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Investigating siRNA delivery to chronic myeloid leukemia K562 cells with lipophilic polymers for therapeutic BCR-ABL down-regulation

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ABSTRACT

RNAi represents a new alternative for treatment of chronic myeloid leukemia (CML) to overcome the difficulties of current drug treatments such as the acquired resistance. However, potent carriers that can overcome delivery barriers to RNAi agents and have therapeutic efficacy especially in difficult-to-transfect CML cells are needed. Here, we explored the use of lipid-modified polyethylenimines (PEI) of low molecular weights (0.6, 1.2 and 2.0 kDa) in K562 cells and showed that the delivery efficiency was dependent on the type of lipid used for polymer modification, degree of lipid substitution and polymer molecular weight. Among the lipid-substituted polymers investigated, palmitic acid (PA)-substituted 1.2 kDa PEI (~2 lipids/PEI) has proven to be highly efficient in delivering siRNA and silencing of the reporter gene green fluorescent protein (GFP). The silencing efficacy achieved with this polymer was found to be higher than the 25 kDa PEI and is similar to commercial reagent Lipofectamine™ 2000. Moreover, when BCR-ABL protein was targeted in K562 cells, a reduction in the corresponding mRNA levels was observed, as well as an induction of early and late stage apoptosis. The results of this study demonstrated that PA-substitutions on low MW polymers could be useful for siRNA delivery in CML cells for therapeutic purposes.

1. Introduction

Chronic myeloid leukemia (CML) is a cancer of the hematopoietic stem cells arising from chromosomal translocation of chromosomes 9 and 22, which results in a fusion between the Abelson murine leukemia viral oncogene homolog 1 (*ABL*) and breakpoint cluster region (*BCR*) genes [1–3]. Once the normally regulated tyrosine kinase of the ABL protein is constitutively activated by the juxtaposition of the *BCR* sequence, it activates multiple signal transduction pathways, alters cell adhesion to bone marrow stroma, increases cell proliferation and reduces apoptosis, leading to over-population of myeloid cells in the hematopoietic system [3,4]. Current therapies for CML are based on the use of small molecular drugs tyrosine-kinase inhibitors (TKIs) and stem cell transplantation. TKIs such as imatinib mesylate had a major impact on treatment of chronic phase CML; however, TKI monotherapies are not curative and initial and acquired TKI resistance, as well as relapse, remain significant challenges [3–5]. There are multiple mechanisms that confer TKI resistance, including increased expression of *BCR-ABL* and its tyrosine kinase activity, and/or point mutations in the tyrosine kinase domain that affect drug binding to its target [2,6]. In addition, primary CML stem cells, including primitive quiescent cells, are not effectively targeted or eradicated by TKIs and hence constitute a critical population of cells in setbacks upon IM discontinuation and in generating IM-resistant clones [3,4]. Stem-cell transplantation therapy is an option when treatment with TKIs fails; however, this therapy has a substantial risk of mortality due to chronic graft-versus-host disease [4,5].

The shortcomings of current treatments call for alternative and more efficacious therapies for CML. Owing to increasing knowledge on molecular changes in CML, gene-based therapy is becoming a promising approach since it can specifically address the underlying cause of the disease. Synthetic small interfering RNA (siRNA) delivered into cytoplasm of transformed cells can interact with a desired mRNA for down-regulation of specific proteins involved in processes such as cellular over-growth and inactivation of apoptosis. Although numerous potential molecular targets have been identified for siRNA delivery in CML cells [7–10], a functional carrier is needed for effective intracellular delivery of siRNA [11–13], since the anionic siRNA is incapable of traversing plasma membrane on its own. Physical methods, such as electroporation, result in high cell death [14,15] and cannot be practised

Abbreviations: CML, chronic myeloid leukemia; GFP, green fluorescent protein; LA, linoleic acid; MFI, mean fluorescence intensity; MW, molecular weight; PA, palmitic acid; PEI, polyethylenimine; RNAi, RNA interference; siRNA, short interfering RNA.

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in vivo. While viral vectors have been effectively used for manipulation of leukemic cells, they represent a significant safety risk because of their capacity to integrate to the host's genome and/or cause lethal immune responses and inflammation [12,16]. Cationic polymers, which are actively explored for siRNA delivery [17], are a safer alternative to viruses, especially considering that they are readily amenable for engineering to match the needs of the application. Lipid-substituted polyethylenimines (PEIs), in particular, have been developed for this purpose and were tailored to deliver plasmid DNA as well as siRNA to a variety of cell types. Unlike high molecular weight (MW) PEI, which acts as an effective "proton-sponge" for endosomal escape [18] but displays excessive toxicity, we focused on low MW PEIs due to the low cytotoxicity of these polymers [19]. By employing the amine groups of PEI for substitutions, we found that the relatively nontoxic but ineffective 2 kDa PEI (PEI2) polymer could be transformed into an effective nucleic acid carrier as a result of lipid substitution on these groups [19,20].

In this study, we explored the efficacy of lipid-substituted PEIs for siRNA delivery to CML cells for the first time. By using K562 cells as a CML model, we investigated the structural features of lipid-substituted PEIs that influenced the siRNA delivery. Since the physicochemical properties of the polymers were reported previously in Reference [21], we focused on siRNA delivery and the resulting silencing activity in the chosen cell model. The latter was explored based on a reporter gene (green fluorescent protein, GFP) that was virally incorporated into CML cells, and the endogenous *BCR-ABL* oncogene.

2. Materials and methods

2.1. Materials

Branched PEIs with MWs of 0.6 (PEI0.6) and 1.2 kDa (PEI1.2) were purchased from Polysciences, Inc. (Warrington, PA). PEI with MWs of 2 (PEI2) and 25 kDa (PEI25), anhydrous dimethylsulfoxide (DMSO), *N*,*N*-dimethylformanide (DMF), linoleyl chloride (C18:2 9Z,12Z; 99%), trypsin/EDTA and were obtained from Sigma-Aldrich Corporation (St. Louis. MO). Stearoyl chloride (C18; >98.5%) was obtained from Fluka. Caproyl chloride (C8; >99%), palmitoyl chloride (C16; 98%) and octanoyl chloride (C18:2 9Z; 99%) were purchased from Aldrich. Unlabeled scrambled siRNA, 5′-carboxyfluorescein (FAM)-labeled scrambled siRNA and M-MLV reverse transcriptase were purchased from Invitrogen (Burlington, ON). A GFP siRNA (GFP-22) was from Qiagen (Toronto, ON). A custom-synthesized BCR-ABL siRNA (5′-GCA GAGUUCAAAAGCCCTT-3′ and 3′-TTCGUCUCAAGUUUUCGGG-5′) was obtained from Integrated DNA Technologies, Inc. (IDT) (Coralville, IA), while two other BCR-ABL siRNAs were obtained from Allele Biotechnology (San Diego, CA; catalog numbers: ABP-Ri-VAsi-D09 and ABP-Ri-VAsi-D10). The RPMI Medium 1640 medium with L-glutamine, low-glucose DMEM, Opti-MEM® I reduced serum medium, penicillin (10,000 U/mL), streptomycin (10 mg/mL) were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was purchased from PAA Laboratories Inc. (Etobicoke, ON). Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX Reagent are from Invitrogen, Metafectamine Pro from Biontex (San Diego, CA), FuGENE HD from Roche Diagnostics (Laval, QC) and HiPerFect Transfection Reagent from Qiagen (Mississauga, ON). Annexin V-FITC apoptosis detection kit I was purchased from BD Biosciences (San Jose, CA). RNAeasy Mini Kit was from Qiagen (Toronto, ON).

2.2. Synthesis and characterization of lipid-substituted polymers

In this study, we used two different lipid-substituted polymer libraries that were previously synthesized and characterized in-house. For the first library, PEI2 was N-acylated with lipids of varying carbon chains: caprylic acid (CA), myristic acid (MA), palmitic acid (PA), stearic acid (SA), oleic acid and (OA) and linoleic acid (LA), as originally described in [19]. In brief, lipid chlorides individually dissolved in 1 ml of DMF were added drop-wise to 100 mg of PEI in 1 ml of DMSO. Three different lipid:PEI amine ratios (0.066, 0.1 and 0.2) were used during synthesis to control the level of substitutions. After 24 h at room temperature under argon, polymers were precipitated and washed with excess ethyl ether and vacuum-dried at room temperature. The polymers were analyzed by ^{1}H NMR in D_2O using the characteristic proton shifts of lipids (δ ~ 0.8 ppm; $-C\underline{H}_3$) and PEI (δ ~ 2.5–2.8 ppm; NH–$C\underline{H}_2$–$C\underline{H}_2$–NH–) to calculate the lipid substitution levels. Reference [19] provides a summary of the degree of lipid substitutions.

For the second library, PA substitutions were performed on 0.6, 1.2 and 2 kDa PEIs [21]. Briefly, 60 mg of PEI0.6, 120 mg of PEI1.2 and 200 mg of PEI2 were dissolved individually in 200 ml of chloroform and 160 μl of 1.15 mmol of triethylamine was added. Three different amounts of palmitoyl chloride were added drop-wise to each polymer solution and stirred at room temperature for 12 h. The final products of the three different lipid:PEI amine feed ratios (1, 2 and 4) used for each polymer were precipitated and washed with excess ethyl ether. Based on ^{1}H NMR analysis, reference [21] provides a summary of degree of lipid substitutions on these polymers.

2.3. Cell model of CML

K562 cells, a *BCR-ABL* positive cell line established from a CML patient in blast crisis [22], was used as the CML model. A GFP-expressing K562 cell line (GFP-K562) was generated by transduction of cells with a retroviral vector containing the green fluorescent protein (GFP) gene [23] and used as the silencing model due to convenience of assessing GFP silencing. K562 and GFP-K562 cells were maintained in RPMI medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin under incubation (37 °C, 5% CO_2). Every third day in culture, spent medium was removed by centrifugation (600 rpm, 5 min) and cells were diluted 10 times (or 1×10^6 cells) in 25 ml of fresh medium for cell expansion or seeded at 1×10^5 cell/ml in multi-well plates one day before prior testing. MDA-MB-231 cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 37 °C, 5% CO_2. When the cells were confluent (~80% of the plate surface covered), they were de-attached from the surface by HBSS-washing and a 5-min incubation with Trypsin/EDTA at room temperature. The suspended cells were then collected by centrifugation and sub-cultured at a 10% concentration of the original count.

2.4. siRNA/lipid-modified polymer complex preparation

For the preparation of siRNA/polymers complexes, an aliquot of siRNA stock solution (10 μM in RNAse-free water) was first dissolved in a 150 mM NaCl solution in polypropylene sterile tubes. Typically, ~0.25 μg of siRNA was added to 150 mM NaCl for a final siRNA concentration of 36 nM in cell suspension. The polymers (dissolved at 1 mg/ml in ddH_2O) were then added to the siRNA solution to give the desired polymer:siRNA weight ratios (2:1, 4:1, 8:1 and 12:1), bringing the final volume to 60 μl. After a 30-min incubation at room temperature, complexes (20 μl/well) were added in triplicate to the cells seeded on 24-well plates one day before. A similar procedure was used to prepare the Lipofectamine™ 2000 complexes, except that Lipofectamine™ 2000 was diluted separately and then mixed with the siRNA solution at specific siRNA:carrier ratio; the buffer used for Lipofectamine™ 2000 complex preparation was either 150 mM NaCl or Opti-MEM (the recommended medium for Lipofectamine™ 2000 formulations). The 2:1, 4:1, 8:1 and 12:1 polymer:siRNA ratios used for polymer complexes corresponded to 13.3:1, 26.7:1, 53.3:1 and 80.1:1 N:P ratio, respectively (assuming 43 Da for PEI single unit, 22 bp for siRNA with 2 phosphates per base pair).

For silencing studies with PA-based polymers, complexes were prepared as described above but the siRNA and polymers were diluted in 300 μl (triplicate) of RPMI medium. 100 μl of complex

solution was added to empty wells and a cell suspension of 100,000 cells in 500 μl of complete medium was added on the top of the well with the complexes.

2.5. Delivery of siRNA to K562 cells

One day prior to transfection, 0.5 ml of K562 cells (at 1×10^5 cells/ml) was seeded in 24-well plates. K562 cells were transfected with complexes prepared with FAM-labeled and non-labeled scrambled siRNA (as negative control) as described above. At the indicated times, cells were transferred to tubes, centrifuged (1400 rpm for 5 min), washed twice with HBSS and re-suspended in a fixed volume of 3.7% formalin. The cell-associated FAM-siRNA was quantified by flow cytometry (Cell Lab Quanta SC; Beckman Coulter) using the FL1 channel and calibrating the instrument so that the negative control (i.e., no-treated cells) gave ~1% of positive cells as the background. siRNA delivery in K562 cells was determined by measuring the mean fluorescence of cells and percentage of FAM-siRNA positive cells. The cell concentration of the total population was also determined.

2.6. GFP silencing in GFP-K562 cells

Silencing effect of complexes was evaluated by quantifying the reduction of the GFP fluorescence in the GFP-K562 cells. Complexes were prepared with scrambled (as negative control) and GFP siRNA at the indicated siRNA concentrations and polymer:siRNA ratios. At the indicated time after transfection, cells were transferred to tubes, centrifuged (1400 rpm, 5 min), washed twice with HBSS and re-suspended in 3.7% formalin. GFP silencing as well as cell concentration were assessed by flow cytometry using the FL-1 channel. The GFP-expressing cell population is shown on the third quadrant under the FL1+ region of the histograms, while the GFP-negative cell population can be seen shifted towards the left when there is a silencing effect (see Fig. S1). Percent decrease in mean fluorescence was calculated as follows: 100-([Mean FL1 of cells treated with GFP siRNA/polymer] / [Mean FL1 of cells treated with scrambled siRNA/polymer] × %). Percent decrease in GFP-positive cells was calculated as follows: [% of GFP-negative cells of cells treated with GFP siRNA/polymer] − [% of GFP-negative cells of cells treated with scrambled siRNA/polymer].

A comparison of the transfection efficiency between lipid-modified polymers and commercial transfection reagents (PEI25, Lipofectamine™ 2000, Lipofectamine™ RNAiMax, DOTAP, Metafectene, Interferin, Ibofect and Fugene HD) was also performed. Twenty-four hours prior to transfection, 15,000 cells were seeded in 100 μl of complete medium in 96-well plates. On the day of the transfection, complexes were prepared following the manufacturer's instructions as closely as possible using GFP-siRNA and scrambled siRNA as it follows: first, the siRNA and the carriers were diluted in 75 μl Opti-MEM separately to make carrier:siRNA weight ratios of 2:1, 4:1 and 8:1 and a final siRNA concentration of 24 nM. After a 5-min incubation of the reagents in OptiMEM, siRNA and reagent solutions were mixed by pipetting few times, except Interferin that was vortexed. PEI25 complexes were incubated for 30 min, Lipofectamine™ 2000, Lipofectamine™ RNAiMax, DOTAP, Metafectene, and Interferin were incubated for 20 min and Fugene HD was incubated for 15 min prior to drop-wise addition (50 μl) of complex solution to cells. The differences in complexation protocols (as well as relative ratios of carrier:siRNA) were due to differences in recommendations of each manufacturer, which was adopted as closely as possible rather than re-optimizing the formulations to match the polymer formulations developed in this study. GFP silencing was assessed by flow cytometry 72 h post-transfection as described above.

2.7. Apoptosis analysis

For the apoptosis assay, K562 cells were seeded 24 h prior to transfection on 24-well plates at a 1×10^5 cells/ml. On the day of the transfection, complexes were prepared with control siRNA and a mixture of three BCR-ABL siRNAs at 50 and 100 nM (one third of each siRNA) and PEI1.2-PA (1.98 PA per PEI) with polymer:siRNA ratio of 4:1 as described in the siRNA/lipid-modified polymer complex preparation section. After 1, 2 and 3 days after transfection, apoptosis was assessed by Annexin kit following the manufacturer's protocol. For this, cells were collected in tubes, washed twice with cold HBSS and, aliquots of 1×10^5 cells diluted in 100 μl of 1× were incubated in dark for 15 min at room temperature with 5 μl of FITC-Annexin V and 5 μl of Propidium Iodide (PI). This solution was dissolved in 400 μl of 1× Binding Buffer and analyzed with a BD LSR Fortessa flow cytometer (Franklin Lakes, NJ) (Flow cytometry facility, University of Alberta).

2.8. BCR-ABL silencing in K562 cells

K562 cells seeded on 6-well plates in 2.5 ml of complete medium were treated with complexes prepared with control and BCR-ABL siRNA (catalog number: ABP-Ri-VAsi-D09 from Allele Biotechnology) at a polymer:siRNA weight ratio of 4:1 and a 100 nM siRNA concentration. Twenty-four and 48 h after transfection, levels of *BCR-ABL* were assessed at the mRNA level. First, treated cells were transferred to tubes, washed twice with HBSS and the total RNA was extracted using the RNAeasy Mini Kit following manufacturer's instructions except that 2-mercaptoethanol was omitted from the extraction. The integrity of the RNA extracted was then checked by spectrophotometry (GE Nanovue). For each sample, 500 ng of RNA were then reverse-transcribed with M-MLV reverse transcriptase, following the manufacturer's instructions. Oligo (dT) as well as random primers were used for the cRNA synthesis [24,25]. Finally, for real-time PCR analysis, 2× SYBR green master mix with ROX (MAF Center, University of Alberta) was used to follow the fluorescence intensity. Specific forward and reverse primers used to detect expression levels are the following: beta-actin (housekeeping endogenous gene): 5′-CCA CCC CAC TTC TCT CTA AGG A-3′ and 5′-AAT TTA CAC GAA AGC AAT GCT ATC A-3′ [24], BCR-ABL: 5′-CAT TCC GCT GAC CAT CAA TAA G-3′; 5′-GAT GCT ACT GGC CGC TGA AG-3′ [23]. A 10 μl volume containing 5 μl of 2× master mix SYBR Green, 2.5 μl of 3.2 μM primer and 2.5 μl of (10 ng/μl) cDNA template for each sample in triplicate were transferred to a Fast Optical 96-well plate. Using an Applied Biosystems StepOnePlus instrument, reaction mixtures were heated for 2 min at 95 °C before going through 40 cycles of a denaturation step (15 s at 95 °C) and an annealing/elongation step (1 min at 60 °C). Analysis to determine differences in gene expression was performed by $2^{-\Delta\Delta CT}$ method using the no-treatment groups as the calibrator. *BCR-ABL* C_T was normalized against Beta-actin C_T and the results are expressed as a relative quantity of the targeted mRNA.

3. Results and discussion

A library of PEI2 polymers modified with CA, MA, PA, SA, OA, and LA at different lipid substitutions was previously described in Reference [19]. During the synthesis, the lipid:PEI mole ratio was controlled so as to control the extent of lipid substitutions (determined by ^{1}H NMR), which was generally increased with increasing lipid:PEI ratio. Similarly, a library of PA-substituted PEIs was previously described in Reference [19–21] where the PA substitution was again controlled by the lipid:PEI ratios during the synthesis. The resultant polymers were readily dissolved in water, which made them suitable for siRNA complexation under aqueous conditions. A variability in siRNA delivery efficiency of lipid-modified polymers was previously reported among anchorage-dependent cell lines in References [20,26–28], letting us to

believe that the most effective lipo-polymer is needed to be tailored for individual cell type. In the absence of previous experience with CML cells, we first set out to determine the delivery and silencing efficiency of lipid-substituted PEI2 polymers.

3.1. Comparison of siRNA delivery to K562 and MDA-MB-231 using lipid-substituted PEI2s

Since lipid-modified PEI2 mediated effective delivery of siRNA to attachment-dependent MDA-MB-231 breast cancer cells [29], we first compared siRNA delivery efficiency to K562 and MDA-MB-231 cells head-to-head in this study. The siRNA delivery with the lipid-modified polymers was substantially higher in MDA-MB-231 cells: siRNA delivery was >45-fold greater than the no carrier (siRNA alone) group with most of the polymers (Fig. 1A). As in previous results in Reference [29], PEI2LA again gave the highest delivery in MDA-MB-231 cells (mean delivery ~400-fold greater than no carrier group). Although siRNA delivery to K562 cells was lower, PEI2LA again had the highest delivery efficiency in these cells (mean delivery >29-fold greater than no carrier group; Fig. 1A). Almost all of MDA-MB-231 cells were positive for the siRNA delivered with lipid-substituted PEIs (>85% with most effective polymers), while the highest levels of siRNA-positive K562 cells were obtained only with PEI25 and PEI2LA polymers (~76% and 67%, respectively) (Fig. 1B). A strong correlation in siRNA cellular delivery was evident between MDA-MB-231 and K562 cells (Fig. 1C; $r = 0.843$, based on mean fluorescence values from Fig. 1A). Although this is suggestive of the similar performance of each polymer in both cell types, the difference was more evident in the absolute quantities of siRNA delivered (Fig. 1A).

We next explored the influence of cell density on siRNA delivery to K562 cells. Cells were seeded at initial densities of 0.4, 0.7, 1, 1.5 and 2×10^5 cells/ml and treated with siRNA complexes for 24 h (Fig. 1D). Although there was a trend of decreasing siRNA delivery with increasing cell concentration for two of the polymers (PEI25 and PEI2LA), these trends were not strong enough to lead to a significant change in siRNA delivery. It was conceivable that delivery of siRNA to K562 cells was limited due to their higher cell density in these cells (since they undergo rapid proliferation) as compared to MDA-MB-231 cells, but this did not appear to be the reason for the reduced level of siRNA delivery to K562 cells.

The difficulty of delivering nucleic acids to suspension growing cells is generally appreciated in the field [30–33]. It is likely that the interaction of the complexes with the adherent cells is facilitated by the monolayer formation by these cells; once the complexes settle to the bottom of tissue culture plates, they have a larger cell membrane area to which they can interact with. In contrast, the interaction of complexes with suspension cells could be reduced since both components are in suspension and electrostatic charges between the complexes and cells might not be strong enough for tight binding and subsequent endocytosis [30]. The increased gene delivery efficiency in adherent cells in comparison with suspension cells was also explained by the interaction of cationic particles with the components of the cell membrane involved in cell anchoring to the extracellular matrix, which are absent in suspension cells [30,34]. For this reason, suspension cells such as hematopoietic and T lymphocytes cells have been made to adhere to monolayer of cells to improve the liposomal-mediated transfection efficiency [30].

Previous studies in Reference [20,24] indicated that LA-substitution sustained most silencing among the lipid-substituted PEI2s for down-regulation of GAPDH and P-glycoprotein expression in MDA-MB-435 MDR [20], as well as GFP and CXCR4 in acute myeloid leukemia cells (THP-1) as described in reference [24]. Combined with the fact that LA substitution again gave the most siRNA delivery to K562 cells, this polymer was further investigated for silencing efficiency in K562 cells.

3.2. GFP silencing with PEI2 polymers and Lipofectamine™ 2000 in K562 cells

The silencing efficiency of siRNA delivered with PEI2LA was assessed in GFP-K562 cells (Fig. 2). The GFP silencing was evaluated after 48 and 72 h post-transfection by comparing complexes prepared in either

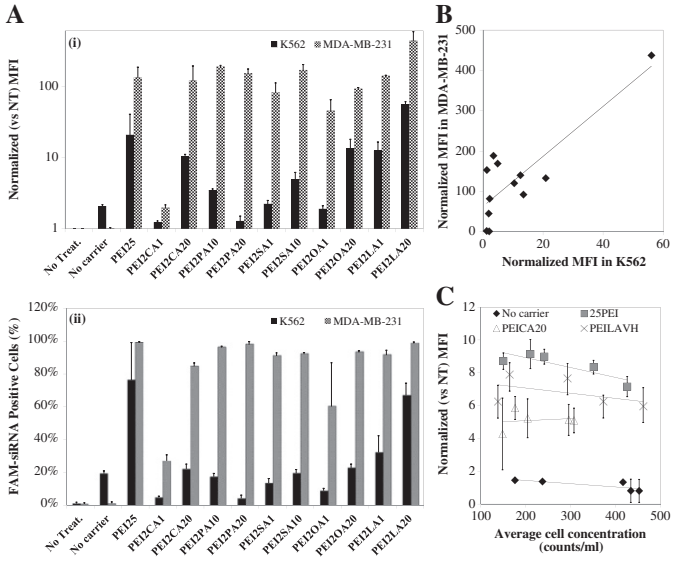

Fig. 1. (A) Cellular uptake of polymer/siRNA complexes in K562 and MDA-MB-231 cells. (i) Mean fluorescence intensity (MFI) of treated cells normalized against MFI of non-treated (NT) cells after 24 h exposure to complexes prepared with FAM-labeled siRNA and the indicated polymers. Two levels of substitutions were used for each lipid and complexes were prepared at a polymer:siRNA ratio of 8:1 and added to cells at siRNA concentration of 36 nM. (ii) Percentage of FAM-siRNA positive K562 and MDA-MB-231 cells after 24 h exposure to complexes. Non-treated cells were set at 1% positive cell as a background. (B) Correlation of siRNA cellular delivery between K562 and MDA-MB-231 cells with a correlation coefficient of 0.843 (based on MFI values from (Ai); $p = 0.001$). (C) Effect of cell seeding density on MFI of K562 cells exposed to FAM-siRNA complexes for 24 h (polymer:siRNA ratio of 8:1 and 36 nM siRNA concentration). Averages of final cell densities counted by flow cytometer were plotted against the normalized MFI.

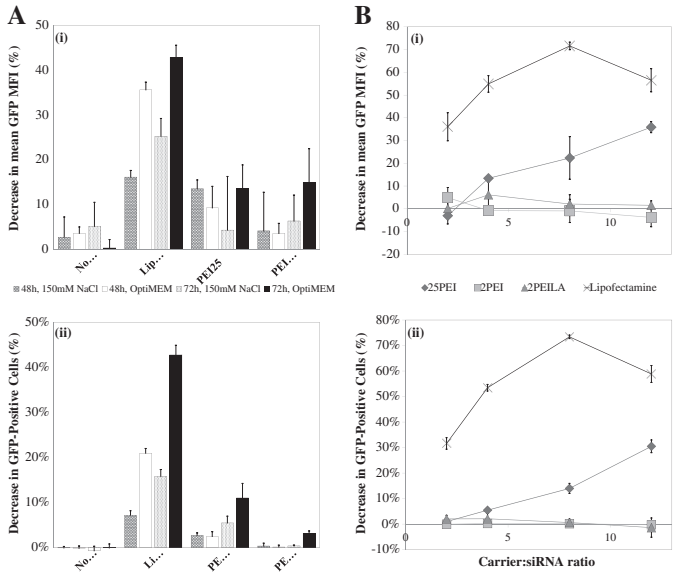

Fig. 2. GFP silencing in GFP-K562 cells after 48 and 72 h of siRNA treatment. (A) GFP silencing of complexes prepared in 150 mM NaCl and Opti-MEM and presented as (i) decrease in mean GFP fluorescence and (ii) decrease in GFP-positive cell population. The carriers used were Lipofectamine™ 2000, PEI25 and PEI2LA (2.1 LA/PEI) at a carrier:siRNA ratio of 8:1 (36 nM siRNA concentration). (B) GFP silencing in GFP-K562 cells treated with PEI25, PEI2, PEI2LA and Lipofectamine™ 2000 at carrier:siRNA ratios of 2:1, 4:1, 8:1 and 12:1 (36 nM siRNA concentration). GFP silencing was analyzed by flow cytometry 72 h after siRNA treatment and summarized as (i) decrease in mean GFP fluorescence and (ii) decrease in GFP-positive cell population.

Opti-MEM or 150 mM NaCl at a carrier:siRNA ratio of 8:1 and at 36 nM siRNA concentration. The carriers additionally used were the unmodified PEI2, PEI25 and Lipofectamine™ 2000. The latter was chosen since it was found to be among the most effective commercial reagents tested for GFP-silencing in GFP-K562 cells at a range of carrier:siRNA ratios (2:1, 4:1 and 8:1) and using a final siRNA concentration of 24 nM (Fig. S2). The range of carrier:siRNA ratios used in this initial screening fell into the manufacturer's suggestion for optimization of the reagents. Lipofectamine™ 2000 was chosen since, in addition to being most effective in this study (along with PEI25), it is widely used in the field and it has a relatively lower cost in comparison to other highly efficient carrier, Lipofectamine™ RNAiMAX.

The GFP silencing was summarized both as a percent decrease in mean GFP fluorescence (Fig. 2Ai) and the decrease in proportion of GFP-positive cells (Fig. 2Aii). Since control (scrambled) siRNA generated a minor (insignificant) change in the GFP fluorescence distinct for each carrier (see Fig. S3 for this data from day 3 assessment), silencing in cells treated with GFP-specific siRNA complexes was normalized against the control siRNA complexes. PEI2LA was found to have a small effect in decreasing the mean GFP fluorescence (<10% at both time points), while PEI25-delivered siRNA reduced the GFP expression by ~10% after three days of transfection. PEI25 appeared to perform better than PEI-LA based on the reduction of GFP-positive cells (Fig. 2Aii). No significant changes were found in GFP silencing with complexes prepared with 150 mM NaCl and Opti-MEM (for PEI25 and PEI2LA) (Fig. 2Ai). Since the silencing with complexes prepared in Opti-MEM was only increased by a minor amount (both for PEI25 and PEI2LA at 72 h post-transfection; Fig. 2Aii), we decided to prepare the complexes with 150 mM NaCl in subsequent studies. On the other hand, Lipofectamine™ 2000 gave a significant reduction in GFP (as much as 43% in mean GFP fluorescence), which was higher for the complexes prepared in Opti-MEM. The GFP silencing was also increased after 72 h post-transfection (from 16% to 25% for 150 mM NaCl vs. from 36% to 43% for Opti-MEM; Fig. 2Ai). The stronger silencing effect for Lipofectamine™ 2000 was also evident in the changes in the percentage of GFP-positive cells after 48 and 72 h post-transfection (from 21% to 43% for Opti-MEM Fig. 2Aii).

To evaluate whether the silencing efficiency of PEI2LA could be increased, the carrier:siRNA ratio was next varied since this ratio influenced the silencing efficiency of the complexes in other cell types [35,36]. While keeping siRNA concentration constant (36 nM), the varying of carrier:siRNA ratio was found to affect the efficiency of PEI25 and Lipofectamine™ 2000 (Fig. 2B). Complexes prepared with PEI25 led to increasing GFP silencing at increasing carrier:siRNA ratio, based on both mean decrease in GFP fluorescence and GFP-positive cell population, ultimately reaching to a ~30% silencing (based on mean GFP fluorescence) with ratio 12:1. However, the concentration of PEI25-treated cells was reduced at this ratio to ~78% in comparison with no-treated cells (from flow cytometry; not shown), indicating the cytotoxicity of this carrier. With Lipofectamine™ 2000, GFP silencing reached its peak level at the carrier:siRNA ratio of 8:1 (~70% silencing) with no apparent changes in cell concentration (not shown). PEI2 and PEI2LA polymers gave insignificant silencing at the ratios evaluated (Fig. 2Bi and 2Bii).

The less than optimal performance of LA-substituted PEI2 was surprising since this polymer showed effective silencing in several attachment-dependent cells in previous studies [20,29,37,38] as well as in the acute myeloid leukemia THP-1 cells [24]. The low levels of siRNA delivery observed to K562 cells with respect to attachment dependent cells might partly explain this result. The possibility of K562 cells not being conducive to siRNA-mediated silencing was also considered (e.g., due to low RISC complex), but the fact that Lipofectamine™ 2000 provided effective silencing argues against this possibility. To better explore the relationship between siRNA delivery and silencing efficiency, a more detailed comparison of PEI2LA against Lipofectamine™ 2000 was next conducted.

3.3. Correlating siRNA delivery to GFP silencing

The comparison of siRNA delivery and GFP silencing efficiencies was performed with wild-type K562 and GFP-K562 cells. PEI2LA, PEI25 and Lipofectamine™ 2000 carriers were used for this purpose to prepare the complexes at an increasing range of siRNA concentrations (36, 72 and 140 nM). A general increase in siRNA delivery was evident for all carriers at increasing siRNA concentration, with Lipofectamine™ 2000 showing the highest siRNA delivery. A positive correlation between the siRNA delivery and GFP silencing was found for PEI25 and Lipofectamine™ 2000: i.e., increasing delivery resulted in increasing GFP silencing (Fig. 3A and B). Although PEI25 was effective in silencing (~40% decrease in mean GFP fluorescence and ~25% in GFP-positive cell population at 140 nM siRNA; Fig. 3Bi and Bii), a significant (~50%) decrease in viable cell concentration was evident in both delivery (Fig. 3Aiii) and silencing studies (Fig. 3Biii), again indicating the high cytotoxicity induced by this polymer. The cytotoxicity of Lipofectamine™ 2000 was less than the one of PEI25 (Fig. 3Aiii and 3Biii). Although the siRNA delivery efficiency of PEI2LA (Fig. 3Ai and Aii) was nearly as high as the PEI25, the GFP silencing efficiency was considerably lower (Fig. 3Bi and Bii). No major changes in cell concentration were found with PEI2LA (Fig. 3Aiii and Biii), consistent with the previously published compatibility of this polymer with different cell types [20,24].

These results indicate that increasing siRNA delivery to K562 cells with PEI2LA did not necessarily lead to GFP silencing. This was unlike Lipofectamine™ 2000, which gave the most significant siRNA delivery as well as the silencing efficiency. It is likely that other barriers exist to the successful silencing in the case of PEI2LA. Among the likely reasons are a poor binding of the complexes to the cell membrane that does not promote endocytosis [39], endosomal entrapment followed by lysosomal

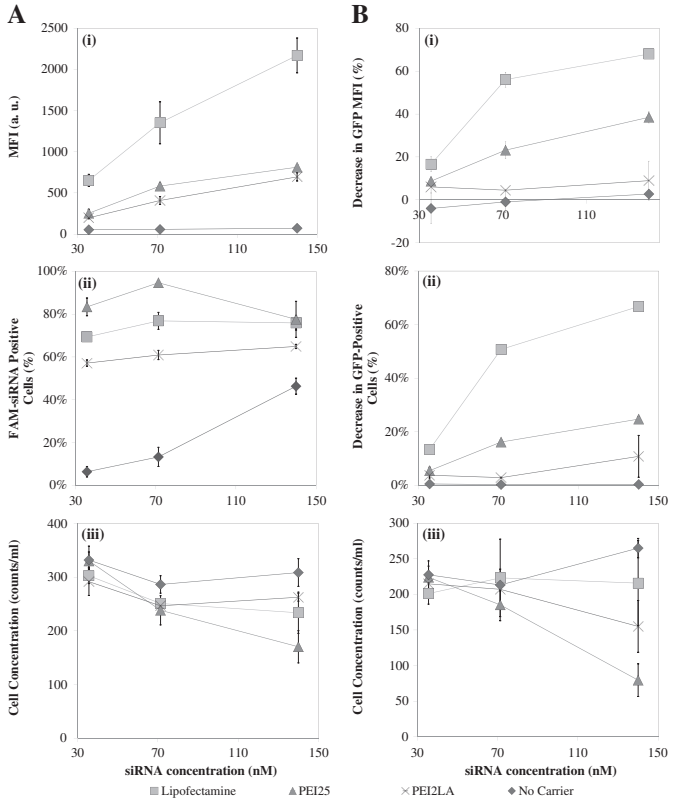

Fig. 3. FAM-siRNA uptake in K562 cells (A) and GFP silencing in GFP-K562 cells (B). The cells were exposed to siRNA complexes for 24 h (for uptake studies in A) or 72 h (for GFP silencing in B). The complexes were prepared with FAM-siRNA (A) and GFP-siRNA (B), and exposed to cells at siRNA concentrations of 36, 72 and 140 nM. A carrier:siRNA ratio of 4:1 was used for 25PEI and PEI2LA (2.1 LA/PEI) complexes, and 2:1 for Lipofectamine™ 2000 complexes. Cell concentrations obtained after the siRNA treatment periods, as determined by the counts from flow cytometry, are summarized in (iii) in A and B.

degradation in cytoplasm [18], and/or a lack of dissociation of the siRNA from the complex due to tight binding of the siRNA molecules to the polymer backbone [35,40]. Since Lipofectamine™ 2000 is a small cationic lipid (exact structure not disclosed by the manufacturer) that forms relatively large and less tight complexes with nucleic acids [27] for a better siRNA release, we next explored a readily available library of PA-modified PEI polymers of lower MW to evaluate whether the lipid modification on 0.6 and 1.2 kDa PEI can deliver siRNA more efficiently in K562 cells than the 2 kDa counterpart.

3.4. GFP silencing with PA-modified PEIs in K562 cells

A library of PA-substituted PEIs was next explored, which was constructed by using PEIs of different backbones (0.6 vs. 1.2 vs. 2.0 kDa; [21]). The lower MW PEIs were particularly appealing to us since the small molecular lipid Lipofectamine™ 2000 (structure and molecular weight not known to us) was effective in GFP silencing in K562 cells, as shown in Fig. 3. Previous studies with this library indicated that plasmid DNA delivery to immortal 293 T cells was achievable upon PA substitution on the ineffective native polymers as described in Reference [21]. The higher MW PEIs (1.2 and 2.0) performed generally better in plasmid DNA delivery and transgene expression, since they afforded a higher degree of PA substitution (up to 2 for 1.2 kDa PEI and up to 3.0 for 2 kDa PEI) as compared to 0.6 kDa PEI (<0.5 PA per PEI). The ζ-potentials of complexes prepared with these PA-modified polymers were found to be strongly positive and higher than those of unmodified PEI25 complexes [21]. However, these polymers were not previously evaluated for siRNA delivery.

For GFP silencing efficacies of PA-modified PEIs in GFP-K562 cells, the complexes were formed at a polymer:siRNA ratio of 8:1 and the cells were treated at a 72 nM siRNA concentration for 3 days. As expected, PEI25 showed ~54% decrease in mean GFP fluorescence (Fig. 4Ai) and ~40% in percentage of GFP-positive cells (Fig. 4Aii). However, this was accompanied by a severe reduction in cell concentration (~22% of non-treated cells, Fig. 4Aiii). Among the PA-substituted PEIs, the PEI1.2PAIII (with a lipid substitution of 1.98 PEI/PA) stood out in GFP silencing, in that it showed a ~63% decrease in the mean GFP fluorescence (Fig. 4Ai) and a ~55% decrease in GFP-positive cells (Fig. 4Aii), while giving a milder effect on cell concentration in comparison with PEI25 (~62% of non-treated cells, Fig. 4Aiii). The rest of the polymers had <30% decrease in mean GFP fluorescence and <20% decrease in GFP-positive cells. The extent of GFP silencing (based on mean GFP values) was correlated to the extent of lipid substitution for each type of PEI; a positive correlation was evident between the extent of PA substitution and GFP silencing (Fig. 4B). Among the three different MW PEIs evaluated, the GFP silencing was more sensitive to PA substitution for smaller PEIs, as compared to the 2 kDa PEI, in which silencing efficiency was not as strong as the lower MW PEI at equivalent PA substitution.

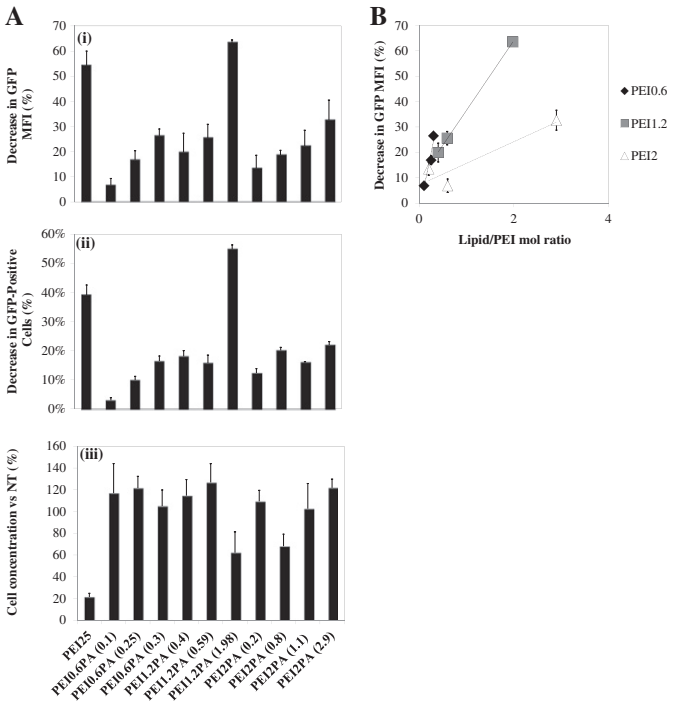

Fig. 4. GFP silencing in GFP-K562 cells with PA-substituted polymers. Reduction in GFP MFI (Ai), percent decrease in the GFP-positive population (Aii) and cell concentration/ml (as a percentage of non-treated cells (NT); Aiii) were assessed by flow cytometry 3 days after siRNA treatment. The complexes were prepared at a polymer:siRNA ratio of 8:1 and used at 72 nM GFP-siRNA concentration. (B) Correlation between the percent decrease in GFP MFI (data from Ai) and the extent of lipid substitution based on the number of lipids per PEI (shown in parenthesis in the polymer labels of Aiii). Correlation for each MW PEI is shown separately.

A more detailed comparison of the GFP silencing of PEI1.2PAIII with PEI25 and Lipofectamine™ 2000 was conducted (Fig. 5). The siRNA concentrations of 50 (Fig. 5A) and 100 nM (Fig. 5B) were tested using low and high carrier:siRNA ratios of 4:1 and 8:1 for the polymers and 2:1 and 4:1 for Lipofectamine™ 2000. At 50 nM siRNA and high ratio (8:1 for PEI1.2PAIII with PEI25 and 4:1 for Lipofectamine™ 2000) (Fig. 5A), PEI25 showed a ~34% decrease in the mean GFP fluorescence and ~16% decrease in GFP-positive cells; in contrast, PEI1.2PAIII and Lipofectamine™ 2000 had similar performances, giving 61% and 60% decrease in the mean GFP fluorescence (Fig. 5Ai) and 52% and 58% decrease in GFP-positive cells (Fig. 5Aii), respectively. The cell concentration under the same conditions was decreased to ~55% in comparison to non-treated group for the three carriers (Fig, 5Aiii). At the low ratio (4:1 for PEI1.2PAIII with PEI25 and 2:1 for Lipofectamine™ 2000), the decrease in mean GFP fluorescence at 50 nM siRNA was increased when a 100 nM concentration was used: going from 8.3% to 41.4% with PEI1.2PAIII, from ~21.3% to ~34% with PEI25 and from ~38% to ~61% with Lipofectamine™ 2000 (Fig. 5Ai and Bi). As expected, as the GFP silencing levels increased from 50 to 100 nM at the low ratio, an opposite effect was found in the cell concentration: going from a cell concentration of ~90% in comparison to no-treated cells at 50 nM for the three carriers (Fig. 5Aiii) to cell concentrations of ~67% for PEI1.2PAIII, ~53% for PEI25 and ~73% for Lipofectamine™ 2000 when 100 nM of siRNA were used (Fig. 5Biii). Finally, when the high ratios (8:1 for polymers and 4:1 for Lipofectamine™ 2000) were used at 100 nM siRNA, the decrease in mean GFP increased to ~75% with the three carriers (Fig. 5Bi) Again, a decrease in cell concentration associated with high GFP silencing effect was seen: the cell concentration after Lipofectamine™ 2000 treatment was decreased to ~50%; however, the cell concentration with PEI1.2PAIII and PEI25 polymers was decreased severely (to ~1% for both polymers, Fig. 5Biii) in comparison with non-treated cells. These results show that siRNA concentration as well as carrier:siRNA ratio are key factors for optimization of the transfection in order to find a balance between silencing efficacy and toxicity for each of the carriers.

The general relationship between the extent of lipid substitution and delivery efficiency for nucleic acids has been reported for a number of polymers [19–21,24,41]. This relationship has been particularly well studied in our hands with PEI2 polymers for siRNA delivery in attachment dependent cells [20,24]. The role of lipid substituent was previously explored by using different lipids, where we noted longer lipids (e.g., LA) to be more effective for silencing certain targets/cells (e.g., P-gp in MDA-MB-435 cells) as described in Reference [20], whereas shorter lipids (e.g., CA) were more effective for other targets/cells (e.g., survivin in MDA-MB-231 cells) as described in Reference [29]. We now report that lower MW backbone will be more beneficial for suspension-growing cells such as K562 cells. This outcome might be related to dissociation of complexes; as the MW of PEI backbone is decreased, we expect a better dissociation of complexes inside the cells. Another reason might be related to the strongly positive ζ-potentials of

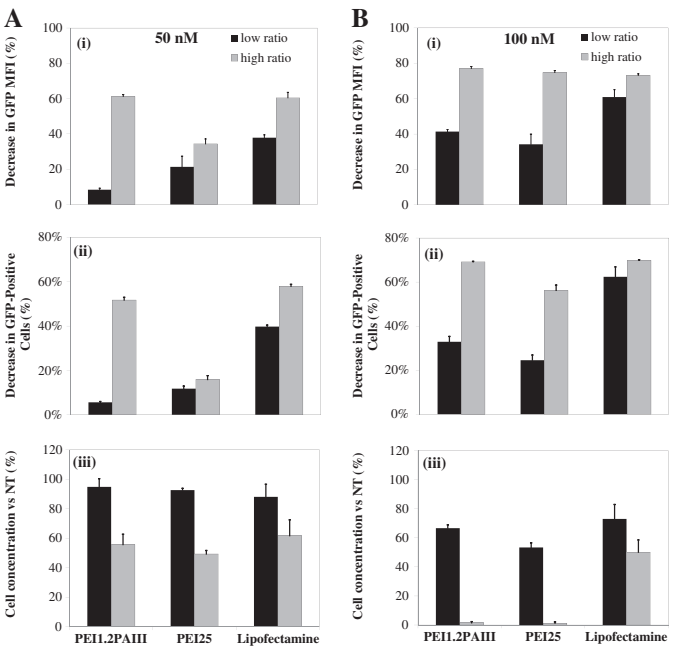

Fig. 5. GFP silencing in GFP-K562 cells transfected with PEI1.2PAIII (1.98 PA/PEI) and the commercial reagents PEI25 and Lipofectamine™ 2000 at siRNA concentrations of 50 nM (A) and 100 nM (B). Low carrier:siRNA ratios corresponds to 4:1 for PEI1.2PAIII and PEI25 and, to 2:1 for Lipofectamine™ 2000. High carrier:siRNA ratios corresponds to 8:1 for PEI1.2PAIII and PEI25 and, to 4:1 for Lipofectamine™ 2000. Percent decrease in mean GFP MFI (Ai and Bi), percent decrease in GFP-positive population (Aii and Bii) and cell concentration/ml (as percentage of non-treated cells (NT; Aiii and Biii)) were assessed by flow cytometry 3 days after transfection.

these complexes as it has been shown before with DNA [21] given that this could improve the cell membrane/complex affinity and consequently allow the endocytosis. Further studies to better reveal the role of dissociation are currently underway. It was also interesting to note that when the current PA-substituted PEI library was used in plasmid DNA delivery, the most effective polymer identified here for siRNA delivery was also equally effective in sustaining transgene expression [21]. Modification of 0.6 and 1.2 kDa PEIs with other lipids such as LA, OA and CA could be also effective in K562 cells, but these polymers have not been explored yet.

3.5. BCR-ABL silencing in K562 cells and apoptotic response

Finally, we investigated the ability of PA-substituted polymers to induce apoptosis and to silence the endogenous target *BCR-ABL* product. For this, the cells were treated with control (cr-siRNA) and BCR-ABL specific siRNAs at 50 and 100 nM at a polymer:siRNA weight ratio of 4:1, and assessed the extent of apoptosis after 1, 2 and 3 days post-transfection (Fig. 6A). A ratio of 4:1 was used for these experiments with the purpose of decreasing the cell toxicity due to the polymer at a ratio of 8:1 (as shown above in Fig. 4 and 5). Based on Annexin-positive cells (Fig. 6Ai), a significant elevation of early-apoptotic cell population was evident with 50 nM BCR-ABL siRNA on day 2, and with 100 nM BCR-ABL siRNA on day 2 and day 3. Based on PI-positive cells (Fig. 6Aii), a significant elevation of the late-apoptotic cell population was evident with 100 nM BCR-ABL siRNA on day 2 and day 3. The silencing kinetics was investigated in a parallel study by using q-PCR (Fig. 6B); compared to no treatment samples, treatment with control siRNA did not give any changes in the BCR-ABL mRNA levels on day 1 and day 2. However, BCR-ABL siRNA resulted in a significant reduction of the corresponding mRNA on day 1 (~20%), after which the relative quantity of the specific mRNA became equivalent to other groups.

A transient reduction of BCR-ABL mRNA levels was expected by siRNA delivery given that once these molecules bind and activate the RNA-induced silencing complex (RISC) in cytoplasm, its targeting and cleavage effect last only for a few days [11,39], possibly due to siRNA degradation within the cell [18]. Several other studies have also targeted *BCR-ABL* in K562 cells mediating non-viral delivery, for example: (i) Whithey et al. achieved a 90% transfection efficiency and a 84% protein suppression 48 h after having transfected the cells twice (with a 24-h interval) using Oligofectamine™ (siRNA concentration not specified) [42]; (ii) Arthanari et al. used the Tat-LK15 peptide to deliver siRNA (24 to 729 nM concentration by our calculation) and found a minimum of 70% reduction in p210 BCR-ABL 48 h post-transfection for all concentrations, while no silencing was detected after 48 h (no biological response was evaluated) [16]; (iii) Wilda et al. found that BCR-ABL mRNA levels of Oligofectanime-transfected cells were reduced to ~33% after 48 h post-transfection, and that the number of histone-associated DNA fragments (apoptosis induction) was at the same level in K562 cells treated either by RNAi or with

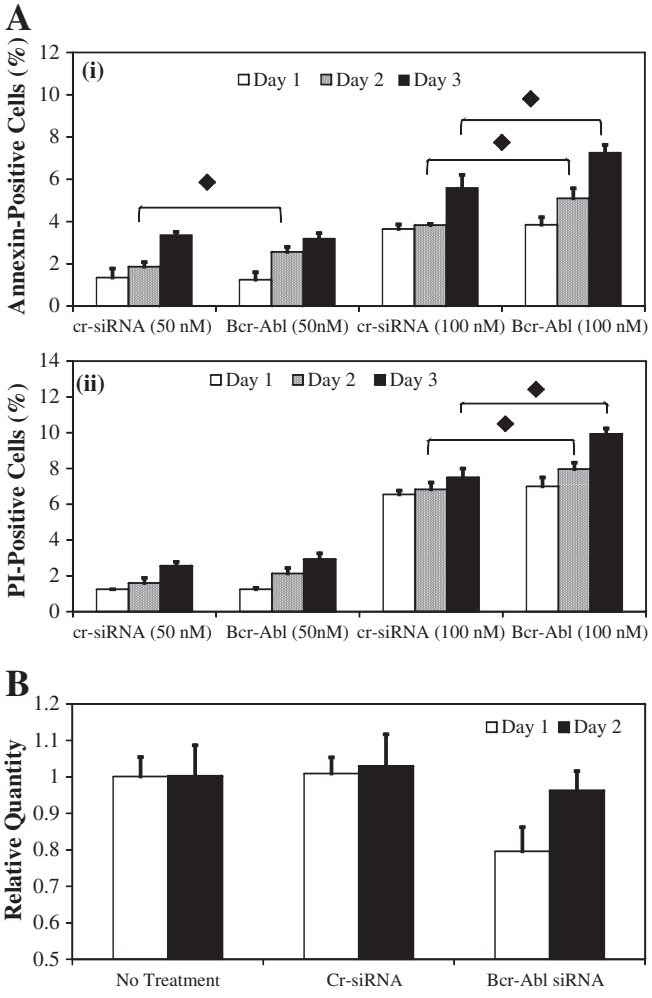

Fig. 6. BCR-ABL-siRNA induced apoptosis and changes in *BCR-ABL* expression. (A) FITC-Annexin V/PI staining of K562 cells exposed to PEI1.2PA (1.98 PA/PEI) complexes for 24, 48 and 72 h. The siRNAs used were either control-siRNA (cr-siRNA) or a mixture of 3 BCR-ABL-siRNAs, with a polymer:siRNA ratio of 4:1 and final siRNA concentration of 50 and 100 nM. Percentage of Annexin-positive cells (i.e., early apoptotic population) was the sum of the percentage of Annexin+/PI− and Annexin+/PI+ populations (Ai). Percentage of PI-positive cells was the sum of percentage of the Annexin-/PI+ and Annexin+/PI+ populations (Aii). Diamond represents a significant increase on early (Ai) or late (Aii) apoptosis ($p < 0.05$). (B) Quantitative PCR results showing BCR-ABL mRNA levels in K562 cells with no-treatment and, cells treated with PEI1.2PA complexes of control siRNA (cr-siRNA) or BCR-ABL siRNA at a polymer:siRNA concentration of 4:1 and a final siRNA concentration of 100 nM. mRNA levels were quantitated 1 and 2 days after siRNA treatment.

Imatinib (1 μM) [10], and; (iv) Zhelev et al. used Lipofectamine-mediated transfection to deliver 3 different siRNAs (3 × 60 nM) every 2 days for a 6 day period and observed a reduction in BCR-ABL mRNA levels, $p210^{BCR-ABL}$ oncoprotein and proliferation capacity by 82%, 64% and 50% by the end of the treatment, respectively [43]. Our PCR studies showed an early decrease of BCR-ABL mRNA levels (24 h post-transfection) and a biological response (apoptosis) to this silencing in K562 cells, albeit apparently at a lower dose and/or frequency of treatment. Although several carriers seem to be functional *in vitro*, it remains to be seen if they are all suitable for use in animal models. The polymeric carriers developed here, being fully described, could act as leads in this respect and offer possibilities for further optimization for animal use.

Finally, we must note that K562 cells used in this study served as a cell model to characterize, understand and improve the siRNA delivery system with lipid-modified polymers in suspension cells representing CML. Studies involving the use of these delivery systems in patient cells to target other over-expressed proteins involved in CML as well as animal studies to test the efficacy of delivery system *in vivo* will need to be carried out to further access the potential of the described polymeric delivery system.

4. Conclusions

Despite the efficient siRNA delivery with LA-substituted PEI2 to K562 cells, the efficiency for GFP silencing proved to be ineffective with these polymers, even when they were evaluated with different lipid substitution levels, and at a range of polymer:siRNA ratios (2:1 to 12:1) and siRNA concentrations (36 to 140 nM). Unlike the LA-substituted PEI2, PA-substituted PEI1.2 (lipid substitution of ~2.0 LA/PEI) conferred a higher GFP silencing and milder cytotoxicity in comparison to the PEI25. Based on GFP silencing studies with Lipofectamine™ 2000, the most effective commercial reagent in our hands, the efficacy achieved with the PA-substituted PEI polymers was at a similar level to commercial reagents. Silencing of *BCR-ABL* expression and induction of apoptosis in K562 cells were demonstrated in this study as a proof-of-principle for the potential of PA-substituted polymers for a functional therapeutic outcome. This effective siRNA delivery system might help to develop alternative strategies for CML therapy that will complement the existing therapies.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.05.014.

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