ORIGINAL ARTICLE

Phospholipid-modified PEI-based nanocarriers for in vivo siRNA therapeutics against multidrug-resistant tumors

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Multidrug resistance (MDR) mediated by P-glycoprotein overexpression in solid tumors is a major factor in the failure of many forms of chemotherapy. Here we evaluated phospholipid-modified, low-molecular-weight polyethylenimine (DOPE-PEI) nanocarriers for intravenous delivery of anti-P-gp siRNA to tumors with the final goal of modulating MDR in breast cancer. First, we studied the biodistribution of DOPE-PEI nanocarriers and the effect of PEG coating in a subcutaneous breast tumor model. Four hours postinjection, PEGylated carriers showed an 8% injected dose (ID) accumulation in solid tumor via the enhanced permeability and retention effect and 22% ID in serum due to a prolonged, PEG-mediated circulation. Second, we established the therapeutic efficacy and safety of DOPE-PEI/siRNA-mediated P-gp downregulation in combination with doxorubicin (Dox) chemotherapy in MCF-7/MDR xenografts. Weekly injection of siRNA nanopreparations and Dox for up to 5 weeks sensitized the tumors to otherwise non-effective doses of Dox and decreased the tumor volume by threefold vs controls. This therapeutic improvement in response to Dox was attributed to the significant, sequence-specific P-gp downregulation in excised tumors mediated by the DOPE-PEI formulations.

INTRODUCTION

Resistance to chemotherapy is a serious challenge for the effective treatment of breast cancer. The development of multidrug resistance (MDR), mediated by the overexpression of the adenosine triphosphate -binding cassette (ABC transporters), is believed to have an important role in the poor efficacy of cancer chemotherapy.1,2 P-glycoprotein (P-gp), a 170-kDa plasma membrane glycoprotein, was the first ABC transporter consistently identified to be overexpressed in breast cancer cell lines displaying MDR. It employs adenosine triphosphate to actively pump cytotoxic drugs out of the cells and is responsible for the efflux of chemotherapeutic drugs such as vinblastine, doxorubicin (Dox) and paclitaxel that leads to a decreased accumulation of drug in tumor cells and, consequently, the failure of chemotherapy.3 To circumvent MDR, P-gp represents an attractive target that is overexpressed up to 30% in newly diagnosed cancers and > 70% in relapsed forms of breast cancer.4

P-gp-mediated MDR can be reversed using small molecules acting as P-gp substrates or inhibitors. However, their clinical use is associated with intolerable side effects and toxicity owing to off-target distribution and pharmacokinetic interactions with anticancer drugs.5–11 An alternative approach to inhibition of P-gp activity is to downregulate its expression at the transcriptional level by using short double-stranded RNAs, the so-called siRNA, that trigger the catalytic degradation of complementary mRNAs.12–14 Clear advantages of the siRNA approach compared with chemical inhibitors include its reduced toxicity toward non-specific tissues (as siRNA cannot diffuse passively through cellular membranes) and its high specificity. However, the targeted delivery of siRNA into tumor cells following a systemic application is difficult due to its poor in vivo stability (rapid degradation in plasma and cellular cytoplasm) and poor cellular uptake.15

The development of parenteral siRNA formulations that are stable in the circulation, promote tumor accumulation and that lack carrier-related toxicities is essential for the clinical use of siRNA-based drugs. One of the most investigated carriers is polyethyleneimine (PEI), which functions as a transfection reagent based on its ability to compact siRNA into nano-scale complexes. This feature offers protection from harsh enzymatic degradation and improves both intracellular delivery of siRNA and trafficking uptake through endocytosis and escape from lysosomes.16,17 The transfection efficiency/cytotoxicity profile of PEIs is largely influenced by their molecular weight. With the increase in their molecular weight, branched PEIs exhibit increased transfection that also correlates with increased non-specific toxicity due to excessive interactions with cell membranes by the cationic charges of PEI. Low-molecular-weight PEIs have low toxicity and are hemocompatible but display poor transfection.18–20 We previously reported that chemical conjugation of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) phospholipid to low-molecular-weight PEI (1.8 kDa) significantly improved the intracellular siRNA delivery and the gene silencing of non-modified PEI while keeping cytotoxicity levels low.21,22 The present study reports on the in vivo utility of these DOPE-PEI nanocarriers. In particular, we evaluated their tissue distribution upon intravenous injection in tumor-bearing mice and the effect of PEG coating as a strategy to improve their stability in circulation and enhance permeability and retention by the tumor.23–25 In a second step, we evaluated the anti-cancer activity of DOPE-PEI/anti-P-gp siRNA nanopreparations and Dox combinations in resistant breast cancer MCF-7/ADR xenografts. The scheme of the proposed experiment is depicted in Figure 1. We used different dosing regimens, that is, simultaneous vs sequential administration of siRNA and Dox to optimize their anti-cancer activity. To the best of our knowledge,
this is the first study that demonstrates the utility of low-molecular-weight PEI (1.8 kDa) for intravenous siRNA delivery to tumors for the reversal of P-gp resistance to Dox and investigates the impact of different delivery modes on the therapeutic outcome of siRNA/drug combinations.

RESULTS

Biophysical characterization of the formulations

DOPE-PEI/siRNA complexes were prepared as described. The complexes were also mixed with the micelle-forming material, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethyleneglycol)-2000] (PEG-PE), to improve stability. The biophysical characterizations of DOPE-PEI/siRNA and DOPE-PEI/PEG/siRNA are shown in Figure 2. The detailed structure and the effect of PEG addition on the morphology of the formulations was studied by dynamic light scattering (DLS), atomic force microscopy (AFM) and transmission electronic microscopy (TEM). DLS was used to determine the hydrodynamic diameter of the complexes, whereas AFM and TEM were chosen as methods that allowed sample visualization, measurement of the real size (not the hydrodynamic) of the complexes and additionally gave a three-dimensional profile of the sample (in the case of AFM). The formation of the PEG-PE corona around the DOPE-PEI/siRNA core was proposed based on the fact that the zeta potential drastically changed from an average of about +48 mV for DOPE-PEI/siRNA complexes to about +13 mV after the addition of PEG-PE, suggesting a cationic charge-shielding effect, whereas the mean size measured by DLS was not affected by the addition of PEG (Figure 2a). The zeta potential for free PEG-PE micelles was −27 ± 8 mV in accordance with reported values. By AFM analysis, the DOPE-PEI/siRNA complexes appeared as well-developed, individualized, rounded particles with a broad size distribution ranging from 50 to 500 nm. DOPE-PEI/PEG/siRNA particles exhibited an almost spherical shape and a narrower size distribution. TEM analysis of DOPE-PEI/siRNA and DOPE-PEI/PEG/siRNA complexes confirmed the protective and stabilizing effect of the PEG-PE corona. The DOPE-PEI/siRNA complexes appeared like ovoid particles. Some concentric ovoid structures were also visualized with a mean particle size of 110.4 ± 20.4 nm. The DOPE-PEI/PEG/siRNA micelles appeared as almost spherical particles (Figure 2b). The mean particle size for the DOPE-PEI/PEG/siRNA complex was 95.8 ± 38.7 nm.

We also monitored the stability of the DOPE-PEI/siRNA nanocarriers (PEGylated and non-PEGylated) in 150 mM NaCl. The benefit of PEGylation was clear: the PEGylated nanocarrier exhibited no signs of aggregation in contrast to the non-PEGylated nanocarrier (Figure 2c). Although in vitro experiments with highly concentrated formulations (40 μg siRNA per 100 μl of formulation) demonstrated fast aggregation for the non-PEGylated formulation, we proposed that in vivo, due to the rapid dilution with the volume of blood, that aggregation would be prevented and physiological activity preserved. This was confirmed in the efficacy study (see further).

Biodistribution study

For biodistribution studies, 4T1 murine breast cancer cells were chosen to induce tumors as this is a well-established animal model closely mimicking human metastatic breast cancer, and it is fairly simple to induce these tumors quickly in female Balb/c mice. Figure 3 depicts the biodistribution 4 h postinjection of fluorescent siRNA loading with different formulations. Our data indicate an enhanced blood circulation for DOPE-PEI/PEG/siRNA complexes when compared with DOPE-PEI/siRNA complexes (22% injected dose (ID) vs 9% ID) and naked siRNA, for which no detectable fluorescence signal was measured. PEGylated formulations had significantly better tumor accumulation as compared with naked siRNA (8% ID vs 4% ID). DOPE-PEI nanocarriers delivered higher siRNA doses in the lung and liver as compared with PEGylated formulations (14% ID vs 6% ID and 19% ID vs 13% ID, respectively). Inclusion of PEG-PE in formulations shielded the non-specific interactions with these organs and led to % ID values similar to those of the naked siRNA. The highest fluorescent signal of about 20% ID was detected in the kidney where no difference between formulated and non-formulated siRNA accumulation was observed.

Development of MCF7/ADR and MCF7/S orthotopic and subcutaneous tumor models and quantitative reverse transcriptase–PCR (qRT-PCR) characterization

Due to the conflicting literature regarding the development of MCF-7/ADR tumors and the need for β-estradiol supplementation
for the growth of MCF7/ADR xenografts, we decided to first optimize tumorigenic conditions for MCF7/ADR cells in female nude mice. Mice were injected orthotopically in the mammary fat pad or subcutaneously over the right flank with these cells as described in the Materials and methods section. The tumors were allowed to develop for 5 weeks after which the mice were killed, and their tumors were excised. The tumors sizes in the two orthotopic tumors MCF-7/ADR were similar with or without estrogen supplementation (mean volume approximately 40 mm$^3$). The qRT-PCR data clearly showed that the MDR1 levels in the MCF7/ADR orthotopic tumors in the presence or absence of the estradiol were very similar suggesting that, in contrast to orthotopic MCF7/S tumors, the growth of MCF7/ADR orthotopic tumors was estrogen independent. The implantation of the estradiol pellet did not affect tumor volume or MDR1 gene expression 5 weeks posttumor inoculation. On the other hand, the subcutaneous tumors (about 100 mm$^3$) had a lower MDR1 level compared with the MCF7/ADR orthotopic tumors, albeit they had significantly higher MDR1 levels than the orthotopic MCF7/S tumors (Figure 4). The subcutaneous model was chosen for the final therapeutic efficacy experiments as larger tumors were obtained in a shorter period of time, and the tumors retained high MDR1 levels.

Therapeutic efficacy study
The therapeutic effect of siRNA targeting P-glycoprotein (siMDR1) formulated in DOPE-PEI nanocarriers (PEGylated and non-PEGylated) in combination with Dox was evaluated in resistant breast MCF-7/ADR tumors (Figure 5). The mice were randomized for treatment after the subcutaneous tumor volume reached 60 mm$^3$. The dosing regimen was one naked siRNA or formulation containing siRNA injection followed by one Dox injection after 48 h (sequential regimen). This dosing regimen was carried on for 5 weeks. Five days after the final dose, the mice were killed. The Dox effect was assessed by monitoring the tumor volume during the treatment and by the final weight of the postmortem excised tumors. Although, the primary goal of the study was to show improved therapeutic outcome using the formulation and not to elucidate the detailed mechanism, we clearly show the P-gp downregulation by quantifying the P-gp levels (mRNA and

Figure 2. Biophysical characterization of DOPE-PEI nanocarriers. (a) Schematic illustration of the nanocarriers’ assembly with the mean diameter and the zeta potential of the formulations. (b) Detailed structure and morphology of free siRNA, DOPE-PEI and PEG-PE components and assembled nanocarriers by AFM and TEM. (c) Stability of nanocarriers indicated by the change in size after incubation with 150 mM NaCl for up to 1 h at RT.

Figure 3. Biodistribution of fluorescein-labeled siRNA either naked or complexed in formulations 4 h postinjection. Results are expressed as the mean percentage of injected dose (% IDg) of tissue or ml of serum ± s.d. ($n=5$); *$P<0.05$, ***$P<0.001$ vs the naked siRNA group.
protein) as shown in previous studies by Lee et al., Gao et al., and Jiang et al.

The combination of DOPE-PEI or DOPE-PEI/PEG/siMDR1 and Dox resulted in sensitization of resistant tumors to Dox and improved its anti-cancer activity. Both treatments significantly inhibited the tumor growth relative to all control groups, including buffer control, free siMDR1+Dox and DOPE-PEI/scramble siRNA +Dox ($P < 0.001$). No significant difference was observed between PEGylated and non-PEGylated formulations. The treated mice showed a threefold reduction in tumor volume (mean relative tumor volume (RTV/m)) as compared with the control groups (Figure 5a) that was consistent with the half-reduction in tumor weight measured after the killing of the animals (Figure 5b). DOPE-PEI/scrambled siRNA had no therapeutic advantage, which demonstrates the relevance of the MDR1 siRNA sequence specificity for therapeutic efficacy. Free siMDR1 in combination with Dox did not show any significant difference with respect to the buffer control in terms of tumor inhibition, confirming our previous in vitro studies showing that the association of siRNA with DOPE-PEI nanocarriers is essential to deliver and internalize siRNA into tumor cells, mediate P-gp downregulation and restore Dox resistance.

To confirm P-gp (MDR1) downregulation in excised tumors, qRT-PCR was performed for evaluation of the transcriptional mRNA levels of the MDR1 gene (Figure 5c), and flow cytometry was performed to check the P-gp protein level expression on the surface of the tumor cells (Figure 5d). Free siMDR1 and DOPE-PEI/scrambled siRNA did not show any significant differences in P-gp suppression compared with buffer control. However, MDR1 mRNA levels were reduced by half in those tumors treated with DOPE-PEI/siMDR1 and almost completely abrogated after DOPE-PEI/PEG/siMDR1 treatment. Similarly, the overexpression of P-gp protein in resistant tumors was restored to the levels observed in sensitive tumor cells after DOPE-PEI/PEG/siMDR1 and DOPE-PEI/siMDR1 treatments. The slightly greater P-gp downregulation found for PEGylated nanopreparations did not correlate with a better response of tumors to Dox.

A second therapeutic experiment was performed to check the effect of the dosing regimen. In particular, we evaluated whether the different administration of the DOPE-PEI/siRNA nanocarrier and Dox, sequential or simultaneous, would have a different effect

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**Figure 4.** Levels of mRNA MDR1 in orthotopic and subcutaneous tumors measured 5 weeks after tumor implantation by qRT-PCR.

**Figure 5.** Anti-tumor effect of siRNA targeting P-glycoprotein (siMDR1) in DOPE-PEI nanocarriers and Dox using nude mice bearing MCF-7/ADR tumors. Mice were intravenously injected once per week with BHG, free siRNA or different DOPE-PEI formulations followed by Dox injection (after 48 h) for 5 weeks. (a) RTV and (b) tumor weight ratio for the different treatments. Results are plotted as mean ± s.d. ($n = 5$). (c) Levels of mRNA MDR1 and (d) P-glycoprotein at the final time point. Results are plotted as mean ± s.d. ($n = 5$, performed in duplicates and triplicates for panels (c) and (d), respectively). *$P < 0.001$ vs BHG, free siMDR1 and DOPE-PEI/scramble siRNA.
on P-gp suppression and tumor growth. With this in mind, three groups of female nude mice (n = 5) bearing MCF7/ADR tumor xenografts were treated as follows in a manner similar to the conditions in the previous therapeutic efficacy study: Group 1—naked therapeutic MDR1 siRNA+Dox administration at +48 h; Group 2—DOPE-PEI/MDR1 siRNA+Dox administration at +48 h (sequential); and Group 3—DOPE-PEI/MDR1 siRNA followed by immediate Dox injection (simultaneous). At day 20, the sequential administration of the treatments resulted in significantly better tumor inhibition than their simultaneous administration (fivefold and twofold tumor volume reduction, respectively, as compared with buffer control). However, at the end of the study (day 40), both regimes turned out to be equally effective with a threefold tumor volume reduction as compared with free siRNA+Dox treatment (Figure 6a). The anti-cancer activity was associated with significant downregulation of MDR1 observed in the excised tumor and was higher in the case of the co-administration group (Figures 6b and c).

To monitor the overall health of the animals, animal weights were recorded throughout the study. The levels of amino transferases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) in serum were also analyzed as indicator of repeated dosing toxicity in liver.37 The animals tolerated the treatments well. No significant loss in body weight or evidence of hepatotoxicity was observed (Tables 1 and 2).

**DISCUSSION**

Resistance to chemotherapy is a major cause of treatment failure and relapse of many cancer types, including breast cancer.

![Figure 6](image-url) Anti-tumor effect of siRNA targeting P-glycoprotein (siMDR1) in DOPE-PEI nanocarriers with Dox sequentially or simultaneously administered in nude mice bearing MCF-7/ADR tumors. (a) RTV for the different treatments. (b) Levels of mRNA MDR1 and (c) P-glycoprotein at the final time point. Results are plotted as mean ± s.d. n = 5, performed in duplicates and triplicates for panels (b) and (c), respectively. *P < 0.001 **P < 0.01 vs free siMDR1+Dox.

| Treatment                        | AST (IU l⁻¹) | ALT (IU l⁻¹) | % Change in body weight (from day 0) |
|----------------------------------|--------------|--------------|------------------------------------|
| Control                          | 11.2 ± 0.1   | 15.0 ± 0.1   | 1%                                 |
| Free siMDR1                      | 14.1 ± 0.3   | 16.9 ± 0.1   | 1%                                 |
| DOPE-PEI/scramble                | 12.9 ± 0.1   | 16.2 ± 0.1   | 1%                                 |
| DOPE-PEI/siMDR1                  | 13.2 ± 0.2   | 19.4 ± 0.3   | 7%                                 |
| DOPE-PEI/PEG/siMDR1              | 20.3 ± 0.5   | 21.2 ± 0.3   | 0.30%                              |

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Dox, doxorubicin; MDR, multidrug resistance; PEI, polyethylenimine.

Approaches based on a combination of chemotherapeutics with gene-silencing molecules to suppress the expression of efflux transporters, such as P-glycoprotein, are currently among the most widely investigated drug–nucleic acid combinations for cancer therapy.23,36,38–42 The translation of such combinations to animal models has been challenging due to an unfavorable siRNA pharmacokinetic profile, low tumor accumulation and a lack of safe and efficient delivery systems.

We recently showed that DOPE–PEI conjugates based on low-molecular-weight PEI self-assembled within micellar structures that condensed siRNA, had high transfection efficiency and had a better toxicity profile than PEI 25 kDa and Lipofectamine. The DOPE-PEI/anti-P-gp siRNA nanopreparations inhibited P-gp
expression and enhanced the intracellular delivery and the cell killing by Dox in resistant breast cancer cells (MCF-7/ADR).22 Here we tested their utility for systemic delivery of anti-P-gp siRNA to tumors in combination with Dox chemotherapy. We incorporated PEG-PE in the carriers to achieve enhanced tumor delivery and to improve their performance upon intravenous injection. We proposed that PEG-PE would spontaneously incorporate into DOPE-PEI due to the hydrophobic interactions and additionally provide some steric stabilization of complexes by forming a protective barrier, shielding the positive charge and promoting increased stability in high-salt conditions. The DOPE-PEI complexes with PEG-PE were stable in 150 mM NaCl with no significant change in size (Figure 2c). Hydrophobic interactions between the lipid moieties in DOPE-PEI and those in PEG-PE resulted in their self-assembly into micelle-like particles (Figure 2b). A similar hydrophobic interaction was suggested between the amphiphilic Pluronic chains of Pluronic-grafted PEI, which form a micelle-like structure around the polyplex core and unmodified Pluronic A biodistribution study was performed to investigate the in vivo behavior (circulation, tissue distribution, absence of acute toxicity) of DOPE-PEI nanocarriers and to test whether the effects of PEG inclusion, mainly hindrance of positive charge and improved stability, would be beneficial in vivo (Figure 3). The results of biodistribution studies of the different formulations of siRNA at 4 h postinjection have clearly confirmed prolonged circulation and good tumor accumulation for DOPE-PEI/PEG/siRNA preparations as compared with naked siRNA for which no fluorescence signal in the blood and low tumor accumulation were found, and our numbers are in good agreement with previous reported data,45–47 which showed tumor accumulation from 0.5% to 10% of the ID of labeled-free siRNA at similar time point despite its fast degradation and complete elimination from the blood. A decreased interaction with serum proteins, a lower accumulation in the lung and a decreased mononuclear phagocyte system uptake allowed the formulation to circulate in vivo for a longer time and resulted in greater accumulation in the tumor via the enhanced permeability and retention effect.23–25 Recently, two studies have reported on the biodistribution of PEG-grafted PEI 25 kDa/siRNA complexes in healthy mice.47,48 Similar to our observations, inclusion of PEG in PEI 25 kDa/siRNA formulations stabilized siRNA in the blood and decreased non-specific interactions with the lung and liver by shielding the positive charge of PEI complexes that otherwise can cause acute toxicity (erythrocyte aggregation, pulmonary embolism, hepatotoxicity).47,48 We found high levels of siRNA in the kidney (about 20% ID) regardless of the formulation. This is an expected phenomenon as naked siRNA is rapidly excreted by the kidneys.49 In the case of complexes, interaction between the negatively charged basal membrane of the Bowman’s capsule and the cationic complexes occurs.50

Once we evaluated the biodistribution of DOPE-PEI nanocarriers and confirmed that the inclusion of PEG improved the biodistribution pattern of siRNA (not only in terms of better tumor delivery but also by decreasing DOPE-PEI/siRNA non-specific interactions with the lung and liver), we moved to optimize the MCF-7/ADR animal model to test the therapeutic application of the nanocarriers using P-glycoprotein-siRNA/Dox combinations that previously showed positive results in MCF-7/ADR cell culture.22 Combination of DOPE-PEI or DOPE-PEI/PEG/siMDR1 and Dox resulted in sensitization of MCF-7/ADR-resistant tumors to Dox and improved in vivo anti-cancer activity. Weekly administration of 2 mg kg−1 of Dox-Free siMDR1 had no therapeutic effect, whereas the sequential administration of anti-P-gp nanopreparations and Dox mediated specific down-regulation of P-gp in the tumors and inhibited their growth (Figures 5a and b). The elevated P-gp levels exhibited by the resistant tumors were lowered to levels comparable to the MCF-7 sensitive cells (Figure 5d). In the case of DOPE-PEI/PEG/siMDR1, P-gp downregulation was even more pronounced (Figures 5c and d). Interestingly, the slightly greater P-gp downregulation found for PEGylated nanopreparations did not produce a greater anti-cancer effect. Both treatments significantly inhibited the tumor growth relative to all the control groups, but no significant difference was observed between the PEGylated and non-PEGylated formulations. A possible explanation for the absence of differences may be the use of low doses of Dox. The non-PEGylated formulation produced a robust suppression of P-gp. If the maximum Dox anti-cancer effect had already been achieved for this P-gp suppression, incremental P-gp suppression by a PEGylated formulation would not lead to an incremental anti-cancer effect. For this proof-of-concept study, we employed non-effective low doses of Dox (2 mg kg−1) to show the effectiveness of the addition of siRNA nanopreparations. Future experiments will test higher doses of Dox to help to discriminate different levels of P-gp down-regulation and optimize their anti-cancer activity. Jiang et al.36 demonstrated significant inhibition of tumor growth in the same MCF-7/ADR tumor model by using RGD-modified liposomes containing anti-P-gp siRNA (2 mg kg−1) or Dox (4 mg kg−1). Similar to our results, they found a threefold decrease in the RTVm compared with buffer controls and a twofold decrease in RTVm when compared with liposomal Dox alone. However, systemic toxicity (a 20% loss in weight) resulted from Dox treatments. In the case of MCF-7 sensitive tumors, Dox doses in the range of 1.5–5 mg kg−1 produced 50–60% tumor-inhibition rates.51–53

The rationale for the sequential administration of anti-P-gp siRNA and chemotherapy is that the inhibition of efflux mechanisms has to precede the chemotherapy treatment for the maximum anti-cancer effect. Therefore, a time lapse (24–48 h) between P-gp siRNA and the chemotherapy drug is usually included in the majority of the experimental protocols to allow for sufficient target protein downregulation. This notion is supported in vivo by studies in MDR tumors.30,34,55 On the other hand, the simultaneous delivery of siRNA and chemotherapy, mostly by the co-loading of the agents in a single carrier, has also been shown to effectively reverse MDR.41,56,57 The rationale behind this approach is that temporary co-localization of the anti-cancer drug against P-gp in the same cancer cell can promote a better anti-cancer activity. To date, in vivo studies that have investigated the effects of multiple injections and different regimes of administration on gene suppression and therapeutic responses mediated by siRNA/drug combinations are rare.58 There is a great need of a better understanding of the mechanistic and kinetic aspects involved in these anti-cancer combinations. The optimal sequence of administration of siRNA and drug for the maximum anti-cancer responses has not been elucidated yet. With this in mind, we compared the effect of sequentially or simultaneously administered siRNA nanopreparations and Dox on P-gp downregulation and anti-tumor activity over a period of 5 weeks of treatment (Figure 6). The administration mode had no

| Table 2. Evaluation of toxicity in mice of DOPE-PEI/siRNA nanopreparations+Dox after repeated dosing of treatments (sequentially or simultaneously administered) by measurement of changes in serum AST, ALT levels and body weight |
| Treatment | AST (IU l−1) | ALT (IU l−1) | % Change in body weight (from day 0) |
|-----------|-------------|-------------|----------------------------------|
| Free siMDR1+Dox | 14.0 ± 0.3 | 17.0 ± 0.1 | 1% |
| Sequential | 13.2 ± 0.2 | 19.4 ± 0.3 | 7.20% |
| Simultaneous | 11.0 ± 0.5 | 17.0 ± 0.2 | 0.19% |

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Dox, doxorubicin; MDR, multidrug resistance; PEI, polyethylenimine.
impact on the final therapeutic outcome. Both regimens turned out to be equally effective in inhibiting tumor growth. This is in agreement with our previous findings performed with MCF-7/ADR cells, where the activity of Dox was significantly increased by anti-P-gp siRNA/DOPE-PEI preparations regardless of the time lag (0–48 h) between the treatments. On the other hand, the co-administration of siRNA nanopreparations and Dox showed higher downregulation of P-gp in the tumors (Figures 6b and c). At low doses, Dox behaves like an anti-angiogenic agent, expanding the interstitial space, vessel diameter and blood perfused area. In the co-administration group, it is possible that the carrier penetration was improved by this phenomenon, especially if the carrier and the drug co-existed spatially. It has been shown that free Dox injected intravenously has a plasma half-life of about 16 ± 3 h in nude tumor-bearing mice, and our biodistribution data prove that DOPE-PEI nanoparciors can deliver siRNA to the tumor within 4 h (5% ID in tumor and 10% ID in the blood, Figure 3).

In conclusion, we demonstrated the usefulness of DOPE-PEI nanoparciors for intravenous delivery of siRNA to tumors. We showed that DOPE-PEI nanoparciors loaded anti-P-gp siRNA can be used to suppress P-gp activity and restore Dox sensitivity in resistant human breast cancer tumors. The nanopreparations had small particle sizes (<150 nm) compatible with parental administration and showed improved colloidal stability when PEG was incorporated in the formulation. In addition, PEGylated formulations showed prolonged circulation and improved siRNA tumor delivery via an enhanced permeability and retention effect as compared with non-PEGylated ones. DOPE-PEI–based nanopreparations, regardless of the presence of PEG, deliver sufficiently high amounts of siRNA to mediate specific P-gp downregulation in resistant tumors and restore their sensitivity to an otherwise non-effective doses of Dox. Simultaneous or sequential weekly administration of anti-P-gp siRNA nanopreparations and Dox were equally effective in inhibiting tumor growth over a period of 5 weeks and were well tolerated by the animals.

MATERIALS AND METHODS

Materials

All materials were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. Branched PEI60 with a molecular weight of 1.8 kDa was purchased from Polysciences, Inc. (Warrington, PA, USA). PEG-PE and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All siRNA duplexes were purchased from Dharmacon (Lafayette, CO, USA), namely, siRNA targeting the human MDR1 gene (sense) 5′-GGdTdT-3′ and (anti) 5′-AGUACUGCUUACGAUAC-3′. FITC-labeled P-glycoprotein antibody (UIC2) was purchased from Chemicon International (Temecula, CA, USA). A phospholipid-PEI/siRNA complex solution was prepared by adding a DOPE-PEI/siRNA complex solution at room temperature. A PEGylated formulation, namely, DOPE-PEI/PEG/siRNA (DOPE-PEI:PEG-PE was 1:10 w/w) and allowed to stand at room temperature for 30 min with intermittent shaking. The formulations were characterized for particle size distribution and zeta potential by DLS using a Malvern Zetasizer ZS90 Nano instrument (Malvern Instruments, Westborough, MA, USA). Briefly, 100 μl of freshly prepared formulation was diluted suitably in nuclease-free water, and the size and zeta measurements were obtained. The morphological properties of the nanopreparations were also evaluated using AFM and TEM.

Transmission electron microscopy

For TEM, 10 μl of the sample was placed on the Formvar-coated copper grids (Electron Microscopy Science, Hatfield, PA, USA) and negatively stained with 50 μl of 1% (w/v) uranyl acetate for 2–5 min. A Whatman filter paper was used to drain excess liquid; the grids were allowed to air-dry for a minute. Images were acquired using a JEOL 100lx transmission electron microscope (JEOL USA Inc, Peabody, MA, USA).

In vivo studies

Animals. Female Balb/c mice (6–8 weeks) were purchased from Charles River Laboratory (Wilmington, MA, USA). Animals were housed in sterile cages with ad libitum access to sterile food and water on a 12:12 light/dark cycle. All experiments were approved by the Northeastern University-Institutional Animal Care and Use Committee (NU-IACUC).

Biodistribution study

Female Balb/c mice were inoculated with 4T1 tumors by resuspending 2 × 10^7 cultured cells into sterile phosphate-buffered saline (100 μl) injected subcutaneously into the right hind flank of anesthetized animals. Once tumors had reached a palpable volume of at least 100 mm^3, mice were randomly assigned to one of the four treatment groups (naked siRNA, PEG-siRNA, DOPE-PEI/siRNA and DOPE-PEI/PEG/siRNA) all complexed with FL-siRNA at N/P 16 in a manner similar to that described previously under sample preparation. The siRNA dose was 1.2 mg kg^-1 (40 μg per animal). All the formulations were prepared in nuclease-free, filtered HEPES buffered glucose (HBG), pH 7.4. Mice were killed 4 h after treatment.
Organs were harvested, and the blood was collected via the cardiac puncture method. The organs were homogenized in cold HBSS, centrifuged (15 000 g for 20 min at 4 °C) to obtain a clear supernatant, and the serum was collected from the derived blood. The organ homogenates and serum were transferred to a 96-well plate, and the fluorescence from the samples was read in a fluorescence plate reader at λex 485/20 and λem 528/20. Fluorescence values were converted into percentage of ID per gm tissue (% ID g⁻¹ tissue or % ID ml⁻¹ of serum) from standard curves plotted separately for every tissue type, obtained by spiking varying amounts of FL-siRNA in the organ supernatants or serum.16,46 The percentage of recovery was approximately 80% for all the organs except for the tumor (56%).

Comparative evaluation of MDR1 levels in orthotopic vs subcutaneous MCF7/ADR tumor xenografts

MCF7 wild-type (MCF7/S) or MCF7/ADR cells were inoculated in three different groups (n = 3) of nude female mice. For each mouse, 5 × 10⁶ cells in complete DMEM media were resuspended into Matrigel from BD Biosciences (San Jose, CA, USA) to yield a total volume of 100 μl.

For the first group, this mixture was injected into the mammary fat pad of isoflurane anesthetized animals after which they were left to rest until tumors reached a palpable volume. For the second group, silastic tubing was cut into 1 cm × 1 cm lengths and one end was plugged with a silicone rubber sealant. Estradiol was mixed with cholesterol at a weight ratio of 1:1 and ground in a mortar and pestle. About 2.3 mg of the mixture was added into each tube, followed by sealing as before.35 The filled implants were wiped clean, sterilized with ultraviolet radiation overnight and stored sterile before use. These were then implanted surgically at the back of the neck of the three nude female mice. A couple of days were allowed for the implants to develop. The implants were then excised and processed for RT-PCR.

The treatment used were (1) Sterile HEPES buffered glucose 5% (HBG); (2) Free siMDR1+Dox; (3) DOPE-PEI/siMDR1+Dox; (4) DOPE-PEI/scramble siRNA)+Dox and (5) DOPE-PEI/PEG/siMDR1+Dox.

Toxicity evaluation in mice

One-way analysis of variance Bonferroni post hoc test was conducted which included a group of nude female mice that were simultaneously injected with DOPE-PEI/siRNA and Dox. We performed the same evaluations that were carried out in the previous therapeutic efficacy study, namely, RTVm, body weight monitoring, AST/ALT, MDR1 and P-gp expression. The treatments used were (1) Free siMDR1+Dox; (2) DOPE-PEI/siMDR1+Dox (sequential); and (3) DOPE-PEI/siMDR1+Dox (simultaneous).

Statistical analysis

Results are presented as mean ± s.d. Statistical analysis was performed using GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, CA, USA). One-way analysis of variance Bonferroni’s multiple comparisons test or two-way analysis of variance were used for data analysis. A P-value < 0.05 was considered to be statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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