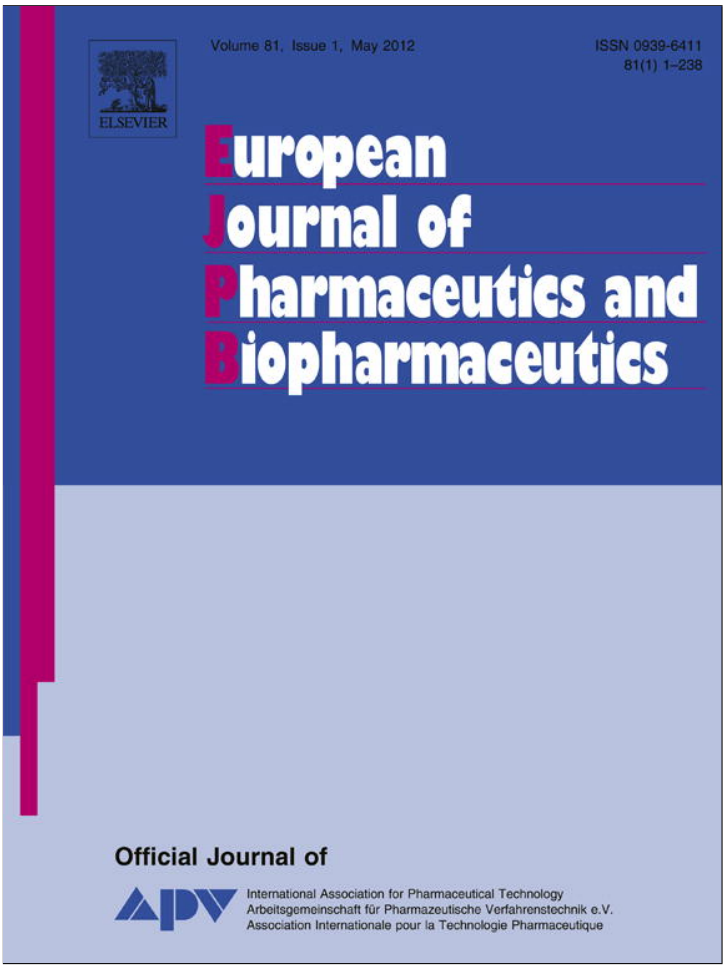

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Research paper

Effective down-regulation of Breast Cancer Resistance Protein (BCRP) by siRNA delivery using lipid-substituted aliphatic polymers

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ABSTRACT

Breast Cancer Resistance Protein (BCRP, ABCG2) is an efflux protein whose aberrant activity has been linked to multidrug resistance in cancer. Although siRNA delivery to down-regulate BCRP expression is promising to sensitize tumor cells against drugs, therapeutic use of siRNA requires effective carriers that can deliver siRNA intracellularly with minimal toxicity on target cells. This study explored the feasibility of special class of cationic polymers, namely lipid-substituted low molecular weight (2 kDa) polyethyleneimine (PEI), as a carrier for siRNA-mediated BCRP down-regulation. Structure–function studies methodically evaluated the effect of a range of lipophilic substitutions for siRNA delivery and BCRP down-regulation. Our results showed a significant increase in siRNA delivery as a function of lipid substitution for a range of lipids ranging from C8 to C18. The BCRP silencing was correlated to siRNA delivery efficiency of the polymers, and effectively lasted for ~5 days after a single treatment of siRNA. BCRP down-regulation sensitized the drug-resistant cells to cytotoxic effect of mitoxantrone by a ~14-fold decrease in the IC_{50} value, whose effect was evident even after 14 days. This study demonstrated the possibility of functional siRNA delivery by lipid-modified low molecular weight PEI and highlighted the importance of the extent and nature of lipid substitution in effective siRNA delivery.

1. Introduction

Down-regulating the activity of drug efflux proteins is a feasible strategy to reverse multidrug resistance (MDR) commonly observed in cancer patients [1,2]. These transporters efflux the drugs accumulate in tumor cells and reduce the intracellular concentration of therapeutic agents administered in patients, thereby reducing the effectiveness of the chemotherapy. Since the efflux proteins can accommodate a diverse class of compounds, the resistance is manifested against a multitude of drugs, rather than a specific drug employed in the chemotherapy. The expression of the efflux proteins is often up-regulated in response to elevated concentration of the substrates (i.e., drugs). Among the drug efflux proteins linked to clinical manifestation of MDR is the Breast Cancer Resistance Protein (BCRP) [3]. BCRP is a naturally occurring protein whose normal physiological distribution is consistent with its excretory role for its substrates [4]. Role of BCRP in limiting distribution of drugs into the central nervous system at Blood–Brain Barrier (BBB) has also been reported [5]. It has been shown that BCRP can play a major role in resistance to many drugs, including leflunomide and its metabolite A771726 (teriflunomide) [6], methotrexate and its main toxic metabolite (7-hydroxymethotrexate) [7], riluzole [8], and mitoxantrone (MTX) [9–11].

To diminish the BCRP activity, one can employ conventional inhibitory substrates that can reduce the efflux activity of the pump [12]. This approach, however, is limited by non-specific effects of these substrates on other cellular targets. A more specific approach could be short interfering RNA (siRNA)-based RNA interference (RNAi), where the expression of BCRP protein is directly suppressed at the translational level. RNAi is an evolutionary process of post-transcriptional gene silencing that can be triggered by siRNAs to mediate sequence-specific mRNA degradation [13]. Since the initial report in 1998 [14], RNAi has rapidly developed into an effective tool to down-regulate specific gene expression in a wide variety of target cells. Delivering siRNA to intracellular targets, however, has proven to be a challenging task. The rapid degradation of siRNAs in the extracellular environment with RNase A type nucleases combined with rapid renal clearance results in serum half-life of <30 min [15]. In addition, the poor cellular uptake of siRNA has made it a practically incompetent silencing agent. Advanced materials are needed for therapeutic siRNA delivery, and

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cationic polymers are attractive for this purpose since they can be tailored to neutralize the anionic charge of nucleic acids and do not raise the safety concerns associated with viral carriers.

The electrostatic interaction between the anionic phosphates in siRNA and cationic polymer can assemble siRNA molecules into nanoparticles suitable for cellular uptake. High molecular weight polyethyleneimine (PEIs, >25 kDa) is one class of polymers that have been shown to be effective siRNA delivery agents [16–18]. Unprotonated amines on the PEI structure create an opportunity for endosomal escape due to the "proton sponge effect" [19]. The high density of positive charges on PEI also facilitates strong binding to siRNA, which in turn creates a stronger protection effect against enzymatic degradation. However, the toxicity of high molecular weight PEIs has been an important obstacle for their clinical use [20–22]. Low molecular weights PEIs (<5 KDa) present acceptable toxicity profiles, but, unfortunately, the small polymers do not display efficacious siRNA delivery into cells. Incorporating hydrophobic moieties into polymer structure has been investigated to improve siRNA delivery, since hydrophobic substituents increase the interaction of polymers with lipophilic cell membranes and facilitate siRNA uptake (reviewed in [23]). A cholesterol-substituted 1.8 kDa PEI was previously reported to be effective for siRNA delivery [24], but the role of the lipid substituent on siRNA delivery could not be assessed due to lack of comparative studies with unmodified polymers. We recently reported lipid-substituted PEIs from a low molecular weight PEI (2 kDa; PEI2) and a range of fatty acids with varying chain lengths (from C8 to C18) as lipophilic substitutions [25]. The lipid-substituted PEI2s were employed for plasmid delivery *in vitro,* and the effect of hydrophobic modifications on transgene expression was carefully elucidated in that report [25].

In this study, we hypothesize that hydrophobic modification of PEI2 could increase the siRNA delivery efficiency and enable effective down-regulation of BCRP levels. Using a BCRP over-expressing cell model [26], we undertook a systematic approach to investigate the toxicity profile of the designed polymers, the siRNA delivery efficiency, and the BCRP down-regulation *in vitro*. The effect of siRNA treatment on IC_{50} (Inhibitory Concentration for 50% cell death) of a well-known BCRP substrate, MTX, was investigated in order to elucidate the functional outcome of the BCRP silencing.

2. Experimental procedures

2.1. Materials

The 2 kDa PEI (PEI2) (M_n: 1.8 kDa, M_w: 2 kDa), 25 kDa PEI (PEI25), anhydrous dimethylsulfoxide (DMSO), Caproyl chloride (C8; >99%), Palmitoyl chloride (C16; 98%), Octanoyl chloride (C18:1 9Z, 12Z; 99%), Linoleyl chloride (C18:2 9Z,12Z; 99%), Hanks' Balanced Salt Solution (HBSS with phenol red), trypsin/EDTA, heparin, EDTA, ethidium bromide, mitoxantrone dihydrochloride (MTX), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from SIGMA (St. Louis, MO). Stearoyl chloride (C18; >98.5%) was purchased from Fluka (St. Louis, MO). Clear HBSS (phenol red free) was prepared in house. Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX™-l, penicillin (10,000 U/mL), ultrapure agarose, and streptomycin (10 mg/mL) were from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was from VWR Laboratories (Ottawa, Ontario). The scrambled siRNAs used as controls were supplied by Gene Pharma Co. LTD (Shanghai, China). The Silencer® siRNAs versus ATP-binding cassette sub-family G member 2 (ABCG2; Breast Cancer Resistance Protein gene) were purchased from Ambion (catalog numbers: s18056, s18057, and s18058). The Phycoerythrin-labeled monoclonal anti-human BCRP antibody (catalog number: FAB995P) was purchased from R&D Systems Inc. (Minneapolis, MN).

2.2. Cell Line

Wild-type and BCRP-transfected Madin–Darby Canine Kidney (MDCK) cells were kindly provided by Dr. Alfred H. Schinkel (The Netherlands Cancer Institute). The preparation and characterization of the BCRP-expressing cell line was previously reported [26], where an IRES promoter was used to derive co-expression of BCRP and the reporter Green Fluorescent Protein (GFP). The cells were grown in 37 °C and 5% CO_2 using a high glucose DMEM medium with L-glutamine substituted with GlutaMAX™-l on a molar equivalent basis, with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin added. Cell culture was considered confluent when a monolayer of cells covered more than 80% of the flask surface ($\sim 8 \times 10^5$ cells). To propagate the cells, monolayer was washed with HBSS and subsequently incubated with 0.05% Trypsin/EDTA for 10 min at 37 °C. The suspended cells were centrifuged at 600 rpm for 4 min and were re-suspended in the medium after the removal of the supernatant. The suspended cells were sub-cultured at 10% of the original count.

2.3. Synthesis and characterization of lipid-substituted polymers

The synthesis of lipid-substituted polymers was described elsewhere [25]. Briefly, a 50% PEI2 solution was purified by freeze-drying, and substitution was performed by N-acylation of PEI with commercially available lipid chlorides. Acid chlorides were typically added to 100 mg of PEI in anhydrous DMSO at given lipid:PEI amine ratios (0.066, 0.1, and 0.2). The mixture was allowed to react for 24 h at room temperature under argon, after which excess ethyl ether was added to precipitate and wash the polymers. The substituted polymers were dried under vacuum at ambient temperature overnight. Polymers were analyzed by ^{1}H NMR (Bruker 300 MHz; Billerica, MA) in D_2O. The characteristic proton shift of lipids ($\delta \sim 0.8$ ppm; —CH_3) and PEI ($\delta \sim 2.5$–2.8 ppm; NH—CH_2—CH_2—NH—) were integrated, normalized for the number of protons in each peak, and used to determine the extent of lipid substitutions on polymers. The polymers used in this study were designated as PEI2-XXY.Y, where XX refers to the lipid substituted and Y.Y to the level of substitution (e.g., PEI-CA6.9 refers to CA substitution at 6.9 lipids/PEI2).

The binding affinity to scrambled siRNA, the hydrodynamic diameter and ζ-potential of the siRNA complexes, as well as the stability of siRNA complexes after exposure to serum (as an indication of the ability of the polymer to protect siRNA against degradation) were measured for all synthesized polymers, and the results are reported elsewhere [27].

2.4. Evaluation of polymer cytotoxicity

The cytotoxicity of the polymers was evaluated in both wild-type and BCRP-positive MDCK cells using the MTT assay. Confluent cell cultures were trypsinized, seeded in 48 well plates with 0.2 mL medium in each well, and allowed to reach ~80% confluence ($\sim 8 \times 10^5$ cells) (1–2 days). Polymer/siRNA complexes were prepared using the scrambled siRNA at polymer:siRNA ratio of 8:1 and were added to the wells to give final polymer concentrations of 1.25, 2.5, 5, and 10 μg/mL in triplicate wells. Cells were incubated for 24 h in their normal maintenance conditions and then 40 μL of MTT solution (5 mg/mL in HBSS) was added to each well. After 2 h of incubation in 37 °C, the medium was removed, and 500 μL of DMSO was added to each well to dissolve the crystals formed. The optical density of the wells was measured with an ELx800 Universal Microplate Reader (BioTek Instruments; Winooski, VT, USA) with cell-less medium as blank. The absorbance of polymer-treated cells was compared to untreated cells (as 100% viability), and %cell viability was calculated. The results are summarized as a function of total

polymer concentration (including lipid weights) or as PEI concentration (excluding lipid weights).

2.5. Cellular uptake of siRNA

The cell uptake of the complexes was only evaluated in the wild-type cell line, due to the strong green fluorescence of the BCRP-transfected cells that interfered with the analytical method. Confluent wild-type MDCK cell cultures were trypsinized and re-suspended as described before, and seeded in 48 well plates (0.35 mL in each well) at ~50% confluency (~5×10^5 cells). After 24 h, medium was removed from all cells and 200 μL fresh medium was added to each well, followed by the addition of polymer/siRNA complexes. The complexes were prepared in sterile tubes using both FAM-labeled scrambled siRNA and non-labeled scrambled siRNA (as a negative control) with polymer:siRNA ratios of 2:1 and 8:1 (corresponding 36 nM siRNA and 1 and 4 μg/mL polymer in culture medium). The N:P ratio for the indicated weight ratios was ~15.5 and ~61.9 for native PEIs, respectively (assuming 1 μg of siRNA has 3 nMol of phosphate, and 1 μg PEI has 23.2 nMol of amine nitrogen) [28]. The N:P ratios for the other complex preparations are provided in Supplementary Fig. 1. The prepared complexes were added to wells in triplicates and were incubated in 37 °C for 24 h. After the incubation period, cells were washed with HBSS (×3) and trypsinized. A 3.7% formaldehyde solution was added to suspended cells, and the siRNA uptake was quantified by a Beckman Coulter QUANTA SC flow cytometer using the FL1 channel to detect cell-associated fluorescence. The percentage of cells showing FAM-fluorescence, and the mean fluorescence in the total cell population was determined. Analysis was performed by calibrating gating to the negative control (i.e., "No Treatment" group) such that the autofluorescent cell population represented 1–2% of the total cell population.

2.6. BCRP knockdown in BCRP-positive MDCK cells

Confluent cell cultures were trypsinized and seeded in 24 well plates (500 μL in each well) at ~50% confluency (~5×10^5 cells). After 24 h, the medium was removed and 200 μL of fresh medium was added to each well. The polymer/siRNA complexes were prepared in sterile tubes using both scrambled siRNA (as a negative control) and a cocktail of the three different BCRP-specific siRNAs with polymer:siRNA ratios of 2:1 and 8:1 (corresponding to a total of 36 nM siRNA with 12 nM of each BCRP-specific siRNA, with 1 and 4 μg/mL polymer in cell culture medium, respectively) and were added to the wells in triplicates. The plates were incubated in 37 °C for 48 h, after which the medium was removed and cells were washed with HBSS and trypsinized, and transferred to separate tubes for each well. Cells were then centrifuged at 1200 g for 4 min to remove the supernatant and were then washed (×3) with PBS supplemented with 0.5% Bovine Serum Albumin (BSA). Cells were then re-suspended in 50 μL of the same PBS/BSA buffer, and 4 μL of the Phycoerythrin-labeled anti-human BCRP antibody was added to each tube. Tubes were incubated at 2–8 °C for 45 min, were washed (×2) with PBS/BSSA buffer, and then were fixed with 3.7% formaldehyde solution. The BCRP down-regulation was quantified by the flow cytometer using the FL1 channel to detect the fluorescence of GFP and FL2 channel for the antibody label. The percentage of cells positive for the label and the mean fluorescence in the total cell population were determined. Analysis was performed by calibrating gating to the negative control (i.e., "No Treatment" group) such that the autofluorescent cell population represented 1–2% of the total cell population.

2.7. Cytotoxicity of MTX

The cytotoxicity of MTX was evaluated in wild-type and BCRP-positive cells. The cells were exposed to polymers alone as well as polymer/siRNA complexes under same conditions. MTX was dissolved in isotonic NaCl for a stock solution of 1 mg/mL. Confluent wild-type and BCRP-positive MDCK cell cultures were trypsinized and re-suspended as described before, and seeded in 48 well plates (0.35 mL in each well) at ~50% confluency (~5×10^5 cells). After 24 h, 200 μL fresh medium was added to each well, followed by the addition of MTX in different concentrations ranging from 12.5 to 100,000 ng/mL. Cells were incubated for 24 h in their normal maintenance conditions, and then the MTT assay was performed as described for polymer:siRNA cytotoxicity evaluation. Inhibitory Concentration for 50% cell death (IC_{50}) was calculated based on the cell viability curve. To evaluate the effect of BCRP down-regulation on the IC_{50} of MTX, BCRP-positive cells were treated with the polymer/siRNA complexes as explained before, and the cytotoxicity assay was performed after 48 h exposure to siRNA. The sensitivity factor and residual resistance were calculated using the following equation:

$$\text{sensitivity factor} = IC_{50}(\text{MTX in BCRP cells})/IC_{50}(\text{MTX} + \text{siRNA in BCRP cells}),\ \text{and}$$

$$\text{residual resistance} = IC_{50}(\text{MTX} + \text{siRNA in BCRP cells})/IC_{50}(\text{MTX in wild-type cells}).$$

2.8. Statistics

The compiled data were presented as mean ± SD. Where feasible, the data were analyzed for statistical significance by unpaired Student's *t*-test (assuming unequal variance). The level of significance was set at $\alpha = 0.05$. Correlation factor was also calculated for the interrelated data to evaluate any potential causal relationship(s).

3. Results

Three levels of lipid substitutions (with lipid:PEI amine mole ratios of 0.066, 0.1 and 0.2) were performed on the PEI2 with caprylic acid (CA), palmitic acid (PA), oleic acid (OA), and linoleic acid (LA) based on the methodology described elsewhere [25]. A general increase in lipid substitution was observed as the lipid:-PEI ratio was increased during the synthesis (Supplementary Fig. 1), and the highest number of lipids substituted was achieved with CA at lipid:PEI amine ratio of 0.2 (6.9 CAs/PEI2, corresponding to modification of 6.9 amines out of possible 14 primary amines on PEI). Based on a SYBR Green II dye binding assay [29], complete siRNA binding was achieved typically at polymer:siRNA ratio of ~0.5 for all polymers. At polymer:siRNA ratio of 1:1, all lipid-substituted polymers showed complete protection against degradation, while naked siRNA was readily degraded (<5% intact siRNA remaining) and only ~68% of siRNA bound with PEI2 remained intact. Particle size analysis showed a range of 300–600 nm for siRNA particles formed with all lipid-substituted polymers with no clear effect of lipid substitution on particle sizes. Our previous report provides detailed information on these characteristics [27].

Since cytotoxicity is a major concern for polymers employed for siRNA delivery, the MTT assay was performed with current cell

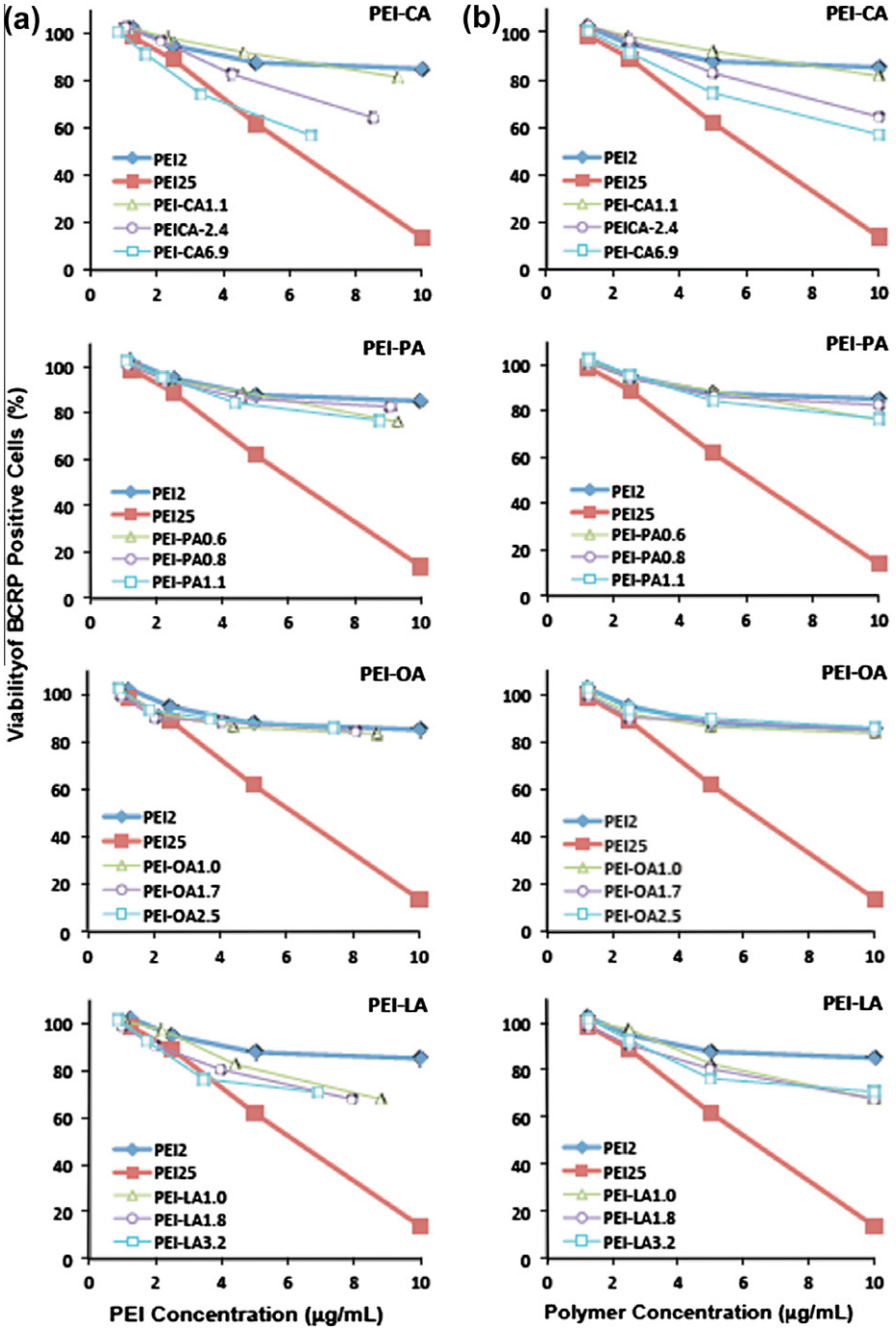


Fig. 1. Polymer toxicity. The viability of the BCRP-transfected MDCK cells after 24 h exposure to polymer/siRNA complexes prepared using increasing polymer concentrations and scrambled siRNA. The polymer concentrations were expressed as either effective PEI concentration (excluding the lipid component (a) or total polymer concentration (including the lipid component (b). While PEI25 was obviously toxic to the cells at concentrations above 2.5 μg/mL, the toxicity profiles of the lipid-substituted polymers were generally similar to the relatively non-toxic PEI2. The CA-substituted polymers showed most toxicity among the lipid-substituted polymers and, at low PEI concentrations, matched the toxicity of the PEI25. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

models to determine the toxicity of the synthesized polymers compared to PEI2 and PEI25. Fig. 1 summarizes the cell viability of the BCRP-positive MDCK cells after 24 h exposure to a range of polymer concentrations as complexes with scrambled siRNA. The toxicity data are summarized as a function of effective PEI concentration (Fig. 1a) or total polymer concentration (Fig. 1b). PEI2 showed little toxicity even at 10 μg/mL (>85% cell viability), while the toxic effect of PEI25 was evident at 5 μg/mL, and only 13.8% BCRP-positive cells survived at 10 μg/mL PEI25. Lipid substitution did not affect the toxicity of OA- and PA-substituted polymers, but an increase in toxicity was evident for CA- and LA-substituted PEI2. With CA, increasing substitutions gave higher toxicity and even matched the toxicity of PEI25 for the highest substituted CA (6.9 CAs/PEI2). A lower toxicity of CA-substituted polymers (compared to PEI25) was evident when one considered the total polymer concentration exposed to the cells (Fig. 1b). The

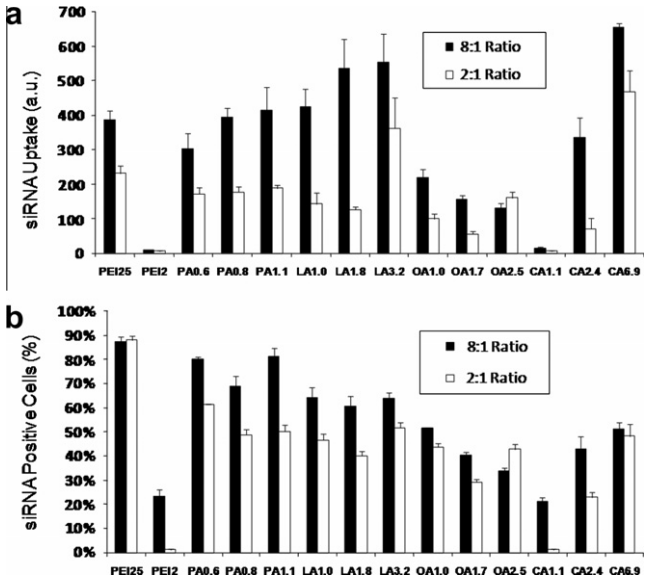


Fig. 2. Cellular uptake of polymer/siRNA complexes; (a) The mean fluorescence of the wild-type MDCK cells after 24 h exposure to complexes formed with FITC-labeled siRNA at polymer:siRNA ratios of 2:1 and 8:1 (weight/weight). (b) The percentage of cells positive for FITC-siRNA after 24 h exposure to siRNA complexes. Hydrophobic modification enhanced the siRNA cellular uptake significantly, even more than the uptake with PEI25 (in case of LA-substituted polymers and PEI2-CA6.9). In general, siRNA uptake was more significant with the polymer:siRNA ratio of 8:1.

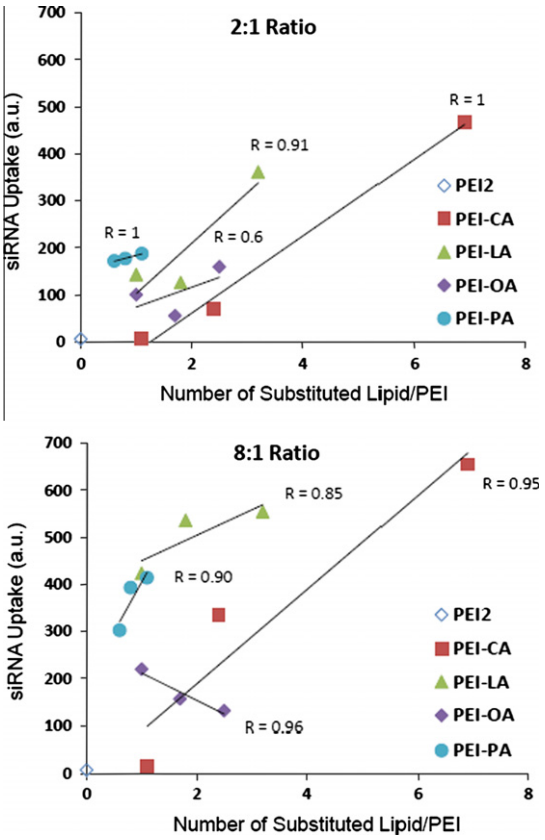
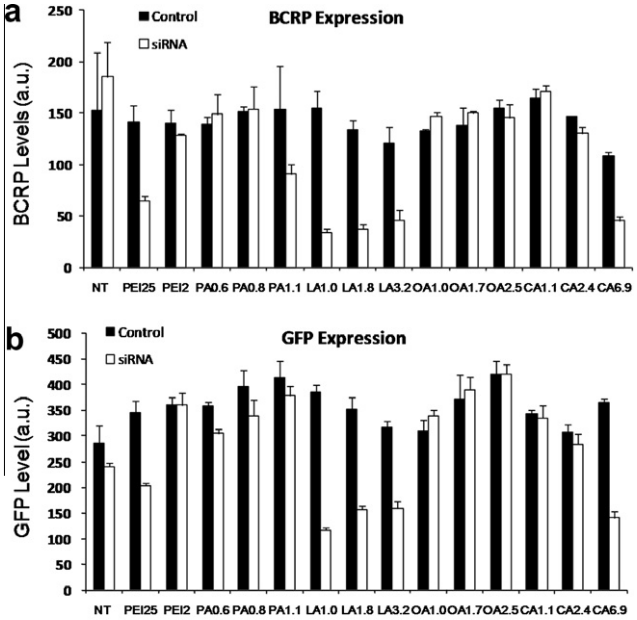


Fig. 3. Correlation between polymer substitution level and siRNA cellular uptake of polymer/siRNA complexes; (a) The correlation between the extent of cellular uptake (i.e., mean fluorescence from Fig. 2a) and the extent of lipid substitution based on the number of lipids per PEI for polymer:siRNA ratios of 2:1 (a), and 8:1 (b). There was a positive correlation between the substitution level and uptake at ratio of 2:1 for all hydrophobic moieties; such a correlation was not observed for OA-substituted polymers at 8:1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

LA-substituted PEI2 also gave equivalent toxicity to PEI25 at low concentration, which did not cause additional toxicity at higher concentrations. At the highest total polymer concentrations (10 μg/mL), >60% cell viability was retained for all lipid-substituted polymers, indicating an overall better response as compared to PEI25. Similar observations were noted when the toxicity studies were conducted with wild-type cells (Supplementary Fig. 2).

The efficiency of lipid-substituted polymers for siRNA delivery was evaluated at the polymer:siRNA ratios of 2:1 and 8:1 (Fig. 2). The PEI2 showed minimal siRNA delivery capability at both ratios, while PEI25 was among the effective polymers. The polymer:siRNA ratio of 8:1 was generally more effective in siRNA delivery to the cells as compared to the 2:1 ratio. Among the lipid-substituted polymers, LA- and CA-substituted polymers showed the highest siRNA delivery, and OA-substituted polymers had the lowest efficacy. For the effective polymers, the highest level of substitution (3.2 LA/PEI2 and 6.9 CA/PEI2) showed the highest delivery efficacy. Note that these complexes also had lowest N/P ratios among the complexes with the same weight ratio (Supplementary Fig. 1), indicating the lipid component rather than N-content affecting the uptake. Fig. 2b summarizes the percentage of siRNA-positive cells for the evaluated polymers. Polymer:siRNA ratio of 8:1 showed higher percentages of cells with siRNA, and lipid-substituted polymers gave a maximum of ~80% siRNA-positive cells. The correlation between the cellular siRNA uptake (based on mean fluorescence of cells) and the level of substitution (based on number of substituted lipids per PEI) is summarized for ratios of 2:1 and 8:1 in Fig. 3a and b, respectively. For polymer:siRNA ratio of 2:1, a relatively strong correlation was observed for all substituted lipids (the lowest correlation factor was 0.6), indicating a higher siRNA delivery with increased lipid substitution. At the polymer:siRNA ratio of 8:1, however, OA-substituted polymers (which were the least effective polymers) showed a reverse trend, showing a lower uptake with an increase in substitution level ($R = 0.96$). For the rest of the polymers, a positive correlation between the level of substitution and siRNA delivery was observed. Significant differences observed in siRNA uptake between PEI2, PEI25, and PEI-LA were also confirmed by fluorescent microscopy (Supplementary Fig. 3).

The feasibility of down-regulating BCRP levels was evaluated after 48 h of treatment with polymer/siRNA complexes prepared at 8:1 ratio. Since the cells were prepared by an IRES plasmid expressing both GFP and BCRP, a strong GFP fluorescence was observed with the BCRP-positive cells in flow cytometry. Figs. 4a and b show the BCRP and GFP protein levels, respectively, after the treatment of BCRP-positive cells with siRNA complexes of different polymers. We observed that BCRP-specific siRNA delivery also caused a parallel down-regulation of GFP levels for select polymers. Similar to siRNA delivery results, PEI2 had minimal effect on the BCRP and GFP levels, while PEI25 was effective in down-regulating both GFP and BCRP protein levels. Among the lipid-substituted polymers, PEI-LA polymers and PEI-CA6.9 were the only efficient carriers for down-regulating the protein levels. Similar results were also obtained based on the analysis of BCRP-positive cell population obtained after siRNA treatment (Supplementary Fig. 4): the LA-substituted polymers gave the most effective BCRP down-regulation, and PA- and OA-substituted polymers were least effective. The extent of BCRP and GFP down-regulations obtained is summarized in Fig. 4c (calculated as a percentage of BCRP/GFP levels from specific siRNA delivery with respect to non-specific siRNA delivery). Increasing the lipid substitution level had a significant impact on improving siRNA efficacy for CA- and PA-substituted polymers, where only the highest CA- and PA-substituted polymers



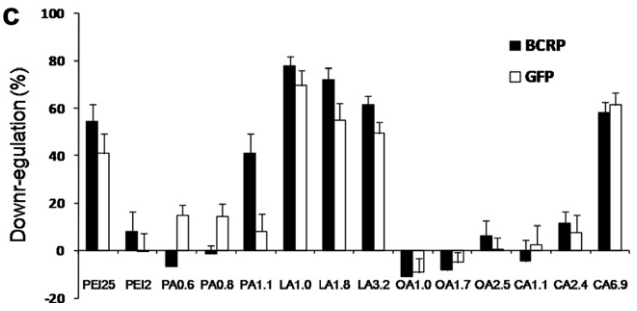


Fig. 4. Down-regulation of BCRP AND GFP expression by polymer/siRNA complexes; a and b. The BCRP (a) and GFP (b) levels in BCRP-transfected cells after 48 h exposure to polymer/siRNA complexes at polymer:siRNA ratio 8:1. The black bars represent BCRP levels for scrambled siRNA-treated cells, whereas the white bars represent cells treated with BCRP-specific siRNA cocktail. NT (No Treatment) refers to cells treated with buffer alone. (c) The level of BCRP and GFP down-regulation in BCRP-transfected MDCK cells after 48 h exposure to complexes. The level of down-regulation was calculated as a percentage of protein levels in cells treated with scrambled siRNA complexes (calculated based on data presented in panels a and b).

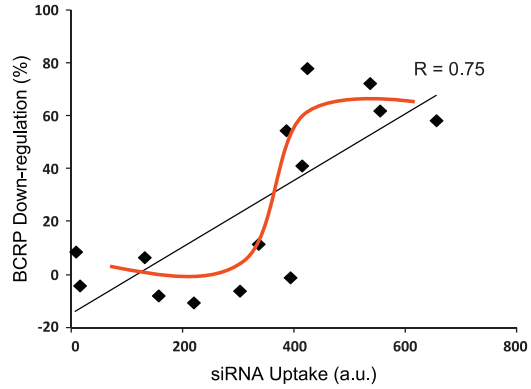


Fig. 5. Correlation between siRNA cellular uptake and BCRP down-regulation; The correlation between the extent of the siRNA cellular uptake (calculated based on the mean fluorescence presented in Fig. 2a) and BCRP down-regulation (data from Fig. 4c) for the polymer:siRNA ratio of 8:1. A strong correlation ($R = 0.75$) was observed indicating a higher down-regulation achieved with an increase in siRNA cellular delivery. However, a sigmoidal shape for the correlation curve also indicates a lag phase for low cellular uptake levels and a plateau for high cellular uptakes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

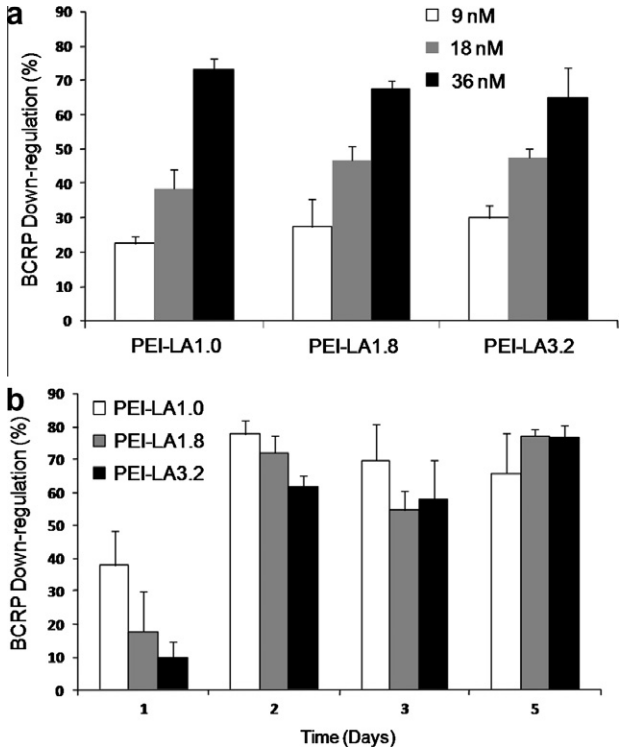


Fig. 6. Down-regulation of BCRP expression evaluated with different siRNA doses and at different time points; (a) The BCRP down-regulation levels in BCRP-transfected cells after 48 h exposure to polymer/siRNA complexes at polymer:siRNA ratios of 8:1 and siRNA doses of 9, 18, and 36 nM. Data indicate a linear dose–effect relationship for siRNA silencing of BCRP in the evaluated siRNA dose range. (b) The BCRP down-regulation levels in BCRP-transfected cells after 24, 48, and 72 h, and 5 days exposure to polymer/siRNA complexes at polymer:siRNA ratios of 8:1 and siRNA dose of 36 nM. The down-regulating effect seems to peak after 48 h and plateau for the time period evaluated in this study.

were effective in silencing BCRP expression. The LA-substituted polymers were all effective, but a reverse trend between the substitution level and down-regulation was obtained: PEI-LA3.2 gave a lower BCRP down-regulation (61.7%) compared to PEI-LA1.0 (77.8% down-regulation) and PEI-LA1.8 (72.1% down-regulation). OA-substituted polymers were ineffective in BCRP down-regulation. The LA-substituted polymers (i.e., the most successful polymers) were also evaluated at the polymer:siRNA ratio of 2:1 as well. Only PEI-LA3.2 showed a small but significant down-regulation of BCRP/GFP, and other polymers (including PEI25) showed no significant BCRP/GFP down-regulation (Supplementary Fig. 5).

The correlation between the BCRP down-regulation (based on mean fluorescence of cells) and the siRNA uptake (from Fig. 2a for ratio 8:1) is summarized in Fig. 5. A strong correlation ($R = 0.75$) between the BCRP down-regulation and siRNA uptake was observed when all polymers were considered. However, a closer look at the data revealed a sigmoid pattern that showed little change for BCRP down-regulation when the siRNA uptake was less than 400 (a.u.). After a sharp increase at this threshold, BCRP down-regulation plateaued for siRNA uptakes higher than ~450 (a.u.).

In order to evaluate the effect of siRNA dose on BCRP down-regulating, we conducted a separate experiment with three different siRNA concentrations (9, 18, and 36 nM) with the LA-substituted polymers and determined the BCRP protein levels after 48 h (Fig. 6a). There was no significant difference between the three different polymers studied at any of the doses. The data indicate a linear relationship between the siRNA concentration and BCRP down-regulation in the studied range (lowest $R^2 = 0.962$). A similar study was designed to evaluate the kinetics of BCRP down-

Table 1
IC_{50} and sensitivity factor for MTX alone in wild-type and BCRP-positive cell lines, and in combination with polymer:siRNA complexes and controls in the BCRP-positive cell line ($n = 3$).

	IC_{50}	Sensitivity factor[a]	Residual resistance[b]
MTX in wild-type cells	2.71	–	–
MTX in BCRP-positive cells	79.68	–	29.40
MTX + PEI-LA1.0/Scrambled siRNA	81.95	–	30.24
MTX + PEI-LA1.8/Scrambled siRNA	77.36	–	28.55
MTX + PEI-LA3.2/Scrambled siRNA	72.24	–	26.66
MTX + PEI-CA6.9/Scrambled siRNA	73.48	–	27.11
MTX + PEI-LA1.0/BCRP siRNA	6.68	11.93	2.46
MTX + PEI-LA1.8/BCRP siRNA	5.72	13.92	2.11
MTX + PEI-LA3.2/BCRP siRNA	17.77	4.48	6.55
MTX + PEI-CA6.9/BCRP siRNA	16.42	4.85	6.05

[a] Sensitivity factor = IC_{50} (MTX in BCRP cells)/IC_{50} (MTX + siRNA in BCRP cells).
[b] Residual resistance = IC_{50} (MTX + siRNA in BCRP cells)/IC_{50} (MTX in wild-type cells).

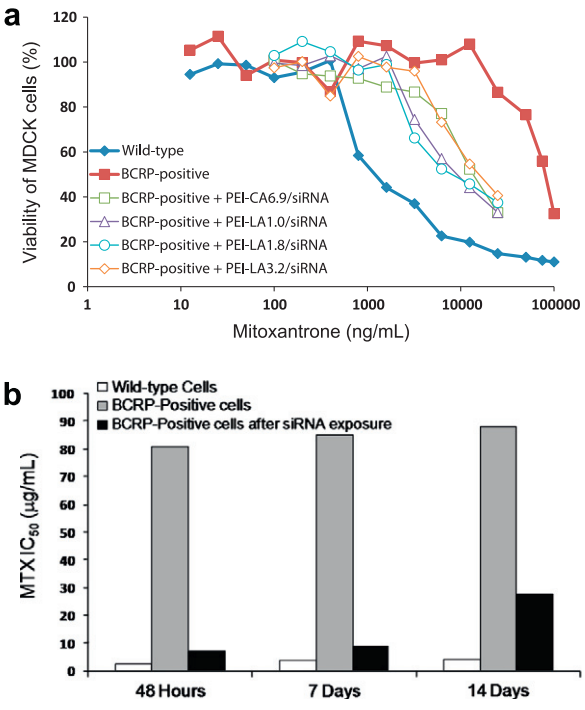


Fig. 7. The effect of BCRP down-regulation on mitoxantrone (MTX) cytotoxicity. (a) The IC_{50} of MTX in wild-type and BCRP-transfected MDCK cells, and the IC_{50} of MTX in BCRP-transfected MDCK cells after exposure to polymer/siRNA complexes prepared at polymer:siRNA ratios of 8:1, siRNA dose of 36 nM, and different polymers. A significant decrease in MTX IC_{50} after siRNA exposure indicates a sensitizing effect for the cytotoxic effect of MTX. (b) The IC_{50} of MTX in wild-type and BCRP-transfected cells, and the IC_{50} of MTX in BCRP-transfected cells after 48 h, 7 and 14 days exposure to a single dose of polymer/siRNA complexes prepared at polymer:siRNA ratios of 8:1, siRNA dose of 36 nM, and PEI2-LA1.0 polymers. A slight increase in IC_{50} of MTX in siRNA-treated cells after 7 days indicates a "wearing off" in the silencing effect, which is confirmed with the significant increase in IC_{50} of MTX after 14 days of exposure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

regulation, by determining the BCRP levels at different time points after a single exposure to siRNA complexes with LA-substituted polymers (Fig. 6b). Relatively, little (<38%) down-regulation was evident after 24 h exposure to siRNA (again with an inverse trend between the level of LA substitution and BCRP down-regulation). The BCRP down-regulation peaked after 48 h and remained constant up to 5 days. There was no significant difference between the down-regulation achieved with different polymers in this period.

The cytotoxic effect of MTX and its IC_{50} was evaluated in both wild-type and BCRP-positive MDCK cells using a wide range of MTX concentrations (12.5–10.0000 ng/mL), and the results are summarized in Table 1 and Fig. 7a. As expected, the efficacy of MTX in the BCRP-positive MDCK cells was significantly compromised, with an IC_{50} that was almost 30-folds higher than the wild-type cells. Pre-exposure of BCRP-positive cells to polymer/scrambled siRNA did not change the IC_{50} of MTX significantly (a range of 73–82 μg/mL for the studied polymers compared to 80 μg/mL for no treatment cells). However, exposure of BCRP-positive cells to BCRP-specific siRNA complexes (36 nM siRNA) for 48 h before the addition of MTX decreased the IC_{50} of MTX significantly. The sensitivity factor was increased by 11.9 and 13.9 for complexes prepared with PEI-LA1.0 and PEI-LA1.8, respectively (Table 1). To determine the stability of BCRP-mediated drug sensitization, the cells were exposed to a single dose of siRNA complexes (36 nM) with PEI-LA1.0 and the MTX treatment was performed after 2, 7, and 14 days (Fig. 7b). After 2 days, the results were similar to previous data summarized in Table 1 (IC_{50} of 2.7, 80.9, and 7.0 μg/mL for wild-type cells, BCRP-positive cells and siRNA-treated BCRP-positive cells, respectively). While the IC_{50} on day 7 was similar to the IC_{50} on day 2 for wild-type (4.0 μg/mL) and BCRP-positive cells (84.9 μg/mL), the IC_{50} was slightly increased in siRNA-treated BCRP-positive cells (9.1 μg/mL compared to 7.0 μg/mL at 2 days). This change in IC_{50} was more significant after 14 days, where the IC_{50} for siRNA-treated BCRP-positive cells was increased to 27.5 μg/mL. However, even after 14 days, there was a significant increase in the sensitivity factor (3.2) for the siRNA-treated BCRP-positive cells.

4. Discussion

The role of BCRP in MDR has been closely scrutinized in recent years. Although BCRP was discovered in a breast cancer cell line (MCF7/AdrVp; [30]), this membrane protein has been shown to be expressed in different types of leukemia and solid tumor cells [31,32]. The endogenous BCRP has been postulated to have an excretory role to protect body against harmful xenobiotics [4]. The specific substrates for each of the ABC family of transports have been elucidated [33,34], and MTX and topotecan are among the anticancer agents that are substrates for BCRP [32]. The BCRP was additionally proposed to play a key role in chemoresistance in cancer stem cells [35], so that this membrane protein might be critical for long-term resiliency of the transformed cells. In this manuscript, we report our studies to elucidate the effectiveness of lipid-substituted polymers to down-regulate BCRP expression as a strategy to sensitize drug-resistant cells to chemotherapeutic agents. We previously reported the synthesis and characterization of a set of polymers prepared by hydrophobic modification of a low molecular weight PEI [25]. For the present study, a BCRP-transfected cell line was employed to evaluate the siRNA-mediated silencing effect of our delivery system.

It has been hypothesized that PEI and similar polycationic polymers increase cellular uptake of nucleic acids via the formation of transient nanoscale holes in cell membrane, which allows material exchange across the cell membrane [36]. The same destabilizing effect has been proposed as the mechanism of cytotoxicity of these polymers [37]. It is, therefore, not surprising that the more efficient polymers in delivering nucleic acid are also more cytotoxic in general. Hydrophobic modification has been reported as a strategy to increase the efficacy of polymeric carriers for delivering nucleic acids [23]. Hydrophobic moieties are speculated to enhance the

interaction between the polymeric carrier and the lipophilic cell membrane, which in turn increase the cellular uptake of the polymer-associated nucleic acid. There are several reports on increased toxicity of low molecular weight PEIs with hydrophobic modification [25,38,39]. We previously reported the toxicity profile of the same modified polymers in a P-glycoprotein (P-gp) over-expressing breast cancer cell line (MDA-MB-435/MDR), where lipid substitution on PEI2 increased the toxicity of the complexes, especially for CA- and OA-substituted PEIs; however, the observed toxicity of the complexes with lipid-substituted polymers was significantly lower than the PEI25 complexes [27]. The same pattern was observed in this study in both wild-type and BCRP-positive MDCK cells, so that a similar pattern of toxicity seemed to be emerging in different cell lines. The enhanced toxicity in this study manifested itself at the low concentrations, which did not proportionally increase at higher concentrations. Since the effective BCRP silencing was achieved with total polymer concentration of ~4 μg/mL, the modified polymers proved to be relatively safe at the effective concentrations.

We observed that some hydrophobically modified polymers (not all) were quite effective in siRNA delivery into MDCK cells, which was more significant for complexes prepared with a higher polymer:siRNA ratio. This could be due to more effective interaction between the cell membrane and complexes with higher portion of lipid-substituted polymers. Hydrophobic modification of PEI with cholesterol and its derivatives has been reported to have a positive effect on intracellular delivery of siRNA [24,40] or down-regulating target protein expression [38,41,42]. However, no correlations between the extent of cholesterol substitution and the obtained silencing were reported in these studies. This is important to unequivocally establish the beneficial effect of lipid substituents. Probing the correlation between the level of lipid substitution and siRNA delivery in this study indicated a strong correlation between these two parameters, especially with polymer:siRNA ratio of 2:1 (Fig. 3a). Such a strong relationship was not observed when a higher ratio (8:1) was used for complex formation (Fig. 3b), and in fact, a reverse trend was observed with OA-substituted polymers. This could be partly due to significantly higher siRNA delivery levels at this ratio, which might saturate the siRNA uptake mechanism and obscure any correlation that might have existed. The N/P ratio did not also correlate with siRNA uptake at each weight ratio used (not shown).

The polymers that enhanced the cellular uptake the most (i.e., LA- and CA-substituted PEI2s) were the most effective in the BCRP down-regulation. This was confirmed by a correlation between the cellular uptake and down-regulation level with a sigmoidal shape that plateaued after a certain level of cellular uptake (~450 a.u.) (Fig. 5). We note that the BCRP detection procedure employed in this study detects membrane-bound protein and changes in mRNA level of the protein needs to be performed to definitively conclude silencing of the gene expression. However, down-regulation of GFP was also observed along with BCRP down-regulation; this was expected due to the transfection of the cells with an IRES plasmid that yielded a single mRNA for translating both proteins. Silencing GFP with BCRP-specific siRNAs provides indirect evidence for the BCRP inhibition at the mRNA level. Other studies have been reported on siRNA silencing of BCRP expression. Beck et al. reported down-regulation for endogenous BCRP in BeWo cells and exogenous BCRP in transfected HeLa cells by commercially available LipofectAMINE; as much as 50% down-regulation of BCRP expression was observed by delivery of 10 μg specific siRNA to each well (exact siRNA concentration not reported) [43], compared to ~0.5 μg/mL (36 nM) siRNA in our experiment. The highest BCRP down-regulation achieved in our experiments was ~78%, which is significantly higher than the value reported in that study. However, we note that differences in the siRNA sequences, carriers, and cell targets (i.e., endogenous mRNA level and its turnover rate) between these studies make the underlying reason(s) for the observed difference difficult to pinpoint. A similar end goal has been pursued by short hairpin RNA (shRNA) transfection in MCF-7 and JAR cells [44], where the BCRP expression was reduced as a result of stable shRNA expression in that study (analyzed by western blot and not quantified). It must be stated that stable expression of shRNA was obtained under 'selection pressures' in that study. Although this approach was useful to reveal the importance of BCRP activity in drug tolerance, it is not clinically feasible to apply such selective pressures. The LA-substituted PEI2s were the most effective polymers in this study, but a clear effect of the lipid substitution on silencing was not evident among these polymers (even though such an effect was evident with all polymers considered); the level of substitutions ranged from 1.1 to 3.2 LA/PEI2 and it seemed that as little as ~1 LA/PEI2 was sufficient to improve the performance of the PEI2. The OA has the same number of lipid carbons as LA but with one double bond (vs. two found in LA), and the OA-substituted PEI2s had similar substitutions levels to that of LA-substituted PEI2s, so that subtle differences between the lipid substituents seem to be critical for the design of effectively functioning carrier. These observations also eliminated any possibility of chemical modification *per se* (acylation) as the reason for better siRNA delivery with modified PEI2s.

A dose–response analysis revealed a linear increase in the siRNA-mediated BCRP down-regulation in the studied range, which further confirms the desired siRNA-mediated silencing mechanism. Different studies have pointed out the importance of an optimal siRNA concentration required for an effective target down-regulation. Unspecific up-regulation and/or down-regulation of un-related genes associated with high concentrations of siRNA are a general concern [45–47]. Twenty to 50 nM concentrations of siRNA are considered desirable for this reason and we have shown a significant BCRP down-regulation (~70%) with 36 nM siRNA in our experimental system. The lower dose of 18 nM siRNA showed ~50% BCRP down-regulation, which was lower than the silencing achieved with 36 nM. While 18 nM would be a safer concentration in regard to the risk of unspecific gene regulations, the 36 nM concentration routinely employed in this study still seems to be in the acceptable dose range.

In order to confirm the desired pharmacological (functional) effect of BCRP suppression, cytotoxicity analysis with MTX was conducted. MTX is an anthraquinone that inhibits topoisomerase II, and therefore interferes with DNA repair, which is essential for genomic stability [48]. The drug has been used in metastatic breast cancer [49–51], prostate cancer [52], and acute myeloid leukemias [53]. Many studies have shown that MTX is a BCRP substrate, and in fact, BCRP is also known as mitoxantrone resistance gene [9]. As expected, a significant difference was observed in the MTX IC_{50} in the studied cell lines, which indicates resistance to cytotoxic effect of MTX in BCRP-positive cells due to over-expression of BCRP and reduced intracellular concentration of the cytotoxic drug. This was also confirmed by a reversal of resistance with BCRP down-regulation after treatment with siRNA complexes. The efficiency of the different polymeric carriers was reflective of their ability in down-regulating the target protein, which was observed in the earlier experiments. The IC_{50} of MTX was decreased 10.5-folds after siRNA exposure in previous studies employing LipofectAMINE [40], whereas we achieved a 13.9-fold decrease in MTX IC_{50}. Using a shRNA, the IC_{50} of MTX was decreased by 14.6-folds [44].

The peak effect of BCRP silencing occurred after 48 h and remained relatively constant up to 5 days after treatment. Our previous studies have shown a similar timeline for the maximum silencing effect for P-gp and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 48 and 72 h, respectively) [27]. However, the down-regulation obtained with P-gp expression with

the same delivery system was not as long lasting, and the level of down-regulation disappeared after 5 days [54]. A long-lasting silencing could play an important role in the clinical setting by providing a wider window of opportunity for the optimum cytotoxic effect of chemotherapy on the sensitized cells. The duration of BCRP down-regulation was also evaluated from a functional perspective by assessing for changes in IC_{50} for MTX. Consistent with BCRP silencing, the sensitizing effect of siRNA persisted for 7 days with our best delivery system (i.e., siRNA complexes prepared with PEI-LA1.0) at which point the first signs of "wearing off" was observed with a slight increase in IC_{50} of MTX in siRNA-treated cells. After 14 days, the MTX IC_{50} was increased in siRNA-treated cells; however, it was still less than half of the IC_{50} calculated in BCRP-positive cell line (Fig. 7b). This seems to be a more persistent effect compared to what P-gp down-regulation reported from our laboratory [54]. However, the transcriptional and translational rates of the targeted genes, as well as the turnover rates (half-lives) of the targeted proteins may play an important role in the persistence of the silencing effect and therefore should be considered for further conclusions. Nevertheless, such a prolonged silencing obtained with polymeric delivery systems and target-specific siRNAs will facilitate clinical application of transporter-specific siRNAs as a general approach of sensitizing the tumors to chemotherapy.

5. Conclusions

In this study, we reported effective and relatively long-lasting down-regulation of an efflux protein (BCRP) involved in multidrug resistance in cancer. This was made possible by the modification of the non-cytotoxic but ineffective PEI2 with different hydrophobic (lipid) moieties. The resultant polymers showed a significant capability for cellular delivery of BCRP-specific siRNA, which in turn translated into a reversal in resistance to an anticancer agent (MTX) known to be a substrate for the targeted protein. The levels of siRNA uptake and protein down-regulations were generally higher with higher level of lipid substitution for most of the substituted polymers. The LA-substituted polymers were more effective than the other lipids used in this study, and as little as ~1 LA/PEI was sufficient for effective siRNA delivery. This study yielded a promising delivery system for safe and effective siRNA delivery, which should further stimulate investigation into BCRP silencing in preclinical animal models.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2012.01.011.

References

[1] L. Gatti, G.L. Beretta, G. Cossa, F. Zunino, P. Perego, ABC transporters as potential targets for modulation of drug resistance, Mini Rev. Med. Chem. 9 (2009) 1102–1112.
[2] Y.C. Mayur, G.J. Peters, V.V. Prasad, C. Lemo, N.K. Sathish, Design of new drug molecules to be used in reversing multidrug resistance in cancer cells, Curr. Cancer Drug Targets 9 (2009) 298–306.
[3] Z. Ni, Z. Bikadi, M.F. Rosenberg, Q. Mao, Structure and function of the human breast cancer resistance protein (BCRP/ABCG2), Curr Drug Metab. 11 (2010) 603–617.
[4] M. Maliepaard, G.L. Scheffer, I.F. Faneyte, M.A. van Gastelen, A.C. Pijnenborg, A.H. Schinkel, M.J. van De Vijver, R.J. Scheper, J.H. Schellens, Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues, Cancer Res. 61 (2001) 3458–3464.
[5] L. Zhou, K. Schmidt, F.R. Nelson, V. Zelesky, M.D. Troutman, B. Feng, The effect of breast cancer resistance protein and P-glycoprotein on the brain penetration of flavopiridol, imatinib mesylate (Gleevec), prazosin, and 2-methoxy-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl)propanoic acid (PF-407288) in mice, Drug Metab. Dispos. 37 (2009) 946–955.
[6] E. Kis, T. Nagy, M. Jani, E. Molnar, J. Janossy, O. Ujhellyi, K. Nemet, K. Heredi-Szabo, P. Krajcsi, Leflunomide and its metabolite A771726 are high affinity substrates of BCRP: implications for drug resistance, Ann. Rheum. Dis. 68 (2009) 1201–1207.
[7] M.L. Vlaming, Z. Pala, A. van Esch, E. Wagenaar, D.R. de Waart, K. van de Wetering, C.M. van der Kruijssen, R.P. Oude Elferink, O. van Tellingen, A.H. Schinkel, Functionally overlapping roles of Abcg2 (Bcrp1) and Abcc2 (Mrp2) in the elimination of methotrexate and its main toxic metabolite 7-hydroxymethotrexate in vivo, Clin. Cancer Res. 15 (2009) 3084–3093.
[8] A. Milane, S. Vautier, H. Chacun, V. Meininger, G. Bensimon, R. Farinotti, C. Fernandez, Interactions between riluzole and ABCG2/BCRP transporter, Neurosci. Lett. 452 (2009) 12–16.
[9] L.A. Doyle, W. Yang, L.V. Abruzzo, T. Krogmann, Y. Gao, A.K. Rishi, D.D. Ross, A multidrug resistance transporter from human MCF-7 breast cancer cells, Proc. Natl. Acad. Sci. USA 95 (1998) 15665–15670.
[10] T. Litman, M. Brangi, E. Hudson, P. Fetsch, A. Abati, D.D. Ross, K. Miyake, J.H. Resau, S.E. Bates, The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2), J. Cell. Sci. 113 (2000) 2011–2021.
[11] S.K. Rabindran, H. He, M. Singh, E. Brown, K.I. Collins, T. Annable, L.M. Greenberger, Reversal of a novel multidrug resistance mechanism in human colon carcinoma cells by fumitremorgin C, Cancer Res. 58 (1998) 5850–5858.
[12] Y.A. Gandhi, M.E. Morris, Structure-activity relationships and quantitative structure-activity relationships for breast cancer resistance protein (ABCG2), AAPS J. 11 (2009) 541–552.
[13] M. Scherr, D. Steinmann, M. Eder, RNA interference (RNAi) in hematology, Ann. Hematol. 83 (2004) 1–8.
[14] A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, Nature 391 (1998) 806–811.
[15] J. Haupenthal, C. Baehr, S. Kiermayer, S. Zeuzem, A. Piiper, Inhibition of RNAse A family enzymes prevents degradation and loss of silencing activity of siRNAs in serum, Biochem. Pharmacol. 71 (2006) 702–710.
[16] W.T. Godbey, K.K. Wu, A.G. Mikos, Poly(ethyleneimine) and its role in gene delivery, J. Control. Release 60 (1999) 149–160.
[17] J.C. Bologna, G. Dorn, F. Natt, J. Weiler, Linear polyethyleneimine as a tool for comparative studies of antisense and short double-stranded RNA oligonucleotides, Nucleos. Nucleot. Nucleic Acids 22 (2003) 1729–1731.
[18] B. Urban-Klein, S. Werth, S. Abuharbeid, F. Czubayko, A. Aigner, RNAi-mediated gene-targeting through systemic application of polyethyleneimine (PEI)-complexed siRNA in vivo, Gene Ther. 12 (2005) 461–466.
[19] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. USA 92 (1995) 7297–7301.
[20] W.T. Godbey, K.K. Wu, A.G. Mikos, Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery, Proc. Natl. Acad. Sci. USA 96 (1999) 5177–5181.
[21] J. Kloeckner, S. Bruzzano, M. Ogris, E. Wagner, Gene carriers based on hexanediol diacrylate linked oligoethylenimine: effect of chemical structure of polymer on biological properties, Bioconjug. Chem. 17 (2006) 1339–1345.
[22] J.H. Jeong, S.H. Song, D.W. Lim, H. Lee, T.G. Park, DNA transfection using linear poly(ethylenimine) prepared by controlled acid hydrolysis of poly(2-ethyl-2-oxazoline), J. Control. Release 73 (2001) 391–399.
[23] V. Incani, A. Lavasanifar, H. Uludag, Lipid and hydrophobic modification of cationic carriers on route to superior gene vectors, Soft Matter 6 (2010) 2124–2138.
[24] W.J. Kim, C.W. Chang, M. Lee, S.W. Kim, Efficient siRNA delivery using water soluble lipopolymer for anti-angiogenic gene therapy, J. Control. Release 118 (2007) 357–363.
[25] A. Neamnark, O. Suwantong, R.K. Bahadur, C.Y. Hsu, P. Supaphol, H. Uludag, Aliphatic lipid substitution on 2 kDa polyethyleneimine improves plasmid delivery and transgene expression. Mol. Pharm. 6, 1798–1815, Mol. Pharm. 6 (2009) 1798–1815 (Correction in Mol. Pharm. 2010, 7, 618).
[26] P. Pavek, G. Merino, E. Wagenaar, E. Bolscher, M. Novotna, J.W. Jonker, A.H. Schinkel, Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine, J. Pharmacol. Exp. Ther. 312 (2005) 144–152.
[27] H. Montazeri Aliabadi, B. Landry, R.K. Bahadur, A. Neamnark, O. Suwantong, H. Uludag, Impact of lipid-substitution on assembly and delivery of siRNA by cationic polymers, Macromol. Biosci. 11 (2011) 662–672.
[28] A. Maleka, O. Merkelb, L. Finkc, F. Czubaykoa, T. Kisselb, A. Aigner, In vivo pharmacokinetics, tissue distribution and underlying mechanisms of various PEI(−PEG)/siRNA complexes, Toxicol. Appl. Pharmacol. 236 (2009) 97–108.

[29] D. Jing, J. Agnew, W.F. Patton, J. Hendrickson, J.M. Beechem, A sensitive two-color electrophoretic mobility shift assay for detecting both nucleic acids and protein in gels, Proteomics 3 (2003) 1172–1180.
[30] L.A. Doyle, D.D. Ross, Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2), Oncogene 22 (2003) 7340–7358.
[31] J.D. Allen, A.H. Schinkel, Multidrug resistance and pharmacological protection mediated by the breast cancer resistance protein (BCRP/ABCG2), Mol. Cancer Ther. 1 (2002) 427–434.
[32] M. Dean, T. Fojo, S. Bates, Tumour stem cells and drug resistance, Nat. Rev. Cancer 5 (2005) 275–284.
[33] M.M. Gottesman, T. Fojo, S.E. Bates, Multidrug resistance in cancer: role of ATP-dependent transporters, Nat. Rev. Cancer 2 (2002) 48–58.
[34] M. Dean, A. Rzhetsky, R. Allikmets, The human ATP-binding cassette (ABC) transporter superfamily, Genome Res. 11 (2001) 1156–1166.
[35] Y. An, W.M. Ongkeko, ABCG2: the key to chemoresistance in cancer stem cells?, Expert Opin Drug Metab. Toxicol. 5 (2009) 1529–1542.
[36] S. Hong, P.R. Leroueil, E.K. Janus, J.L. Peters, M.M. Kober, M.T. Islam, B.G. Orr, J.R. Baker Jr, M.M. Banaszak Holl, Interaction of polycationic polymers with supported lipid bilayers and cells: nanoscale hole formation and enhanced membrane permeability, Bioconjug. Chem. 17 (2006) 728–734.
[37] U. Lungwitz, M. Breunig, T. Blunk, A. Gopferich, Polyethyleneimine-based non-viral gene delivery systems, Eur. J. Pharm. Biopharm. 60 (2005) 247–266.
[38] D.A. Wang, A.S. Narang, M. Kotb, A.O. Gaber, D.D. Miller, S.W. Kim, R.I. Mahat, Novel branched poly(ethylenimine)-cholesterol water-soluble lipopolymers for gene delivery, Biomacromolecules 3 (2002) 1197–1207.
[39] S. Kim, J.S. Choi, H.S. Jang, H. Suh, J. Park, Hydrophobic modification of polyethyleneimine for gene transfectants, Bull. Kor. Chem. Soc. 22 (2001) 1069–1075.
[40] D.Y. Furgeson, R.N. Cohen, R.I. Mahato, S.W. Kim, Novel water insoluble lipoparticulates for gene delivery, Pharm. Res. 19 (2002) 382–390.
[41] S. Han, R.I. Mahato, S.W. Kim, Water-soluble lipopolymer for gene delivery, Bioconjug. Chem. 12 (2001) 337–345.
[42] Y.T. Ko, A. Kale, W.C. Hartner, B. Papahadjopoulos-Sternberg, V.P. Torchilin, Self-assembling micelle-like nanoparticles based on phospholipid-polyethyleneimine conjugates for systemic gene delivery, J. Control. Release 133 (2009) 132–138.
[43] P.L. Ee, X. He, D.D. Ross, W.T. Beck, Modulation of breast cancer resistance protein (BCRP/ABCG2) gene expression using RNA interference, Mol. Cancer Ther. 3 (2004) 1577–1583.
[44] H. Lv, Z. He, X. Liu, J. Yuan, Y. Yu, Z. Chen, Reversal of BCRP-mediated multidrug resistance by stable expression of small interfering RNAs, J. Cell. Biochem. 102 (2007) 75–81.
[45] A. Kumar, J. Haque, J. Lacoste, J. Hiscott, B.R. Williams, Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B, Proc. Natl. Acad. Sci. USA 91 (1994) 6288–6292.
[46] S.P. Persengiev, X. Zhu, M.R. Green, Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs), RNA 10 (2004) 12–18.
[47] D. Semizarov, L. Frost, A. Sarthy, P. Kroeger, D.N. Halbert, S.W. Fesik, Specificity of short interfering RNA determined through gene expression signatures, Proc. Natl. Acad. Sci. USA 100 (2003) 6347–6352.
[48] H.M. Coley, Mechanisms and strategies to overcome chemotherapy resistance in metastatic breast cancer, Cancer Treat. Rev. 34 (2008) 378–390.
[49] G. Fried, M.E. Stein, A. Kuten, M. Quigley, A. Gershuny, N. Siegelmann-Danieli, J. Zaidan, N. Haim, Vinorelbine/VP-16 (etoposide) in metastatic breast cancer: a phase II study, J. BU ON 10 (2005) 201–204.
[50] A. Onyenadum, H. Gogas, C. Markopoulos, D. Bafaloukos, G. Aravantinos, M. Mantzourani, A. Koutras, E. Tzorakoelefterakis, N. Xiros, T. Makatsoris, G. Fountzilas, H.P. Kalofonos, Mitoxantrone plus vinorelbine in pretreated patients with metastatic breast cancer, J. Chemother. 19 (2007) 582–589.
[51] B. Vahid, P.E. Marik, Pulmonary complications of novel antineoplastic agents for solid tumors, Chest 133 (2008) 528–538.
[52] R. Dreicer, Current status of cytotoxic chemotherapy in patients with metastatic prostate cancer, Urol. Oncol. 26 (2008) 426–429.
[53] C. Parker, R. Waters, C. Leighton, J. Hancock, R. Sutton, A.V. Moorman, P. Ancliff, M. Morgan, A. Masurekar, N. Goulden, N. Green, T. Revesz, P. Darbyshire, S. Love, V. Saha, Effect of mitoxantrone on outcome of children with first relapse of acute lymphoblastic leukaemia (ALL R3): an open-label randomised trial, Lancet 376 (2010) 2009–2017.
[54] M. Abbasi, A. Lavasanifar, L.G. Berthiaume, M. Weinfeld, H. Uludag, Cationic polymer-mediated small interfering RNA delivery for P-glycoprotein down-regulation in tumor cells, Cancer 116 (2010) 5544–5554.