72 hours (Richert et al., 1988). It has been shown that the degradation rate of P-gp may vary depending on the cell line and such factors as serum deprivation or high cell density (Muller et al., 1995). *mdr1* overexpression is the main cause of MDR, and selective modulation of the expression of this gene through antisense (Alahari et al., 1996; Bertram et al., 1995; Liu et al., 1996; Brigui et al., 2003) and triple-helix strategies (Labroille et al., 1998; Scaggiante et al., 1994) has been shown. Some first stud-

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ies using siRNAs have been published recently (Wu et al., 2003; Nieth et al., 2003). In the present paper, we examined the efficiency of siRNAs in silencing *mdr1* in two cell lines: NIH-MDR-G185 cells stably transfected with the human *mdr1* gene and the doxorubicin-selected human cell line MCF7-R. Both cell lines overexpressed P-gp. We hypothesized that the efficiency of siRNA inhibition of P-gp would be improved during the exponential phase of cell growth. As a consequence, we proposed that cells with a long cycle duration are a better adapted model, as it is possible to favor their exponential growth while minimizing siRNA dilution effects.

MATERIALS AND METHODS

Cell culture

NIH-3T3 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). NIH-MDR-G185 cells stably transfected with a plasmid containing the human *mdr1* gene (pSK1 MDR) were a gift from M. Gottesman (Bethesda, MD) (Kane et al., 1989). MCF7-S (the parental human mammary adenocarcinoma cell line) and MCF7-R (the doxorubicin-resistant line) were a gift from M.F. Poupon (Paris, France) (Raynaud et al., 1999). Cells were grown in DMEM (RPMI for MCF7 cells) medium supplemented with 10% decomplemented fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine in a 5% CO_2 atmosphere. In order to maintain P-gp expression, the medium of NIH-MDR-G185 cells was supplemented with 60 ng/ml colchicine.

siRNAs and antisense oligonucleotide

RNAs (21 nt) were synthesized and PAGE purified by Eurogentec (Seraing, Belgium). They were solubilized in TE buffer (10 mM Tris, 1 mM EDTA in diethyl pyrocarbonate [DEPC]-treated water), pH 7.8. The duplexes were formed according to the supplier protocol. Single strands (20 μM) were incubated in annealing buffer (50 mM Tris, pH 7.5–8.0, 100 mM NaCl in DEPC-treated water) for 2 minutes at 95°C, then slowly cooled down to ambient temperature. Duplex formation was confirmed by electrophoresis (SYBR gold nucleic acid gel stain [Molecular Probes, Eugene, OR] detection). si1 was an siRNA targeting the human *mdr1* mRNA (Chen et al., 1990) at the level of the region 88–108 relative to the start codon (antisense, 5′-UACACUGACAGUUGGUUUCdTdT; sense, 5′-GAAACCAACUGUCAGUGUAdTdT). si2 was directed toward the region 162–182 (antisense, 5′-UGGAUGAUGGCAGCCAAAGdTdT; sense, 5′-CUUUGGCUGCCAUCAUCCAdTdT). si3 was a control without target on *mdr1* (antisense, 5′-UACGGUGUCAAUUCGUAUCdTdT; sense, 5′ GAUACGAAUUGACACCGU-AdTdT). An all-phosphorothioate antisense (AS) oligonucleotide, 5′-d(CCATCCCGACCTCGCGCTCC), was directed toward the region of the start codon (−16–+3) (Alahari et al., 1996; Brigui et al., 2003). An siRNA directed toward the same region was also checked (antisense, 5′ UCCAUCCCGACCUCGCGCUdTdT; sense, 5′ AGCGCGAGGUCGGGAUGGAdTdT). In fluorescence imaging experiments, the sense strand was 5′-d(AAAGCGCGAGGTCGGGATGGA)**F**, protected by two phosphorothioate residues at its 5′-end and labeled by a fluorescein at its 3′-end. A BLAST search (NCBI database) was carried out to check that the only target of si1 and si2 was on *mdr1*, that they had no target in the mouse genome (NIH3T3 cells), and that si3 had no target at all.

Transfection

Transfection with Oligofectamine (Invitrogen, San Diego, CA) was carried out as directed by the manufacturer with the modifications proposed by Elbashir et al. (2002). Cells (3×10^5/well) were plated in 6-well plates. They were transfected at 30% confluence (exponential phase). The final concentration in siRNAs was 100 nM (Elbashir et al., 2002) or 20 nM; that of AS was 100 nM or 500 nM. Transfection was performed in OPTI-MEM1 (Invitrogen) without serum or antibiotics in a final volume of 2.5 ml. After 4 hours incubation at 37°C, FBS (10% final concentration), 50 U/ml penicillin, and 50 μg/ml streptomycin were added. In experiments carried out under limited serum conditions, this medium was replaced by OPTI-MEM1 supplemented with 2% FBS and antibiotics after an additional 2 hours incubation.

Cell lysis

Cells were lysed 48 hours or 72 hours after transfection. Nontreated NIH-MDR-G185 (MCF7-R) cells and NIH-3T3 (MCF7-S) cells were used as a reference of resistant or sensitive cells. Cells were trypsinized, washed in phosphate-buffered saline (PBS), counted, and resuspended in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet-P40, 0.5% deoxycholate) containing 5 mM EDTA and protease inhibitors at the ratio of 100 μl buffer for 3×10^6 cells. After 30 minutes on ice with some vortexing, the lysates were centrifuged at 13,000*g* for 20 minutes at 4°C. Protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, CA).

Western blotting

Equal amounts of proteins (20 μg) were mixed with SDS reducing buffer. Protein samples were separated on 7.5% acrylamide SDS-PAGE, then transferred onto PVDF membrane (Hybond-P, Amersham Pharmacia Biotech, Orsay, France). The membrane was blocked with 5% nonfat dry milk in 0.1% Tween-PBS and treated with

0.65 μg/ml C219 monoclonal anti-P-gp antibody (Dako, Carpinteria, CA) or 2 μg/ml AC-74 monoclonal anti-β-actin antibody (Sigma, St. Louis, MO). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL plus, Amersham Pharmacia Biotech). P-gp expression was quantified by NIH Image software. The error has been evaluated to 5%. All experiments were performed at least in duplicate, and three Western blots were performed for each cell lysis preparation.

RT-PCR

Forty-eight or seventy-two hours after transfection, total RNA was extracted (RNeasy kit, Qiagen, Chatsworth, CA), and cDNA was synthesized using a one-step RT-PCR kit (Qiagen). Amplification was kept in its exponential phase (22 cycles). The primers used in the case of *mdr1* amplification were: sense, 5′-d(TCTTGAAGGGC-CTGAACCTG); reverse, 5′-d(AGTCATAGGCATTG-GCTTCC). The products obtained from PCR amplification were separated on 1.8% agarose gel stained with ethidium bromide.

siRNA uptake by fluorescence imaging

After the 4 hours incubation at 37°C without serum of a fluorescently labeled double-stranded (ds) oligonucleotide internalized with Oligofectamine, NIH-MDR-G185 cells were observed by fluorescence imaging to verify the efficiency of transfection.

RESULTS

Figure 1 shows the efficiency of two siRNAs (si1 and si2) directed against *mdr1* in MCF7-R and NIH-MDR-G185 cell lines growing under limited serum conditions (2%). si3 was a control siRNA, and AS was an antisense directed toward the region of the start codon. The siRNAs and AS concentrations were 100 nM (Elbashir et al., 2002). Such limited serum conditions (2% instead of 10%, as in a regular cell culture) were chosen in order to reduce cellular division and the resulting dilution or absence of oligonucleotides in the daughter cells. In a previous work (Brigui et al., 2003), an almost complete P-gp extinction was obtained in NIH-MDR-G185 after treatment with the all-phosphorothioate antisense oligonucleotide (AS) (1 μM) in a 2% serum medium. This is no longer the case when using siRNAs (Western blots in Fig. 1A). With MCF7-R cells, a 72-hour treatment was necessary to get the best silencing. A histogram showing the mean of the results of several experiments is shown (Figure 1B). si1 and si2 had the same efficiency (about 40% P-gp remaining expression). The control si3 was without effect; there was no detectable P-gp expression in parental MCF7-S cells (data not shown). In contrast to silencing in MCF7-R cells, silencing in NIH-MDR-G185 cells growing in limited serum conditions sometimes required a 48-hour treatment and sometimes a 72-hour treatment. Figure 1C shows the results of three different experiments. As in MCF7-R cells, the best silencing efficiency resulted in approximately 40% remaining P-gp expression but si1 had a greater effect than si2 (Fig. 1C, 45% remaining P-gp expression vs. about 60% in a; about 60% vs. 80% in b; and 50% vs. 70% in c). Depending on the experiment, the efficiency of siRNAs in NIH-MDR-G185 cells was more or less significant (45% remaining P-gp expression with si1 in Fig. 1Ca or about 60% after 72 hours in b), more or less fast (about 50% P-gp remaining expression at 48 hours in c with si1 or only after 72 hours in a). Si3 and AS (100 nM as for siRNAs) had no significant effect. The siRNA directed toward the initiation codon was ineffective; there was no detectable P-gp expression in parental cells (data not shown).

A possible correlation between silencing and the manner of cell growth was examined by counting the cells before cell lysis (Fig. 1C, inset). Each improvement in silencing was directly preceded by a fast growth phase. When the number of cells increased 2.5 times between 48 and 72 hours after transfection (Fig. 1C, inset, a), the remaining P-gp expression after treatment with si1 decreased from 69% to 45% during the same period. When the number of cells increased only very slightly (Fig. 1C, inset, b), there was less improvement in P-gp reversal (decrease from 79% to 63%). Some cells had grown particularly fast (Fig. 1C, inset, c, same number of cells at 48 hours as usually found at 72 hours), and a 51% remaining expression was obtained as early as 48 hours.

siRNA efficiency was greatly improved when cells grew in a regular 10% serum medium (Fig. 2). MCF7-R and NIH-3T3-G185 cells differ by the duration of their cell cycle. We measured (Fig. 2, inset) a cell cycle of about 48 hours for MCF7-R cells and only about 24 hours for NIH-MDR-G185 cells. These values are comparable to previously published results (Marin et al., 2004; Hitomi and Stacey, 1999). siRNA efficiency was examined at siRNA concentrations of 100 nM (Fig. 2A) and 20 nM (Fig. 2B). After treatment with 100 nM si1, only about 10% P-gp remaining expression was observed in MCF7-R cells vs. 20% in NIH-3T3-G185 cells. (Fig. 3 summarizes, for each cell line, the smallest remaining P-gp expression values as a function of serum and siRNA concentration, whatever the necessary duration of treatment.) si2 demonstrated a lower efficiency, about 30% remaining P-gp expression in MCF7-R cells vs. 50% in NIH-3T3-G185 cells. A 72-hour treatment was once more necessary for MCF7-R cells, whereas a 48 or 72-hour treatment led to comparable results in the case of NIH-3T3-G185 cells (Fig. 2A).

With 20 nM siRNA concentration (Fig. 2B), a 72-hour treatment led to a very efficient decrease in P-gp expression in MCF7-R cells (about 20% remaining P-gp ex-

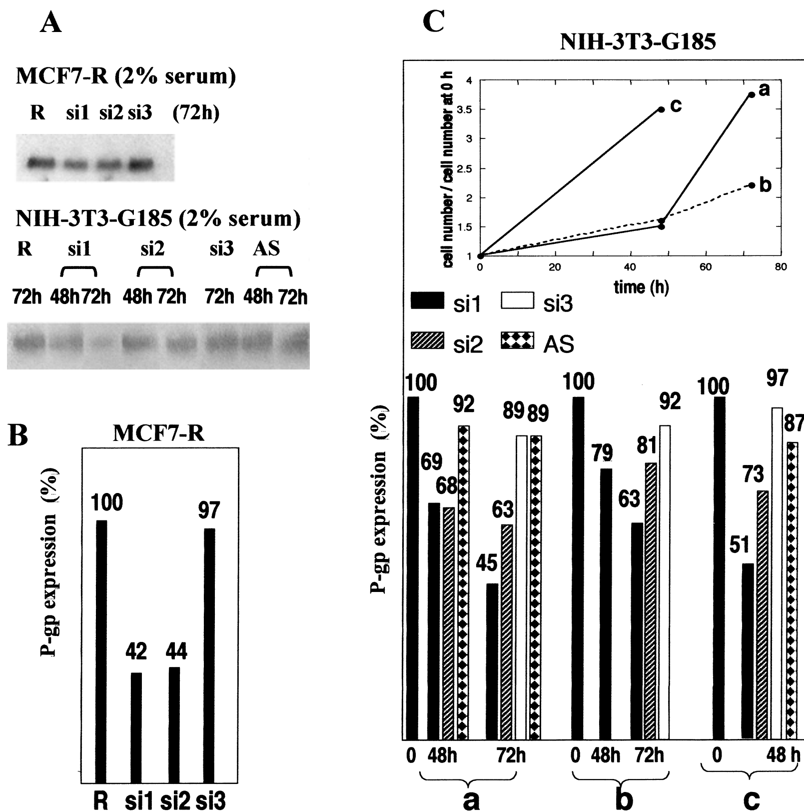

FIG. 1. P-gp reduced expression after siRNA treatment in a 2% serum medium. (**A**) P-gp detection by Western blotting in MCF7-R or NIH-MDR-G185 cells without treatment (R) or treated with si1, si2, or si3, or AS for 48 or 72 hours (siRNA = AS = 100 nM). (**B**) MCF7-R cells. The histogram presents a mean of the results of several experiments. (**C**) NIH-3T3-G185 cells. The histogram shows a set of three experiments. (Inset) Cell growth as a function of time.

pression with si1 and si2 (Fig. 3). It is noticeable that 500 nM AS led to silencing (about 30% remaining P-gp expression), comparable to results obtained with as low as 20 nM siRNA. In NIH-3T3-G185 cells, about 40% remaining expression was observed after si1 or si2 treatment, sometimes after a 48-hour (Fig. 2B,a) and sometimes after a 72-hour (Fig. 2B,b) treatment. Whereas si1 was more efficient than si2 in decreasing P-gp expression when used at 100 nM in regular 10% serum medium, both siRNAs had comparable efficiencies at 20 nM (Fig. 3). si3 was inefficient and even seemed to lead to some increase in P-gp expression when used at 100 nM in a regular cell medium. siRNA treatment did not affect the β-actin expression in NIH-3T3-G185 cells (Fig. 2B,b). The decrease in *mdr1* mRNA after siRNA treatment was checked at 48 hours and 72 hours (RT-PCR). After treatment with si1 and si2, a 50% decrease in the amount of *mdr1* mRNA was observed at 48 hours compared with nontreated or si3-treated cells (Fig. 2B,b). β-Actin encoding mRNA was not affected. The initial level of *mdr1* mRNA was restored at 72 hours. The 24-hour gap between reduction of mRNA and reduction of protein resulted from the P-gp long half-life and was described previously (Nieth et al., 2003).

The inset in Figure 3 shows the incorporation of a ds oligonucleotide (labeled with fluorescein) after transfection with Oligofectamine. Fluorescence imaging has been recorded at the end of the 4-hour incubation period in the absence of serum. This shows that according to the manufacturer, this incubation period allowed the internalization of ds oligonucleotides complexed to Oligofectamine.

DISCUSSION

In this study, the efficiency of siRNAs directed against *mdr1* coding to the long half-life protein P-gp was examined in two cell lines overexpressing P-gp (NIH-MDR-G185 stably transfected with human *mdr1* and a doxorubicin-selected human line MCF7-R). Difficulties in the siRNA strategy were expected when targeting proteins with such unusually long half-lives (Harborth et al., 2001).

Our results showed a very high efficiency of siRNAs against *mdr1*. This efficiency was dependent on cell line (better in MCF7-R cells than in NIH-3T3-G185 cells) and on cell growth conditions (10% serum preferable to 2% serum). A 2% serum medium was *a priori* assumed to allow improved silencing to be obtained by lowering

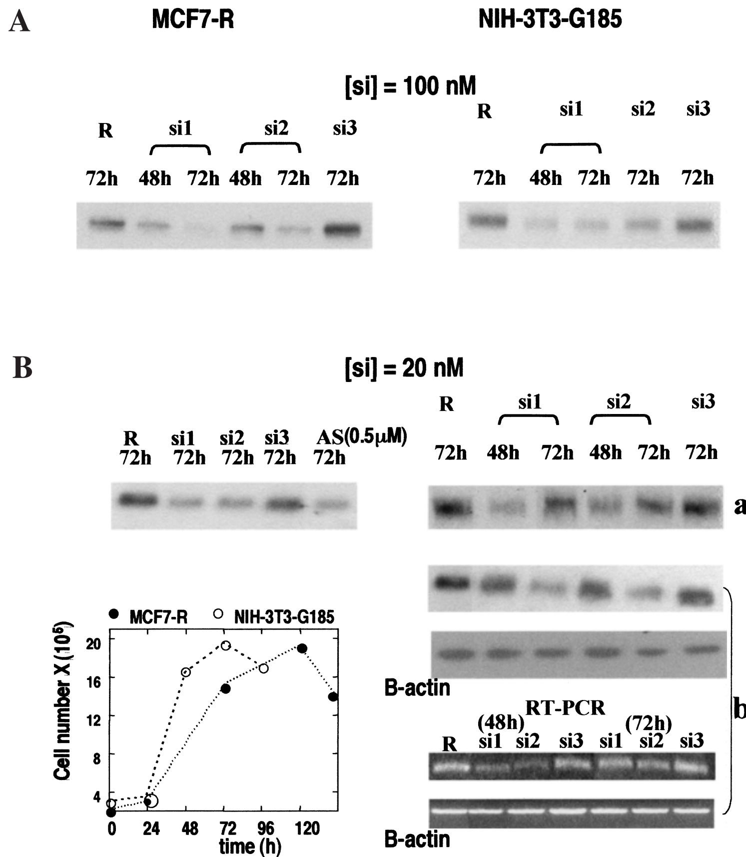

FIG. 2. P-gp reduced expression after siRNA treatment in a 10% serum medium. (**A**) P-gp detection by Western blotting in MCF7-R and NIH-MDR-G185 cells without treatment (R) or treated with si1, si2, or si3 for 48 or 72 hours (siRNA = 100 nM). (**B**) The same treatment as in **A** with siRNA = 20 nM, AS = 500 nM. *mdr1* mRNA was quantified by RT-PCR. β-Actin was a control for Western blotting and RT-PCR. (Inset) MCF7-R and NIH-MDR-G185 cell growth in 10% serum medium.

cellular division and the resulting dilution or even absence of siRNAs inside the daughter cells. Nevertheless, this was not the case, and silencing was more efficient in a regular 10% serum medium. Difference in siRNA efficiency between 2% and 10% serum media cannot be assigned to differences in cell penetration of siRNAs. It was shown (fluorescence imaging) that siRNA uptake in cells occurred during the first 4 hours of incubation in a medium devoid of serum, that is, before the serum concentration was adjusted to 2% or 10%.

Outstanding extinction (Fig. 3) was obtained in MCF7-R cells growing in a 10% serum medium (about 10% P-gp remaining expression at 100 nM si1 and 20% at 20 nM). This latter value was comparable to that obtained with 500 nM AS (30% P-gp remaining expression). Two first studies of siRNA-directed *mdr1* silencing were published recently (Wu et al., 2003; Nieth et al., 2003). They were carried out in regular culture media, respectively, at 200 nM and 100 nM siRNA. Both groups used an siRNA directed against the same *mdr1* mRNA region as si1. The 60% P-gp extinction obtained by Wu et al. (2003) on MCF7-R cells was inferior to the results obtained here in a 10% serum medium at 100 nM or even 20 nM siRNA. They were comparable to those obtained in a 2% culture medium (Fig. 3). In human pancreatic carcinoma cells, Nieth et al. (2003) observed a nearly complete P-gp extinction, which is comparable to the results presented here in the case of MCF7-R cells growing in a regular 10% culture cell medium and treated with 100 nM siRNA. In gastric carcinoma cells (Nieth et al., 2003), the siRNA was slightly less efficient and led to results comparable to those obtained in the present study when MCF7-R cells were treated with as low as 20 nM si1 or when NIH-3T3-G185 cells were treated with 100 nM si1.

Variation in the duration of treatment necessary to obtain the best silencing could be seen. Nieth et al. (2003) observed a peak of protein reduction only after 3–5 days, whereas Wu et al. (2003) obtained the best silencing after a 24-hour treatment (MCF7-R cells) and showed extinction decrease for further incubation times. In the present paper, it was shown that a 72-hour treatment was always necessary in the case of MCF7-R cells, whatever the cell growth medium and the siRNA concentration. For NIH-3T3-G185 cells, best results were obtained after only a 48-hour treatment or after a 72-hour treatment, apparently without relation to cell growth medium or siRNA amount.

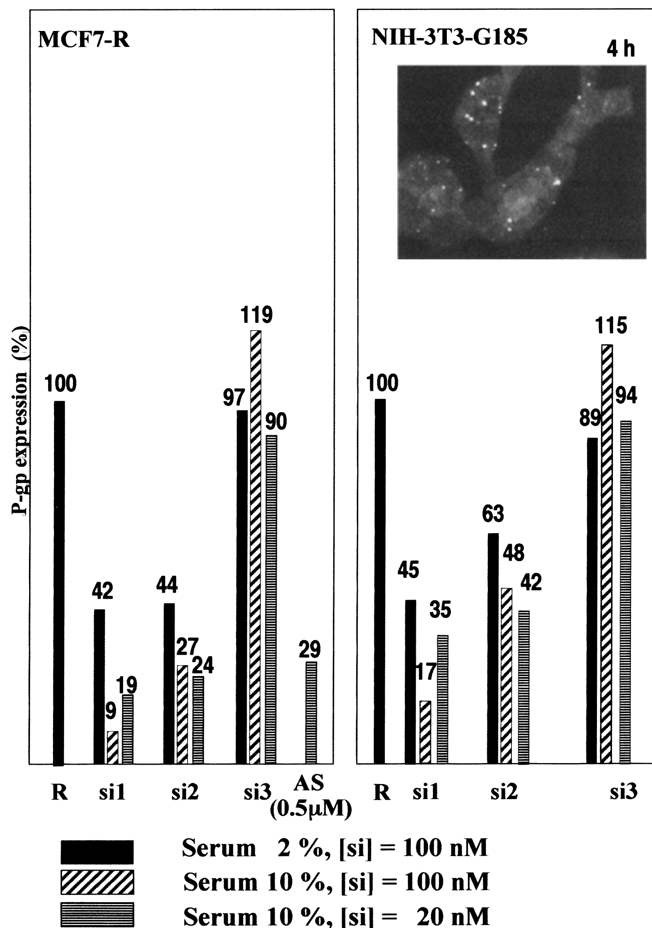

FIG. 3. P-gp reduced expression after siRNA or AS treatment (MCF7-R and NIH-MDR-G185 cells). The histograms shows a mean of the results obtained by Western blotting. Serum and oligonucleotide concentrations were as specified. The duration of treatment was that which allowed best results to be obtained. (Inset) Transfection with Oligofectamine of a fluorescent ds oligonucleotide (4 hours incubation).

Because the cell cycle duration is about 48 hours for MCF7-R cells and only 24 hours for NIH-MDR-G185 cells, dilution effects were minimum for MCF7-R cells during the time of the experiment (72 hour) compared with NIH-3T3-G185 cells. In addition, the degradation rate of P-gp has been shown to depend on the cell line (Muller et al., 1995). In some strains, particularly of epithelial origin (e.g., MCF7 cells), it has been shown that the P-gp half-life in exponentially growing cells is only 14–17 hours but dramatically increases (72 hours) in conditions of serum deprivation or high cell density (Muller et al., 1995). NIH3T3 cells are also sensitive to serum conditions. (To be synchronized, these cells are arrested in G_0 by culture in 0.5% serum medium for 48 hours, then released into the cell cycling by the addition of serum to a final concentration of 10%) (Zerfass et al., 1995). We can thus assume that the variation in siRNA efficiency directed toward *mdr1* results, on one hand, from dilution effects due to cellular division and, on the other hand, from P-gp half-life differences according to cell growth phase.

This explains the improved silencing obtained in 10% serum with MCF7-R cells compared with NIH-3T3-G185 cells. The positive effect due to the relatively short P-gp half-life (14–17 hours) (Muller et al., 1995) during exponential growth of cells was not counterbalanced by cell division. On the contrary, cell division led to dilution of the siRNA effect in the case of NIH-3T3-G185 cells. When growing in a 2% serum medium, MCF7-R cells left the exponential growth phase, and P-gp half-life was increased, leading to less efficient silencing. In more drastic serum deprivation conditions (0.5% serum instead of 2%), the P-gp half-life would tend toward 72 hours (Muller et al., 1995). In the case of NIH-MDR-G185 cells (cell cycle of 24 hours), exponential growth (shorter P-gp half-life in favor of the siRNA efficiency) and cell division (dilution of the siRNA efficiency) led to opposite effects. We assume this is the cause of the variability

in the duration of treatment necessary to obtain the best silencing. In a 2% serum medium, both effects are reduced (longer P-gp half-life but also less dilution), but they are still of comparable potencies, leading to enhanced P-gp remaining expression but maintaining uncertainty about the duration of treatment. Correlation between silencing and the manner of cell growth had been established. It was shown that growth of NIH-MDR-G185 cells in a 2% medium was not steady but consisted of periods of cell doubling and periods of slight increase in cell numbers. Each improvement of P-gp silencing was directly preceded by a fast growth phase (see Results). This could be an indication that the parameter, exponential growth, is more important than the parameter, dilution.

Consequently, in such a specific case, several parameters are able to increase or decrease the siRNA efficiency. It is preferable to avoid parameters depending on the cell line and to keep only those due to the protein to be silenced itself. Consequently, NIH-MDR-G185 cells do not appear as an adapted model for this study.

Increased efficiency of si1 compared with si2 was expected because of its better agreement with Tuschl's rules (Elbashir et al., 2002), according to which the region recognized by an siRNA should be preferably located 50–100 nt downstream of the start codon of mRNA. This was the case for si1, whereas si2 was directed against a region slightly further downstream (162–182 nt). (The siRNA recognizing the same region as AS, i.e., the start codon region, was without effect, as expected.) Nevertheless, it was only at 100 nM (in 10% serum for both cell lines and in 2% serum for NIH-MDR-G185 cells) (Fig. 3) that si1 led to a better silencing than si2. The efficiencies of si1 and si2 were the same at 20 nM. As it has already been shown that siRNAs that are efficient at 100 nM are still active at 20 nM (Semizarov et al., 2003) and that nonspecific effects can occur with as low as 100 nM siRNA concentration, si1 and si2 can both be considered as efficient siRNAs. Therefore, comparison of siRNA efficiency should be done only at low siRNA concentration (20 nM). Nonspecific effects occurring at 100 nM could have produced the increase in remaining P-gp expression in cells treated with 100 nM si3.

The discrepancy between our results and those of Wu et al. (2003) about the necessary duration of treatment in the case of MCF7-R cells could, thus, be due to nonspecific effects. These authors used as much as 200 nM siRNA. Such nonspecific effects could have led to an apparently faster efficiency and to the absence of a 24-hour gap between reduction in *mdr1* mRNA and reduction in P-gp in Wu's study (2003).

In conclusion, the siRNA strategy is efficient in silencing a protein with an unusually long half-life, such as P-gp. This efficiency is improved in some strains at the time of growth in an exponential phase, in the course of which P-gp half-life is minimized. It is also possible that such exponential growth phases allow the optimal amount of proteins necessary to form the RISC complex to occur, reinforcing the efficiency of the system. This improved efficiency will be more obvious because of the long duration of the cell cycle.

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