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RESEARCH PAPER

ShRNA-mediated knock-down of CD200 using the self-assembled nanoparticle-forming derivative of polyethylenimine

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ABSTRACT

Objective(s): ShRNA-mediated silencing strategy is considered to be a potent therapeutic approach. The present study aimed to assess the ability of the previously prepared polyethylenimine (PEI) derivative for the shRNA knock-down of the CD200 gene on the cells obtained from the patients with chronic lymphocytic leukemia (CLL).

Materials and Methods: Since there are several investigations regarding the role of CD200 over-expression in the progression of several malignancies (e.g., CLL), polyplexes were prepared using succinylated PEI and the plasmid encoding anti-CD200 shRNA. The ability of the nanoparticles for CD200 silencing at the levels of protein and mRNA, as well as the apoptotic effects induced by unmodified PEI and its derivative, were evaluated.

Results: Conjugation of succinic acid using the primary amines of PEI reduced the cell-induced toxicity of the polymer. Under such circumstances, 92.1% of the cells remained alive after treatment with the nanoparticles based on modified PEI. In addition, CD200 knock-down evaluations demonstrated a 50% reduction in the expression of the gene in the samples obtained from patients with CLL, while using the same formulation on the cells obtained from healthy donors decreased the CD200+ cells up to 10%. The results of CD200 silencing at the mRNA level revealed that the shRNA formulation could reduce the CD200 level in the cells of the patients by 3.2-6.06-fold relative to the cells transfected with non-effective, scrambled shRNA.

Conclusion: Our findings supported the application of succinylated PEI for the down-regulation of the CD200 gene in the upcoming attempts to develop nano-carriers for gene therapy.

Keywords: *CD200, Gene Delivery, Knock-down, Nanoparticle, Polyethylenimine*

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INTRODUCTION

Recently, great attention has been paid to immunotherapy as a new paradigm for cancer treatment [1, 2]. The groundbreaking advances in cancer immunotherapy have led researchers to seek novel molecular targets in order to enable the immune system to combat cancerous cells [3, 4]. According to reports, over-expressed immunoregulatory molecules on tumor cells lead to the reduction or suppression of antitumor immune responses [5, 6]. CD200 (formerly known as OX-2) is a type 1a transmembrane protein, which is expressed on numerous cells, including hematopoietic and non-hematopoietic cells [7]. On the other hand, the CD200 receptor (CD200R) is expressed on the monocyte/macrophage lineage and T lymphocytes. The interaction of CD200-CR200R sends an inhibitory signal, switching the cytokine production from Th1 to Th2, which in turn leads to the suppression of the T cell-mediated immune responses [5]. Growing evidence confirms the immunosuppressive role of CD200 in several malignancies, including chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma, and other B-cell lymphoproliferative disorders, such as hairy cell leukemia [8-10].

CLL is the most prevalent leukemia in western countries, the mortality rate of which has been estimated at 5,000 cases per year in the United

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States [11]. Despite the use of conventional chemotherapeutics (e.g., fludarabine) for the treatment of CLL, immunotherapy is considered to be a potential synergistic approach to the augmentation of the immune responses against leukemic cells. Rituximab and alemtuzumab are the most commonly used monoclonal antibodies in the treatment of CLL, which target CD20 and CD52, respectively [12]. Samalizumab is the first-in-human phase I monoclonal antibody to target CD200, which has demonstrated great tolerability and changes in the immune responses against CLL [13]. However, the laborious and costly methods for the large-scale production of monoclonal antibodies have caused researchers to consider gene therapy approaches (e.g., shRNA plasmid silencing strategy) to down-regulate the expression of CD200 and the subsequent CD200-induced immunosuppression.

RNA interference (RNAi) has shown great therapeutic ability in the treatment of several diseases [14]. Post-transcriptional gene silencing using RNAi is mediated by double-stranded small interfering RNA (siRNA) and short hairpin RNA (shRNA), which interact with an enzyme complex known as the RNA induced-silencing complex (RISC). The sequence-specific degradation of mRNAs occurs following the formation of RISC, and shRNA molecules could be produced inside the cells using intracellular expression cassettes (plasmid DNA) with prominent advantages over siRNA in terms of the lower off-target effects and co-expression with a reporter gene, as well as the possibility to use inducible promoters to control the expression of shRNA molecules [15]. Therefore, the shRNA plasmid silencing strategy could be applied as a potent and specific knock-down approach.

The delivery of genetic materials into the cells is considered to be a major challenge in gene therapy. Most of the clinical trials regarding gene therapy have been performed using viral carriers [16]. However, there are concerns for their wide clinical applications due to the associated immunogenicity, potential oncogenicity, and risk of insertional mutagenesis, as well as the high costs and difficult production procedures. Therefore, non-viral gene carriers (e.g., polycationic compounds) might be considered as the alternative vehicles with lower immunogenicity compared to viral vectors [17].

Among various polycationic carriers, polyethylenimine (PEI) is the most extensively investigated polymer for gene delivery [18, 19]. Due to high density of its amine content, PEI is able to condense genetic materials outside the cells, protecting them against nucleases. Furthermore, the high amine content of the polymer causes the 'proton sponge effect', which enables the polymer/gene complex (i.e., polyplex) to escape the endo/lysosomal compartments before degradation is initiated [20].

Despite the controversial reports regarding the importance of the proton sponge effect, it is the most acknowledged mechanism to explain the PEI transfection efficiency [21]. However, the high positive-charge density and the subsequent apoptotic effect of this polymer, as well as its unfavorable hydrophilic-hydrophobic balance, are considered to be the major limitations in the wide clinical application of PEI. In other words, PEI must be modified with various chemical conjugation strategies so as to be improved in terms of cell-induced toxicity [22-30].

Various conjugation strategies have been assessed in our previous investigations aiming to improve the PEI properties in gene delivery, such as the substitution of the PEI primary amines with different hydrophobic moieties [20, 26, 31]. Accordingly, the succinylation of PEI was the most effective conjugation method to enhance the gene delivery efficiency of this polymer and reduce its cytotoxicity. These zwitterion-like derivatives of PEI may overcome the low transfection efficiency and biocompatibility of unmodified PEI. Therefore, we hypothesized that succinylated PEI (suc-PEI) could be considered an efficient non-viral carrier for the shRNA plasmid-induced silencing of CD200.

In the present study, suc-PEI was evaluated based on our previous investigations considering its ability to transfer the plasmid encoding shRNA against the *CD200* gene in cell lines, as well as the cells obtained from patients with CLL patients. Moreover, the apoptotic effects of modified PEI were compared with the unmodified parent polymer in different conditions.

MATERIALS AND METHODS

Experimental materials

N-[2-hydroxythylpiperazine-N-[2-ethanesulfonic acid] (HEPES) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Munich, Germany). CD200 human shRNA plasmid kit consisting of four unique 29-mer shRNA constructs in retroviral GFP

vector was obtained from OriGene (Rockville, MD, USA), and the non-effective 29-mer scrambled shRNA cassette in pGFP-V-RS Vector was used as well (OriGene, Rockville, MD). FITC Annexin V apoptosis detection kit was obtained from BioLegend (Fell, Germany). In addition, cDNA synthesis and real-time polymerase chain reaction (PCR) were performed using the PrimeScript™ RT reagent kit (Perfect Real Time, TaKaRa, Dalian, China) and RealQ Plus 2x Master Mix Green High ROX™ (AmpliQon, Denmark).

The cell culture experiments were performed using fetal bovine serum (FBS) and RPMI 1640 (Gibco, Gaithersburg, MD, USA). All the solvents and chemicals used in the study were obtained from Sigma-Aldrich (Munich, Germany) with the highest purity available.

Nanoparticle preparation

Polymer/plasmid complexes (polyplexes) were prepared based on the weight/weigh ratio (C/P ratio) by adding 50 microliters of a solution containing the PEI conjugate at proper concentrations to the same volume of plasmid DNA (40 µg/ml) in the HBG buffer (HEPES-buffered glucose solution; HEPES: 20 mM, 5% glucose, pH=7.2). Afterwards, the mixture was incubated at room temperature for 30 minutes to form stable nanoparticles.

The polymer conjugate used in this investigation was the succinylated derivative of PEI (suc-PEI), which was synthesized by adding proper levels of succinic anhydride to the PEI solution, followed by incubation at room temperature for three hours [26, 31]. At the next stage, the conjugate was purified and characterized in terms of the buffering capacity, particle size, zeta potential, pDNA condensation ability, and protection against enzymatic degradation as described in our previous investigations [26, 31]. Accordingly, maximum transgene expression was obtained by the succinylated PEI at the conjugation degree of 30%. Therefore, 30% suc-PEI was selected for the current research.

Cell culture and MTT assay

CLL-CII chronic lymphocytic leukemia cell (C582, NCBI, Pasteur Institute, Iran) and Waco3-CD5 B-cell chronic lymphocytic leukemia cell (C547, NCBI, Pasteur Institute, Iran) were incubated at the temperature of 37°C in a humidified atmosphere with 5% CO_2 in 100 microliters of RMPI 1640, supplemented with 10% FBS, streptomycin (100 µg/ml), and penicillin (100 IU/ml). The cells were cultured at 1×10^4 cells per well in 96-well plates for 24 hours before transfection and performing the toxicity assay. For the MTT assay, the PEI conjugates were examined at the conjugation degrees of 10%, 20%, 30%, and 40% in order to determine the compounds with lower toxicity for further gene silencing experiments.

To prepare the nanoparticles at various C/P ratios (range: 0.25-8), proper concentrations of PEI and its derivatives were prepared in HBG buffer. Following that, pDNA solutions (40 µg/ml) were prepared in the same buffer in separate tubes. Prior to the MTT assay, 50 microliters of the PEI solution was added to the plasmid solution so as to prepare the nanoparticles at the C/P ratios of 0.25, 4, and 8 with the final volume of 100 microliters. The MTT assay was carried out by adding 10 microliters of the prepared formulations to the 96-well plates, followed by the replacement of the medium with 100 microliters of a fresh medium containing FBS after four hours. The final concentration of shRNA plasmid was adjusted to 200 ng pDNA/well.

After incubation for 24 hours, the medium was aspirated, and the MTT solution (5 mg/ml) was added to each well and incubated for another 1.5 hours. Finally, the formed formazan crystals were dissolved in dimethyl sulfoxide (100 µl/well), and the absorbance was measured using an ELISA reader (ELx800, BioTek, Germany) at the wavelength of 590 nanometers. In addition, the background was corrected at 630 nanometers. Data were expressed as mean and standard deviation (SD; n=3).

Apoptosis assay

In order to assess the apoptotic effects of the PEI conjugates, FITC Annexin V apoptosis detection kit with 7-AAD was used in accordance with the instructions of the manufacturer. In this experiment, 30% suc-PEI was compared with the unmodified parent polymer at the C/P ratio of eight, in which the highest transfection efficiency was achieved based on our previous study. In brief, the cells were seeded into a six-well plate at the density of 1×10^6 cells/well. Afterwards, the cells were treated with the polymer alone or polyplexes (polymer/pDNA complex) to demonstrate the effects of polyplex formation on the apoptosis of polycationic compounds. In order to evaluate the effect of the incubation time on apoptosis, the

assay was carried out four and 24 hours after the treatment.

At the next stage, the cells were suspended in Annexin V binding buffer, and five microliters of FITC Annexin V and the same amount of 7-AAD viability staining solution were added and incubated at room temperature in the dark for 15 minutes. Following the addition of 400 microliters of Annexin V binding buffer, apoptosis was measured based on flow cytometry (FACS Calibur, Becton Dickinson, CA) using the FlowJo software (TreeStar Inc., CA). All the measurements were performed in triplicate, and the cells treated with no polymer, plasmid, and polyplex (i.e., medium only) were considered as negative control.

Green fluorescent protein (GFP) expression

In order to demonstrate the ability of the PEI conjugate to transfer plasmid DNA to the cells, the transfection experiment was carried out on the WACO-3 and CLL-CII cell lines. The cells were seeded in six-well trays at the density of 1×10^6 cells/well and treated with the polyplexes containing the CD200 human shRNA plasmid expressing the green fluorescent protein (GFP). The transfection test was performed at the C/P ratio of eight, and the polyplexes were prepared as previously described (*vide supra*). Transfection efficiency was analyzed based on flow cytometry (FACS Calibur, Becton Dickinson, Mountain View, CA) using the FlowJo software (TreeStar Inc., San Carlos, CA). In addition, the plasmid alone (i.e., without carrier) was considered as negative control.

CD200 silencing experiment

In order to evaluate the efficiency of PEI and its derivative in the silencing of the *CD200* gene at the proteome level, knock-down experiments were performed on the $CD200^+$ cells obtained from patients with CLL, as well as the CLL-CII cell line. The heparin-treated whole blood of 15 volunteer donors with CLL was used to obtain the human peripheral blood mononuclear cell using the Ficoll-Paque PLUS gradients (Amersham Biosciences, Uppsala, Sweden). Moreover, the same procedure was carried out to obtain lymphocytes from five healthy volunteer. In these experiments, $CD5^+CD19^+$ primary CLL cells were considered as the target cells for the silencing evaluations.

The polyplexes were prepared at the C/P ratio of eight using unmodified PEI or 30% suc-PEI as previously described. Since the CD200 human shRNA plasmid kit contains four unique 29-mer shRNA constructs that target various sequences, these oligonucleotides were designated as *CD200* shRNA#1, *CD200* shRNA#2, *CD200* shRNA#3, and *CD200* shRNA#4. At the next stage, 10 microliters of the polyplex formulation was added to each well following incubation at the temperature of 37°C for four hours. Afterwards, the medium was replaced with fresh RPMI 1640 containing 10% FBS and incubated for an additional 48 hours. At the end of the transfection procedure, the cells were harvested and suspended in FACS buffer (phosphate buffered saline, 2% FBS, and 0.1% Na_3N) at 5×10^6 cells/mm. Following that, five microliters of APC anti-human CD200 antibody (Biolegend, Germany) was added, followed by incubation on ice for 5-10 minutes in the dark. Finally, the cells were centrifuged and resuspended in an appropriate buffer and analyzed based on flow cytometry (FACS Calibur, Becton Dickinson, Mountain View, CA) using the FlowJo software (TreeStar Inc., San Carlos, CA). The cells treated with no plasmid or polyplex (i.e., medium only) were considered as negative control.

To assess the *CD200* knock-down at the mRNA level, real-time quantitative PCR was performed [20]. RNA extraction was carried out using the RNA isolation kit (Jena Bioscience, Germany) in accordance with the instructions of the manufacturer. Following the isolation of RNA and treatment with DNase (Thermo Fisher Scientific, Waltham, MA, USA), the levels of *CD200* transcripts were quantified using the PrimeScript™ RT reagent kit (Perfect Real Time, TaKaRa, Dalian, China) and RealQ Plus 2x Master Mix Green High ROX™ kit (AmpliQon, Denmark). The *CD200* primers used in this study were as follows: forward: 5′-AATACCTTTGTTTTTGGGAAGATCT-3′,reverse: 5′-GGTGGTCTTCAGAGAATTTGTAGTGA-3′Transcript levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (forward: 5′-ACTTCAACAGCGACACCCACT-3′, reverse: 5′-GCCAAATTCGTTGTCATACCAG-3′).

All the reactions were performed using MJ mini thermal cycler (BIO-RAD, Germany), and the relative gene expression of *CD200* was calculated using the $2^{-(\Delta\Delta CT)}$ method based on GAPDH as the reference gene.

Statistical analysis

Data analysis was performed using student's t-test at the significance level of less than 0.05. The obtained results were expressed as mean and SD.

RESULTS

Preparation of the nanoparticles

In our previous study, PEI reacted with succinic anhydride at various conjugation degrees based on the mole percentage of the estimated number of the primary amines. According to the obtained results, 30% suc-PEI showed maximum transfection efficiency at the C/P ratio of eight. Meanwhile, the polyplexes formed by the electrostatic interaction between 30% suc-PEI and the plasmid could condense pDNA effectively, forming nanoparticles with the particle size of approximately 125 nanometers and zeta potential of 15 mV. In the present study, PEI succinylation at the substitution degree of 30% could significantly reduce the hemolytic activity of the polycationic compound, while the substantial protection ability against nuclease digestion was denoted [26]. Therefore, it could be concluded that 30% suc-PEI was the candidate polyplex formulation for shRNA plasmid delivery into the cell lines, as well as the $CD200^+$ cells obtained from patients with CLL.

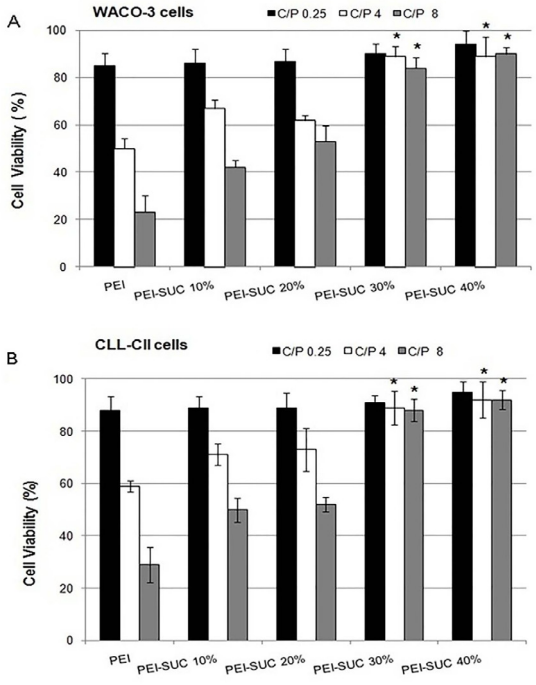

Fig 1. Cellular Cytotoxicity Induced by Unmodified PEI and Its Derivatives at Various Carrier:Plasmid Ratios (Cytotoxicity was assayed by MTT method and expressed as percentage of cell viability; *P<0.05 for modified PEI derivatives versus unmodified PEI at same C/P ratio)

MTT assay

Cell viability was evaluated following the treatment of the WACO-3 and CLL-CII cell lines with various conjugates of PEI at different C/P ratios using the MTT assay. The MTT assay was carried out to determine the conjugate with minimum toxic effects on these cell lines for further investigations. As is depicted in Fig 1, the cell-induced toxicity at the lowest C/P ratio (0.25) was negligible in both cell lines, while the toxic effects increased by increasing the amount of the polymer used for polyplex formation. In other words, the cell viability of the polyplexes formed with unmodified PEI decreased from 85% to 25% by increasing the C/P ratio from 0.25 to eight.

On the other hand, increasing the conjugation degree to 30-40% significantly decreased the effect of polymer concentration on cell-induced toxicity. These conjugates did not induce toxicity even at the highest C/P ratios evaluated in the current investigation, while the PEI conjugates with lower conjugation degrees (10-20%) showed toxicity in a concentration-dependent manner. Moreover, the cell viability for 30% suc-PEI at the C/P ratio of eight was approximately 85%, while the unmodified PEI led to the cell death of approximately 80% at the same C/P ratio.

Apoptosis assay

The apoptotic effects of unmodified PEI and its succinylated derivative were assessed using the Annexin V/7-AAD assay. The FITC-conjugated Annexin V was attached to phosphatidylserine and showed early apoptosis, while late apoptosis was detected by the binding of 7-AAD to the nuclear DNA. Figs 2 and 3 depict the apoptotic effects of unmodified PEI and 30% suc-PEI, respectively.

In order to demonstrate the effects of nanoparticle formation on the induced apoptosis, the apoptosis by the polymer along and along with the polyplexes (polymer/pDNA complexes) was measured four and 24 hours after the treatment. As is shown in Fig 2, the unmodified PEI polymer resulted in apoptosis in 75.8% and 85.7% of the cells four and 24 hours after the treatment, respectively. In other words, no significant changes were observed in the apoptotic effects of the polymer alone four and 24 hours after the cell treatment. On the other hand, nanoparticle formation using the polymer decreased apoptosis induction from 86.6% to 58.5%. At 24 hours after the treatment of the cells with the nanoparticles, 28.1% of the cells remained alive, while the percentage of the live cells after the treatment with unmodified polymer reduced to 4.3%.

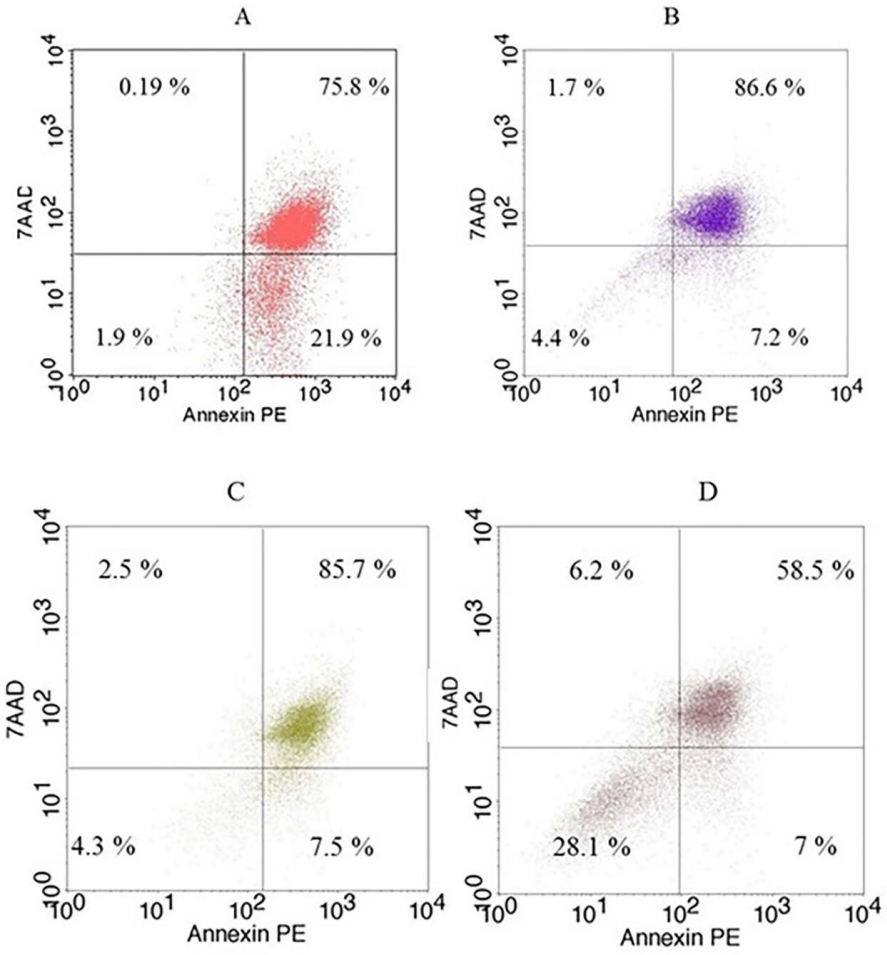

Fig 2. FITC AnnexinV/7-AAD Flow Cytometry Demonstrating Apoptosis Induction Post-transfection by A) Unmodified PEI Four Hours after Treatment, B) Unmodified PEI 24 Hours after Treatment, C) Unmodified PEI/pDNA Polyplex Four Hours after Treatment, and D) Unmodified PEI/pDNA Polyplex 24 Hours after Treatment

The apoptosis assay was also used for 30% suc-PEI four and 24 hours after the treatment (Fig 3). The obtained results demonstrated that after the treatment of the cells with the conjugate alone, only 32.3% of the cells entered the late apoptosis stage after four hours, while the percentage of the cells in late apoptosis phase decreased to 17.5 following the treatment with the polyplexes formed by 30% suc-PEI.

Furthermore, apoptosis was evaluated 24 hours after the treatment with either the PEI conjugate or polyplexes containing the PEI derivative.

According to the obtained results, 92.1% of the cells remained alive after the treatment with 30% suc-PEI nanoparticles.

In other words, only 28.1% of the cells did not enter the apoptosis stages following the treatment with the unmodified PEI polyplexes after 24 hours, while the percentage of the live cells after the addition of 30% suc-PEI polyplexes increased to 92.1%.

GFP expression analysis

The ability of PEI and its succinylated derivative for pDNA delivery was evaluated on the WACO-3 and CLL-CII cell lines.

The polyplexes were prepared at the C/P ratio of eight, and the expression of GFP was assessed by flow cytometry.

As is depicted in Fig 4, the unmodified PEI polyplexes resulted in the transfection efficiency of 10.8 and 12.4 on the WACO-3 and CLL-CII cell lines, respectively.

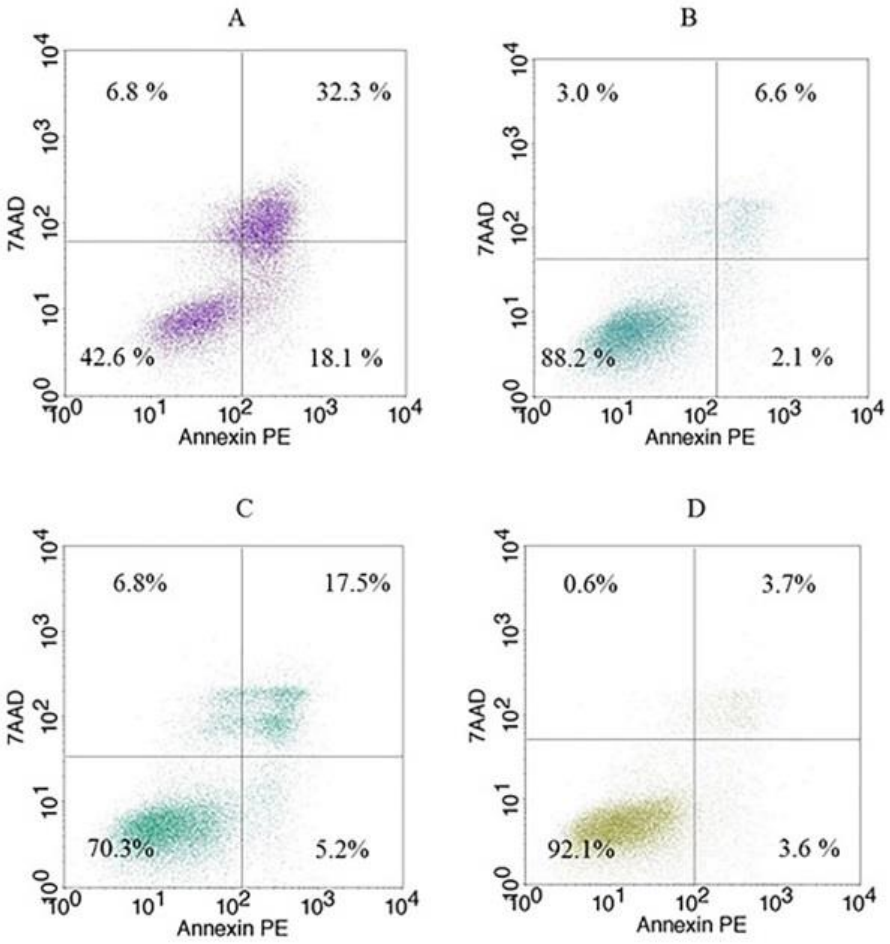

Fig 3. FITC AnnexinV/7-AAD Flow Cytometry Demonstrating Apoptosis Induction Post-Transfection by A) Suc-PEI Derivative Four Hours after Treatment, B) Suc-PEI Derivative 24 Hours after Treatment, C) Suc-PEI/pDNA Polyplex Four Hours after Treatment, and D) Suc-PEI/pDNA Polyplex 24 Hours after Treatment

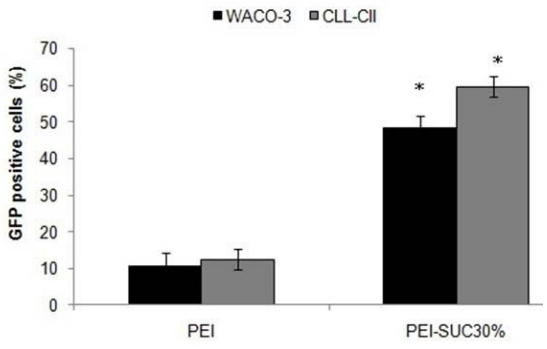

Fig 4. GFP Expression Measured by Flow Cytometry on WACO-3 and CLL-CLL Cell Lines Using Polyplexes Prepared by Unmodified PEI or Its Derivative (*P<0.05, PEI derivative compared to unmodified parent polymer at same C/P ratio (n=3; error bars represent mean±standard deviation)

Following the treatment of the cell lines by the succinylated derivative of PEI, the transfection efficiency on the WACO-3 cells increased to 48.2%. In addition, the maximum gene transfer efficiency was observed on the CLL-CII cell line, while GFP expression increased to 59.6%. In other words, the succinylated PEI derivative could improve GFP expression 4.8-fold at the C/P ratio of eight.

CD200 silencing tests

In order to evaluate the efficiency of the PEI derivative in the down-regulation of *CD200*, four unique commercial 29-mer shRNA constructs were used, and the *CD200* expression was determined at the protein and mRNA levels. Initially, $CD5^{+}CD19^{+}$ primary CLL cells were assessed to determine the most efficient shRNA construct.

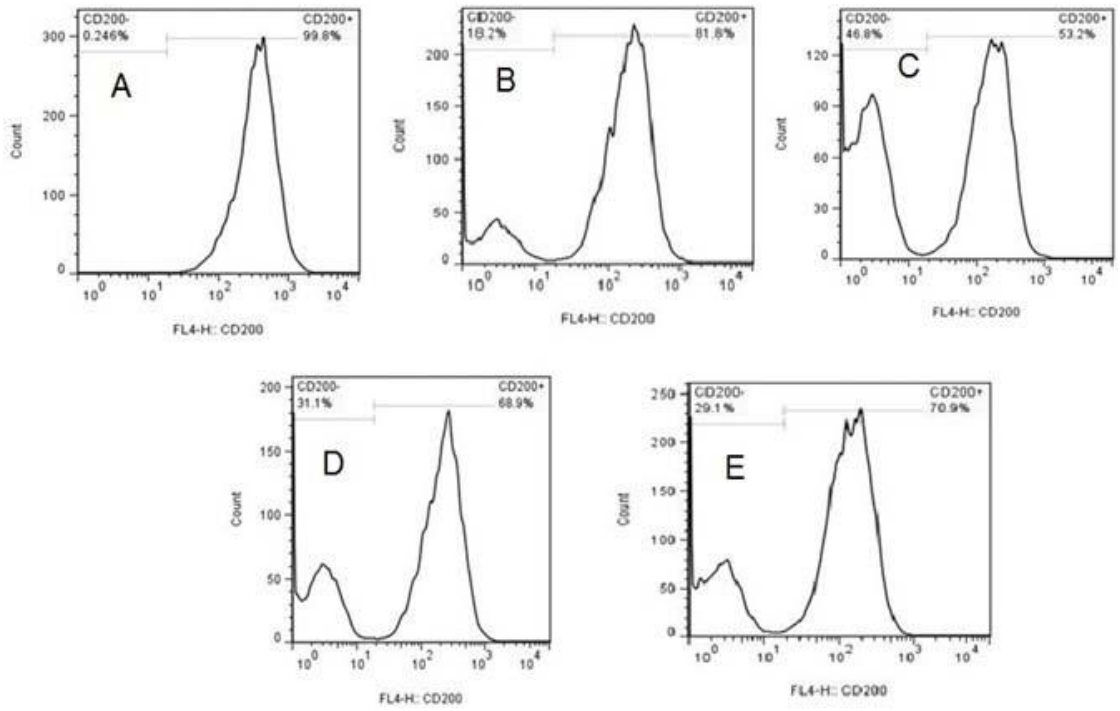

Fig 5. CD200 Expression Level Measured by Flow Cytometry on CD5+CD19+ Primary CLL Cells Obtained from A) Patients with CLL and Treatment with Polyplexes Prepared by 30% suc-PEI, B) shRNA#1, C) shRNA#2, D) shRNA#3, and E) shRNA#4 at C/P Ratio of Eight

Optimal silencing was achieved by shRNA#2 (Fig 5), in which the number of the $CD200^+$ cells decreased from 99.8% to 55.3%. In other words, a 45% decrease in the number of the $CD200^+$ cells was observed using the shRNA#3, which was formulated with 30% suc-PEI at the C/P ratio of eight.

The other shRNA constructs decreased the number of the $CD200^+$ cells to the range of 81.8-68.9%.

Therefore, shRNA#2 was considered to be most efficient and applied for further investigation to down-regulate the *CD200* gene on the cells obtained from patients with CLL.

According to the results of *CD200* down-regulation on the cells obtained from patients with CLL and healthy donors, the number of the $CD200^+$ cells in the healthy donors was up to 25.7%, while the protein was over-expressed on at least 61% of the $CD5^+CD19^+$ cells in patients with CLL (Fig 6-A). In other words, a 2.4-4-fold increase was observed in the number of the over-expressed CD200 cells obtained from patients with CLL compared to the cells obtained from healthy donors.

Following the treatment of all the cells using shRNA#2, which was formulated with 30% suc-PEI, the number of the cells over-expressing the CD200 protein reduced significantly.

Furthermore, the down-regulation of *CD200* resulted in a 50% reduction in the expression of the gene in the samples obtained from patients with CLL. However, using the same formulation on the cells obtained from healthy donors decreased the CD200 cells by approximately 10%.

The down-regulation experiment was also carried out at the mRNA level in order to demonstrate the decreased CD200 mRNA following the treatment of the cells by the same formulations (Fig 6-B).

The results of CD200 silencing at the mRNA level indicated that the shRNA formulation could reduce the CD200 levels of the cells in patients with CLL 3.2-6.06 folds compared to the cells transfected with the non-effective scrambled shRNA.

The results of the down-regulation at the transcriptome and proteome levels were consistent, showing a significant decrease in the $CD5^+CD19^+$ cells over-expressing *CD200* on their surfaces.

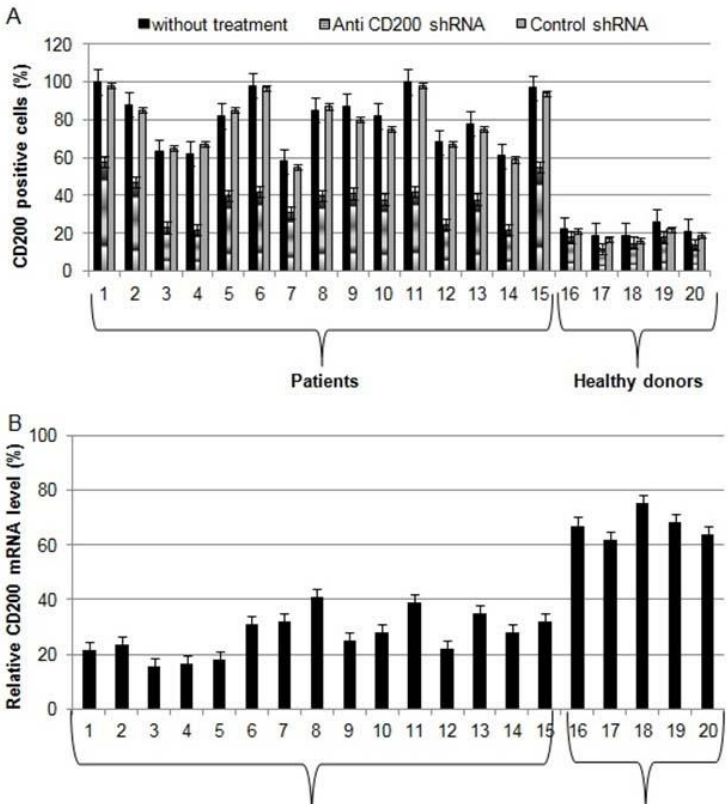

Fig 6. CD200 Expression Level Measured by Flow Cytometry on CD5+CD19+ Primary CLL Cells Obtained from CLL Patients and Healthy Donors at A) Protein Level Measured by Flow Cytometry and B) mRNA Level by RT-PCR Following 24 Hours of Transfection

DISCUSSION

CLL is considered to be the most prevalent leukemia in the adults in western countries, accounting for 9% of all cancers and 30% of the leukemia cases [11]. Several strategies have been proposed for the treatment of CLL, including the use of chemotherapeutic agents and monoclonal antibodies [11, 13]. Recently, great attention has been paid to gene-based therapies, such as antisense technology, aptamers, ribozymes, and RNA interference via siRNA or shRNA [15]. However, the wide clinical applications of these therapies have remained unfulfilled due to insurmountable delivery problems.

PEI is a promising non-viral delivery system for oligonucleotides, which interacts electrostatically with the negatively charged backbone of nucleic acid materials, condensing them into nano-sized particles and protecting them against enzymatic degradation. However, PEI toxicity might be considered the most significant restrictive factor in the clinical applications of this agent [18, 24, 32, 33].

Various strategies have been adopted to reduce PEI toxicity, while maintaining or even increasing its transfection efficiency [18]. An extensively studied conjugation method for the reduction of the cell-induced toxicity of PEI is the conjugation of hydrophobic moieties through the surface amines [22, 26, 31, 34, 35]. These amines play a key role in the high ability of the polymer to transfer genetic materials through the proton sponge effect. Additionally, positively charged amines are essential to interaction with negatively charged nucleic acids and nanoparticle formation. The conjugation of hydrophobic moieties may modulate the hydrophobic-hydrophilic balance of the polymer, which could act as a prerequisite for efficient gene delivery [36]. These conjugations not only improve the hydrophobic-hydrophilic balance of PEI, but they also may modulate the positive charge on the surface of the polymer,

which is considered to be the major influential factor for the induction of cytotoxicity. In other words, modulation of positive charge on the surface of the polymer is an effective approach to reducing cell toxicity [31].

In our previous investigations, several substitutions were assessed to improve PEI toxicity. Accordingly, the succinylation of PEI at the conjugation degrees of 10-20% could elevate siRNA delivery, while the higher degrees of substitution (30-40%) could enhance its plasmid delivery ability [26, 31]. Increased transfection efficiency in both cases is the result of reduced toxicity, which allows researchers to use more polymers for transfection experiments. However, it seems that the most efficient vectors for pDNA are not essentially the most potent for siRNA delivery [31]. Due to the differences in the structure, size, and negative charge of pDNA and siRNA or shRNA, various modifications or conjugation degrees might be used to increase the efficiency of the vector in each case.

According to the results of the MTT assay in the present study, PEI toxicity was largely dependent on the concentration of the polymer used in polyplex preparation. In other words, increasing the concentration of the polymer in the final formulation significantly decreased cell viability, which could be attributed to the effect of the higher positive charge on cell damage. The positive charge on the polymer surface plays a pivotal role in the interaction of polyplexes with the negatively charged components of the cell membrane and the subsequent adsorptive endocytosis [37]. However, higher positive charges may disrupt the integrity of the cell membrane, thereby leading to cell death [38, 39].

According to the current research, succinylation of PEI (particularly at higher substitution degrees) could significantly increase cell viability in both cell lines, which could be attributed to the effect of positive charge reduction. Reduction of the positive charge could be an efficient route for the improvement of gene delivery with the persistence of the other characteristics of PEI, such as nucleic acid condensation, buffering capacity, nanoparticle formation, and protection against nuclease digestion. According to the previous studies on PEI succinylation, grafting at the substitution degrees of 30-40% is still acceptable for an efficient gene carrier, while reducing its toxicity.

The results of apoptosis assay in the present study clearly indicated the impact of charge reduction on the apoptotic effects of PEI. Some reports in this regard have suggested a two-stage mechanism for PEI toxicity [39]. Correspondingly, the necrotic stage starts within 30 minutes after exposure to positively charged polyplexes. This stage largely depends on the association between the positively charged complexes and negatively charged components of the plasma membrane, which leads to the permeabilization of the cell membrane. The second stage of toxicity is the apoptotic stage, which occurs 24 hours after exposure. It has been suggested that the second stage is the result of mitochondrially mediated apoptotic programs. In this stage, the polyplexes that cross the cell membrane may interact with the membrane of the mitochondria and form channels in the outer membrane, thereby resulting in the activation of caspases and apoptosis induction [39]. However, the results of apoptosis analysis in the current research revealed no significant difference between the apoptotic effects of the unmodified polymer alone four and 24 hours after exposure.

In the present study, the interaction of PEI with pDNA and formation of polyplexes diminished the induced apoptotic effects after 24 hours, which could be due to charge neutralization following the interaction with negatively charged nucleic acids. Following the conjugation of hydrophobic moieties on the PEI structure, a significant reduction was observed in the apoptotic effects, which was not only the effect of charge neutralization and polyplex formation, but it was also the impact of positive charge modulation on the polymer. Furthermore, this was consistent with the zeta potential measurements of the polyplexes formed by the unmodified PEI and PEI derivatives [26].

The results of GFP expression and down-regulation of *CD200* also demonstrated the elevated transfection efficiency of succinylated PEI compared to the unmodified polymer. The increased gene transfer ability might be associated with several determinants of transfection efficiency. Highly positive polycationic compounds are able to effectively condense nucleic acid materials outside the cells, manifesting high protection efficiency [35]. However, some reports in this regard have indicated that looser polyplexes have higher transfection efficiencies compared to strong complexes, which could be attributed to the effect of the dissociation of nucleic acids

from the cargo [24, 40]. Either for plasmid DNA or siRNA, the free form of nucleic acids is required for the expression or down-regulation of the target proteins. Therefore, facilitating vector un-packaging might be a promising route to increase transfection efficiency.

Overall, the optimization of PEI to act as a potent, non-toxic gene carrier requires the balance between several factors, including the condensation ability, particle size, and zeta potential, as well as the buffering capacity and protective effects of the polymer against nuclease digestion.

CONCLUSION

According to the results, the shRNA-mediated silencing of the *CD200* gene was achieved using the PEI derivative that was produced using a simple conjugation method. The most probable reason for the enhanced gene delivery of PEI could be the reduced toxicity resulting from the positive charge modulation and introduction of negatively charged moieties onto the polymer surface.

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