molecular pharmaceutics

ARTICLE

pubs.acs.org/molecularpharmaceutics

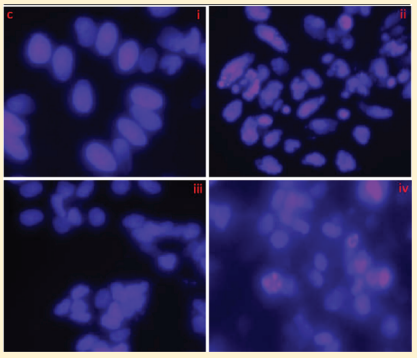
Induction of Apoptosis by Survivin Silencing through siRNA Delivery in a Human Breast Cancer Cell Line

Hamidreza Montazeri Aliabadi,[†] Breanne Landry,[†] Parvin Mahdipoor, and Hasan Uludağ[*,†,‡,§]

[†]Department of Chemical & Materials Engineering, Faculty of Engineering, [‡]Faculty of Pharmacy and Pharmaceutical Sciences, and [§]Department of Biomedical Engineering, Faculty of Medicine, University of Alberta, Edmonton, AB, Canada

Supporting Information

ABSTRACT: Post-transcriptional silencing of antiapoptotic genes is a promising strategy for cancer therapy, but delivering short interfering RNA (siRNA) molecules against such targets is challenging due to inability of anionic siRNA to cross cellular membranes. Lipid substitution on small molecular weight, nontoxic polyethylenimine (PEI) has been investigated as a promising approach for effective siRNA delivery. In this study, we report on the ability of low molecular weight, lipid-substituted PEI to deliver siRNA against the antiapoptotic protein survivin. Toxicity of a library of lipid-substituted PEIs, as well as their siRNA delivery and survivin silencing efficiency, was evaluated in MDA-MB-231 human breast cancer cells. A significant increase in cellular delivery of siRNA was observed as a result of lipid substitution. Most significant downregulation of survivin was established by caprylic acid-substituted polymers, which resulted in significant levels of apoptosis induction and resultant loss of cell viability. Survivin downregulation prior to anticancer drug treatment decreased the IC_{50} of several drugs by 50- to 120-fold. Our experiments indicated an effective downregulation of survivin, a cell protective protein upregulated in tumor cells, by delivering siRNA with hydrophobically modified PEI. This study introduces a promising delivery system for safe and effective siRNA delivery that will be suitable for further investigation in preclinical animal models.



KEYWORDS: survivin, siRNA, polycationic polymers, hydrophobic modification

INTRODUCTION

Programmed cell death, or apoptosis, is executed by the activation of caspase family of enzymes that cleave cellular proteins.[1] Activation of caspases could be triggered by intrinsic and extrinsic pathways, and ultimately results in cleavage of substrates essential for cell survival, such as cytoskeletal proteins, DNA repair proteins, and inhibitory subunits of endonucleases and, subsequently, cell death.[2] Apoptosis is tightly regulated by a delicate balance between proapoptotic and antiapoptotic factors in normal cells. Transformed cells, on the other hand, typically display resistance to normal apoptotic triggers as well as cytotoxic drugs employed in cancer therapy.

One class of molecules that block apoptosis by direct binding to caspases is the inhibitor of apoptosis proteins (IAP[3]). Survivin, discovered in 1997, is a member of IAP family and has been implicated in multiple essential functions, including cell division, apoptosis, cellular stress response, and checkpoint mechanisms of genomic integrity.[4] Survivin has multiple functions including cytoprotection, inhibition of cell death, and cell-cycle regulation, especially at the mitotic process stage, all of which favor cancer survival. Survivin is expressed during the G2/M phase of the cell cycle and is hypothesized to inhibit a default apoptotic cascade initiated in mitosis.[5] There are conflicting reports on the interaction between survivin and effector caspases, and therefore, it is hypothesized that mechanisms other than the caspase inhibition are involved in the antiapoptosis effect of survivin.[6,7] Survivin may antagonize cell death upstream of the effector caspases since it was shown to inhibit caspase 9[8] and to bind to hepatitis B X-interacting protein (HBXIP) complex bound to pro-caspase-9 to prevent recruitment of Apaf-1 to the apoptosome.[9] Several studies have shown that survivin expression is upregulated in human cancers,[10−14] is associated with resistance to chemotherapy or radiation therapy, and is linked to poor prognosis.[5] Survivin could be an attractive target for anticancer therapy, since (a) survivin expression is low in normal cells unlike the malignant cells; (b) survivin is involved in multiple signaling mechanisms controlling tumor maintenance; and (c) survivin may block angiogenesis as well as tumor growth.[15] Small molecular weight antagonists for survivin, such as YM155,[16] shepherdin,[17] and terameprocol,[18,19] were explored in cancer therapy, but, being small organic entities, they have the potential for undesired activities due to poor specificity of small organic entities.

Post-transcriptional gene silencing by RNA interference (RNAi) has been recognized as an effective silencing mechanism

Received: April 8, 2011
Accepted: August 14, 2011
Revised: August 2, 2011
Published: August 15, 2011

in cancer therapy. Since the initial discovery of double-stranded RNA (dsRNA) to be at least 10-fold more potent in silencing than sense or antisense RNAs alone,[20] RNAi has rapidly emerged as a promising tool to downregulate the expression of specific target proteins in a wide variety of cells. It has the potential to act more specifically as compared to conventional survivin antagonists. Delivering siRNA to intracellular targets, however, has proven to be a challenge. RNAs are rapidly degraded in the extracellular environment with RNase A type nucleases,[21] and it cannot traverse cell surface membrane due to the anionic nature of both the RNA and cell surface.[22]

Cationic polymers are attractive for siRNA delivery, since they can be tailored to neutralize the anionic charge of nucleic acids and do not raise the safety concerns associated with viral carriers. The electrostatic interaction between the anionic phosphates in siRNA and the cationic polymer also assembles nanoparticles that are more suitable for cellular uptake. High molecular weight polyethylenimines (PEIs) are one class of polymers that have been shown to be effective siRNA delivery agents.[23−25] These polymers create an opportunity for endosomal escape after cellular uptake, due to the presence of unprotonated PEI amines that act as "proton sponges".[26] However, the toxicity of high molecular weight PEIs has been an important obstacle for their clinical use.[27−32] Lower molecular weight PEIs present acceptable toxicity profiles but, unfortunately, do not display efficacious siRNA delivery into cells. We previously reported hydrophobic modification of low molecular weight PEI for siRNA delivery. By using model targets, namely, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and P-glycoprotein involved in multidrug resistance (MDR), the modified polymers were shown to be promising for siRNA delivery.[33] In the present study, we explored feasibility of using these carriers in order to silence survivin and to determine the biological consequences of survivin downregulation on the potency of several anticancer drugs. Our results showed that survivin is an effective target for silencing to improve the pharmacological response to chemotherapeutic agents.

MATERIALS AND METHODS

Materials. The 2 kilodalton (kDa) PEI (PEI2; M_n, 1.8 kDa; M_w, 2 kDa), 25 kDa PEI (PEI25; M_n, 10 kDa; M_w, 25 kDa), anhydrous dimethyl sulfoxide (DMSO), caproyl chloride (C8; >99%), palmitoyl chloride (C16; 98%), octanoyl chloride (C18:1 9*Z*, 12*Z*; 99%), linoleyl chloride (C18:2 9*Z*,12*Z*; 99%), Hanks' balanced salt solution (HBSS with phenol red), trypsin/EDTA, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), paclitaxel, doxorubicin hydrochloride, and mitoxantrone hydrochloride were obtained from SIGMA (St. Louis, MO). Clear HBSS (phenol red free) was prepared in house. Dulbecco's modified Eagle's medium (DMEM; low glucose), penicillin (10000 U/mL) and streptomycin (10 mg/mL) were from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was from PAA Laboratories (Etobicoke, Ontario). The scrambled siRNAs were AllStars Negative siRNA Fluorescein (catalog number: 1027290) and AllStars Negative Control siRNA (catalog number: 1027281), both from Qiagen (Huntsville, AL, USA). The human survivin siRNA (catalog number 29499) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-human survivin-fluorescein monoclonal antibody (catalog number: IC6472F) and FlowTACS Apoptosis Detection Kit (catalog number: 4817−60-K) were provided by R&D Systems Inc. (Minneapolis, MN, USA).

Cell Line. M. D. Anderson human metastatic breast cancer 231 (MDA-MB-231) cells were a generous gift from Dr. Michael Weinfeld (Cross Cancer Institute, Edmonton, AB). The cells were cultured in DMEM with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin added at 37 °C and 5% CO_2. Cell culture was considered confluent when a monolayer of cells covered more than 80% of the flask surface. To propagate the cells, the monolayer was washed with HBSS, and subsequently incubated with 0.05% Trypsin/EDTA for 5 min at room temperature. The suspended cells were centrifuged at 600 rpm for 4 min, and were resuspended in the medium after removal of the supernatant. The suspended cells were subcultured at 10% of the original count.

Synthesis of Lipid-Substituted Polymers . The synthesis of lipid-substituted polymers has been described elsewhere.[34] Briefly, a 50% 2 kDa PEI solution was purified by freeze-drying, and substitution was performed by N-acylation of PEI with commercially available lipid (CA, PA, OA and LA) chlorides. Acid chlorides were typically added to 100 mg of PEI in anhydrous DMSO. The lipid:PEI ratios were systematically varied between 0.066 and 0.2. The mixture was allowed to react for 24 h at room temperature under argon, after which excess ethyl ether was added to precipitate and wash the polymers. The substituted polymers were dried under vacuum at ambient temperature overnight. Polymers were analyzed by ^{1}H NMR (Bruker 300 MHz; Billerica, MA) in D_2O. The characteristic proton shifts of lipids (δ ~0.8 ppm; $-CH_3$) and PEI (δ ~2.5−2.8 ppm; $NH-CH_2-CH_2-NH-$) were integrated, normalized for the number of protons in each peak, and used to determine the extent of lipid substitutions on polymers. Characterization of the polymers, including the binding affinity to siRNA, complex size and zeta (ζ)-potential, and stability of the siRNA in the complexes after exposure to serum are reported elsewhere.[33] The polymers evaluated in this study were designated as PEI2-XX*Y.Y*, where XX refers to the substituted moiety and *Y.Y* to the level of substitution (e.g., PEI-LA3.2 refers to LA substitution at 3.2 lipids/PEI2).

Evaluation of Polymer Cytotoxicity. The cytotoxicity of the polymers on MDA-MB-231 cells was evaluated using the MTT assay in 48-well flat-bottomed plates. Confluent cell cultures were trypsinized, seeded in 48-well plates with 0.2 mL medium in each well, and allowed to reach ~80% confluence (1−2 days). Polymer/siRNA complexes were prepared using the scrambled siRNA at a polymer:siRNA weight ratio of 8:1 and were added to the wells to give final polymer concentrations of 1.25, 2.5, 5, and 10 μL/mL in triplicate wells. Cells were incubated for 24 h in their normal maintenance conditions, and then 40 μL of MTT solution (5 mg/mL in HBSS) was added to each well. After 2 h of incubation in 37 °C, the medium was removed, and 500 μL of DMSO was added to each well to dissolve the crystals formed. The optical density of the wells were measured at 570 nm with an ELx800 Universal Microplate Reader (BioTek Instruments; Winooski, VT, USA) with cell-less medium as blank. The absorbance of polymer-treated cells was compared to untreated cells (as 100% viability), and the % cell viability was calculated for each concentration of polymers.

Cellular Uptake of siRNA. Confluent MDA-MB-231 cell cultures were trypsinized and resuspended as described before,

and seeded in 48-well plates (0.35 mL in each well) at ~50% confluency. After 24 h, medium was removed from all cells and 200 μL of fresh medium was added to each well, followed by the addition of polymer/siRNA complexes. The complexes were prepared in sterile tubes using both FAM-labeled scrambled siRNA and nonlabeled scrambled siRNA (as a negative control) with polymer:siRNA ratios of 2:1 and 8:1 (corresponding to 36 nM siRNA and 1 and 4 μg/mL polymer in culture medium). The prepared complexes were added to wells in triplicate and were incubated at 37 °C for 24 h. After the incubation period, cells were washed with HBSS (×3) and trypsinized. A 3.7% formaldehyde solution was added to suspended cells, and the siRNA uptake was quantified by a Beckman Coulter QUANTA SC flow cytometer using the FL1 channel to detect cell-associated fluorescence. The percentage of cells showing FAM-fluorescence, the mean fluorescence in the positive cells, and the mean fluorescence in the total cell population were determined. Calibration was performed by gating with the negative control (i.e., "No Treatment") group such that the autofluorescent cell population represented 1−2% of the total cell population.

Evaluation of Survivin Expression. Confluent cell cultures were trypsinized and seeded in 24-well plates (500 μL in each well) at ~50% confluence. After 24 h, the medium was removed and 200 μL of fresh medium was added to each well. The polymer/siRNA complexes were prepared in sterile tubes using both scrambled siRNA (as a negative control) and survivin siRNA with polymer:siRNA weight ratio of 2:1 (corresponding 54 nM siRNA and 1 μg/mL polymer in cell culture medium), and were added to the wells in triplicate. Plates were then incubated in 37 °C for 72 h, after which the medium was removed, and cells were trypsinized, fixed with 3.7% formaldehyde solution, and transferred to tubes. Cells were then washed with HBSS and permeabilized for intracellular staining with a 0.1% solution of Triton X100 in HBSS. Permeabilized cells were exposed to fluorescein-conjugated antisurvivin monoclonal antibody for 45 min, and then were washed with the same permeabilizing solution twice before being resuspended in HBSS for flow cytometry assay. The percentage of cells showing FAM-fluorescence, the mean fluorescence in the positive cells, and the mean fluorescence in the total cell population were determined by fluorescence measurement in FL1 channel. Calibration was performed by gating with the negative control such that the autofluorescent cell population represented 1−2% of the total cell population.

Detection of Apoptosis Induction by siRNA Downregulation. Apoptosis among the survivin siRNA-treated cells and the control groups was evaluated by the FlowTACS Apoptosis Detection Kit and fluorescent microscopy. Cleavage of DNA generates free 3′-hydroxyl residues that can be utilized by terminal deoxynucleotidyl transferase (TdT) in end-labeling reactions. Incorporation of biotinylated nucleotides into the DNA by TdT allows detection with streptavidin−FITC conjugates by flow cytometry. Positive control was prepared by adding nuclease to the cells before performing the detection test according to the manufacturer's instructions. To microscopically visualize the nucleus and the fragmentation resulting from the survivin siRNA treatment, the cells were stained with both Hoechst and SYBR Green I dyes.

Cell Viability after Survivin Downregulation. Confluent cell cultures were trypsinized and seeded in 24-well plates (500 μL in each well) at ~50% confluence. After 24 h, the medium was removed and 200 μL of fresh medium was added to each well. The polymer/siRNA complexes were prepared in sterile tubes using both scrambled siRNA (as a negative control) and survivin siRNA with polymer:siRNA weight ratio of 2:1, 4:1, and 8:1 (corresponding to 18, 36, and 54 nM siRNA and 1, 2, and 4 μg/mL polymer in cell culture medium), and were added to the wells in triplicate. The plates were incubated at 37 °C for 72 h, after which the medium was removed and the MTT assay was performed as described in Evaluation of Polymer Cytotoxicity to determine the cell viability for each group.

Effect of Survivin siRNA on Cytotoxicity of Anticancer Agents. The cytotoxicity of doxorubicin, paclitaxel and mitoxantrone was evaluated in MDA-MB-231 cells using a standard cell viability test based on the MTT assay.[35] The siRNA-treated and untreated cells were exposed to a wide range of doxorubicin, paclitaxel, and mitoxantrone concentrations for 24 h. The medium was then removed and the MTT assay was performed (as described in Evaluation of Polymer Cytotoxicity) to determine the cell viability. The inhibitory concentration for 50% cell viability (IC_{50}) was calculated based on the cell viability curves generated.

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

RESULTS

Three series of lipid substitutions (with lipid:PEI amine mole ratios of 0.066, 0.1 and 0.2) were performed on the PEI2 with caprylic acid (CA), palmitic acid (PA), oleic acid (OA), and linoleic acid (LA).[36] Characteristics of the polymers used in this study (Figure S1 in the Supporting Information) have been reported elsewhere.[33] All polymers employed for this study remained water-soluble; a general increase in lipid substitution was observed as the lipid:PEI ratio was increased during the synthesis and the highest number of lipids substituted was achieved with CA at lipid:PEI amine ratio of 0.2 (6.9 CAs/PEI, corresponding to modification of 6.9 amines out of a possible 14 primary amines on PEI2). Based on a SYBR Green II dye binding assay,[34] complete siRNA binding was achieved at polymer:siRNA ratio of ~0.5 for all polymers. Particle size analysis showed a range of 300 to 600 nm for siRNA particles formed with all lipid-substituted polymers with no clear effect of lipid substitution on particle sizes. At a polymer:siRNA ratio of 1:1, all lipid-substituted polymers showed complete protection against degradation, while unprotected siRNA was readily degraded (<5% intact siRNA remaining) and only ~68% of siRNA bound with PEI2 remained intact in serum-containing medium after 24 h of incubation.

Since the cytotoxicity of carriers is a major concern for polymeric systems employed for siRNA delivery, the MTT assay was performed to determine the toxicity of the lipid-substituted polymers on the chosen cell target. Figure 1 summarizes the cell viability of MDA-MB-231 cells after 24 h exposure to a range of polymer concentrations (0−10 μg/mL) added as complexes with scrambled siRNA. PEI2, as expected, showed little toxicity in this cell line, and even at 10 μg/mL, ~92% of the cells were still viable. However, the toxic effect of PEI25 was evident at 5 and 10 μg/mL, with only ~19% of cells surviving the polymer exposure.

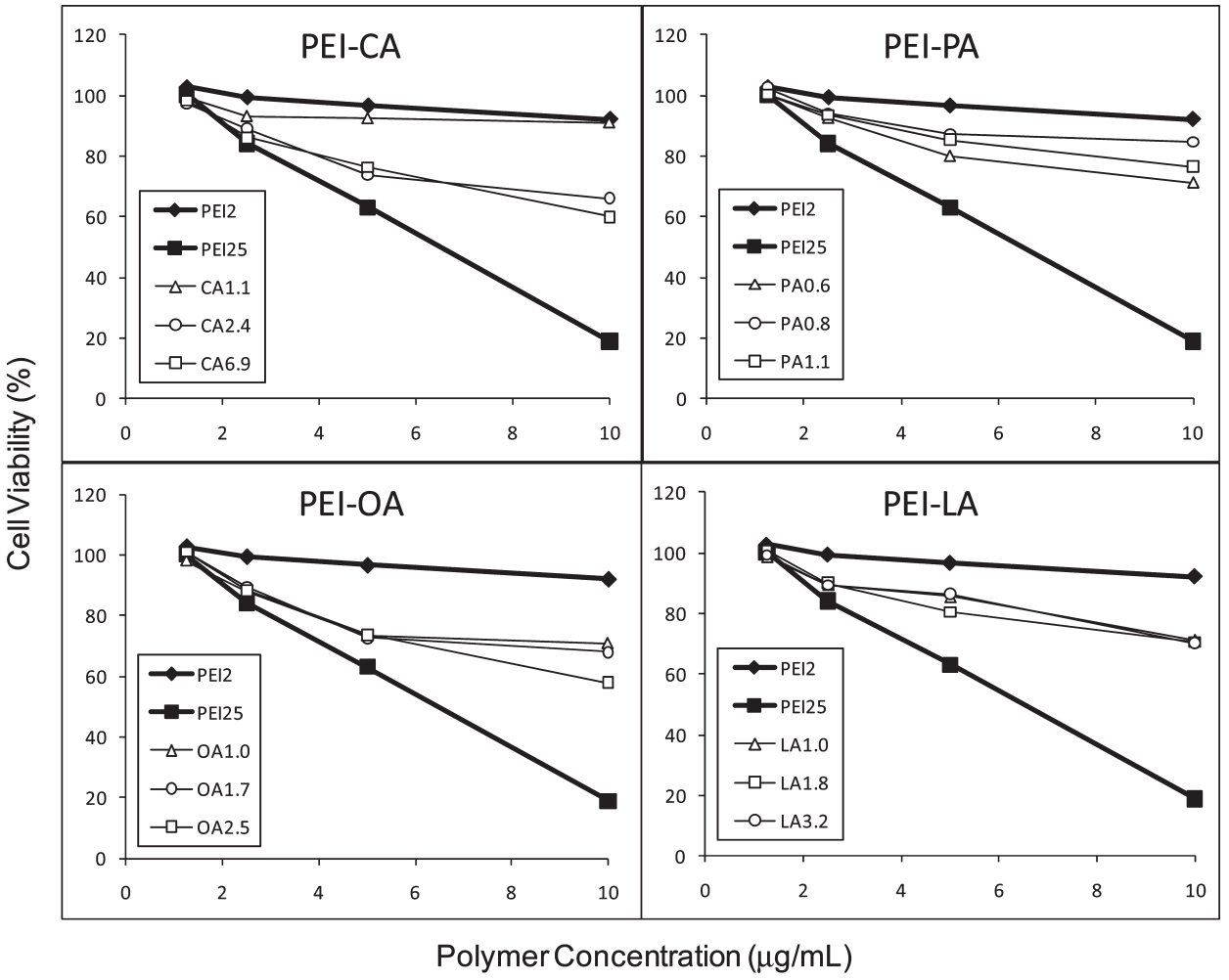


Figure 1. Polymer toxicity. The viability of MDA-MB-231 cells after 24 h exposure to polymer/siRNA complexes prepared using increasing polymer concentrations and scrambled siRNA. While PEI25 was obviously toxic to the cells at concentrations above 2.5 μg/mL, the toxicity profiles of the lipid substituted polymers were more acceptable. The OA- and CA-substituted polymers showed the most toxicity among the lipid-substituted polymers in both cell lines.

Lipid substitution increased the toxicity of PEI2 for all of the evaluated polymers, which was most significant for OA-substituted lipids; however, the observed toxicity was significantly lower than the toxicity of PEI25 (>50% cell viability for all lipid-substituted polymers at 10 μg/mL).

The efficiency of lipid-substituted polymers for siRNA delivery was evaluated at two different polymer:siRNA ratios (2:1 and 8:1; Figure 2a). Expectedly, PEI2 showed minimal delivery capability at both ratios, while PEI25 was among the effective polymers. While the ratio of 8:1 was significantly more effective in siRNA delivery (compared to the 2:1 ratio) for LA- and OA-substituted polymers (based on the mean fluorescence of the cells), such a significant difference was not evident for other polymers. Among the lipid-substituted polymers, LA-substituted polymers provided the highest cellular uptake, while other polymers gave lower siRNA delivery. Figure 2b demonstrates the percentage of siRNA-positive cells for the evaluated polymers. A polymer:siRNA ratio of 8:1 showed higher percentages of siRNA uptake for LA-substituted polymers, and lipid-substituted polymers showed a maximum of ~96% of cells with siRNA delivery (achieved with LA1.0). The correlation between the cellular uptake (calculated based on the mean fluorescence of the cells) and the level of substitution (calculated based on the number of substituted lipids per PEI2, as well as the number of lipid methylenes per PEI2) are summarized in Figure 2c for the ratios of 2:1 and 8:1. For the polymer:siRNA ratio of 2:1, strong correlations were observed for all substituted lipids (the lowest R was 0.96), and the overall results showed a stronger uptake with increased lipid substitution level at this polymer:siRNA ratio. At the polymer:siRNA ratio of 8:1, however, mixed results were observed. The PA-substituted polymers showed a reverse trend, showing a lower uptake with an increase in substitution level (R = 0.99). While a decisive correlation between the substitution level and siRNA uptake was not observed for LA- and OA-substituted PEI2, the CA-substituted polymers gave a positive correlation between the level of substitution and the siRNA uptake.

The lipid-modified PEI2s were then used to deliver survivin specific siRNA, and cell viabilities were evaluated using the MTT assay. The CA- and LA-substituted polymers were selected for further investigation based on the result of this initial experiment (data not shown). Figure 3a shows the results of MDA-MB-231 viabilities after treatment with 3 different siRNA concentrations (18, 36, and 54 nM) at three different polymer:siRNA ratios with

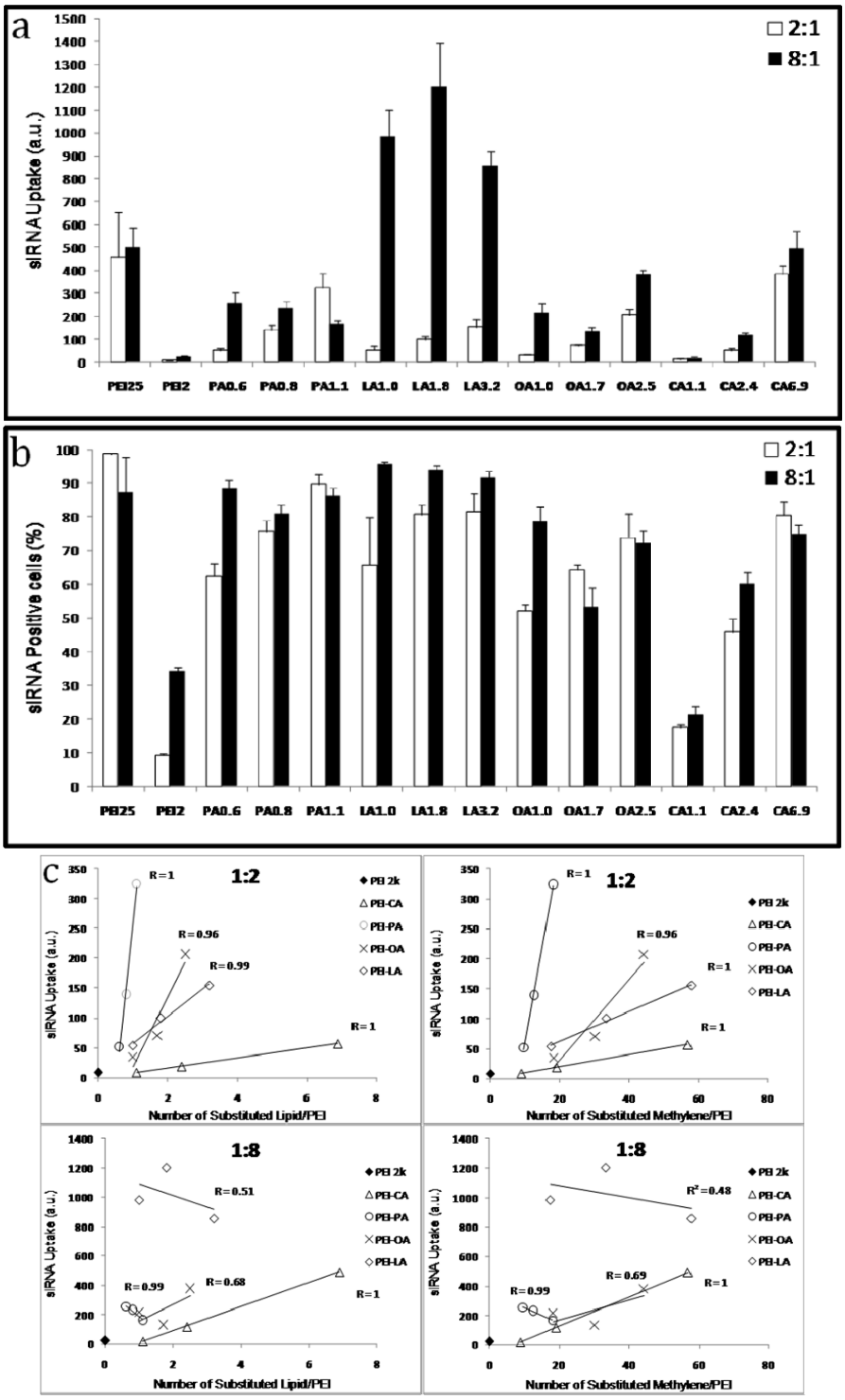


Figure 2. Cellular uptake of polymer/siRNA complexes. (a) The mean fluorescence of the MDA-MB-231 cells after 24 h exposure to complexes formed with FITC-labeled scrambled siRNA at polymer:siRNA ratios of 2:1 and 8:1 (weight/weight). (b) The percentage of cells positive for FITC-siRNA after 24 h exposure to siRNA complexes. Hydrophobic modification enhanced the siRNA cellular uptake significantly, even more than the uptake with PEI25 (in case of LA-substituted polymers at ratio of 8:1). In general, siRNA uptake was more significant with the polymer:siRNA ratio of 8:1. (c) Correlation between the polymer substitution level (based on number of lipids per PEI chain [panels on left] and number of substituted methylenes per PEI [panels on right]) and siRNA cellular uptake of polymer/siRNA complexes (calculated based on mean fluorescence presented in panel a). There was a positive correlation between the substitution level and uptake at ratio of 2:1 for all hydrophobic moieties.

the PEI2-LA3.2 polymer. The toxic effect of the polymer was evident at the higher ratios (i.e., for cells treated with control siRNA complexes). A clear survivin siRNA-induced cell death was not evident for cells treated with the PEI2-LA polymers under these conditions (Figure 3a, inset). Figure 3b shows the results of MDA-MB-231 viabilities after siRNA delivery with

dx.doi.org/10.1021/mp200176v |*Mol. Pharmaceutics* 2011, 8, 1821–1830

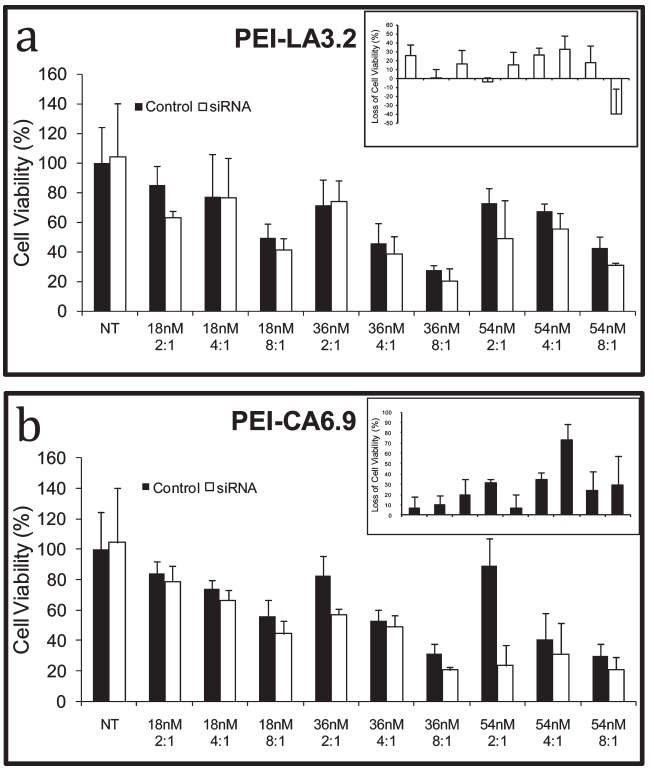


Figure 3. Effect of survivin downregulation on cell viability. Cell viability after treatment with three different siRNA concentrations (18, 36, and 54 nM) at three different polymer:siRNA ratios (2:1, 4:1, and 8:1) using PEI2-LA3.2 (a) and PEI-CA6.9 (b). The toxic effect of the polymers is evident at the higher ratios. A clear survivin siRNA-induced cell death was not evident for cells treated with the PEI2-LA3.2 polymer under these conditions. A significant drop in cell viability was observed at a polymer:siRNA ratio of 2:1 at 36 and 54 nM siRNA concentrations for PEI2-CA6.9. Insets represent the loss in cell viability (%) in survivin siRNA-treated cells compared to cells exposed to scrambled siRNA as control.

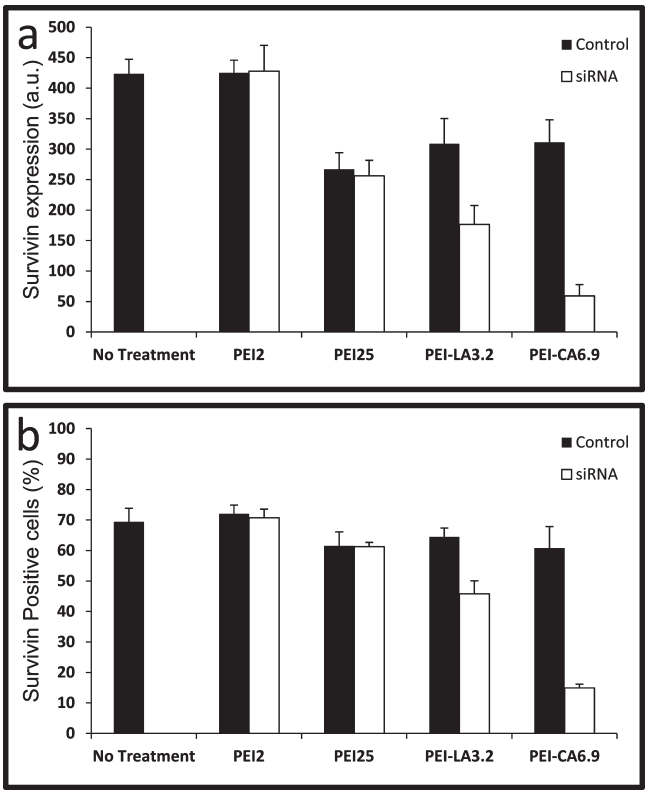


Figure 4. Downregulation of survivin expression by polymer/siRNA complexes. (a) The survivin levels in MDA-MB-231 cells after 72 h exposure to siRNA complexes prepared with unmodified PEIs, PEI-LA3.2, and PEI-CA6.9 at a polymer:siRNA ratio of 2:1. The black bars represent survivin levels for scrambled siRNA treated cells, whereas the white bars represent cells treated with survivin specific siRNA. NT (No Treatment) refers to cells treated with buffer alone. (b) The level of survivin expression presented as percentage of survivin-positive cells after 72 h exposure to the same complexes. Survivin downregulation was observed for both polymers; however, the level of downregulation was more significant for PEI-CA6.9 polymer, which is in correlation with the cell viability results presented in Figure 3.

PEI2-CA6.9. A significant drop in cell viability was also observed at high polymer:siRNA ratios; however, a specific drop in cell viability was especially noted at the polymer:siRNA ratio of 2:1 at 36 and 54 nM siRNA concentrations (Figure 3b). The Figure 3b inset summarizes the decrease in cell viability as a percentage of the viable cells compared to the control siRNA treatment, which clearly shows the effective groups for siRNA delivery. The unmodified PEIs (even high MW PEI25) did not induce any specific cell death, even with the highest dose of 54 nM siRNA (Figure S2 in the Supporting Information). To ensure that 2:1 is the optimum polymer:siRNA ratio, this experiment was repeated with a polymer:siRNA ratio of 1:1, but the loss of cell viability was significantly less than that for the 2:1 ratio (results not shown). Based on these results, a polymer:siRNA ratio of 2:1 and siRNA concentration of 56 nM were selected for the rest of the experiments.

The effect of siRNA delivery on survivin protein downregulation was evaluated after 72 h of treatment with the polymer/siRNA complexes. Figures 4a summarizes the mean survivin levels analyzed by flow cytometry after treatment with siRNA complexes of LA-substituted polymers and PEI2-CA6.9, as opposed to unmodified PEIs. While LA- and CA-modified polymers showed a decrease in survivin levels in comparison to the cells treated with scrambled siRNA, PEI2-CA6.9 gave the most significant downregulation in survivin levels (~82% vs 25−40% for PEI2-LA polymers). The unmodified PEIs were ineffective in survivin silencing (Figure S2 in the Supporting Information). Figure 4b represents the percentage of survivin-positive cells from the same experiment, which again confirmed the superior effect of PEI2-CA6.9 to silence survivin expression.

In order to determine if survivin downregulation caused apoptosis induction, apoptosis levels in cell populations were evaluated based on DNA fragmentation. Figure 5 summarizes the results of apoptosis assay on scrambled and survivin siRNA-treated cells, along with negative (cells exposed to saline) and positive controls (cells treated with a nuclease). The data is summarized as the mean apoptosis level (Figure 5a), or as the percentage of cells positive for apoptosis (Figure 5b). There was no significant increase in apoptosis induction in cells exposed to polymer/scrambled siRNA complexes; however, cells treated with survivin siRNA complexes with PEI2-LA3.2 and PEI2-CA6.9 displayed 2- and 3.5-fold increased apoptosis levels, respectively (Figure 5a). While the nuclease treatment resulted in ~54.2% apoptotic cells (compared to ~10% in

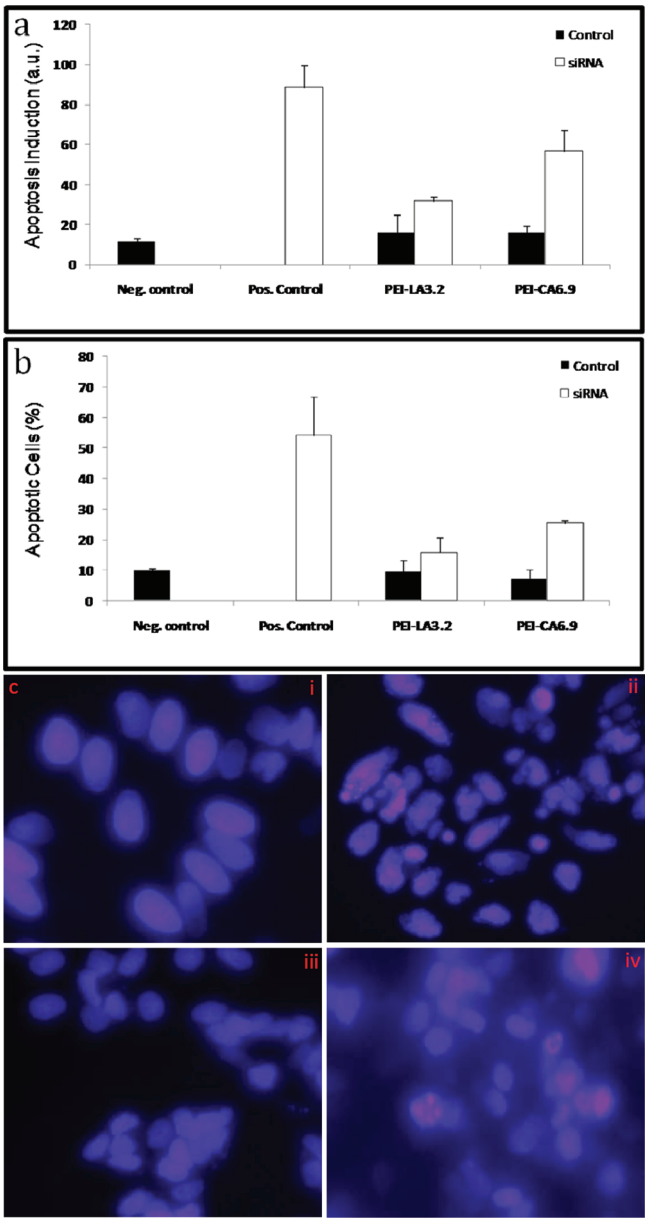


Figure 5. Apoptosis induction in MDA-MB-231 cells by survivin downregulation. (a) The apoptosis levels in MDA-MB-231 cells after 72 h exposure to siRNA complexes prepared with LA3.2 and CA6.9 at a polymer:siRNA ratio of 2:1, calculated based on mean fluorescence after analysis with apoptosis evaluation kit. The black bars represent survivin levels for scrambled siRNA treated cells, whereas the white bars represent cells treated with survivin specific siRNA. NT (No Treatment) refers to cells treated with buffer alone. Positive control represents apoptosis in cells exposed to nuclease. (b) The level of apoptosis presented as percentage of apoptotic cells after 72 h exposure to same complexes. Apoptosis levels were significantly higher for cells exposed to PEI-CA6.9/survivin siRNA, which is in correlation with data presented in Figures 3 and 4. (c) Samples of MDA-MB-231 cells stained with Hoechst dye to detect nucleus fragmentation after exposure to saline (i), 10 μg/mL paclitaxel for 24 h (ii), scrambled siRNA/PEI-CA6.9 complexes (iii), and survivin siRNA/PEI-CA6.9 (iv): Nucleus fragmentation was observed after survivin downregulation.

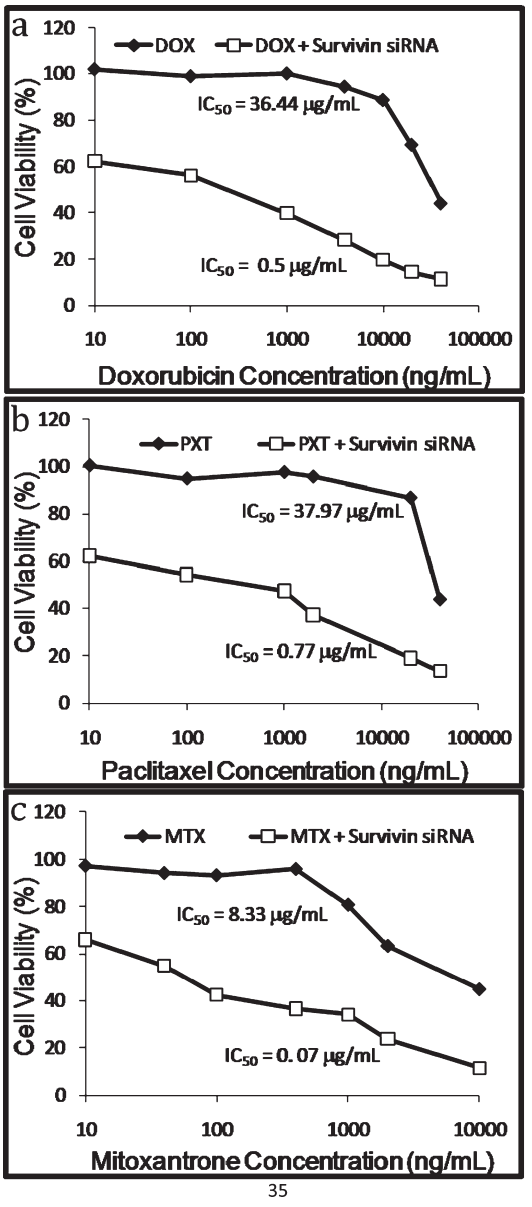


Figure 6. The effect of survivin downregulation on the cytotoxic effect of a variety of commonly used anticancer drugs. Viability of MDA-MB-231 cells exposed to increasing concentrations of doxorubicin (a), paclitaxel (b), and mitoxantrone (c): Pretreatment of the MDA-MB-231 cells with PEI2-CA6.9/survivin siRNA complexes for 48 h before exposure to anticancer drug (for 24 h) increased the efficacy of the anticancer agent significantly, as represented by a significant drop in the IC_{50} of the anticancer agent.

negative control), survivin siRNA delivered by PEI2-LA3.2 and PEI2-CA6.9 induced 15.8% (compared to 9.5% for scrambled siRNA; not significantly different) and 25.3% (compared to 7.4% for scrambled siRNA; $p < 0.001$) apoptotic cells, respectively (Figure 5b).

Microscopic analysis of the cells was also conducted to confirm nuclear fragmentation (Figures 5c). Cells exposed to saline (Figure 5c-i) or complexes prepared with scrambled siRNA (Figure 5c-ii) showed no nuclear fragmentation. However, nuclear fragmentation was observed in cells treated with PEI2-CA6.9/survivin siRNA complexes in both Hoechst (Figure 5c-iii) and SYBR Green I (not shown) stained cells. A positive control was prepared by exposing the cells to 10 μg/mL

paclitaxel for 24 h, which also yielded significant nuclear fragmentation (Figure 5c-iv).

The effect of survivin downregulation on the cytotoxic effect of three common anticancer drugs was evaluated using the MTT assay. Cells were exposed to increasing concentrations of doxorubicin, paclitaxel, and mitoxantrone, and the resultant cell viabilities are summarized in Figures 6a, 6b and 6c, respectively. Pretreatment of the MDA-MB-231 cells with PEI2-CA6.9/survivin siRNA complexes for 48 h before exposure to the drugs increased the efficacy of the anticancer agent significantly, as represented by a 72.8-, 49.6-, and 123-fold decrease in the IC_{50} of doxorubicin, paclitaxel, and mitoxantrone, respectively.

DISCUSSION

Apoptosis induction, through stimulating apoptotic pathways or inhibiting antiapoptotic mechanisms, is actively explored as a strategy in cancer therapy. Survivin was observed to be involved in multiple intracellular mechanisms, all of which favor cell survival.[37] All IAP proteins share two to three common structures of baculovirus IAP repeat (BIR) domains to bind and inactivate caspases, except survivin, the smallest human IAP protein which contains only a single BIR repeat.[38] Overexpression of survivin has been demonstrated in many different cancer cells, and nuclear expression of survivin has been attributed to its role in cell division via controlling microtubule stability of the normal mitotic spindle.[12] Survivin has been also suggested to be responsible for resistance of colorectal cancer to clinical therapies.[39] Based on these observations, we considered survivin downregulation to be a promising anticancer strategy and employed a previously reported library of lipid-modified polymers[33,36] to determine the feasibility of survivin silencing through siRNA delivery with these new carriers.

The present study was initiated with a toxicity evaluation of the modified polymers on the selected cell line; while PEI25 was extremely toxic to the cells, the PEI2 did not affect cell viability at the evaluated concentration range. Since the destabilizing effect of PEI on cell membrane has been suggested as the mechanism responsible for both cytotoxic and cargo delivery effects,[40,41] a severe toxic effect was expected for the highly cell-interactive PEI25. Hydrophobic moieties are speculated to enhance the interaction between the polymeric carrier and the lipophilic cell membrane, which in turn could increase the cellular uptake of the cargo, but may also result in higher cytotoxicity. Our results showed a slight increase in toxicity compared to PEI2 for the modified polymers; however, at a concentration proven to be effective for survivin silencing (1.5 μg/mL), minimal cytotoxicity was evident for all lipid-modified polymers, which confirms the safety of the designed delivery system.

Most of the modified polymers showed promising capability for cellular delivery of siRNA. At a polymer:siRNA ratio of 2:1, PEI25 was more effective in siRNA delivery than all of the modified polymers. However, increasing this ratio to 8:1 increased siRNA cellular uptake for LA-substituted polymers significantly, and therefore, all PEI2-LA polymers showed a higher siRNA delivery compared to PEI25 at this ratio. PEI2-CA6.9 showed a comparable siRNA delivery capability to PEI25 at both ratios. This could be explained by a more effective interaction between the polymer/siRNA complexes and the cell membrane by using a higher portion of the lipid-substituted polymers. The beneficial role of hydrophobic substitution was also confirmed by the strong correlation obtained between the level of substitution and siRNA delivery for all substituting moieties in polymer:siRNA ratio of 2:1 (Figure 2c). At the 8:1 polymer:siRNA ratio, this correlation was seen for only CA-substituted polymer and not for other polymers, which could be due to significantly higher uptake levels that may have saturated the uptake mechanism(s).

The survivin downregulation was initially assessed indirectly by the MTT cell viability assay. The siRNA complexes prepared with the CA- and LA-substituted polymers were shown to decrease the viability of MDA-MB-231 cells more significantly, which were the polymers that gave the highest siRNA delivery as well (Figure 2a). However, a closer look at different polymer:siRNA ratios showed the most significant downregulation to be achieved with PEI-CA6.9 at a 2:1 ratio, which did not correlate with the results of the siRNA delivery, where the highest delivery was accomplished with LA-substituted polymers and at the 8:1 ratio. While a higher portion of polymer seems to increase the uptake by enhanced interactions with the cell membrane, this ratio could also form more stable complexes, which becomes an obstacle for siRNA release after internalization. Therefore, even though the PEI2-CA6.9 complexes gave a lower siRNA uptake, a higher intracellular siRNA release may result in their superior efficacy for survivin downregulation. Our previous study had shown that CA-substituted polymer had the lowest binding affinity to siRNA,[33] due to the fact that about 43% of the primary amines in the polymer chain were substituted with CA moieties (compared to 31% and less for other polymers included in this study). This means that fewer amine groups are available for nucleic acid binding and, therefore, the affinity of CA-substituted PEI for binding to siRNA is lower compared to LA-PEI, which in turn could explain this hypothesis. On the other hand, the fact that PEI25 was unable to create an effective downregulation, despite significant siRNA delivery, was supportive of this hypothesis (Figure S2 in the Supporting Information and Figures 4a and 4b).

The changes in cell viability as a result of survivin siRNA delivery were correlated with the analysis of survivin protein levels (Figure 4) and apoptosis induction (Figure 5). In fact, siRNA delivery with PEI2-CA6.9 caused >80% survivin downregulation and 3.5-fold increased apoptosis induction. Several studies have demonstrated that survivin knockdown, accomplished through different methods of RNA interference, is able to induce apoptosis in various human tumor cell models.[42−45] Survivin inhibition in endothelial cells caused both a proapoptotic effect and an interruption of tumor angiogenesis,[46] and induced apoptosis in osteosarcoma cells,[47] while in xenografts, survivin knockdown decreased tumor formation and reduced angiogenesis development;[48,49] however, all these studies were conducted using small hairpin RNA (shRNA) expression *in situ*, which is not practical in clinics. Among the studies conducted with siRNA, Paduano et al. was the only group to report survivin downregulation quantitatively,[45] and neither of these studies reported the level of apoptosis in the cells to confirm the survivin downregulation as the underlying basis of decreased cell viability. Paduano et al. study shows ~75% survivin downregulation by siRNA transfection using LipofectAMINE 2000 and Oligofectamine, but with a relatively high concentration of ~100 nM siRNA. Different studies have pointed out the importance of an optimal siRNA concentration required for an effective target downregulation. Unspecific upregulation and downregulation of

unrelated genes associated with high concentrations of siRNA are general concerns, and a 20−50 nM concentration range of siRNA is considered desirable for therapeutic silencing.[50−52] It is difficult to compare the relative efficacy of these commercial carriers to the polymers synthesized in this study, given the differences in experimental system, but the reported polymers were able to induce an apoptotic response by using siRNA concentrations in the desired range.

Survivin downregulation was previously reported to enhance the apoptotic response of tumor cells to different drugs, including adriamycin and cisplatin in KB squamous cell carcinoma cells,[53,54] etoposide, cisplatin and doxorubicin in osteosarcoma cells,[47] and doxorubicin or TNF-α in different cancer cells.[48] However, all of these studies employed shRNA expression *in situ*. We evaluated the effect of more clinically relevant siRNA-induced survivin silencing on the IC_{50} of three cytotoxic drugs in the MDA-MB-231 cells. Since the viability of the siRNA-treated cells was only ∼60% of the control cells in the absence of any drugs, a significant drop in the IC_{50} of the evaluated anticancer agents was expected. In fact ∼73-, ∼50-, and ∼123-fold drops in IC_{50} were observed for doxorubicin, paclitaxel, and mitoxantrone, respectively (Figure 6). While "sensitizing" resistant cancer cells to specific anticancer agents has been a major strategy in the past, inducing apoptosis through a more general pathway such as survivin downregulation has the advantage of being applicable to wider range of tumor cells. Our findings regarding the IC_{50} of the anticancer agents are a significant observation, which could decrease the minimum drug concentration required for the effective treatment significantly and, therefore, minimize the toxic side effects of chemotherapy. We and others have attempted to lower the drug IC_{50} by delivering siRNA against drug transporters, such as P-glycoprotein,[55,56] and the strategy pursued in this study focusing on survivin might provide an alternative approach for sensitizing the cells to drug therapy.

CONCLUSION

This manuscript reported effective downregulation of survivin, which is a member of the IAP family of proteins shown to be overexpressed in cancer cells with an antiapoptosis role. This was accomplished by delivering survivin-specific siRNAs by using hydrophobically modified PEI2, obtained by substituting different lipophilic moieties on the polymer chain. The PEI-CA6.9 with the highest caprylic acid substitution was shown to be the most effective polymer in downregulation of survivin, and induction of apoptosis in a human breast cancer cell line, which, in turn, decreased the IC_{50} of different anticancer agents. This study introduces an effective and nontoxic delivery system for survivin downregulation for further testing and sheds more light on the importance of this protein in cell survival and cancer therapy.

ASSOCIATED CONTENT

Supporting Information. Figure S1 on substitution level for the lipid-substituted library, and Figure S2 on effect of unmodified PEIs on cell viability. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*#2-020 RTF, Chemical & Materials Engineering Department, Faculty of Engineering, University of Alberta, Edmonton, Alberta, Canada T6G 2G6. Tel: (780) 492-0988. Fax: (780) 492-2881. E-mail: hasan.uludag@ualberta.ca.

ACKNOWLEDGMENT

This project was financially supported by operating grants from Alberta Advanced Education and Technology, Canadian Institute of Health Research (CIHR) and Natural Science and Engineering Research Council of Canada (NESRC). The equipment support was provided by Alberta Heritage Foundation for Medical Research (AHFMR). We thank Dr. Michael Weinfeld (Cross Cancer Institute, Edmonton, AB) for providing the cell line for this study. H.M.A. is supported by an AHFMR Fellowship. B.L. is supported by an AFC Graduate Studentship.

REFERENCES

(1) Kerr, J. F.; Wyllie, A. H.; Currie, A. R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **1972**, *26*, 239–257.

(2) Cryns, V.; Yuan, J. Proteases to die for. *Genes Dev.* **1998**, *12*, 1551–1570.

(3) Schimmer, A. D. Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. *Cancer Res.* **2004**, *64*, 7183–7190.

(4) Ambrosini, G.; Adida, C.; Altieri, D. C. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat. Med.* **1997**, *3*, 917–921.

(5) Kanwar, R. K.; Cheung, C. H.; Chang, J. Y.; Kanwar, J. R. Recent advances in anti-survivin treatments for cancer. *Curr. Med. Chem.* **2010**, *17*, 1509–1515.

(6) Altieri, D. C. Survivin, cancer networks and pathway-directed drug discovery. *Nat. Rev. Cancer* **2008**, *8*, 61–70.

(7) Eckelman, B. P.; Salvesen, G. S.; Scott, F. L. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep.* **2006**, *7*, 988–94.

(8) Dohi, T.; Beltrami, E.; Wall, N. R.; Plescia, J.; Altieri, D. C. Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis. *J. Clin. Invest.* **2004**, *114*, 1117–1127.

(9) Marusawa, H.; Matsuzawa, S.; Welsh, K.; Zou, H.; Armstrong, R.; Tamm, I.; Reed, J. C. HBXIP functions as a cofactor of survivin in apoptosis suppression. *EMBO J.* **2003**, *22*, 2729–2740.

(10) Altieri, D. C. Survivin, versatile modulation of cell division and apoptosis in cancer. *Oncogene* **2003**, *22*, 8581–8589.

(11) Espinosa, M.; Cantu, D.; Herrera, N.; Lopez, C. M.; De la Garza, J. G.; Maldonado, V.; Melendez-Zajgla, J. Inhibitors of apoptosis proteins in human cervical cancer. *BMC Cancer* **2006**, *6*, 45.

(12) Giodini, A.; Kallio, M. J.; Wall, N. R.; Gorbsky, G. J.; Tognin, S.; Marchisio, P. C.; Symons, M.; Altieri, D. C. Regulation of microtubule stability and mitotic progression by survivin. *Cancer Res.* **2002**, *62*, 2462–2467.

(13) Kania, J.; Konturek, S. J.; Marlicz, K.; Hahn, E. G.; Konturek, P. C. Expression of survivin and caspase-3 in gastric cancer. *Dig. Dis. Sci.* **2003**, *48*, 266–271.

(14) Lu, S.; Zhang, B.; Wang, Z. Expression of survivin, cyclinD1, p21(WAF1), caspase-3 in cervical cancer and its relation with prognosis. *J. Huazhong Univ. Sci. Technol., Med. Sci.* **2005**, *25*, 78–81.

(15) Ryan, B. M.; O'Donovan, N.; Duffy, M. J. Survivin: a new target for anti-cancer therapy. *Cancer Treat. Rev.* **2009**, *35*, 553–562.

(16) Nakahara, T.; Takeuchi, M.; Kinoyama, I.; Minematsu, T.; Shirasuna, K.; Matsuhisa, A.; Kita, A.; Tominaga, F.; Yamanaka, K.; Kudoh, M.; Sasamata, M. YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. *Cancer Res.* **2007**, *67*, 8014–8021.

(17) Plescia, J.; Salz, W.; Xia, F.; Pennati, M.; Zaffaroni, N.; Daidone, M. G.; Meli, M.; Dohi, T.; Fortugno, P.; Nefedova, Y.; Gabrilovich, D. I.;

Colombo, G.; Altieri, D. C. Rational design of shepherdin, a novel anticancer agent. *Cancer Cell* **2005**, *7*, 457–468.

(18) Chang, C. C.; Heller, J. D.; Kuo, J.; Huang, R. C. Tetra-O-methyl nordihydroguaiaretic acid induces growth arrest and cellular apoptosis by inhibiting Cdc2 and survivin expression. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 13239–13244.

(19) Smolewski, P. Terameprocol, a novel site-specific transcription inhibitor with anticancer activity. *IDrugs* **2008**, *11*, 204–214.

(20) Hannon, G. J. RNA interference. *Nature* **2002**, *418*, 244–251.

(21) Haupenthal, J.; Baehr, C.; Kiermayer, S.; Zeuzem, S.; Piiper, A. Inhibition of RNAse A family enzymes prevents degradation and loss of silencing activity of siRNAs in serum. *Biochem. Pharmacol.* **2006**, *71*, 702–710.

(22) Wiethoff, C. M.; Middaugh, C. R. Barriers to nonviral gene delivery. *J. Pharm. Sci.* **2003**, *92*, 203–217.

(23) Bologna, J. C.; Dorn, G.; Natt, F.; Weiler, J. Linear polyethylenimine as a tool for comparative studies of antisense and short double-stranded RNA oligonucleotides. *Nucleosides, Nucleotides Nucleic Acids* **2003**, *22*, 1729–1731.

(24) Godbey, W. T.; Wu, K. K.; Mikos, A. G. Poly(ethylenimine) and its role in gene delivery. *J. Controlled Release* **1999**, *60*, 149–160.

(25) Urban-Klein, B.; Werth, S.; Abuharbeid, S.; Czubayko, F.; Aigner, A. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. *Gene Ther.* **2005**, *12*, 461–466.

(26) Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297–7301.

(27) Coll, J. L.; Chollet, P.; Brambilla, E.; Desplanques, D.; Behr, J. P.; Favrot, M. In vivo delivery to tumors of DNA complexed with linear polyethylenimine. *Hum. Gene Ther.* **1999**, *10*, 1659–1666.

(28) Ferrari, S.; Moro, E.; Pettenazzo, A.; Behr, J. P.; Zacchello, F.; Scarpa, M. ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. *Gene Ther.* **1997**, *4*, 1100–1106.

(29) Godbey, W. T.; Wu, K. K.; Wu, Mikos, A. G. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 5177–5181.

(30) Jeong, J. H.; Song, S. H.; Lim, D. W.; Lee, H.; Park, T. G. DNA transfection using linear poly(ethylenimine) prepared by controlled acid hydrolysis of poly(2-ethyl-2-oxazoline). *J. Controlled Release* **2001**, *73*, 391–399.

(31) Kloeckner, J.; Bruzzano, S.; Ogris, M.; Wagner, E. Gene carriers based on hexanediol diacrylate linked oligoethylenimine: effect of chemical structure of polymer on biological properties. *Bioconjugate Chem.* **2006**, *17*, 1339–1345.

(32) Wightman, L.; Kircheis, R.; Rossler, V.; Carotta, S.; Ruzicka, R.; Kursa, M.; Wagner, E. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J. Gene Med.* **2001**, *3*, 362–372.

(33) Montazeri Aliabadi, H.; Landry, B.; Bahadur, R. K.; Neamnark, A.; Suwantong, O.; Uludag, H. Impact of Lipid Substitution on Assembly and Delivery of siRNA by Cationic Polymers. *Macromol. Biosci.* **2011**, *11*, 662–672.

(34) Neamnark, A.; Suwantong, O.; Bahadur, R. K.; Hsu, C. Y.; Supaphol, P.; Uludag, H. Aliphatic lipid substitution on 2 kDa polyethylenimine improves plasmid delivery and transgene expression. *Mol. Pharmaceutics* **2009**, *6*, 1798–1815. Correction:**2010**, *7*, 618.

(35) Brigulova, K.; Cervinka, M.; Tosner, J.; Sedlakova, I. Chemoresistance testing of human ovarian cancer cells and its in vitro model. *Toxicol. in Vitro* **2010**, *24*, 2108–2115.

(36) Incani, V.; Tunis, E.; Clements, B. A.; Olson, C.; Kucharski, C.; Lavasanifar, A.; Uludag, H. Palmitic acid substitution on cationic polymers for effective delivery of plasmid DNA to bone marrow stromal cells. *J. Biomed. Mater. Res. A* **2007**, *81*, 493–504.

(37) Yamamoto, H.; Ngan, C. Y.; Monden, M. Cancer cells survive with survivin. *Cancer Sci.* **2008**, *99*, 1709–1714.

(38) Salvesen, G. S.; Duckett, C. S. IAP proteins: blocking the road to death's door. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 401–410.

(39) Rodel, F.; Frey, B.; Leitmann, W.; Capalbo, G.; Weiss, C.; Rodel, C. Survivin antisense oligonucleotides effectively radiosensitize colorectal cancer cells in both tissue culture and murine xenograft models. *Int. J. Radiat. Oncol. Biol. Phys.* **2008**, *71*, 247–255.

(40) Hong, S.; Leroueil, P. R.; Janus, E. K.; Peters, J. L.; Kober, M. M.; Islam, M. T.; Orr, B. G.; Baker, J. R., Jr.; Banaszak Holl, M. M. Interaction of polycationic polymers with supported lipid bilayers and cells: nanoscale hole formation and enhanced membrane permeability. *Bioconjugate Chem.* **2006**, *17*, 728–734.

(41) Lungwitz, U.; Breunig, M.; Blunk, T.; Gopferich, A. Polyethylenimine-based non-viral gene delivery systems. *Eur. J. Pharm. Biopharm.* **2005**, *60*, 247–266.

(42) Ai, Z.; Yin, L.; Zhou, X.; Zhu, Y.; Zhu, D.; Yu, Y.; Feng, Y. Inhibition of survivin reduces cell proliferation and induces apoptosis in human endometrial cancer. *Cancer* **2006**, *107*, 746–756.

(43) Lu, Y. H.; Luo, X. G.; Tao, X. Survivin gene RNA interference induces apoptosis in human HL60 leukemia cell lines. *Cancer Biother. Radiopharm.* **2007**, *22*, 819–825.

(44) Nakao, K.; Hamasaki, K.; Ichikawa, T.; Arima, K.; Eguchi, K.; Ishii, N. Survivin downregulation by siRNA sensitizes human hepatoma cells to TRAIL-induced apoptosis. *Oncol. Rep.* **2006**, *16*, 389–392.

(45) Paduano, F.; Villa, R.; Pennati, M.; Folini, M.; Binda, M.; Daidone, M. G.; Zaffaroni, N. Silencing of survivin gene by small interfering RNAs produces supra-additive growth suppression in combination with 17-allylamino-17-demethoxygeldanamycin in human prostate cancer cells. *Mol. Cancer Ther.* **2006**, *5*, 179–186.

(46) Coma, S.; Noe, V.; Lavarino, C.; Adan, J.; Rivas, M.; Lopez-Matas, M.; Pagan, R.; Mitjans, F.; Vilaro, S.; Piulats, J.; Ciudad, C. J. Use of siRNAs and antisense oligonucleotides against survivin RNA to inhibit steps leading to tumor angiogenesis. *Oligonucleotides* **2004**, *14*, 100–113.

(47) Zou, J.; Gan, M; Mao, N.; Zhu, X.; Shi, Q.; Yang, H. Sensitization of osteosarcoma cell line SaOS-2 to chemotherapy by downregulating survivin. *Arch. Med. Res.* **2010**, *41*, 162–169.

(48) Li, Q. X.; Zhao, J.; Liu, J. Y.; Jia, L. T.; Huang, H. Y.; Xu, Y. M.; Zhang, Y.; Zhang, R.; Wang, C. J.; Yao, L. B.; Chen, S. Y.; Yang, A. G. Survivin stable knockdown by siRNA inhibits tumor cell growth and angiogenesis in breast and cervical cancers. *Cancer Biol. Ther.* **2006**, *5*, 860–866.

(49) Zhen, H. N.; Li, L. W.; Zhang, W.; Fei, Z.; Shi, C. H.; Yang, T. T.; Bai, W. T.; Zhang, X. Short hairpin RNA targeting survivin inhibits growth and angiogenesis of glioma U251 cells. *Int. J. Oncol.* **2007**, *31*, 1111–1117.

(50) Kumar, A.; Haque, J.; Lacoste, J.; Hiscott, J.; Williams, B. R. Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6288–6292.

(51) Persengiev, S. P.; Zhu, X.; Green, M. R. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *RNA* **2004**, *10*, 12–18.

(52) Semizarov, D.; Frost, L.; Sarthy, A.; Kroeger, P.; Halbert, D. N.; Fesik, S. W. Specificity of short interfering RNA determined through gene expression signatures. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6347–6352.

(53) Jiang, G.; Li, J.; Zeng, Z.; Xian, L. Lentivirus-mediated gene therapy by suppressing survivin in BALB/c nude mice bearing oral squamous cell carcinoma. *Cancer Biol. Ther.* **2006**, *5*, 435–440.

(54) Kojima, H.; Iida, M.; Yaguchi, Y.; Suzuki, R.; Hayashi, N.; Moriyama, H.; Manome, Y. Enhancement of Cisplatin sensitivity in squamous cell carcinoma of the head and neck transfected with a survivin antisense gene. *Arch. Otolaryngol. Head Neck Surg.* **2006**, *132*, 682–685.

(55) Abbasi, M.; Lavasanifar, A.; Berthiaume, L. G.; Weinfeld, M.; Uludag, H. Cationic polymer-mediated small interfering RNA delivery for P-glycoprotein down-regulation in tumor cells. *Cancer* **2010**, *116*, 5544–5554.

(56) Abbasi, M.; Lavasanifar, A.; Uludag, H. Recent attempts at RNAi-mediated P-glycoprotein downregulation for reversal of multidrug resistance in cancer. *Med. Res. Rev.* **2011**, DOI: 10.1002/med.20244.