Modulation of Drug Resistance in Ovarian Adenocarcinoma by Enhancing Intracellular Ceramide Using Tamoxifen-Loaded Biodegradable Polymeric Nanoparticles

Harikrishna Devalapally,1 Zhenfeng Duan,2 Michael V. Seiden,2 and Mansoor M. Amiji1

Abstract

Purpose: To modulate intracellular ceramide levels and lower the apoptotic threshold in multidrug-resistant ovarian adenocarcinoma, we have examined the efficacy and preliminary safety of tamoxifen coadministration with paclitaxel in biodegradable poly(ethylene oxide)–modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles.

Experimental Design: In vitro cytotoxicity and proapoptotic activity of paclitaxel and tamoxifen, either as single agent or in combination, was examined in wild-type (SKOV3) and MDR-1–positive (SKOV3TR) human ovarian adenocarcinoma cells. Subcutaneous SKOV3 and SKOV3TR xenografts were established in female nu/nu mice, and this model was used to evaluate the antitumor efficacy and preliminary safety. Paclitaxel (20 mg/kg) and tamoxifen (70 mg/kg) were administered i.v. either as a single agent or in combination in aqueous solution and in PEO-PCL nanoparticles.

Results: In vitro cytotoxicity results showed that administration of paclitaxel and tamoxifen in combination lowered the IC50 of paclitaxel by 10-fold in SKOV3 cells and by >3-fold in SKOV3TR cells. The combination paclitaxel/tamoxifen co-therapy showed even more pronounced effect when administered in nanoparticle formulations. Upon i.v. administration of paclitaxel/tamoxifen combination in PEO-PCL nanoparticle formulations, significant enhancement in antitumor efficacy was observed. Furthermore, the combination paclitaxel/tamoxifen therapy did not induce any acute toxicity as measured by body weight changes, blood cell counts, and hepatotoxicity.

Conclusions: The results of this study show that combination of paclitaxel and tamoxifen in biodegradable PEO-PCL nanoparticles can serve as an effective clinically translatable strategy to overcome multidrug resistance in ovarian cancer.

Ovarian cancer is the most common gynecologic malignancy in women with more than 23,000 cases per year in the United States. Despite aggressive chemotherapy, the mortality rate of ovarian cancer remains relatively high (1). This is due to initial diagnosis of the disease at late stages, when there is significant dissemination in organs of the peritoneal cavity and failure of therapy due to intrinsic and acquired resistance development (2). Taxanes (e.g., paclitaxel) and platinum drugs (e.g., cisplatin and carboplatin) are the first-line choice for chemotherapy in ovarian cancer (3). Unfortunately, paclitaxel resistance is seen in >70% of patients at the time of initial diagnosis and almost all upon relapse (4). Acquisition of drug resistance to a multitude of chemotherapeutic drugs occurs due to poor availability of systemically administered drugs and phenotypic alterations in cancer cells due to microenvironmental selection pressures (5, 6).

The expression of MDR-1 gene in tumor multidrug resistance (MDR) leads to the presence of membrane-bound ATP-binding cassette family of transporters, including P-glycoprotein (7). Additional phenotypic alternations in MDR leads to enhanced DNA repair, rapid metabolism of drugs by cytochrome P-450 and glutathione S-transferase enzymes, and alteration in the apoptotic signaling cascade (8, 9). Recent studies have shown that intracellular ceramide, a secondary lipid messenger, levels in MDR cells are significantly altered due to inhibition of transport from the endoplasmic reticulum and overexpression of ceramide-metabolizing enzymes, such as glucosylceramide synthase (GCS; refs. 10, 11). Lower levels of intracellular ceramide and correspondingly higher levels of GCS have been shown in a variety of MDR models. Using different MDR-reversing agents, such as verapamil (a calcium channel blocker; ref. 12), quinidine and other antiarrhythmics (13), cyclosporine A (an immunosuppressive agent; ref. 14), and tamoxifen (the selective estrogen response modifier; ref. 12, 15), Cabot et al. have observed that GCS inhibition can be a very effective strategy to overcome tumor MDR (16, 17). Although there are a number of GCS inhibitors, including the three-1-phenyl-2-decanoylamino-3-morpholino-1-propanol and

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exogenous C6-ceramide administration using biodegradable studies, we have shown that combination of paclitaxel and type by lowering the tumor apoptotic threshold. In previous combination strategy that involves improvement in systemic the intracellular ceramide levels, is considered to be the most important strategy for MDR reversal (17).

We hypothesized that tumor MDR can be augmented with a combination strategy that involves improvement in systemic drug delivery efficiency and alterations in the cellular phenotype by lowering the tumor apoptotic threshold. In previous studies, we have shown that combination of paclitaxel and exogenous C6-ceramide administration using biodegradable polymeric nanoparticles can significantly enhance cytotoxicity and proapoptotic activity in vitro and in vivo in human tumor xenograft models (23, 24). Using poly(ethylene oxide)–modified poly(epsilon-caprolactone) (PEO-PCL) nanoparticles (100-300 nm in diameter), we observed preferential drug accumulation in tumors after i.v. administration due to the hyperpermeability of the microvasculature and lack of lymphatic drainage.

To further evaluate the role of intracellular ceramide modulation as an effective strategy to reverse tumor MDR, in the present study, we have examined in vitro cytotoxicity and proapoptotic activity, as well as in vivo efficacy and safety of paclitaxel and tamoxifen coadministration in PEO-PCL nanoparticles in wild-type (drug-sensitive) and MDR-1–expressing (drug-resistant) SKOV3 human ovarian adenocarcinoma models. Previous studies in our laboratory have shown that PEO-PCL nanoparticles can efficiently encapsulate hydrophobic anticancer therapeutics, including paclitaxel and tamoxifen (25, 26). When administered in MDA-MB-231 human breast tumor–bearing female nu/nu mice, >15% to 20% of the recovered radiolabeled nanoparticle dose was found to accumulate at the tumor site. Interestingly, the PEO-PCL nanoparticles afford longer residence of the drug at the tumor site due to slow diffusion-mediated and degradation-mediated release (27).

Materials and Methods

Preparation and characterization of PEO-PCL nanoparticles

PEO-PCL nanoparticles were prepared by the solvent displacement method as previously described (25, 27). Briefly, a solution of PCL (85 mg) and Pluronic F-108 (15 mg) was prepared in acetone and was introduced into a precooled (<15°C) purified water maintained under vigorous magnetic stirring. For preparation of drug-loaded nanoparticles, paclitaxel (10 mg) or tamoxifen (20 mg) was dissolved along with the polymeric blends of PCL-Pluronic FI08 in the organic phase before introduction into the aqueous medium. Pure paclitaxel and tamoxifen were purchased from ICN Biomedicals, and Taxol (i.e., paclitaxel in Cremophore EL-ethanol solution) was obtained from Bristol-Myers Squibb. After overnight stirring, to allow the organic solvent to evaporate, the nanoparticle suspension was centrifuged at 15,000 rpm for 10 min and the supernatant was discarded. The nanoparticle pellet was lyophilized, resuspended, and diluted suitably in deionized distilled water, appropriate buffer, or cell culture medium.

Each batch of the control (without any drugs) and drug-loaded PEO-PCL nanoparticles formed were characterized for particle size, size distribution, and surface charge. Particle size and surface charge (ζ potential) measurements were done with 90-Plus ZetaPALS instrument (Brookhaven Instruments). Scanning electron microscopy was used to determine the nanoparticle morphology and surface characteristics. Additionally, the drug loading was determined by dissolving a known amount of the nanoparticles in acetone and assaying for the amount of drug incorporated using previously published assay procedures.

In vitro cytotoxicity and apoptosis studies

Cell culture. Human hypodiploid ovarian adenocarcinoma cell line, SKOV3, sensitive cells were procured from American Type Culture Collection, and MDR-1–positive phenotype SKOV3MDR cells were kindly supplied from Dr. Michael Seiden’s laboratory at the Massachusetts General Hospital. Both types of cells were grown in RPMI 1640 supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin combination. The SKOV3MDR cells have been fully characterized and are known to overexpress MDR-1 gene (ABCD-1) and GCS (28, 29). Cells were cultured in a humidified atmosphere of 95% of air and 5% CO2 at 37°C. For the subculture, cells growing as monolayer were detached from the tissue flasks by treatment with 0.05% trypsin/EDTA. The viability and cell count were monitored routinely using trypan blue dye exclusion method. The cells were harvested during the logarithmic growth phase and resuspended in serum-free medium before incubations and formulations.

Cytotoxicity studies. Approximately 3,000 cells per well were seeded into 96-well plates and allowed to adhere overnight. The growth medium was replaced with serum-free medium and the solutions containing graded concentrations of paclitaxel and tamoxifen, either alone or in combination, in aqueous solution (i.e., 0.1% DMSO in water) and in PEO-PCL nanoparticles. Eight wells were used for each experimental condition by varying the drug concentrations, and the incubation period was kept constant for 5 d. After this, the medium was replaced with a mixture of RPMI 1640 and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), which was kept for 4 h. MTS solutions were prepared according to the manufacturer’s instructions. Stock phenazine methosulfate (electron coupling reagent; Sigma) was dissolved in PBS (pH 7.4) at a concentration of 0.92 mg/ml. DBPS (0.92 mg/ml phenazine methosulfate in DBPS is also included with the CellTiter 96 AQueous, Assay System from Promega). The solutions were then stored in light-protected tubes at -20°C. MTS and phenazine methosulfate detection reagents were mixed, using a ratio of 20:1 (MTS/phenazine methosulfate), immediately before addition to the cell culture at a ratio of 1:5 (detection reagents/cell culture medium). Viable cells converted the MTS into soluble formazan product, which has an optical absorbance at 490 nm. The absorbance of control and treated cells was measured using a microplate reader (Synergy HT, Bio-Tek Instruments), and the percentage cell viability values were calculated relative to untreated (0.1% DMSO) cells as a negative control and 0.2 mg/mL poly(ethyleneimine) (molecular weight, 10 kDa), a known cytotoxic cationic polymer, as a positive control.

Paclitaxel and tamoxifen combination index (CI) was determined with the classic isobologram equation of Chou and Talalay (30, 31). CI = a / A + b / B, wherein a is the paclitaxel IC50 (concentration resulting in 50% cell death) in combination with tamoxifen at concentration b, A is the paclitaxel IC50 without tamoxifen, and B is the tamoxifen IC50 in the absence of paclitaxel. According to this formula, when CI < 1, the interaction is synergistic; when CI = 1, the interaction is additive; and when CI > 1, the two agents are antagonistic.

Quantitative and qualitative apoptosis studies. For the determination of enhanced apoptotic activity upon coadministration of paclitaxel and tamoxifen in solution or in PEO-PCL nanoparticles, caspase-3/caspase-7 activation and the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were used as quantitative and qualitative indicators of apoptosis, respectively.
Caspase-3 and caspase-7 enzyme activities were detected after 6-h incubation with the Apo-ONE homogenous caspase-3/caspase-7 assay (Promega). This assay uses a profluorescent substrate that is selectively cleaved by caspase-3 and caspase-7. At each time point, fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm on Synergy HT plate reader. Blank values were subtracted and fold increase in activity was calculated based on the activity measured from untreated cells.

For the TUNEL assay, cells were then fixed with 4% paraformaldehyde in PBS for 25 min and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After washing with PBS, the coverslips were incubated with biotinylated nucleotide mixture with terminal deoxynucleotidyl transferase enzyme; incorporated nucleotides were detected using diaminobenzidine and hydrogen peroxide and developed until there was a light brown background. Apoptotic nuclei were stained dark brown.

In vivo antitumor efficacy studies

Animal model. The experimental protocol involving use of animals for this research was approved by the Institutional Animal Care and Use Committee of Northeastern University. Female athymic mice (nu/nu strain), 4 to 6 wk old, weighing ~25 g, were purchased from Charles River Laboratories and were housed under controlled laboratory conditions in polycarbonate cages, having free access to sterilized rodent pellet diet and acidified drinking water. The animals were allowed to acclimate for at least 48 h before any experiments.

Tumor inoculation and drug administration protocols. Approximately 4 and 7 million SKOV3 or SKOV3TR cells, respectively, suspended in 100 µL of serum-free medium were implanted s.c. on the flanks of mice under light isoflurane anesthesia. Palpable solid tumors developed within 10 to 15 d posttumor cell inoculation, and as soon as tumor volume reached ~130 mm³, the animals were randomly allotted to seven different control and treatment groups (i.e., vehicle-treated control, paclitaxel in aqueous solution, tamoxifen in aqueous solution, combination of paclitaxel and tamoxifen in solution, paclitaxel in PEO-PCL nanoparticles, tamoxifen in PEO-PCL nanoparticles, and combination of paclitaxel and tamoxifen in PEO-PCL nanoparticles). An aqueous solution of paclitaxel was prepared by diluting the drug stock solution in Cremophore EL/ethanol (1:1) mixture with normal saline.

Paclitaxel and tamoxifen, either as single agent or in combination, were administered i.v. through the tail vein in aqueous solution or in PEO-PCL nanoparticles to lightly (isoflurane)-anesthetized tumor-bearing animals. A total of two doses of each agent were administered on day 1 and day 24. Paclitaxel was given at a dose of 20 mg/kg, and tamoxifen was given at a dose of 70 mg/kg in 100-µL volumes for single agents. For the combination therapy, the paclitaxel and tamoxifen aqueous solutions and nanoparticle formulations were mixed before administration and were injected together in 200-µL volumes.

Evaluation of tumor growth suppression. The tumor diameters were measured twice weekly with a Vernier caliper in two dimensions. Individual tumor volumes (V) were calculated using the formula: 

\[ V = \left( \frac{L \times W^2}{2} \right) \]

wherein length (L) is the longest diameter and width (W) is the shortest diameter perpendicular to length. Growth curves for groups of tumors are presented as the mean volume relative to the values on the first day of the treatment. The time taken for the tumor volume to triple was determined. The difference between mean values of this variable for individual tumors in the control and treatment groups was examined using Student’s t test. The data were considered to be statistically significant if the P value of <0.05 (i.e., 95% confidence interval) was considered to be statistically significant.

Evaluation of in vivo GCS levels in tumor tissues. We studied GCS expression in the tumor tissue cryosections by immunohistochemical staining. Tumor cryosections (10 µm thick) were deparaffinized in ice-cold acetone and hydrated in PBS (pH 7.4). After blocking endogenous peroxidase and nonspecific antibody binding, mouse anti-human GCS monoclonal antibody was diluted at a ratio of 1:100 in 1% bovine albumin–containing PBS and the tumor sections were incubated for 1 h at room temperature. A secondary biotinylated antimouse antibody (dilution 1:200) was added, followed by dianminobenzidine as a chromogen. The sections were lightly counterstained with hematoxylin and examined under light microscopy at >20 magnification. GCS expression was considered positive if >25% of the cells in representative paraffin sections showed positive immunostaining.

Evaluation of in vivo apoptotic activity. Enhancement in apoptosis upon coadministration of paclitaxel and tamoxifen in solution and in PEO-PCL nanoparticle formulations was confirmed by histologic analysis of the tumor tissue cryosections by the TUNEL assay. The TUNEL reaction was done using the DeadEnd Colorimetric Apoptosis Detection System (Promega) according to the protocol described by the supplier. Random tumor cryosections were selected from each sample, and the sections were fixed with 4% (w/v) paraformaldehyde/PBS for 15 min and treated with 20 µg/mL proteinase K for 10 min at room temperature. Subsequently, the sections were postfixed with 4% (w/v) paraformaldehyde/PBS for 5 min. The terminal deoxynucleotidyl transferase reaction was done at 37°C for 60 min. Biotinylated nucleotide is incorporated at the 3′-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase. Endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide/PBS for 10 min at room temperature. Horseradish peroxidase–labeled streptavidin is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate hydrogen peroxide and the stable chromogen diaminobenzidine. Diaminobenzidine staining was done for 30 min at room temperature in the dark, resulting in an insoluble brown-colored substrate at the site of DNA fragmentation.

Preliminary in vivo safety studies

Preliminary evaluations of safety with the paclitaxel and tamoxifen single and combination therapy was assessed by measurements of body weight changes, WBC and platelet counts, liver enzyme levels, and liver tissue histology. Each mouse was weighed every alternate day, and the body weight was reported as percentage change upon normalization to the weight at the start of therapy. WBC and platelet counts were measured using a hemocytometer slide at the time of sacrifice. Additionally, alanine aminotransferase and aspartate aminotransferase levels in the serum at the time of sacrifice were measured using an ELISA. Last, the liver tissue was cryosectioned and stained with H&E for tissue histologic evaluations.

Data analysis

Data sets were analyzed using a commercially available software package (InStat v2.03, GraphPad Software, Inc.). A Student’s t test was used to determine the validity of the differences between the control and treatment data sets. The P value of <0.05 (i.e., 95% confidence interval) was considered to be statistically significant.

Results

Preparation and characterization of nanoparticle formulations. Spherical PEO-PCL nanoparticles having a smooth surface and distinct boundary were reproducibly obtained by the solvent displacement method (Fig. 1). PEO-PCL nanoparticles were internalized by SKOV3 cells by nonspecific endocytosis and can efficiently deliver the encapsulated payload. The blank particles obtained were in the range of 195 to 225 nm with a mean diameter of 200 nm, and the payload. The blank particles obtained were in the range of 195 to 225 nm with a mean diameter of 200 nm, and the surface charge remained at -25.1 mV and -42.6 mV, respectively. Lack of change in particle size and
surface charge is indicative that the drug was distributed in the polymer matrix rather than adsorbed to the surface of the nanoparticle. Tamoxifen nanoparticles were obtained in the size range of 201 nm and surface charge about +22.7 mV. The positive surface charge with tamoxifen-loaded nanoparticles could be due to surface localization of some of the drug. When paclitaxel was added to the polymer solution in acetone at 10% (w/w) and tamoxifen at 20% (w/w) due to the polymer hydrophobicity, >99.5% of both added drugs were encapsulated in the PEO-PCL nanoparticles.

In vitro drug sensitivity assessments. We have recently reported that MDR subculture SKOV3TR cells overexpress GCS and the classic MDR marker P-glycoprotein, in contrast to the drug-sensitive SKOV3 cells. Furthermore, dose-response studies against paclitaxel revealed that the SKOV3TR line is at least 300-fold more resistant to paclitaxel than its drug-sensitive counterpart as seen by the right shift in dose-response curve (Fig. 2A). The experimental $IC_{50}$ for the SKOV3 cells was set at 9.89 nmol/L whereas the $IC_{50}$ for the MDR subculture (SKOV3TR) was set at ~300-fold higher at 3.0 μmol/L. Additionally, for tamoxifen, the differences in the $IC_{50}$ values against both cell lines were ~2-fold different (i.e., 11.9 and 19.2 μmol/L). Concomitantly, with administration of PEO-modified PCL-paclitaxel nanoparticle formulation, $IC_{50}$ value differences decreased even further by ~100-fold (1.53 nmol/L and 1.62 μmol/L), and with PCL-tamoxifen nanoparticle formulation, there was no change in the $IC_{50}$ value difference (4.96 and 10.82 μmol/L). Upon treatment with nanoparticle formulations, the $IC_{50}$ was reduced ~10-fold and ~2-fold in SKOV3 and SKOV3TR cells, respectively, compared with solution, whereas with tamoxifen nanoparticle formulation, the difference was ~2-fold in both the cell lines. To verify the therapeutic potential of tamoxifen in sensitizing the MDR cells to paclitaxel, we carried out the combination study. The results revealed that, in the presence of tamoxifen, the $IC_{50}$ of paclitaxel was reduced to ~3-fold in SKOV3TR cells. Overall, the use of paclitaxel and tamoxifen together resulted in synergy with the CI values of <1 (i.e., CI, 0.4 and 0.7) in SKOV3 and SKOV3TR cells, respectively.

Figure 2B shows the relative caspase-3/caspase-7 activities upon treatment with single and combination paclitaxel and tamoxifen therapy in aqueous solution and PEO-PCL nanoparticles. As compared with paclitaxel + tamoxifen in aqueous solution, PEO-PCL nanoparticle–mediated delivery of combination therapy significantly increased caspase-3/caspase-7 activities in SKOV3 and SKOV3TR cells. Additionally, qualitative analysis of cellular apoptosis after single and combination therapy was evaluated using TUNEL assay. As shown in Fig. 2C, the positively stained cells seemed to have dark brown nuclei.

In vivo antitumor efficacy studies of single and combination therapy. The results of tumor volume changes as a function of time with paclitaxel and tamoxifen administration, either as single agents or in combination in aqueous solution and in PEO-PCL nanoparticle formulations, are shown in Fig. 3A. In both SKOV3 and SKOV3TR models, the enhanced efficacy of paclitaxel and tamoxifen combination when administered in nanoparticle formulations was clearly observed. In SKOV3-sensitive model, the average tumor volumes at day 24 after initiation of therapy were as follows: 2,497 mm$^3$ for untreated control, 1,575 mm$^3$ for paclitaxel in aqueous solution, 2,066 mm$^3$ for tamoxifen in aqueous solution, 892 mm$^3$ for the combination of paclitaxel and tamoxifen in solution, 1,294 mm$^3$ for paclitaxel in PEO-PCL nanoparticles, 1,921 mm$^3$ for tamoxifen in PEO-PCL nanoparticles, and 460 mm$^3$ for the combination of paclitaxel and tamoxifen in PEO-PCL nanoparticles. It is important to note that there was no significant difference between tamoxifen in aqueous solution and tamoxifen in PEO-PCL nanoparticles. In SKOV3TR drug-resistant model, the effect of paclitaxel and tamoxifen administration in PEO-PCL nanoparticles was even more pronounced compared with SKOV3 model. On day 28 posttherapy, the average volumes in SKOV3TR tumor-bearing animals were as follows: 1,004 mm$^3$ for untreated control, 919 mm$^3$ for paclitaxel in aqueous solution, 804 mm$^3$ for tamoxifen in aqueous solution, 437 mm$^3$ for combination of paclitaxel and tamoxifen in solution, 826 mm$^3$ for paclitaxel in PEO-PCL nanoparticles, 752 mm$^3$ for tamoxifen in PEO-PCL nanoparticles, and 336 mm$^3$ for the combination of paclitaxel and tamoxifen in PEO-PCL nanoparticles. In the SKOV3TR model, at day 26, the effect of tamoxifen in PEO-PCL nanoparticles was significantly greater than that of paclitaxel in PEO-PCL nanoparticles. In both models, after administration of second dose, significant tumor growth suppression was observed compared with solution treatment with combination therapy.

The pharmacodynamic variables of antitumor response to therapy were determined by measuring the growth delay and...
the volume doubling times (Table 1). When administered as single agents, paclitaxel and tamoxifen aqueous solution–treated animals showed a growth delay of 6.0 and 4.1 days, respectively, in SKOV3 model. When treated with PEO-PCL nanoparticles, the growth delay was 6.8 and 4.3 days for paclitaxel and tamoxifen, respectively. Combination of paclitaxel + tamoxifen in solution and nanoparticles had a growth delay of 7.9 and 18.1 days, respectively. The tumor volume doubling time for paclitaxel and tamoxifen as single agent in aqueous solution in SKOV3 model was 9.1 and 7.1 days, respectively. When paclitaxel and tamoxifen were administered in PEO-PCL nanoparticles, the volume doubling time increased to 9.4 and 7.3 days, respectively. Most impressive was the tumor volume doubling time of 14.1 days when paclitaxel + tamoxifen were administered in nanoparticle formulations. A similar trend was also observed with the SKOV3TR model. The tumor growth delay time with paclitaxel and tamoxifen as single agents in aqueous solution was 11.0 and 13.0 days, whereas with nanoparticles it changed to 13.3 and 13.9 days, respectively. The tumor volume doubling time with paclitaxel and tamoxifen in aqueous solution was not significantly different from the control (9.0 days for paclitaxel and tamoxifen compared with 8.1 days for untreated control).

However, treatment of SKOV3TR model with paclitaxel and tamoxifen in PEO-PCL nanoparticles increased the tumor growth delay time to 20.3 and 49.0 days, respectively. In contrast, the combination of paclitaxel and tamoxifen in aqueous solution and nanoparticles had the volume doubling time of 26.3 and 32.9 days, respectively.

On the day of sacrifice, the tumor mass was excised and imaged. The weights of excised tumor mass from the SKOV-3 and SKOV-3TR models after treatment with paclitaxel and tamoxifen as single agents and in combination are shown in Fig. 3B. The untreated tumor from SKOV3 model weighed 1.20 g, whereas the animals treated with paclitaxel and tamoxifen in aqueous solution had tumor weights of 0.97 and 1.40 g, respectively. When the combination of paclitaxel and tamoxifen was administered in aqueous solution, the tumor weight was 0.55 g. Administration of paclitaxel and tamoxifen in PEO-PCL nanoparticles decreased the weight of tumor to 0.71 and 1.36 g, respectively. However, the least weight of tumor of 0.33 g was observed when paclitaxel and tamoxifen were administered in combination in PEO-PCL nanoparticles. Similar results were also obtained with the tumor weights from sacrificed animals bearing SKOV3TR xenograft. The control tumor weight at the time of sacrifice was 1.06 g, whereas those

![Graphs showing in vitro cytotoxicity and apoptosis with single and combination nanoparticle–delivered therapy.](https://www.aacrjournals.org/doi/abs/10.1158/1078-0432.CCR-07-3434)

**Fig. 2.** In vitro cytotoxicity and apoptosis with single and combination nanoparticle–delivered therapy. A, percentage cell viability upon administration of paclitaxel and tamoxifen, either alone or in combination, in solution and in PEO-PCL nanoparticles (NP) to enhance cytotoxicity in SKOV3 and SKOV3TR cells. B, activation of caspase-3/caspase-7 upon treatment with single and combination therapy. C, the micrographs at ×20 magnification of SKOV3 and SKOV3TR cells showing TUNEL staining of apoptotic nuclei upon treatment with paclitaxel and tamoxifen, either alone or in combination, in aqueous solution and in PEO-PCL nanoparticle formulations.
animals treated with paclitaxel and tamoxifen in aqueous solution had tumor weight of 0.94 and 0.82 g, respectively. The combination of paclitaxel and tamoxifen in aqueous solution resulted in the tumor weight of 0.40 g. When paclitaxel and tamoxifen were administered in PEO-PCL nanoparticles as single agents, the weights were 0.82 and 0.80 g, respectively, whereas combination of paclitaxel and tamoxifen in PEO-PCL nanoparticles resulted in tumor weight of only 0.21 g. In addition, after sacrifice, the tumor mass was excised and imaged as well.

We further investigated whether the elevated levels of GCS expression correlates with the drug resistance and tumor growth suppression as a result of combination therapy. Figure 3C shows that marked brown staining indicates elevated expression of GCS, which is characteristic of the drug-resistant phenotype. Qualitative analysis of GCS expression using tissue immunohistology confirmed that the levels were significantly reduced in paclitaxel and tamoxifen nanoparticle–treated animals. To further evaluate that the tumor suppression observed with combination of paclitaxel and tamoxifen therapy was due to enhancement in apoptotic activity, the TUNEL assay was done on tumor sections. As shown in Fig. 3D, the positively stained tumor tissue sections appeared dark brown. In contrast, in tumor sections from animals receiving single-agent therapy or in aqueous solution, the level of apoptosis was not as pronounced.

Preliminary evaluations of safety with combination therapy. Acute safety profiles of paclitaxel and tamoxifen coadministration in aqueous solution and in the PEO-PCL nanoparticles formulations was evaluated by measuring the changes in body weight as a function of time and the blood cell counts at the time of sacrifice. As shown in Fig. 4A, in the SKOV3 group, there was no significant difference in the body weight of tumor-bearing mice after the second dose treatment with nanoparticle formulations in contrast to solution treatment. For instance, in paclitaxel and tamoxifen combination in aqueous solution–treated animals, the change in body weight was 2.0 g compared with formulation treatments. In the SKOV3TR group, there was a significant decrease in body weight of control (untreated) tumor-bearing animals from 29.0 g on day 1 to 26.5 g on day 24 posttherapy. Administration of paclitaxel and tamoxifen combination in aqueous solution or in nanoparticle formulation did not affect the body weights of tumor-bearing animals. However, i.v. administration of the same dose of paclitaxel and
tumorexifen or combination or the two treatments in the Cremophor EL-ethanol (1:1) solution resulted in development of immediate ataxia, decreased activity, and enhanced respiration, which were reversed after 5 minutes.

Additionally, we have examined the safety profile of paclitaxel and tamoxifen combination therapy to overcome drug resistance in solid tumor by measuring the blood cell counts 2 days before sacrificing the animals in each group. The results in Fig. 4B showed that there was a significant increase in WBC count in control (untreated) tumor-bearing animals compared with the baseline cell counts in normal animals that did not have tumor xenografts. However, upon treatment with paclitaxel and tamoxifen, either alone or in combination, in aqueous solution and in PEO-PCL nanoparticle formulations, the WBC count returned to baseline levels. Similar increase in WBC count between normal and untreated tumor-bearing animals was observed with SKOV3TR model as well. However, in this case, the WBC counts remained relatively higher compared with normal levels after treatment. There was no significant difference in the WBC counts with respect to each of the treatment approaches. Platelet counts were also used as indicator of hematologic effect after treatment with paclitaxel and tamoxifen, either alone or in combination, in aqueous solution and nanoparticle formulations (Fig. 4C). A similar trend was observed in both models upon administration of paclitaxel and/or tamoxifen administration either in solution or formulation treatment.

Liver toxicity profiles. From a previous work by our group, it is known that these PEO-PCL nanoparticles, loaded with paclitaxel, exhibit nonspecific accumulation mostly within the liver, a common site of therapeutic toxicity. To evaluate potential for toxicity in the liver after single and combination therapy, we have examined alanine aminotransferase and aspartate aminotransferase levels in plasma within the liver, a common site of therapeutic toxicity. To evaluate potential for toxicity in the liver after single and combination therapy, we have examined alanine aminotransferase and aspartate aminotransferase levels in plasma at the time of sacrifice. As shown in Fig. 5A and B, there was no significant difference in the enzyme levels, either with solution or nanoparticle treatments in both models. In addition, the liver tissue histology was done to determine cellular infiltration due to inflammatory response. There was no evidence of acute liver toxicity from either single or combination therapy using aqueous solution and PEO-PCL nanoparticles (Fig. 5C).

Discussion

One of the major obstacles that account for limited progress made in cancer chemotherapy is acquired drug resistance. Cancers that respond to chemotherapeutics (e.g., ovarian, breast, and colon cancer) or antihormonal drugs (e.g., breast and prostate cancer) almost always relapse in a resistant form at a later stage (4). Paclitaxel is a frontline agent against ovarian cancer; however, the incidence of resistance to paclitaxel therapy occurs in ~70% of patients at onset and eventually in all of them with recurrent disease (3). Several mechanisms of MDR are responsible for the lack of therapeutic efficacy, with many of them ultimately leading to interruption in the apoptotic signal cascade. Intracellular sphingolipids and, in particular, ceramide have been shown to promote apoptosis in many different types of mammalian cells and in vivo (5). Enhancement of endogenous ceramide levels by GCS inhibitors (e.g., tamoxifen) has shown to augment proapoptotic activity of a number of anticancer agents, including paclitaxel (33).

Among various biodegradable polymers used in pharmaceutical products intended for systemic administration, PCL possesses unique properties, such as higher hydrophobicity and neutral biodegradation end products, which do not disturb the pH balance of the degradation medium (34, 35). In the present study, we have used Pluronic F108, an ABA triblock copolymer, having 56 residues of propylene oxide and 122 ethylene oxide residues for surface modification of the nanoparticles. When the two polymers are blended in organic solvent, the resulting solid nanoparticle is formed by an assembly where the central poly(propylene oxide) groups of Pluronic associate with hydrophobic PCL matrix, whereas the two hydrophilic PEO side arms in a mobile state extend outward from the particle surface in aqueous medium and provide stability to the particle suspension by a repulsion effect that involves both enthalpic and entropic contributions.

### Table 1. Tumor growth delay and volume doubling times after paclitaxel and tamoxifen treatment with the control and PEO-PCL nanoparticles in SKOV3 wild-type and SKOV3TR multidrug-resistant subcutaneous ovarian adenocarcinoma xenografts in female nu/nu mice

<table>
<thead>
<tr>
<th>Treatment option</th>
<th>SKOV3 (wild-type)</th>
<th>SKOV3TR (multidrug resistant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor growth delay time (d)</td>
<td>Tumor volume doubling time (d)</td>
</tr>
<tr>
<td>Untreated control</td>
<td>3.8 ± 0.4</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>PTX in aqueous solution</td>
<td>6.0 ± 0.5</td>
<td>9.1 ± 1.1*</td>
</tr>
<tr>
<td>TAM in aqueous solution</td>
<td>4.1 ± 0.5</td>
<td>7.1 ± 0.6</td>
</tr>
<tr>
<td>PTX + TAM in aqueous Solution</td>
<td>7.9 ± 0.9*</td>
<td>9.9 ± 0.8*</td>
</tr>
<tr>
<td>PTX in PEO-PCL nanoparticles</td>
<td>6.8 ± 0.8*</td>
<td>9.4 ± 0.9*</td>
</tr>
<tr>
<td>TAM in PEO-PCL nanoparticles</td>
<td>4.3 ± 0.5</td>
<td>7.3 ± 1.8</td>
</tr>
<tr>
<td>PTX + TAM in PEO-PCL nanoparticles</td>
<td>18.1 ± 3.6†</td>
<td>14.1 ± 0.9†</td>
</tr>
</tbody>
</table>

NOTE: The tumor growth delay and volume doubling times were calculated for each group of control and treated mice using the formula described in Materials and Methods.

Abbreviations: PTX, paclitaxel; TAM, tamoxifen.

*Statistical significance at $P < 0.05$ compared with control.

†Statistical significance at $P < 0.05$ compared with solution-treated animals.
The end result is a nanoparticulate system that has high capacity to encapsulate hydrophobic agents, is slower-eroding, and is less phagocyte prone (hence, long circulating) in the systemic circulation. Once accumulated within the tumor interstitium by exploiting vascular abnormalities that allows free access to the tumor mass, the PEO-PCL nanoparticle system would increase the drug concentration inside the tumor cells as a result of nonspecific endocytic process, followed by gradual release of the drug. When administered in vivo in MDA-MB-231 human breast tumor-bearing female nu/nu mice, for instance, paclitaxel levels in the plasma and in the tumor tissue after 5 hours postadministration were found to be 5-fold and 10-fold higher, respectively, when administered in nanoparticle formulation relative to control aqueous solution (27). In addition, the area-under-the-curve of plasma concentration versus time profile was at least 2-fold higher (27). PEO-PCL nanoparticles were found to efficiently deliver up to 20% of the i.v. injected dose to the tumor mass in vivo and promote the residence of paclitaxel for up to 12 hours (27). With tamoxifen-loaded PEO-PCL nanoparticles, the plasma and

Fig. 4. Changes in body weight and blood cell counts with single and combination nanoparticle–delivered therapy. A, body weight changes over time in control and treated SKOV3 and SKOV3TR subcutaneous tumor-bearing female nu/nu mice upon treatment with single and combination of paclitaxel and tamoxifen in aqueous solution or in PEO-PCL nanoparticle formulations. B, WBC counts in blood from control and treated animals upon sacrifice after 40 d postadministration of paclitaxel and tamoxifen, either alone or in combination, in aqueous solution or in PEO-PCL nanoparticles. C, platelet counts in blood from control and treated animals upon sacrifice after 40 d postadministration of paclitaxel and tamoxifen, either alone or in combination, in aqueous solution and in PEO-PCL nanoparticles. Paclitaxel and tamoxifen were administered i.v. via the tail vein at doses of 20 and 70 mg/kg, respectively, in all of the formulations. Normal, cell counts from animals that did not have a tumor burden; control, cell counts from untreated animals that had the tumor burden. For combination therapy, the paclitaxel-loaded and tamoxifen-loaded PEO-PCL nanoparticles were premixed and administered together in a single injection.
tumor concentrations after 6-hour postadministration were 4-fold and 5-fold higher, respectively (26).

In this study, we hypothesized that inhibition of GCS by tamoxifen enhances the accumulation of intracellular ceramide levels, which would result in the lowering of the apoptotic threshold necessary for augmenting the efficacy of paclitaxel in a drug-resistant model. Paclitaxel and tamoxifen were efficiently encapsulated at up to 10% (w/w) and 20% (w/w) concentrations, respectively, in PEO-PCL nanoparticles of 100 to 200 nm in diameter. The resulting delivery system was initially evaluated in wild-type SKOV3 and multidrug-resistant SKOV3TR human ovarian adenocarcinoma cell cultures for paclitaxel drug sensitivity and reversal of its resistance in the presence of tamoxifen. Furthermore, we evaluated for therapeutic efficacy and safety profile in tumor-bearing female nu/nu mice. Aqueous solutions and PEO-PCL nanoparticle formulations of paclitaxel and tamoxifen were either individually administered or given in combination via the tail vein to tumor-bearing mice.

The results of the cytotoxicity study revealed that nanoparticle administration resulted in 10-fold and 2-fold lower IC50 in SKOV3 and SKOV3TR cell lines, respectively, compared with solution treatment. This effect was further significantly reduced in presence of tamoxifen administration. This is due to blockade of glucosylceramide formation and resultant cerebroside and ganglioside biosynthesis in drug-resistant cancer cells. Previously, we have shown that the SKOV3TR cells used in these studies express P-glycoprotein and GCS (24). In vivo study showed that paclitaxel and tamoxifen coadministration in PEO-PCL nanoparticles effectively suppressed tumor growth.

Fig. 5. Liver toxicity profile with nanoparticle-delivered therapy. As an indicator of acute liver toxicity, (A) alanine aminotransferase (ALT) levels and (B) aspartate aminotransferase (AST) levels were measured in plasma from control and treated animals. Normal, enzyme levels from animals that did not have a tumor burden; control, enzyme levels from untreated animals that had the tumor burden. C, the micrographs of hematoxylin-stained liver tissue sections at ×20 magnification from control and treated animals upon sacrifice after 40 d postadministration of paclitaxel and tamoxifen, either alone or in combination, in aqueous solution and in PEO-PCL nanoparticles. Paclitaxel and tamoxifen were administered i.v. via the tail vein at doses of 20 and 70 mg/kg, respectively, in all of the formulations. For combination therapy, the paclitaxel-loaded and tamoxifen-loaded PEO-PCL nanoparticles were premixed and administered together in a single injection.
in SKOV3 and SKOV3TR models. Paclitaxel and tamoxifen at 20 and 70 mg/kg, respectively, administered on day 1 and day 24, suppressed tumor growth for up to 40 days in both the sensitive and drug-resistant models. The tumor growth delay and volume doubling times were significantly higher ($P < 0.05$) with the combination of paclitaxel and tamoxifen in PEO-PCL nanoparticles compared with all other treatment methods. These results were further supported by the observation of the excised tumor mass and the tumor weights at the times of sacrifice. The smallest tumors were excised from animals treated with the combination of paclitaxel and tamoxifen in PEO-PCL nanoparticles.

To examine the apoptotic activity, the cells were grown on coverslips and incubated with different formulations in vitro; for in vivo experiments, 10-µm-thick cryosections of excised tumor mass were fixed and stained. Using the TUNEL procedure, apoptotic nuclei were stained dark brown. The control tumor cryosections show blue nuclei, whereas the tumor sections from paclitaxel and tamoxifen treatment increasingly show brown nuclei. There was a significant increase in brown color formation in tumor sections from animals that received the combination of paclitaxel and tamoxifen in PEO-PCL nanoparticles. It is evident that tamoxifen administration promotes ceramide accumulation in cells by inhibiting GCS that would otherwise scavenge ceramide. Limiting GCS activity by tamoxifen treatment down-regulates the expression of P-glycoprotein. In addition, GCS inhibition retards clearance of ceramide generated in response to paclitaxel.

For safety evaluations of the PEO-PCL nanoparticulate formulations for exogenous delivery of tamoxifen, we observed that the mice tolerated two i.v. doses of paclitaxel and tamoxifen or combination of the two agents with no evidence of gross toxicity. In addition, the change in body weight as a function of time in tumor-bearing animals was used as one of the marker of safety. The results showed that there was a slight decrease (<8%) in the body weights of solution-treated animals in SKOV3 model. Tumor-bearing animals treated with paclitaxel, tamoxifen, and the combination of the two either in aqueous solution or in PEO-PCL nanoparticles did not show any appreciable loss in body weight over the period of this study. Additional indicator of treatment safety was based on the hematologic profile upon the time of sacrifice by measurements of WBC and platelet counts. There was no significant effect on WBC and platelet counts upon treatment with single or combination agent.

**Conclusions**

Ovarian cancer treatment is associated with a high degree of relapse due to acquisition of drug-resistant phenotype. In this study, we have investigated the potential for biodegradable polymeric nanoparticle-based delivery of combination paclitaxel and tamoxifen therapy to overcome drug resistance in ovarian cancer. In vitro studies in SKOV3 ovarian adenocarcinoma cells showed that there was significant enhancement in cytotoxicity effect of paclitaxel when administered in nanoparticles compared with solution formulations. In addition, paclitaxel and tamoxifen nanoparticle coadministration resulted in sensitization of drug-resistant SKOV3TR cells to paclitaxel. The combination of paclitaxel and tamoxifen when administered in PEO-PCL nanoparticles led to significant enhancement in tumor growth suppression and cellular apoptosis in sensitive SKOV3 and drug-resistant SKOV3TR xenograft models. PEO-PCL nanoparticles also provided an opportunity for administration of the combination therapy with less toxicity as measured by acute changes in body weight, blood cell counts, and hepatotoxicity profile. Based on these results, systemic administration of paclitaxel and tamoxifen in biodegradable polymeric nanoparticle delivery system promises to be a very effective strategy to enhance the cytotoxicity of paclitaxel and to overcome drug resistance in ovarian cancer patients. However, additional studies, especially upon chronic administration, are necessary to evaluate the preclinical efficacy and safety of paclitaxel and tamoxifen co-therapy when administered in PEO-PCL nanoparticles before clinical evaluations in cancer patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


Modulation of Drug Resistance in Ovarian Adenocarcinoma by Enhancing Intracellular Ceramide Using Tamoxifen-Loaded Biodegradable Polymeric Nanoparticles

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