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Models and Technologies

Molecular Cancer Therapeutics

Multifunctional Polymeric Micelles Co-loaded with Anti–Survivin siRNA and Paclitaxel Overcome Drug Resistance in an Animal Model of Ovarian Cancer

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Abstract

Ovarian cancer is a dreadful disease estimated to be the second most common gynecologic malignancy worldwide. Its current therapy, based on cytoreductive surgery followed by the combination of platinum and taxanes, is frequently complicated by the onset of multidrug resistance (MDR). The discovery that survivin, a small antiapoptotic protein, is involved in chemoresistance provided a new prospect to overcome MDR in cancer, because siRNA could be used to inhibit the expression of survivin in cancer cells. With this in mind, we have developed self-assembly polymeric micelles (PM) able to efficiently co-load an anti–survivin siRNA and a chemotherapeutic agent, such as paclitaxel (PXL; survivin siRNA/PXL PM). Previously, we have successfully demonstrated that the downregulation of survivin by using siRNA-containing PM strongly sensitizes different cancer cells to paclitaxel. Here, we have evaluated the applicability of the developed multifunctional PM *in vivo*. Changes in survivin expression, therapeutic efficacy, and biologic effects of the nanopreparation were investigated in an animal model of paclitaxel-resistant ovarian cancer. The results obtained in mice xenografed with SKOV3-tr revealed a significant downregulation of survivin expression in tumor tissues together with a potent anticancer activity of survivin siRNA/PXL PM, while the tumors remained unaffected with the same quantity of free paclitaxel. These promising results introduce a novel type of nontoxic and easy-to-obtain nanodevice for the combined therapy of siRNA and anticancer agents in the treatment of chemoresistant tumors. *Mol Cancer Ther; 14(4); 1075–84. ©2015 AACR.*

Introduction

Ovarian cancer, the most deadly gynecologic malignancies, is often diagnosed at late stages (1). The current therapy of advanced ovarian carcinomas consists of platinum and paclitaxel-based combination chemotherapy (2). However, invariably, after an unpredictable time of response to therapy, a significant percentage of patients undergo to a resistant phase. Recently, a correlation between chemoresistance and expression of survivin in cancer has been reported (3). Survivin is a small antiapoptotic protein expressed only in embryonal and fetal tissues (4). However, high survivin levels have been detected in many cancer tissues (5), especially in advanced stages. The overexpression of survivin has been associated with poor prognosis and aggressiveness of the tumors (6). In advanced ovarian carcinomas, it has been found that the expression of survivin directly correlates with a clinical resistance to taxane chemotherapy (7). The treatments that suppress survivin expression can induce apoptosis, inhibit cancer growth, and enhance the sensitivity of cancer cells to chemotherapy and radiotherapy (8). From here, new strategies based on the inhibition of survivin in tumor tissues should represent a powerful tool to enhance the chemosensitivity in patients with drug-resistance ovarian cancer.

The use of small interfering RNA (siRNA) offers a valid and efficient approaches to selectively inhibit the expression of survivin *in vitro* (9). Because the unfavorable pharmacokinetic profile of the siRNA hampers its direct use in the clinic, earlier we have designed stable nanopreparations of siRNA. In particular, we have developed and characterized polyethyelene glycol2000-phosphatidyl ethanolamine (PEG_{2000}-PE)-based polymeric micelles (PM) containing an anti–survivin siRNA reversibly conjugated with phospholipid (phosphatidylethanolamine, PE) via a disulfide linkage (survivin siRNA-S-S-PE; refs. 10, 11). This chemical conjugation was designed to increase the stability of the siRNA in biologic fluids and allow for siRNA liberation in free form in cancer cells due to the reduction of the disulfide bond with high concentration of intracellular glutathione. We found an effective stabilization of the modified siRNA in PM against nucleolytic degradation *in vitro* (10). In addition, the incorporation of the modified survivin siRNA-S-S-PE into PEG_{2000}-PE–based PM allowed to efficiently deliver the siRNA in the cells. As a result, in different cancer cell lines, a significant downregulation of survivin protein expression, and a decrease in the cell viability were observed (11).

Then, we attempted combining anti–survivin siRNA and paclitaxel within one multifunctional nano-assembly by encapsulating both into PM to achieve a better anticancer effect of the two agents for the treatment of aggressive ovarian cancer. Clear

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evidences are given by preclinical and early clinical trials that the combined delivery of siRNA and chemotherapeutic agents within one nanoparticulated system are indeed more efficient in inhibiting the tumor growth and overcoming the drug resistance compared with nanoparticles containing single agents (12). In our preliminary study, survivin siRNA/PXL PM effectively coencapsulated chemotherapeutic agent and siRNA and showed high cytotoxicity against SKOV3-tr cell line (11). In this article, we have investigated the *in vivo* therapeutic potential of the developed survivin siRNA/PXL PM in mice with xenografts of paclitaxel-resistant ovarian carcinoma, SKOV3-tr. We have also investigated the downregulation of survivin synthesis in tumor cells and the biochemical effects of the formulation.

Materials and Methods

Materials

Unless otherwise stated, all chemicals were from Sigma-Aldrich. Survivin siRNA 5′-GCAUUCGUCCGGUUGCGCUdT-dT-3′ and a scrambled siRNA 5′-AUGAACUUCAGGGUCAG-CUdTdT-3′ have been used. Both siRNAs modified at the 3′-end of the sense strand with the N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) group were purchased from Thermo Scientific Dharmacon. Paclitaxel was purchased from LC Laboratories. The Paclitaxel Oregon Green (P22310) was from Invitrogen. The 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (PE-SH, MW 731) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-{methoxy[poly(ethyleneglycol)]-2000} (PEG_{2000}-PE) were from Avanti Polar Lipids. The RNeasy kit for mRNA isolation was obtained from Qiagen. The First-Strand cDNA Synthesis Kit and the SYBR green kit for qRT-PCR were purchased from Roche. Primers for the survivin gene (5′CTGCCTGGCAGCCCTTT-3′) and (5′CCTCCAGAAGGGCCA-3′) and for β-actin were obtained from Invitrogen. The aspartate aminotransferase (AST)/alanine aminotransferase (ALT) assay kit was purchased from the biomedical research service center at SUNY Buffalo. The rabbit anti–survivin antibody, AF886, was from R&D Systems. β-Tubulin antibody (G-8) was from Santa Cruz Biotechnology. Texas red-x goat anti-rabbit IgG (T6391) and Alexa Fluor 488 goat anti-mouse IgG, IgA, IgM (H+L) were from Life Technologies. Hoechst 33342 trihydrochloride, trihydrate, was purchased from Molecular Probes. Vecta Shield mounting medium for fluorescence, H-1000, was from Vector Laboratories, Inc. DeadEnd Fluorometric TUNEL System from Promega Corporation.

Preparation and characterization of PM coencapsulating survivin siRNA and paclitaxel

The survivin siRNA-S-S-PE conjugate was synthesized as described earlier (11). The PEG_{2000}-PE micelles containing survivin siRNA-S-S-PE and paclitaxel were prepared as reported previously, with slight modifications for the *in vivo* translation (11). Briefly, an organic solution of paclitaxel in methanol (1 mg/mL) was added to the PEG_{2000}-PE solution (60 mg/mL) in chloroform. The initial quantity of paclitaxel relative to the main micelle-forming component was 2% *w*/*w*. The resulting solution was added to a 50-mL round-bottom flask, and the organic solvent was removed under the reduced pressure on a rotary evaporator under the nitrogen, followed by freeze-drying. Then, the polymeric film formed was hydrated with 1 mL of the solution of survivin siRNA-S-S-PE in PBS at pH 7.4 at PEG_{2000}-PE/siRNA-S-S-PE weight ratio of 600:1. The resulting dispersion was gently vortexed to form mixed survivin siRNA/PXL PM. PEG_{2000}-PE–based PM containing survivin siRNA-S-S-PE alone and scrambled siRNA-S-S-PE in combination with paclitaxel were prepared similarly. Each formulation was prepared in triplicate. For intratumor accumulation studies, survivin siRNA/PXL PM containing 0.1% (*w*/*w*) of Oregon Green–labeled paclitaxel were prepared similarly.

Characterization of survivin siRNA/PXL PM

The mean diameter of PM containing survivin siRNA-S-S-PE in combination with paclitaxel, was determined at 20°C by the dynamic light scattering (DLS) using a Zeta Plus Instrument (Brookhaven Instrument Co.). Briefly, each sample was diluted in deionized/filtered water and analyzed with detector at the 90° angle. As a measure of the particle size distribution, polydispersity index (P.I.) was used. For each batch, mean diameter and size distribution were the mean of three measurements. For each formulation, the mean diameter and P.I. were calculated as the mean of three different batches. The quantitative analysis of survivin siRNA-S-S-PE and paclitaxel content in PM was performed by the size-exclusion high-performance liquid chromatography (SEC-HPLC) and reversed-phase-HPLC, respectively, as reported previously by Salzano and colleagues (11).

Cell culture

Human ovarian adenocarcinoma-resistant cell line, SKOV3-tr paclitaxel-resistant cells, were kindly provided and tested by Dr. Zhenfeng Duan at the Massachusetts General Hospital (Boston, MA) immediately before the *in vivo* study. The phenotype of SKOV3-tr has been deeply characterized by Duan and colleagues (13) by high-density Affymetrix HG-U95Av2 microarrays to quantify the gene expression. In detail, SKOV3 cells, obtained from the ATCC, were selected to be paclitaxel resistant by continuous exposure of cells with increasing concentration of paclitaxel for 8 months. A significant and stable overexpression of the MDR-1 gene, associated with acquired paclitaxel resistance, was identified. In addition, the paclitaxel-resistant phenotype and the MDR-1 expression did not changed when the SKOV3-tr cells were grown for 6 months without paclitaxel. In our laboratory, we have routinely monitored the IC_{50} of paclitaxel in SKOV3-tr by CellTiter Blue assay (CTB). CTB assay showed that SKOV3-tr were at least 100-fold more resistant than the sensitive SKOV3 cell line.

SKOV3-tr cells were grown in RPMI-1640 medium supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), and 100 U/mL penicillin G sodium and 100 mg/mL streptomycin sulfate (complete medium), in a humidified atmosphere of 95% air 5% CO_2 at 37°C. For the subculture, cells growing as a monolayer were detached from the tissue flasks by the treatment with trypsin/EDTA. The viability and cell count were monitored routinely using the Trypan blue dye exclusion method. The cells were harvested during the logarithmic growth phase and resuspended in serum-free medium before inoculation in animals.

Subcutaneous tumor xenograft development

The experimental protocol involving the use of animals was approved by the Institutional Animal Care and Use Committee of Northeastern University. SCID female nude mice (nu/nu), 6 to 8 weeks old and weighing 20 to 25 g were purchased from Charles River Laboratories, and were housed under controlled laboratory conditions in polycarbonate cages. The animals were allowed to

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acclimate for at least 48 hours before any experiment. Approximately 7 million of SKOV3-tr cells, suspended in 100 μL of Matrigel (in free serum media 1:1 volume ratio), were injected subcutaneously in the left flank of each mouse under light isoflurane anesthesia. Palpable solid tumors developed within 15 days after tumor cell inoculation, and as soon as tumor volume reached 150 to 200 mm^3, the animals were randomly allocated to five different control and treatment groups [i.e., PBS, paclitaxel in Cremophor EL1-ethanol (1:1) mixture with normal saline (Taxol), PM containing scrambled siRNA-S-S-PE conjugate, and paclitaxel in combination (scrambled siRNA/PXL PM), PM containing survivin siRNA-S-S-PE conjugate (survivin siRNA PM) and PM containing survivin siRNA-S-S-PE and paclitaxel in combination (survivin siRNA/PXL PM)]. Six animals per group were used. All controls and micelle formulations were diluted and suspended in sterile PBS. Each tumor-bearing animal received siRNA-S-S-PE at a dose of 20 μg, corresponding to 1 mg/kg per injection, and paclitaxel at a dose 10 mg/kg both in Cremophor solution or in PM by intravenous administration through the tail vein once per week for 5 consecutive weeks.

Evaluation of therapeutic efficacy

The tumor diameters were measured three times weekly with a vernier calipers in two dimensions. Individual tumor volumes (V) were calculated using the formula (14):

$$V = [length \times (width)2]/2$$

where length (L) is the longest diameter and width (W) is the shortest diameter perpendicular to length.

Growth curves for tumors are presented as the relative tumor volume (RTV), defined as V_n/V_o, where V_n was the tumor volume in mm^3 on day "n" (V_n) and V_o at the start of the treatment plotted versus time in days. Mean RTV (mRTV) and standard deviation were calculated per each group. At the end of the experiment, the animals were sacrificed by cervical dislocation, and the tumor mass was harvested and weighed.

Evaluation of repeated dose toxicity in mice

For safety evaluation of the controls and survivin siRNA/PXL PM formulation, the body weight of each mouse was determined three times per week and related to the first day weight as percentage change in body weight. In addition, blood samples were collected via the cardiac puncture prior to the sacrifice, and the levels of serum AST and ALT were measured. The serum was obtained by centrifugation of the freshly collected blood samples at 2,000 × g for 30 minutes at 4°C. Then, AST and ALT were measured using the manufacturer's standard kinetic assay protocol (Biomedical Research Service).

Collection of tumor tissues

Tumors were excised, dissected free of the skin and body tissue, and weighed on a digital balance. Then, the tumors were immediately snap-frozen in liquid nitrogen and maintained at −80°C until ready for sectioning. For immunofluorescence analysis, frozen sections (6 μm) were cut on a Cryostats microtome (Thermo Scientific), placed on glass slides and stored at −20°C until they were used.

Tumor cell apoptosis

Tumor sections were stained by Hoechst 33342, and the level of the tumor cell apoptosis was analyzed by DeadEnd Fluorometric TUNEL System, according to the protocol described by the supplier. The pictures were taken by the confocal microscopy. The slides were visualized by light microscopy at 10× magnification.

Evaluation of survivin mRNA expression with RT-PCR

Survivin mRNA expression was assayed from different tumor tissues by performing the quantitative real-time PCR method, as described previously (15). Briefly, tumors were vortexed in a 1.5-mL tube containing 1 mL of cold PBS. Total RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. This isolated RNA was treated with DNase, followed by RNA quantification using a ND-1000 NanoDrop spectrophotometer. cDNA synthesis and subsequent PCR amplification were performed using 1 μg of the isolated RNA, random hexamers, and reverse transcriptase enzyme as per the First-Strand cDNA Synthesis Kit from Roche. qRT-PCR assay was performed on triplicate samples the SYBR Green I Master from Roche on the LightCycler 480 qRT-PCR machine from Roche as described previously (15). The following survivin primer sequence: human survivin: (forward, 5′-CTGCCTGGCAGCCC-TTT-3′) and (reverse, 5′-CCTCCAAGAAGGGCCAGTTC-3′; 16), β-actin; (forward, 5′-ACCGAGCGCGGCTACAGT-3′) and (reverse, 5′CTTAATGTCACGCACGATTTC-3′) was used as an internal control. All custom primers were designed using the Invitrogen OligoPerfect Designer to have between 50% and 60% GC content, an annealing temperature of approximately 60°C and a length of 20 bases. No template controls (NTC) were run on each plate as well as done to verify that there was neither unspecific amplification nor the formation of primer dimers. Data were analyzed using the Roche quantification method $\Delta(\Delta C_t)$ and were normalized to β-actin and compared with control levels. The relative expression levels are expressed as a percentage of the indicated control.

Determination of survivin protein expression by immunofluorescence analysis

Survivin immunofluorescence analysis was performed on frozen tumor sections. Tumor sections were fixed with 4% of paraformaldehyde for 20 minutes at room temperature. After washing twice with PBS, slides were immersed in 0.5% of H_2O_2/PBS solution at room temperature for 10 minutes to block the endogenous peroxidase activity. The slides were rinsed in PBS solution with two changes, 5 minutes each, and then, incubated in 1% of Triton X-100 solution for 10 minutes. Enzymatic activity and nonspecific binding sites were blocked by incubating the slides in 10% of FBS for 1 hour at room temperature in a humidified chamber. Subsequently, replicate sections were incubated at 4°C overnight with a primary rabbit anti–survivin antibody (AF886; R&D Systems) at the final concentration of 10 μg/mL. Thorough rinsing was followed by the incubation with the red-fluorescent dye-labeled anti-rabbit IgG (10 μg/mL; T6391; Life Technologies) for 1 hour. Negative controls for each tissue section were performed leaving out the primary antibody. Finally, the nuclei were stained with Hoechst 33342 (5 μmol/L) for 15 minutes at room temperature and the stained sections were observed and photographed using a Nikon Eclipse E400 microscope and a Spot Advanced software (Spot Imaging). To quantify the survivin protein levels from the images, the cell fluorescent intensity was measured with the ImageJ.

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Evaluation of the simultaneous intratumor accumulation of paclitaxel and downregulation of survivin expression in tumor sections

For this experiment, mice ($n = 3$) were injected once with survivin siRNA/PXL PM. After 48 hours, the animals were injected with survivin siRNA/PXL PM containing Oregon Green–labeled paclitaxel (0.1% w/w). After 1 hour, the animals were sacrificed. Tumors were excised and processed as above under light protection. At the same time, the intratumor accumulation of Oregon Green–labeled paclitaxel and survivin protein expression were evaluated. The survivin protein expression was evaluated by the immunofluorescence analysis as described above. The nuclei were stained with Hoechst 33342. Negative control, such as untreated tumor sections and sections exposed to the secondary antibody only, were processed as described above. Images were recorded by confocal microscopy.

Tubulin immunostaining

Frozen sections were processed as above and incubated with a monoclonal antibody against β-tubulin (G-8; Santa Cruz Biotechnology; dilution 1:50) for overnight at 4°C. After washing, the sections were incubated with a secondary Alexa Fluor 488–labeled goat anti-mouse IgG targeting antibody (dilution 1:100) for 1 hour at room temperature. Then, after washing, the sections were incubated with Hoechst 33342 (5 μmol/L) for nuclear staining. The slides were mounted on glass slides with Fluoromount-G (Fisher Scientific) medium and sealed using a nail lacquer. Negative control sections were exposed to the secondary antibody only and processed as described above. The slides were observed with a Zeiss LSM 700 inverted confocal microscope (Carl Zeiss Co. Ltd.) equipped with a 63×, 1.4-numerical aperture plan-apochromat oil-immersion objective. The images were analyzed using the ImageJ version 1.42.

Statistical analysis

For comparison of several groups, one-way ANOVA for multiple groups, followed by the Newmane–Keuls test if $P < 0.05$ was performed using the GraphPad Prism version 5.0 software (GraphPad Software, Inc.). All numerical data are expressed as mean ± SD; $n = 3$ or more, from 3 different experiments. Any P value less than 0.05 was considered statistically significant.

Results

The characteristics of the developed multifunctional PM are summarized in the Table 1. Survivin siRNA/PXL PM have a mean diameter of about 25 nm and a very narrow size distribution (P.I. ≤ 0.2). The chromatographic analysis of the amount of survivin siRNA-S-S-PE and paclitaxel entrapped in the same PM, was performed as previously reported by Salzano and colleagues (11). HPLC analysis of nonincorporated siRNA and paclitaxel showed an actual loading of approximately 1 μg of survivin siRNA-S-S-PE/mg polymer and approximately 22 μg of paclitaxel/mg polymer, corresponding to an incorporation efficiency of approximately 50% and 90%, respectively.

In vivo antitumor activity of survivin siRNA/PXL PM in xenografted mice

The antitumor efficiency of the codelivery of anti–survivin siRNA and paclitaxel in the same PM was evaluated in an animal model of SKOV3-tr xenografts. As shown in Fig. 1A, paclitaxel alone, in Cremophor solution (Taxol) or incorporated in PM (scrambled siRNA/PXL PM), did not induce a significant effect on the tumor growth. In contrast, following the treatment of the animals with PM containing survivin siRNA alone or in combination with paclitaxel, the therapeutic outcome was significantly enhanced. The treatment with survivin siRNA alone was able to induce a significant slowing of the tumor growth. This effect was even more pronounced in the survivin siRNA/PXL PM–treated group. The treatment of animals with the combination of siRNA and paclitaxel in PM elicited the highest antitumor activity, showing the least RTV among all the treatment groups ($P < 0.05$). Moreover, the postmortem tumor weights of mice treated with this schedule were significantly reduced compared with all the control groups ($P < 0.01$; Fig. 1B). None of the agents caused any noticeable toxicity, because we did not detect significant changes in body weight (Fig. 1D), toxic adverse events or deaths, confirming low nonspecific toxicity of the treatments. To monitor the general toxicity after repeated doses of the treatments, the serum levels of ALT and AST were measured. As presented in Fig. 1C, there was no significant decrease in ALT and AST levels in serum following all the treatment procedures, suggesting the absence of liver toxicity induced by the treatments.

Tumor cell apoptosis

To investigate the cellular mechanisms of the tumor growth inhibition in mice treated with survivin siRNA/PXL PM, the level of the apoptosis in tumor tissues was evaluated. Tumor sections of tumors dissected from the previous experiment were stained by Hoechst 33342, and the level of the apoptosis was analyzed by the TUNEL assay under the confocal microscopy. As shown in Fig. 2, the codelivery of paclitaxel and survivin siRNA leads to the highest rate of cell apoptosis (Fig. 2E), which was clearly superior to all the other treatment groups (Fig. 2A–D).

Downregulation of survivin mRNA expression *in vivo*

To provide the evidence that the inhibition of the tumor growth by using survivin siRNA/PXL PM was due to the ability of this preparation (siRNA component) to downregulate survivin *in vivo*, the transcriptional mRNA of the survivin gene expression was evaluated in tumor tissues by RT-PCR. Experiments were repeated three times. The relative levels of survivin mRNA in tumor tissues were normalized against mRNA of an internal control gene, β-actin, performed in the same run. As shown in Fig. 3, the relative levels of survivin mRNA in mice treated with survivin siRNA/PXL PM (0.09 ± 0.03) were significantly decreased compared with those in untreated (0.99 ± 0.007) and scrambled siRNA/PXL PM (0.86 ± 0.05) animal groups. The codelivery of anti–survivin siRNA and paclitaxel showed an inhibitory rate of survivin mRNA of about 90%. Interestingly, the treatment with Taxol could

Table 1. Physical characteristics of PM-based formulations

Formulation	Mean diameter (nm ± SD)	P.I. ± SD	Survivin siRNA incorporation efficiency (% ± SD)	PXL incorporation efficiency (% ± SD)
Survivin siRNA/PXL PM	25.0 ± 3.6	0.190 ± 0.07	51.0 ± 2.5	89.9 ± 3.5
Survivin siRNA PM	23.0 ± 2.1	0.182 ± 0.10	50.6 ± 1.5	—
Scrambled siRNA/PXL PM	22.9 ± 1.5	0.195 ± 0.05	52.2 ± 3.0	90.1 ± 2.4

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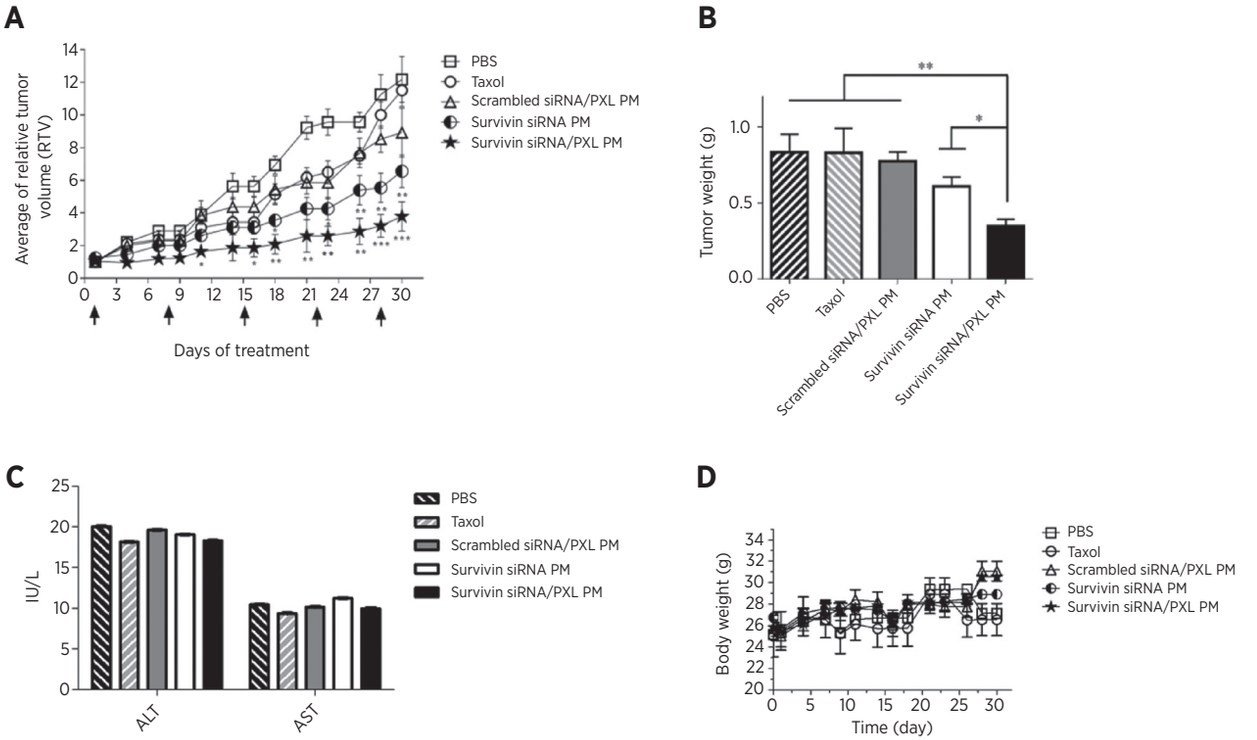

Figure 1.
A, *in vivo* antitumor activity of survivin siRNA/PXL PM in SKOV3-tr xenografts. Survivin siRNA/PXL PM were administered at a final concentration of anti–survivin siRNA and paclitaxel of 1 and 10 mg/kg, respectively, once per week for 5 consecutive weeks. RTV values [tumor volume in mm^3 on day "*n*" (V_n))/tumor volume at the start of the treatment (V_o) plotted versus time in days] are reported. Data are given as mean ± SD for each treatment group. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$ were obtained by comparing each treatment group with the Taxol group. B, postmortem tumor weights. On day 30, heterotopically implanted tumors were weighed and plotted. *, $P < 0.05$ and **, $P < 0.01$ were considered significant and very significant, respectively, and were obtained by comparing each treatment group with the survivin siRNA/PXL group. C, evaluation of repeated dosing toxicity in mice by measurement of changes in serum levels of transaminase (AST/ALT). Data are given as mean ± SD for each treatment group. D, changes in body weight by measurement of the body weight of the mice three times a week for 5 consecutive weeks. Data are given as mean ± SD for each treatment group.

also reduce the survivin expression in tumors to a certain extent (Fig. 3). In according with the results previously reported by Hu and colleagues (17), free paclitaxel temporarily reduces the expression of survivin as a result of mitosis inhibition. These results indicated that the combination of an anti–survivin siRNA with a chemotherapeutic agent, such as paclitaxel, with effective silencing propriety on survivin expression, could be a powerful approach to treat multidrug resistance (MDR) tumors.

Espression of survivin protein detected by immunofluorescence analysis

To confirm the results obtained by qRT-PCR analysis, protein levels of survivin in tumor tissues were investigated by immunofluorescence analysis. As shown in Fig. 4A and B, the microscopic examination of stained tumor sections showed strong immunoreactivity for survivin (red color) in the untreated and scrambled siRNA/PXL PM groups. In contrast, the intensity of survivin signal was dramatically decreased in the survivin siRNA PM and survivin siRNA/PXL PM–treated groups (Fig. 4A and B). Furthermore, the simultaneous delivery of paclitaxel and siRNA in tumors was examined by the confocal laser scanning microscope. For confocal microscopy observation, the immunostaining for survivin was used to evaluate the gene silencing, while the Oregon Green–labeled paclitaxel was used to follow the drug. The mice were treated once with survivin siRNA/PXL PM. After 48 hours, the animals were injected with survivin siRNA/PXL PM containing Oregon Green–labeled paclitaxel. One hour later, the animals were sacrificed and tumor sections were processed under light protection. The confocal microscopy study showed clearly that Oregon Green–labeled paclitaxel was transported in the tumor tissues and survivin was significantly downregulated (Fig. 4C). It is interesting to note that, a single administration of survivin siRNA/PXL PM was able to efficiently downregulate survivin expression in tumors, as suggested by the almost complete absence of the survivin red signal in the sections.

Effect of survivin siRNA/PXL PM on microtubule conformation of ovarian cancer xenografts

Previously, we have shown that survivin downregulation enhanced the paclitaxel activity on microtubule organization in SKOV3-tr cells (11). After downregulation of survivin levels by treating cells with survivin siRNA PM, paclitaxel was able to destabilize the microtubule organization at a very low concentration and exposure time. Here, to assess the effect of survivin siRNA/PXL PM on microtubule organization *in vivo*, SKOV3-tr tumor sections were incubated with an antitubulin fluorescent antibody. As shown in Fig. 5A, in untreated mice, tumor cells exhibited staining of elongated microtubule fibers, demonstrative of an intact microtubule network. No significant differences were observed in all the control groups (Fig. 5B and C). At the same

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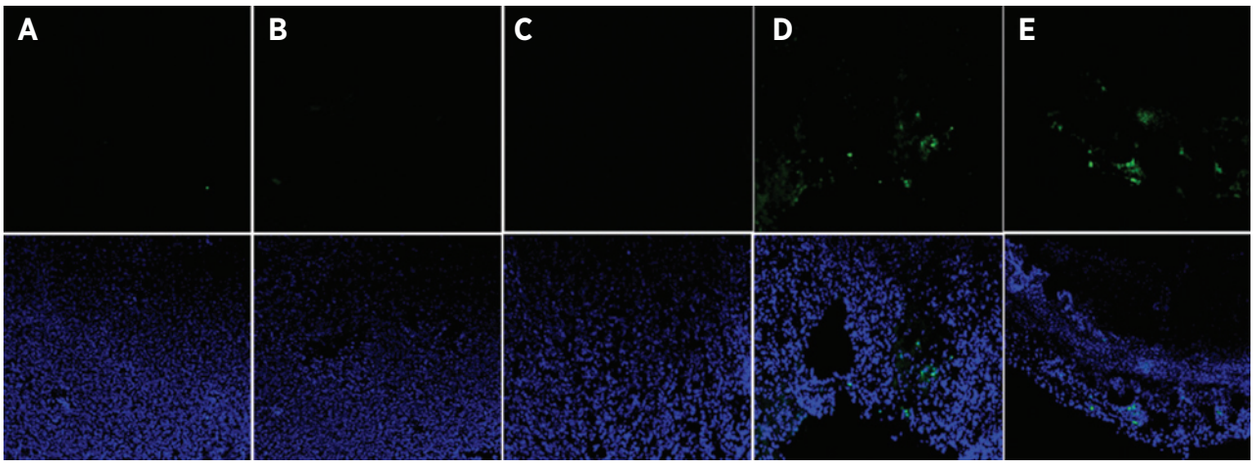

Figure 2.
Apoptosis analysis on tumor sections by the TUNEL assay. Pictures were taken by the confocal microscopy (×10 magnification). The nuclei were stained for Hoechst 33342 (blue) and apoptotic cells (green) for TUNEL. Representative images of untreated (A), scrambled siRNA/PXL PM (B), Taxol (C), survivin siRNA PM (D), and survivin siRNA/PXL PM groups (E).

time, the survivin siRNA/PXL PM group showed a microtubule-staining organization that was markedly different from all the other treatments (Fig. 5E). In particular, as indicated by the arrows, in survivin siRNA/PXL PM-treated mice, the tumor cells exhibited diffuse organization of microtubules (Fig. 5E). This result can be demonstrative of a compromised microtubule network, as previously reported *in vitro* (11).

Discussion

Resistance to chemotherapy is a major cause of treatment failure and relapse of many cancer types, including ovarian cancer. Survivin is one of the most tumor-specific molecules, which is selectively overexpressed in cancer tissues where it antagonizes apoptosis, stimulates tumor-associated angiogenesis, and promotes cell growth by stabilizing the microtubules organization (18). In the last years, the retrospective analysis of patient specimens and xenografts tumor data are pointing out that there is a clear connection between the existence of high levels of survivin protein and the resistance to therapy and poor prognosis in multiple tumor types (19–22). In the particular case of ovarian cancer, the prognostic role of survivin is not clear due to some conflicting results (23–26). However, the potential of survivin as a target for apoptosis-based therapy is well-established (27). The inhibition of survivin using the transcriptional repressor, YM155 sensitized primary cell cultures to cisplatin and decreased the tumor size of OVCa ovarian cancer xenografts (28). The use of siRNA to downregulate the survivin expression has been proposed as an alternative strategy to sensitize and strengthen the tumor response. The combination of paclitaxel and survivin RNAi down-regulation induced synergistic apoptosis *in vitro* and inhibited tumor growth in ovarian cancer SKOV-3 xenografts (17, 29). Despite, the exact mechanism of such a significant chemosensitization effect is still not clear, there are evidences that upregulated levels of survivin preserve the microtubules network (30). The interaction of survivin with microtubules of the mitotic spindle results to a protection of cancer cells from paclitaxel-mediated apoptosis. The disruption of survivin–microtubule interactions and the downregulation of survivin by using siRNA can results in loss of survivin-mediated resistance to apoptosis. The translation of paclitaxel and anti–survivin siRNA combinations in the clinical settings is challenging because of the lack of appropriate delivery systems. On the one hand, siRNA has a poor pharmacokinetic profile upon intravenous injection, that is, fast degradation, fast clearance, and it cannot cross cellular membranes *per se*. This impairs tumor accumulation and the subsequent cellular internalization of siRNA. Current siRNA carriers, such as cationic lipidic or polymeric nanoparticles, fail in their *in vivo* stability and safety (31–33). On the other hand, paclitaxel has a very low solubility in aqueous solution and, to be intravenously injected, requires to be formulated with ethanol and Cremophor, the commercial formulation Taxol, which is associated with serious side effects including hypersensitivity, myelosupression, and

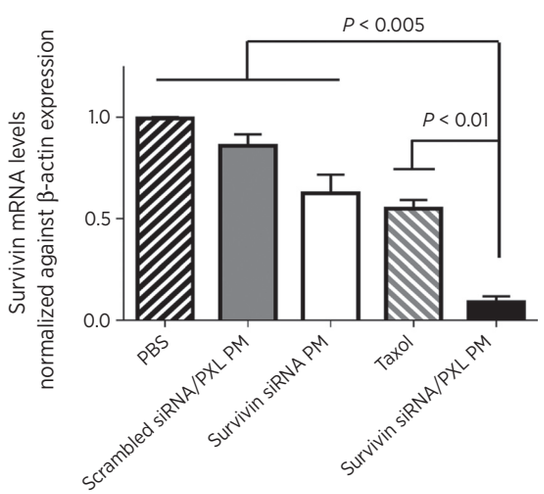

Figure 3.
Survivin mRNA levels in tumor tissues by rt-PCR analysis. Data are given as mean ± SD for each treatment group. $P < 0.005$ and $P < 0.01$ were obtained by comparing each treatment group with the survivin siRNA/PXL group.

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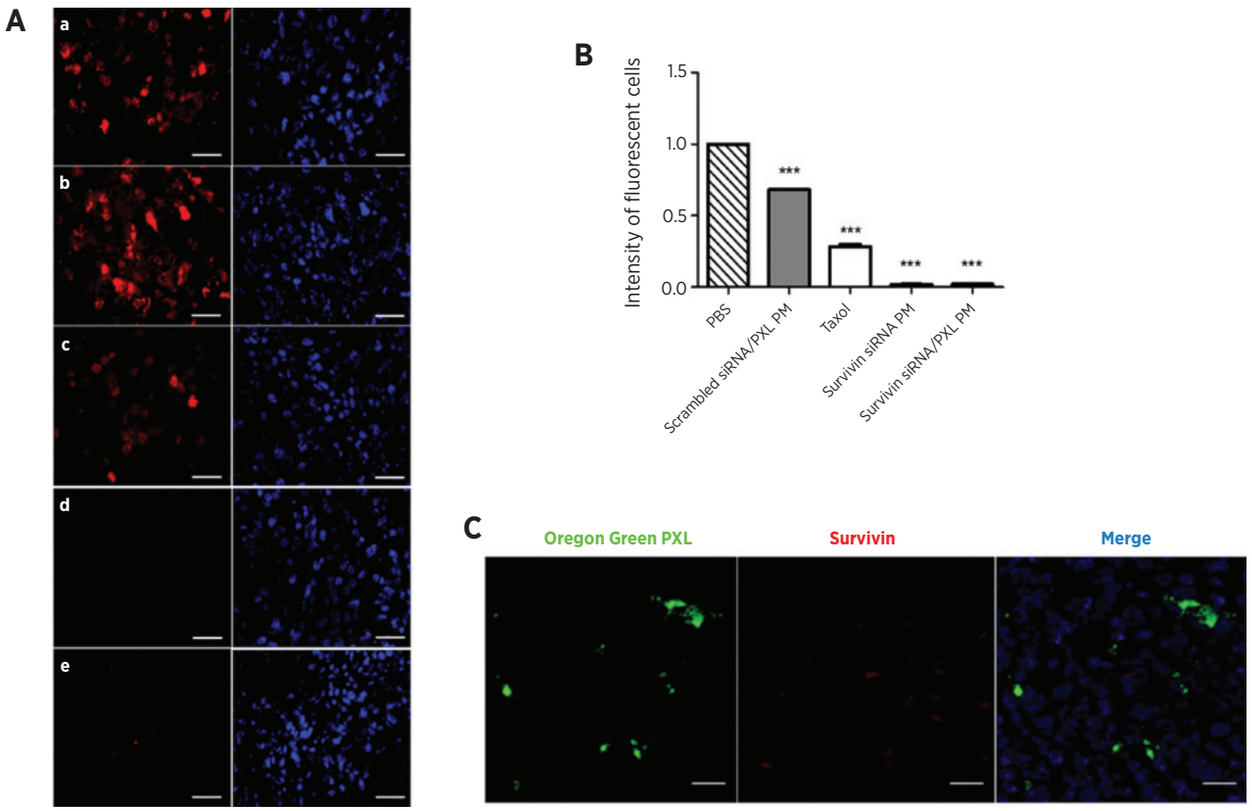

Figure 4.
A, immunohistochemistry analysis. Survivin protein levels were evaluated by the fluorescent microscopy (50×). Representative images of three independent experiments showing survivin expression (in red) and nuclei staining with Hoechst 33342 (blue). Untreated group (a), scrambled siRNA/PXL PM group (b), Taxol group (c), survivin siRNA PM group (d), and survivin siRNA/PXL PM group (e). Scale bar, 20 μm. B, cell fluorescent intensity of the survivin protein levels measured with ImageJ. ***, $P < 0.005$ was obtained by comparing the cell fluorescence of each treatment group with the PBS group (C). Simultaneous downregulation of survivin expression and paclitaxel penetration in tumor tissues by using survivin siRNA/PXL PM. At the same time, the intratumor accumulation of Oregon Green–labeled paclitaxel (left) and survivin protein expression (middle) was evaluated on tumor sections by confocal microscopy (magnification 63×). Scale bar, 20 μm.

neurotoxicity (34–36). Therefore, there is a clear need of alternative formulations for both siRNA and paclitaxel that improve their stability, are suitable for intravenous injection, and lack of carrier-related toxicities.

We previously reported on bioreductive PM for siRNA delivery based on siRNA conjugated to PE via a disulfide linkage. The inclusion of siRNA-PE conjugate into PEG_{2000}-PE micelles further stabilized the siRNA preventing its nucleolytic degradation and allowing its release in free and biologically active form inside the cells by intracellular glutathione (10). *In vitro* cytotoxicity and survivin protein levels studies revealed the ability of survivin siRNA PM to downregulate the survivin in different cancer cell lines and sensitize the cells to paclitaxel. In addition, paclitaxel and survivin siRNA simultaneously delivered in PM were especially active in resistant ovarian cancer cells, leading to superior cytotoxicity compared with their sequential administration (11). This study aims to validate the utility and safety of these nanopreparations *in vivo* for intravenous codelivery of siRNA and paclitaxel to distant tumors and verify their activity in a resistant ovarian cancer model.

For the preparation of PM containing anti–survivin siRNA and paclitaxel, we first reversibly modified anti–survivin siRNA with a PE lipid moiety via disulfide linkage by a facile chemical conjugation. Then, paclitaxel and conjugated siRNA were incorporated into PEG_{2000}-PE PM by one-step polymer film hydratation. The PM showed high colloidal stability, high drug incorporation efficiency (90% and 50%, for paclitaxel and siRNA, respectively), and small particle sizes. Usually, cationic nanoparticles commonly used for siRNA formulation need to be freshly prepared right before injection to avoid premature precipitation and loss of activity. The PM retained their technological properties for hours and even days after their preparation and do not require the use of toxic excipients for their preparation. Intravenous injection of PM was well tolerated by the mice. We did not observe any signs of blood aggregation or hemolytic activity, pulmonary embolism, or fatigue reported in the case of cationic carriers (33), or cremophor-like hypersensitivity reactions (37). In addition to their safety, the small particle size of PM (25 nm) ensured extravasation and accumulation of the PXL/siRNA cargo in the tumor by the enhanced permeability and retention (EPR) effect (38, 39). We detected the presence of paclitaxel in tumor sections 2 hours after injection of PM (Fig. 4B). We also verified the release of free and active siRNA inside tumoral cells by the strong survivin

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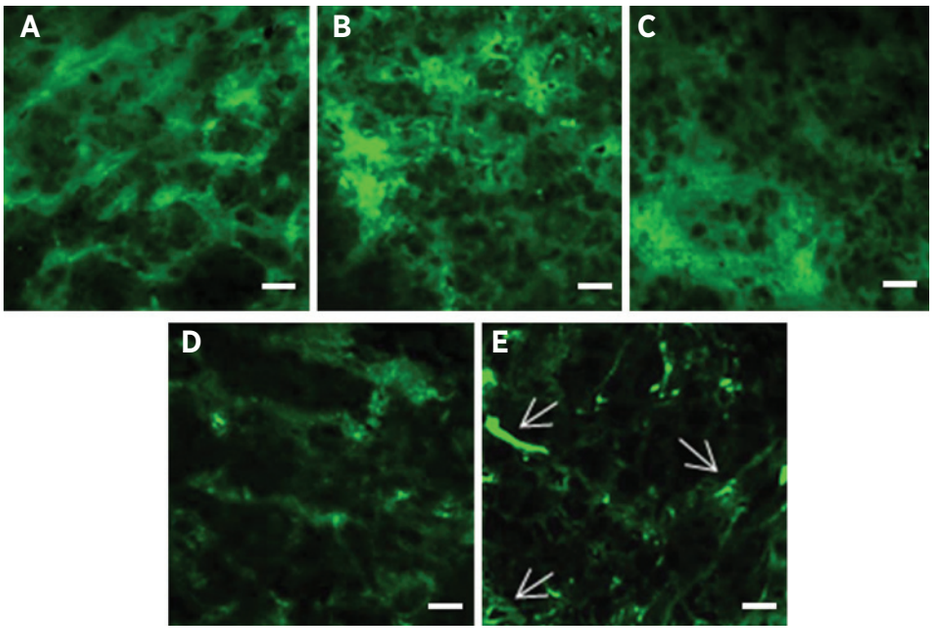

Figure 5.
Microtubule organization after treatment with survivin siRNA/PXL PM *in vivo*. SKOV3-tr tumor sections were stained for β-tubulin (green). A–E, representative images of three independent experiments showing the organization of microtubules (magnification, ×63). Untreated group (A), scrambled siRNA/PXL PM group (B), Taxol group (C), survivin siRNA PM group (D), and survivin siRNA/PXL PM group (E). Scale bar, 10 μm.

downregulation 24 hours after single-injection of anti–survivin siRNA PM (Fig. 4B).

The antitumor effect of anti–survivin siRNA and paclitaxel co-loaded in PM was demonstrated in nude mice bearing SKOV3-tr tumors. The animals were treated with Taxol, scrambled siRNA/PXL PM, survivin siRNA PM, and survivin siRNA/PXL PM once a week for five consecutive weeks at doses of 1 and 10 mg/kg for siRNA and paclitaxel, respectively. During the treatment, the overall health of the animals was good. No weight loss or evident hepatoxicity was found after repeated doses of the different PM formulations or even Taxol (Fig. 1C and D). It is worth noticing that intravenous injections of Taxol (paclitaxel $\sim$ 10 mg/kg) thrice a week produced significant body loss in SKOV-3 xenografted mice with poor improvement in the therapeutic outcome.

Combination of paclitaxel and survivin siRNA in PM resulted in sensitization of resistant tumors to paclitaxel and improved anticancer activity. As shown in Fig. 1A, the codelivery of anti–survivin siRNA and paclitaxel in PM inhibited tumor growth and exceeded the therapeutic effect of the single agents. Mice treated with survivin siRNA/PXL PM showed a 4-fold tumor volume reduction as compared with saline control that was consistent with the half-reduction in tumor weight after sacrifice the animals. Survivin downregulation by survivin siRNA PM exhibited certain anticancer activity although lesser than the one obtained after treatment with survivin siRNA/PXL PM. This intrinsic anticancer activity was already described in SKOV3 animal models after direct tumor injection of survivin shRNA (29). Finally, the lack of therapeutic response of paclitaxel either as Taxol or incorporated into scrambled siRNA/PXL PM demonstrated that survivin downregulation mediated the sensitization of resistant tumors to noneffective doses of paclitaxel.

Sequence-specific downregulation by survivin siRNA in PM was confirmed in excised tumors (Fig. 3). Taxol-treated tumors had significant decreased levels of survivin mRNA as previously described (40). Only those nanopreparations containing anti–survivin siRNA were able to consistently decrease survivin mRNA and protein levels at the same extent.

We further characterized the tumor response to survivin siRNA and paclitaxel combination by the detection of apoptosis in tumor sections. The degree of apoptosis (Fig. 2) correlated well with the tumor growth curves. The highest level of tumor apoptosis was found for survivin siRNA/PXL PM followed by survivin siRNA PM, whereas no significant apoptosis increase was found for paclitaxel formulations (Fig. 2). Finally, the restoration of paclitaxel sensitivity by survivin siRNA/PXL PM treatment was also noticed by the changes in the microtubule network in tubulin-stained tumor sections (Fig. 5). Similar to what we observed *in vitro* (11), survivin siRNA/PXL PM treatment resulted in more intensively stained tubulin as compared with the rest of the preparations, consistent with an improvement of the microtubule-stabilizing activity of paclitaxel (41).

Conclusions

We have developed a micellar nanopreparation (PM) containing anti–survivin siRNA as siRNA-S-S-PE conjugate and paclitaxel for the treatment of ovarian cancer. The developed system allows for easy and highly efficient coencapsulation of chemotherapeutic drugs and siRNA, showed high colloidal stability and had small particle sizes compatible with parenteral administration. The micelles accumulate in distal tumors and delivered anti–survivin siRNA and paclitaxel in sufficiently high amounts to mediate a potent and specific survivin downregulation and improved anticancer activity as compared with single agents. Survivin downregulation by anti–survivin siRNA/PXL PM mediated the sensitization of the resistant ovarian tumor to noneffective doses of paclitaxel. Finally, the system avoids the use of toxic excipients and is well tolerated by the animals even after repeated dosing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The authors alone are responsible for the content and writing of this article.

Authors' Contributions

Conception and design: G. Salzano, G. Navarro, V.P. Torchilin
Development of methodology: G. Salzano, G. Navarro
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Salzano, G. Navarro, M.S. Trivedi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Salzano, G. Navarro, M.S. Trivedi
Writing, review, and/or revision of the manuscript: G. Salzano, G. Navarro, G. De Rosa, V.P. Torchilin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.S. Trivedi
Study supervision: G. Salzano, G. Navarro, V.P. Torchilin

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Molecular Cancer Therapeutics

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