Research Article

Development of Novel Docetaxel Phospholipid Nanoparticles for Intravenous Administration: Quality by Design Approach

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Abstract. The objective of this study was to develop novel docetaxel phospholipid nanoparticles (NDPNs) for intravenous administration. Modified solvent diffusion-evaporation method was adopted in the NDPN preparation. Central composite design (CCD) was employed in the optimization of the critical formulation factor (drug content) and process variable (stirring rate) to obtain NDPNs with 215.53±1.9-nm particle size, 0.329±0.02 polydispersity index (PDI), and 75.41±4.81% entrapment efficiency. The morphological examination by transmission electron microscopy revealed spherical structure composed of a drug core stabilized within the phospholipid shell. Enhanced cell uptake of coumarin-6-loaded phospholipid nanoparticles by MCF-7 cell line indicated NDPN-efficient cell uptake. *In vitro* hemolysis test confirmed the safety of the phospholipid nanoparticles. NDPNs exhibited increased area under the curve (AUC) and mean residence time (MRT) by 3.0- and 3.3-fold, respectively, in comparison with the existing docetaxel parenteral formulation (Taxotere®), indicating a potential for sustained action. Thus, the novel NDPNs exhibit an ability to be an intravenous docetaxel formulation with enhanced uptake, decreased toxicity, and prolonged activity.

KEY WORDS: cancer; delivery vehicle; docetaxel phospholipid nanoparticles; nanoparticles; pharmacokinetics.

INTRODUCTION

Cancer is one of the major causes of death in the world. According to World Health Organization (WHO), around 7.6 million people die due to cancer all over the world. Cancer accounts for 13% of the total death in the world, principally due to lung, breast, stomach, liver, and colon cancer. It continues to claim lives with about 13.1 million deaths in 2030. In 2013, breast, lung, bronchus, and colorectal cancers were the most prevalent cancer in women, accounting for 51% of the estimated cancer cases. Breast cancer alone accounts for 29% of cancer in women and is the leading cause of death from cancer [1, 2].

Docetaxel (DTX) is one of the most potent anticancer agents to treat a wide range of tumors, especially breast, ovarian, lung, and head and neck cancers. DTX is a taxane derivative, structurally similar to paclitaxel, but more potent as an inhibitor of microtubule depolymerization. Since DTX exhibits very low water solubility, its marketed formulation (Taxotere®, Sanofi Aventis, Dagenham, UK) for intravenous administration contains a high concentration of Polysorbate 80. The presence of Polysorbate 80 in Taxotere is reported to cause numerous adverse effects, including acute hypersensitivity reactions, fluid retention, and peripheral neuropathy [3–5]. Hence, several approaches have been investigated to eliminate the addition of the Polysorbate 80 and increase DTX solubility and efficacy. They include PLGA nanospheres, PEgylated nanoliposomes, liposomes, nanostructured lipid carriers (NLCs), chitosan nanoparticles, oral solid dispersion, conjugates, and β-cyclodextrin complex [6–14]. Although these systems exhibit specific advantages, limitations exemplified by low drug loading, drug leakage, and polymer toxicity necessitate the development of a safe and effective DTX intravenous formulation.

Phospholipids are endogenous amphiphilic biocompatible macromolecules having a neutral charge. They form complexes with hydrophilic and lipophilic drug molecules, and the resulting colloidal dispersion has been called phytosomes and pharmacosomes [15–17]. Abelcet®, the phospholipid complex of Amphotericin B, forms a nanoassembly when added to aqueous medium. Upon intravenous administration, they have been found to accumulate in tumor tissue, attributed to the enhanced permeability and retention (EPR) effect associated with the poorly developed tumor vasculature that allows the 200–600-nm particles to diffuse freely into the tumor microenvironment [18, 19].

In the present study, we developed novel docetaxel phospholipid nanoparticles (NDPNs) with improved pharmacokinetics which can be safely administered intravenously. NDPNs eliminate the need for surfactants in preparation of parenteral formulation. A combination of phospholipids, distearoyl phosphatidylcholine (DSPC), and distearoyl phosphatidylglycerol

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(DSPG) (1:1) was selected in NDPNs. We adopted a novel modified solvent diffusion-evaporation method in NDPN preparation. Central composite design was employed for optimization of critical formulation and process parameters, identified by initial experimentation, to obtain optimum particle size, polydispersity index, and entrapment efficiency. The nanoparticle structure was determined by transmission electron microscopy (TEM). A simple hemolysis test was adopted to compare the toxicity of NDPNs with Taxotere. In addition, we compared the pharmacokinetics of NDPN and Taxotere in Wistar rats.

MATERIALS AND METHODS

Materials

Docetaxel trihydrate and paclitaxel were generous gift samples from Mac-Chem Product Pvt. Ltd. (Thane, India). Parenteral grade phospholipids, namely, dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol sodium salt (DMPG-Na), distearoyl phosphatidylglycerol sodium salt (DSPG-Na), distearoyl phosphatidylethanolamine (DSPE), distearoyl phosphatidylcholine (DSPC), Lipoid S 75–3 (saturated phosphatidylcholine 70%), Lipoid E 80-S (phosphatidylcholine 70%), and Lipoid S PC-3 (phosphatidylcholine ≥98%), were obtained as gift samples from Lipoid (GmbH, Germany). Coumarin-6 was purchased from Sigma-Aldrich, Mumbai, India. Dulbecco's modified Eagle's medium (DMEM) (high glucose) was purchased from HiMedia, Mumbai, India. Fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from Invitrogen, NY, USA. All other chemicals used were of analytical grade.

HPLC Analysis of Docetaxel

A HPLC method was validated for DTX estimation in all the samples, including in solubility and release experiments. The HPLC system comprised of a Waters 2695 separation module equipped with a quaternary pump, an auto sampler unit, and a Waters 2996 photodiode array detector (PDA). Phenomenex-C18 (5 μm; 250 mm×4.6 mm) analytical column was used for the estimation. The mobile phase consisted of acetonitrile and water in 65:35 (*v/v*) proportions. The flow rate was maintained at 1 ml/min and the PDA detector was set at 230 nm. The Empower HPLC software was employed for analysis of the results. The procedure was performed in triplicate, and the average and standard deviations were calculated.

Selection of Phospholipids

Several phospholipids were screened in order to select the optimum phospholipid on the basis of DTX solubility. Briefly, 1:2 molar ratios of DTX and phospholipid in distilled water were dispersed and agitated at 100 rpm for 24 h at 37°C [20]. The amount of dissolved DTX was quantified by HPLC.

Preparation of NDPN

Modified solvent diffusion-evaporation method was employed in the preparation of NDPNs. Weighed quantities of DTX and DSPC/DSPG (1:1) were separately dissolved in 5 and 10 ml methanol, respectively. Both the solutions were mixed at 40°C to obtain a clear solution and added dropwise in to 15 ml aqueous phase with stirring (RH basic 1 IKAMAG® heating plate, IKA, India) at 750 rpm. The solvent was evaporated (Rotavapor® R-210, Buchi, Switzerland) at 35°C under very low pressure (~50 mbar) to obtain NDPN dispersion. Free DTX in dispersion was separated by centrifugation at 8000 rpm for 10 min. The supernatant containing NDPNs was freeze-dried and stored at 2–8°C [21, 22].

The coumarin-6 phospholipid nanoparticles were prepared by the same method, as described above.

Design of Experiments (DoE)

In the screening trials, we evaluated the NDPN formulation and process parameters, including drug content, the ratio of combinations of phospholipids, volumes of the organic and aqueous phases, and stirring and evaporation rates. NDPN particle size, polydispersity index (PDI), and entrapment efficiency formed the responses; these are the critical quality attributes which affect the performance of the nanoparticle dispersion. They were significantly influenced by drug content (critical formulation parameter) and stirring rate (critical process parameter). Therefore, we selected drug content and stirring rate as the critical parameters for optimization by central composite design (CCD) (Design Expert 8.0.7.1) (Table I). The parameters were evaluated at three levels, high, middle, and low (+1, 0, and −1). Other parameters such as the volumes of organic and aqueous phases, evaporation rate, ratios of phospholipids, and amount of phospholipids were kept constant during the optimization study. A total of 13 random experimental trials were designed by the software with five center points, and four axial and four factorial points.

Freeze Drying of NDPNs

NDPNs were freeze-dried with 5%*w/v* PEG-6000 as a cryoprotectant. NDPNs were rapidly frozen in a shell freezer and lyophilized by continuous freeze drying for 48 h. Temperature at −74°C and pressure of 0.045 to 0.055 TORR were maintained in the freeze dryer (Allied frost FD-3, India) during the operation, and the freeze-dried NDPNs were stored at 2–8°C.

Table I. Formulation and Process Factors for CCD Study

Independent factor		Design level	
Drug content (% *w/w*)	A	35.0	−1
		52.5	0
		70.0	+1
Stirring rate (rpm)	B	750.0	−1
		1050	0
		1350	+1

Characterization of Nanoparticles

Particle Size, PDI, and Zeta Potential

The size and size distribution of NDPN particles were determined by dynamic light scattering (DLS) (Nano ZS, Malvern Instruments, UK), considering the average of ten measurements. The estimation of zeta potential was based on electrophoretic mobility in an electric field. The values are an average of 20 measurements.

Shape and Morphology

The shape and morphology of NDPNs were studied by transmission electron microscopy (TEM) (Technai F20, FEI, Netherlands). The sample was stained with 1.5% phospho-tungstic acid and placed on 400-mesh carbon-coated grid and held at RT for 90 s. The sample was viewed at an accelerating voltage of 100–200 kV.

Entrapment Efficiency

An indirect method was adopted for estimating the percentage of drug encapsulated in NDPNs. Briefly, NDPN dispersion was centrifuged at 8000 rpm for 10 min to sediment the free drug. The supernatant, containing the nanoparticles, was subjected to ultracentrifugation at 40,000 rpm for 30 min. The entrapment efficiency was calculated by the following formula:

$$\text{Entrapment efficiency}\ (\%)$$
$$= \frac{\text{weight of drug taken} - \text{weight of free drug}}{\text{weight of drug taken}} \times 100$$

Differential Scanning Calorimetry (DSC)

DSC thermograms of the freeze-dried NDPNs, physical mixture, PEG-6000, and DTX were recorded (Mettler Toledo, DSC, 821e, Switzerland). The system was calibrated with indium before the study. The samples (2–8 mg) were weighed, placed in aluminum pans, and heated at a rate of 10°C/min in an atmosphere of nitrogen. The samples were heated over a temperature range of 25–200°C and analyzed with empty pan as a reference.

Powder X-Ray Diffraction (PXRD)

The X-ray diffraction pattern of freeze-dried NDPNs, physical mixture, PEG-6000, and DTX was acquired from X-ray diffractometer (Bruker D8 Advance, Bruker, Germany). Measurements were performed at a voltage of 40 kV and 25 mA. The scanned angle was set from $3° < 2\theta > 40°$ and scanned at rate of 1 min^{-1}.

In Vitro Release Kinetics

In vitro release of NDPNs was studied in 0.01 M phosphate-buffered saline (PBS, pH 7.4, 3% Tween 80). The release profile of NDPNs was compared with the marketed formulation (Taxotere). Briefly, 2 ml of the sample (~3 mg of DTX) was filled into a dialysis bag (MWCS 12 kDa). The bag was suspended in beaker containing PBS (20 ml) and agitated in a shaker water bath at 100 rpm at 37°C [23]. At regular intervals, samples were withdrawn (200 μl) and quantified by HPLC. To analyze the release kinetics and mechanism, the data were fitted to zero-order, first-order, Hixson Crowell, Weibull, Higuchi's, Baker-Lonsdale, and Korsemeyer-Peppas release model:

$$\text{Release}\ (\%) = \frac{\text{Released DTX}}{\text{Total DTX}} \times 100$$

Cell Culture Experiment

Cell Line

Human breast cancer cell line MCF-7 was purchased from National Center for Cell Sciences, Pune, India. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, NY, USA) containing 10% fetal bovine serum.

Cell Uptake Study

The cell uptake potential of the phospholipid nanoparticles was evaluated in MCF-7 human adenocarcinoma cells. The cells were cultivated in the eight-well cover glass chamber until 70% confluence. Coumarin-6-loaded phospholipid nanoparticles, at 0.250 mg/ml concentration in DMEM, were added to the wells [9] and incubated for 30 min. After which, the cells were rinsed three times with phosphate-buffered saline (PBS) and observed under a fluorescent microscope (Nikon Eclipse, Te2000-S).

Hemolysis Study

NDPNs and Taxotere were evaluated for toxicity at 50 μg/ml concentration. Freshly collected blood samples from Wistar rat were washed three times with PBS and centrifuged at 2800 rpm for 5 min. The erythrocyte suspension (100 μl) was added to the formulations (900 μl) and incubated for 60 min at 37°C with intermittent shaking. The samples were centrifuged at 3000 rpm for 60 min, and the hemoglobin released in the supernatant was quantified by UV–vis spectroscopy at 540 nm. Erythrocytes incubated with saline and distilled water served as negative (0%) and positive controls (100%), respectively, and the data is represented as the percentage of complete hemolysis. The experiments were run in triplicate. The percent hemolysis rate (% HR) was calculated from the following Eq. (5):

$$\%\ HR = \frac{At - Anc}{Apc - Anc} \times 100$$

where At, Anc, and Apc are the absorbance of test samples, and negative and positive controls, respectively.

In Vivo Pharmacokinetic Study

Animals and Dosing

Female Wistar rats of 100–120 g were obtained from the central animal facility (CAF), NIPER, S.A.S. Nagar, India. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC), National Institute of

Pharmaceutical Education & Research (NIPER), S.A.S Nagar, India. The animals were maintained at 25±2°C and 50–60% relative humidity (RH) under natural light/dark conditions for 1 week before the experiment.

Rats were randomly divided into two groups of six animals each. They were administered (dose of 4 mg/kg) Taxotere injection and NDPNs in normal saline intravenously [23]. Blood samples were collected from the retro-orbital plexus under mild anesthesia into microcentrifuge tubes containing heparin (40 IU/ml blood) at 5-, 15-, 30-, 60-, 240-, 480-, and 720-min time points. Plasma was separated by centrifuging the blood samples at 7000 rpm for 5 min and stored at −20°C prior to analysis.

Quantification of DTX in Plasma Samples

DTX was quantified by the HPLC (Waters 2695). Briefly, an aliquot (100 µl) of plasma was mixed with 10 µl of internal standard solution (paclitaxel 20 µg/ml) and 50 µl of drug solution. After vortexing for 45 s, chilled acetonitrile (150 µl) was added as protein precipitating agent and vortexed for 3 min. The mixture was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was filtered (0.45 µm PVDF syringe filter, Millipore Millex-HV) and the filtrate (80 µl) was analyzed by HPLC system. Phenomenex-C18 (5 µm; 250 mm×4.6 mm) analytical column at temperature of 40°C was employed for the estimation. The mobile phase consisted of acetonitrile and water (pH 4.0) in 48:52 (*v/v*) proportions, maintained at 1.5 ml/min flow rate. DTX was quantified at 230 nm using PDA detector (Waters 2996). Calibration curve was obtained from 50–2000 ng/ml with r^2 value of 0.993.

Statistical Analysis

The results of *in vivo* experiments are reported as mean ±SD (*n*=6). The difference between the groups was tested by paired *t* test using GraphPad Prism 5.0 software. The difference was considered to be statistically significant when *P*<0.05.

RESULTS AND DISCUSSION

Selection of Phospholipids

The aim of the solubility study was to select the phospholipid in which DTX exhibited minimum solubility (Fig. 1). Although, in Lipoid S 75–3, DSPG-Na, DSPE, DSPC, and Lipoid S PC-3, DTX exhibited minimum solubility, we selected combination of DSPC and DSPG-Na (1:1) in NDPNs. DTX formed a stable dispersion with DSPC and DSPG-Na. The dispersion retained its size and integrity, which could be due to the negative charge on DSPG-Na, which stabilizes NDPNs and minimizes its aggregation.

Mechanistic Understanding of Particle Formation by Solvent Diffusion-Evaporation Method

In the marketed DTX formulation (Taxotere), Polysorbate 80 and ethanol (50:50*v/v*) added as co-solvents cause hypersensitivity reaction and fluid retention [24]. Hence, in order to avoid these adverse reactions, NDPNs were formulated by modified solvent diffusion-evaporation
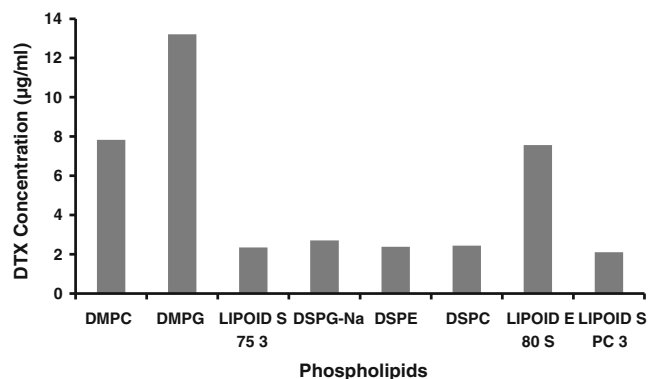

Fig. 1. Solubility of DTX in presence of different phospholipids

method. The nanodispersion is formed by a difference in the surface tension of the organic and aqueous phases. The force exerted by the liquid exhibiting high surface tension (water) on the surrounding liquid is stronger than the force exerted by methanol, which exhibits lesser surface tension. This difference results in turbulence and thermal inequalities in the system, leading to the continuous formation of eddies of methanol at the liquid interface, which generates interfacial convective flows. Consequently, violent spreading is observed due to miscibility of methanol and water, which again break down into smaller droplets and so on until it forms submicron droplets. The solvent present in the submicron droplets flow away because of its low surface tension and precipitate submicron phospholipid nanoparticles [25].

Optimization of Nanoparticles

In order to optimize the NDPN formulation and its process, the relation between the components of the formulation and process variables were studied and optimized by adopting the QbD principles. The DoE was adopted to optimize the critical formulation and process factors based on their effect on critical quality attributes (CQAs), those characteristics that affect the performance of NDPNs [26]. The CQAs of NDPNs include particle size, polydispersity index (PDI), and entrapment efficiency.

Effect of Independent Factors on Particle Size (y_1), PDI (y_2), and Entrapment Efficiency (y_3)

Thirteen experiments were conducted to optimize the drug content and stirring rate on NDPN particle size (y_1), PDI (y_2), and entrapment efficiency (y_3). From the study, particle size ranged from 170 to 508 nm, PDI from 0.270 to 0.534, and entrapment efficiency from 7.06 to 62.68%. Table II presents the data of CCD experimental runs.

ANOVA was applied to determine the significance and the magnitude of the effects of the main variables and interaction between variables. The results (Table III) confirm the adequacy of the models (*P* value <0.05). It identified the significant factors that affect the responses y_1, y_2, and y_3 of NDPNs. In particle size and PDI, the drug content (A) and stirring rate (B) were not significant; however, their interaction was significant (AB, AB^2, and A^2). In entrapment efficiency, stirring rate was the significant factor.

The final mathematical models obtained from the design are as follows:

Table II. Results of CCD Experiments

Run	Drug content (% w/w)	Stirring rate (rpm)	Particle size (y₁) (nm)	PDI (y₂)	Entrapment efficiency (%) (y₃)
1	52.5	1050	243.3	0.270	16.51
2	27.7	1050	419.5	0.448	7.060
3	52.5	1050	338.0	0.405	56.41
4	52.5	625	209.1	0.279	54.68
5	35.0	750	239.8	0.420	41.75
6	52.5	1050	253.2	0.348	44.38
7	35.0	1350	170.4	0.435	21.28
8	52.5	1050	246.2	0.468	47.38
9	77.2	1050	291.1	0.534	62.38
10	52.5	1050	342.8	0.370	49.30
11	70.0	750	270.0	0.328	56.87
12	70.0	1350	515.0	0.528	13.30
13	52.5	1474	333.2	0.447	45.73

$$\text{Particle size}(y_1) = 306.04 - 45.38A + 46.78B + 78.6AB - 11.28B^2 + 139.06AB^2$$
$$\text{PDI}\ (y_2) = 0.369 + 0.015\ A + 0.057\ B + 0.059\ A^2 \qquad (2)$$

$$\text{Entrapment efficiency}(y_3) = 37.54 + 10.67A\text{-}14.71B \qquad (3)$$

The positive sign indicates a synergistic effect, while the negative sign indicates an antagonistic effect.

Equation 1 illustrates that particle size decreases with an increase in drug content. However, the size increases with an increase in stirring rate. The interaction of drug content and stirring rate increases the particle size. A similar response was obtained with interaction of drug content with B^2.

Equation 2 illustrates that both drug content and stirring rate increased PDI. Similar response was observed with A^2.

Equation 3 illustrates that drug content increases the entrapment efficiency while stirring rate caused a reduction.

The 3D surface plots further explain the relationship between the variables and responses. Figure 2a presents the effect of stirring rate and drug content on particle size. The optimum particle size was obtained at decreased stirring rate and drug content. Figure 2b presents the effect of drug content and stirring rate on PDI.

Minimum PDI was obtained at decreased stirring rate and drug content of 51.63% w/w. Similarly, optimum entrapment efficiency at decreased stirring rate and at maximum drug content was observed (Fig. 2c).

The stirring rate at 750 rpm resulted in the optimum size, PDI, and entrapment efficiency in NDPNs. At lower stirring rates, the collision between the solvent droplets is less, providing enough time to disperse into submicron-sized particles. At higher stirring rates, although the particle size decreases, there is increase in PDI, probably due to breakage of the nanoparticles being formed due to attrition leading to smaller particles but with increased PDI (Fig. 2b). Drug content at 51.63% w/w resulted in optimum size, PDI, and entrapment efficiency in NDPNs. When drug content is 51.63% w/w, there is sufficient phospholipid to envelope the drug molecule. Further increase in drug content increases the percentage of drug entrapment which reaches an optimum (Fig. 2c).

The optimum conditions in NDPN formulation predicted by the model are drug content of 51.63% and stirring rate of 750 rpm to obtain particle size from 170 to 275 nm, PDI from 0.27 to 0.35, and entrapment efficiency within 40 to 62%. NDPNs exhibited optimized zeta potential of −37.05±0.77 mV. Experimental batches pre-

Table III. ANOVA for Particle Size (y₁), PDI (y₂), and Entrapment Efficiency (y₂)

Source	Sum of squares			Degree of freedom			F value			Prob>F P value		
	y₁	y₂	y₃	y₁	y₂	y₃	y₁	y₂	y₃	y₁	y₂	y₃
Model	83705.0	0.051	2173.85	5	3	2	5.67	4.29	5.00	<u>0.028</u>	<u>0.04</u>	<u>0.034</u>
A-Drug content (% w/w)	08239.4	0.001	911.090	1	1	1	2.79	0.47	4.19	0.145	0.51	0.070
B-Stirring rate	10799.8	0.018	1262.75	1	1	1	3.66	4.68	5.81	0.104	0.06	<u>0.039</u>
A^2	–	0.023	–	–	1	–	–	5.86	–	–	<u>0.04</u>	–
AB	24716.5	–	–	1	–	–	8.37	–	–	<u>0.027</u>	–	–
B^2	0538.54	–	–	1	–	–	0.18	–	–	0.684	–	–
AB^2	38677.1	–	–	1	–	–	13.1	–	–	<u>0.011</u>	–	–
Residual	0.031	2.029	1953.44	6	8	9	–	–	–	–	–	–
Lack of fit	0.010	0.033	1011.32	2	4	5	1.40	0.49	0.85	0.345	0.74	0.570
Pure error	0.021	0.025	942.110	4	4	4	–	–	–	–	–	–
Cor total	0.083	0.001	4127.29	11	11	11	–	–	–	–	–	–

Underlined entries are significant model terms with P value <0.05
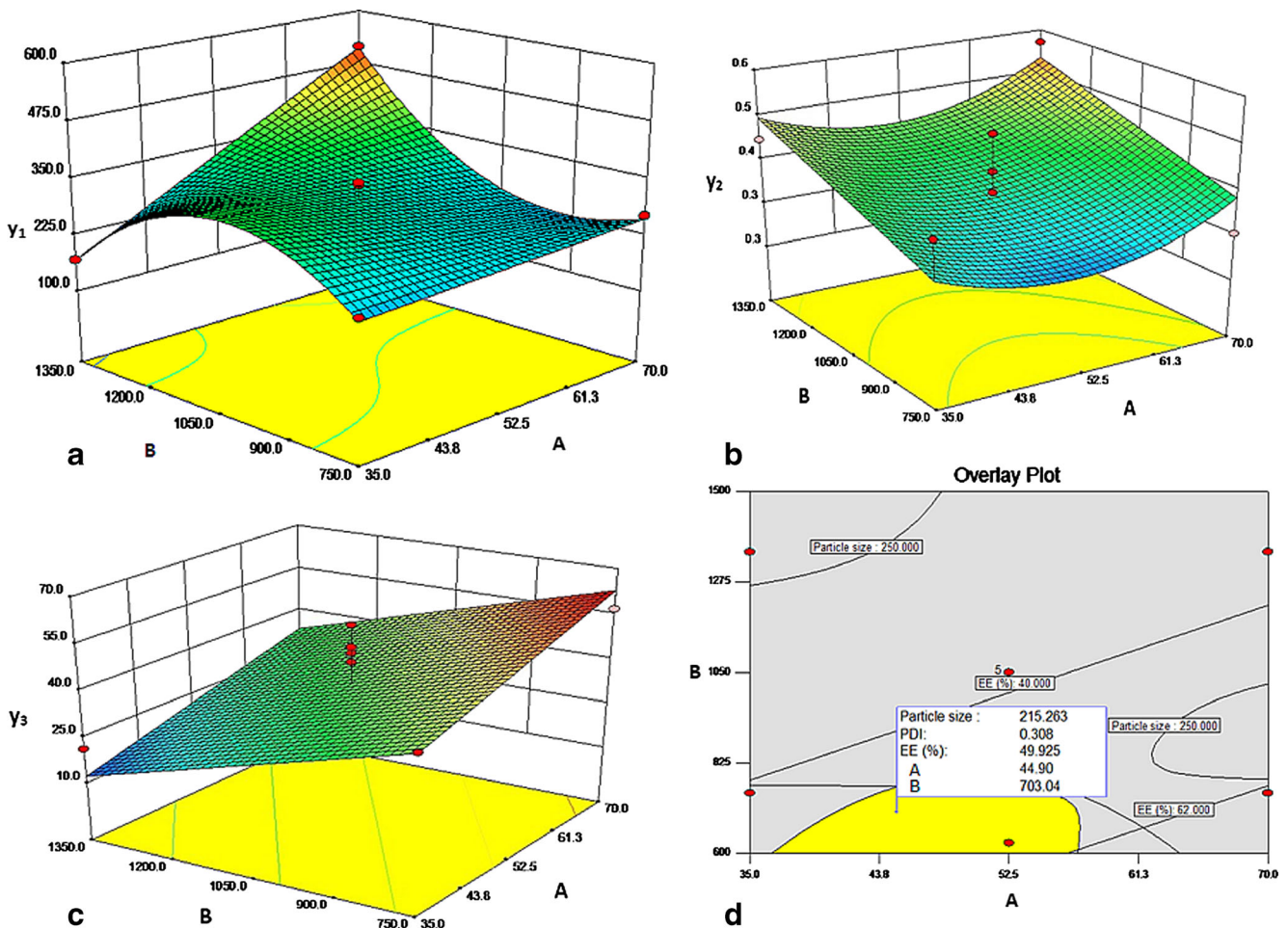
Fig. 2. Effect of drug content (**a**) and stirring rate (**b**) on particle size (**a**), PDI (**b**), and entrapment efficiency (**c**). Overlay plot for optimized parameters of NDPN (**d**)

pared with the optimum values yield results within the predicted limits, thus confirming the reliability of the optimization process. Figure 2d is an overlay plot with the design space (yellow area) for obtaining the nanoparticles.

Freeze Drying of NDPNs

NDPNs were freeze-dried with PEG-6000 (5%*w/v*) as a cryoprotectant to form to an intact fluffy cake which easily re-dispersed in water upon shaking with no significant ($P>0.05$)
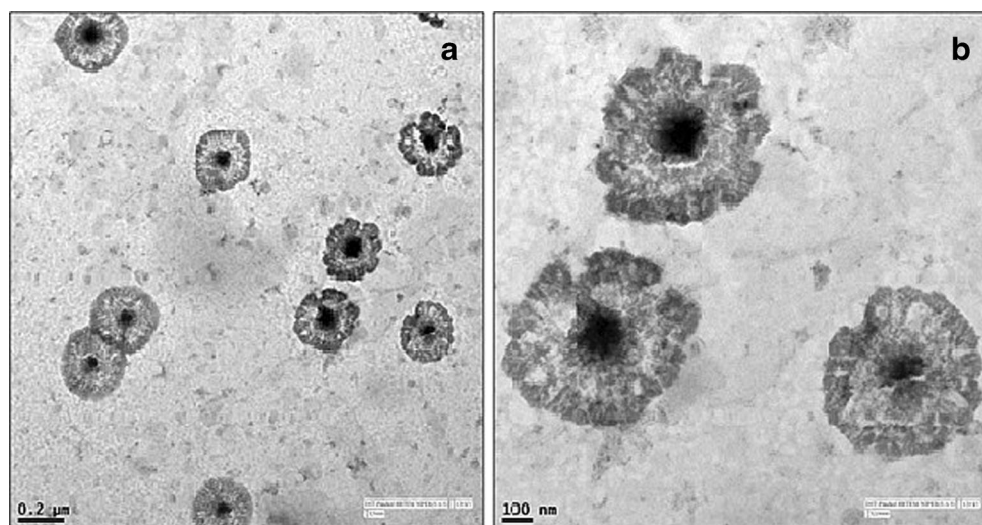
Fig. 3. TEM image of NDPNs at **a** ×5000 and **b** ×19,000
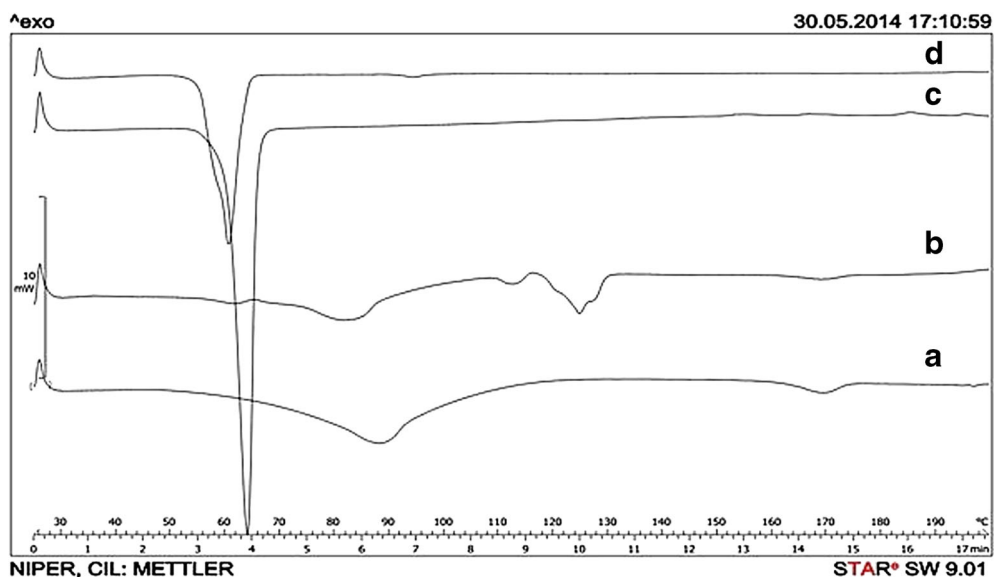
Fig. 4. DSC thermograms of DTX (**a**), physical mixture (**b**), PEG-6000 (**c**), and freeze-dried NDPNs (**d**)

changes in particle size ((217.60±3.66 nm before and 295.96 ±57.53 nm after freeze drying).

Characterization of Nanoparticles

Shape and Morphology

TEM image of NDPNs presents a spherical structure with a transparent layer of phospholipids around drug particles (Fig. 3). The polar groups of the phospholipid molecules are oriented toward the surface, while the non-polar tails face the core in which the drug appears to be present. A good correlation was obtained in the values of particle size obtained by Zetasizer and TEM.

DSC Analysis

DSC study was undertaken to determine the physical state of drug in the nanoparticles. The physical mixture exhibits endothermic peaks at 61.84°C (movement of phospholipid head group), 82.14°C (loss of water), 112.89°C (melting
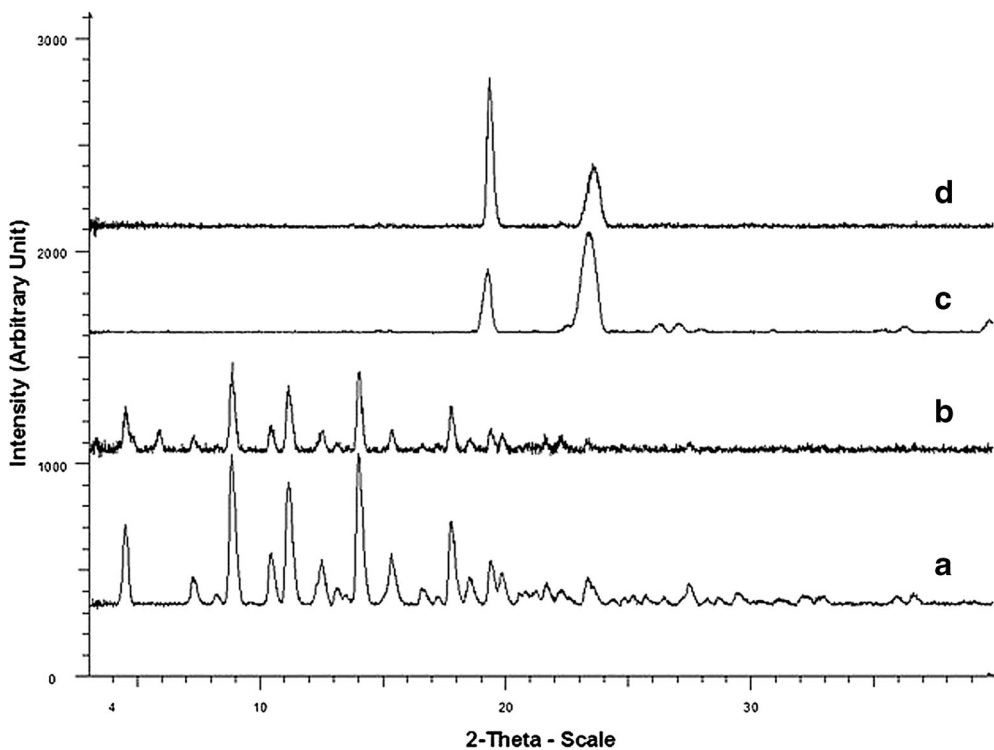

Fig. 5. X-ray diffraction pattern of DTX (**a**), physical mixture (**b**), PEG-6000 (**c**), and freeze-dried NDPNs (**d**)
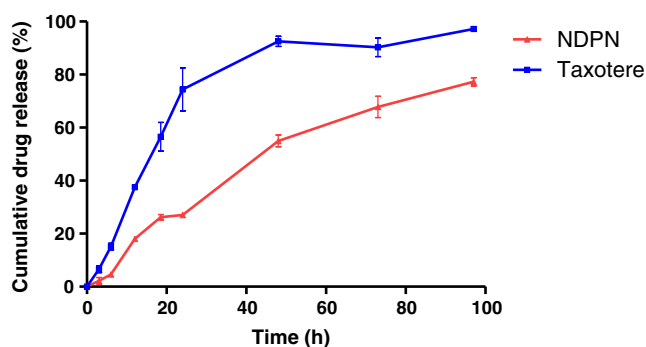
Fig. 6. Release profile of NDPNs

of DSPC), 124.82°C (melting of DSPG), and 169.24°C (melting of DTX). PEG-6000 exhibits a peak at 63.47°C (Fig. 4). In NDPNs, DTX endothermic peak at 169.24°C was absent, indicating possible entrapment of DTX within the phospholipid nanoparticle.

PXRD Analysis

X-ray diffraction (XRD) study was undertaken to determine the effect of the nanoparticle on the crystallinity of DTX. Figure 5 presents the XRD data of freeze-dried NDPNs, physical mixture, PEG-6000, and DTX. DTX exhibits characteristic XRD patterns, which were absent in NDPNs. This pattern could be due to DTX entrapment in the core of the phospholipid nanoparticles. Our finding was supported by the DSC analysis where the drug's endotherm peak has disappeared.

Release Kinetics

Figure 6 presents the drug release profiles from NDPNs and Taxotere. NDPNs exhibited a biphasic release profile with 26.43% of drug released in 24 h followed by prolonged release of up to 3 days (78.35%), while Taxotere released almost 80% of drug within 20 h. The release data was fitted into different release models. Weibull model was best suited (r^2=0.9972) for the release of DTX from NDPNs. This is an empirical model which has been widely employed to study the release data of both rapid and extended release drug delivery systems. It is applicable for any drug release mechanism involving
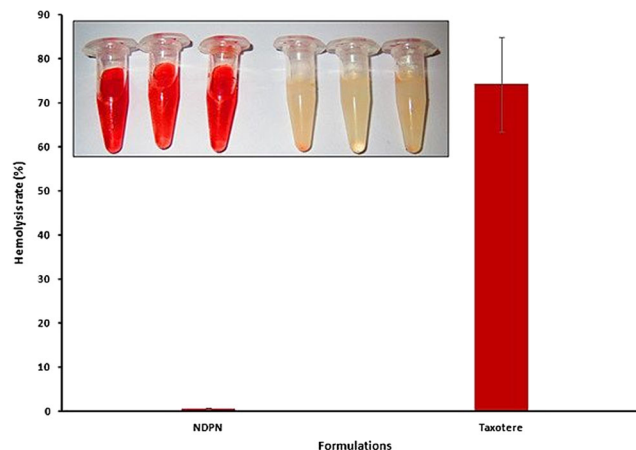

Fig. 8. *In vitro* hemolysis of NDPNs and Taxotere. Insert images (from *left*): the *first three tubes* are Taxotere and *last three tubes* are NDPNs (*n*=3)

dissolution, diffusion, and mixed dissolution-diffusion rate limited process [27]. The slope of model equation (shape factor) is an indicator of the mechanism of transport of drug through the delivery system. The estimated value of shape factor ≤0.75 implies Fickian diffusion while value in the range of 0.75–1 indicates a combined mechanism of Fickian diffusion and swelling control release [28]. From NDPNs, DTX exhibited a shape factor value of 0.8 indicating a combined mechanism of release. Sustained release of DTX from NDPNs would lead to enhanced plasma concentration and reduced biodistribution in comparison to Taxotere, which in turn may reduce the harmful effects of DTX to normal tissues.

Cell Uptake Study

The cell uptake potential of NDPNs was studied in human adenocarcinoma (MCF-7) cells by fluorescence microscopy. Since DTX does not exhibit fluorescence, NDPNs of coumarin-6, a fluorescent hydrophobic dye with poor aqueous solubility (0.25 µg/ml), were employed. The dye has been commonly employed as a model for studying cell uptake and internalization potential of nanoparticles [29, 30]. The MCF-7 cells were selected for the uptake study considering that the
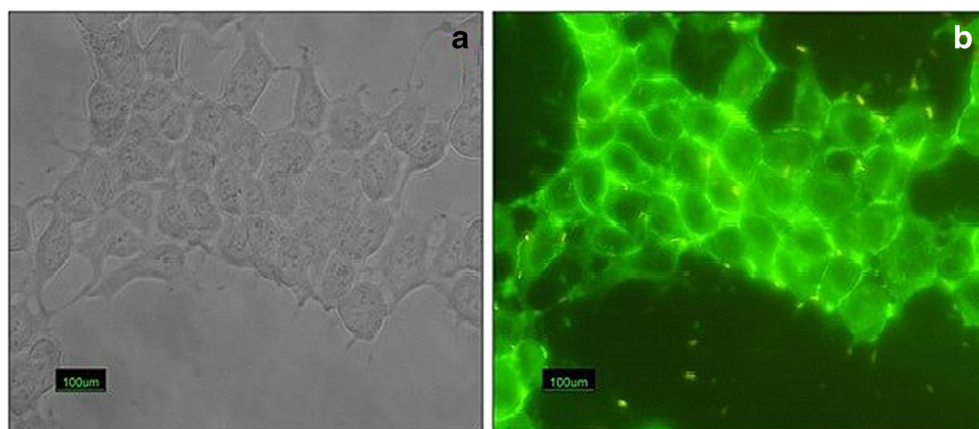

Fig. 7. Cell uptake study in MCF-7 cells. **a** Transmitted light channel and **b** FITC channel
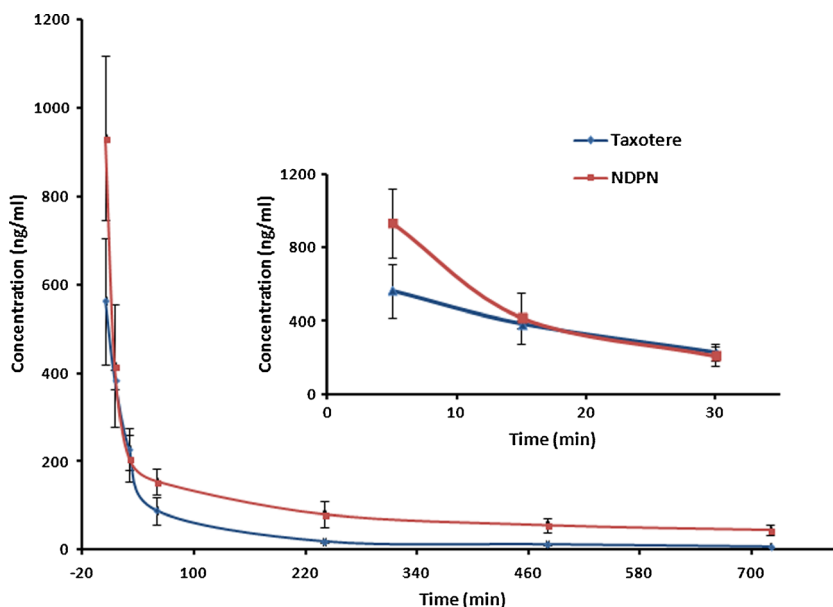
Fig. 9. Plasma concentrations of DTX after intravenous administration of Taxotere and NDPNs to rats (4 mg/kg) (*n*=6). Insert figure is the detail view of mean DTX plasma concentration-time profiles from above two formulations over period from 5 to 30 min

ultimate goal of NDPN formulation was in optimization of breast cancer treatment. Figure 7 presents the internalization of the coumarin-6 phospholipid nanoparticles in the MCF-7 cells. There was an enhanced uptake of the nanoparticles, indicated visually by the intense fluorescence observed within the cells, confirming the potential of NDPN formulation for increased uptake by cancer cells.

Hemolysis Study

This study was undertaken to evaluate the hemolytic activities of Taxotere and NDPNs. Surfactants have been reported to interact with the lipid bilayer of red blood cells and cause hemolysis [31]. NDPNs exhibited significantly less ($P<0.05$) hemolytic activity (50 μg/ml DTX) in comparison to Taxotere (Fig. 8). Polysorbate 80 present in the commercial formulation as solubilizing agent causes severe hemolysis. The biocompatibility of NDPNs can be attributed to phospholipid, which is a component of biological membranes. Hence, NDPNs are a novel formulation for safe intravenous administration of docetaxel.

Table IV. Pharmacokinetic Parameters of DTX After Intravenous Administration of Taxotere and NDPNs to Rats (4 mg/kg)

Parameters	Taxotere	NDPN
C_{max} (ng/ml)	562.86±143.19	932.20±186.05*
Cl (l/h/kg)	6.73±0.81	2.21±0.36*
$T_{1/2}$(h)	5.57±0.86	10.42±4.35*
MRT(h)	3.58±0.20	11.8±3.89*
AUC_{0-12h} (ng/ml·h)	598.48±72.34	1806.29±282.94*

Data are represented as mean value±SD, *n*=6
C_{max} maximum plasma concentration, *Cl* clearance, *$T_{1/2}$* biological half life, *MRT* mean residence time, *AUC_{0-12h}* area under the curve
*$P<0.05$, significant difference from Taxotere

Pharmacokinetic Study

The comparative effect of NDPNs and Taxotere on the DTX pharmacokinetic parameters was evaluated. Figure 9 presents the plasma concentration-time profiles after intravenous administration of the two formulations. NDPNs exhibited enhanced DTX plasma concentrations with enhancement in the mean residence time (MRT) (11.85±3.89 *vs*. 3.58±0.20 h), area under the curve (AUC_{0-12h}), and prolonged $T_{1/2}$ (10.42±4.35 *vs*. 5.57±0.86 h) in comparison to Taxotere (Table IV). NDPNs exhibited considerably reduced clearance (2.21±0.36 *vs*. 6.73 ±0.81 l/h/kg) in comparison to Taxotere. NDPNs were detectable even after 12 h while Taxotere concentration was undetectable after 4 h in the blood plasma. This enhancement in plasma DTX concentration for an extended period of time can be attributed to the reduction in clearance owing to the presence of phospholipid, which probably retards the elimination rate of the drug. Thus, NDPNs aid in safe intravenous delivery of DTX with prolonged circulating time, and enhanced cell uptake has the potential for increased efficacy.

CONCLUSIONS

In the present study, we developed novel docetaxel phospholipid nanoparticles. Docetaxel is lipophilic molecule necessitating the need of solubilizing agents in the development of parenteral formulation. The phospholipid nanoparticles proved to be suitable for intravenous administration with added advantages of safety, cell uptake with a potential for prolonged duration of action. The oral bioavailability enhancement of drug phospholipid complex has been widely reported, including studies from our laboratory; however, the viability of phospholipid nanoparticles for intravenous administration with reduced toxicity

and enhanced bioavailability indicates its potential for effective parenteral delivery, as well.

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