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Combined Chemo-immunotherapy as a Prospective Strategy To Combat Cancer: A Nanoparticle Based Approach

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Abstract: The prime objective of this study was to develop a combined chemo-immunotherapeutic formulation which could directly kill cancer cells as well as activate the immunosuppressed tumor microenvironment to mount a robust antitumor immune response. Paclitaxel (PTX) and SP-LPS (nontoxic derivative of lipopolysaccharide) were selected as anticancer drug and immunostimulant respectively. Poly(lactic-*co*-glycolic acid) (PLGA) based PTX and SP-LPS containing nanoparticles (TLNP) were prepared by the double-emulsion method (w/o/w) and characterized in terms of size, zeta potential and transmission electron microscopy (TEM). The release behavior of PTX and SP-LPS from the TLNP exhibited a biphasic pattern characterized by an initial burst followed by slow continuous release. In vitro anticancer activity of TLNP was found to be higher compared to PTX when studied in a tumor cell−splenocyte coculture system. TLNP activated murine monocytes induced the secretion of various proinflammatory cytokines. After iv administration of TLNP in tumor bearing C57BL/6 mice, the amount of PTX in the tumor mass was found to be higher in TLNP treated mice as compared to commercial Taxol group at all time points studied. In vitro studies suggest that nanoparticles containing PTX and SP-LPS have both direct cytotoxicity and immunostimulatory activity. Hence this might have potential as a chemo-immunotherapeutic formulation against cancer with advantage over present day chemotherapy with Taxol, in terms of tumor targeting, less toxicity and immunostimulation.

Keywords: Chemo-immunotherapy; nanoparticles; immunostimulation; PLGA; paclitaxel; SP-LPS; tumor targeting

Introduction

Paclitaxel (PTX) is a promising drug against a wide array of solid cancers, viz., breast cancer, lung cancer, ovarian cancer, head and neck cancer and acute leukemia.[1] It is known to promote tubulin polymerization to a hyperstabilized state, interfering with the normal dynamics necessary for cell division, leading to cell death.[2] The use of PTX is however limited by the drug's toxicity and its poor aqueous solubility.[3] The current commercial formulation of PTX, i.e., Taxol, is a 1:1 (v/v) mixture of Chremophor EL and dehydrated alcohol. Some of the side effects are due to the Chremophor EL[4] while others are due to the limited availability in tumor mass and distribution to other tissues when administered via the iv route, reducing the effective dose of the drug.[5−7] Several attempts have been made to develop alternate

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paclitaxel formulations with reduced systemic toxicity and enhanced therapeutic index.[8]

The inability of chemotherapy to completely root out cancer cells might be due to development of resistance against the cytotoxic drugs. This is because of the genetic plasticity of tumor cells, which helps a subgroup of cells to mutate and evade the chemotherapy. Increasing the dose of drugs does not guarantee elimination of this subset of cells, but would eventually lead to systemic toxicity. To target these resistant cells, a combined chemo-immunotherapeutic approach can be beneficial. It is well established that tumor suppresses the anticancer immune response at its local microenvironment to facilitate its growth, leading to cancer progression and metastasis.[9] Immunotherapy, on the other hand, stimulates the immune system and helps to induce an effective antitumor response. Combined chemo-immunotherapy has multifaceted advantages. First, chemotherapy induced cell death can enhance cross-priming of immune cells by providing them with important cancer specific antigens, thereby increasing the antitumor T cell response.[10] Second, chemotherapeutic drugs increase the expression of class I MHC molecules on the tumor cells.[11] Third, chemotherapy can also be used to manipulate pathways of immune tolerance and regulation.[12]

Since long ago, bacterial endotoxins have been used against cancer, which anticancer effect is attributed to their toll-like receptor (TLR) agonistic activity.[13] TLR agonists play crucial roles in orchestrating both innate and adaptive immune response against cancer. Bacterial lipopolysaccharide (LPS) is a potent TLR-4 agonist, but due to its systemic toxicity, it is of limited use in clinical setup. Several detoxified LPS derivatives have been prepared like OM-174,[14] ONO-4007,[15] monophosphoryl lipid A (MPL),[16] and 7-acyl lipid A.[17] One of such derivative is SP-LPS,[18] which is a high molecular weight, sodium phthalate salt of parent LPS, which has been used in this study. Because of its high molecular weight polymer like structure, it is less possible for the drug to leak into the systemic circulation after tumor targeted delivery.

Colloidal drug delivery systems like polymeric micelles, liposomes, surface modified particles or nanoparticles have attracted great interest for clinical administration of antitumor drugs. The efficacy of these drug delivery systems has been attributed to their small size, low toxicity, controlled drug release, fewer systemic side effects, modified drug pharmacokinetics and biodistribution. Several nanoparticle formulations carrying PTX have been prepared and tested.[19−21] Nanoparticles (NP) prepared from poly(lactic-*co*-glycolic acid) (PLGA) have the advantage of good mechanical strength, biodegradability, drug compatibility and ease of processing.[22] As our aim was to coadminister PTX and SP-LPS, the approach taken in this study was to encapsulate both of them as a single delivery system in the form of PLGA nanoparticles and evaluate their anticancer potential. As PTX

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Table 1. Formulation Parameters and Characteristics of the Different Formulations of Nanoparticles Prepared Using Varying Concentration of PVA and PLGA

					encapsulation efficiency	
PVA (%)	PLGA (mg)	hydrodynamic diam (nm)	PDI	ζ potential (mV)	% paclitaxel	% SP-LPS
2	100	250 ± 5	0.05 ± 0.01	-26.1 ± 3.2	77 ± 5	5.2 ± 0.2
5	100	244 ± 6	0.05 ± 0.02	-21.8 ± 0.4	79 ± 4	12.7 ± 0.1
10	100	236 ± 2	0.06 ± 0.01	-17.6 ± 0.8	80 ± 4	15.1 ± 1.2
5	200	293 ± 12	0.17 ± 0.03	-21.0 ± 0.7	85 ± 6	20.8 ± 2.7

is lipophilic and SP-LPS is hydrophilic, a delivery system was required which could coadminister both the moieties together. Hence, the double emulsion method (w/o/w) was chosen, as the internal aqueous phase could accommodate SP-LPS, while the oil phase could retain PTX. As PLGA is biodegradable, we hypothesized that both the moieties would be released within the tumor mass. The PTX would have its direct cytotoxic effect on the cancer cells whereas SP-LPS would activate tumor residing macrophages by binding with TLR-4.

The anticancer therapeutic efficacy of this nanoparticle formulation was evaluated on B16-F10 melanoma cells. Their immunomodulatory activity was evaluated on a murine macrophage cell line as well as with a primary culture of splenocytes. Results confirmed the presence of both anticancer activity of PTX and the immunostimulatory property of SP-LPS in the nanoparticle formulation. In vitro biological activity of PTX−SP-LPS nanoparticle (TLNP) was evaluated by coculture study which confirmed the chemo-immunotherapeutic property of the nanoparticle preparation. Treatment of TLNP in a coculture of splenocytes and cancer cells exhibited an increase in the death of tumor cells compared to treatment with only PTX. The biodistribution study of TLNP in a mouse model of cancer showed higher retention of the drug in the tumor mass. Taken together these results suggest the development of a novel combined chemo-immunotherapeutic approach which has the potential to open up new avenues for cancer therapy in the near future.

Methods

Cell Lines. B16-F10 and J774.1, which are murine melanoma and macrophage cell lines respectively, were purchased from ATCC. They were grown in DMEM supplemented with 10% FBS and 1% antibiotic antimycotic solution in a 37 °C incubator with 5% CO_2/95% humidified air. Splenocytes were isolated from C57BL/6 mice and plated in a 24 well plate at a cell density of 1 million cells per well.

Animals. Inbred C57BL/6 mice at 6 weeks of age were obtained from the animal facility of the National Institute of Immunology, New Delhi, India, where animals were bred and housed in agreement with the guidelines of the Institute's Animal Ethics Committee.

General Materials. Poly(lactic-*co*-glycolic acid) (PLGA) copolymer (50/50; Resomer, molecular weight 45,000−70,000, viscosity 0.4 dL/g) was a gift from PURAC biochem. Sucrose, polyvinyl alcohol (PVA) (MW 30,000), propidium iodide (PI) and lipopolysaccharide from *Salmonella enterica* serotype *minnesota* (LPS) were purchased from Sigma Aldrich. Intaxel (Taxol) and pure dry powder of paclitaxel were purchased from Dabur and 21CEC Pharma Limited, Shanghai, respectively. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Biological Industries, Israel, while 100X antibiotic antimycotic solutions and enzyme free cell dissociation buffer were from Himedia. Mouse TNF-α and IFN-γ ELISA kits were from eBiosciences whereas IL-12, IL-10, IL-1β ELISA kits and annexin V apoptosis detection kit were purchased from BD Biosciences. Acetonitrile, dichloromethane and ethyl acetate (HPLC grade) were obtained from Merck. Water was purified by reverse osmosis (Milli-Q, Millipore). Wherever PTX was used alone, it was dissolved in DMSO as a primary stock of concentration 2 mg/mL. Subsequent dilutions were made in complete media.

Synthesis of SP-LPS. SP-LPS was prepared from lipopolysaccharide derived from *Salmonella enterica* serotype *minnesota* by the method of McIntire et al.[18] Briefly, 10 mg of LPS was mixed with 100 mg of phthalic anhydride and dried. One milliliter each of dry pyridine and dry formamide was added and reacted for 36 h. After the completion of the reaction, it was dialyzed using a 30 kDa cutoff membrane, first against Milli Q water, then against 0.1 M sodium bicarbonate and finally against Milli Q water. The dialyzed sample was then lyophilized to obtain dry powdered SP-LPS.

Preparation of Double Emulsion Nanoparticles Coencapsulating Paclitaxel and SP-LPS (TLNP) and Their Characterization. TLNP was prepared using a w/o/w double emulsion/solvent evaporation method.[23] Briefly 10 mg of SP-LPS was dissolved in 200 μL of water. This was emulsified in 2 mL of DCM containing 100 or 200 mg of PLGA and 10 mg of PTX by sonication (20W, 40% duty cycle, 20 cycles, 2 min) (Bandellin Sonifier). The primary emulsion thus formed was added dropwise to an external aqueous phase (EAP) containing different concentrations of emulsifier PVA 2%/5%/10% (w/v) (Table 1) and 10% (w/v) sucrose as lyoprotectant, in Milli-Q water, and sonicated (20 W, 40% duty cycle, 20 cycles) for 2 min, leading to formation of

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secondary emulsion. It was then stirred overnight to evaporate DCM. The particles thus formed were collected by centrifugation at 14,000 rpm for 30 min (Sorvall Evolution RC, Kendro), washed thrice with Milli Q water and lyophilized (−80 °C and <10 μm of mercury pressure, Labconco, Kansas City, MO) to obtain dry TLNP in powdered form. The amounts of polymer and emulsifier used for the preparation of different batches of particles are shown in Table 1.

Particle size distribution (hydrodynamic diameter, polydispersity index (PDI)) and zeta potential of the nanoparticles were measured by dynamic light scattering (DLS) using a Malvern NanoZS (Malvern Instruments, U.K.). To determine the particle size and zeta potential, a dilute suspension of particles (100 μg/mL) was prepared in PBS (20 mM; pH 7.4). For each sample the mean diameter of 3 determinations was calculated. Values reported are the mean ± standard deviation of at least 3 different batches of nanoparticles.

Quantification of Encapsulated Paclitaxel and SP-LPS. PTX and SP-LPS loading in the TLNP was determined by HPLC after extraction of the drug from the nanoparticles. Two milligrams of lyophilized TLNP was dissolved in 1 mL of acetonitrile and vortexed vigorously to break the particles followed by centrifugation for 5 min at 10000*g*. PTX, being soluble in acetonitrile, was present in the supernatant while SP-LPS remained in the pellet. Pellet was extracted again with acetonitrile for any residual PTX. The pooled supernatant and pellet were used for PTX and SP-LPS quantification respectively. The supernatant was evaporated to dryness under nitrogen. Dried residue was reconstituted in 200 μL of acetonitrile, and the quantity of PTX was determined in duplicate by HPLC. The mobile phase consisted of 60:40 (v/v), acetonitrile:water and delivered at a flow rate of 1.0 mL/min. Detection was done at 228 nm. The reverse phase column used was a Phenomenex RPC18 column (300 × 5 mm, pore size 5 μm).

SP-LPS was quantified from the acetonitrile extracted pellet. The pellet was redissolved in 100 μL of phosphate buffer (20 mM, pH 7.4). HPLC was performed using a size exclusion column Phenomenex BioSep-SEC-S 2000 (300 × 7.8 mm, pore size 5 μm) with 20 mM phosphate buffer as the mobile phase. The flow rate was 1.0 mL/minute, and the detection was done at 232 nm. The encapsulation efficiency was estimated by the following equation:

$$\text{encapsulation efficiency (\%)} = \frac{\text{mass of the drug in nanoparticles}}{\text{mass of the drug used in formulation}} \times 100$$

Transmission Electron Microscopy. Morphology of the TLNP was determined by transmission electron microscopy (CM10, Phillips, Holland) after coating the particles with 1% uranyl acetate over a copper grid (Polysciences, Warrington, PA). TEM images were obtained using software image capture engine (version 5.42.391).

Nanoparticles prepared with 200 mg PLGA and 5% PVA were selected on the basis of highest encapsulation, and all further experiments were performed using this formulation.

Release Profile of Paclitaxel and SP-LPS. For the estimation of in vitro cumulative drug release, 2 mg of freeze-dried TLNP was suspended in 1 mL of PBS (20 mM, pH 7.4) in a tube. 0.1% Tween 80 was added to maintain the sink condition. The tubes were placed in a shaker at 37 °C at 120 rotations per minute. At specific time intervals 0.5 mL of released medium was taken out. 0.5 mL of fresh medium (PBS with Tween 80) was added to the tubes for continuous release studies. 0.5 mL of dichloromethane was added to the withdrawn medium, vortexed and centrifuged. The DCM layer was collected and was allowed to evaporate completely. The residue was reconstituted in acetonitrile for HPLC analysis of PTX by the above-mentioned method. The aqueous layer was lyophilized to dryness. The powder was redissolved in 100 μL of phosphate buffer (20 mM; pH 7.4) and quantified for the SP-LPS by HPLC method as mentioned before.

In Vitro Anticancer Activity of TLNP. B16-F10 cells were plated at a density of 0.2 million cells per well in a 6 well plate and incubated overnight for attachment. The cells were then treated with PTX/blank NP/TLNP containing different concentration of PTX (0.025, 0.25, 2.5 μg/mL) for 24 and 48 h. At the determined time, the cells were aspirated out and centrifuged at 1500 rpm for 5 min and the pellet was resuspended in 500 μL of sterile PBS. Cell death was determined by PI uptake using a flow cytometer (BD LSR, BD Bioscience), and data were analyzed using WinMDI 2.8.

In Vitro Apoptosis Inducing Activity of TLNP. For in vitro apoptosis study, 0.2 million B16-F10 cells were plated in a 6 well plate. After overnight incubation for adherence, they were given treatment with PTX/blank NP/TLNP containing PTX concentrations of 0.0025, 0.025, and 0.25 μg/mL. After 24 h, cells were dislodged and labeled with annexin V−FITC conjugate and PI. Apoptosis was determined by flow cytometry (BD LSR, BD Bioscience), and data were analyzed using WinMDI 2.8.

in Vitro Macrophage Stimulatory Activity. 0.2 million macrophage cells (J744.1) were plated in a 24 well plate and left for adherence for 24 h. Following this, treatment was given with SP-LPS solution/TLNP containing SP-LPS at concentrations of 0.005, 0.05, 0.1, and 0.2 μg/mL. The supernatant was withdrawn at 30 min, 1, 3, 6, and 9 h and estimated for TNF-α by ELISA. For IL-12 assay, 0.2 million J744.1 cells were given treatment with SP-LPS solution/TLNP at 0.2 μg/mL SP-LPS concentration and incubated for 24 h. The culture supernatant was withdrawn and assayed for IL-12 by ELISA.

Immunomodulatory Activity of TLNP with Splenocyte Culture. Splenocytes were isolated from C57BL/6 mice and plated in a 24 well plate at a cell density of 1 million cells per well. These were stimulated with SP-LPS solution and TLNP at a concentration of 0.1 μg of SP-LPS/mL. Culture supernatant was withdrawn twice at 18 and 48 h. As TNF-α and IL-12 are secreted by macrophages, they were detected from the supernatant withdrawn at 18 h while the rest of the cytokines IFN-γ, IL-10 and IL-1β were measured from the treated splenocyte supernatant withdrawn at 48 h.

Table 2. Biodistribution Analysis of PTX (μg/mg tissue) Delivered as Commercial Taxol or TLNP at the Dose of 10 mg/kg PTX equivalent in Different Organs of Tumor Bearing C57BL/6 Mice after Iv Injection

time (h)	plasma	heart	lung	spleen	kidney	tumor	liver
				TLNP			
0.05	3.0 ± 1.7	10.2 ± 3.2	20.8 ± 6.3	14.6 ± 5.3	16.0 ± 7.2	12.2 ± 3.9	12.9 ± 4.3
0.5	1.5 ± 0.8	9.0 ± 3.0	17.3 ± 4.2	19.5 ± 7.3	12.5 ± 3.7	13.7 ± 5.8	12.4 ± 3.9
1	0.3 ± 0.3	6.3 ± 0.7	15.0 ± 6.5	23.4 ± 9.1	10.6 ± 3.2	14.1 ± 4.2	8.7 ± 3.3
2	0.3 ± 0.2	5.4 ± 1.0	13.8 ± 5.6	17.1 ± 6.4	5.2 ± 2.9	15.4 ± 5.3	7.9 ± 2.2
4	nd	5.2 ± 2.6	10.3 ± 3.1	17.1 ± 6.8	5.9 ± 2.8	15.6 ± 5.8	8.5 ± 3.2
8	nd	4.2 ± 0.1	6.3 ± 2.6	14.6 ± 5.2	3.2 ± 1.0	16.4 ± 3.2	6.2 ± 2.2
12	nd	3.1 ± 1.1	5.2 ± 3.1	7.7 ± 6.3	3.0 ± 2.0	14.1 ± 3.8	1.7 ± 1.1
24	nd	2.0 ± 0.1	1.9 ± 0.7	2.6 ± 0.1	0.4 ± 0.2	7.4 ± 2.9	0.6 ± 0.6
				Taxol			
0.05	10.2 ± 3.3	15.6 ± 7.0	20.3 ± 7.5	25.1 ± 9.9	12.7 ± 3.2	4.0 ± 1.3	11.8 ± 4.2
0.5	3.7 ± 1.5	5.9 ± 1.3	17.9 ± 5.2	25.7 ± 9.1	8.9 ± 1.3	4.1 ± 2.3	10.1 ± 3.2
1	0.8 ± 0.6	6.6 ± 3.4	16.1 ± 3.7	21.4 ± 7.3	8.6 ± 3.4	5.3 ± 1.2	12.4 ± 4.2
2	0.4 ± 0.2	5.4 ± 3.2	12.5 ± 3.2	20.8 ± 8.3	5.9 ± 1.3	6.1 ± 2.4	8.2 ± 3.1
4	nd	5.2 ± 3.5	10.0 ± 8.3	20.5 ± 5.1	4.1 ± 2.0	6.9 ± 2.0	6.9 ± 2.2
8	nd	5.9 ± 1.3	7.7 ± 6.2	14.7 ± 7.1	2.8 ±1.0	8.0 ± 3.2	4.2 ± 0.2
12	nd	5.9 ± 1.3	6.2 ± 1.0	12.2 ± 4.1	2.6 ± 2.0	7.8 ± 3.2	2.0 ± 1.1
24	nd	4.7 ± 1.3	5.1 ± 2.1	10.7 ± 5.1	2.8 ± 3.2	2.6 ± 2.1	0.8 ± 0.2

Study of Chemo-immunotherapeutic Activity of TLNP in Tumor Cell−Splenocyte Coculture. In the coculture experiment, the chemo-immunotherapeutic potential of TLNP was analyzed. Briefly, 0.2 million B16-F10 cells were labeled with CFSE (5 μM) and cocultured with 5 million splenocytes isolated from C57BL/6 mice. These were treated with PTX and TLNP at the concentration of 0.25 μg of PTX/mL. After 24 h, the death of tumor cells was determined by the PI uptake method using flow cytometer (BD LSR, BD Bioscience) and data were analyzed using WinMDI 2.8.

Biodistribution Study. For the biodistribution study, male C57BL/6 mice were injected subcutaneously with 1 million B16-F10 cells in the right flanking region. When tumors were about 300−400 mm^3 in size, the mice were injected intravenously through the tail vein with commercially available Taxol or TLNP at the dose of 10 mg/kg PTX equivalent. Three mice were sacrificed at each time point, viz., 5 and 30 min and 1, 2, 4, 8, 12, and 24 h after drug administration. Samples of plasma, liver, kidneys, spleen, lungs, heart and tumor were harvested and stored at −50 °C until analyzed. Tissues were homogenized in PBS (with 10% BSA) followed by PTX extraction using ethyl acetate. The extract was then evaporated to dryness under nitrogen, and the residue was analyzed for amount of PTX present by HPLC as described previously.

Statistical Analysis. Data are presented as the mean ± standard deviation except for Figure 6 and Table 2, where it is expressed as mean ± standard error of the mean. Statistical analyses were performed by a two-tailed Student *t* test. The statistical analysis was performed with GraphPad InStat software (GraphPad Software Inc. version 3.05).

Results

Physicochemical Characterization of Nanoparticles. In order to predict the in vivo behavior of the prepared nanoparticles, their mean hydrodynamic diameter, polydispersity index (PDI), zeta potential and morphology were evaluated. The mean diameter of the nanoparticles prepared with different amounts of PLGA and PVA varied from 236 to 293 nm (Table 1). While particles prepared with 100 mg of PLGA were more uniform in size (236−250 nm), the diameter was larger in preparations where we used 200 mg of PLGA (286−294 nm). There was a moderate effect of concentration of PVA on particle size. Average particle size reduced from 250 to 236 nm for NPs prepared with 2% and 10% PVA respectively. Zeta potential also varied moderately (−17 mV to −26 mV) with the amount of PVA used. Particles prepared with a higher amount of PVA had less surface charge compared to NPs prepared using less PVA.

Transmission Electron Microscopy (TEM). Analysis done by TEM showed uniform particles with smooth surface morphology. No breakage or tear was noticed. The size of the particles was also reconfirmed from the TEM data (Figure 1).

Paclitaxel and SP-LPS Loading in Nanoparticles. The loading efficiency of PTX and SP-LPS was found to be 85% and 20% respectively in the nanoparticles with the 200 mg of PLGA and 5% PVA batch. With different amount of PLGA and PVA used we got varying degree of encapsulation of SP-LPS (Table 1). Variation in encapsulation was less in the case of PTX (77−85%). This can be explained by the high lipophilicity of PTX, minimizing loss of the drug into the external aqueous phase. Encapsulation of SP-LPS was dependent on both the amount of PVA and PLGA. With 2% PVA, less SP-LPS got entrapped (5%) in the NP preparation, while with increasing PVA concentration, efficiency of SP-LPS encapsulation increased (15%) with constant amount of PLGA (100 mg). The amount of PLGA used also played a crucial role. With 100 mg of PLGA and 5% PVA, encapsulation was about 12%, but with 200 mg of PLGA,

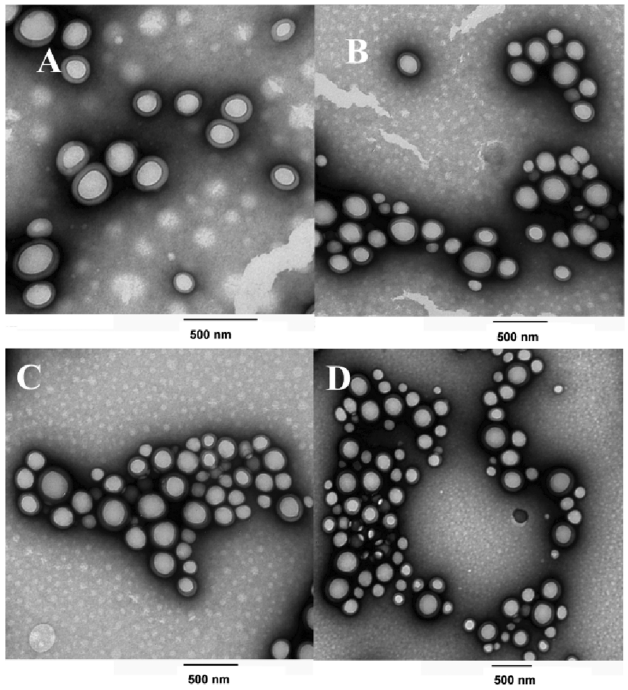

Figure 1. Transmission electron microscopic images of different nanoparticles prepared: (A) 2% PVA, 100 mg of PLGA; (B) 5% PVA, 100 mg of PLGA; (C) 10% PVA, 100 mg of PLGA; (D) 5% PVA, 200 mg of PLGA.

encapsulation increased to 20%. This can be attributed to the emulsifying effect of PVA as well as the matrix forming property of PLGA. NPs prepared with more PVA would be having a thicker interphasic barrier of PVA, which was also reflected in the zeta potential of the NPs. This would prevent SP-LPS from leaching out from the NPs during preparation and hence resulted in more encapsulation. NPs prepared with less PLGA would make a more porous preparation, which may not be able to entrap the water-soluble SP-LPS.

Finally we selected 200 mg of PLGA and 5% PVA concentration for the NP preparation, since higher encapsulation of SP-LPS was achieved in this formulation. And all further experiments were performed with nanoparticles prepared with this composition.

In Vitro Cumulative Release Study. In vitro cumulative release analysis of PTX and SP-LPS from TLNP was done for 15 days. The release characteristics of both the drugs from the NPs exhibited a biphasic pattern, characterized by a fast initial release during the first 24 h, followed by a slow and continuous release (Figure 2). The reason for this may be that initially the drug deposited at the NP interface is released at a faster rate via diffusion through the water channels created due to evaporation of the internal aqueous phase (IAP) in the NPs. The slower and sustained release of the drug at later stages could be due to the diffusion/erosion of polymeric matrix which releases the encapsulated drug. The release of SP-LPS was found to be faster compared to PTX. About 65% of total SP-LPS got released in the first 48 h, while it was about 38% for PTX. In 15 days, the total release was found to be around 70% for both of the drugs.

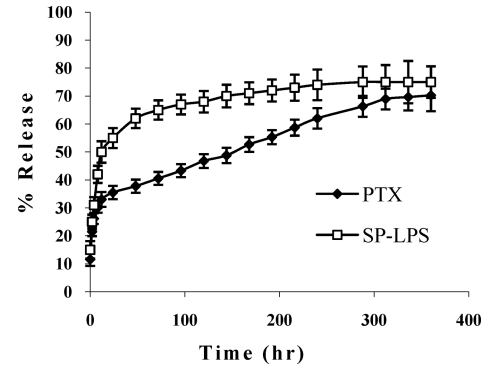

Figure 2. Cumulative release profile of PTX and SP-LPS from TLNP made with 5% PVA and 200 mg of PLGA. Particles were suspended in 20 mM PBS (pH 7.4). Released medium was analyzed by HPLC for amount of both drugs at different time points.

Direct Cytotoxicity. In vitro cytotoxic activity of PTX and TLNP was compared using B16-F10 melanoma cell line. The range of concentration of PTX (2.5, 0.25, 0.025 μg/mL) was selected because it corresponds to plasma levels of the drug achievable in humans.[24]

As can be seen from Figure 3A, both PTX and TLNP exhibited a similar dose-dependent cytotoxic activity, with TLNP showing a marginally higher cytotoxicity at the lower concentration. The results indicate PTX encapsulated in TLNP is bioavailable and its cytotoxic activity on tumor cells is comparable to PTX in soluble form, which is consistent with other published data.[20,25]

Induction of Apoptosis. As PTX causes cell death mainly via apoptotic pathway,[26] the apoptosis inducing property of the TLNP preparation was studied and compared with PTX. The data corresponds with the direct cytotoxicity data (Figure 3B). The results show significantly high percentage of cells undergoing apoptosis as compared to the control group. Among the two treatment groups, TLNP have shown slightly higher apoptosis than PTX treated cells. The degree of apoptosis increased with increase in drug concentration.

In Vitro Macrophage Stimulating Activity. To evaluate the immunomodulatory property of the TLNP containing SP-LPS and PTX, we studied the macrophage stimulating activity of the same. LPS exhibits its immunostimulatory activity mainly by binding with TLR-4 on macrophages.[27] TNF-α is a signature cytokine of LPS activated macrophages which has a direct cytotoxic effect on cancer cells.[28] The

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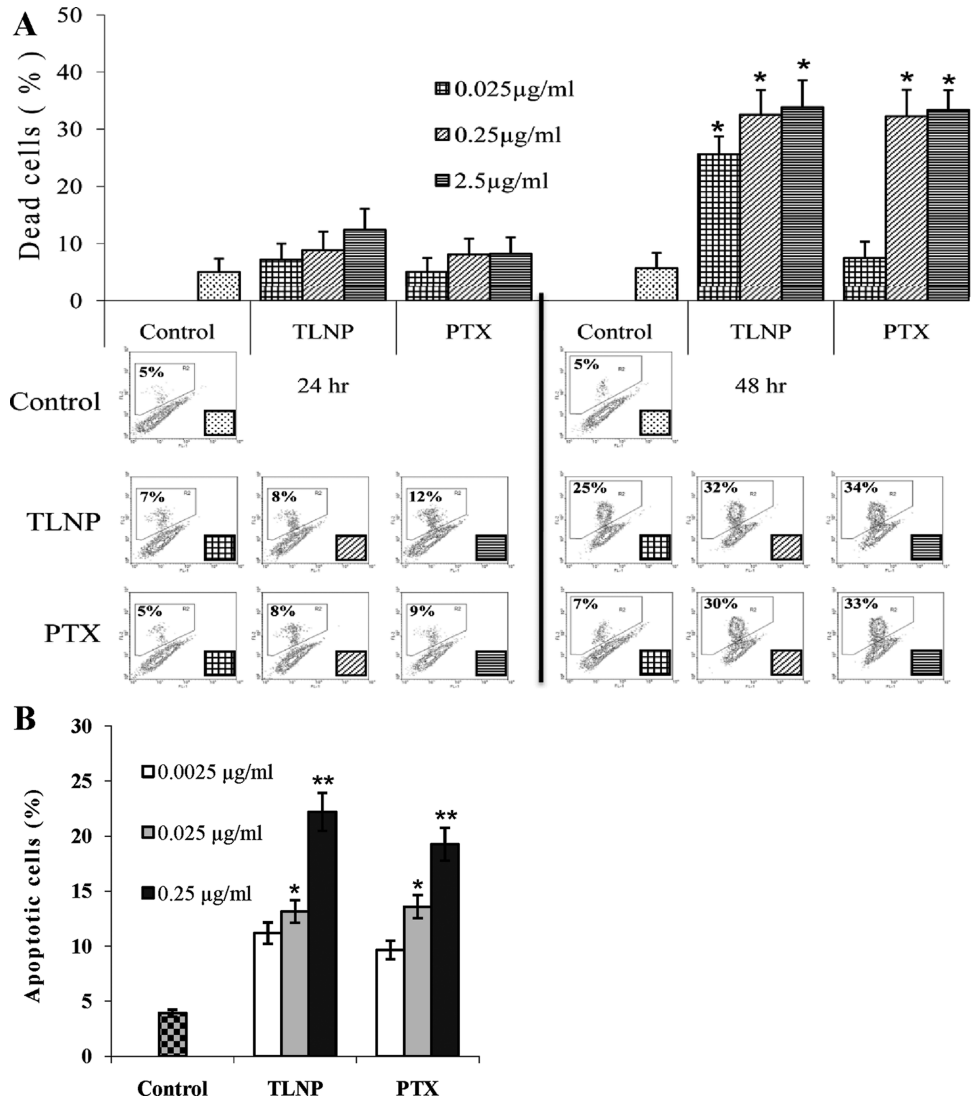

Figure 3. Comparison of direct cytotoxic and apoptotic activity of TLNPs and PTX on B16-F10 melanoma cells: (A) percentage of tumor cell death after treatment with TLNP or PTX for 24 and 48 h; (B) percent apoptotic cells after treatment with TLNP or PTX for 24 h. *$p < 0.05$; **$p < 0.01$.

time kinetics of TNF-α secretion from J774.1 macrophage cells treated with SP-LPS as well as TLNP is shown in Figure 4A. It started increasing after 1 h and reached saturation by 9 h. A significantly high amount of TNF-α secretion was found in both SP-LPS and TLNP treated macrophages compared to control.

IL-12 is an important stimulatory cytokine which activates different types of T cells.[29] The amount of IL-12 secreted was estimated after 24 h incubation. Both SP-LPS solution and TLNP induced a similar amount of IL-12 release (Figure 4A inset), but it was significantly higher compared to control. These results suggest that SP-LPS encapsulated in TLNP is in bioactive form and activates macrophages which in turn secrete proinflammatory cytokines important for antitumor immune response.

Immunostimulatory Activity of TLNP with Splenocyte Culture. Next, the immunostimulatory property of TLNP was studied in the primary culture of mice splenocytes. As splenocytes have all types of immune cells, the cross talk between macrophages and T cells and macrophage mediated T cell activation can be analyzed using this as a model system. For anticancer immune response, T cell activation is very important. Activated macrophages secrete IL-12,

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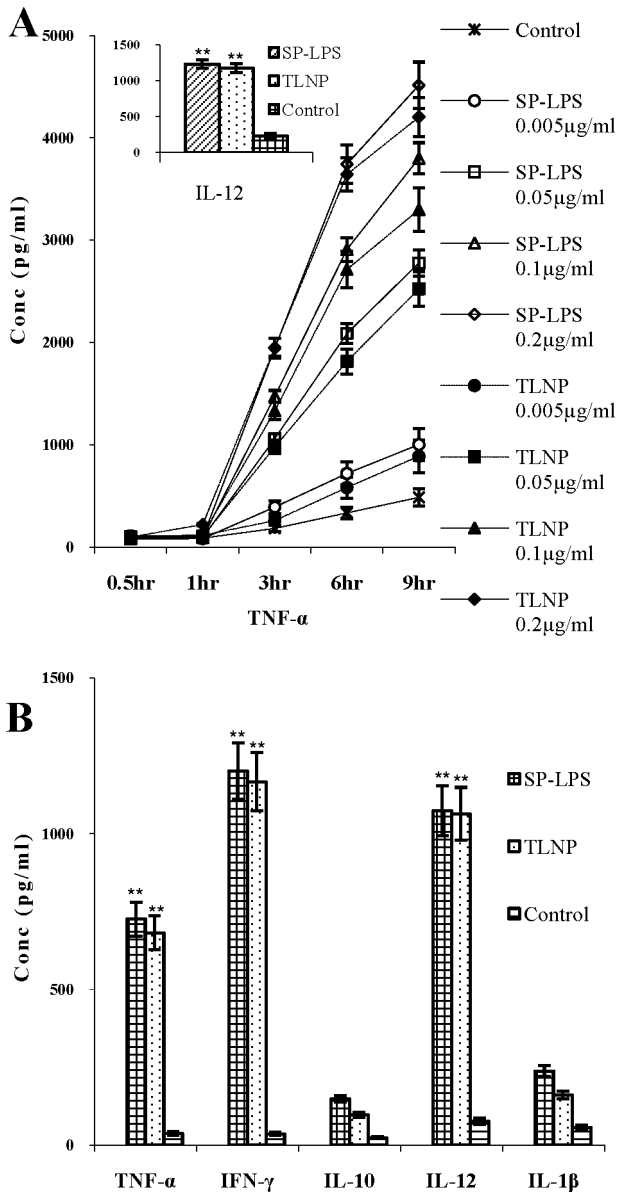

Figure 4. Comparison of immune stimulatory activity of TLNP and SP-LPS. (A) Release kinetics of TNF-α from macrophage cell line (J774.1), when stimulated with different concentrations of TLNP or SP-LPS solution. Inset shows release of IL-12 after 24 h incubation with SP-LPS or TLNP at 0.2 μg/mL SP-LPS equivalent concentration. (B) Induction of different cytokines by splenocytes after stimulation with SP-LPS solution or TLNP at the concentration of 0.2 μg/mL SP-LPS equivalent. $^{**}p < 0.01$.

which further activates T cells.[29] A high amount of IL-12 secretion was observed in the TLNP treated macrophages (Figure 4A, inset). To further confirm these findings in the primary culture of immune cells, IL-12 and TNF-α concentration was again measured from the culture supernatant of SP-LPS and TLNP treated splenocytes (Figure 4B). As expected, equally high concentration of both cytokines was found in SP-LPS solution as well as TLNP treated splenocytes. To analyze the T cell activation status, IFN-γ concentration was measured in the culture supernatant, as this is the signature cytokine of Th1 type of immune response.[30] A very high amount of IFN-γ secretion was observed in the treated groups as compared to control, providing evidence of T cell activation. Another stimulatory cytokine, IL-1β, was also secreted in a moderate amount in the treated groups. A very low amount of immune regulatory cytokine IL-10 was found in the culture supernatant of treated and control groups, and the difference was not significant. A higher concentration of IL-10 could have an inhibitory effect on the proinflammatory cytokines[31] and can down-regulate the antitumor immune response. Hence, these results establish that TLNP can effectively modulate the immune cells to an activated state to induce an efficient antitumor response.

Chemo-immunotherapeutic Activity of TLNP in Tumor Cell−Splenocyte Coculture System. The hypothesis of combined chemo-immunotherapy was confirmed by the coculture analysis, where B16-F10 cells were incubated with splenocytes derived from C57BL/6 mice. This coculture was used as a model system to analyze combined chemo-immunotherapeutic activity. Splenocyte stimulatory activity of TLNP was observed where TLNP activate the macrophages, which in turn stimulate other effector cells downstream in the immune cascade. In this coculture experiment, treatment with TLNP had shown increase in the death of the target cells as compared to PTX (Figure 5). The data suggest that, with TLNP treatment, chemotherapy and immunotherapy are acting in a synergistic manner to facilitate death of the cancer cells.

Biodistribution. The objective of the biodistribution study was to evaluate distribution of PTX when given as nanoparticles compared to commercial paclitaxel (Taxol). The drug (PTX) distributed in all the organs analyzed, in both the groups (Table 2). The plasma concentration of the drug varied significantly between the two groups (Figure 6A). The plasma C_{max}, which was attained at 5 min in both the groups, was about 3-fold less in the TLNP treated group compared to Taxol. The AUC of the Taxol group was found to be more than 2-fold compared to the TLNP group, which reflects rapid biodistribution of the drug to tissues in the TLNP treated group (Figure 6A). In the TLNP treated group, a higher amount of PTX accumulated in the tumor even at 5 min after injection (Figure 6B). The drug concentration in the tumor was found to be higher at all time points studied. This signifies rapid accumulation and retention of the drug in tumor, when administered as TLNP. The AUC of the drug in tumor was calculated to be 312.75 and 147.75 for TLNP

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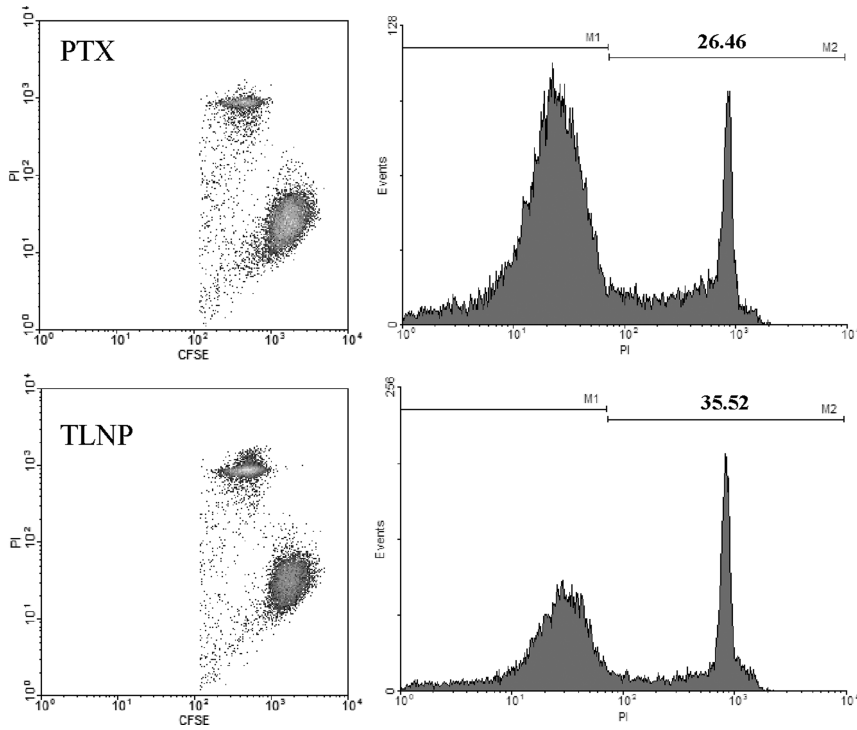

Figure 5. Effect of PTX or TLNP containing equivalent amount of PTX on tumor cell death when added in a coculture of splenocytes and CFSE labeled tumor cells. Target cells were gated and percentage of cell death was analyzed by PI uptake method.

and Taxol treatment respectively (Figure 6B). This indicates that the tumor cells were exposed to a significantly higher amount of the drug when delivered as TLNP in comparison to Taxol. The amount of PTX present in the liver at different time points was found to be similar in both the groups. The major pathway of metabolism of PTX is via hepatobiliary system. Comparable concentration in liver meant the metabolism of PTX was similar when delivered as NP dosage form or as commercial formulation.

Discussion

Although the current clinical formulation of paclitaxel (Taxol) has a promising clinical activity against a wide variety of tumors, it has significant toxic side effects, some of which are due to the solvent in which it is formulated and others due to the limited availability of drug in tumor mass. The toxicity of paclitaxel is related to the time that the drug exceeds plasma threshold levels in the range 50−100 nm.[5] The therapeutic dose of paclitaxel can be reduced by improving its availability in tumor mass (tumor targeting), which in turn will reduce its plasma concentration. Simultaneously its anticancer activity can be supported by activating the immune cells, so that combination of chemotherapeutic and immunotherapeutic activity could attack the tumor cells.

In this study we prepared the nanoparticles using PLGA and PVA was used as stabilizer. SP-LPS was present in the inner aqueous phase while PTX was in the oil phase. Amount of PVA used influenced the size of the nanoparticles as well as the encapsulation efficiency of both SP-LPS and PTX. Higher concentration of the PVA reduced the particle size moderately. This could be due to increase in the encapsulation of both PTX and SP-LPS with increase in PVA concentration, which prevented further reduction in the size of the particles. PVA also influenced the surface charge of the particles. Particles prepared with higher amount of PVA had less surface charge compared to NPs prepared using less PVA. This might be because the thickness of the interphasic layer of PVA varies proportionately with the amount of PVA used and this coating directly influences the zeta potential.[32] Nanoparticles prepared were uniform in size as evident from TEM images and low polydispersity index.

Release of SP-LPS from the TLNP was quite rapid: about 65% release was observed in the first 48 h. As SP-LPS was dissolved in the internal aqueous phase, the solvent water might have come out during the lyophilization process from the core of the particles and in the process made narrow channels, depositing SP-LPS on those channels as well as on the surface of the particles. This would have made this hydrophilic compound readily accessible to the aqueous release medium (PBS), resulting in rapid release.

Paclitaxel has been reported to have immunostimulatory activity in different model systems.[33] At subpharmacological dose, it increases the tumor immunogenicity by increasing

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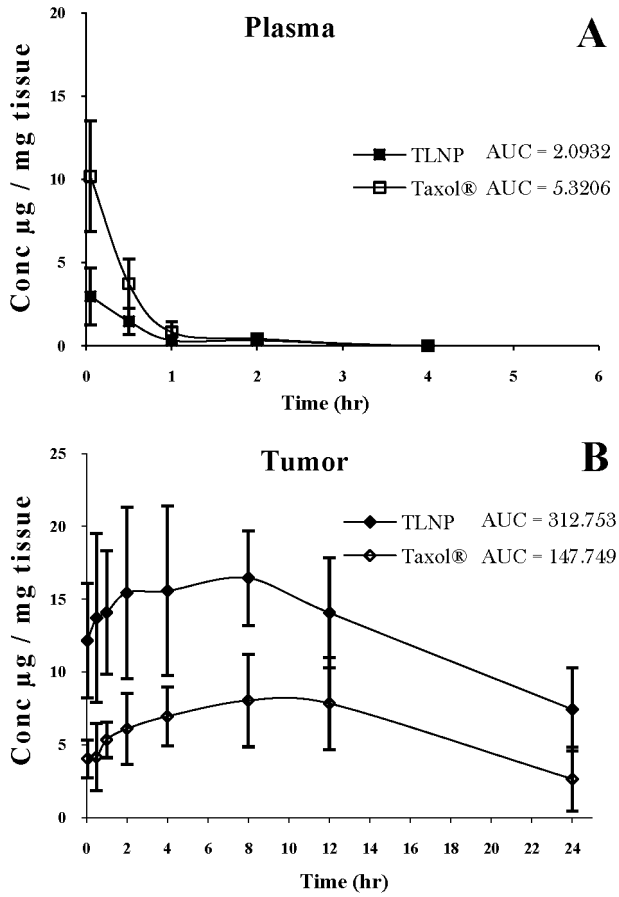

Figure 6. Amount of PTX in plasma (A) and tumor (B) at different time points after iv injection of Taxol or TLNP at the dose of 10 mg/kg PTX equivalent. Respective area under the curve (AUC) was calculated and is shown in the figure.

the amount of tumor antigen available.[34] In another study, pretreatment with low dose paclitaxel in vivo improved the antitumor potential of dendritic cell vaccine given intratumorally.[35] We found similar immunostimulatory activity of SP-LPS solution and TLNP containing equivalent amount of SP-LPS. Presence of PTX in TLNP did not contribute to the immunostimulatory activity of the SP-LPS. Although PTX is known to activate macrophages to secrete TNF-α, the concentration of the drug needed (3 μM)[36] for the secretion of a measurable quantity of the cytokine is much higher than that delivered in this experiment (0.8 μM; highest amount of PTX present in TLNP delivering 0.2 μg/mL SP-LPS). Hence, expectedly, there was no significant difference in the amount of TNF-α released between the two groups. In the splenocyte stimulation experiment also, TLNP induced significantly high TNF-α secretion. A high amount of IFN-γ was also induced, which indicates activation of effector T cells with IL-12 secreted by activated macrophages. These activated macrophages and T cells could target the tumor cells by direct cytotoxic action and also through the secreted cytokines.

In order to better understand the combined chemo-immunotherapeutic effect of TLNP, tumor cell death was analyzed in a coculture system where tumor cells and mononuclear immune cells treated with PTX and TLNP were coincubated. The results demonstrated that TLNP had high anticancer activity compared to PTX in the coculture model, due to combined effect of chemotherapy and immunstimulation. The proposed mechanism of the higher tumor cell death observed could be explained as, while the PTX would have direct tumor cell cytotoxicity, the SP-LPS would activate the macrophages which in turn would activate effector immune cells in the splenocytes to induce an efficient antitumor immunotherapeutic response. Activated effector cells like cytotoxic T cells and NK cells as well as TNF-α secreted from macrophages would contribute in killing the tumor cells.[37]

After iv administration of TLNP, the plasma concentration of free PTX was found to be significantly less while the tumor concentration was found to be higher compared to the Taxol group. This provides evidence of tumor targeted delivery of TLNP, which would result in less systemic toxicity. Simon et al. have reported that the plasma half-life of larger particles was much less than that of smaller particles.[38] While 90% of the particles in the size range 300−1000 nm were cleared from the circulation in less than 5 min after iv injection, it takes 15 min for 100 nm particles to get cleared. In another study the plasma concentration of the PTX was found to be much less when delivered as the micelle formulation (size 240 nm) and exhibited rapid tissue distribution.[39] Consistent with these previous studies, in the

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present study, TLNP which are about 290 nm in size have shown low plasma concentration and rapid distribution to tumor and other tissues.

In this study, following administration of TLNP, plasma concentration of free PTX was relatively low. Since the toxicity of PTX is related to the time that free drug exceeds a threshold concentration in plasma,[5] toxicity associated with TLNP treatment is expected to be less than that associated with administration of an equivalent dose of Taxol.

The results demonstrate that TLNP have high anticancer activity due to combined effect of chemotherapy and immunstimulation compared to PTX. With this combined therapy, we can reduce the amount of PTX required which in turn would reduce the side effects associated with its use. These results set the stage for targeted codelivery of chemotherapeutic drug and immunostimulant for future in vivo testing.

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