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Effective down-regulation of signal transducer and activator of transcription 3 (STAT3) by polyplexes of siRNA and lipid-substituted polyethyleneimine for sensitization of breast tumor cells to conventional chemotherapy

Arash Falamarzian,[1] Hamidreza Montazeri Aliabadi,[2] Ommoleila Molavi,[3] John M. Seubert,[1,4] Raymond Lai,[3] Hasan Uludağ,[1,2] Afsaneh Lavasanifar[1,2]

[1]Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E1
[2]Department of Chemical & Materials Engineering, Faculty of Engineering, University of Alberta, Edmonton, Alberta, Canada T6G 2V4
[3]Department of Laboratory Medicine and Pathology, Cross Cancer Institute, Edmonton, Alberta, Canada T6G 1Z2
[4]Department of Pharmacology, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Received 4 August 2013; revised 6 October 2013; accepted 8 October 2013
Published online 25 October 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.34992

Abstract: Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that plays a major role in the development of resistance to conventional anti-cancer drugs in many types of cancer, when constitutively activated. Inhibition of STAT3 is considered as a promising strategy for inhibition of tumor growth and overcoming the drug resistance manifested. In this study, the capability of STAT3 knockdown by lipid substituted low molecular weight (2 kDa) polyethyleneimine (PEI2) complexes of STAT3–siRNA was assessed. The efficiency of PEI/STAT3-siRNA polyplexes in the induction of STAT3 associated cell death in wild type and drug-resistant MDA-MB-435 breast cancer cells as monotherapy and upon combination with chemotherapeutic agents, doxorubicin and paclitaxel, was also investigated. Our results identified linoleic acid-substituted (PEI-LA) polymer as the most efficient carrier among different lipid substituted PEI2 for siRNA delivery, leading to most STAT3 associated loss of cell viability in MDA-MB-435 cells. STAT3-siRNA delivery by the PEI-LA polymer resulted in efficient down-regulation of STAT3 at both mRNA and protein levels. Furthermore, pre-treatment of cancer cells with STAT3–siRNA formulation increased the cytotoxic effect of doxorubicin and paclitaxel in both wild type and drug resistant MDA-MB-435 cells. The results of this study point to the potential of PEI-LA polyplexes of STAT3–siRNA as inhibitors of STAT3 expression in breast tumor cells. The results also demonstrate an improved efficacy for chemotherapeutic drugs in combination with lipid-substituted low molecular weight PEI-LA/STAT3-siRNA complexes in comparison to drug therapy alone. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 102A: 3216–3228, 2014.

Key Words: STAT3, siRNA, polycationic polymers, hydrophobic modification, chemotherapy

How to cite this article: Falamarzian A, Aliabadi HM, Molavi O, Seubert JM, Lai R, Uludağ H, Lavasanifar A. 2014. Effective down-regulation of signal transducer and activator of transcription 3 (STAT3) by polyplexes of siRNA and lipid-substituted polyethyleneimine for sensitization of breast tumor cells to conventional chemotherapy. J Biomed Mater Res Part A 2014:102A:3216–3228.

INTRODUCTION

Signal transducers and activator of transcriptions (STATs) are a family of cytoplasmic proteins that convey signals from cell membrane to the nucleus.[1] STAT3 is an important member of this family that is constitutively activated in different malignancies including breast, head and neck, and prostate cancers.[2–4] STAT3 transduces signals from various oncogenic proteins and pathways. It can be activated by different kinds of cytokines and growth factors including interleukin (IL) 6, interferon (IFN) β, IFN γ, fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF).[5–10] Following activation, STAT3 monomers dimerize through reciprocal phosphotyrosine–SH2 interactions, translocate to the nucleus, and bind to STAT3-specific sites on the promoters of target genes to induce gene transcription.[11] Many proteins that are important for tumor-cell proliferation and survival are regulated by STAT3, including BCL-X_L, MCL1, Survivin, MYC, and Cyclin D1.[12] Pro-angiogenic factors including HGF, basic fibroblast growth factor (bFGF), and matrix metalloproteinase 2 (MMP2) are also shown to be

Correspondence to: H. Uludağ; e-mail: huludag@ualberta.ca or A. Lavasanifar; e-mail: afsaneh@ualberta.ca
Contract grant sponsors: Alberta Cancer Foundation (ACF), Canadian Institute of Health research (CIHR)

up-regulated by STAT3.[13,14] The capability of STAT3 to participate in many features of oncogenesis including cell proliferation, survival, angiogenesis, invasion, and tumor induced immune-suppression has made it a desirable target in cancer therapy.[15] Several small-molecule drugs have been tested to inhibit STAT3 and provoke apoptosis in cancer cells. Their mechanism of action includes upstream inhibition of growth factors and cytokines, disruption of STAT3 dimerziation, blocking of STAT3 nuclear translocation, and inhibition of STAT3 DNA-binding and transcriptional activity.[1,16–21] Clinical use of many of these compounds, however, has been halted because of poor solubility, low bioavailability, limited biological stability, and undesirable side effects caused by their non-specific anti-STAT3 activity.

An alternative approach for anti-STAT3 therapy to the use of small molecule inhibitors of STAT3 function would be the use of small interfering RNA (siRNA) that inhibit the expression of STAT3.[11,22,23] The purpose of such therapy is to specifically induce the cleavage of STAT3 mRNA for efficient gene silencing. RNA interference (RNAi) is a sequence specific, evolutionary mechanism that can be employed to suppress any specific gene, giving it an edge over classic low molecular weight drugs that may cause broad spectrum of side effects as a result of activity on undesired molecular targets.[24] However, advancement of this technology as a ground-breaking therapy with a high degree of specificity for disease related genes has been relatively slow, mainly due to problems associated with its safe and effective delivery in a clinical setting.[25] Delivering siRNA to the desired site of action constitutes a major challenge due to its rapid degradation by nucleases, uptake by the reticuloendothelial system and speedy renal excretion, all of which can lead to early elimination of the siRNA from biological system.[26,27] In addition, the polyanionic nature of siRNA and its large molecular weight causes poor cellular uptake, restricting its access to intracellular siRNA target.[28]

In order to alleviate these obstacles, different delivery materials including viral vectors, lipids, and polymeric nanocarriers have been examined for siRNA delivery. High immunogenicity and unsatisfactory safety profile of viral vectors has limited the application of such delivery systems in clinical settings.[29,30] Cationic lipids and polymers can be used to electrostatically bind and form effective complexes with negatively charged siRNA. Polymeric systems have the advantage of structural flexibility over their lipid counterparts and can be chemically tailored in order to obtain desirable physiochemical properties.[31] Among cationic polymers, high molecular weight- polyethyleneimines (PEIs) (MW $>$ 25 kDa) have shown to be efficient in protection and siRNA delivery.[32,33] These polymers have high cationic charge density and are able to non-covalently bind siRNA and protect it against enzymatic degradation.[34] Unprotonated amines of the PEI generate a so-called "proton-sponge effect" causing enhanced influx of protons and water, endosomal rapture, and release of complexes to the cytoplasm.[35] The toxicity of high molecular weight PEIs has been a major hurdle for their clinical use. Lower molecular weight PEIs might be more suitable due to better safety profiles but, these polymers have shown low transfection efficiency.[36] Hydrophobic modifications of low MW PEIs have been tried in order to develop more effective delivery systems for siRNA. These hydrophobic moieties are expected to increase the interaction of polymers with lipophilic membrane of cells and ease the uptake of complexed siRNA.[37] In previous studies, aliphatic lipid-substituted 2 kDa PEIs with an array of fatty acids with different chain lengths (from C8 to C18) have been explored for plasmid DNA delivery. An equivalent transfection ability to that of 25 kDa PEI was observed for lipid substituted 2kDa PEIs, without the toxic effect associated with the former polymer.[36] In separate studies, the lipid-substituted 2 kDa PEIs have been utilized for siRNA delivery and shown to improve the cellular uptake of siRNA compared to unmodified 2 kDa PEIs while demonstrating negligible toxicity. Effective silencing of target P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP) by relevant siRNAs complexes of lipid modified PEI 2kDa in P-gp transfected MDA-MB-435/MDR cells and BCRP-transfected MDCK cells have also been demonstrated, respectively.[37,38] BCRP silencing caused a reversal in resistance to an anticancer agent, mitoxantrone, and a 14-fold reduction of its IC_{50} value in drug resistant cells.

In the current study, we utilized a library of lipid-substituted 2 kDa PEIs polymers for efficient siRNA delivery and silencing of STAT3 in a triple negative breast cancer cell line, that is, MDA-MB-435, and investigated the potential benefit of this approach in sensitization of wild type (WT) and resistant (RES) phenotypes to cytotoxic effects of conventional anti-cancer drugs, doxorubicin (DOX), and paclitaxel (PTX).

EXPERIMENTAL SECTION

Materials

The 2 kDa PEI (PEI2) (Mn: 1.8 kDa, Mw: 2 kDa), 25 kDa PEI (PEI25K), anhydrous dimethylsulfoxide (DMSO), caproyl chloride (C8; $>$99%), oleyl chloride (C18:1 9Z, 12Z; 99%), linoleyl chloride (C18:2 9Z,12Z; 99%), Hanks' Balanced Salt Solution (HBSS), 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). Cell culture media RPMI 1640, DMEM, penicillin–streptomycin, fetal bovine serum, HEPES buffer solution (1*M*) and trypsin/ethylenediaminetetraacetate were purchased from GIBCO, Invitrogen Corp (USA). The scrambled siRNA used as control and Silencer® FAM™ labeled Negative siRNA were supplied from Ambion (catalog numbers: AM4636). The Silencer siRNAs against STAT3 was purchased from Qiagen (catalog numbers: SI02662338, sequence: CAGCCTCTCTGCAGAATTCAA). Doxorubicin and paclitaxel were purchased from Ontario chemicals Inc. and LC laboratories, respectively.

Cell line

The wild-type MDA-MB-435 (MDA-MB-435/WT) cells were originally obtained as a gift from the laboratory of Dr. Robert Clark (Georgetown University, USA). The MDA-MB-435 resistant cells (referred to as DOX/RES) were developed

TABLE I. Lipid-Substituted PEI 2K Library

Polymer	Substituted Lipid	Lipid:PEI ratio[a]	Lipid/PEI[b]	Methylene/PEI[c]
PEI-SA0.5	Stearic acid	0.066	0.5	8.4
PEI-SA3.6		0.1	3.6	66.6
PEI-OA1	Oleic acid	0.066	1.0	18.1
PEI-OA1.7		0.1	1.7	30.0
PEI-OA2.5		0.2	2.5	44.1
PEI-CA6.9	Caprylic acid	0.2	6.9	56.8
PEI-LA1.5	Linoleic acid	0.2	1.5	27.2
PEI-LA2.1		0.2	2.1	37.9

[a]Molar ratios used for synthesis.
[b]Extent of lipid substitution per PEI calculated from ^{1}H NMR analysis.
[c]Extent of methylene substitution per PEI, calculated based on the extent of substitution (from ^{1}H-NMR) and number of methylene groups in each lipid.

through exposure of MDA-MB-435/WT cells to DOX with a gradual dose increase, starting from 0.2 μg/mL (~20% of IC50; that is, concentration for 50% cell death), and continuing with 0.5, 0.75, 1.0, 1.5, and 2 μg/mL. Cells were exposed to each dose for three passages or one week (whichever longer), and frozen at the end of each stage. MDA-MB-435 WT and DOX/RES cells were cultured in RPMI 1640 media with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C and 5% CO_2. DOX/RES cells were cultured in the presence of 2 μg/mL of DOX in culture media at all times. Cell cultures were considered confluent when a monolayer of cells covered more than 80% of the flask surface. To propagate the cells, a monolayer was washed with HBSS, and subsequently incubated with 0.05% trypsin/EDTA for 3 min at room temperature. The suspended cells were centrifuged at 650 rpm for 5 min, and were re-suspended in the medium after removal of the supernatant. The suspended cells were either sub-cultured at 10% of the original count or seeded in multi-well plates for testing.

Synthesis of lipid-substituted polymers

Lipid-substituted polymers were synthesized according to the process which has been described elsewhere.[36,39] Briefly, a 50% PEI2 solution (in water) 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. The lipid:PEI molar ratios were systemically varied between [0.066 and 0.2]. The mixture was allowed to react for 24 h at room temperature under nitrogen, after which excess ethyl ether was added to precipitate and washed 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 ($\delta \approx 0.8$; —CH3) and PEI ($\delta \approx 2.5$–2.8; NH—CH2—CH2—NH—) were integrated, normalized for the number of protons in each peak, and used to determine the extent of lipid substitutions on polymers (Table I). PEI-SA0.5 and PEI-SA3.6 refer to stearic acid substitution at 0.5 and 3.6 lipids/PEI2. PEI-OA1, PEI-OA1.7, and PEI-OA2.5 refer to oleic acid substitution at 1, 1.7, and 2.5 lipids/PEI2. PEI-CA6.9 refers to caprylic acid substitution at 6.9 lipids/PEI2. PEI-LA1.5 and PEI-LA2.1 refer to linoleic acid substitution at 1.5 and 2.1 lipids/PEI2 accordingly.

Assessing the cellular association of siRNA polyplexes by flow cytometry

To assess the ability of polymers to transfer siRNA into WT and DOX/RES cells, complexes were prepared using 5-carboxyfluorescein (FAM)-labeled scrambled siRNA at polymer:siRNA ratios of 8:1 (wt/wt) by incubation in water (corresponding 36 n*M* siRNA and 4 μg/mL polymer in culture medium). Confluent cell cultures were trypsinized, re-suspended as described before and seeded in 24-well plates (0.6 mL in each well) at 50% confluence. After 24 h, 400 mL fresh medium was added to each well, followed by the addition of siRNA polyplexes. The prepared polyplexes were added to wells in triplicates and were incubated at 37°C for 24 h. After the incubation period, cells were washed with HBSS and trypsinized. A 3.7% formaldehyde solution was added to suspend the cells and the siRNA uptake was quantified by a Beckman Coulter QUANTA 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.

Assessing the cellular uptake and distribution of selected siRNA polyplexes by fluorescence microscopy

Fluorescent microscopy was used to assess the intracellular trafficking of siRNA in WT cells. FAM-labeled siRNA was complexed with PEI-LA1.5, PEI-LA2.1, PEI25K at polymer:-siRNA ratio of 8:1 (wt/wt) and were added to the wells (final polymer and siRNA concentration of 6 μg/mL and 54 n*M* per well). Cells grown on the glass-bottom Petri dishes were incubated with the polyplexes for 3 and 24 h, respectively. At the end of incubation period, the cells were washed three times with phosphate buffered saline (PBS). For nucleus labeling, cells were incubated with DAPI (Molecular Probes, Invitrogen Co., OR) for 15 min. Localization of complexes in cells was visualized by an epiflourescence microscope (Carl Zeiss Microscope systems, Jena, Germany) with identical settings for each study.

In vitro cytotoxicity studies

The cytotoxicity of STAT3 and scrambled siRNA complexed with different polymers within the PEI library was evaluated in MDA-MB-435 WT and DOX/RES cells using MTT assay. Confluent cell cultures were trypsinized and re-suspended as described before, and seeded in 24-well plates (0.6 mL in each well) at 50% confluency. After 24 h, 400 mL fresh media was added to each well. siRNA polyplexes were prepared using the scrambled and STAT3-siRNA at polymer:siRNA ratio of 8:1 (wt/wt) and were added to the wells (final polymer and siRNA concentration of 6 μg/mL and 54 n*M* per well in triplicate wells). Cells were incubated for 72 h in their normal maintenance conditions and then 60 μL of MTT solution (5 mg/mL in HBSS) was added to each well. After 2 h of incubation at 37°C, the medium was removed, and 300 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) with cell-less medium as blank. The absorbance of polyplex-treated cells was compared to untreated cells and % cell viability was calculated using the following equation.

$$\%\ \text{cell viability} = (\text{absorbance of siRNA polyplex treated cells} / \text{absorbance of untreated cells}) \times 100$$

To determine the optimum polymer:siRNA ratio, confluent cell cultures of WT and DOX/RES cells were trypsinized and seeded in 24-well plates (600 mL in each well) at 50% confluence. After 24 h, 400 mL fresh medium was added to each well. The PEI-LA/siRNA polyplexes were prepared at different ratios of polymer:siRNA in sterile tubes using the STAT3-siRNA and scrambled siRNA with polymer:siRNA ratios of 2:1, 4:1, and 8:1 (wt/wt) (corresponding to 54 n*M* siRNA and 1.5, 3 and 6 μg/mL polymer in cell culture medium), and were added to the wells in triplicates. Cells were incubated for 72 h in their normal maintenance conditions, followed by MTT assay as described earlier.

Furthermore, in order to evaluate the siRNA dose response as part for PEI-LA based polyplexes, confluent cell cultures of WT and DOX/RES cells were trypsinized and seeded in 24-well plates (600 mL in each well) at 50% confluence. After 24 h, 400 mL fresh medium was added to each well. The PEI-LA2.1/siRNA polyplexes were prepared using the scrambled and STAT3-siRNA at the ratio of 8:1 (wt/wt) and were added to the wells to give final siRNA concentrations of 9, 18, 27, 45, 54, and 72 n*M* per well in triplicate. Cells were incubated for 72 h in their normal maintenance conditions, followed by MTT assay as described earlier.

Assessing the silencing activity of STAT3-siRNA polyplexes by real-time PCR

Real-time (RT) PCR was carried out to determine STAT3 knock-down at the mRNA level. Confluent cell cultures were trypsinized and re-suspended as described before, and seeded in 6-well plates (2.4 mL in each well) at 50% confluency. After 24 h, 1.8 mL fresh medium was added to each well. siRNA polyplexes were prepared using scrambled and STAT3-siRNA at polymer:siRNA ratio of 8:1 (wt/wt) and were added to the wells (final polymer and siRNA concentration of 6 μg/mL and 54 n*M* per well) in triplicate. After 48 h, total RNA was extracted using RNeasy spin columns (Qiagen, Mississauga, ON, Canada) according to the manufacturer's recommendations. cDNA was synthesized following Invitrogen's protocol, briefly adding 2 μL master mix 1 (0.5 μL Oligo$(dT)_{12-18}$ Primer), 0.5 μL random primer and 1 μL (10 mM MdNTP's per sample) to 10 μL of RNA (5000 ng) and then heated to 65°C for 5 min. About 7 μL of Master Mix 2 (4 μL 5 × Synthesis Buffer, 2 μL DTT (0.1*M*) and 1 μL RNAout RNase inhibitor (1.8 U/μL)) was then added and the samples heated at 37°C for 2 min. About 1 μL of M-MLV RT enzyme was then added per sample and they were heated at 25°C for 10 min, 37°C for 50 min, and 70°C for 15 min. Real time PCR was performed on a StepOnePlus™ RT-PCR system (ABI) with GAPDH (Forward: 5′-CAC ATG GCC TCC AAG GAG TAA-3′) and (Reverse: 5′-TGA GGG TCT CTC TCT TCC TCT TGT-3′) as the endogenous housekeeping gene and the specific STAT3 primers (Forward: 5′-AAG TTT ATC TGT GTG ACA CCA ACG A-3′) and (Reverse: 5′-CTT CAC CAT TAT TTC CAA ACT GCA T-3′). 7.5 μL of master mix containing 5 μL of SYBR® Green ROX™ qPCR Mastermix (Qiagen, Mississauga, ON, Canada) and 2.5 μL primer (3.2 μM; per sample) was added to each well. Then 2.5 μL of template of each sample was added in triplicate. Levels of mRNA were measured as CT threshold levels and normalized with the individual GAPDH control CT values. Altered mRNA levels in cells are indicated as a "fold change" compared with control cells. Each sample was measured at least three times.

Assessing the silencing activity of STAT3-siRNA polyplexes by Western blot

Western blot was carried out to determine STAT3 knock-down at the protein level. Confluent cell cultures were trypsinized and re-suspended as described before, and seeded in 6-well plates (2.4 mL in each well) at 50% confluence. After 24 h, 1.8 mL fresh medium was added to each well. siRNA polyplexes were prepared using the scrambled and STAT3-siRNA at polymer:siRNA ratio of 8:1 and were added to the wells (final polymer and siRNA concentration of 6 μg/mL and 54 n*M* per well) in triplicate wells. After 48 h incubation, the cells were washed with cold phosphate buffered saline (PBS) and lysed using RIPA cell lytic buffer supplemented with 0.1 m*M* phenylmethylsulfonylfluoride (PMSF) (Sigma Aldrich), a protease inhibitor cocktail (Nacalai Inc, San Diego, CA) and a phosphatase inhibitor cocktail (Calbiochem, EMD Biosciences, Darmstadt, Germany). The lysate was then incubated on ice for 30 min which was followed by centrifugation at 17,000*g* for 15 min to remove genomic DNA. Protein quantification was determined by the BCA protein assay kit (Pierce, Rockford, IL) and equal amounts of protein (50 μg) were loaded in 4–20% Tris-HCl precast gel (Bio-Rad, Mississauga, Ontario). After gel electrophoresis, the proteins were transferred to a nitrocellulose

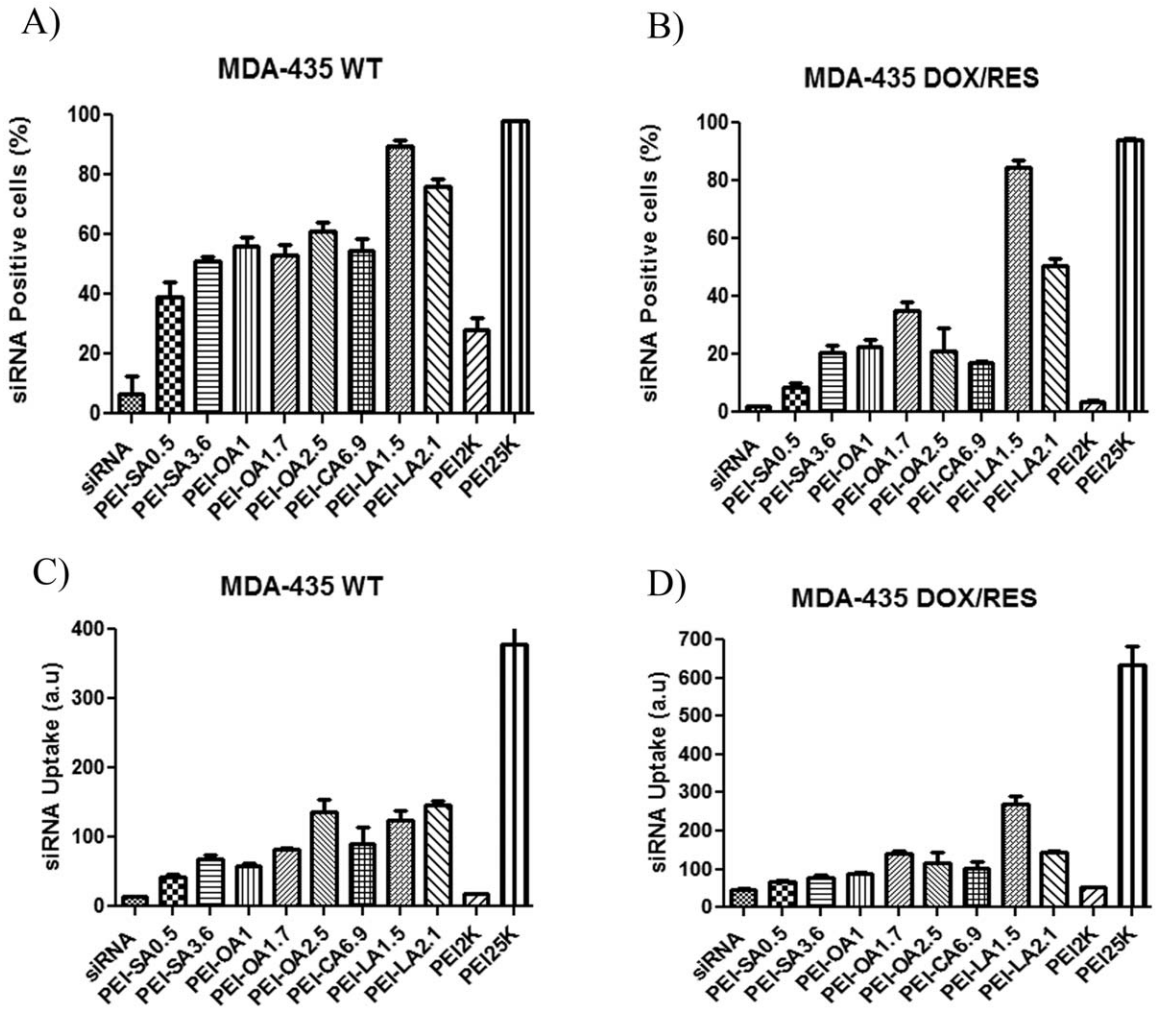

FIGURE 1. Cellular uptake of siRNA polyplexes by flow cytometry. Cellular uptake of polymer/FAM-siRNA complexes by MDA-MB-435 WT and DOX/RES cells. A, B: The percentage of cells positive for FAM-siRNA after 24 h exposure to siRNA complexes at polymer:siRNA ratios of 8:1 (weight/weight). C, D: The mean fluorescence of the cells after 24 h exposure to complexes. The complexes were added to the wells to give final polymer and siRNA concentration of 4 μg/mL and 36 n*M* per well. The data are the mean ± SD for $n = 3$.

membrane and stained with 0.05% Ponceau S (Sigma-Aldrich) to ensure equivalent protein loading per lane. The membrane was probed with antibodies against STAT3 and p-STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were then detected using peroxidase-conjugated anti-mouse IgG and visualized by enhanced chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Scientific, Rockford, IL). Optical intensity of STAT3 and p-STAT3 band was quantified and normalized to actin protein band using Adobe Photoshop software.

Assessing the effect of STAT3-siRNA/PEI-LA pre-treatment on the cytotoxicity of doxorubicin and paclitaxel in WT and DOX/RES breast cancer cells

The cytotoxicity of free DOX and PTX against WT and DOX/RES cells was evaluated. Confluent cell cultures of WT and DOX/RES cells were trypsinized and seeded in 24-well plates (600 mL in each well) at 50% confluence. After 24 h, 400 mL fresh medium was added to each well. DOX and PTX solutions were prepared at different concentrations in sterile tubes and added to the wells in triplicates. Cells were incubated for 24 h in their normal maintenance conditions, followed by MTT assay as described earlier. These experiments were followed by evaluating the effect of STAT3 silencing on cytotoxicity of these anticancer drugs against WT and DOX/RES cells. Confluent cell cultures of WT and DOX/RES cells were trypsinized and seeded in 24-well plates (600 mL in each well) at 50% confluence. After 24 h, 400 mL fresh medium was added to each well. The siRNA polyplexes were prepared in sterile tubes using STAT3 or scrambled siRNA with PEI-LA2.1 at polymer:-siRNA ratios of 8:1 (wt/wt) (corresponding to 54 n*M* siRNA and 6 μg/mL polymer in cell culture medium), and were added to the wells in triplicates and kept for 24 h. DOX or PTX were added separately to the cells in different amount. Cells were incubated for another 24 h in their normal maintenance conditions followed by MTT assay as described earlier.

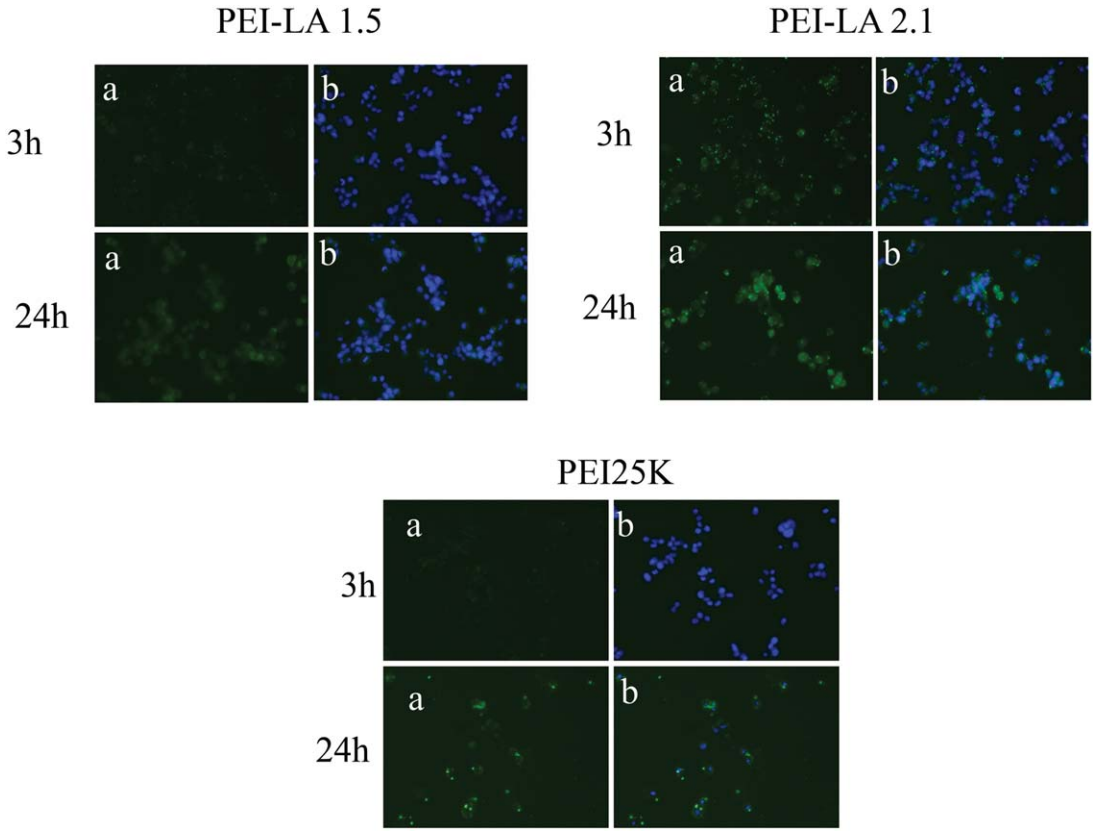

FIGURE 2. Cellular distribution of selected siRNA polyplexes by fluorescence microscopy. Uptake and intracellular distribution of FAM-siRNA formulated in polymer by MDA-MB-435 WT cells using fluorescence microscopy. The observation was done after 3 and 24 h exposure to siRNA complexes at polymer:siRNA ratios of 8:1 (weight/weight). The complexes were added to the wells to give final polymer and siRNA concentration of 6 μg/mL and 54 n*M* per well. The nucleus was stained with DAPI (blue). (a) Images represent FAM-siRNA (green) alone. (b) Images represent both FAM-siRNA (green) and nucleus (blue). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Statistics

Compiled data were presented as means ± standard deviation (SD). Where feasible, the data were analyzed for statistical significance using unpaired student's *t*-test, one-way analysis of variance followed by Tukey test as noted in the results section. The level of significance was set at $\alpha \leq 0.05$.

RESULTS

Characterization of lipid-substituted polymers

A library of lipid substituted PEI2 polymers (with lipid:PEI2 ratios varying between 0.066 and 0.2) with SA, OA, CA and LA have been synthesized for siRNA delivery, based on method described elsewhere.[36] The characteristics of prepared polymers are shown in Table I. Among the synthesized polymers, PEI-CA6.9 contained the highest number of lipid substitution (corresponding to CA substitution at 6.9 lipids/PEI2.[37]

Cellular association and uptake studies

The uptake of polymer/siRNA polyplexes was determined in WT and DOX/RES cells at 8:1 polymer:siRNA ratio (wt/wt). Based on these results PEI25 was the most effective polymer for siRNA delivery, while PEI2 had the least efficacy in both cell lines (Fig. 1). Overall, substitution of lipids on PEI2 increased the association of siRNA complexes with breast cancer cells (Fig. 1). PEI-LA/siRNA polyplexes achieved the highest percentage of siRNA positive cells (~80%) among lipid substituted polymers in both WT and DOX/RES cells [Fig. 1(A,B)]. This level was close to the number of siRNA positive cells achieved by PEI25 in both WT and DOX/RES cells. Lipid substituted polymers showed a higher percentage of cells with siRNA in WT cells compared to DOX/RES cells. Among lipid substituted siRNA polyplexes, PEI-LA polymers showed the highest siRNA delivery (mean fluorescence intensity) in both cell lines while PEI-OA and PEI-SA polymers showed the least uptake, respectively [Fig. 1(C,D)]. Among different lipid substituted PEI2s, PEI-LA2.1, PEI-LA1.5, and PEI-OA2.5 exhibited the highest uptake in WT cells. In DOX/RES cells, PEI-LA1.5 showed the highest uptake which was significantly higher than other lipid substituted polymers under study (one way ANOVA followed by *post hoc* Tukey test, $p < 0.05$).

Furthermore, the cellular distribution of selected polymer/siRNA complexes was investigated in WT cells by fluorescence microscopy (Fig. 2). Very lucid fluorescence was observed in cytoplasm for PEI-LA2.1/siRNA polyplex compared to PEI-LA1.5/siRNA polyplex at both 3 and 24 h. Surprisingly, PEI25/siRNA polyplex exhibited less fluorescence

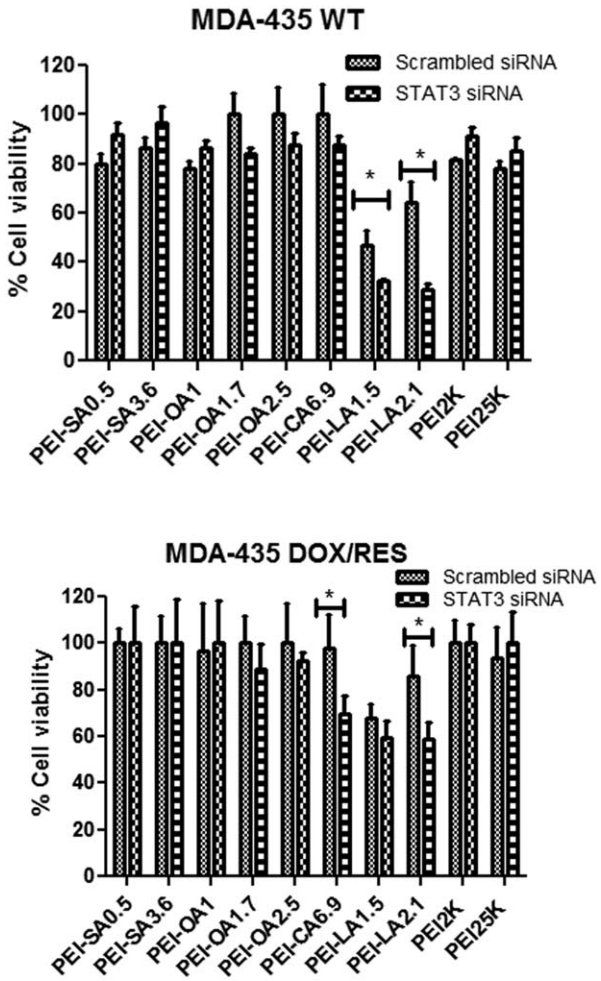

FIGURE 3. Cytotoxicity of STAT3-siRNA versus scrambled siRNA polyplexes. The viability of the MDA-MB-435 WT and DOX/RES cells after 72 h exposure to polymer/siRNA polyplexes prepared using the scrambled and STAT3-siRNA at polymer:siRNA ratio of 8:1 (weight/weight). The complexes were added to the wells to give final polymer and siRNA concentration of 6 μg/mL and 54 n*M* per well, respectively. The data are the mean ± SD for $n = 3$. *Significantly different (unpaired student's *t*-test, $p < 0.05$).

compared to PEI-LA2.1/siRNA polyplex. siRNA delivered with PEI25 appeared to remain in more distinct punctuate (particle) shape, whereas a more disperse pattern was observed for siRNA delivered with PEI-LA polymers.

Cytotoxicity of STAT3 and scrambled siRNA polyplexes

The cytotoxic effect of STAT3-siRNA complexed with different polymers within the PEI library was investigated 72 h after treatment with MDA-MB-435 cells using MTT (Fig. 3). Scrambled siRNA containing polyplexes did not cause any considerable non-specific cell death in both cell lines except when complexed with PEI-LA polymers. PEI-LA1.5 and PEI-LA2.1 complexed with scrambled siRNA caused ~50% and 35% cell death in MDA-435 WT cells, respectively. PEI-LA polymers complexed with STAT3-siRNA exhibited higher level of cell death in WT cells compared to non-specific siRNA complexes, while other polymers in the library did not show any cell death associated with STAT3-siRNA. PEI-LA2.1/STAT3-siRNA polyplex caused ~72% cell death, while PEI-LA1.5/STAT3-siRNA polyplex caused ~68% which were significantly different compared to the same polymer complexes with scrambled siRNA (unpaired students' *t*-test, $p < 0.05$). PEI-LA2.1 was the most efficient in terms of STAT3 associated cell death in WT cells. In DOX/RES cells, PEI-CA6.9 and PEI-LA2.1 complexed with STAT3-siRNA showed ~30 and ~40% cell deaths which were significantly higher ($p < 0.05$) compared to cell deaths caused by scrambled siRNA polyplexes. Overall, these results showed the superiority of PEI-LA2.1 polymer in terms of STAT3 associated toxicity over other polymers under study in both cell lines.

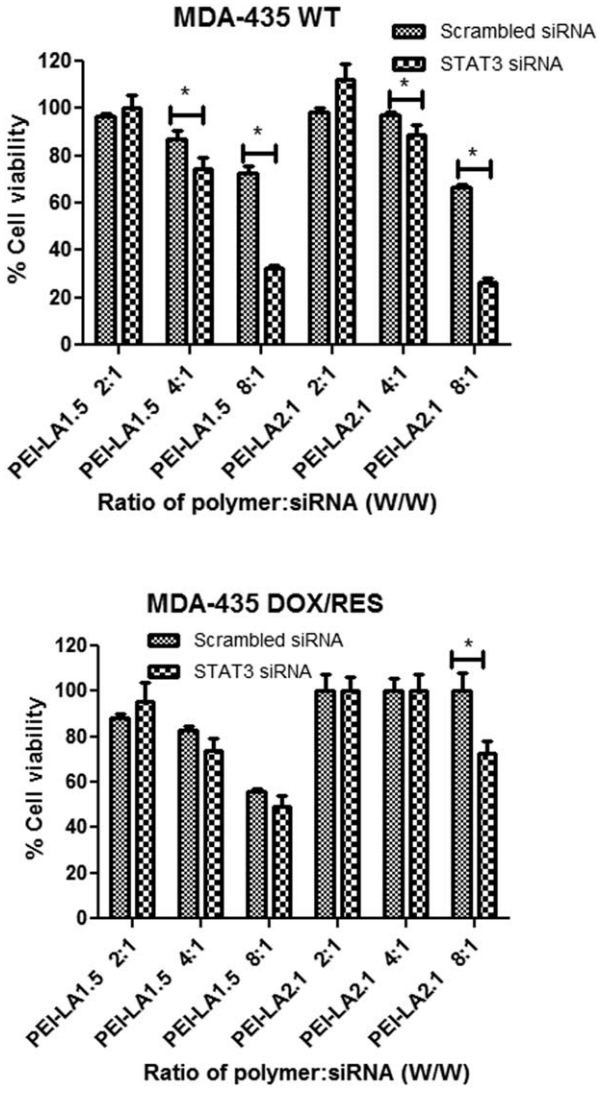

FIGURE 4. PEI-LA polymer efficiency. The viability of the MDA-MB-435 WT and DOX/RES cells after 72 h exposure to PEI-LA/siRNA complexes prepared using the scrambled and STAT3-siRNA at polymer:siRNA ratios of 2:1, 4:1, and 8:1 (weight/weight). The complexes were added to the wells to give final polymer concentrations of 1.5, 3 and 6 μg/mL and siRNA concentration 54 n*M* per well. The data are the mean ± SD for $n = 3$. *Significantly different (unpaired student's *t*-test, $p < 0.05$).

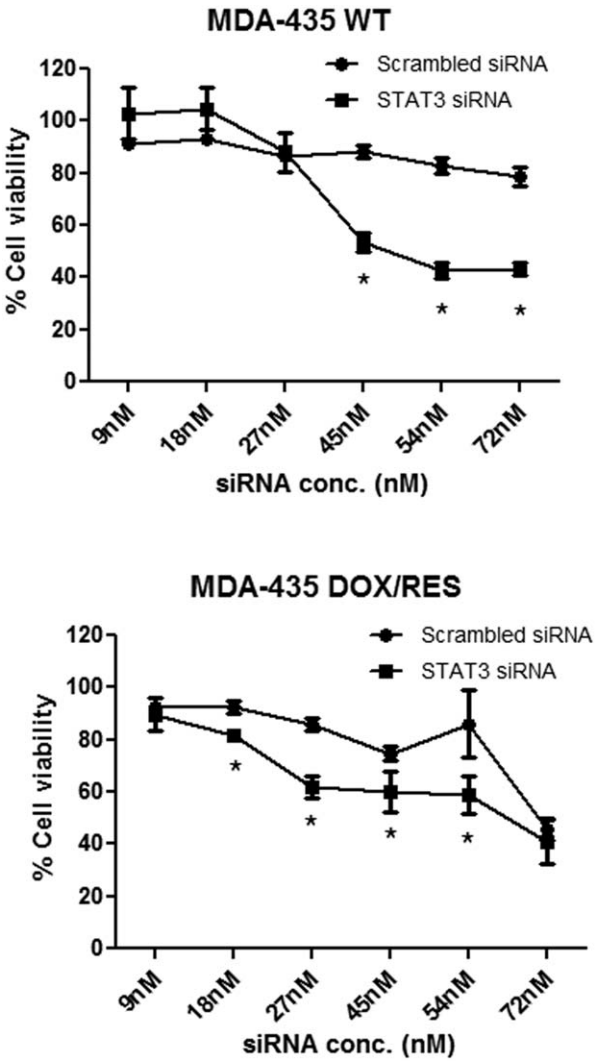

FIGURE 5. siRNA dose response with PEI-LA2.1 polymer. The viability of the MDA-MB-435 WT and MDA-435 DOX/RES cells after 72 h exposure to PEI-LA2.1/siRNA complexes prepared using the scrambled and STAT3-siRNA at polymer:siRNA ratios of 8:1 (weight/weight). The complexes were added to the wells to give final siRNA concentrations 9, 18, 27, 45, 54, and 72 n*M* per well. The data are the mean ± SD for $n = 3$. *Significantly different from their identical scrambled siRNA polyplexes (unpaired student's *t*-test, $p < 0.05$).

Cytotoxicity at different PEI-LA:siRNA ratios

In order to find the optimum PEI-LA polymer:siRNA ratio, cytotoxicity of STAT3 and scrambled siRNA complexed with PEI-LA polymers at different polymer:siRNA ratios were investigated 72 h after treatment (Fig. 4). The polymer:-siRNA ratios were set at 2:1, 4:1, and 8:1 (wt/wt). Scrambled siRNA containing complexes did not cause considerable non-specific cell death in both cell lines except for PEI-LA polymer/siRNA complexes at polymer:siRNA ratio of 8:1 (wt/wt). PEI-LA1.5 and PEI-LA2.1 complexed with STAT3-siRNA exhibited the highest STAT3 associated cell death at 8:1 polymer:siRNA (wt/wt) ratio in WT cells showing ~68% and ~74% cell death, respectively, compared to 27 and 34% cell death for same polymer polyplexes with scrambled siRNA. In DOX/RES cells, PEI-LA2.1/STAT3-siRNA polyplex at 8:1 polymer:siRNA (wt/wt) ratio was the only effective polyplex in exhibiting STAT3 associated cell death (at ~28%). The PEI-LA2.1/scrambled siRNA polyplexes did not cause cell death in RES/DOX cells. These results proved the superiority of PEI-LA2.1 polymer/siRNA complex at 8:1 (wt/wt) ratios in terms of STAT3 associated toxicity over other ratios under study in both cell lines.

siRNA dose response with PEI-LA2.1 polymer

To evaluate the optimum siRNA dose for STAT3 specific cell death, WT and DOX/RES cells were treated with PEI-LA2.1/STAT3-siRNA polyplexes with varying siRNA doses ranging from 9 to 72 n*M* per well (Fig. 5). PEI-LA2.1/STAT3-siRNA complexes with siRNA doses of 45, 54, and 72 n*M* exhibited STAT3 associated cell death which were significantly different from cell death observed by their identical scrambled siRNA polyplexes (unpaired students' *t*-test, $p < 0.05$). The highest STAT3 associated cell death in WT cells was achieved with 54 n*M* siRNA/polymer complex, where STAT3-siRNA polyplexes caused ~58% cell compared to ~17% cell death by scrambled siRNA polyplexes. In DOX/RES cells, STAT3 associated cell death was evident at doses of 18, 27, 45, and 54 (~19, 39, 40, and 60% cell death, respectively). This was significantly different from cell death caused by identical scrambled siRNA polyplex doses (~8, 14, 26, and 14% cell death, respectively). These results implied that PEI-LA2.1 polymer/siRNA complex with 54 n*M* siRNA dose to be the most effective in terms of STAT3 associated toxicity compared to other doses in both cell lines.

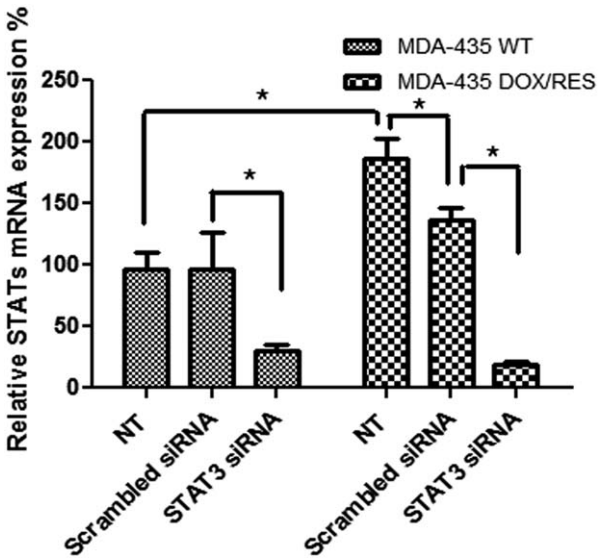

FIGURE 6. Silencing activity of STAT3-siRNA polyplexes by RT-PCR. STAT3 silencing activity of the STAT3-siRNA at mRNA level in MDA-MB-435 WT and DOX/RES cells after transfection with STAT3-siRNA formulated in PEI-LA2.1/siRNA polyplexes prepared using polymer:-siRNA ratios of 8:1 (weight/weight). The complexes were added to the wells to give final polymer and siRNA concentrations 6 μg/mL and 54 n*M* per well. Values are relative to non-treated controls (NT).The data are the mean ± SD for $n = 3$. *Significantly different (one way Anova followed by Tukey test, $p < 0.05$).

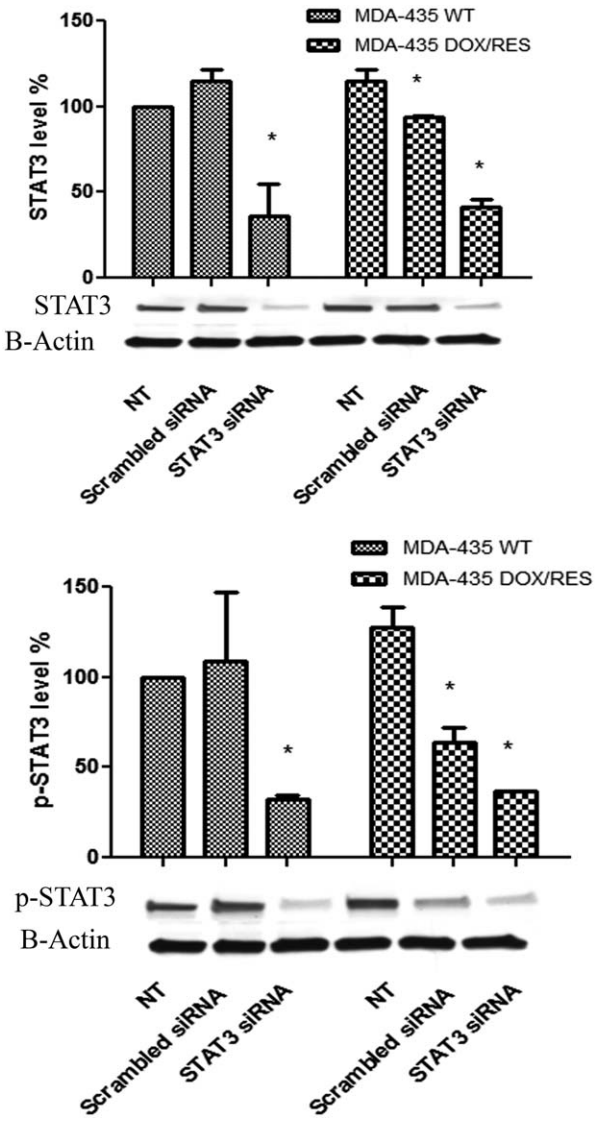

FIGURE 7. Silencing activity of STAT3-siRNA polyplexes by Western blot. STAT3 silencing activity of the STAT3-siRNA at protein level in MDA-MB-435 WT and DOX/RES cells after transfection with STAT3-siRNA formulated in PEI-LA2.1/siRNA polyplexes prepared using polymer:siRNA ratios of 8:1 (weight/weight). The complexes were added to the wells to give final polymer and siRNA concentrations 6 μg/mL and 54 n*M* per well. Values are relative to non-treated controls (NT). *Significantly different from its non-treated control (one way Anova followed by Tukey test, $p < 0.05$).

STAT3 knockdown by siRNA complexes

To evaluate the ability of PEI-LA2.1/STAT3-siRNA polyplexes for STAT3 silencing at mRNA level, WT and DOX/RES were treated for 48 h with siRNA dose of 54 n*M* and polymer:-siRNA ratio of 8:1 (wt/wt) (Fig. 6). In WT cells, the level of STAT3 mRNA expression after incubation with STAT3-siRNA polyplex was reduced by ~70% compared to its corresponding non-treated (NT) control group. Identical polyplexes with scrambled siRNA did not decrease STAT3 mRNA expression. In DOX/RES cells, STAT3-siRNA polyplex caused a ~90% decrease in mRNA expression compared to its non-treated control group. STAT3-siRNA polyplex also caused a significant reduction (~85%) in STAT3 mRNA expression compared to identical scrambled siRNA polyplexes. Interestingly, the level of STAT3 mRNA expression has almost doubled in non-treated DOX/RES cells when compared to non-treated WT cells.

STAT3 and p-STAT3 protein levels were measured after 48 h treatment with PEI-LA2.1/siRNA polyplexes prepared at 8:1 ratio (wt/wt) and 54 n*M* siRNA dose (Fig. 7). In WT cells, both STAT3 and p-STAT3 levels decreased significantly when treated with STAT3-siRNA polyplex compared to NT control group. Identical polyplexes with scrambled siRNA did not cause a reduction of STAT3 protein compared to NT group. In DOX/RES cells, the level of both STAT3 and p-STAT3 decreased significantly upon treatment with STAT3-siRNA when compared to their NT control groups. A non-specific reduction in STAT3 and p-STAT3 protein expression was observed with identical scrambled siRNA polyplexes in RES/DOX cells.

Effect of STAT3 silencing on the cytotoxicity of DOX and PTX

The cytotoxicity of free DOX and PTX alone were evaluated in both WT and DOX/RES cells. A wide range of concentrations was used for DOX [0.1–200 μg/mL; Fig. 8(A)] and PTX [0.2–1638 ng/mL; Fig. 8(B)], respectively. As expected, the efficacy of both DOX and PTX in DOX/RES cells were significantly hampered. In light of previous results, we then examined the effect of STAT3 silencing on the cytotoxicity of DOX and PTX. As shown in Figure 8(C), compared to WT cells treated with DOX alone (0.02–0.2 μg/mL), those pre-exposed to STAT3-siRNA/PEI-LA2.1 polyplexes and then treated with DOX at similar concentrations, showed significant increase in cell death (by ~25%). Pre-exposure of WT cells to scrambled siRNA polyplexes followed by DOX treatment did not cause changes in cytotoxic effect of drugs. A similar trend was observed in DOX/RES cells when pre-exposed to STAT3-siRNA polyplexes [Fig 8(D)]. Treatment with STAT3-siRNA formulation, followed by 50 μg/mL DOX treatment caused ~18% more cell death compared to identical dose of DOX alone.

In the case of PTX, a ~25% reduction in cell viability was observed when WT cells were pre-exposed to STAT3-siRNA polyplexes, and then treated with PTX (0.1–1.6 ng/mL) compared to PTX treatment alone [Fig. 8(E)]. Non-specific toxicity was not observed when cells were treated with scrambled siRNA formulations. In DOX/RES cells, ~40% cell death was observed when cells were exposed to STAT3-siRNA formulation and PTX (25.6 ng/mL) combined [Fig. 8(F)]. This was in contrast to PTX alone treatment at the same concentration that led to 2% cell death in DOX/RES MDA-MB-435 cells upon 24 h incubation. These observations point to capability of silencing STAT3 expression as potential therapeutic modality and its ability to potentiate anti-cancer drug activity in both sensitive and resistant phenotype.

DISCUSSION

STAT3 is persistently tyrosine-phosphorylated in 50% of primary breast carcinomas and tumor-derived cell lines.[40,41]

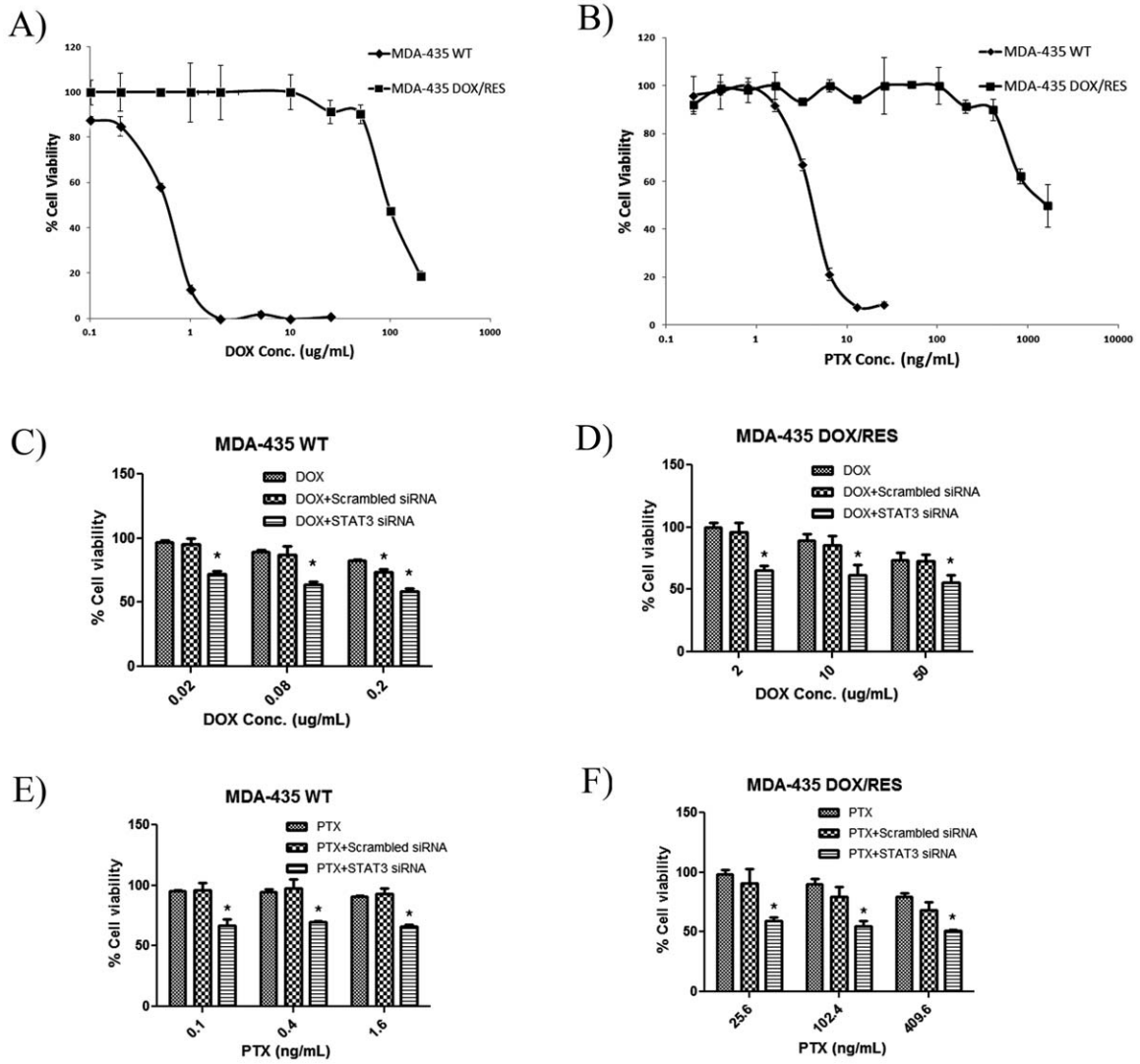

FIGURE 8. Effect of STAT3-siRNA/PEI-LA pre-treatment on the cytotoxicity of doxorubicin and paclitaxel in WT and resistant breast cancer cells. A: The viability of the MDA-MB-435 WT and DOX/RES cells after 48 h exposure to DOX alone. B: The viability of the MDA-MB-435 WT and DOX/RES cells after 48 h exposure to PTX alone. The viability of the MDA-435 WT and MDA-435 DOX/RES cells after 48 h exposure to PEI-LA2.1/siRNA complexes and doxorubicin (C and D) or Paclitaxel (E and F). The complexes were added to the wells to give final polymer concentrations of 6 μg/mL and siRNA concentration 54 n*M* per well. The data are the mean ± SD for $n = 3$. *Significantly different from DOX or PTX alone (one way Anova followed by Tukey test, $p < 0.05$).

Clinical studies demonstrated that elevated levels of tyrosine-phosphorylated Stat3 (p-STAT3) to be a poor prognostic feature in breast cancer patients and correlates with an incomplete response to neoadjuvant chemotherapy.[42,43] Recently, Marotta et al. discovered a network of 15 genes that are required for cell growth and proliferation in $CD44^{+}CD24^{-}$ human stem cell-like breast cancer cells. STAT3 has been emphasized to have a critical role as key downstream transcriptional effector in this network. They found that inhibition of several of these genes such as IL6, PTGIS, HAS1, CXCL3, and PFKFB3 down-regulated STAT3 activation. It was proposed that a STAT3 inactivation treatment in combination with other chemotherapeutic drugs may circumvent therapeutic resistance and lower the side effects of cancer treatment.[44] Inhibition of STAT3 as a molecular target in breast cancer models has been mostly pursued through application of small molecule inhibitors. In this context, Turkson et al. identified ISS 610, a peptidomimetic analog of the tripeptide PY*L, which binds to STAT3 SH2 domain. This molecule was shown to inhibit constitutively active STAT3 and caused selective growth blockage and initiation of apoptosis in MDAMB-231 and MDA-MB-435 human breast carcinoma cells that contain persistently active STAT3.[45] A small molecule inhibitor of STAT3 dimerziation, STA-21 has shown to inhibit growth and survival of MDA-MB-231, MDA-MB-435, and MDA-MB-468 breast cancer cells with constitutive STAT3 signaling.[46] In another study, platinum compounds CPA-1, CPA-7, and platinum (IV) tetrachloride have been used to block STAT3 activity. These compounds inhibited STAT3 DNA binding, its mediated gene

regulation, and caused growth inhibition and apoptosis in MDA-MB-231 and MDA-MB-435 human breast cells.[47] Inhibition of STAT3 activation is shown to be an effective strategy in inhibition of breast cancer growth; however, many functions of STAT3 protein is regulated through its interaction with other transcription factors by mechanisms that are independent of phosphorylated status of STAT3 protein.

An alternative approach has sought inhibiting the expression of STAT3 protein (rather than inhibition of its activation) using oligonucleotides,[48,49] and siRNAs[50] for application in cancer therapy. RNAi has been investigated for targeting STAT3 expression as a more explicit modality. However, advancement of this technology has been relatively slow, mainly due to difficulties associated with its safe and effective delivery in a clinical setting.[24] Different nonviral delivery materials including lipids and polymeric nanocarriers have been examined for STAT3 siRNA delivery in different cancer models. For instance, inhibition of STAT3 by siRNA, inducing apoptosis in B16 melanoma tumor tissue has been achieved using nanoparticles based on polyethylenimine (PEI) 25 kDa modified with stearic acid (StA) for siRNA delivery by our group. In that study, at 50 n*M* siRNA, PEI–StA complexes showed up to 60% reduction in p-STAT3 protein level compared to non-treated control. This has led to a significant regression in tumor growth after multi-dose treatments both *in vitro* and *in vivo* (upon intra-tumoral administration). Factors associated with STAT3 activity, that is IL-6 level and caspase-3 activity were increased, while a reduction of VEGF level has been achieved.[11] In another study, STAT3 down-regulation using siRNA-Lipofectamine™ 2000 complex has been demonstrated to hinder cell motility and invasion, as well as inducing cell death in human DU145 and PC3 prostate cancer cells *in vitro*.[51] Zhang et al. used targeted STAT3 with siRNA expressing plasmid in human hepatocellular carcinoma Bel-7402 cells. A siRNA and rapamycin combined treatment enhanced apoptosis and up-regulated cleaved caspase 3 in Bel-7402 cells.[52]

In the current study, we investigated application of lipid-substituted (low molecular weight) PEI2 polymer–siRNA complexes for STAT3 down-regulation in a model triple negative human breast cancer (TNBC) cell line, MDA-MB-435 cells. The possible efficacy of this approach in reducing the viability of WT and DOX/RES phenotypes alone or in combination with common anticancer agents used in therapy of TNBC (i.e., DOX and PTX) was also investigated. Lipid modification of polymers has been pursued as a method to enhance the efficacy of polymeric complexes in delivering siRNA to cells. The lipid substitution of polymers is suggested to help the cellular uptake of siRNA complexes due to increased interaction of polyplexes with cell membrane.[53] Our results demonstrated that some of lipid-substituted polymers under study (not all) were quite effective for siRNA delivery into cells (Fig. 1). Cellular association of siRNA was highest when complexed with PEI-LA polymer as compared to other lipid-substituted PEI2s. The results was in line with previous findings by Aliabadi et al. who also reported that LA-substituted PEI2 to be the most effective carrier for siRNA delivery among lipid-substituted PEI2s.[37] Fluorescence microscope images confirmed the results of flow cytometry revealing better intracellular uptake of siRNA by PEI-LA2.1 polyplexes (Fig. 2). PEI-LA2.1 appeared to be successful in delivery of compelxed siRNA into cytoplasm, whereas siRNA complexed by PEI25 appeared as distinct particle in cytoplasm as well as in the nucleus. Accumulation of siRNA in released not particulate form and in the cytoplasm where its target mRNA locates (rather than nucleus) provides advantage for the lipid-substituted PEI2 over PEI25 for siRNA delivery. We have then screened the library of lipid-PEI2K forming polyplexes with scrambled and STAT3-siRNA for induction of non-specific and STAT3 associated cell death, respectively, in both WT and DOX/RES human breast cancer cells. STAT3 is known to regulate the expression of anti-apoptotic factors such as BCL-2, BCL-xL, MCL-1, and Survivin[54–58] in breast cancer cells; hence inhibition of STAT3 is expected to lead to cancer cell death. Among lipid-substituted polymers under study, PEI-LA2.1 exhibited the most effective results in causing a significantly higher level of cytotoxicity after delivery of STAT3-siRNA compared to scrambled siRNA polyplexes in both cell lines (Fig. 3). We then tried to maximize the efficiency of the PEI2-LA polyplexes of STAT3-siRNA by assessing the effect of polymer:siRNA (wt/wt) ratio on the cytotoxicity against MDA-MB-435 WT and DOX/RES cells making comparisons with identical polyplexes of scrambled siRNA. The complexes prepared at the ratio of 8:1 generally showed better efficiency (more STAT3 associated cell cytotoxicity) compared to the 2:1 and 4:1 ratios (Fig. 4). This can be explained by higher association of lipid-substituted polyplexes with the cell membrane at higher ratios of polymer to siRNA. A siRNA dose-response experiment showed a siRNA dose of 54 n*M* to be the most effective dose for STAT3 mediated cell cytotoxicity effect in both WT and DOX/RES cells. At this dose, PEI-LA2.1/STAT3-siRNA polyplex also yielded significant silencing of STAT3 mRNA and protein in both WT and DOX/RES cell lines compared to untreated cells or cells treated with scrambled siRNA polyplexes (Figs. 6 and 7). In DOX/RES cells, an unspecific STAT3 mRNA silencing as well as STAT3 and p-STAT3 protein down-regulation for the polyplexes made from scrambled siRNA was seen (Figs. 6 and 7). The reason for this observation is not clear. Interestingly, relative mRNA expression almost doubled in DOX/RES cells compared to WT cells (Fig. 6). This may imply a possible role for up-regulation of STAT3 expression, as a mechanism of drug resistance in DOX/RES breast cancer cells. Conventional anti-neoplastic drugs, that is, DOX and PTX, are included in the majority of chemotherapy regimens for breast cancer patients.[59,60] However, nonspecific distribution leading to intolerable adverse effects, restricted access, and accumulation of these anticancer agents to tumor site upon systemic administration and development of drug resistance have limited their clinical success specially in advanced stages of the disease. Inhibition of STAT3 expression and/or activity in breast tumors was hypothesized to reduce the threshold cytotoxic dose of standard anti-cancer agents in both WT and RES breast cancer phenotypes. In this study, this

hypothesis was examined through STAT3 silencing by PEI-LA polyplexes in breast cancer cells. Then, cytotoxicity analysis was carried out combining STAT3-siRNA polyplexes with DOX and PTX treatment in WT and DOX/RES human breast cancer cells. As expected, the efficacy of both DOX and PTX in DOX/RES cells were significantly decreased compared to WT cells [Fig. 8(A,B)]. Overexpression of STAT3 might be responsible for this resistance, partly. Our data showed an increase in the cytotoxic efficacy when STAT3-siRNA/PEI-LA polyplexes were combined with DOX and PTX. Overall, the results of our studies provides proof for the benefit of STAT3 silencing in enhancing the potency of chemotherapeutic drugs in breast tumors or their anti-cancer activity at given doses. A combination approach in therapy can take care of $STAT3^+$ RES population in heterogeneous breast tumor population and enhance the effect of standard chemotherapy in primary or reoccurring breast tumors.

CONCLUSION

In this study, we reported on effective silencing of STAT3 that is involved in cancer proliferation and survival, angiogenesis and invasion, and tumor induced immune-suppression by lipid-substituted low molecular weight PEI polyplexes of specific siRNA. Effective delivery of STAT3-siRNA into MDA-MB-435 cell translated into efficient down-regulation of STAT3 mRNA and protein, and subsequent cell death. STAT3 down-regulation additionally increased the cytotoxic capability of model anti-cancer drugs, that is DOX and PTX, in WT and DOX/RES breast tumor phenotypes. The result of this study provided proof of concept for combination therapy approaches combining STAT3 silencing with conventional chemotherapy as means to improve the clinical benefit of breast cancer chemotherapy in both WT and RES breast tumor phenotypes.

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