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RESEARCH PAPER

siRNA-Mediated Down-Regulation of P-glycoprotein in a Xenograft Tumor Model in NOD-SCID Mice

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ABSTRACT

Purpose The efficacy of chemotherapy is decreased due to over-expression of the drug transporter P-glycoprotein (P-gp). This study was conducted to determine the feasibility of down-regulating tumor P-gp levels with non-viral siRNA delivery in order to sensitize the tumors to drug therapy.

Methods P-gp over-expressing MDA435/LCC6 MDR1 cells were used to establish xenografts in NOD-SCID mouse. Cationic polymers polyethylenimine (PEI) and stearic acid-substituted poly-L-lysine (PLL-StA) were formulated with P-gp-specific siRNAs and delivered intratumorally to explore the feasibility of P-gp down-regulation in tumors. Intravenous Doxil™ was administered to investigate tumor growth.

Results PEI and PLL-StA effectively delivered siRNA to MDA435/LCC6 MDR1 cells *in vitro* to reduce P-gp expression for 3 days. Intratumoral injection of siRNA with the carriers resulted in 60-80% and 20–32% of siRNA retention in tumors after 24 and 96 hr, respectively. This led to ~29.0% and ~61.5% P-gp down-regulation with PEI- and PLL-StA-mediated siRNA delivery, respectively. The P-gp down-regulation by intratumoral siRNA injection led to better response to systemic Doxil™ treatment, resulting in slowed tumor growth in originally doxorubicin-resistant tumors.

Conclusion Effective P-gp down-regulation was feasible with polymeric siRNA delivery in a xenograft model, resulting in an enhanced response to the drug therapy.

KEY WORDS multidrug resistance · non-viral siRNA delivery · P-glycoprotein · polymeric biomaterials · xenograft

ABBREVIATIONS

DOX	doxorubicin
HBSS	Hank's Balanced Salt Solution
MDR	multi-drug resistance
MDR1	multi-drug resistance gene 1 expressing cells
NOD-SCID	non-obese/severe combined immunodeficient
PEI	polyethylenimine
P-gp	P-glycoprotein
PLL-StA	stearic acid substituted poly-L-lysine
siRNA	short interfering RNA
WT	wild-type cells

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INTRODUCTION

Multidrug resistance (MDR) displayed by tumor cells against hydrophobic drugs is among the major factors limiting the efficiency of chemotherapy in various types of cancers (1). MDR is defined as simultaneous resistance to different drugs with different targets and chemical structures (2). MDR is consistently detected in tissues undergoing chemotherapy in several types of cancers (3,4). The MDR often arises from elevated presence of ATP cassette transporter (ABC) proteins on cell surfaces that act as an ATP-dependent efflux pump. The expression of the efflux pumps is up-regulated in tumor cells undergoing chemotherapy (5), resulting in increased efflux of hydrophobic chemotherapeutic drugs out of the cells. Among these transporters, P-glycoprotein (P-gp) is believed to be the most common protein causing MDR (6,7). P-gp is normally present at the apical surface of epithelium lining the colon, small intestine, bile ductules, and kidney proximal tubules, where it secretes xenobiotics and metabolites into bile, urine, and the lumen of gastrointestinal tracks. It is also present in the endothelial cells of the blood-brain barrier, blood-testis barrier, and blood-ear barrier, where it protects these sensitive organs from toxic xenobiotics (8). Chemical P-gp inhibitors have been the choice for inhibiting P-gp activity for more than 30 years; however, they produce undesirable off-target activities, leading to unacceptable toxicities, and they have yielded limited clinical success (9,10).

Small interfering RNAs (siRNAs) have the potential to suppress the expression of any specific gene at the RNA level and prevent expression of proteins that lead to mortal diseases such as cancer (11). To efficiently serve as a cancer therapy, specific siRNA sequences need to be delivered against 'aberrant' mRNA molecules in tumor cells, facilitating the degradation of the targeted mRNA and resulting in sustained suppression of undesired gene(s). *In vitro* suppression of P-gp using various siRNA delivery and siRNA expression systems has been relatively successful (12–17), and, in some cases (i.e., under selection pressures), complete knockdown of target genes has been feasible (18,19). However, translation of these results to animal models has been challenging, since additional limitations exist in *in vivo* siRNA delivery. Systemic delivery of siRNA can lead to undesired side effects, caused by non-specific siRNA activity on mRNA sequences with partial complementary sequences (20). Localized injection, on the other hand, could limit the diffusion of the injected siRNA into non-specific sites and is therefore beneficial for topical tumors (21). Another limitation is the rapid siRNA degradation in the physiological milieu (22). Cationic carriers have been developed for non-viral siRNA delivery by complexing with siRNA via electrostatic interactions, providing protection against degradation. Among the non-viral delivery systems previously used for P-gp down-regulation are lipid-based carriers such as Lipofectamine™ 2000, Oligofectamine™, and liposomes (12–16). Despite their success *in vitro, in vivo* use of lipid-based carriers has been questioned due to considerable side effects. Acute inflammatory reactions have been reported in animals treated with intravenous injection of lipoplexes (23), and significant toxicities have been associated with lipid-mediated gene delivery (24,25).

An alternative non-viral carrier for siRNA delivery is cationic polymers. These carriers posses a strong positive charge and are able to form tight complexes with the negatively charged siRNAs. This interaction can effectively formulate siRNA molecules into 'nano'-sized particles, which facilitate intracellular uptake of siRNA. A major benefit of using polymers is that they can be engineered to increase biocompatibility and to minimize host immune reactions. A drawback of these carriers is their low effectiveness, which will hamper their utility in a clinical setting. In a previous report (26), we described an efficient polymeric carrier obtained as a result of stearic acid substitution on poly-*L*-lysine (PLL-StA). The polymer effectively interacted with siRNAs, protected against nuclease degradation, and delivered a high concentration of siRNA into the drug-resistant MDA435/LCC6 MDR1 cells (26). We showed that ~55% P-gp down-regulation was feasible by using this carrier *in vitro* and that intracellular drug concentrations could be effectively increased as a result of P-gp down-regulation. This study, however, was limited to *in vitro* observations; it was not known whether P-gp expressed in tumors could be down-regualted by this approach and whether such an intervention would yield a better response to drug therapy.

The current study employed the previously described siRNA carrier PLL-StA and explored the feasibility of siRNA-mediated P-gp down-regulation in an animal model. A commonly used cationic carrier of nucleic acids, namely branched 25 kDa polyethylenimine (PEI), was additionally utilized as a reference carrier, since this polymer was previously employed for successful siRNA delivery (27,28). A xenograft tumor model derived from P-gp over-expressing human cells in NOD-SCID mice were employed to assess the feasibility of P-gp down-regulation.

MATERIALS AND METHODS

Materials

Branched polyethylenimine (PEI, 25,000 Da) was purchased from Aldrich (Milwaukee, WI). The synthesis and characterization of the PLL-StA (degree of substitution: ~10 stearic

acids per PLL) were described previously (29). Hanks' Balanced Salt Solution (HBSS), trypsin/EDTA, and heparin sulfate were obtained from Sigma (St. Louis, MO). Clear HBSS (phenol red free) was prepared in house. RPMI 1640 medium, penicillin (10000 U/mL), and streptomycin (10 mg/mL) were from Gibco Invitrogen (Burlington, ON). Fetal bovine serum (FBS) was from VWR International (Mississauga, ON). The carboxyfluorescein (FAM)-labeled negative control siRNA was purchased from Gene Pharma Co. LTD (Shanghai, China). The fluorescein isothiocyanide (FITC)-labeled mouse anti-human P-gp antibody was purchased from BD Biosciences Pharmingen™ (San Diego, CA). A siRNA against P-gp (siRNA-1, Cat No: SI00018732; sense: 5′-CAGAAAGCUUAGUACCAAAdTdT, antisense: UUUGGUACUAAGCUUUCUGTC-3′) and a nonspecific control siRNA were purchased from QIAGEN (Mississauga, Ontario). Two other siRNAs, siRNA-2 (sense: 5′-GUAUUGACAGCUAUUCGAAGAGUG, antisense: CCACUCUUCGAAUAGCUGUCAAUAC-3′) and siRNA-3 (sense: 5′ GAAACCAACUGUCA GUGUA, antisense: UACACUGACAGUUGGUUUC-3′) were custom synthesized by Integrated DNA Technologies (Toronto, ON). The Pegylated liposomal doxorubicin (DOX) hydrochloride (Doxil™) was from Schering-Plough Canada Inc. A lysis buffer was prepared by mixing 0.1% Tween in 50 mM Tris-HCl and 150 mM NaCl. The Shandon Cryomatrix™ used for histological embedding was purchased from Thermo Scientific (Pittsburgh, PA).

Cells Culture and Tumor Model

Wild-type MDA-435/LCC6 cells (referred to as WT cells) and their MDR1 (P-gp) transfected phenotype (referred to as MDR1 cells) were kindly provided by Dr. R. Clarke (Georgetown University Medical School, Washington, DC). The cells were initially classified as breast cancer cell type, but recent evidence classified the cells of melanoma origin (30). The WT and MDR1 over-expressing MDA-435/LCC6 cells were propagated in RPMI medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C. Cells were grown and expanded in 75 mL flasks. For harvesting, the cells were washed with HBSS, and trypsinization was performed with 2 mL of trypsin per flask. Cell counts were taken by a hemocytometer. Female NOD-SCID mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All experiments were performed in accordance with the University of Alberta guidelines for the care and use of laboratory animals. All experiments were performed using 4–6-week-old female mice. For tumor formation, ~2 million MDA-435/LCC6 WT or MDR1 cells were injected into the right flank of the mice. After ~3 weeks, at tumor size of ~150 mm^3, intratumoral injections of siRNA and/or tail vein injection of Doxil™ were performed (see below for details).

Cellular Uptake of Complexes by Confocal Microscopy

The MDA-435/LCC6 MDR1 cells were seeded on glass cover glasses in 12-well plates (0.5 mL medium). Twenty four hours later, the cells were treated with FAM-labeled or non-labeled siRNA, combined with PLL-StA and PEI. The complexes were prepared by mixing 2.5 μL of FAM-siRNA solution (0.14 mg/mL) with 3.5 μL of polymer solution (10 mg/mL) in 60 μL of 150 mM NaCl (giving siRNA:polymer ratio of 1:10). The complexes were incubated for 30 min before addition to the cells in triplicate (20 μL/well). After 1, 4, 8, 24, 72 and 144 h, the cells were fixed, and the cell nuclei were stained with 300 ng/mL Hoechst 33258 for 30 min. A Leica TCS-SP2 multiphoton confocal laser scanning microscope (TCS-MP) was used to detect cell-associated FAM-siRNA.

In Vitro P-gp Down-Regulation with siRNA

MDA-435/LCC6 MDR1 cells in 24-well plates (with 0.25 mL of medium) were incubated with the chosen siRNAs either alone or in combination (see Fig. 2). To form complexes, 2.5 μL of siRNA (0.14 μg/mL) was incubated with 3.5 μg of carrier in 60 μL of 150 mM NaCl to form complexes for 30 min. Complexes were then added to cells in triplicates either once (after 24 h), twice (after 24 and 48 h), or three times (after 24, 48 and 72 h) at a concentration of 20 nM siRNA (0.35 mg/ml). At indicated time points, 10 μL of the FITC-labeled anti-human P-gp antibody was added to the cells. The cells were then washed with clear HBSS, trypsinized, and suspended in HBSS with 3.7% formalin. The amount of P-gp in cells was quantified by Beckman-Coulter flow cytometer (Cell Lab Quanta) using the FL-1 detection channel to detect the mean fluorescence of the cells (~5000 events/sample). The mean fluorescence of the FL1 population (an indicative of P-gp amount in all cells) was normalized against the mean fluorescence of the non-treated cells and used as %P-gp expression.

In Vivo Tumor Cell Uptake of siRNA

Upon tumor formation in NOD-SCID mice, intratumoral injections of siRNA with and without polymers were performed. 6.5 μg of FAM-labeled siRNA with or without 32 μg of polymer was injected intratumorally at a final volume of 40 μL into the tumors formed at the right flank of NOD-SCID mice. As a control, unlabeled scrambled

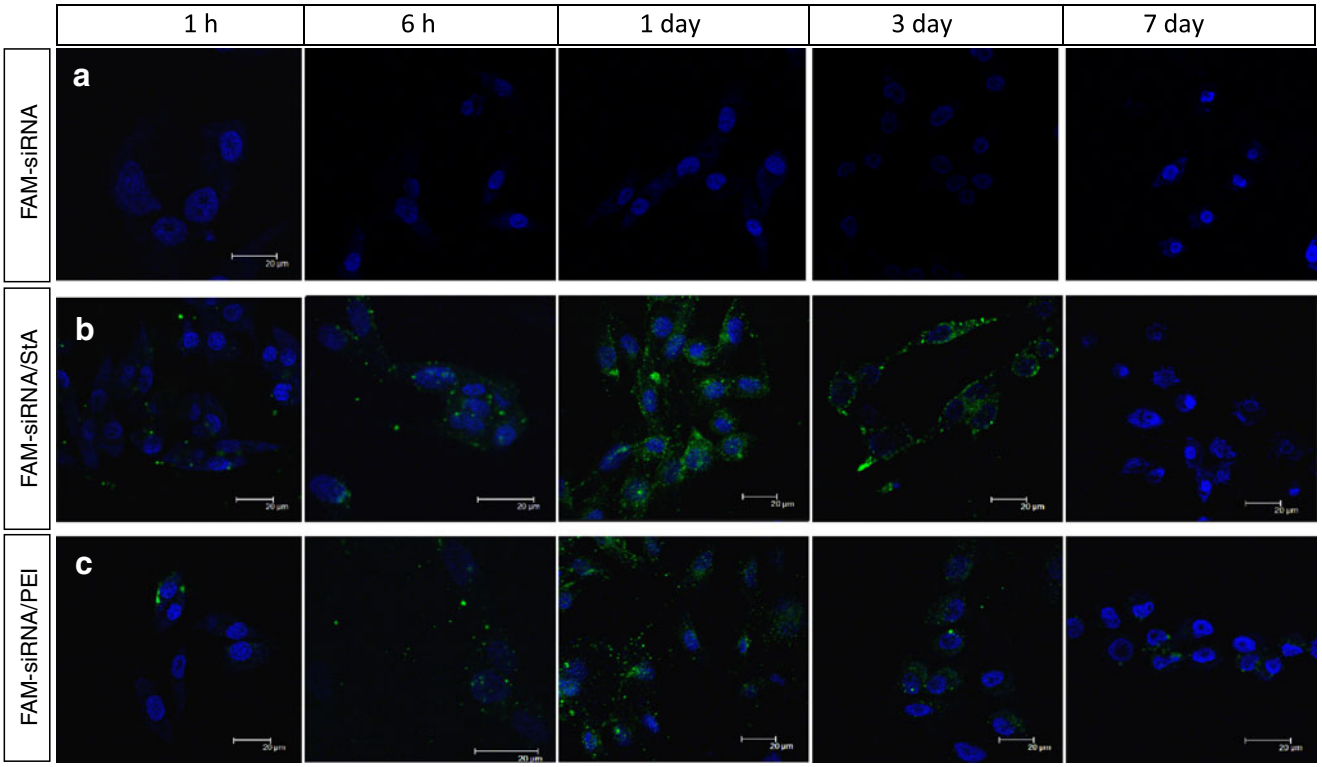

Fig. 1 Confocal microscope assessment of siRNA uptake in MDA-435/LCC6 MDR1 cells. The cells are exposed to free FAM-siRNA (**a**), PLL-StA/FAM-siRNA (**b**) or PEI/FAM-siRNA complexes (**c**) for a period of 1 h, 6 h, 1 day, 3 days and 7 days. The Hoechst 33258 stained cell nuclei are detected in blue, and the FAM-siRNA is detected in green. Note the lack of siRNA association with cells in the absence of a carrier, and larger and more separated siRNA particles at the 1 h and 6 h time points. There was no detectable siRNA in the cells after 7 days.

siRNA (C-siRNA) and its polymeric complexes were injected in order to account for any non-specific effects that might be induced as a result of injection *per se*. After 24 h, the mice were euthanized by CO_2 aphyxsiation, and the tumors were surgically excised. Half of the tumors were initially used to assess the siRNA uptake of tumor cells by using fluorescence microscopy. For this, tumors were extracted and cut in half, and one-half was preserved in Shandon Cryomatrix™ and frozen. Sectioning of the tumor was performed by a Leica CM-3050-S Cryostat and stained by DAPI for nuclear staining. The FAM-siRNA uptake of cells was assessed using a FSX 100 Olympus fluorescent microscope.

The other half of the tumor was used to assess quantitative siRNA uptake by flow cytometry and gel electrophoresis. For flow cytometry, approximately one-quarter of the excised tumors was homogenized by a Pyrex™ Tissue Grinder, filtered through a 35-μm mesh, and suspended in 3.7% formalin in HBSS. The cells displaying FAM-labeled siRNA uptake were determined by a Beckman-Coulter flow cytometer using the FL-1 detection channel to assess the FAM-siRNA-positive cells (~10000 events/sample). The instrument was calibrated so that the negative control sample (i.e., non-treated cells) gave 1–2% cells positive for FAM-siRNA.

Finally, the siRNA uptake was determined by recovering the siRNA from MDA-435/LCC6 MDR1 tumors and assessing its integrity by gel electrophoresis. For this, the cells extracted from one quarter of the tumors were washed with HBSS and centrifuged, and the cell pellets were lyzed with 40 μL lysis buffer. Cells were then placed on a shaker for 30 min, and the solutions obtained after cell lysis were treated with heparin (0.625%) for 20 min. Four μL of 6× diluted loading buffer was added to the samples, and the samples were run on a 1.5% agarose gel (120 V for 35 min). As a reference standard, an equivalent amount of the FAM-labeled siRNA (i.e., amount equal to the total amount injected to the tumors) was run on the gel. The FAM-labeled siRNA was detected by a Fuji FLA-5000 flat-bed scanner using the LD blue laser (485 nm), and percent recovery was calculated based on the spot densitometry as follows: 100%×[recovered siRNA amount/injected siRNA amount].

In Vivo Tumor Cell Uptake of DOX

After establishing MDR1 and WT tumors in NOD-SCID mice, 100 μL of 51 μg of Doxil™ diluted in saline (equivalent to 3 mg/kg) was injected via the tail vein of

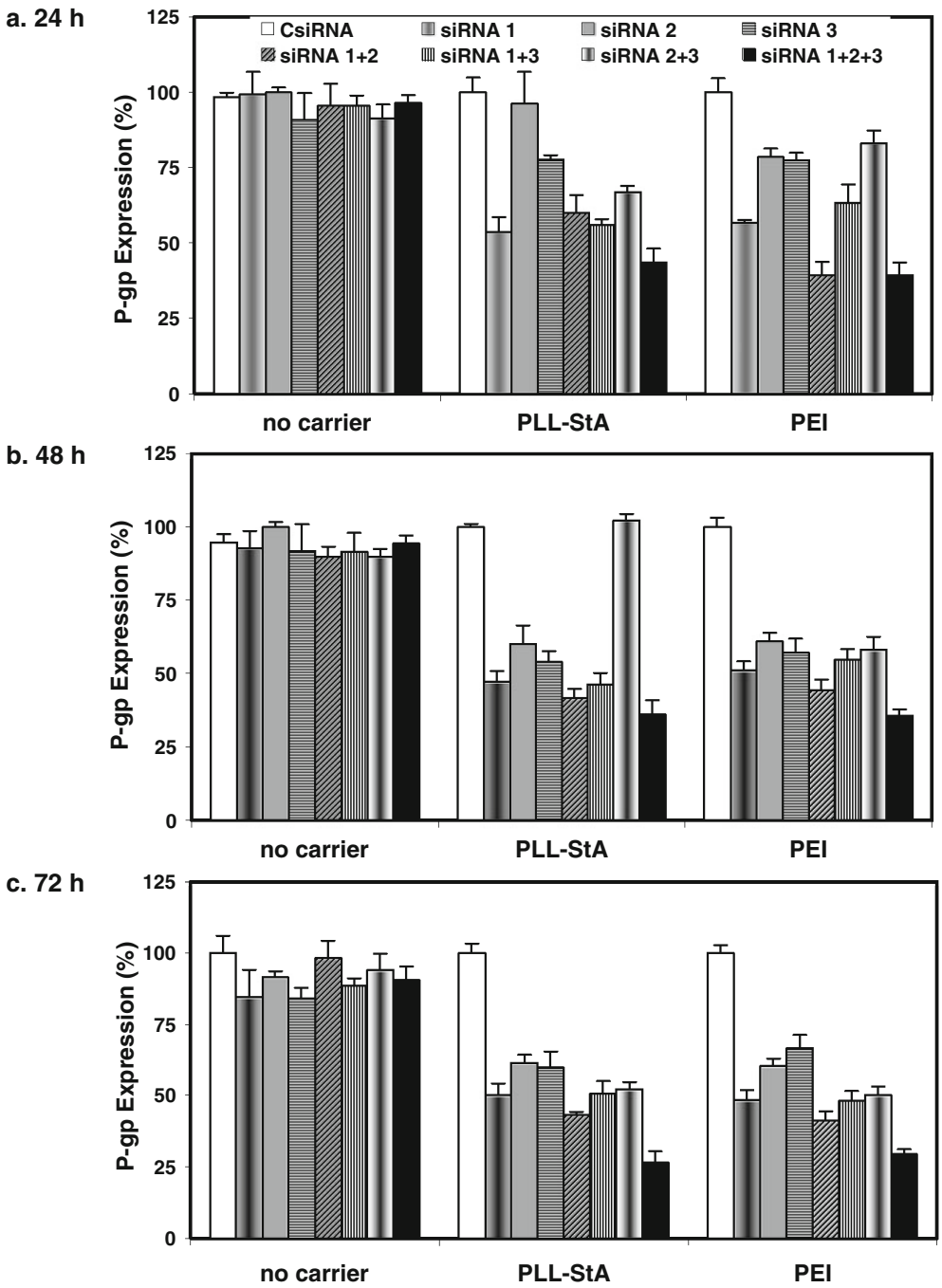

Fig. 2 P-gp down-regulation in MDR1 cells. The cells were treated with siRNA-1, siRNA-2, and siRNA-3 alone (i.e., without a carrier), or a combination of the siRNAs complexed with PLL-StA and PEI. The level of P-gp was detected by flow cytometry after 24 (**a**), 48 (**b**) and 72 (**c**) hours of treatment and normalized with respect to the untreated cells (not shown). The siRNAs in the absence of a carrier did not give any P-gp down-regulation. The PLL-StA and PEI showed similar efficiency in siRNA-mediated P-gp down-regulation. siRNA-1 showed ~52% P-gp suppression at day 1 and ~47% P-gp suppression at days 2 and 3. The efficiency of siRNA-1/-2 and siRNA-1/-3 for P-gp suppression was similar to the efficiency of siRNA-1 alone. The combination of all siRNAs was the most effective, leading to ~65% P-gp suppression at day 1, ~59% P-gp suppression at day 2, and ~73% P-gp suppression at day 3. The siRNA-2, siRNA-3, and their combination (siRNA-2/-3) were the least effective in P-gp suppression.

mice. After 24 h, the mice were euthanized, tumors were extracted, and the extracted tumors were homogenized by a Pyrex™ Tissue Grinder, filtered through a 35-μm mesh and suspended in 3.7% formalin in HBSS. DOX uptake of cells was quantified by the flow cytometer using the FL-2 detection channel to determine percentage of DOX-positive cells and mean DOX fluorescence in cells (~10000 events/sample). The instrument was calibrated so that the negative control sample (i.e., non-treated cells) gave 1–2% DOX-positive cells.

In Vivo P-gp Suppression and DOX Uptake

The complexes of PLL-StA/ABCB1-siRNA and PEI/ABCB1-siRNA were injected intratumorally by using the combination of three siRNA sequences (siRNA-1/-2/-3). The siRNA and polymer doses were 6.5 μg (equal amount of each siRNA) and 65 μg, respectively, per 50 μL of injection volume per mouse. After 24 h, 51 μg of Doxil™ (3 mg/kg) was injected intravenously into the tail vein of the mice. After a further 24 h, mice were euthanized, and the

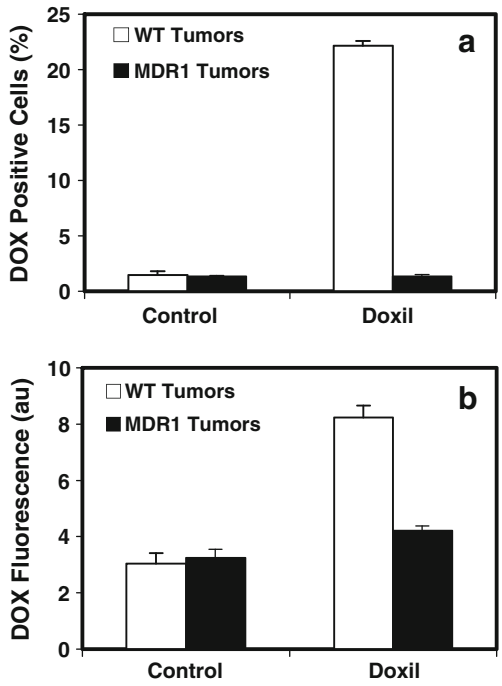

Fig. 3 Flow cytometric analysis of DOX uptake in the MDR1 and WT tumors 24 h after tail vein injection of saline (control) or Doxil™. The results are summarized as the percentage of DOX-positive cells (**a**) or mean DOX fluorescence in cell population (**b**). A clear difference between the MDR1 and WT tumor cells was evident in the DOX uptake, where the cells from WT tumors displayed enhanced DOX uptake unlike the cells from MDR! tumors.

tumors were recovered, processed, filtered as above, and suspended in 3.7% formalin in HBSS. Ten µL of FITC P-gp antibody was added to the cells, and the cells were analyzed by flow cytometry using using the FL-1 detection channel to assess the FAM-siRNA-positive cells (~10000 events/sample) and FL2 channel to assess the DOX-positive cells. The flow cytometry was previously optimized for simultaneous detection of DOX and P-gp using *in vitro* cultured cells.

P-gp Suppression and Tumor Growth with Doxil™ Therapy

The MDR1 tumors were established in 24 NOD-SCID mice ~3 weeks after injections of the cells, and tumor dimensions were measured in each mouse before the study onset. The longest dimension (l) and the width of the perpendicular dimension (w) were used to estimate the tumor volume (= $1 \times w^2 \times 0.4$). Saline or PLL-StA/siRNA complexes in saline were then injected intratumorally into two groups of 12 mice each. The siRNA used was a combination of siRNA-1/-2/-3; each mouse received 6.5 µg siRNA cocktail (equal sum of all 3 siRNAs) complexed with 40 µg PLL-StA in 50 µL saline. After 24 h, each group received either intravenous saline injection or 51 µg of Doxil™ (3 mg/kg) injection via the tail vein of the mice. This design gave four study groups: (i) intratumoral saline and systemic saline injection (6 mice),

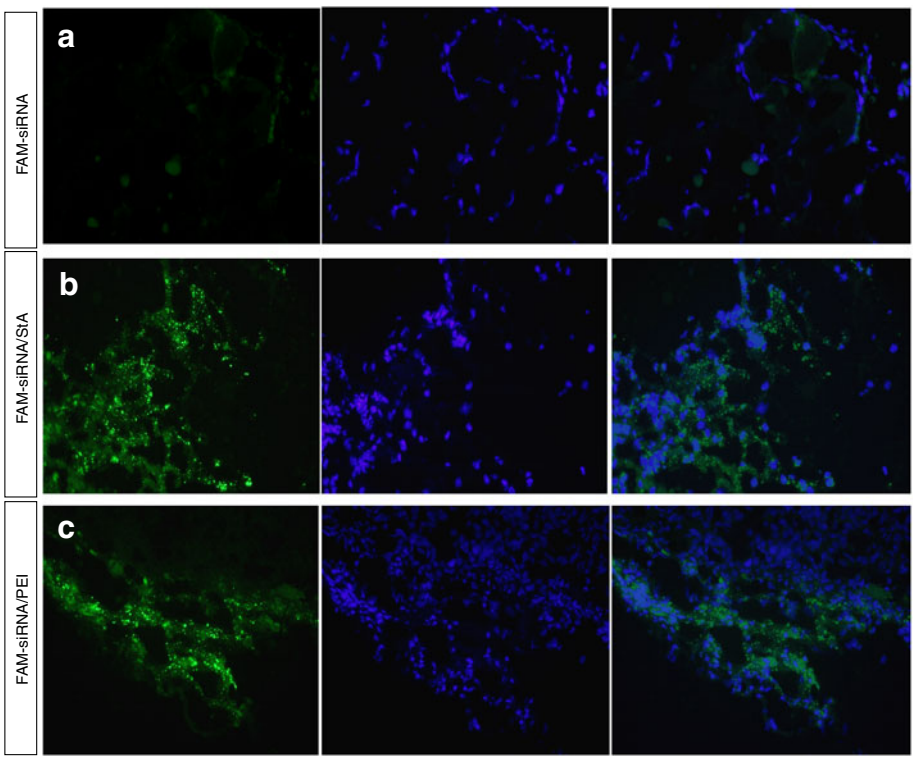

Fig. 4 Epifluorescent microscopic assessment of the sectioned tumor tissue injected with free FAM-siRNA (**a**), PLL-StA/FAM-siRNA complexes (**b**) and PEI/FAM-siRNA complexes (**c**). DAPI stained images in blue, FAM-siRNAs in green, and merged images are presented. The free FAM-siRNA injected tumors showed no obvious FAM-siRNA retention, whereas the PLL-StA/FAM-siRNA and PEI/FAM-siRNA injected tumors showed significant siRNA retention at the site.

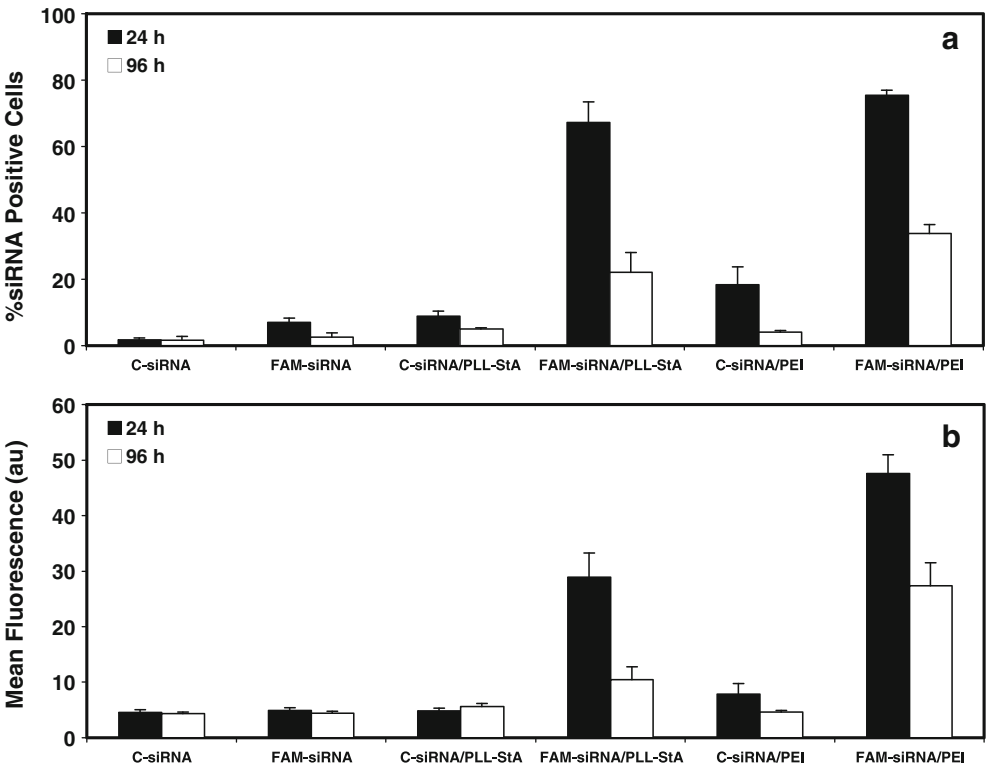

Fig. 5 FAM-siRNA uptake in tumor cells analyzed by flow cytometry. Unlabeled, control siRNA (C-siRNA) and FAM-labeled scrambled siRNA (FAM-siRNA) were injected into the MDR1 tumors either without a carrier or as a complex with PLL-StA and PEI. 24 and 96 h after injection, the tumors were recovered, and siRNA uptake was assessed by flow cytometry. The results are summarized as either percentage of cells positive for siRNA (**a**) or mean fluorescence of the cell population (**b**). At 24 h, PEI proved the most efficient, with ~75% of cells positive for FAM-siRNA, whereas PLL-StA gave ~67% FAM-siRNA positive cells. At 96 h, PEI yielded ~33% FAM-siRNA positive cells whereas PLL-StA gave ~22% FAM-siRNA positive cells. The mean fluorescence of the cells shown in (**b**) indicated a similar result, where the PEI appeared to retain a higher percentage of FAM-siRNA in tumors.

(ii) intratumoral saline and systemic Doxil™ injection (6 mice), (iii) intratumoral PLL-StA/siRNA-1 and systemic saline injection (6 mice), and (iv) intratumoral PLL-StA/siRNA-1 and systemic Doxil™ injection (6 mice). PEI was

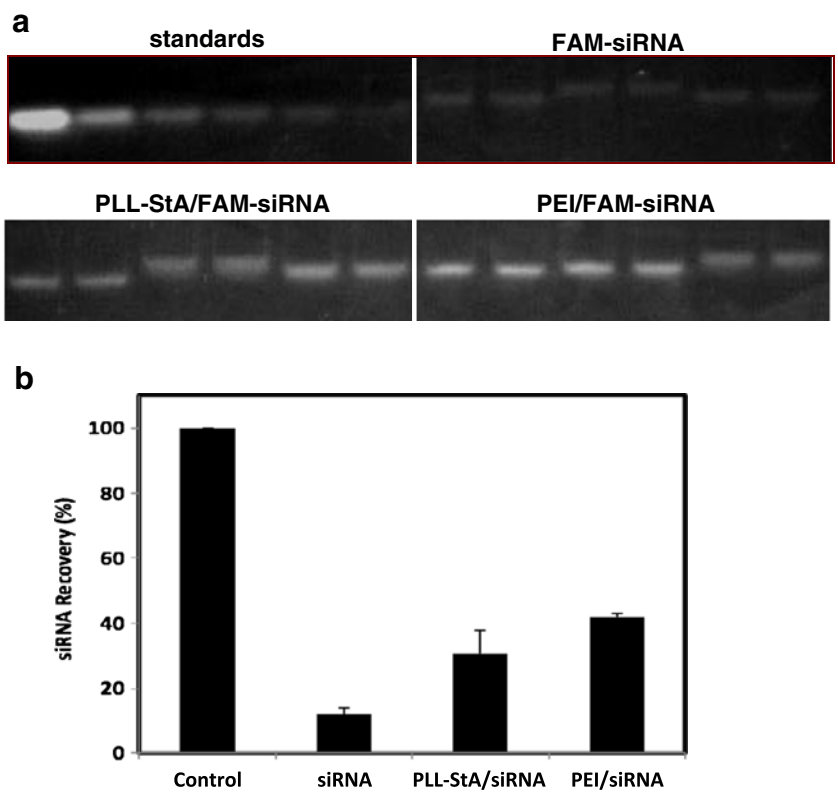

Fig. 6 The recovery of FAM-siRNA from the MDR1 tumors after 24 h. (**a**) FAM-siRNA bands as visualized in agarose gels. Standards refer to a calibration curve of FAM-siRNA generated with an un-injected sample (same dose as injected samples), which was serially diluted (x2) to generate a calibration curve. Tumors from 3 mice was extracted and analyzed in duplicate. (**b**) Quantitative recovery of FAM-siRNA obtained from the densitometric analysis of FAM-siRNA bands in (**a**). Control refers to the sample that was not injected and taken as 100% recover. The PEI gave the highest siRNA recovery (~42%), while the tumors injected with PLL-StA gave ~30% FAM-siRNA recovery.

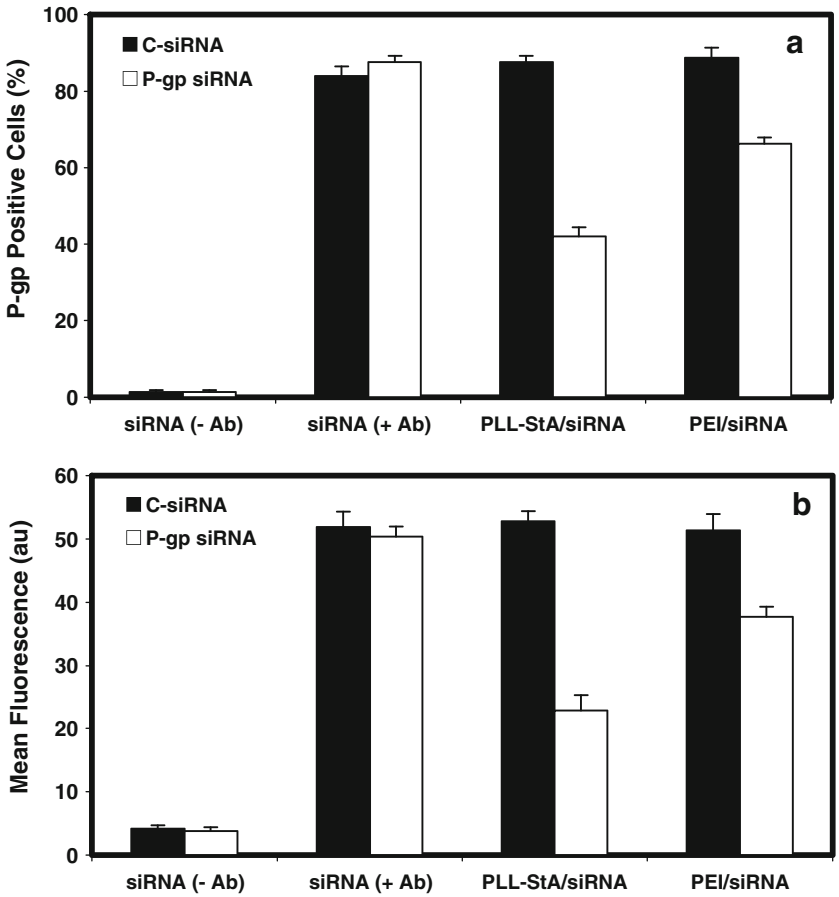

Fig. 7 *In vivo* P-gp down-regulation by P-gp siRNA after 24 h of intratumoral injection into MDR1 tumors. The siRNA used was either a scrambled control siRNA (C-siRNA) or a mixture of P-gp-specific siRNA-1/-2/-3. The siRNAs were injected either without a carrier or as complexes with PLL-StA and PEI. The analysis was carried out with flow cytometry, and the cells from tumors injected with siRNA only were analyzed without and with P-gp-specific antibody (−Ab and +Ab, respectively). The results are summarized as percentage of P-gp-positive cells (**a**) and mean P-gp levels in the cell population (**b**). The tumors treated with P-gp-specific siRNA/PLL-StA complexes gave the highest P-gp down-regulation, showing ~58% down-regulation, while the tumors treated with P-gp specific siRNA/PEI showed ~34% down-regulation. The tumors treated with C-siRNA showed no P-gp down-regulation whether a carrier was used or not. The results from the mean fluorescence of the population also confirmed a similar result.

not evaluated for siRNA delivery in this study to limit study scope. Tumor volumes were measured periodically for 12 days. A second injection of saline or PLL-StA/siRNA-1/-2-3 complexes in saline was performed at this point, followed by intravenous saline or Doxil™ (3 mg/kg) injection after 24 hours. The changes in tumor volume were monitored for another 18 days, after which the animals were euthanized, tumors excised, and tumor weights measured. There was an excellent correlation between calculated tumor volumes at the sacrifice time and the excised tumor weights ($r^2=0.926$). The tumor volumes of individual mouse at different times were normalized with the tumor volume at the study onset to obtain a relative volume for each tumor. Mean±standard deviation (SD) of relative tumor volumes in each group were calculated and plotted as a function of time.

Statistical Analysis

Where indicated, the results are summarized as mean±SD of the indicated number of replicates. Variations between the group means were analyzed by either ANOVA for multiple group comparisons or Student's *t*-test for comparison of two study groups ($p<0.05$).

RESULTS

In Vitro siRNA Uptake Kinetics

An initial study was conducted to investigate the fate of siRNA delivered by the cationic polymers, with the purpose of determining siRNA persistence in MDA-435/LCC6 MDR1 cells. The cells were exposed to a single dose of siRNA complexes for 24 h, after which the siRNAs were removed. Confocal microscopy images of the cells exposed to free FAM-siRNA or polymer/FAM-siRNA complexes are shown in Fig. 1 over a period of 7 days. In cells treated with free FAM-siRNA, no siRNA was detected at any of the time points (Fig. 1a), indicating lack of cellular uptake in the absence of a polymeric carrier. The PLL-StA/FAM- gave some uptake after 1 h cell-associated FAM-siRNA particles at this time point (Fig. 1b), but the PEI/FAM-siRNA-treated cells showed limited, if any, siRNA uptake (Fig. 1c). The uptake of PLL-StA/FAM-siRNA and PEI/FAM-siRNA complexes was qualitatively similar at the subsequent time points: the cell-associated FAM-siRNA particles increased significantly at 6 h; after 1 day, a uniform distribution of particles in the cytoplasm was detected for both polymers. After 3 days, the particles were

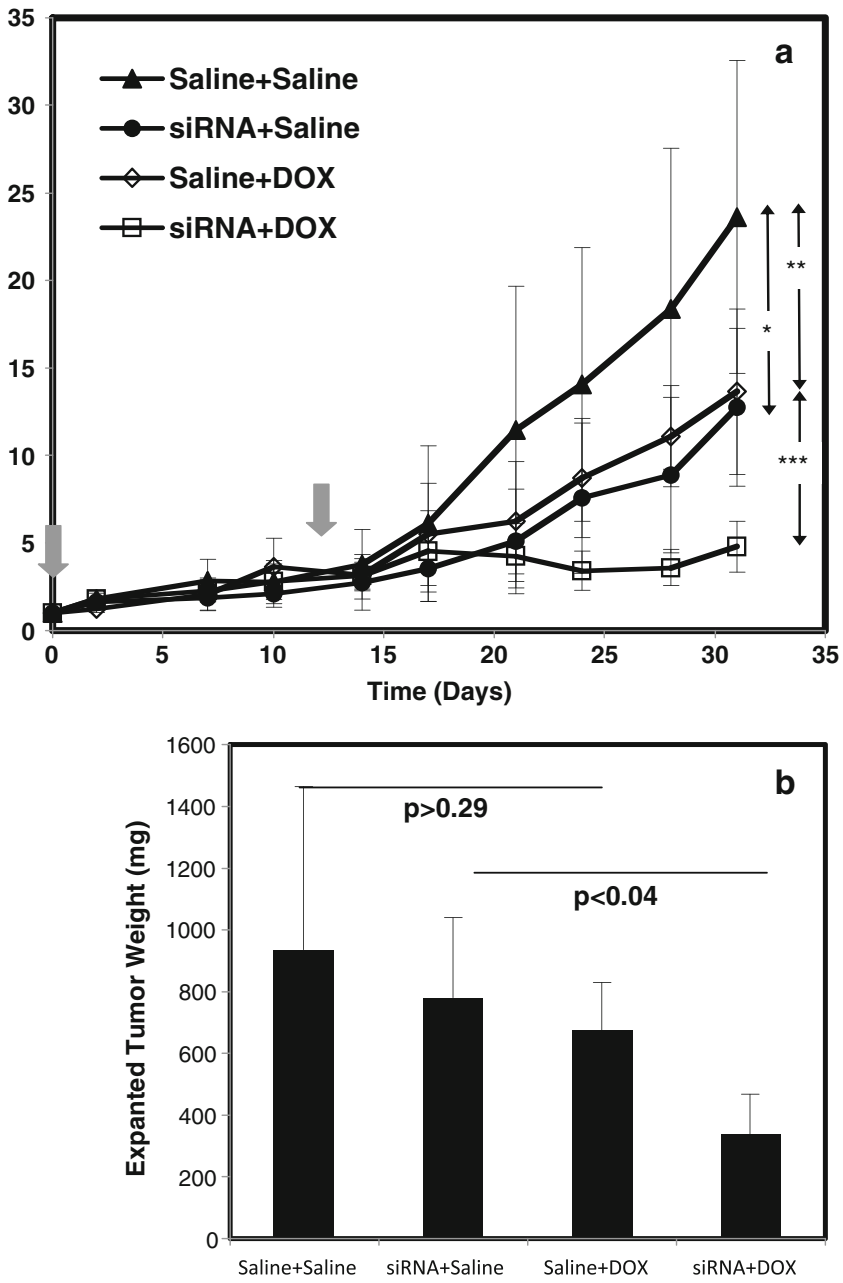

Fig. 8 (**a**) Changes in the relative volume of MDR1 tumors that received injection of saline or P-gp-specific PLL-StA/siRNAs (day 0 and day 13; block arrows). After 24 hr, each treatment group was injected with saline or Doxil™. The tumor volume in individual NOD-SCID mouse was measured at the indicated time points and divided by the tumor volume at the study onset to obtain a relative tumor volume. Tumors receiving intratumoral saline, followed by intravenous saline injection (i.e., no treatment) displayed the highest growth as expected. Doxil™ injection appeared to retard the growth, but the relative volume was not significantly different from saline-injected control at the study end (*: $p > 0.07$). Intratumoral injection of PLL-StA/siRNA reduced the relative volume as well at the study end, but this was again not significant (**: $p > 0.05$). The tumors that received P-gp-specific PLL-StA/siRNA complexes followed by Doxil™ injection gave the smallest tumor weights at the end of the study period (***: $p < 0.04$ *vs.* saline injected). (**b**) excised tumor weight at the end of the study period ($n = 6$, $n = 4$, $n = 4$ and $n = 5$ for each group from left to right). The study groups received either saline or P-gp-specific siRNA, each subsequently receiving either saline or DOX. Intravenous Doxil™ injection did not reduce the weights of tumors receiving intratumoral saline ($p > 0.29$, *t*-test) but did significantly reduce the weight of tumors receiving P-gp-specific PLL-StA/siRNAs ($p < 0.04$, *t*-test).

concentrated around the nuclear membrane, and the particles completely disappeared after 7 days.

In Vitro P-gp Down-Regulation

The suppression of P-gp by the three different siRNAs at days 1, 2 and 3 are shown in Fig. 2. The treatment of cells with siRNA in the absence of a carrier did not lead to P-gp suppression at any of the time points, consistent with the lack of siRNA uptake by the MDR1 cells. The siRNA delivery with PLL-StA and PEI showed similar efficiency in P-gp suppression at all time points. The siRNA-2, siRNA-3, and their combination (siRNA-2/-3) were the least effective in P-gp suppression, leading to ~23% P-gp suppression at day 1, and ~41% P-gp suppression at days 2 and 3. The siRNA-1 delivery with the polymers gave ~52% P-gp suppression at day 1 and ~47% P-gp suppression at days 2 and 3. The efficiency of siRNA-1/-2 and siRNA-1/-3 combinations for P-gp suppression was similar to the efficiency of siRNA 1 at the investigated time points. The siRNA-1/-2/-3 was the most effective siRNA combination in P-gp suppression, leading to ~65% P-gp suppression at day 1, ~59% P-gp suppression at day 2, and ~73% P-gp suppression at day 3.

DOX Uptake in Tumors after Doxil™ Injection

For tumor formation, ~2×10^6 MDA-435/LCC6 WT or MDR1 cells were injected into the right flank of the mice. After 3 weeks where an average tumor size of ~150 mm^3 was reached, systemic injection of tumor-bearing mice with Doxil™ was performed to assess tumor localization of the drug. The Doxil™ injection in mice bearing the WT tumors resulted in 22±1% DOX-positive tumor cells ($p<0.001$ *vs.* control), but Doxil™ injection in MDR1 tumors resulted no measurable uptake of the drug ($p>0.39$ *vs.* control; Fig. 3a), consistent with the phenotypic features of the tumor reconstituting cells. The mean fluorescence of the WT and MDR1 tumor cells showed a similar trend as well, where the WT tumors gave the highest amount of DOX uptake, and MDR1 tumors displayed no apparent uptake (Fig. 3b).

In Vivo siRNA Uptake in Tumors

Intratumoral injections of siRNA and polymer/siRNA complexes were performed to assess siRNA localization in tumors. The siRNA uptake on extracted tumors was initially analyzed by epifluorescent microscopy one day after siRNA injection. The FAM-siRNA treated cells (i.e., without a carrier) gave limited siRNA localization at the tumors (Fig. 4a), presumably due to rapid diffusion of siRNA from the injection site. The injection of PLL-StA/FAM-siRNA complexes showed a relatively high amount of siRNA dispersed evenly throughout the tumor (Fig. 4b). The PEI/FAM-siRNA-treated cells also showed a high amount of siRNA uptake, but the siRNA was more concentrated in certain areas of tumors and appeared to be aggregated and/or less dispersed (Fig. 4c).

The siRNA uptake in tumor cells was quantitated by flow cytometry. The PEI/FAM-siRNA and PLL-StA/FAM-siRNA-treated cells showed a similarly high percentage of siRNA-positive cells (70–75%) after 24 h of injection. The PEI/C-siRNA and PLL-StA/C-siRNA-treated cells showed 18±5% and 9±2% siRNA-positive cells, indicating some level of autofluorescence as a result of siRNA injection by PEI and PLL-StA polymers. The FAM-siRNA-treated cells showed 7±1% siRNA-positive cells at 24 h post-injection. At 96 h, the percentage of siRNA-positive cells was reduced significantly, yielding 39±3% siRNA-positive cells for the PEI/FAM-siRNA-treated tumors and 22±6% for the PLL-StA/FAM-siRNA-treated tumors (Fig. 5a). The amount of siRNA uptake (as determined by the mean fluorescence) was higher in the PEI/FAM-siRNA-treated tumors compared to the PLL-StA/FAM-siRNA-treated tumors at the 24 and 96 h time points (Fig. 5b; 1.6-fold and 2.5-fold, respectively).

The siRNA recovery from tumors was also assessed by gel electrophoresis after extracting the siRNA from the tissue mass. Since this approach is based on intact siRNA quantitation after gel migration, it provides a measure of intact siRNA in the tumor tissue. The PEI/FAM-siRNA-treated tumor cells showed the highest siRNA recovery after 24 h (42±1% of injected dose). The PLL-StA/FAM-siRNA-treated cells showed 31±7% recovery of the injected dose ($p<0.004$ *vs.* PEI/FAM-siRNA-treated tumors; one-tailed *t*-test), and the naked FAM-siRNA-treated cells showed 12±2% siRNA recovery (Fig. 6).

In Vivo P-gp Down-Regulation

Intratumoral injection of P-gp-specific siRNAs was performed to assess P-gp down-regulation in the MDR1 tumors. The siRNA used for this purpose was the combination of the three siRNAs (siRNA-1/-2/-3), as this was the most efficient siRNA combination tested *in vitro*. As the baseline, untreated tumors yielded 88±2% P-gp-positive cells from the tumors. The tumors treated with free P-gp-specific siRNA (i.e., without any polymeric carrier) and polymer/C-siRNA complexes showed no significant down-regulation of P-gp, based on lack of changes in either the extent of percentage of P-gp-positive tumor cells (Fig. 7a) or the mean P-gp level in tumor cells (Fig. 7a and b, respectively; see Supplementary Material for typical flow cytometry histograms). The tumors treated with P-gp-specific PEI/siRNA and PLL-StA/siRNA complexes had 66±1% and 42±2% P-gp-specific cells ($p<0.01$), respectively. The changes in mean fluorescence of the cell population (indicating the relative levels of P-gp) confirmed the above results, as the tumors treated with P-gp-specific siRNA complex of PLL-StA showed higher extent of P-gp down-regulation as compared to P-gp-specific siRNA delivery with PEI (61.5 *vs.* 29.0%, respectively).

Systemic Doxil™ injections were also performed in mice bearing MDR1 tumors pre-treated with polymer complexes of C-siRNA and P-gp-specific siRNA. After siRNA injection, 24 h were allowed before the injection of Doxil™ and DOX uptake in tumor-derived cells were compared to the tumors receiving no Doxil™. No DOX uptake was detected in the extracted MDR1 tumor cells pre-treated with either C-siRNA or P-gp-specific siRNA/polymer complexes (not shown), despite a significant down-regulation of P-gp in the latter case.

Tumor Growth after P-gp Inhibition and Doxil™ Treatment

The effect of systemic Doxil™ on the growth of MDR1 tumors was lastly investigated. The tumors were treated with intratumoral injection of saline or P-gp-specific PLL-StA/siRNA complex on days 1 and 15. The tumors injected with

saline which received no systemic Doxil™ displayed the most rapid growth during the 31-day study period (Fig. 8a). Upon Doxil™ injection, these tumors displayed retarded growth, and the excised tumor weights were ~28% lower than tumors receiving no Doxil™ (Fig. 8b). However, the excised tumor weights ($p>0.29$) or relative volume at the study end ($p>0.07$) for Doxil™-injected animals were not significantly different from the control (saline treatment) group. Intratumoral injection of P-gp-specific PLL-StA/siRNA complexes appeared to reduce tumor growth on its own without Doxil™ injection (Fig. 8a), but the recovered weights ($p>0.55$) or the relative tumor volume at the end of the study ($p>0.05$) were not significantly different from the intratumoral saline injected tumors. Tumors injected with P-gp-specific PLL-StA/siRNA complexes and treated with Doxil™, on the other hand, displayed the most retarded growth, especially after the second injection of siRNA complexes and Doxil™, where no more tumor growth was observed. Doxil™ treatment resulted in a ~58.9% reduction in the weight of tumors injected with P-gp-specific siRNA/PLL-StA complexes, as compared to tumors injected with P-gp-specific siRNA/PLL-StA complexes but receiving saline alone ($p<0.04$).

DISCUSSION

The resistance of tumor cells to chemotherapy is a major drawback in the therapy of breast (12), ovarian (15,16), gastric (31), and pancreatic cancer (32). Attempts to reverse the MDR have mainly focused on P-gp, the protein most consistently linked to efflux of therapeutic drugs (6,7). The polymeric carriers previously found promising for *in vitro* down-regulation of P-gp (26) were further investigated in this study in a xenograft model. Confocal microscopy studies were initially conducted to determine the intracellular fate of siRNA after uptake into MDA-435/LCC6 MDR1 cells. Using PLL-StA and PEI for siRNA delivery, uptake into cells was detected within 1 h of complex exposure, and by 24 h exposure, the FAM-siRNA particles were spread evenly in large frequency within cells. Significant intracellular trafficking was evident subsequently since the siRNA was concentrated at the intracellular and nuclear membranes on day 3, after which the siRNA disappeared by day 7. It could be seen that siRNA particles detected at the 1 and 6 h time points were larger, more compact, and more dispersed as compared to particles at later time points. This may suggest that the siRNA particles at early time points were localized in endosomes, whereas the siRNA particles detected after 24 h were released from the endosomes and were spread in the cytosol.

Our previous studies on *in vitro* P-gp down-regulation using PLL-StA resulted in a maximum of ~55% P-gp reduction at the protein level, using a relatively low siRNA concentration (20 nM with siRNA-1) (26). The need to employ low siRNA concentrations to limit interference with non-specific genes is well established (33,34). In this study, an attempt was made to improve on our previous results to increase the efficiency and duration of P-gp down-regulation by using two other siRNA sequences. The G/C content of siRNA molecules has been reported to be significant in siRNA-mediated silencing efficiency. It has been reported that efficient siRNA sequences contain 30–52% G/C content (35). Our siRNA-1 contained 38% G/C, siRNA-2 contained 44% G/C, and siRNA-3 contained 42% G/C, so that all three siRNAs were expected and shown to be effective at P-gp down-regulation. Using a combination of two or three specific siRNAs did lead to a significant increase in gene silencing in a study by Ji *et al.* (36), while Holen *et al.* saw no beneficial effect with this approach (37). Stierlé *et al.* observed that the delivery of a very efficient and a slightly efficient siRNA molecules resulted in gene silencing equivalent to that of more efficient siRNA alone (12). We observed similar results to Stierlé *et al.*, as the combination of most efficient siRNA sequence (siRNA-1) with the less efficient siRNA sequences (siRNA-2 and −3) resulted in a similar efficiency to that of siRNA-1. As expected, the combination of siRNA-2/-3 did not prove beneficial; however, the combination of all three siRNA sequences resulted in a significant increase in efficiency (maximum of ~75% P-gp protein down-regulation). It should be noted that a higher dose of siRNA is applied when a combination of multiple siRNA sequences is used, 40 nM in the case of two siRNAs and 60 nM in the case of three siRNAs. Changes in the siRNA concentration, rather than the nature of siRNA *per se*, are also likely to contribute to increased P-gp down-regulation efficiency. To increase the duration of P-gp knockdown, repeated exposure of siRNA/polymer complexes to cells was attempted. Our previous study had indicated the maximum silencing at 24 h after a single treatment and loss of the silencing after 72 h of siRNA/polymer exposure (26). We therefore exposed siRNA complexes to cells every 24 h for a period of 3 days to increase the duration of P-gp knockdown and were able to prolong P-gp down-regulation for a period of 72 h. Such a prolonged incubation with siRNA was presumably more representative of *in vivo* situation, where the tumor-deposited siRNA complexes will remain at the site for a longer time.

The *in vivo* siRNA uptake studies with flow cytometry confirmed the presence of FAM-siRNA in injected tumors. Both polymers were able to deliver siRNA into cells, but PEI appeared to be more effective than the PLL-StA in this

respect. This was in line with our siRNA recovery results from gel electrophoresis, where ~42% of injected siRNA was recovered from the PEI/FAM-siRNA-injected tumors as compared to ~30% by PLL-StA. It is likely that the siRNA bound to PLL-StA was dissociated faster than the PEI-bound siRNA, causing faster disappearance of the siRNA. As expected, there was a significant reduction in siRNA levels in tumors after 96 h, more so for the PLL-StA/FAM-siRNA-treated tumors. This level of reduction was in line with our previous *in vitro* results (26), where a more significant reduction was evident for the PLL-StA delivered siRNA in MDA-435/LCC6 MDR1 cells (~50% *vs.* ~20% for PLL-StA *vs.* PEI delivered FAM-siRNA from 24 to 72 h). Considering that free siRNA is degraded within 24 h in the presence of serum (unpublished), the FAM-siRNA that is detected at tumor cells at the 96 h time point is expected to be in a complex with carriers. None of the other studies focusing on P-gp down-regulation in animal models explored *in situ* siRNA pharmacokinetics (14–21), and our studies provide useful information in this regard.

Upon siRNA delivery by the PLL-StA and PEI carriers, a higher P-gp down-regulation efficiency was detected for PLL-StA compared to PEI. It was unexpected that the PLL-StA was more effective *in vivo*, since (i) we had a similar efficiency for the two carriers in *in vitro* P-gp down-regulation and (ii) *in vivo* siRNA localization was larger for PEI. One reason for this difference might be more aggregated nature of the PEI/siRNA complexes in tumors, retaining the siRNA complexes extracellularly and reducing the relative bioavailability of siRNA inside tumor cells. This might not be a significant consideration for cells in culture, which is readily accessible to siRNA particles due to two-dimensional monolayer culture. Another reason could be the tighter complex formation between the PEI and siRNA compared to PLL-StA (26). Efficient complex, dissociation in the cytosol is required for siRNA binding to the RISC complex, and reduced dissociation of siRNA from PEI may reduce siRNA bioavailability in the cytosol, leading to a lower P-gp mRNA knockdown and ultimately a lower P-gp down regulation. It is possible that the dissociation is not limiting *in vitro* efficiency, unlike the case in the tumors. The P-gp down-regulation in MDR1 tumors was expected to lead to an increase in cellular DOX accumulation after systemic Doxil™ injection. However, there was still no DOX uptake in P-gp down-regulated MDR1 tumor cells (not shown). Since WT tumors displayed ~23% of cells with DOX uptake, and based on previous studies comparing DOX uptake in WT *vs.* MDR cells in culture (26), only ~3% of the cells was expected to be positive in the case of MDR1 tumors. This is relatively low and was probably not distinguishable from the background. The Doxil™ concentration employed in this study corresponded to typical therapeutic doses in rats; increasing the injected dose might be one approach to detect DOX uptake in future studies, as well as employing a local injection route (e.g., subcutaneous site) for Doxil™.

A limited number of research groups have attempted *in vivo* down-regulation of P-gp and MDR reversal. Early studies employed models that stably expressed short hairpin RNA to demonstrate RNAi-mediated down-regulation of P-gp (38,39). This approach, however, is not clinically feasible. In a more relevant approach, Xiao *et al.* (40) used a Stealth™ RNAi delivery system for siRNA interference in the human lung carcinoma cells NCI-H460. Stealth™ RNAi shares the same interfering properties of siRNA but shows less off-target activities due to chemical inactivation of the sense strand. Nude mice were used in their study, and 80 μM of the Stealth™ RNAi was delivered by electroporation directly into tumors (without a carrier). The maximal P-gp down-regulation obtained was ~80%. This value was slightly higher then the P-gp down-regulation obtained in this study (~61.5% based on mean P-gp levels in tumors). After P-gp down-regulation, treatment by vinorelbine tartrate resulted in maximal reduction in tumor size by ~60% after 13 days (41), whereas the current study gave a similar reduction (~58.9%) in tumor volume after 31 days. In another study, Patil *et al.* used biotin-decorated poly(D,L-lactide-co-glycolide) nanoparticles for simultaneous delivery of siRNA and paclitaxel (PTX). After a single injection of the nanoparticles (20 mg/kg PTX and 20 μg of the P-gp-specific siRNA), the volume of the induced breast cancer tumors was reduced by ~50% after 16 days (41). The extent of P-gp down-regulation was not investigated in that study. Significant differences in the nature of the cell models, tumor volumes on study, injection methods, and the nature/concentration of injected siRNAs are all likely factors that may have contributed to some of the differences observed in the final outcomes. The DOX accumulation was not reported in the other studies as well. This will be critical to link the local drug concentrations to the obtained reductions in tumor growth.

An interesting observation on the tumor growth results reported here was the relative effectiveness of the second dose of siRNA and Doxil™ injection; it appeared that the tumor growth was not retarded significantly after the first injected dose, but significant differences appeared after the second injection. Although the exact reason(s) for this observation remains to be determined, it is likely that the larger tumor mass resulted in better retention of the siRNA complexes (yielding better P-gp down-regulation) and/or larger tumors were more responsive to systemic Doxil™ injections. Increased vascularization of larger tumors might have enabled increased DOX delivery to tumors at the time of second Doxil™ injection. The relatively small size of the study groups ($n=6$) did not allow us to explore a relationship between the initial tumor volume and the response to drug theapy.

It is likely that P-gp silencing was achieved for relatively short duration with siRNA administration (1–3 days), but this might be sufficient to sensitize the tumors to Doxil™ therapy administered on the next day and give long-lasting effect(s) due to close administration of P-gp-specific siRNA and Doxil™.

In conclusion, this study investigated the intracellular as well as intratumoral fate of siRNA complexes in drug-resistant MDA-435/LCC6 cells. In addition to *in vitro* down-regulation of P-gp, effective P-gp down-regulation in MDA-435/LCC6 MDR1 tumors was demonstrated by using the cationic polymeric carriers PEI and PLL-StA. The MDA-435/LCC6 WT tumors displayed the expected DOX accumulation after systemic Doxil™ injection, but tumors derived from MDA-435/LCC6 MDR1 cells did not yield DOX accumulation with or without P-gp down-regulation, possibly due to the low levels of DOX in tumors that were below the detection limit of the assessment technique. The MDR1 tumors, however, responded well to systemic Doxil™ administration, and the extent of tumor reduction was higher, especially with P-gp down-regulation. We conclude that siRNA delivery by polymeric carriers is a feasible approach to effective siRNA delivery in xenograft models, and P-gp down-regulation in such tumors led to a functional reduction in tumor growth with systemic drug therapy.

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