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Lapatinib/Paclitaxel polyelectrolyte nanocapsules for overcoming multidrug resistance in ovarian cancer

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Abstract

The sonication-assisted layer-by-layer (SLBL) technology was developed to combine necessary factors for an efficient drug-delivery system: (i) control of nanocolloid size within 100 – 300 nm, (ii) high drug content (70% wt), (iii) shell biocompatibility and biodegradability, (iv) sustained controlled release, and (v) multidrug-loaded system. Stable nanocolloids of Paclitaxel (PTX) and lapatinib were prepared by the SLBL method. In a multidrug-resistant (MDR) ovarian cancer cell line, OVCAR-3, lapatinib/PTX nanocolloids mediated an enhanced cell growth inhibition in comparison with the PTX-only treatment. A series of in vitro cell assays were used to test the efficacy of these formulations. The small size and functional versatility of these nanoparticles, combined with their ability to incorporate various drugs, indicates that lapatinib/PTX nanocolloids may have in vivo therapeutic applications.

From the Clinical Editor: The efficacy of Lapatinib/Paclitaxel polyelectrolyte nanocapsules is described in this study in cell cultures of multidrug-resistant ovarian cancer. If in vivo studies also result in similar efficacy and low toxicity, this may represent a viable avenue to address such malignancies.

Key words: Ovarian cancer; Lapatinib; Paclitaxel; Nanocapsules; Sonication-assisted Layer-by-Layer

Ovarian cancer is one of the most common gynecological malignancies in women and the leading cause of gynecological cancer-related deaths in developing countries.[1] Due to the absence of early symptoms, the disease is often undiagnosed until the advanced stage, when it has already spread to distant sites. The picture is complicated by the failure of the currently available therapies to prove effective. The major obstacle consists of a broad variety of acquired drug-resistance mechanisms, some of which have been well described for many of the chemotherapeutics currently used in clinical practice, including Paclitaxel (PTX).[2] PTX is a drug of natural origin isolated from the bark of *Taxus brevifolia* and used for ovarian cancer and breast cancer clinical treatment.[3] PTX promotes microtubule assembly and stability, thus leading to the disruption of the normal microtubule network required for mitosis.

In past years, many efforts have been made to overcome drug resistance associated with PTX treatment, and a number of specific factors affecting the drug response have been identified at many levels.[4-8] However, this has not yet resulted in the cure of patients with recurrent disease. Furthermore, PTX shows poor

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water solubility, and it is formulated for its current clinical administration in a mixture of Cremophor EL/absolute ethanol (50% v/v) that has been associated with several side effects, including nephrotoxicity and neurotoxicity.[9]

To overcome toxicity, to increase bioavailability, and to control drug release, several approaches for packaging PTX are under investigation with numerous advantages over conventional methods and early nanoparticle (NP) products.[10-13] Among the wide variety of novel formulations, previous studies have shown that sonication-assisted layer-by-layer (SLBL) nanoassembly technique can be used efficiently for the nanoencapsulation of poorly soluble anticancer drugs.[14,15]

Under powerful ultrasonication, the air bubble that was dissolved in water underwent the formation and implosion of the cavity, followed by the jet flow. Thus an extreme physicochemical environment was created. Drug crystals were broken into smaller and smaller particles, down to nanoscale. During this process, polyelectrolyte with an opposite charge to that of the pharmaceuticals was added and absorbed onto the drug crystals through electrostatic forces; this prevented any aggregation of the newly formed NPs. Because the ultrasonication is based on the collapse of air bubbles, a bubbling agent such as NH_4HCO_3 was mixed with the polyelectrolyte to increase the intensity of ultrasonication.[14] By using this method, drug NPs of approximately 150 nm ± 30 nm diameter are obtained. In many cases, initial drug particles were negatively charged. After the first layer coating with polyelectrolyte (in this case, polycation), the surface electrical potential become positive and of larger magnitude than the one of the initial particle. Then the second polyelectrolyte (in this case, polyanion) layer was coated, and the surface charge reversed to negative. This regular surface charge alternation demonstrated successful formation of multilayer shell with predetermined architecture and the capability of controlled release by tailoring the number and chemical composition of capsule layers. The SLBL technique can also be applied to combine two drugs in one nanocolloid system for synergistic medical treatment.[16]

We hypothesized that PTX clinical efficacy can be increased through a strategy that combines improvements in PTX cellular delivery and a combination of targeted therapies to inhibit one or more signalling pathways involved in PTX resistance. In particular, ATP-binding cassette (ABC) transporters, such as the multiple drug-resistance transporter multidrug resistance 1 (MDR1),[17] or P-glycoprotein or ABCB1 have been associated with a poor response to chemotherapy.[18] Therefore, MDR1 impairment is likely to have a significant impact on PTX clinical action. However, until now, many P-gp inhibitors failed to show efficacy during preclinical and clinical studies.[19] Recently, lapatinib, an epidermal growth factor receptor (EGFR) and Erb-2 dual tyrosine kinase inhibitor, has been shown to inhibit the function of ABC transporters, including P-gp.[20]

In this study, a multidrug-resistant ovarian cancer cell line, OVCAR-3, has been used to test the efficiency of PTX-loaded nanocolloids in comparison with that of PTX given alone. In addition, we successfully combined PTX and lapatinib in nanocolloids to overcome MDR. Such combination enhanced the cytotoxic efficacy of PTX.

Overall, the results of this study show that PTX-nanocolloids increased in vitro antitumor efficacy of PTX and that the combination with lapatinib can significantly overcome multidrug resistance in ovarian cancer cell lines. These results are encouraging for the development of multifunctional nanocolloids that could be used in the clinical practice.

Methods

Reagents and cell culture

The source of antibodies and inhibitors is provided in Supplementary Materials, available online at http://www.nanomedjournal.com.

The ovarian cancer cell line OVCAR-3 and the breast cancer cell line MCF-7 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovin serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, and cultured at 37°C in a humidified atmosphere of 5% CO_2. Cells were subcultured every 2–3 days.

Preparation of nanocolloids

To build up the nanocapsules for a simultaneous controlled release of two drugs, we used a SLBL technique. Biodegradable chitosan (polycation) and alginic acid (polyanion) were chosen for a biocompatible and biodegradable coating on drug NPs.

PTX nanocolloids

PTX (40 mg), chitosan (6 mg) and NH_4HCO_3 (40 mg) were added to distilled water (30 mL), stirred for 5 minutes and then ultrasonicated in a water/ice bath for 45 minutes (to make PTX/chitosan nanocores). The following LBL self-assembly of alginic acid and chitosan were performed using washless LBL method.[21] After 45 minutes' processing, 4 mL of alginic acid solution (1 mg/mL) was added and sonicated for 25 minutes. This made a second anionic layer in the capsule shell resulting in PTX/chitosan/alginic acid core-shell structure. Chitosan and alginic acid solutions were sequentially added to the PTX nanocolloids maintaining sonication. The coating of chitosan and alginic acid were repeated to make a three-bilayer capsule wall of PTX/(chitosan/alginic acid)$_3$ composition. Particle size and surface electrical ξ-potential were measured after deposition of each next layer, allowing monitoring of the capsule assembly. For each test, the solution samples were centrifuged at 2500 rpm for 20 minutes to remove TiO_2 particle impurities from the sonotrode during powerful ultrasonication.

Dual drug nanocolloids: PTX/chitosan/alginic acid/chitosan/lapatinib/chitosan/alginic acid

The above procedure was used to obtain PTX(chitosan/alginic acid)$_3$ nanocolloids, and 20 mL of lapatinib (0.5 mg/mL) were added followed by the addition of alginic acid (maintaining ultrasonication) to make PTX/(chitosan/alginic acid)$_3$/-lapatinib/alginic acid samples. With this complicated shell architecture, we located PTX in the capsule core and used cationic lapatinib for assembly of the capsule shell. The samples

were centrifuged at 10,000 rpm for 10 minutes to remove the upper liquid.

Nanocolloids characterization

The Brookhaven ZetaPlus Microelectrophoresis Instrument had been applied for monitoring the surface ξ potential and the particle size of drug nanocolloids and nanocapsules. The changes in surface ξ potential allowed monitoring the successful coating of oppositely charged polyelectrolytes.

Hitachi S 4800 field emission scanning electron microscope (FESEM) had been used for imaging morphology and particle size of drug nanocolloids.

Cell viability assay

Cells were seeded at a density of 5×10^3/well in a 96-well plate containing 100 μL of full medium and allowed to adhere to the plate overnight. For determining cell viability, the MTT assay was used. After treatment with PTX or LBL-PTX nanocolloids for 24 hours, the culture medium was aspirated and 100 μL of fresh medium containing 10 μL of MTT solution (stock 5 mg/mL in PBS) was added to each well. Cells were then incubated for further 2–3 hours. After removal of MTT solution, 100 μL of DMSO were added to the wells to dissolve MTT-formazan crystals and maintained in agitation for 15 minutes. Absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 690 nm. The relative cell viability was expressed as a percentage of the untreated control wells. Values are mean ± standard error of the mean (SEM) of three independent experiments performed in triplicate.

Reverse transcription-PCR (RT-PCR)

Total cellular RNA was isolated by Illustra TriplePrep extraction kit following manufacturer's instruction and immediately used. Purified DNA and protein pellet were stored at −80°C for further analysis.

Total RNA (1 μg) was reverse transcribed into cDNA using the high capacity RNA-to-cDNA Master Mix (Applied Biosystems, Monza, Italy). PCR was conducted on a MyCycler thermal cycler (Bio-Rad, Milan, Italy). The final volume of 25 μL included 1 μL of cDNA template, 12.5 μL of PCR Master Mix (Promega, Milan, Italy), and 1 μL of a mix containing primers.

Immunoblotting assay

Whole cell lysates were prepared in RIPA buffer (50 mM Tris-base, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1% protease inhibitor cocktail) and clarified by centrifugation at 13,000 *g* for 15 minutes at 4°C. Protein concentration was determined using the Bradford protein assay. Next, 50 μg of proteins were separated on 10% polyacrylammide gel and transferred to nitrocellulose membranes (Amersham-Biosciences, Milan, Italy). The membranes were blocked overnight in 5% nonfat milk in Tris Buffer Saline and 0.1 % Tween 20 (TBST) buffer at 4°C under agitation, and subsequently probed by the appropriately diluted primary antibodies in blocking buffer. The blots were then incubated with HRP-conjugated secondary antibody for 2 hours at room temperature (20°–25°C). Target proteins were detected by enhanced chemiluminescence reagents and visualized on Hyperfilm ECL films (Amersham-Biosciences).

Confocal microscopy

Exponentially growing ovarian cancer cells were seeded on 25 mM square glass coverslips placed in 35 mM diameter culture dishes. After treatment, cells were fixed for 5 minutes with 3.7% formaldehyde in phosphate-buffered saline (PBS) solution, permeabilized with a 0.1% solution of Triton X-100 in PBS, and incubated for 30 minutes at room temperature with phalloidin-TRITC (Sigma, Milan, Italy). Preparations were mounted in 50% glycerol in PBS and observed under a TCS SP5 laser confocal microscope (Leica Microsystem GmbH, Mannheim, Germany).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.1 (GraphPad Software, San Diego, California). Differences between group means were compared by Student's *t*-test. Data are presented as mean ± SEM. A probability level of $P < 0.05$ was considered statistically significant.

Results

The aim of this study is to develop new strategies based on sonication and LBL technique to overcome multiple drug resistance (MDR) associated with PTX in ovarian cancer. Preliminary experiments were performed to identify cellular targets of PTX action. The identification of these targets will be useful to use them as markers of LBL-nanocolloids action respect to PTX alone.

The ovarian cancer cell line OVCAR-3 was chosen for its drug-resistance phenotype and used as model system in this study. As evaluated by RT-PCR, OVCAR-3 cells constitutively express high levels of TLR-4 (Toll-Like Receptor) and MDR1 (Figure 1, *E*), which were both associated with PTX chemoresistance.[4,6,7] TLR-4 and MDR1 mRNA expression levels in OVCAR-3 were compared with those of the PTX-sensitive breast cancer cell line MCF-7. MDR1 mRNA is greatly expressed in OVCAR-3 with respect to MCF-7 whereas no significant differences were observed for TLR-4, suggesting the involvement of P-gp as the major determinant of PTX resistance in OVCAR-3.

Dose-response studies highlighted the resistant nature of this cell line (Figure 1, *B*). Exponentially growing OVCAR-3 cells were exposed to increasing concentrations of PTX (from 1.5 ng/mL to 5000 ng/mL) for 24 hours and MTT cell viability assay was performed. As shown in Figure 1, the cancer cell line exhibited a characteristic dose-response curve; in fact, treatment with PTX at concentrations above 10 ng/mL did not induce a proportional reduction of cell viability. Comparable findings were obtained in a similar experiment, in which the drug concentration was fixed and the number of cells seeded on the plate was changed (Figure 1, *C*). Ovarian cancer cells seeded at four different concentrations and treated with PTX at 5 μg/mL for 24 hours showed a similar survival ratio for both control and treated cells.

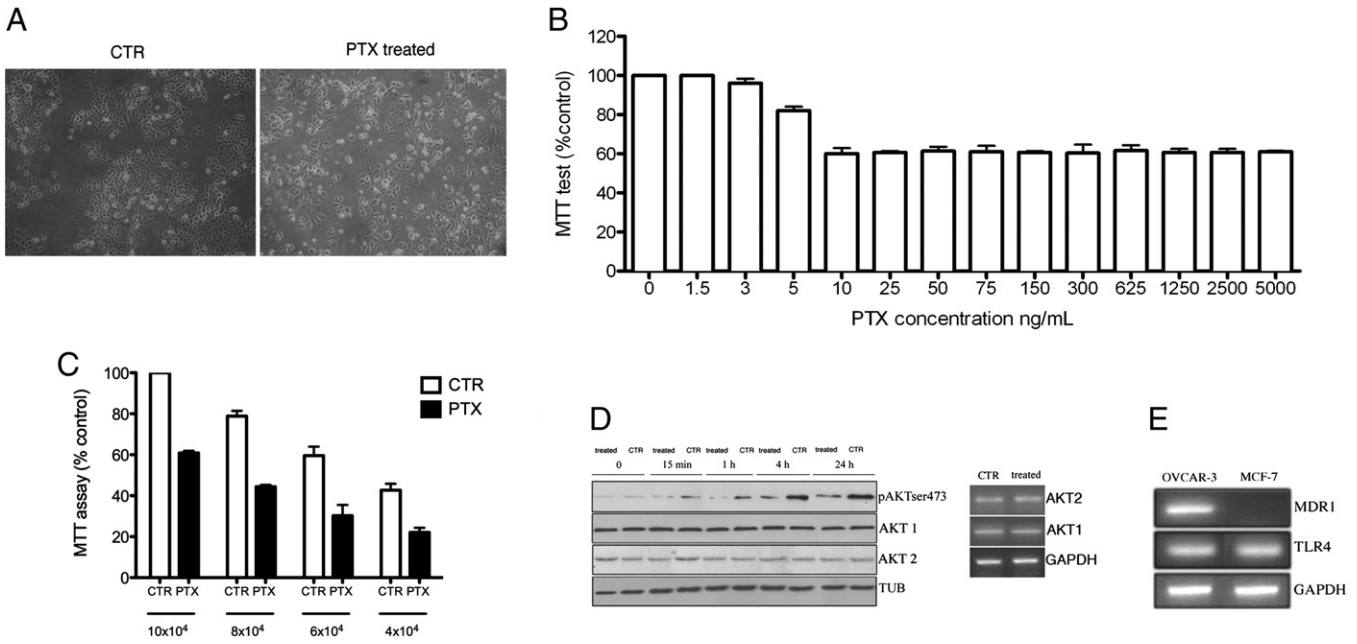

Figure 1. PTX decreased OVCAR-3 cell viability. **(A)** Effects of PTX on OVCAR-3 cell morphology evaluated by phase-contrast microscopy after 24 h of incubation (magnification 10×). Cell death is observed after PTX treatment as evidenced by the increasing number of floating cells. **(B)** PTX reduces cell viability. OVCAR-3 were seeded overnight into 96-well plates and incubated with PTX at the indicated concentrations. After 24 h, cell viability was detected by MTT test. OVCAR-3 demonstrated a plateau in survival at concentrations of PTX above 5 ng/mL. **(C)** PTX reduces cell viability. OVCAR-3 were seeded overnight into 96-well plates at the indicated concentrations and incubated with PTX for 24 h at 5 μg/mL. The response of OVCAR-3 to PTX is independent of the number of cells seeded. **(D)** PTX reduces Akt phosphorylation at ser 473. OVCAR-3 cells were cultured in DMEM supplemented with 10% FBS for 24 h followed by starvation for 12 h in serum-free media. Cells were switched to the media in the absence (CTR) or presence (treated) of PTX 5 μg/mL for 15 min, 1 h, 4 h, and 24 h, respectively. The whole cell lysates were prepared and western blotting was performed as described in the Methods section. Blots were stripped and re-probed with total Akt isoforms. The level of tubulin was used to indicate relative amounts of protein loaded. Experiments were performed three times, and a representative experiment is shown in this figure. Akt1 and Akt2 mRNA expression levels after PTX treatment were detected by RT-PCR. The expression of GAPDH is shown as internal control. Akt primer sets were from Wang J et al.[22] **(E)** Expression of MDR1 and TLR-4 measured by RT-PCR in untreated OVCAR-3 and MCF-7 cells. The expression of GAPDH is shown as internal control. MDR1 primer set was from Wang Y et al.[23]

This finding means that cytotoxicity due to PTX is less dependent on the concentration of the drug at concentrations above 10 ng/mL. In the case of OVCAR-3, the IC_{50} were not determined because even at concentrations of PTX greater than 20 μg/mL, more than 50% of the cells remained viable (data not shown). In contrast, the IC_{50} of PTX was 100–200 ng/mL for the drug-sensitive cell line MCF-7. This suggests that the poor effectiveness of PTX in OVCAR-3 is associated with the expression of the multidrug resistance transporter MDR1.

In recent years, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway has risen to prominence as a regulator of cell survival and growth in many different cell types. In ovarian cancer, constitutive Akt activity or gene amplification was frequently detected in tumor samples and associated with chemoresistance and poor prognosis.[24,25] Akt promotes cell survival and growth through a variety of mechanisms, including the regulation of proapoptotic proteins Bad and caspase-9 and cyclin D1 expression.[26]

To determine the effects of PTX on this signaling pathway, we evaluated by western blot the phosphorylation status of Akt (p-Akt) at ser 473 after PTX treatment. Ovarian cancer cells were grown under normal conditions and were deprived of serum overnight. Cells were then treated or not (control) with PTX in the presence of serum. Western blot analysis showed that levels of p-Akt ser 473 were reduced in comparison with those in control cells that otherwise were increased when stimulated with serum at the indicated times (Figure 1, *D*).

No differences were noted in the levels of total Akt1 and Akt2 isoforms at both mRNA and protein levels suggesting that PTX regulation of this pathway results largely from post-transcriptional regulation.

To investigate whether the encapsulation of PTX in nanocolloids leads to a general improvement of the drug efficacy, we assayed the cytotoxicity of the LBL-PTX toward the OVCAR-3 cell line and compared the results with that of PTX free. To assess this point, two types of LBL-nanocolloids were fabricated and tested: PTX-chitosan and PTX-chitosan-alginic acid. In particular, chitosan is a positively charged natural carbohydrate polymer with minimal toxicity largely used as a biomaterial due to its physicochemical and biological properties (e.g., biodegradability). Chitosan shows strong electrostatic interaction with the negatively charged mucosal surface,[27] a property that can further provide a rationale for using chitosan as delivery system in ovarian tumors considering the higher expression of mucin and other heavily glycosylated extracellular proteins in ovarian tumors in comparison with the surrounding normal tissue.[28]

Both preparations were tested on ovarian cancer cells for their ability to increase PTX efficacy. The results obtained by MTT test were comparable for the two types of nanocolloids and for

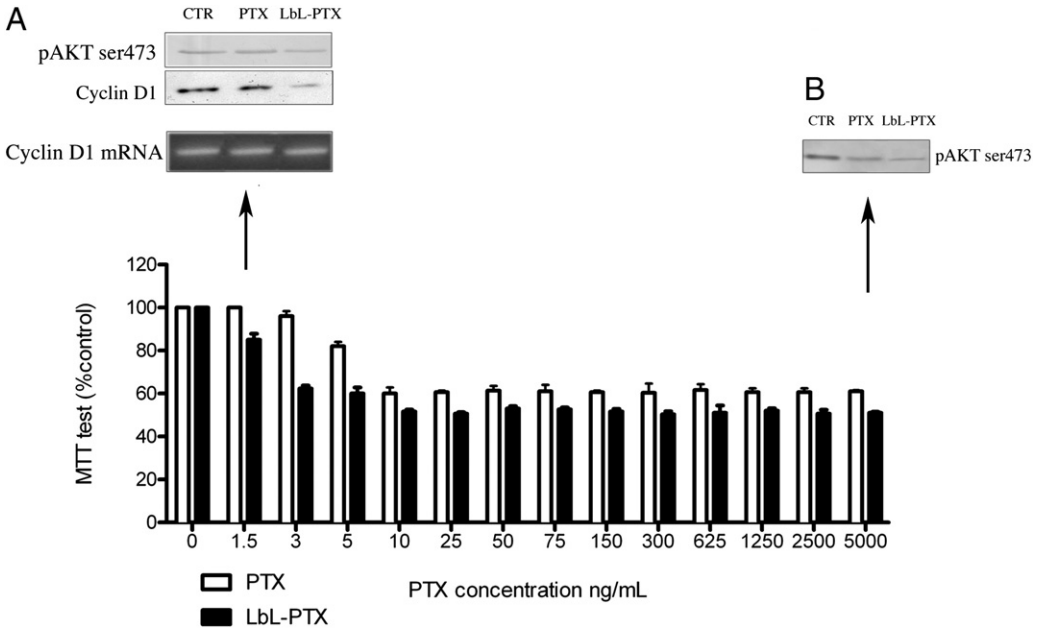

Figure 2. Comparison of cell viability in OVCAR-3 cells after treatment with free PTX and LBL-PTX nanocolloids. Citotoxic effects of PTX alone or LBL-PTX nanocolloids at the indicated concentrations were measured by the MTT assay as described in the Methods section. The cell viability is related to control wells treated with vehicle (DMSO) or empty LBL-nanocolloids (drug free). Effects of PTX and LBL-PTX nanocolloids on the expression of phospho-Akt and cyclin D1. Ovarian cancer cells were treated with PTX or LBL-PTX nanocolloids at the concentration of 1.5 ng/mL **(A)** or 5 μg/mL **(B)** for 24 h. The whole cell lysates (50 μg in each lane) were prepared and subjected to western blotting analysis by using specific antibodies against p-Akt ser 473 and cyclin D1. The Cyclin D1 mRNA level remains unaffected after treatment, suggesting the regulation at the protein level.

this reason, we use the general name of LBL-PTX nanocolloids to designate both formulations. As shown in Figure 2, PTX and LBL-PTX have a similar trend of cytotoxicity in ovarian cells with a higher cytotoxicity for LBL-PTX than for PTX alone. It should be noted that, because cells incubated for 24 hours with empty LBL-nanocolloids (drug free) did not show any significant difference in cell viability in comparison with control cells (data not shown), we exclude the role of empty nanocolloids to explain the difference between PTX and LBL-PTX. Moreover, we observed a significant inhibition of cell growth after treatment of ovarian cancer cells with LBL-PTX at the concentration of 1.5 ng/mL, whereas no change was observed after PTX-free treatment at the same concentration. We then determined whether p-Akt ser 473 and cyclin D1 levels, a downstream target of the Akt signaling pathway,[29] were affected at this concentration of drug. As shown in Figure 2, western blot analysis revealed a downregulation of pAKT ser 473 and cyclin D1 only for LBL-PTX treated cells, further suggesting the sensitivity of OVCAR-3 to LBL-PTX but not PTX at this drug concentration.

We next determined whether the increased cytotoxicity of LBL-PTX nanocolloids with respect to PTX free could be explained by a higher downregulation of the Akt pathway. Western blot analysis of p-Akt ser 473 from ovarian cells treated with PTX and LBL-PTX nanocolloids at 5 μg/mL showed a significant difference in the phosphoprotein level in the control with respect to treated cells with a further downregulation in LBL-PTX treated samples (Figure 2, *B*), a result that correlates with the increased cytotoxicity of nanocolloids in comparison with PTX free. This finding could be explained considering the different mechanisms of drug-nanocolloids uptake in comparison with PTX free. Several mechanisms have been described including the increased accumulation of the NPs in the cells and their entrapment in the endosomes/lysosomes, rendering the drug inaccessible for P-gp and enhancing in this way the sensitivity of cancer cells to the drug.[30]

A possible link between the activation of PI3K/Akt pathway and actin remodeling has been described.[31,32] It has been shown that the PI3K/Akt pathway induced cell migration through the remodeling of actin filaments and that actin is a cellular target of this kinase.[33,34] It is possible that the involvement of this pathway in PTX action could have consequences in the organization of actin cytoskeleton after PTX treatment. TRITC-phalloidin staining of F-actin followed by confocal microscopy analysis revealed some differences in the cell shape and organization of actin filaments for PTX free and LBL-PTX cells in comparison with the control (Figure 3, *A*). Non-treated cells show regularly shaped bodies, with a readily visible actin staining in the periphery, actin protrusions (yellow arrows, Figure 3, *A*), and the absence of bundles of actin filaments. Rounded cells appeared after PTX treatment together with the formation of blebs (green arrows, Figure 3, *A*). No actin protrusions are visible in treated cells. Furthermore, a more distinct net of actin filaments is visible in the cytoplasm of OVCAR-3 treated with LBL-PTX nanocolloids.

The organization of actin filaments is governed by a plethora of proteins that regulate the rate of actin polymerization. One of the key proteins in this scenario is cofilin, which can regulate the

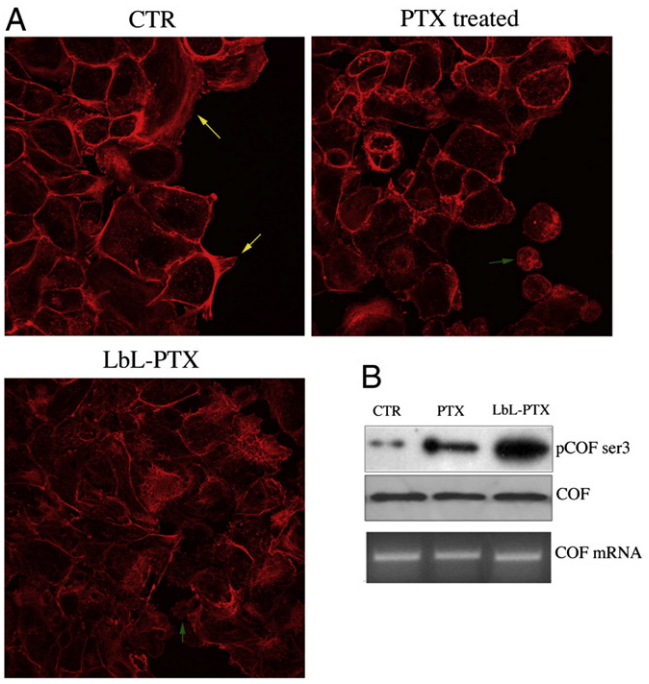

Figure 3. Confocal laser microscopic observation of OVCAR-3 cells. **(A)** Ovarian cancer cells plated on glass coverslips were treated with PTX or LBL-PTX nanocolloids at the concentration of 5 μg/mL for 24 h and then fixed. Cells were stained with TRITC-phalloidin. **(B)** The phosphorylation status of cofilin at ser3 was assessed with a phoshospecific antibody, and then the blot was reprobed for total cofilin levels. The mRNA level of cofilin was assessed by using a specific set of primer.

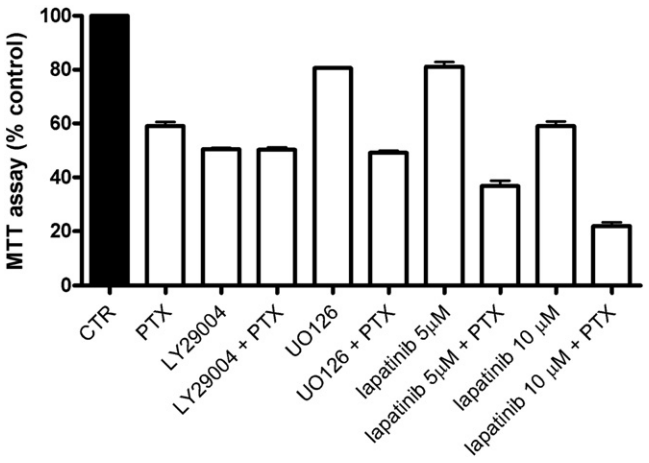

Figure 4. MTT test was used to test the efficacy of PTX (5 μg/mL) in combination with the ERK 1/2 inhibitor UO126 (10 μM), the PI3K inhibitor LY29004 (10 μM) and the drug lapatinib (5 μM and 10 μM) after 24 h of treatment.

rate of actin-filament turnover and the net polymerization of actin. In particular, actin dynamics are regulated by phosphorylation of cofilin at serine, which renders phospho-cofilin inactive towards F-actin.[35] We examined by western blot the phosphorylation status of cofilin after PTX or LBL-PTX nanocolloids treatment. Cofilin phosphorylation increased after PTX treatment and, in particular, after the exposure with LBL-PTX (Figure 3, *B*). This result is consistent with the morphological actin changes observed by confocal microscopy and raises questions about the role of the actin cytoskeleton in mediating PTX sensitivity. Moreover, the significant effect of LBL-NPs on cofilin phosphorylation will prompt us to investigate the role of actin cytoskeleton in mediating the uptake the LBL-NPs into OVCAR-3 cells.

In summary, the Akt and cofilin pathways are targets of PTX and LBL-PTX action and the modulation of Akt after PTX treatment can affect the cell growth as observed by MTT.

Moreover, LBL-PTX nanocolloids lower the mininum dose necessary to obtain a significant reduction of cell viability, an issue that can be important for future possible application in vivo to minimize the adverse side effects associated with PTX.

On the other hand, although the cell growth inhibition by PTX free and LBL-PTX nanocolloids reached a statistical difference, nanocolloids did not produce a sustained growth inhibition in ovarian cancer cells, suggesting that LBL-PTX nanocolloids are susceptible to efflux by P-gp. Thus, the combination with an interrelated drug is required to optimize the therapeutic activity of NP-encapsulated drug.

To overcome the mechanisms of PTX resistance, we tested the cytotoxic activity of PTX in combination with two small molecule-chemical inhibitors, UO126, an extracellular signal-regulated kinase (ERK) 1/2 inhibitor, and LY29004, a PI3K inhibitor. Both signal-pathways have been described and well characterized for their role in drug resistance.[26,36] In addition, the combination with lapatinib, an inhibitor of the intracellular tyrosine kinase domains of both the EGFR and Her-2 receptors, was further evaluated to enhance the cytotoxic efficacy of PTX. The possible combination of lapatinib and PTX is based on a promising phase III clinical trial that demonstrated the efficacy of lapatinib and PTX to significantly improve clinical outcomes in HER-2–positive locally advanced or metastatic breast cancer.[37] Moreover, the ability of lapatinib to reverse multidrug resistance due to ABCB1 and ABCG2 transporters, including P-gp, has been recently described with the potential to increase the cytotoxic effects of several chemoterapeutic drugs including PTX.[20] This finding supports the idea of combining PTX and lapatinib in our cell line, which shows a high expression of P-gp.

As shown in Figure 4, an improvement of PTX efficacy is obtained when the drug is administered in combination with the ERK 1/2 inhibitor UO126, suggesting that this pathway may play a role in the generation of PTX resistance or with lapatinib. On the contrary, there are no significant differences in cell viability between cells treated with LY29004 alone or with LY29004 plus PTX, suggesting that LY29004 alone is the major determinant of the observed decrease in cell viability.

Next, we determined whether the therapeutic potential of LBL-PTX nanocolloids could be further increased by the co-delivery of PTX and lapatinib. For this purpose, nanocolloids containing PTX and lapatinib were prepared by LBL technology.

As shown in Figure 5, the particle size of PTX with one layer coating was 150 ± 50 nm, much smaller in comparison with several micrometers of original PTX powder. After LBL coating with biocompatible and biodegradable layers of chitosan, alginic acid, and lapatinib, the particle diameter was around 250 nm.

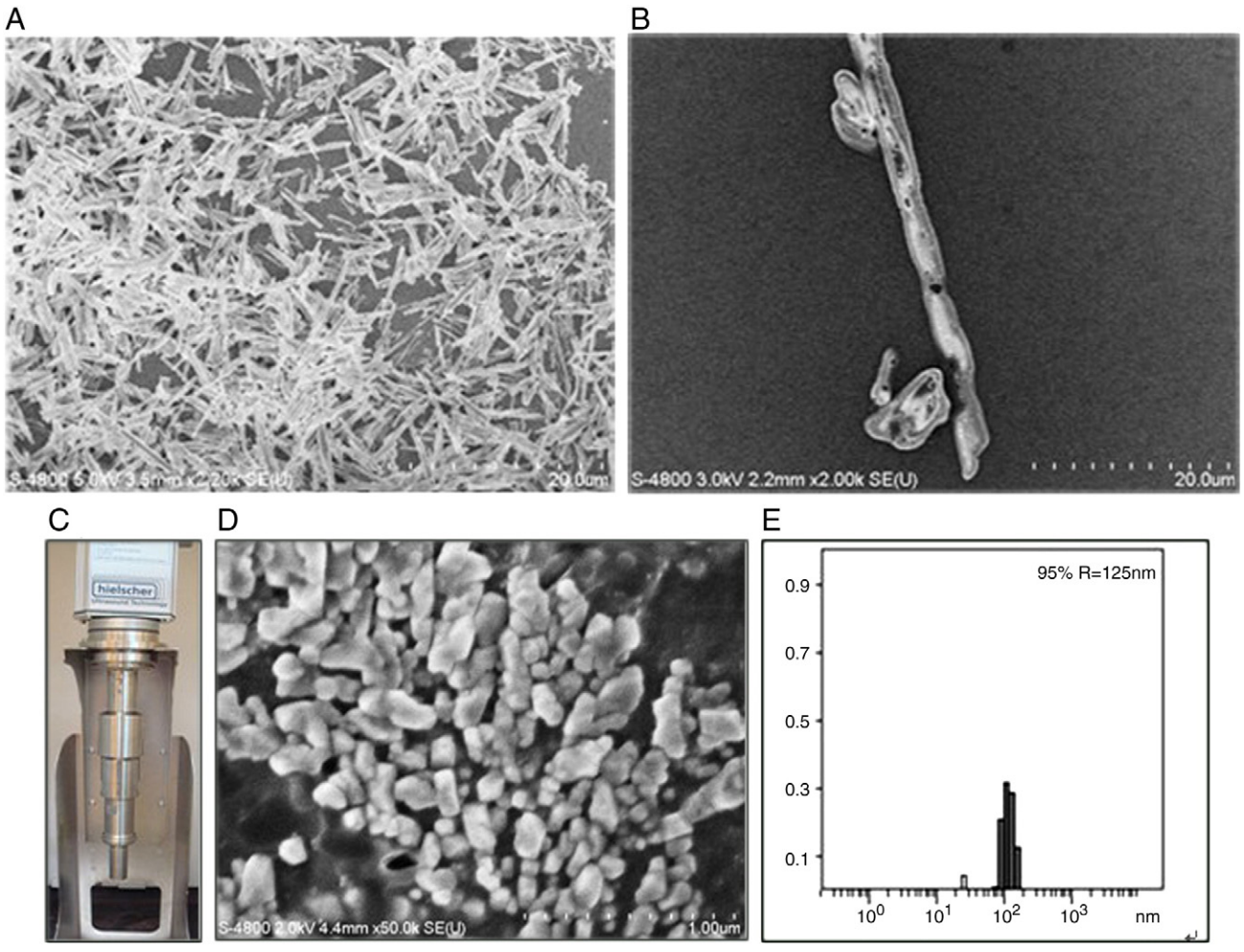

Figure 5. SEM image of PTX **(A)** and lapatinib **(B)** before treatment. Ultrasonication-assisted coating of first layer on PTX: **(C)** sonicator, **(D)** SEM image, **(E)** light scattering result.

Such architectural nanocapsules allowed dual delivery of these two drugs. Results obtained by MTT test confirmed the enhanced cytotoxic activity of these nanopreparations in comparison with free PTX and LBL-PTX (Figure 6).

Discussion

Ovarian cancer still remains one of the most letal malignancies among women. Despite enormous progress in understanding ovarian cancer biology to date, there are no examples of therapies leading to cures. Therefore, improving the efficacy of current therapeutics will have a great impact in the management of the disease. PTX is widely used for the treatment of patients with ovarian cancer, but despite substantial clinical efficacy, the optimal administration regimen remains elusive. Many questions remain concerning the way to administer the drug and the molecular mechanisms at the basis of chemoresistance.

Among the proteins related to the chemoresistance process, the overexpression of P-gp has profound implications in clinical practice. In fact, the presence of drug-efflux pumps that mediate the active efflux of chemotherapeutics is one of the most extensively described mechanisms of drug resistance, and strategies to modulate or block this process have been investigated actively in oncology.[38] In ovarian cancer, the expression of P-gp has been implicated in chemoresistance, correlated inversely with patient survival and associated with resistance to PTX.[29-41]

These observations set the stage for the development of efficacious instruments to increase PTX efficacy by limiting adverse side effects and increasing its cytotoxic action. In this regard, nanotechnology has been recognized as a fundamental tool in cancer research,[42] and the potential of nanocarriers to increase drug efficacy is well described.[43-45]

Here, we describe a SLBL method to efficiently convert PTX into drug NPs. It allows clinicians to combine many necessary factors for an efficient drug-delivery system: i) control of nanocolloid size within 100 – 300 nm, ii) high drug content of approximately 70% wt, iii) shell biocompatibility and biodegradability, and iv) sustained controlled release. Overall, these characteristics, including the small size and the net negative charge (see Supplementary Materials for details), that can be advantageous for their penetration to and within tumors, make NPs attractive candidates for possible in vivo applications.

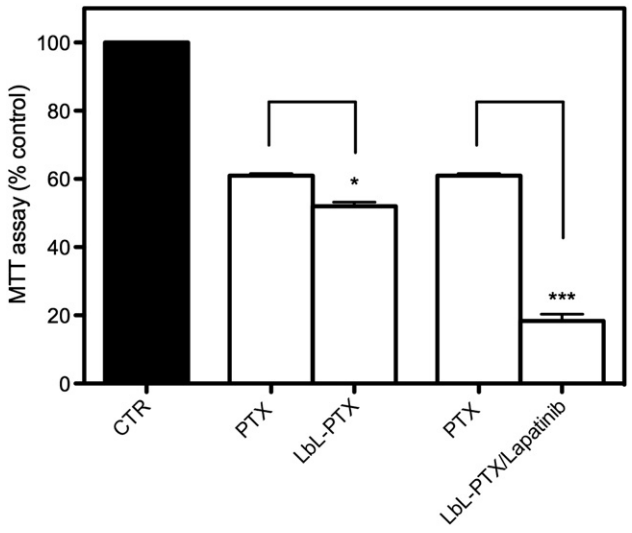

Figure 6. LBL-lapatinib/PTX nanocolloids demonstrate significant cytotoxic activity in P-gp overexpressing ovarian cancer cells as determined by MTT test ($P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***).

In addition, in this research we elaborated nanoformulation of two drugs in one nanocapsule locating PTX in the core and lapatinib on the shell periphery. The rationale for considering combination therapy is to overcome major problems associated with PTX administration, such as the counteraction of PTX resistance and, in combination with dose-escalation, the potential reduction of systemic toxicities. Moreover, with this strategy both drugs can be temporally co-localized in the tumor cells for optimal synergy, limiting possible differences in the pharmacokinetics and tumor accumulation of the two different agents. Given the molecular complexity of cancer, drug combinations are most likely to translate into a significant clinical benefit.

To further increase the therapeutic potential of nanocapsules, a research objective that remains to be explored regards the realization of a target delivery system. Surface functionalization by targeting ligands or antibodies is an attractive opportunity to direct NPs toward cancer-specific cells or tumor-specific clones with substantially greater selectivity in tumor killing versus toxicity to normal host tissues. Several types of targeting ligands should be used for this purpose, including peptides and antibodies. These ligands enable NPs to bind specific receptors and to be internalized by endocytosis, enhancing the intracellular accumulation of drugs. The feasibility of the LBL method makes easy the realization of functionalized NPs by using polymers with free reactive groups for the outer layer of LBL NPs. On the contrary, a relevant concern is the identification of reliable ligands to impart a precise biological function to NPs. Significant research efforts have been made in a recent study from the National Cancer Institute Pilot Project for the acceleration of translational research, where 75 possible tumor antigens were recognized.[46] Some of these tumor-associated antigens, including MUC1, CA 125, NY-ESO-1, and human epidermal GFR 2 (HER2)/neu are potential targets in ovarian cancer. In particular, due to its role in cellular transformation and tumorigenicity, MUC1 received great attention in those years. Recently, a monoclonal antibody anti-MUC1 has been utilized alone or in combination with docetaxel (DTX) in preclinical models of ovarian cancer, leading to a significant increase in survival. Furthermore, a MUC1 aptamer-guided nanoscale drug-delivery system was developed to enhance the PTX delivery to MUC1-overexpressing MCF-7 cells in vitro.[47,48]

To characterize the clinical potential of nanocolloids loaded with PTX and lapatinib, preclinical studies in animal tumor models are necessary, including a detailed evaluation of pharmacokinetics and pharmacodynamics and active intracellular delivery of LBL nanocolloids after intravenous or intraperitoneal administration. Extensive future research is warranted.

Because many women experience recurrences during ovarian cancer therapy due to drug-resistance mechanisms, we postulate that our approach aiming at limiting this problem may serve the purpose of improving the treatment of ovarian tumors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.nano.2011.10.014.

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