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Polymeric Micelles containing Survivin siRNA: a new challenge to overcome the drug resistance in cancer.

Maybe you could change the title since the term novel is not recommended; use of survivin siRNA has already been reported.

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Abstract

Drug resistance is one of the major obstacles for the successful chemotherapy in tumor treatment. The recent discovery that survivin, a small anti-apoptotic protein, is involved in chemoresistance, open a new scenario to overcome the multidrug resistance (MDR) in cancer. Different studies are demonstrating the powerful of the use of siRNA to inhibit efficiently the expression of survivin in cancer cells. However, the clinical use of siRNA is still hampered by an unfavorable pharmacokinetic profile. To address this problem, we developed a new (actually not new since the concept has been published previously, it should be mentioned) system to deliver siRNA in cancer. Namely, we reversibly modified the survivin siRNA with a phosphothioethanol (PE) portion via the a reducible disulfide bond and incorporated the resulting siRNA-S-S-PE conjugate in nanosized PEG-PE-based polymeric micelles (PM), obtained the so-called survivin siRNA PM. Maybe you could write we evaluated xxx The activities of the nanopreparations were determined by survivin protein down-regulation, tumor cell growth inhibition, and chemosensitization of the treated tumor cells to anticancer drugs, such as Paclitaxel (PXL). We found report an important decrease of cell viability and a significant down-regulation of survivin protein levels after treatment with survivin siRNA PM in different several cancer cell lines. In addition, the down regulation of survivin, mediated by treatment with survivin siRNA PM, elicited a significant sensitization of the cells to PXL, ~~also~~ in resistant cancer cell lines. Finally, we demonstrate successful co-delivery of PTX and survivin siRNA in the PM leading to superior therapeutic activity than their sequential administration. Our results support the use of this new platform for the treatment of the most aggressive tumors. Finally, we investigated the possibility to co-encapsulated survivin siRNA and PXL in PM, for multifunctional therapy. Our results suggest that combined treatment of cells with PXL and survivin siRNA resulted in a significantly higher cytotoxicity as compared to individual single drug treatment.

Keywords

Survivin, siRNA, Polymeric micelles, Paclitaxel, Chemoresistance, co-delivery.

Introduction

Survivin, the smallest member of the inhibitors of apoptosis (IAP) family, has gained much attention in recent years as a promising new target in cancer therapy due to its differential expression in tumours versus normal tissues overexpression in tumors compared to xx (Ambrosini G, 1997, Nature). In addition to inhibition of apoptosis, survivin promotes cellular proliferation. Survivin plays an important role in the negative regulation of apoptosis and in cell division (Altieri DC. Nat Rev Cancer. 2003: Yang D et al., PNAS, 2004) Moreover, survivin expression in malignant tissues has been correlated with drug-resistance and evidences demonstrated that survivin, strongly expressed in malignance tissues, causes cellular resistance to drug-induced apoptosis (Lu J et al., Clin Cancer Res. 2009). Accordingly, inhibition of survivin has been a focus of cancer therapy. In the last years, many researchers have proposed different way to counteract survivin in cancer cells with the dual aim to inhibit the tumor growth potential and to sensitize the tumor cells to chemotherapeutic agents (REF). RNA interference (RNAi) offers an attractive and powerful approach to inhibit efficiently the survivin expression in cancer cells (ref Fire and Mello). Carvalho et al., were the first to use siRNA to repress survivin in HeLa cells, showing a specific depletion of survivin in cultures for at least 60 h after transfection with a specific siRNA. Later, Seth S. et al. demonstrated the *in vivo* silencing of survivin and a significant dose-dependent decrease of tumor volumes after intravesical instillation of liposomes containing survivin siRNA in an animal model of bladder cancer. Despite all the potential of siRNA in cancer treatment, selective inhibition of an over-expressed gene via RNAi requires a valid delivery strategy that ameliorates siRNA pharmacokinetic profile. In particular, ~~the siRNA~~ poor stability in biological fluids and ~~the~~ low cellular uptake impaired its direct use in clinical trials. In literature, several approaches for siRNA delivery *in vitro* and *in vivo*, such as viral vectors,(15) hydrodynamic injection, cationic polymer and lipids (REF) are reported. However, only few have demonstrated clinical applicability because of the potential toxicity and poor stability in biological fluids. Therefore, the translation of siRNA to the clinical setting required the development of a suitable delivery system.

We previously reported bioreductive polymeric micelles (PM) for siRNA delivery based on siRNA conjugated to PE via a disulfide linkage Earlier, to prepare stable nanopreparations of siRNA, PEG-PE-based polymeric micelles (PM) containing siRNA reversibly conjugated with phospholipid via a disulfide linkage, have been designed in our lab (Musacchio T et al.). This strategy is based on the dramatically higher concentration of reductases in the tumor micoenvironment over normal tissues (ref) and offers the advantage to protect the siRNA from degradation *in vitro* by a facile chemical conjugation and at the same time. , the cleavable disulfide bonds linked to the siRNA, allow to liberate the original siRNA when inside the cell for target-specific gene silencing. In addition, the siRNA conjugated can be incorporated, by the PE moiety, in a non toxic delivery system, such as PEG-PE PM, previously demonstrated by our group (ref) and others (ref) to be stable in physiological conditions and to accumulate in areas with an abnormal vascularization, i.e. tumors, via the EPR effect.

Here, we use the system for anti survivin siRNA delivery. attempted to formulate a survivin siRNA reversible modified in nanosizer PEG-PE PM for cancer treatment. *In vitro* cytotoxicity and survivin

protein levels studies have revealed the ability of the system developed to inhibit efficiently the cellular growth and to down-regulate the survivin in different cancer cell lines. In a second phase, we investigated the potential of combination therapy with survivin siRNA and a chemotherapeutic agent, PXL, you should use only one abbreviation. The clinical effectiveness of PXT, an agent widely used in clinic for the treatment of several tumors, is often hampered by acquired drug resistance of cells (Singla AK et al., Int J Pharm 2002). Since sensitization to paclitaxel by survivin down-regulation has been reported, we proposed to evaluate co-treatments of PTX and anti survivin siRNA. Cells were either subjected to survivin siRNA before PTX treatment or treated with PM encapsulating PTX and siRNA. There are different studies indicating that siRNA mediated survivin down-regulation is able to reduce tumor cell proliferation and enhanced apoptotic response in tumor cells to PXL (Shen J et al. Biomaterials. 2012, Qinglian Hu, et al., Biomaterials, 2012). In light of these evidences, we investigated the potential of the survivin down regulation by survivin siRNA PM on the chemosensitization of the cells to PXL. Finally, we evaluated the possibility to co-encapsulated survivin siRNA and PXL in PM, for multifunctional therapy in resistant cancer cell lines.

Our results suggest that the newly developed survivin siRNA PM greatly sensitize the cells to PXL treatment and the simultaneous delivery of survivin siRNA and PXL, by using PM, enhance significantly the tumor response to PXL in resistant cancer cell lines.

Materials

A siRNA anti-survivin with the following sense sequence 5'-GCAUUCGUCCGGUUGCGCUdTdT-3' and a siRNA scrambled with the following sense 5'-AUGAACUUCAGGGUCAGCUdTdT-3' sequence were used (ref). Both the siRNA modified at the 3′-end of the sense strand with N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) were purchased from Thermo Scientific Dharmacon (Pittsburgh PA, USA). The Paclitaxel (PXT) was purchased from LC Laboratories (Woburn MA, United States). The 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (PE-SH, MW 731) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethylene glycol))-2000] (PEG-PE_{2000}) were from Avanti Polar Lipids (Alabaster, AL). The d-Salt dextran desalting column was from Pierce (Rockford, IL, USA). Acetonitrile (HPLC grade), analytical grade chloroform (CH3Cl), DMSO, methanol (MeOH) and Triton X-100 were supplied by Sigma Aldrich (Saint Louis, MO). The human total survivin immunoassay, Surveyor IC, was purchased from R&D System (Minneapolis, MN). RNase/DNase-free water was obtained from MP Biomedicals (Solon, OH), the phosphate saline buffer (PBS) 10× solution and bovine serum albumin (Fraction V) were from Fisher Scientific (Fair Lawn, NJ). β-tubulin antibody (G-8) was from Santa Cruz Biotechnology (Dallas, Texas, USA). Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Mouse IgG (H+L) was provided by Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Hoechst 33342 trihydrochloride, trihydrate, was purchased from Molecular Probes (Eugene, Oregon, USA). Vecta Shield mounting medium for fluorescence, H-1000, was from Vector Laboratories, Inc. (Burlingame, CA). The human total survivin immunoassay, Surveyor IC, was purchased from R&D System (Minneapolis, MN, USA). The RNase/DNase-free water was obtained from MP Biomedicals (Solon, OH, USA) and the phosphate saline buffer (PBS) 10× solution was from Fisher Scientific (Fair Lawn, NJ, USA).

Cell Culture

Ref for skov3-tr, Human ovarian cancer cell lines (A2780) and human breast cancer cell lines (MDA-MB231) were grown in DMEM, in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. Human ovarian cancer cell lines sensitive (SKOV3) and multi drug resistant (MDR) (SKOV3-tr) were grown

in RPMI1640 complete, in a humidified atmosphere of 95% air/5% CO2 at 37 °C. DMEM, RPMI1640 and supplements (fetal bovine serum, penicillin, streptomycin, and amphotericin B, Trypan Blue solution and Trypsin were from CellGro (Kansas City, MO).

Methods

Synthesis of survivin siRNA-S−S-PE Conjugate

The survivin siRNA-S−S-PE conjugate was synthesized as previously described by Musacchio T. *et al.* 2010 (REF). Briefly, an aqueous solution of the SPDP-activated survivin siRNA (20 nmol in 120 µL of RNase/DNase-free water), was added dropwise to a solution of PE-SH (2 µmol) in DMSO and $CHCl_3$ (total volume of organic solvents 350 µL). The reaction was carried out for 48 h at room temperature with continuous shaking. The un-reacted reagents were removed by desalting column. The collected samples containing the survivin siRNA-S-S-PE conjugate were freeze-dried for overnight. After freeze-drying, the survivin siRNA-S-S-PE conjugate was hydrated with PBS pH 7.4 at a final siRNA concentration of x and ultracentrifuged for 1 min at 14.5 × 1000 rpm to further remove mixed solvents and/or PE-SH. The survivin siRNA-S−S-PE conjugate was stored at −20 °C. The amount of survivin siRNA-S−S-PE conjugate after purification was determined by absorbance at 260 nm using a Nanodrop (model)Nanodrop at 260 nm.

Incorporation of survivin siRNA-S−S-PE in PEG-PE Micelles

The PEG-PE_{2000} micelles containing survivin siRNA-S-S-PE were prepared by hydration of a thin polymeric film. (Sawant and Torchilin, Chapter 9). In particular, PEG-PE_{2000} was dissolved in chloroform (20 mg/ml) and the resulting solution was added to a 50 ml round-bottom flask. The organic solvent was removed under reduced pressure by a rotary evaporator under nitrogen atmosphere, followed by freeze-drying. Then, the polymeric film was hydrated with 1 ml of survivin siRNA-S-S-PE in phosphate buffer at pH 7.4 at different PEG-PE_{2000}/survivin siRNA-S-S-PE weight ratio (1:200, 1:500, 1:750). The resulting dispersion was gently vortexed to form mixed micelles, so-called survivin siRNA PM. PEG-PE_{2000} PM containing scrambled siRNA-S-S-PE and plain PM were prepared similarly. Each formulation was prepared in triplicate.

Co-encapsulation of PXT and survivin siRNA-S−S-PE in PEG-PE_{2000} PM

PTX was incorporated in survivin siRNA PM as follow. Briefly, an organic solution of PTX in methanol (1 mg/mL) was added to the PE-PEG_{2000} mixture in chloroform. The final concentration of PTX into micelles was 1% w/w compared to the polymer. The incorporation of survivin siRNA-S−S-PE into these micelles was determined as reported above. The encapsulation efficiency of PXT in PM was determined as reported by Torchilin et al. (REF).

Characterization of Polymeric Micelles

The mean diameter of PM containing survivin siRNA-S-S-PE alone or in combination with PXT, was determined at 20°C by dynamic light scattering (DLS) using a Zeta Plus Instrument (Brookhaven Instrument Co., Holtsville, NY). Briefly, each sample was diluted in deionizer/filtered water (0.22 µm pore size, polycarbonate filters, MF-Millipore, Microglass Heim, Italy) and analyzed with detector at 90° angle. As 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 measures. For each formulation, the mean diameter and P.I. were calculated as the mean of three different batches.

siRNA-S−S-PE Conjugate Incorporation Efficiency in PEG-PE_{2000} PM

The quantitative analysis of survivin siRNA-S-S-PE in PM was carried out by high-performance size-exclusion chromatography and size-exclusion high-performance liquid chromatography (SEC-HPLC) and reverse phase HPLC (RP-HPLC). In the case of SEC-HPLC, Waters 510 HPLC pump system) equipped with a Shodex protein KW-804 column (Showa Denko, Japan) and an UV detection at 280 nm, was used. The mobile phase was composed of 50 mM NaCl and 50 mM Tris-HCl (pH 8.0) and the flow rate at 1.0 mL/min. (Hitachi, L-7450A). The RP-HPLC analysis was carried out as reported by Musacchio T. et al. (Bioconjugate Chem., Vol. 21, No. 8, 2010) The siRNA-S-S-PE loading efficiency into PM was evaluated by ratio of the area under the peaks at the same retention time (tr ca. 10 min) of survivin siRNA-S-S-PE not loaded in PM and the siRNA-S−S-PE conjugate theorically added in the PM. As a control, plain PM were also analyzed. To confirm the data collected by HPLC-SEC, we analyzed the same samples also by RP-HPLC. Then, we evaluated the effect of the presence of PXT in PM on the loading efficiency of survivin siRNA-S-S-PE. In particular, the quantitative analysis of survivin siRNA-S-S-PE and PXT was determined by SEC and HPLC, respectively.

Cell viability assay

A2780, MDA-MB 231, SKOV3 and SKOV3-tr cells were seeded at a density of 3×10^3 cells *per* well in 96-well culture plates for 24 h under the condition of 5% CO_2 at 37 °C. After 24 h, the cells were treated with various concentrations of survivin siRNA-S−S-PE free or in PM, scrambled siRNA-S-S-PE in PM and plain PM, in serum contained media. The final concentration of siRNA-S-S-PE was in the range of 200 to 17.6 nM. After 6 h the medium was replaced with fresh media and the cells were incubated until the 48-hour-time point was reached. Cells without treatment were used as control. The cell viability was determined by Cell Titer Blue assay following manufacturer's protocol [REF]. The experiments were done in triplicate on three different sample preparations.

Survivin protein Assay

A2780, MDA-MB231, SKOV3 and SKOV3-tr cells ($3 \cdot 10^4$ cells per well) were seeded into 48-well plates. Cells were treated with survivin siRNA PM at a final concentration of 200 nM in serum containing medium at 37 °C for 6 h. Cells were washed once with fresh media and maintained in fresh medium until 48 h. Cells were rinsed three times with PBS and treated with 200 µl of cold lysis buffer (R&D system). Cells were rinsed three times with PBS and treated with 200 µl of lysis buffer for 30 minutes on ice. Cell lysates were collected, vortexed, and incubated on ice for other 15 minutes twice. Cell debris was removed by centrifugation at 2000g for 5 min, and protein concentrations were determined by BCA assay after 6-fold dilution in PBS. Samples were added into captured antibody pre-coated 96-well plates, and human survivin was assayed by ELISA after 6-fold dilution in assay buffer (R&D system).

Chemosensitization Study

MDA-MB231, SKOV3 and SKOV3-tr cells ($3 \cdot 10^3$ per well) were seeded into 96-well plates. Cells were treated with survivin siRNA PM at the concentration of 200 nM for 6 h. After 6 h the medium was replaced with fresh media and the cells were incubated until 48 h. Cells were then exposed to different concentrations of PXT containing medium for 24 h. Cell viability was detected by Cell Titer Blue assay.

Immunohistochemical staining

SKOV3-tr ($2 \cdot 10^4$ per coverslip) were seeded and grown on glass coverslips coated with 1% gelatin cross-linked with 0.5% glutaraldehyde in a 12-well plate. After 24 h, cells were treated with 200 nM

of survivin siRNA PM for 48 h. Then, cells were exposed to 40 nM of PCL free for 24 h. As control PCL free (40 nM), survivin siRNA PM and untreated cells were used. Cells were washed three times with PBS and then were fixed with 4% paraformaldehyde. Fixed cells were made permeable with 0.2% Triton X-100 in PBS and incubated with a monoclonal antibody against β tubulin (1:10) for 1 h in 1% bovine serum albumin/PBS. After washing, cells were incubated with a secondary FITC-labeled mouse-immunoglobulin G targeting antibody (1:100) for 1 h in 1% bovine serum albumin/PBS. After washing, cells were incubated with Hoechst 33342 (5 μM) for nuclear staining. After three washing steps of 5 minutes per each, coverslips were mounted in Vecta Shield (Vector Laboratories, Burlingame, CA)…**confocal you can write as Swati z-stack imaging was performed using a Zeiss LSM 700 microscope equipped with xx camera.**. Images were captured by a digital camera (Sensicam, Cooke, Tonawanda, NY), applying identical exposure periods, through a 10 or 40 air lens and analyzed and processed with Slidebook software (version 4.0; Intelligent Imaging Innovations, Denver, CO).

***In vitro* cytotoxicity of PM containing survivin siRNA-S-S-PE and PXT in combination.**

SKOV3-tr cells ($3 \cdot 10^3$ cells/well) were seeded in 96 well plates. After 24 h the cells were treated with different concentrations of survivin siRNA /PXT PM for 6 h in serum contained media. After 6 h the medium was replaced with fresh media and incubated until 72 hrs. Same concentrations of PXT free, survivin siRNA PM, scrambled siRNA PM were used as control. The viability was assessed by Cell Titer Blue assay.

Results

Synthesis and Characterization of survivin siRNA-S-S-PE PM

In this study, we proposed a new strategy to stabilize and deliver a siRNA against survivin in cancer cells. In a first part of the work, we synthesized a survivin siRNA conjugated with a phospholipid (PE-SH) by introducing a disulfide linkage at the 3′-end of the modified siRNA SPDP sense strand. We evaluated the binding efficiency of the conjugate and the cleavability of the disulfide linkage under reductive condition. After purification a conjugate binding efficiency of about 90%, corresponding to about 19 nmol/ml, was achieved. Then, survivin siRNA-S-S-PE conjugate was incorporated in PM. In the first phase, we optimized the weight ratio (conjugate to PEG-PE lipid) required to obtain xx tested different weight ratio between survivin siRNA-S-S-PE and PE-PE-PEG_{2000}, in order to obtain PM characterized by a narrow size distribution and the highest siRNA-S-S-PE incorporation efficiency. More specifically, In particular, we prepared PM at a survivin siRNA-S-S-PE/PE-PEG_{2000} weight ratio of 1:200, 1:500 and 1:750, respectively. As reported in Table 1, at the ratio tested, in all the cases, the PM were characterized by a mean diameter of about 20 nm and a narrow size distribution, with a P.I. ≤ 0.2. The incorporation efficiency of survivin siRNA-S-S-PE in PM was determined by SEC-HPLC. The data are summarized in Figure 1. As shown in Table 1, the highest loading efficiency, about 50%, was reached only with PM prepared with the highest amount of polymer, namely with a weight ratio of 1:750. These data were confirmed by RP-HPLC (data not shown). Due to these characteristics, this formulation was selected for the subsequent studies. In the following step, the possibility to co-encapsulate survivin siRNA-S-S-PE and PXT in PM was investigated. In particular, attention was focused on the effect of PXT on the technological characteristic of PM, i.e. size and the survivin siRNA-S-S-PE loading efficiency. As reported in Table 2, when PXT was added to survivin siRNA PM, no change in the size and in the loading efficiency was observed. In particular, PXT-containing PM were characterized by a mean diameter of about 22

nm with a narrow size distribution (PI< 0.2). HPLC analysis of non-encapsulated survivin siRNA-S-S-PE and PXL showed an encapsulation efficiency of about 50% and 70%, respectively.

Here you could show PTX incorporation using fluorophore-labeled PTX followed by HPLC detection of siRNA (UV) and PTX (fluorescence) with same retention time by SEC (Lin has Oregon green PTX). Moreover, you could show delivery of fluorescent PTX by FACS.

Effect of survivin siRNA PM on the viability of cancer cell lines.

The effect of survivin siRNA-S-S-PE incorporated in PM on the growth inhibition of different human cancer cell lines, namely breast (MDA-MB231), ovarian (A2780, SKOV3), taxol resistant ovarian cell (SKOV3-tr), was investigated by Cell Titer Blue assay. In the Figure 2, the cells viability (%) after treatment with 200 nM of survivin siRNA-S-S-PE in PM in the different cell lines analyzed after 48 h, is reported. In all the cases, when considering plain PM, scrambled siRNA-S-S-PE in PM and free survivin siRNA-S-S-PE, any significant cytotoxic effect was observed. On the contrary, when using survivin siRNA-S-S-PE incorporated in PM, we found a significant anti-proliferative effect in all the sensitive cell lines. The highest cell growth inhibition was observed on ovarian cancer cells, especially on A2780, reaching a reduction of cell viability of about 70%. No toxicity of PM or micelles prepared with irrelevant siRNA was observed indicating a RNAi-mediated toxicity. In PTX-resistant SKOV3 tr, no significant reduction of the cell viability was found, inaccordance with (ref).

Survivin protein levels

Then, we evaluated the levels of survivin protein in cells treated with survivin siRNA-S-S-PE in PM by ELISA. As illustrated in Figure 3, after treatment with PM containing survivin siRNA-S-S-PE, a significant down-regulation of survivin, of about 30-40%, was observed in all the cell lines treated. Noteworthy, a significant x% reduction of survivin levels were observed also in survivin – overexpressing SKOV3-tr cells. In cells treated with survivin siRNA-S-S-PE free, and PM containing scrambled siRNA-S-S-PE, any significant down-regulation of the levels of survivin was observed.

Chemosensitization Study.

In order to evaluate whether the down-regulation of survivin mediated by the treatment with survivin siRNA PM is able to sensitize the cell to PXL, chemosensitization studies were performed. In particular, survivin siRNA PM pre-treated MDA-MB-231, SKOV3 and SKOV3-tr cells were exposed to increasing concentrations of PXL and the resultant cell viabilities are summarized in Figures 4 a, b and c, respectively. As detailed in Figure 4, in all the cell lines tested, no toxicity was detected with free PTXat a dose range x the treatment with PXL free at the concentrations between 40 nM and 3.5 nM for 24 h, didn't yield any anti-proliferative effect. Increasing the incubation time to 72h lead to significant toxicity in PTX-sensitive cell lines wherease none was detect with x. At this concentration, a cytotoxic activity is showed only after 72 h of treatment in sensitive cell lines (data not showed). Indeed, in SKOV3-tr, even after 72 h, any significant cytotoxicity was observed. As proposed in our hypothesis, , the down regulation of survivin by pre-treatment of the cells with survivin siRNA PM, strongly sensitized the cells to PXL. In particular, a significant increase of the PXL cytotoxicity was observed after only 24 h of cell exposure to the drug (Figure 4). These results were confirmed by immunofluorescence analysis. Figure 5 shows the influence of the pre-treatment with survivin siRNA PM on the PXL activity on microtubules stabilization in the resistant cancer cell line, SKOV3 tr. In untreated cells, in cells treated for 24 h with 40 nM of PXL and cells treated with survivin siRNA PM for 48 h (figure 5. A,B,C, respectively), microtubules appear "healthy" with elongated and fibrillary extensions from around the nucleus to the cell periphery. In contrast, the

down regulation of survivin mediated by pre-treatment with survivin siRNA PM for 48 h, sensitize SKOV3 tr cells to PXL activity on microtubule organization (Fig. 5D). In particular, microtubules appear disassembled, characterized by a loss of fibrillary extensions as evident by the dense organization of microtubule network around the nucleus (ref). The nucleuses begin to fragment, reflective of cell death. These results confirm the ability of survivin down regulation to induce an improvement of PXL activity on microtubule destabilization.

Multifunctional therapy: survivin siRNA-S-S-PE and PXL co-encapsulated in PM

Finally, we investigated the effect of survivin siRNA-S-S-PE and PXL co-loaded in PM on the viability of a taxol-resistant cancer cell lines, SKOV3-Tr. In particular, we studied the effect of increasing concentrations of survivin siRNA-S-S-PE and PXL co-encapsulated in PM on the cell viability of SKOV3-tr cells. In Figure 6, the cell viability (%) 72 h after the treatment with free PXL, survivin siRNA PM and survivin siRNA/PXL PM, is reported. After treatment with free PXL, any significant inhibition of the cell growth was observed. On the contrary, the simultaneously delivered of PXL and survivin siRNA-S-S-PE by PM to SKOV-3-tr cells lead to significant inhibition in cell growth.

Discussion

Survivin, a member of the IAP family, is considered an attractive target for cancer therapy. Survivin, not usually detected in normal adult tissue, is over-expressed in malignant tumors where plays a key role in hampering apoptosis, promoting cell proliferation, mitosis and angiogenesis (Altieri 2008, REF). Moreover, there are evidences that survivin up-regulation may be a predictive factor to determine drug-resistance of human tumors (REF Survivin KEY REGULATOR OF MITOSIS AND APOPTOSIS). In particular, in different studies, it has been shown that survivin inhibits apoptosis induced by several chemotherapeutic agents (REF). Other approaches reported xx On the contrary, it has been demonstrated that the inhibition of survivin reduces tumor growth potential and enhance tumor cell response to anticancer agents, such as PXL (Biomaterials), doxorubicin (REF) cisplatin (Leaf Huang, Sharma H et al., Cancer Biol Therapy 2005) as well as immunotherapy and ionizing radiation (Kanwar JR et al., J Nat Cancer Inst, 2001, Sah NK et al., Oncol Biol Phys 2006). Recently, , pre-clinical studies targeting survivin with different approaches, including the use of antisense oligonucleotide, (Talbot et al. 2008), small molecules (REF) and siRNA (REF) are in progress. RNAi direct against survivin, can overcome the multidrug resistance and sensitize the human tumors to chemotherapeutic drugs (Shapira A et al., drug resist update 2011). Carvalho et al., firstly used RNAi to repress survivin in HeLa cells, showing the suppression of survivin in the cells for more than 60 hours after transfection with survivin-specific siRNA. Lui *et al.* demonstrated that, survivin siRNA treatment caused a specific and strong decrease of survivin levels that was associated with decreased cell growth and spontaneous apoptosis in human pancreatic cancer cell lines. Unfortunately, siRNA is characterized by a poor stability and low cellular penetration *in vivo*. This disadvantageous pharmacokinetic profile strongly hampers the achievement of effective concentrations in intracellular site of action. Therefore, delivery strategies that enhance intracellular uptake and stabilize siRNA *in vivo* need to be devised. Many researchers proposed different approaches to deliver siRNA to target cells using nano-sized carriers such as cationic liposomes, cationic polymers and lipids [Z. Hassani; B. Urban-Klein; M. Ito; W.J. Kim; C. Zhang]. However, these strategies are restricted to experimental research and still difficult to transfer to clinical applications (check update and reviews). In our lab, a new method addressed to stabilize and deliver the siRNA, was previously proposed. In particular, a

siRNA “reversible” conjugated with a phospholipid by a disulfide linkage was synthesized (Musacchio T., et al.,). The resulting modified siRNA was incorporated in nano-sized PE-PE-PEG_{2000} PM by the hydrophilic moiety. The chemical modification of the siRNA, essential to incorporate the siRNA in PM, increased the *in vitro* stability of the siRNA against nuclease and, at the same time, to release the active siRNA from PM once within the cells. PE-PE-PEG_{2000} micelles has gained in the last years much attention due to their high stability *in vivo*, the possibility to efficiently encapsulate poor soluble drugs, such as PXL (47-49 Torchilin and Sawant), and the ability to accumulate in tumors via the EPR effect. In the present work, a siRNA against survivin was modified with a PE moiety and then incorporated in nanosized PM in order to deliver the survivin siRNA in cancer cells. The survivin siRNA-S-S-PE was synthesized with a 90% of reaction yield. We first optimized the formulation for co-delivery of PTX after selection of parameters xxIn the following phase of the work, siRNA-S-S-PE was incorporated in PM. In this phase of the work, technological parameters, such as concentration of polymer, polymer/survivin siRNA-S-S-PE weight ratio were optimized in order to obtain PM characterized by a colloidal size and a high survivin siRNA encapsulation in PM. We obtained survivin siRNA PM with a mean size of about 20 nm and a narrow size distribution. The survivin siRNA was efficiently incorporated in PM only when a higher amount of PE-PEG_{2000} was used. In particular, the use of a survivin siRNA/PE-PEG_{2000} weight ratio of 1:750 allowed to reach an encapsulation efficiency of about 50 %. Due the increased encapsulation found when increasing the PE-PEG_{2000} concentration, the hydrophobic interaction between the PE of siRNA-S-S-PE and that of the PEGylated lipid was considered the driving force for the siRNA encapsulation into PM. Then, we studied the effect of survivin siRNA PM in different cancer cell lines. Cell culture studies showed a significant cytotoxic effect of survivin siRNA when delivered by PM. In particular, the treatment with survivin siRNA PM, elicited an important decrease of cell viability and a significant down-regulation of survivin protein levels. It is noteworthy that in a resistant cancer cell line (SKOV3-tr), although any cytotoxic effect was observed, a significant down-regulation of survivin was achieved following treatment with survivin siRNA PM. In all the cases, the treatment with a siRNA scrambled conjugate in PM and plain PM didn’t yield any significant effect. In a second phase, we investigated the potential of combination therapy with survivin siRNA and a chemotherapeutic agent, PXL. PXL, exhibit its anticancer activity by promoting tubulin polymerization and stabilizing microtubules, which results in mitotic G2/M arrest and apoptosis (Gallagher BM Jr). PXT provides treatment benefit in a wide range of human tumors (REF). However, the clinical effectiveness of PXT is often hampered by acquiring drug resistance of cells (Singla AK et al., Int J Pharm 2002). There are different lines of evidence indicating that survivin inhibition enhances the antitumor activity of PXL (Shen J et al. Biomaterials. 2012, Qinglian Hu, et al., Biomaterials, 2012). With this in mind, we investigated the potential of the survivin downregulation by survivin siRNA PM on the chemosensitization of the cells to PXL. In particular, we studied the effect of survivin siRNA pre-treatment on the PXL chemosensitization of sensitive and resistance cancer cell lines. In sensitive cancer cell lines (MDA-MB231 and SKOV3) the pre-treatment with survivin siRNA for 48 h before expose the cell to PXL, elicited a significant improvement of the citotoxicity of the drug. In particular, a significant cytotoxic effect of PXL was observed earlier, namely after only 24 h of PXL exposition. In the same condition, but without pre-treatment with survivin siRNA PM, PXL showed a cytotoxic effect after 72 h of treatment. It is noteworthy that, in a human taxol cancer resistant cell line, SKOV3-tr, even after 72 h any significant cytotoxicity was observed following treatment with PXT alone. The pre-treatment of SKOV3-tr with survivin siRNA PM and the consequent down regulation of survivin lead to a significant cell sensitization to PXL. To confirm the key role of the down regulation of survivin on PXL sensitization in SKOV3 tr cell line, we studied the microtubules organization by immunostaining the cells treated for β-tubulin. Survivin has been shows to bind polymerized microtubules in vitro where, presumably, stabilize the mitotic spindle (Li F. et al., Nature

1998). Tran J et al., (2002) demonstrated a role of survivin to maintain the integrity of the microtubule network in HUVECs cells. In particular, they showed that overespression of survivin could preserve the microtubule integrity of HUVECs treated with PXL. Our results, suggest that survivin siRNA PM mediated survivin down regulation, could counteract the PXL chemoresistance in SKOV3 tr cell lines. In fact, in absence of pre treatment with survivin siRNA PM, any change on the microtubules morphology was observed after exposition with PXL. The microtubules appeared like "healthy" cells, with elongated and spread out tubulin filaments. Interestingly, in survivin siRNA PM pre treated cells, the exposition with only 40 nM of PXL for 24 h, yielded a significant change of microtubules morphology. In particular, SKOV3 tr cells displayed a significant lack of microtubule organization reflective of cell death. Our data, therefore, support previous work demonstrating that survivin overexpression can negatively influence the sensitivity of cancer cell line to chemotherapeutic agents, such as PXL. Moreover, the use of a siRNA able to inhibit survivin expression could be a powerful tool to potentiate anticancer strategies based on the use of PXT, especially in the case in which chemioresistance can hamper the success of the chemotherapy. However, as expected, a siRNA administered as such in presence of serum have not effect, thus requiring a delivery system. The PM proposed in this study are able to deliver the anti-survivin siRNA into the cells, as demonstrated by the powerful inhibition of survivin expression. Finally, we investigated if the developed PM could also used to co-encapsulate survivin siRNA and PXL in mixed PM, in order to deliver simultaneously the two agents in cancer cells. In a first step, we evaluated if the presence of PXL could affect the technological characteristics of survivin siRNA PM, in terms of size distribution and survivin siRNA encapsulation efficiency. When PXL was added to survivin siRNA PM, any change in the size and in the encapsulation efficiency was observed. The mixed PM co-loading the two agents, were characterized by a narrow size distribution and an incorporation efficiency of survivin siRNA and PXL of about 50% and 70%, respectively. Then, we investigated the *in vitro* activity of the multifunctional PM in a resistant cancer cell line. The results revealed that the simultaneously delivery of PXL and survivin siRNA in the cells lead a significant enhanced of PXT cytotoxicity. In particular, after 72 h of treatment with PM containing the two agents combined, a strong cytotoxic effect was observed. On the contrary, the treatment with PXL free did not elicit any significant effect.

Taken together, these data demonstrated that the survivin siRNA-S-S-PE PM developed in this work are a promising tool to inhibit survivin expression in cancer cells. Moreover, due to the possibility to co-encapsulate lipophilic drugs, such as PXT, this system could be also used to overcome multidrug resistance in the treatment of chemoresistant tumors.

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Conclusions

In this study, a new self-assembling nano-system for the siRNA delivery in cancer therapy was developed in our lab. The developed system efficiently delivered survivin siRNA into different cancer cells, leading to significant down-regulated survivin expression. Interestingly, cells treated with the survivin siRNA, only delivered with the PM, sensitized cells to PXL treatment. In addition, this system proposes a new platform for stimuli-sensitive co-delivery of x allows to co-encapsulate chemotherapeutic drugs and siRNA for multifunctional therapy. Indeed, Interestingly, the use of PM co-delivered PXT and survivin siRNA simultaneously, was able to reverse drug resistance in an aggressive cancer cell line. yielded a strong inhibition of cells growth in a taxol resistant cancer cell line. These results support further studies able to verify the *in vivo* activity of the developed delivery system.

References

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Table 1. Technological characteristics of survivin siRNA PM

Formulations (weight ratio)	Mean Diameter (nm ± SD)	P.I. ± SD	Survivin siRNA incorporation efficiency (% ± SD)
Survivin siRNA PM (1:200)	18.7 ± 3.5	0.191 ± 0.08	26.0 ± 5.0
Survivin siRNA PM (1:500)	18.3 ± 2.0	0.189 ± 0.06	31.0 ± 3.8
Survivin siRNA PM (1:750)	21.5 ± 3.3	0.160 ± 0.05	52.0 ± 1.6

Figure 1. Incorporation efficiency determined by SEC. The degree of the incorporation efficiency was measured by ratio of the area under the peaks of free survivin siRNA-S-S-PE, not incorporated in PM, and the siRNA-S−S-PE theorically loaded in PM at the same retention time (tr ca. 10 min). As a reference, plain PM were used.

Table 2. Technological characteristics of PM co-loaded survivin siRNA-S-S-PE and PXT.

Formulations	Mean Diameter (nm ± SD)	P.I. ± SD	Survivin siRNA incorporation efficiency (% ± SD)	PXL incorporation efficiency (% ± SD)
Survivin siRNA PM	21.5 ± 3.3	0.160 ± 0.05	50.0 ± 1.0	-
Survivin siRNA/PXL PM	25.0 ± 3.6	0.190 ± 0.07	51.0 ±1.5	69.9 ± 2.5

Figure 2. Cell viability in cancer cell lines. Cells were treated with survivin siRNA PM at a final siRNA concentration of 200 nM in serum-containing medium at 37 °C for 48 h. Cellular proliferation studies in presence of survivin siRNA PM, free survivin siRNA-S-S-PE conjugate, PM containing scrambled siRNA-S-S-PE conjugate and plain PM were investigated by Cell Titer Blue assay.

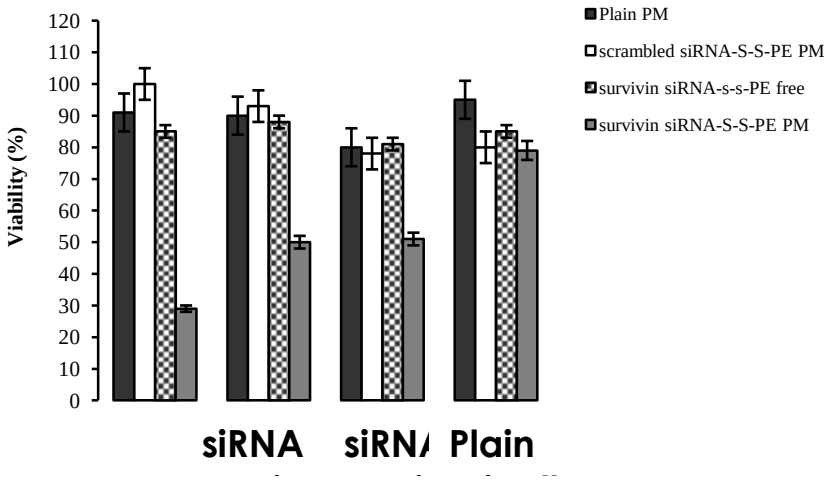

Figure 3. Survivin protein levels in, A2780, MDA-MB231, SKOV3 and SKOV3-tr cells after different treatments. Survivin siRNA was quantified 48 h after treatment by the ELISA method. The data are expressed as ng of survivin protein level on mg of protein.

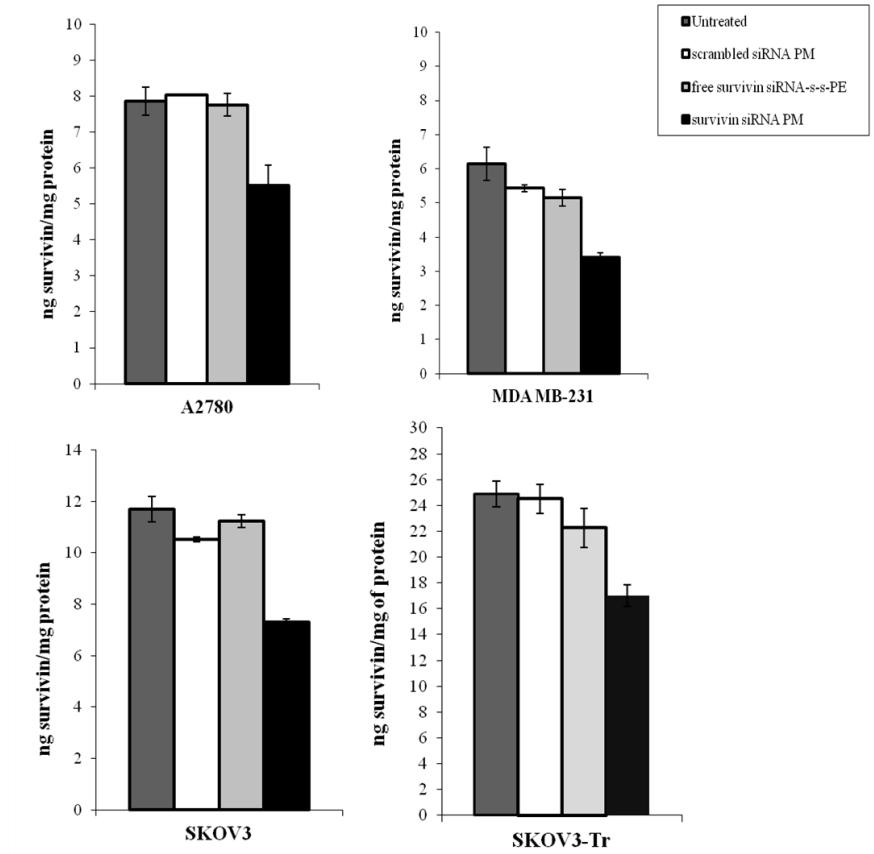

Figure 4. Chemosensitization of MDA MB 231(A), SKOV3 (B) and SKOV3-tr (C) cells mediated by pretreatment of survivin siRNA PM. Cells were incubated with survivin siRNA PM (range of concentration from 200 to 17.5 nM) at 37 °C for 6 h. Forty eight hours later, cells were challenged with various concentrations of paclitaxel. The viability of cells was measured 24 h later by CTB assay.

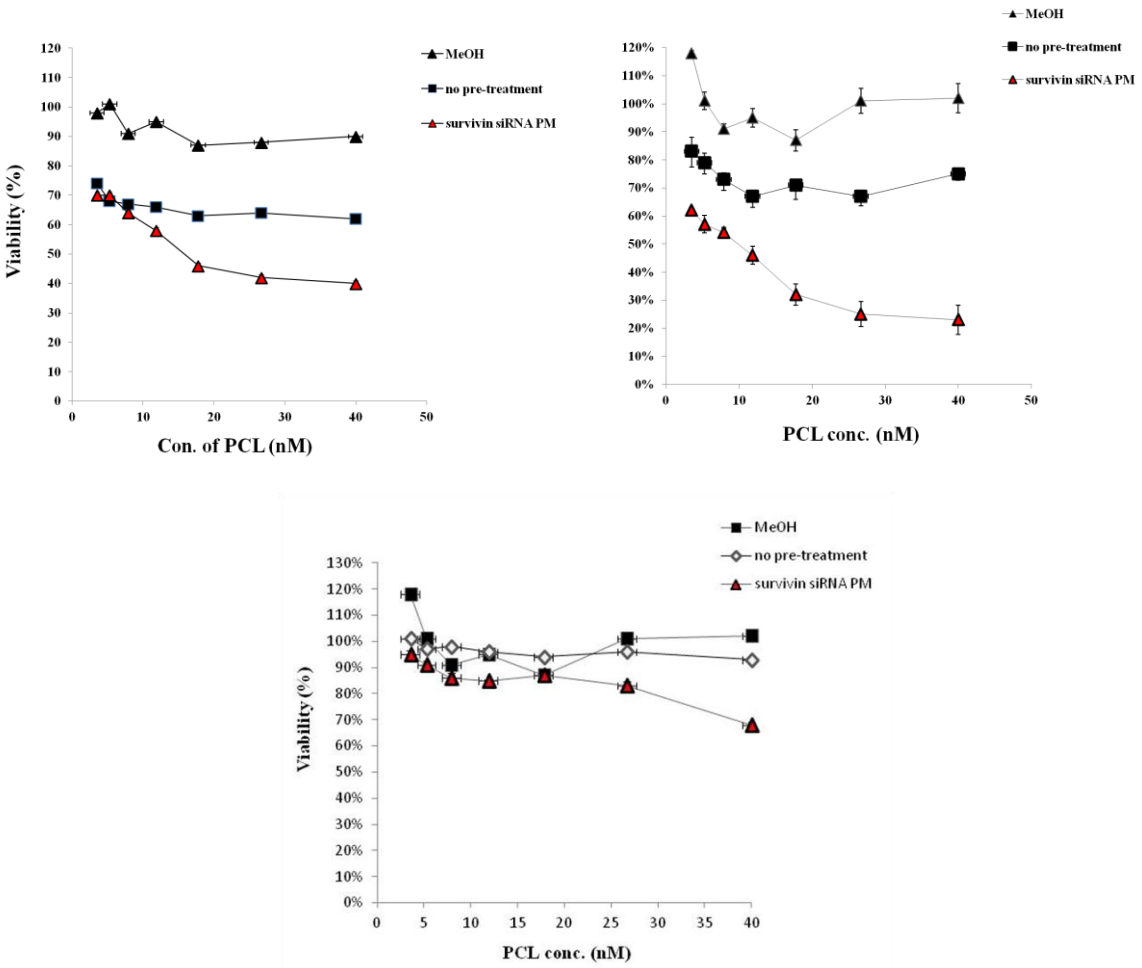

Figure 5. Effect of PXL on microtubule stabilization after down regulation of survivin in SKOV3 tr cells you should add a scale bar

SKOV3 tr cells were pretreated with 200 nM survivin siRNA PM for 48 h followed by treatment with 40 nM of PXT for 24 h. Cells were then stained for β-tubulin (green). The nucleus (blue) is stained with DAPI. A-D: Representative images of three independent experiments showing organization of microtubules.

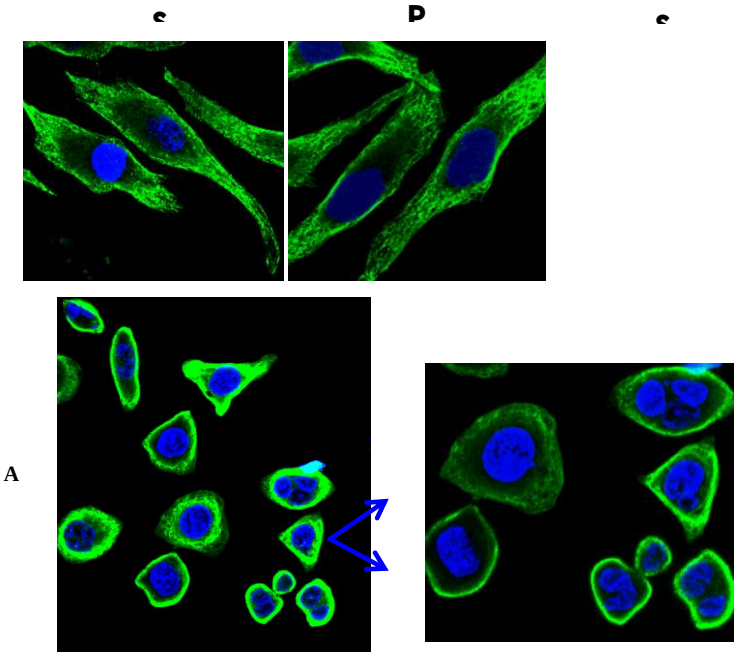
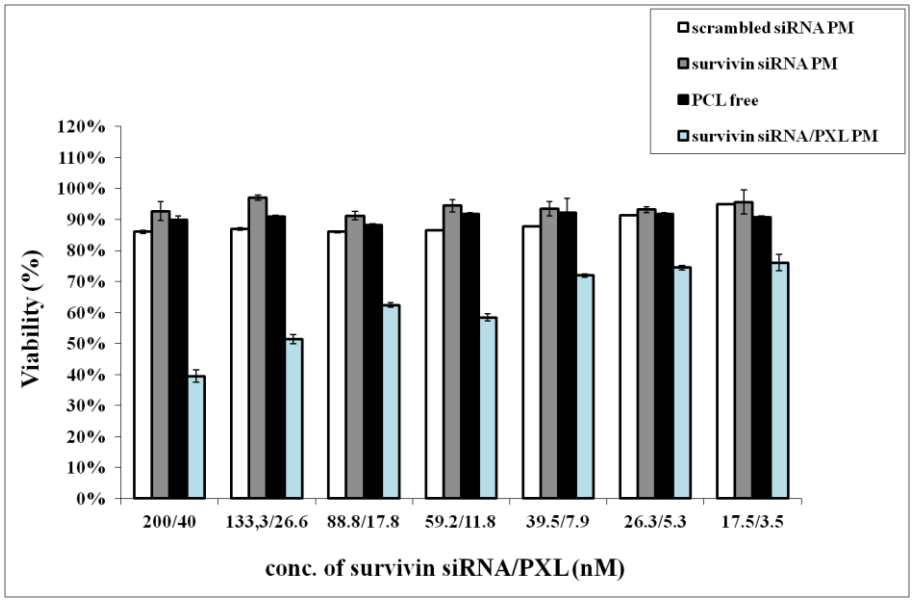
Figure 6. Cell viability in SKOV3 tr cancer cell lines. The cells were treated with the different formulations at 37°C for 6 h. After 72 h the cells viability was measured by Cell Titer Blue assay.