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ORIGINAL ARTICLE

Evaluations of combination *MDR-1* gene silencing and paclitaxel administration in biodegradable polymeric nanoparticle formulations to overcome multidrug resistance in cancer cells

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Abstract In this study, the effect of *MDR-1* gene silencing, using small interfering RNA (siRNA), and paclitaxel (PTX) co-therapy in overcoming tumor multidrug resistance was examined. Poly(ethylene oxide)-modified poly(beta-amino ester) (PEO-PbAE) and PEO-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles were formulated to efficiently encapsulate *MDR-1* silencing siRNA and PTX, respectively. Upon administration in multidrug resistant $SKOV3_{TR}$ human ovarian adenocarcinoma cells, siRNA-mediated *MDR-1* gene silencing was evident at 100 nM dose. Combination of *MDR-1* gene silencing and nanoparticle-mediated delivery significantly influenced the cytotoxic activity of PTX in $SKOV3_{TR}$ cells similar to what was observed in drug sensitive SKOV3 cells. We speculate that the enhancement in cytotoxicity was due to an increase in intracellular drug accumulation upon *MDR-1* gene silencing leading to an apoptotic cell-kill effect. Taken together, these preliminary results are highly encouraging for the development of combination nano-therapeutic strategies that combine gene silencing and drug delivery to provide more potent therapeutic effect, especially in refractory tumors.

Keywords Multidrug resistance · *MDR-1* gene silencing siRNA · Paclitaxel · SKOV3 human ovarian adenocarcinoma cells · Poly(beta-amino ester) · Poly(epsilon-caprolactone)

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Introduction

Multidrug resistance (MDR) in cancer is defined as the ability of cancer cells and tumors to survive in the presence of structurally and functionally dissimilar cystostatic and cytotoxic chemotherapeutic drugs [1, 2]. MDR is termed "intrinsic" when the disease is refractory to the drug from the outset, or it is "acquired" when the disease becomes insensitive to the treatment options upon relapse. Acquired MDR is thought to be the primary reason for the poor efficacy of cancer chemotherapy, especially in ovarian and breast cancers, where up to 50–70% of the patients develop MDR [3, 4]. Acquired resistance is thought to be caused by: (1) a lack of sufficient drug concentration, (2) short residence time of therapeutic agent reaching the tumor mass in vivo, (3) limited drug diffusion into the interstitial spaces of the tumor mass, and (4) the phenotypic alterations in the tumor cells due to various selection pressures.

Cellular mechanisms of drug resistance have been intensively studied and several major classes have been identified [5, 6]. First, drug resistance is due to expression of the *MDR-1* gene that results in the presence of membrane-bound ABC transporter proteins, such as P-glycoprotein (P-gp) that uses ATP to actively pump cytotoxic drugs out from the cell. Alternatively, drug resistance can also be due to reduced influx of drugs into the cell, and hyperactivation of the detoxification mechanisms such as cytochrome-P450 metabolism enzymes and glutathione. Cells can also activate repair mechanisms for drug-induced DNA damage by expression of DNA repair enzymes, such as topoisomerases. Lastly, cellular resistance can also be due to disruptions in the apoptotic signaling pathways that allow the cells to resist drug-induced programmed cell death [7, 8].

RNA interference (RNAi) has emerged as a powerful approach to down-regulate gene function in the cells [9, 10].

The idea of using small interfering RNA (siRNA) for therapeutic purposes has been tested in the last few years with the pioneering work of Elbashir et al. [11, 12]. In cancer therapy, siRNA has been used to suppress tumor growth by silencing various oncogenes, such as *HER2/neu* oncogene [13], inhibition of growth factors including epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) production and neo-angiogenesis [14, 15], as well as down-regulation of P-glycoprotein production by silencing *MDR-1* gene [16, 17]. The major barrier to therapeutic RNAi is efficient delivery of siRNA and other constructs into the cell of interest following systemic administration [18, 19].

Due to the hyper-permeability of the tumor vasculature and the lack of lymphatic drainage, blood-borne macromolecules and colloidal particles are preferentially distributed in the tumor due to the enhanced permeability and retention (EPR) effect [20]. Maeda's group first described the EPR effect of tumor vasculature, which has subsequently been examined and confirmed by other investigators [21]. Using long-circulating liposomes, the effective pore size of cut-off of peripheral tumor vasculature was found to be in the range from 200 to 600 nm in diameter [22].

In previous studies, we have shown that poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles can be used for encapsulation and delivery of hydrophobic agents including tamoxifen, paclitaxel (PTX), and C6-ceramide [23–27]. PEO-PCL nanoparticle formulations, made by blending PCL with Pluronic® F-108, an ABA triblock copolymer of PEO/poly(propylene oxide) (PPO)/PEO, remain stable in vivo, and retain the PEO chains on the surface to increase the circulating half-life and plasma residence time of PTX from a fraction of an hour to 25.3 and 24.0 h, respectively, alongside a nearly 8-fold decrease in total body clearance of the drug [26]. The concentration of PTX inside the tumor mass of mice-bearing human ovarian carcinoma (SKOV3) xenografts, when administered in PEO-PCL nanoparticles, was 8.7-fold higher 5 h post-intravenous administration as compared to mice treated with PTX in aqueous solution [26].

On the other hand, for encapsulating polyanions, such as siRNA, cationic biodegradable polymers offer the advantage of complexation into small nanoparticulates, along with the pH-responsive release characteristics specifically within the acidic environment (pH ~6.5) of the endosome upon cellular internalization. A large library (>5,000) of poly(beta-amino ester)s (PbAE) has been synthesized by parallel synthesis of primary and secondary amines with diol diacrylates [28, 29]. Using a specific PbAE that dissolves at pH 6.5 or below, we have previously developed PEO-modified PbAE nanoparticles to deliver PTX as a novel stimuli-responsive drug delivery system to an in vivo human ovarian cancer model. The results showed that these particles released their drug load not only in a pH-specific manner upon internalization into the tumor cells, but that the resultant therapeutic efficacy increased at least 2-fold over that of non pH-responsive PEO-PCL nanoparticles [30-32].

In this study, we have Pluronic F108®, having 56 residues of propylene oxide and 122 residues of ethylene oxide for surface modification of PbAE and PCL nanoparticles. By blending PbAE and PCL with Pluronic® F108 copolymer in the right proportion, the hydrophilic PEO chains are extended to the particle surface and remain in the mobile state in aqueous media to provide stability to the particle suspension by the steric repulsion mechanism [20, 26, 30]. Additionally, when either PEO-PbAE or PEO-PCL nanoparticles were administered intravenously to tumor-bearing mice, in previous studies, we have observed long-circulation times (typical systemic half-life of >24 h) and preferential tumor accumulation by the EPR effect [26, 31]. Once accumulated within the tumor interstitium by exploiting vascular abnormalities, the PEO-PbAE and PEO-PCL nanocarriers would increase the drug concentration inside the tumor cells as a result of non-specific endocytosis, followed by either pH-responsive triggered release (for PbAE) or sustained release (for PCL) of the encapsulated payload. PbAE also offers additional benefit of stabilizing negatively-charged nucleic acids, such as siRNA, upon cellular internalization. We have fabricated PEO-PbAE nanoparticles for encapsulation and delivery of *MDR-1* silencing siRNA and PEO-PCL nanoparticles for administration of PTX in wild-type (sensitive) and multi-drug resistant SKOV3 human ovarian adenocarcinoma cells. *MDR-1* gene silencing and enhancement of PTX therapeutic effect with this combination therapeutic strategy was examined in vitro.

Materials and methods

Preparation and characterization of PEO-PbAE and PEO-PCL nanoparticles

A hydrophobic representative PbAE (MW ~ 10,000 Da) was synthesized by the addition reaction of 4,4′-trimethyldipiperidine with 1,4-butanediol diacrylate in dimethylformamide for 48 h at 50 C and purified according to the synthesis scheme described earlier. PCL with an average molecular weight of 14,800 Da, as verified by gel permeation chromatography, was purchased from Polysciences, Inc. (Warrington, PA). PEO-PbAE and PEO-PCL, and nanoparticles were prepared according to the method of Shenoy and Amiji [26, 32] using the solvent displacement under controlled temperature and stirring conditions.

The synthetic siRNA sequence targeting human *MDR-1* gene, based on the studies by Wu et al. [33], was custom

synthesized and purchased from Dharmacon (Boulder, CO). The *MDR-1* siRNA duplex with 5′-GGAAAAGA AACCAACUGUCUU-3′ (sense sequence) and 5′-GACA GUUGGUUUCUUUUCCUU-3′ (antisense sequence) was used in this study. *MDR-1* gene silencing siRNA was added to the PbAE and Pluronic® F-108 solution in ethanol. The siRNA-encapsulated PEO-PbAE nanoparticles were prepared as described above and the final concentration of siRNA used for gene silencing was maintained at 100 nM concentration delivered in 2.0 mg of PEO-PbAE nanoparticles. PTX at 10% (w/w) loading was added to the acetone solution of PCL and Pluronic® F-108 and prepared similarly to the blank nanoparticles described above.

Blank PEO-PbAE, siRNA-loaded PEO-PbAE, blank PEO-PCL, and PTX-loaded PEO-PCL nanoparticle samples, dispersed in aqueous medium, were used for determination of mean particle size and size distribution. The samples were analyzed with a Brookhaven Instrument's (Holtsville, NY) ZetaPALS light scattering instrument at 90° scattering angle and 25°C temperature. Surface charge (i.e., zeta potential) values were measured in aqueous suspension using the ZetaPALS instrument as well. Lastly, the shape and surface morphology of freeze-dried PEO-PbAE and PEO-PCL nanoparticle samples were observed with scanning electron microscopy (SEM) as previously described [31].

The loading capacity and efficiency of *MDR-1* gene silencing siRNA in PEO-PbAE nanoparticles was measured indirectly from the amount of siRNA present in the supernatant after the nanoparticle washing with RNAse free deionized distilled water adjusted to pH 8.0 using PicoGreen® (Invitrogen, Carlsbad, CA) fluorescence assay. For the determination of PTX incorporation in PEO-PCL nanoparticles, a known quantity of the nanoparticles were dissolved in acetonitrile and the incorporated drug was assayed by ultraviolet (UV) spectroscopy at 228 nm using a Shimadzu (Columbia, MD) UV/VIS spectrophotometer.

In vitro siRNA and PTX release studies

Fluorescently-labeled siRNA (siGLO® siRNA), purchased from Dharmacon (Boulder, CO), was used for these studies. Fluorescently-labeled siRNA was encapsulated in PEO-PbAE nanoparticles as previously described. A sample of 10 mg of freeze-dried siRNA-containing PEO-PbAE nanoparticles was incubated with 5.0 mL of phosphate-buffered saline (PBS, pH 7.4) and kept at 37°C in a rotating platform. Periodically for the first 45 min, the tubes were centrifuged and 3.0 mL of the release medium was removed and replaced with fresh PBS. After 45 min of release at pH 7.4, the medium was switched to pH 6.5 phosphate buffer to simulate the tumor microenvironment and the release studies were continued for another 60 min. The concentration of siRNA in the release medium was determined from measurement of fluorescence intensity. The intensity was converted to siRNA concentration using a standard curve and the cumulative percent of encapsulated siRNA released at different time points and at different pH values was plotted.

For PTX release from PEO-PCL nanoparticles, 10 mg of freeze-dried drug-loaded nanoparticles was suspended in 5 mL of PBS with 1% (w/v) Tween®-80 at 37°C for the first 6 h of release. Tween®-80, a non-ionic surfactant, was added in the release media to increase the solubility of drug and provide sink-type conditions and to prevent the drug binding to the container surface. At regular time intervals, 3 mL of the release media was removed and replaced with fresh release media. After 6 h of release, the pH of release media was decreased from 7.4 to 6.5 using phosphate buffer with 1% (w/v) Tween®-80 and the release studies continued for another 6 h. PTX released from the PEO-PCL nanoparticles was analyzed by a high performance liquid chromatography (HPLC) assay method, as described by Dordunoo et al. [34], using C_{18} reverse phase column (Zobrax®) as a stationary phase, water and acetonitrile (60:40 volume ratio) as the mobile phase, and PTX was detected at 227 nm using a UV detector.

MDR-1 gene silencing studies by RT-PCR

Wild-type and multidrug resistant cells in culture

Wild-type (sensitive) and *MDR-1* positive SKOV3 ovarian adenocarcinoma cells were kindly provided by Dr. Michael Seiden's group from the Department of Hematology/Oncology at the Massachusetts General Hospital (Boston, MA). The multidrug resistant SKOV3 phenotype cells ($SKOV3_{TR}$) have been previously characterized to overexpress *MDR-1* gene and P-glycoprotein [35, 36]. Both cell lines were maintained in supplemented RPMI-1640, which consists of RPMI®-1640, 10% heat-activated fetal bovine serum, 10 mM HEPES buffer, 1% penicillin–streptomycin, 1 mM sodium pyruvate, and 2-mercaptoethanol. Cell culturing was carried out at 37°C in 5% CO_2 until the cells were at least 70% confluent.

RT-PCR analysis of MDR-1 silencing

Reverse transcription-polymerase chain reaction (RT-PCR) method was used to evaluate the qualitative silencing efficacy of *MDR-1* with siRNA at the transcription level. SKOV3 and $SKOV3_{TR}$ cells were treated with different concentrations of *MDR-1* siRNA (0–100 nM), either in aqueous solution or in PEO-PbAE nanoparticles. After incubation for 24 h, the cells were lysed and whole-cell RNA was collected and quantitated using a UV spectrophotometer. Following reverse transcriptase treatment to obtain

cDNA, the RT-PCR was carried out in one-step method using Superscript® One-Step RT-PCR with Platinum Taq kit (Invitrogen, Carlsbad, CA). A 25 μL of *MDR-1* cDNA was added to a buffer containing 0.2 mM of deoxynucleotide triphosphate, 1.2 mmol/L MgSO4, 1.0 μL Taq DNA polymerase, 0.2 μg of *MDR-1* upstream primer 5′-CC ATCATTGCAATAGCAGG-3′ and 0.2 μg of *MDR-1* downstream primer 5′-GAGCATACATATGTTCAAA CTTC-3′. Polymerase chain reaction, in a total volume of 50 μL, was carried out for 30 cycles in a thermocycler; each cycle comprises of denaturation at 94°C for 30 s, annealing at 55 C for 30 s, and elongation at 72°C for 1 min. RT-PCR products were analyzed by agarose gel electrophoresis with ethidium bromide-stained gels.

Intracellular PTX concentrations measurement

Upon silencing of *MDR-1*, it is expected that the P-gp efflux of PTX will be affected, thereby increasing intracellular concentrations of the drug. Additional, nanoparticle-based endocytotic delivery mechanism appears to bypass P-gp efflux [27]. Intracellular PTX concentrations were measured using the tritiated [^{3}H]-PTX administered either in aqueous solution or in PEO-PCL nanoparticles. Both the sensitive cells (SKOV3) and resistant ($SKOV3_{TR}$) cells were seeded in six-well plates at a density of 100,000 cells per well. After reaching confluency, the cells were dosed with *MDR-1* silencing siRNA encapsulated in PEO-PbAE nanoparticles at a dose of 100 nM. Following addition of siRNA, [^{3}H]-PTX was administered at a 100 nM and 1.0 μM dose in solution or in PEO-PCL nanoparticles. After 6 h of incubation at 37 C, the cells were harvested with Trypsin-EDTA, washed with sterile PBS to ensure removal of adsorbed drug or nanoparticles, and lysed with Triton®-X100. To each 1.0 mL of the cell lysate, 10 mL of the ScintiSafe Econo® scintillation cocktail was added and the samples were allowed to quench for 2 h in the dark before measurement of radioactivity with a liquid scintillation analyzer (TriCarb 1600TR, Packard Instruments, Inc., Meriden, CT). The counts-per-minute of radioactivity were converted into nanomolar PTX concentrations using a standard and normalized with respect to the total protein content in the cell lysate. The total protein content in the lysed cells was determined using the Nano-Orange® protein quantitation kit obtained from Invitrogen (Carlsbad, CA).

Cell viability studies

PTX dose–response studies

The wild-type SKOV3 and *MDR-1* positive $SKOV3_{TR}$ cells were seeded in a 96-well microplate at a seeding density of 5,000 cells per well in 200 μL medium and allowed to adhere overnight. In order to examine the critical dose of PTX necessary for cytotoxic effect in wild-type and *MDR-1* positive cells, they were incubated with drug concentrations ranging from 10 nM to 10 μM either in aqueous solution or in PEO-PCL nanoparticles. After 5 days of incubation, the medium was replaced with the mixture of MTS reagent from Promega (Madison, WI) and incubated for an additional 4 h. We have utilized the 5-day incubation period based on the fact that PTX is a cell cycle specific drug. The absorbance at 490 nm, indicating formation of formazan dye in viable cells, of the control and treated samples was read using a Synergy® HT microplate reader (Bio-Tek Instruments, Winooski, VT). Percent cell viability was calculated from the absorbance of the treated cells relative to untreated cells grown in media alone.

Effect of time lag between siRNA and PTX administration on cell viability

In order to examine the time difference between siRNA and PTX administration for maximum cytotoxicity, wild-type SKOV3 and *MDR-1* positive $SKOV_{TR}$ cells were first treated with siRNA in PEO-PbAE nanoparticles and then with PTX after 0, 2, 4, 6, 8, 12, 24, and 48 h post-siRNA administration. In each case, 1.0 μM final concentration of PTX in aqueous solution or in PEO-PCL nanoparticles was added to each of the wells in a 96-well microplate with 5,000 confluent cells. After 5 days of incubation, the cell culture medium was replaced with the mixture of MTS reagent and the absorbance at 490 nm was measured as described above. Poly(ethyleneimine) (MW 10,000 Da), a cationic cytotoxic linear polymer at 250 μg/mL, was used as a positive control.

siRNA and PTX combination therapy

Finally, to evaluate the effect of *MDR-1* silencing siRNA and PTX administration on the viability of wild-type SKOV3 and *MDR-1* positive $SKOV3_{TR}$ cells, siRNA encapsulated in PEO-PbAE nanoparticles was administered at 100 nM dose. After 24 h, PTX in PEO-PCL nanoparticle was administered and the effect of *MDR-1* silencing was evaluated using MTS assay.

Cellular apoptosis studies

Quantitative apoptosis studies

The Vybrant® apoptosis assay kit #4, containing YO-PRO®-1 and propidium iodide, was used to analyze the apoptotic activity by flow cytometry. The wild-type

SKOV3 and *MDR-1* positive $SKOV3_{TR}$ cells were plated in six well plates at a cell density of 10×10^6 cells per well. The cells were treated with siRNA in PEO-PbAE nanoparticles at a dose of 100 nM and PTX either in aqueous solution or in PEO-PCL nanoparticles at a dose of 1.0 μM. After 12 h of incubation, the cells were harvested with Trypsin-EDTA treatment and washed with sterile PBS. The harvested cells were then suspended in 1.0 mL of PBS adjusting the cell density to 1×10^6 cells/mL. To 1.0 mL of the assay volume, 1.0 μL of the YO-PRO®-1 and 1.0 μL of the propidium iodide solution were added for staining the cells. The cells were then incubated over ice for 30 min. After the incubation, the cells were analyzed over the flow cytometer at an excitation of wavelength of 488 nm and two emission wavelengths; 530 nm for green fluorescence (YO-PRO®-1) and 610 nm for red fluorescence (propidium iodide).

Qualitative evaluation of apoptosis

The DeadEnd® colorimetric apoptosis detection system, purchased from Promega (Madison, WI), which labels fragmented DNA in situ, based on the TUNEL method, was used for qualitative analysis of apoptotic activity in SKOV3 and $SKOV3_{TR}$ cells following treatment with *MDR-1* silencing siRNA and PTX. Cells were plated in six well plates on a sterilized coverslips at the density of 300,000 per well. The cells in each well were dosed with siRNA (100 nM) in PEO-PbAE nanoparticles and after 24 h of silencing period, the cells were treated with PTX (1.0 μM) either in solution or in PEO-PCL nanoparticles for 4 h at 37 C. The coverslips were then washed twice with sterile PBS and were then transferred on to clean glass slides. Cells were fixed by immersing slides in 10% buffered formalin and 4% formaldehyde solution for 25 min at room temperature. The fixed cells were washed with PBS for 5 min and then permeabilized with 0.2% Triton® X100 for 5 min. The reagent from DeadEnd® colorimetric apoptosis detection system was added to the slides according to the manufacturer's instructions and they were mounted in the presence 100% glycerol and imaged with a bright-field microscope.

Data analysis

All of the results are reported as mean ± standard deviation and the differences between the control and test groups were tested using Student's *t* test. Sample size of at least four (4) was used for the analysis. Results were considered statistically significant between the control and test treatment at the level of $P < 0.05$.

Results and discussions

Preparation and characterization of nanoparticle formulations

Using an optimized formulation strategy, we were able to successfully prepare PEO-PbAE and PEO-PCL nanoparticles with encapsulated *MDR-1* gene silencing siRNA and PTX, respectively. PEO-PbAE and PEO-PCL nanoparticles had approximate hydrodynamic diameters of between 200 and 250 nm. Inclusion of *MDR-1* silencing siRNA increased the average particle size of PEO-PbAE by approximately 50 nm and the encapsulation of PTX increased the average particle size of PEO-PCL nanoparticles by approximately 30 nm over the blank nanoparticles. On the other hand, the surface charge (zeta potential) values of blank and siRNA-encapsulated nanoparticles was similar at around positive 9–12 mV, suggesting that the siRNA was not adsorbed on the nanoparticle surface. The positive surface charge is due to ionization of the tertiary amine groups of PbAE at the pH of deionized distilled water. If the siRNA molecules were adsorbed, we would expect the net charge of the particle to decrease substantially. Similarly, the surface charge values of blank and PTX-encapsulated PEO-PCL nanoparticles were also very similar suggesting that the drug was fully dispersed in the PCL matrix rather than migrating to the surface of the nanoparticles. SEM analysis in Fig. 1a shows that the PEO-PbAE nanoparticles were highly prone to aggregation, especially after freeze-drying. However, upon addition of these freeze-dried nanoparticles in aqueous medium for administration in cells, the particles were able to re-disperse well as the average hydrodynamic diameter was similar to the freshly-prepared samples. In contrast, the SEM image of PEO-PCL nanoparticles in Fig. 1b shows uniformly spherical particles with smooth surface morphology.

Both *MDR-1* silencing siRNA and PTX were efficiently loaded in PEO-PbAE and PEO-PCL nanoparticles, respectively. For siRNA, the maximum loading was observed for 51.2 μg per 10 mg of the PEO-PbAE nanoparticles, which represents 0.77 nM of siRNA. The maximum siRNA loading efficiency, therefore, was found to be 77%. From previous studies, PEO-PCL nanoparticles are known to efficiently encapsulate PTX at 10% (w/w) loading. In here, we observed that the loading capacity was 9.32 mg of PTX per 10 mg of PEO-PCL nanoparticles and the loading efficiency was 93.2%.

In vitro release studies were carried out at pH 7.4 to simulate the systemic circulation and pH 6.5 to simulate the tumor microenvironment. As shown in Fig. 1c *MDR-1* silencing siRNA release, approximately 40% of the encapsulated payload was released at pH 7.4, 37°C in the first 45 min. However, when the pH was reduced to 6.5 after

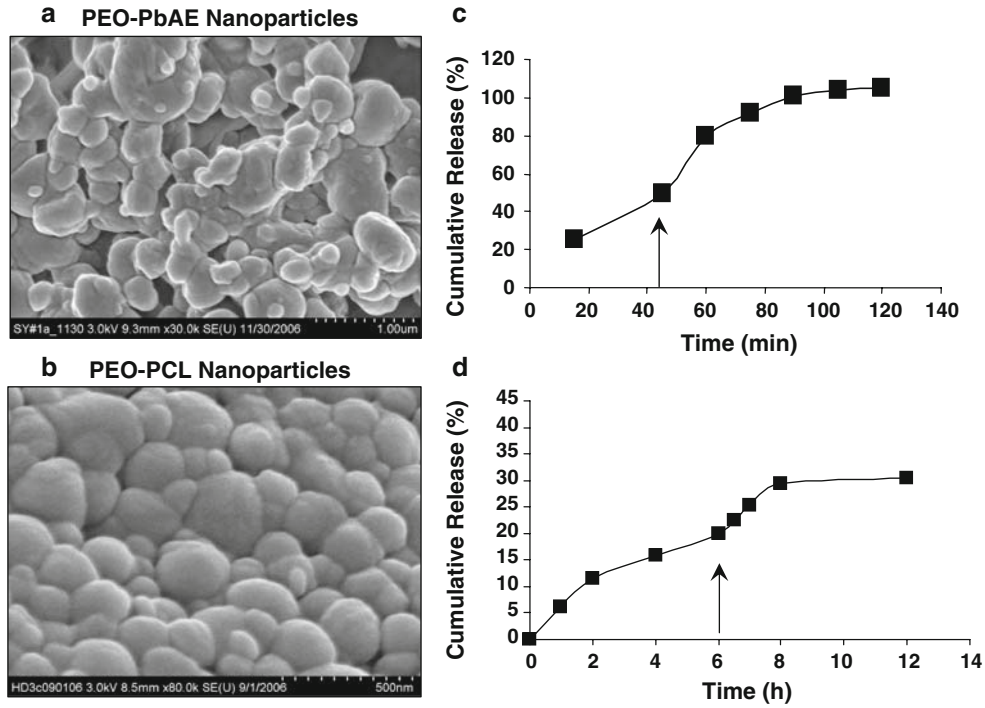

Fig. 1 **a** Scanning electron microscopy (SEM) images of poly(ethylene oxide)-modified poly(beta-amino ester) (PEO-PbAE) and **b** poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles. **c** In vitro release profiles of fluorescently-labeled small interfering RNA (siRNA) from PEO-PbAE nanoparticles and **d** paclitaxel from PEO-PCL nanoparticles at 37 C. The initial release studies for siRNA and paclitaxel was performed with phosphate-buffered saline (PBS, pH 7.4). The *arrows* indicate when the release medium was switched from PBS to pH 6.5 phosphate buffer to simulate the tumor microenvironment. Mean ± SD ($n = 4$)

45 min, all of the remaining siRNA was released rapidly within the next hour. In contrast, PTX release from PEO-PCL nanoparticles (Fig. 1d) was significantly slower with only 10% of the payload released within the first 2 h at pH 7.4, 37°C. Also, after 6 h, when the pH of the released medium was adjusted to 6.5, a relatively small increase in the release was observed for PTX from 22% before the change to about 30% after pH change. These results are consistent with PEO-PCL makeup, which is not responsive to pH differences. In addition, the slow release of PTX from PEO-PCL nanoparticles should also provide a more sustained intracellular delivery profile that could potentially be very useful in drug resistant cells.

MDR-1 gene silencing studies

In order to determine the optimum dosage of siRNA in $SKOV3_{TR}$ cells, we have used RT-PCR as a qualitative assay of *MDR-1* gene silencing efficacy. The siRNA was administered in aqueous solution or in PEO-PbAE nanoparticles at concentrations ranging from 10 to 100 nM. PCR analysis, as shown in Fig. 2, confirm that the wild-type SKOV3 cells did not express the *MDR-1* gene. In $SKOV3_{TR}$ cells, efficiency silencing of *MDR-1* mRNA was observed at 100 nM siRNA dose when administered in PEO-PbAE nanoparticles. The same dose of siRNA in aqueous solution was not effective. PEO-PbAE nanoparticle-mediated siRNA delivery may allow for greater stability and intracellular availability for efficient silencing relative to aqueous solution. For subsequent studies, we have utilized a fixed 100 nM siRNA dose administered in PEO-PbAE nanoparticles.

Intracellular PTX concentration measurements

To evaluate the effect of *MDR-1* gene silencing when delivered intracellularly with PEO-PbAE nanoparticles on PTX accumulation upon administration in aqueous solution or in PEO-PCL nanoparticles, radiolabeled [^{3}H]-PTX was used. The results in Fig. 3, show that although no significant effect in the PTX concentrations were observed with wild-type SKOV3 cells, a significant increase in intracellular PTX levels following *MDR-1* gene silencing in $SKOV3_{TR}$ cells, especially at lower PTX doses (i.e., 100 nM). A 100 nM fixed dose of siRNA was administered in PEO-PbAE nanoparticles. For instance, with PEO-PCL nanoparticles, the intracellular PTX concentration was 1.65 nM per mg of cellular protein when administered at 100 nM dose with siRNA to $SKOV3_{TR}$ cells. At higher PTX doses of 1.0 μM, there was a significant reduction in intracellular concentrations upon addition of siRNA in wild-type SKOV3 cells when PTX was administered in aqueous solution. This

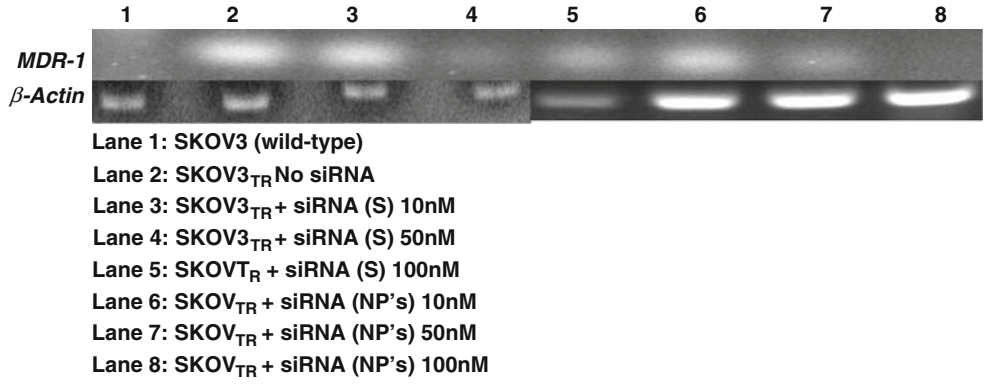

Fig. 2 Reverse transcriptase polymerase chain reaction (RT-PCR) transcription analysis of *MDR-1* gene expression in $SKOV3_{TR}$ multidrug resistant human ovarian adenocarcinoma cells upon treatment with small interfering RNA either in aqueous solution (S) or in poly(ethylene oxide)-modified poly(beta-amino ester) (PEO-PbAE) nanoparticles (NP). Beta-actin isolated from $SKOV3_{TR}$ cells served as a control

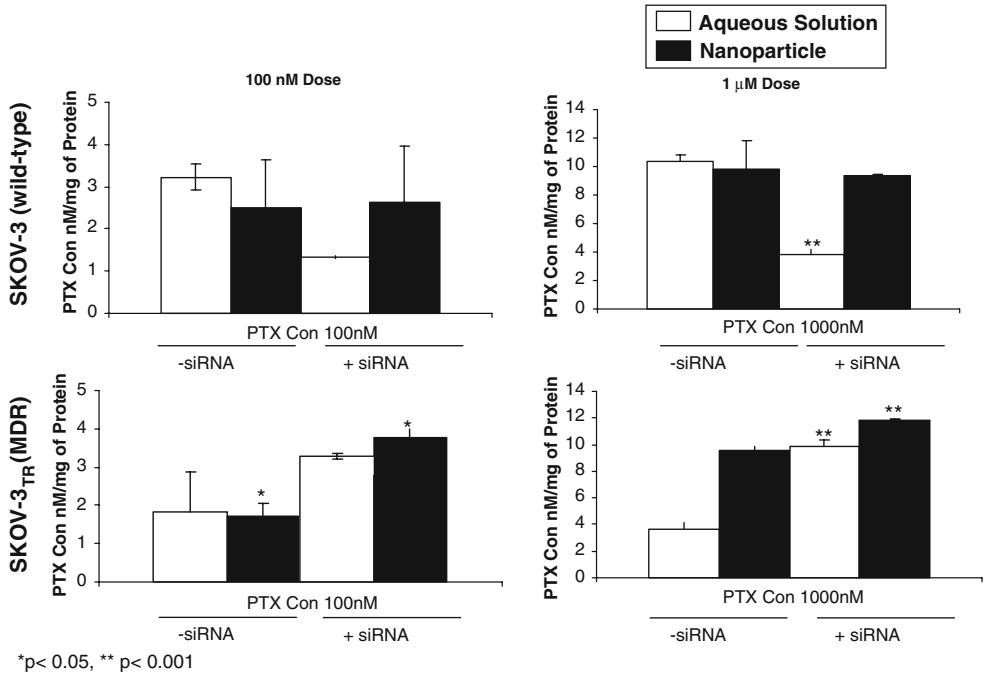

Fig. 3 Intracellular concentration of paclitaxel administered in aqueous solution or in poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles to SKOV3 wild-type (*top*) and $SKOV3_{TR}$ multidrug resistant (*bottom*) human ovarian adenocarcinoma cells. Paclitaxel concentrations were measured at two different doses (100 nM and 1.0 μM) using radioactive scintillation counting of the tritium-labeled drug. *MDR-1* gene silencing small interfering RNA was administered in poly(ethylene oxide)-modified poly(beta-amino ester) (PEO-PbAE) nanoparticles. Mean ± SD ($n = 4$)

effect was not observed with PTX administration in PEO-PCL nanoparticles. However, in $SKOV3_{TR}$ cells, *MDR-1* gene silencing again led to an increase in intracellular PTX concentrations with both aqueous solution and PEO-PCL nanoparticles as compared to the administration in the absence of silencing. For instance, intracellular PTX concentration increased from 8.2 nM per mg of cellular protein in the absence of siRNA to 11.5 nM/mg of cellular protein after gene silencing when administered in PEO-PCL nanoparticles.

Administration of *MDR-1* gene silencing siRNA with PEO-PbAE nanoparticles is shown to significantly influence PTX accumulation in $SKOV3_{TR}$ cells, especially when delivered in PEO-PCL nanoparticles. This result is consistent with prior reports demonstrating P-gp down-regulation upon silencing and decrease in the drug efflux from *MDR-1* positive cells [33]. However, we have previously demonstrated that intracellular PTX levels in $SKOV3_{TR}$ cells are enhanced with PEO-PCL nanoparticles even without *MDR-1* gene silencing, especially at higher doses [27].

This is due to the fact that nanoparticle-mediated delivery proceeds via endosomal uptake away from the membrane-bound P-gp efflux transporter molecules.

Cell viability studies

Given that our results indicate that *MDR-1* silencing siRNA does allow for greater accumulation of PTX in $SKOV3_{TR}$ cells, we decided to examine the effects of *MDR-1* silencing on the enhancement in cytotoxicity of PTX administered in aqueous solution or in PEO-PCL to $SKOV3_{TR}$ cells. In all of these studies, the wild-type (drug sensitive) SKOV3 cells were used as controls. In the first set of experiments, we wanted to examine the dose–response relationship of PTX administered in aqueous solution or in PEO-PCL nanoparticles in SKOV3 and $SKOV3_{TR}$ cells. As shown in Fig. 4, nanoparticle-mediated delivery significantly influences cytotoxicity in drug-sensitive SKOV3 cells, especially at lower doses (<1.0 μM). In $SKOV3_{TR}$ cells, on the other hand, there was significant reduction in the cytotoxicity of PTX when administered in both aqueous solution or in PEO-PCL nanoparticles. Also, the effect of PEO-PCL nanoparticle administration was not as profound in the $SKOV3_{TR}$ cells. It is reasonable to conclude that this decreased effect may be due to rapid P-gp-mediated efflux of the released drug from the cells.

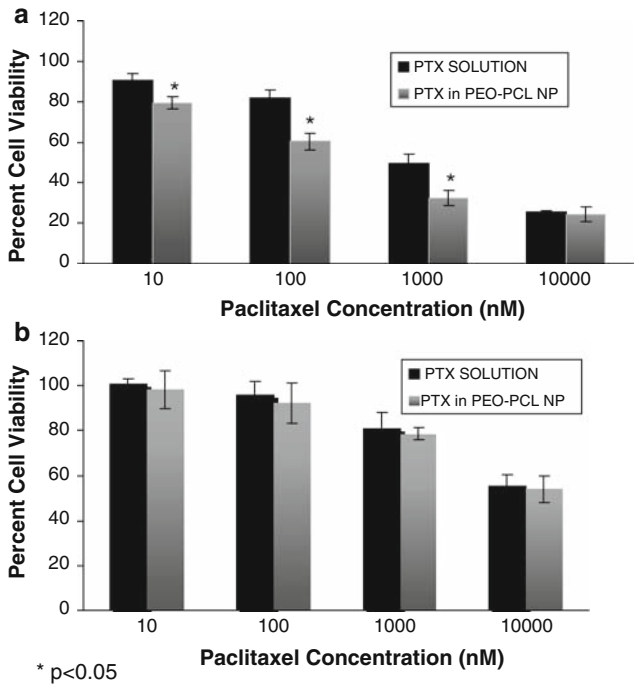

Fig. 4 Evaluation of paclitaxel cytotoxicity when administered either in aqueous solution or in poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles to (**a**) SKOV3 wild-type and (**b**) $SKOV3_{TR}$ multidrug resistant human ovarian adenocarcinoma cells. Mean ± SD ($n = 4$)

In a separate study, the effect of lag-time between *MDR-1* gene silencing with siRNA and PTX administration on the cytotoxicity of wild-type SKOV3 and resistant $SKOV3_{TR}$ cells was examined. A fixed 100 nM siRNA dose was administered in PEO-PbAE nanoparticles, while PTX was administered in PEO-PCL nanoparticles. Using 1.0 μM PTX dose, the effect of separation duration between siRNA and PTX varied from 1 to 48 h. The results, as shown in Fig. 5a, clearly show that there was no significant effect of siRNA administration in wild-type SKOV3 cells. However, a significant enhancement in cytotoxicity was observed in *MDR-1* positive $SKOV3_{TR}$ cells when PTX was administered after 24 h of *MDR-1* gene silencing (Fig. 5b). At 48 h, the effect seemed to reverse as greater percentage of the $SKOV3_{TR}$ cells survived. In all the studies that follow, we

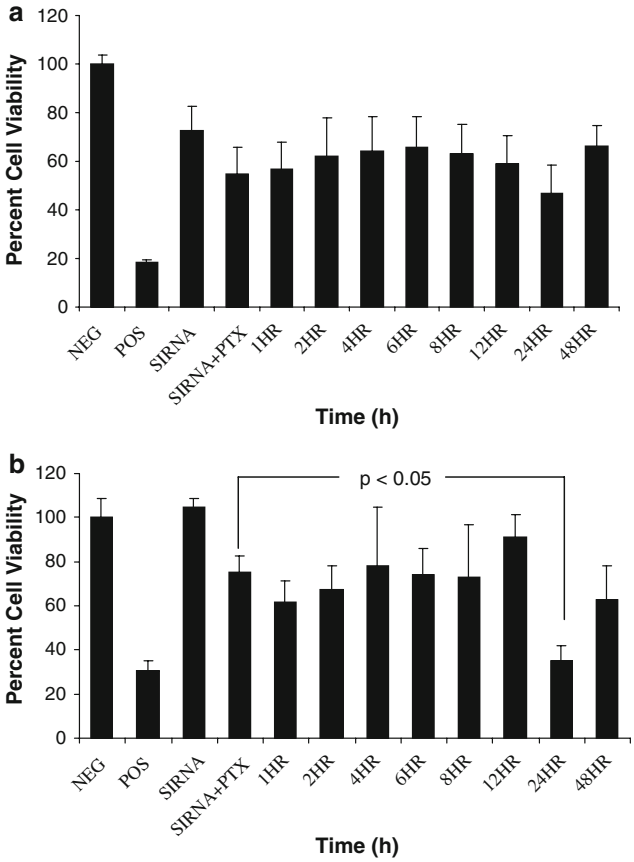

Fig. 5 The effect of time lag between administration of *MDR-1* gene silencing small interfering RNA (siRNA) and administration of paclitaxel on the cytotoxicity in (**a**) SKOV3 wild-type and (**b**) $SKOV3_{TR}$ multidrug resistant human ovarian adenocarcinoma cells. Paclitaxel was administered at 1.0 μM dose in poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles either simultaneously with *MDR-1* silencing (siRNA + PTX) or after a time lag of 1 h up to 48 h. *MDR-1* gene silencing siRNA was administered in poly(ethylene oxide)-modified poly(beta-amino ester) (PEO-PbAE) nanoparticles. NEG and POS represents negative (i.e. media alone) and positive (i.e. poly(ethyleneimine), a cytotoxic cationic polymer, at 250 μg/ml concentration) controls. Mean ± SD ($n = 4$)

have utilized a time lag of 24 h between siRNA treatment and PTX administration based on these observations.

Lastly, we have examined the cytotoxic effects of PTX administration in PEO-PCL nanoparticles with and without siRNA-mediated *MDR-1* gene silencing. The results in Fig. 6 show that there was no significant effect of MDR-1 silencing in wild-type SKOV3 cells. However, in $SKOV3_{TR}$ cells, there was significant enhancement in cytotoxicity of PTX when the cells were pre-treated with 100 nM fixed dose of *MDR-1* silencing siRNA in PEO-PbAE nanoparticles and PTX was administered 24 hours later either in aqueous solution or in PEO-PCL nanoparticles. The positive cell-kill effects of gene silencing and PTX administration in $SKOV3_{TR}$ cells were observed for all of the PTX doses tested ranging from 10 nM to 10 μM. In addition, at 10 and 100 nM PTX doses, co-administration of *MDR-1* silencing siRNA and PTX in SKOV3*TR* cells results in cell viability similar to what was observed in the wild-type SKOV3 cells at the same doses. These results clearly show that *MDR-1* gene silencing with siRNA can have a significant influence on enhancing the chemotherapeutic effects in drug resistant tumor cells.

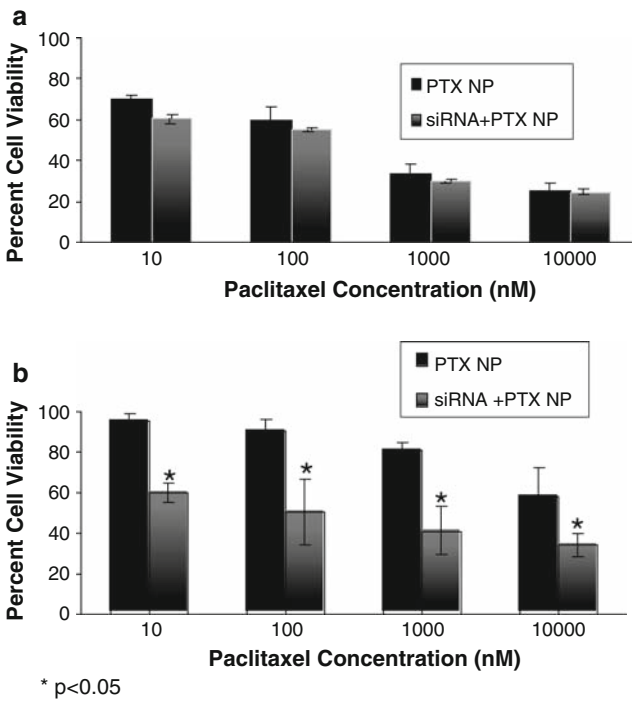

Fig. 6 Evaluation of cytotoxicity of paclitaxel administered with and without *MDR-1* gene silencing small interfering RNA (siRNA) to (**a**) SKOV3 wild-type and (**b**) $SKOV3_{TR}$ multidrug resistant human ovarian adenocarcinoma cells. Paclitaxel was administered in poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles, while siRNA was administered in poly(ethylene oxide)-modified poly(beta-amino ester) (PEO-PbAE) nanoparticles. Paclitaxel in PEO-PCL nanoparticles was administered 24 h after *MDR-1* gene silencing with siRNA administered in PEO-PbAE nanoparticles. Mean ± SD ($n = 4$)

Quantitative and qualitative cellular apoptosis

After microtubule stabilization, PTX is known to exert cytotoxicity by activating the programmed cell death (apoptotic) pathway mechanism and, as such, we have evaluated the enhancement in cellular apoptosis upon co-administration of *MDR-1* silencing siRNA and PTX in both wild-type SKOV3 and resistant $SKOV3_{TR}$ cells. A 100 nM fixed *MDR-1* silencing siRNA dose was administered in PEO-PbAE nanoparticles and PTX was administered either in solution or in PEO-PCL nanoparticles at doses of 100 nM or 1.0 μM.

Quantitative analysis of apoptotic activity in control and treated SKOV3 and $SKOV3_{TR}$ cells was carried out using the Vybrant® apoptosis kit for fluorescence analysis using flow cytometry. In this assay, the YO-PRO dye (green, apoptotic cell permeant) and propidium iodide (red, apoptotic cell impermeant) are used to label and differentiate between the live cells, those undergoing apoptosis, and those that are dead. The flow cytometry scatter plots, shown in Fig. 7, clearly differentiate the apoptotic activities in wild-type SKOV3 cells from the drug resistant $SKOV3_{TR}$ cells. In the case of 1.0 μM PTX dose administered either in aqueous solution or in PEO-PCL nanoparticles, there was no significant difference in the apoptotic activities of the wild-type SKOV3 cells regardless of the absence or presence of pre-treatment with 100 nM *MDR-1* administered in PEO-PbAE nanoparticles. Uniformly, we observed that approximately 77–83% of all wild-type SKOV3 cells were positive for apoptosis. On the other hand, the $SKOV3_{TR}$ cells behaved very differently and there was a marked effect of siRNA pre-treatment on the apoptotic activity in these cells. In the absence of siRNA pre-treatment, only 53 and 47% of the cells underwent apoptotic cell death upon addition of PTX at 1.0 μM dose in aqueous solution and PEO-PCL nanoparticles, respectively. However, upon treatment with *MDR-1* silencing siRNA, the percent apoptotic $SKOV3_{TR}$ cells increased to 60% when PTX was administered in aqueous solution and 77% when PTX was administered in PEO-PCL nanoparticle at 1.0 μM dose.

Additional evidence of enhanced apoptotic activity of combination siRNA-mediated *MDR-1* gene silencing and PTX administration was obtained qualitatively by observing DNA fragmentation in cells after TUNEL staining assay. Figure 8 shows TUNEL-stained bright-field microscopy images of wild-type SKOV3 and drug-resistant $SKOV3_{TR}$ cells upon treatment with 100 nM dose of siRNA in PEO-PbAE nanoparticles and PTX at 1.0 μM dose either in aqueous solution or in PEO-PCL nanoparticle. As anticipated, there was markedly little or no effect of siRNA administration in wild-type SKOV3 cells. However, it is clearly evident from these results that *MDR-1* gene silencing with siRNA administered in PEO-PbAE nanoparticles

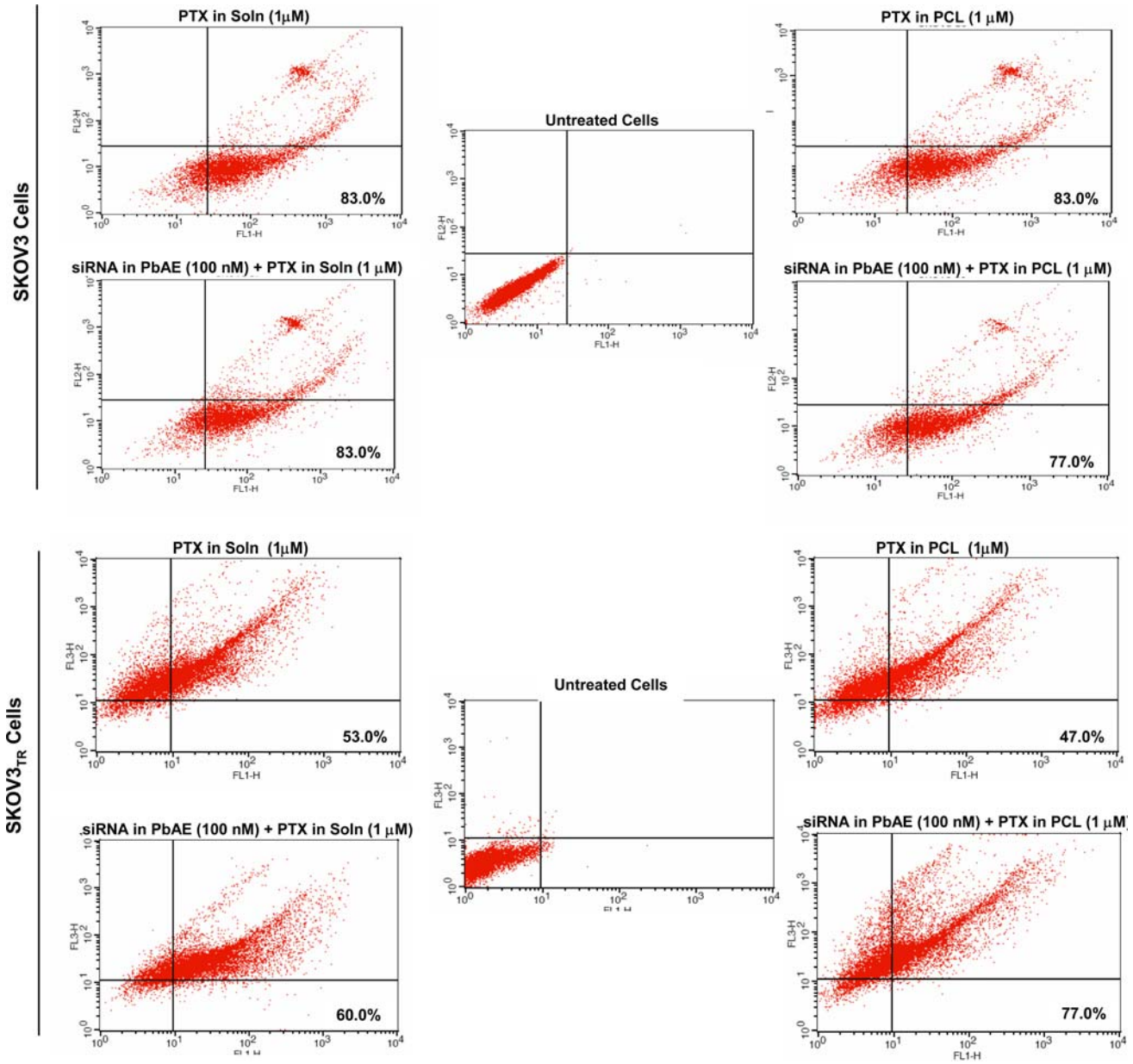

Fig. 7 Flow cytometric analysis for quantitative apoptotic activity in SKOV3 wild-type (*top*) and $SKOV3_{TR}$ multidrug resistant (*bottom*) human ovarian adenocarcinoma cells upon treatment with paclitaxel in solution, paclitaxel in poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles, combination of *MDR-1* gene silencing small interfering RNA (siRNA) encapsulated in poly(ethylene oxide)-modified poly(beta-amino ester) (PEO-PbAE) nanoparticles and paclitaxel in solution, and combination of *MDR-1* silencing siRNA encapsulated in PEO-PbAE nanoparticles and paclitaxel in PEO-PCL nanoparticles. *MDR-1* silencing siRNA and paclitaxel doses were maintained constant at 100 nM and 1.0 μM, respectively. Additionally, paclitaxel was administered 24 hours after *MDR-1* gene silencing

significantly enhances the apoptotic activity of PTX when administered in PEO-PCL nanoparticles in drug-resistant $SKOV3_{TR}$ cells.

Conclusions

In this study, we have examined combination of *MDR-1* gene silencing and PTX administration to overcome MDR in ovarian adenocarcinoma (SKOV3) cells. Using PEO-modified PbAE for siRNA encapsulation and PEO-modified PCL nanoparticles for PTX encapsulation, we have found that combination *MDR-1* silencing enhances PTX accumulation in the resistant cells. Additionally, the enhanced drug accumulation following *MDR-1* silencing was effective in improving the cytotoxic chemotherapeutic effect through induction of cellular apoptosis. Collectively, combination of *MDR-1* gene silencing and cytotoxic drug

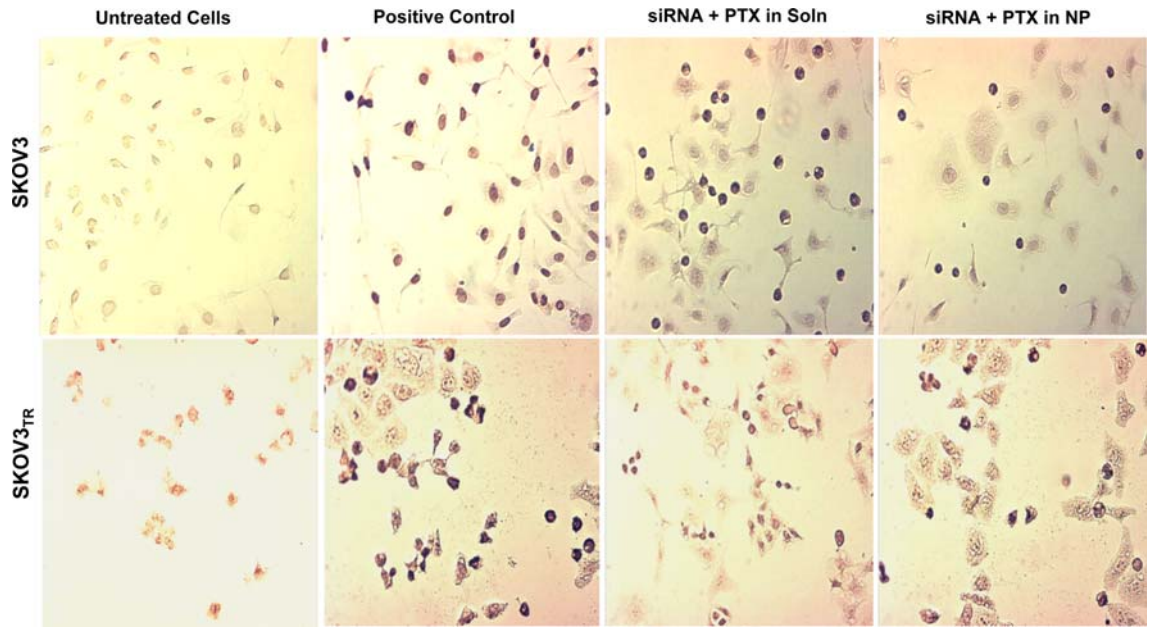

Fig. 8 Analysis of qualitative apoptotic activity in SKOV3 wild-type (*top*) and $SKOV3_{TR}$ multidrug resistant (*bottom*) human ovarian adenocarcinoma cells using the TUNEL assay. *MDR-1* gene silencing small interfering RNA (siRNA) was administered in poly(ethylene oxide)-modified poly(beta-amino ester) (PEO-PbAE) nanoparticles, while paclitaxel was administered in aqueous solution [siRNA + PTX(S)) or in poly(ethylene oxide)-modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles [siRNA + PTX(NP)). *MDR-1* silencing siRNA and paclitaxel doses were maintained constant at 100 nM and 1.0 μM, respectively. Additionally, paclitaxel was administered 24 h after *MDR-1* gene silencing. Positive control samples was prepared as described in the DeadEnd® TUNEL kit assay procedure

delivery using biodegradable and biocompatible polymeric nanoparticles that enhance intracellular availability may be a promising clinical strategy to overcome MDR in solid tumors.

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References

1. Bradley G, Juranka PF, Ling V (1988) Mechanism of multidrug resistance. Biochem Biophys Acta 948:87–128
2. Desoize B, Jardillier JC (2000) Multicellular resistance: a paradigm for clinical resistance? Crit Rev Oncol Hemat 36:193–207
3. Szakacs G et al (2006) Targeting multidrug resistance in cancer. Nat Rev Drug Discov 5:219–234
4. Yusuf RZ et al (2003) Paclitaxel resistance: molecular mechanisms and pharmacologic manipulation. Curr Cancer Drug Targets 3(1):1–19
5. Gottesman MM, Fojo T, Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2:48–58
6. Harris AL, Hochhauser D (1992) Mechanisms of multidrug resistance in cancer treatment. Acta Oncol 31(2):205–213
7. Di Nicolantonio F et al (2005) Cancer cell adaptation to chemotherapy. BMC Cancer 5:78
8. Ganasia-Leymarie V et al (2003) Signal transduction pathways of taxanes-induced apoptosis. Curr Med Chem Anti-Cancer Agents 3(4):291–306
9. Fire AZ (2007) Gene silencing by double-stranded RNA. Cell Death Differ 14(12):1998–2012
10. Mello CC, Conte D Jr (2004) Revealing the world of RNA interference. Nature 431(7006):338–342
11. Elbashir SM et al (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411(6836):494–498
12. Elbashir SM, Lendeckel W, Tuschl T (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 15(2):188–200
13. Faltus T et al (2004) Silencing of the HER2/neu gene by siRNA inhibits proliferation and induces apoptosis in HER2/neu-overexpressing breast cancer cells. Neoplasia 6(6):786–795
14. Oliveira S et al (2007) Photochemical internalization enhances silencing of epidermal growth factor receptor through improved endosomal escape of siRNA. Biochim Biophys Acta 1768(5):1211–1217
15. Schiffelers RM et al (2004) Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. Nucleic Acids Res 32(19):e149
16. Hua J, Mutch DG, Herzog TJ (2005) Stable suppression of MDR-1 gene using siRNA expression vector to reverse drug resistance in a human uterine sarcoma cell line. Gynecol Oncol 98(1):31–38
17. Logashenko EB et al (2004) Silencing of MDR 1 gene in cancer cells by siRNA. Nucleosides Nucleotides Nucleic Acids 23(6–7):861–866
18. Aagaard L, Rossi JJ (2007) RNAi therapeutics: principles, prospects and challenges. Adv Drug Deliv Rev 59(2–3):75–86

19. Akhtar S, Benter IF (2007) Nonviral delivery of synthetic siRNAs in vivo. J Clin Invest 117(12):3623–3632
20. van Vlerken LE, Vyas TK, Amiji MM (2007) Poly(ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery. Pharm Res 24(8):1405–1414
21. Maeda H et al (2000) Tumor vascular permeability and the EPR effect in macromolecular therapuetics: a review. J Control Rel 65(1–2):271–284
22. Monsky WL et al (1999) Augmentation of transvascular transport of macromolecules and nanoparticles in tumors using vascular endothelial growth factor. Cancer Res 59(16):4129–4135
23. Chawla JS, Amiji MM (2002) Biodegradable poly(epsilon-caprolactone) nanoparticles for tumor-targeted delivery of tamoxifen. Int J Pharm 249(1–2):127–138
24. Chawla JS, Amiji MM (2003) Cellular uptake and concentrations of tamoxifen upon administration in poly(epsilon-caprolactone) nanoparticles. AAPS Pharmsci 5(1):E3
25. Devalapally H et al (2007) Paclitaxel and ceramide co-administration in biodegradable polymeric nanoparticulate delivery system to overcome drug resistance in ovarian cancer. Int J Cancer 121:1830–1838
26. Shenoy DB, Amiji MM (2005) Poly(ethylene oxide)-modified poly(epsilon caprolactone) nanoparticles for targeted delivery of tamoxifen in breast cancer. Int J Pharm 293:261–270
27. van Vlerken LE et al (2007) Modulation of intracellular ceramide using polymeric nanoparticles to overcome multidrug resistance in cancer. Cancer Res 67(10):4843–4850
28. Zugates GT et al (2007) Gene delivery properties of end-modified poly(beta-amino ester)s. Bioconjug Chem 18(6):1887–1896
29. Anderson DG et al (2005) Structure/property studies of polymeric gene delivery using a library of poly(beta-amino esters). Mol Ther 11(3):426–434
30. Devalapally H et al (2006) Poly(ethylene oxide)-modified poly(beta-amino ester) nanoparticles as a pH-sensitive system for tumor-targeted delivery of hydrophobic drugs: part 3. Therapeutic efficacy and safety studies in ovarian cancer xenograft model. Cancer Chemother Pharmacol [Epub]
31. Shenoy D et al (2005) Poly(ethylene oxide)-modified poly(beta amino ester) nanoparticles as a pH sensitive system for tumor-targeted delivery of hydrophobic drugs: part 2. In vivo distribution and tumor localization studies. Pharm Res 22(12):2107–2114
32. Shenoy D et al (2005) Poly(ethylene oxide)-modified poly(beta amino ester) nanoparticles as a pH-sensitive system for tumor-targeted delivery of hydrophobic drugs. Part I. Invitro evaluations. Mol Pharmaceutics 2(5):357–366
33. Wu H, Hait WN, Yang JM (2003) Small interfering RNA-induced suppression of MDR1 (P-glycoprotein) restores sensitivity to multidrug-resistant cancer cells. Cancer Res 63(7):1515–1519
34. Dordunoo SK et al (1995) Taxol encapsulation in poly(epsilon-caprolactone) microspheres. Cancer Chemother Pharmacol 36(4):279–282
35. Duan Z et al (2005) Description of paclitaxel resistance-associated genes in ovarian and breast cancer cell lines. Cancer Chemo Pharm 55:277–285
36. Duan Z, Brakora KA, Seiden MV (2004) Inhibition of ABCB1 (MDR1) and ABCB4 (MDR3) expression by small interfering RNA and reversal of paclitaxel resistance in human ovarian cancer cells. Mol Cancer Ther 3(7):833–838