European Journal of Pharmaceutics and Biopharmaceutics 79 (2011) 276–284

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Paclitaxel loaded PEG_{5000}–DSPE micelles as pulmonary delivery platform: Formulation characterization, tissue distribution, plasma pharmacokinetics, and toxicological evaluation

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ARTICLE INFO

Article history:
Received 1 February 2011
Accepted in revised form 27 April 2011
Available online 7 May 2011

Keywords:
PEG_{5000}–DSPE
Sustained release
PEG–lipid micelles
Intratracheal administration
Tissue distribution
Pharmacokinetics

ABSTRACT

The objective of the present study was to evaluate the potential of paclitaxel loaded micelles fabricated from PEG_{5000}–DSPE as a sustained release system following pulmonary delivery. PEG_{5000}–DSPE micelles containing paclitaxel were prepared by solvent evaporation technique followed by investigation of *in vitro* release of paclitaxel in lung simulated fluid. Tissue distribution and plasma pharmacokinetics of the PEG–lipid micelles after intratracheal and intravenous administrations were investigated in addition to intratracheally administered taxol. Finally, toxicological profile of PEG_{5000}–DSPE was investigated. Paclitaxel was successfully formulated in PEG–lipid micelles with encapsulation efficiency of 95%. The PEG–lipid micelles exhibited a sustained release behavior in the simulated lung fluid. Intratracheally administered polymeric micellar paclitaxel showed highest accumulation of paclitaxel in the lungs with AUC_{0-12} in lungs being 45-fold higher than intravenously administered formulation and 3-fold higher than intratracheally delivered taxol. Paclitaxel concentration in other non-targeted tissues and plasma were significantly lower as compared to other groups. Furthermore, toxicity studies showed no significant increase in levels of lung injury markers in PEG_{5000}–DSPE treated group as compared to saline-treated group. PEG_{5000}–DSPE micelles delivered intratracheally were able to sustain highest paclitaxel concentrations in lungs for long periods of time, thus apprehending their suitability as pulmonary drug carriers.

1. Introduction

Pulmonary disorders such as lung cancer, chronic obstructive pulmonary disease, tuberculosis, asthma and many other respiratory infections, can be efficiently treated if high and prolonged drug concentrations are maintained in the lungs [1,2] and delivered via pulmonary route of drug delivery [3,4]. Pulmonary drug delivery for the local treatment of lung disorders offers many advantages over other routes of administration [5–7]. Direct deposition of drug at the diseased site could increase and sustain local drug concentrations [8,9]. Increase in drug retention time may improve the pulmonary receptor occupancy and potentially reduces the overall dose required, thereby avoiding the side effects that result from high doses of drug [10,11]. Furthermore, if pulmonary route of administration is combined with the sustained release potential of novel drug delivery systems, the therapeutic advantages of a drug can be further accentuated. Sustaining the drug in the lungs may result in favorable therapeutic index by prolonging drug action at the target site, reducing its side effects, and enhancing patient compliance [12–14].

A number of micrometer and nanometer-sized drug carrier systems such as liposomes, biodegradable microspheres, lipid nanocapsules, cyclodextrin complexes, and polymeric nanoparticulate systems have been investigated as potential pulmonary sustained-release systems [15,16]. In particular, polymeric nanoparticles fabricated from polymers like poly(lactide-co-glycolide) (PLGA), poly(L-lactic acid) (PLA), gelatin, chitosan, alginate, and poly-L-lysine have been extensively studied [6,7]. When administered, nanoparticles are deposited in the fluid lining the lungs, which protects them from mucociliary clearance until their dissolution [5,17,18]. Majority of these respiratory formulations, however, have not been successful in controlling the pharmacokinetics of inhaled therapeutics beyond a few hours [13]. This is due to the efficient clearance of therapeutics from the deep lung either

Abbreviations: PEG_{5000}–DSPE, polyethylene glycol 5000–distearoylphosphatidylethanolamine; DSC, differential scanning calorimetry; ^1H NMR, proton nuclear magnetic resonance spectroscopy; T_e, targeting efficiency; AUC, area under the curve.

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doi:10.1016/j.ejpb.2011.04.017

through phagocytosis in the alveolar region or via the rapid absorption of the delivered therapeutics by alveoli [14,15].

In order to overcome the limitations of the currently available off-the-shelf polymers, there is a growing need to investigate new polymers for pulmonary delivery that are safe and which may further sustain the drug concentration in the lungs. In 2006, Gaber et al. reported the potential of pulmonary delivery matrices made from pegylated phospholipids to target glucocorticosteroids to inflamed lung tissue [18]. PEG–phospholipids are amphiphilic polymers with a hydrophilic PEG block and a hydrophobic distearoylphosphatidylethanolamine (DSPE) block. In aqueous conditions, this polymer self assembles to form the micellar structures. The hydrophilic PEG end forms the corona of the micelle, which confers the protection from phagocytic uptake, while the lipidic DSPE end forms the core. These polymers are made from phospholipids that are endogenous to the lungs, which make them suitable for pulmonary drug delivery [19]. PEG–DSPE micelles also have the potential to slowly release a molecule over a period of time due to their long fatty acyl chains, which confer less mobility to entrapped drug [19–21]. In addition to this, PEG–DSPE micelles have been shown to be actively accumulated in the Lewis Lung Carcinoma in mice when given intravenously [22]. Therefore, combining the advantages of the pulmonary route of drug administration and the sustained release potential of PEG–DSPE micellar system can be of great therapeutic value in the treatment of local lung disorders, especially lung cancer, which has been traditionally treated with systemically delivered chemotherapeutics. The systemic route of delivering drugs to treat lung disorders has yielded limited results due to the low drug levels attained in the lung tissue and the non-specific uptake by other non-targeted organs.

To date, however, no *in vivo* studies comprehending the proficiency of pulmonary delivered lung-targeted PEG–lipid micelles fabricated from PEG–phospholipids have been reported. Therefore, the objective of this study was to investigate the tissue distribution, and plasma pharmacokinetics of paclitaxel loaded poly(ethylene oxide)-block–distearoyl phosphatdylethanolamine (PEG_{5000}–DSPE) micelles after direct deposition into lungs through intratracheal route in normal Sprague–Dawley rats. Paclitaxel was selected because of its proven therapeutic potential in the treatment of lung cancer [23–25]. Toxicological evaluation of PEG_{5000}–DSPE as a pulmonary drug carrier after intratracheal delivery was also carried out. Overall, this project was undertaken to understand the disposition of paclitaxel and the role of PEG–lipid micelles in improving the body distribution kinetics of paclitaxel when given through the pulmonary route.

2. Materials and methods

2.1. Materials

Paclitaxel and β-N-acetylglucosaminidase assay (NAG) kit were obtained from Sigma–Aldrich Co. (St. Louis, MO). PEG_{5000}–DSPE was obtained from Avanti Polar Lipids (Alabaster, AL). Infasurf was a generous gift from ONY, Inc. (Amherst, NY). Alkaline phosphatase (ALP) assay kit was obtained from Pointe Scientific Inc. (Canton, MI).

2.2. Micelle formation and drug loading

Paclitaxel loaded micelles were prepared by the solvent evaporation method [21,26]. The ratio of drug to polymer was varied in order to achieve maximum encapsulation. The paclitaxel amount was fixed, whereas polymer concentration was increased from 5 to 40 mg/ml. Paclitaxel and PEG_{5000}–DSPE were dissolved in methanol and mixed. The organic solvent was removed by liquid nitrogen at 60 °C to form a thin film of drug–micelle material mixture. This film was further dried under high vacuum to remove any traces of remaining solvent. The dried film was then hydrated in desired volume of distilled water. The mixture was incubated in water bath at 40 °C for 20 min. Non-incorporated paclitaxel was removed by centrifugation at 5000 rpm for 10 min. The supernatant was collected in glass vials and then lyophilized. The amount of paclitaxel encapsulated in the micellar phase was measured by a reversed phase-HPLC method described in the following paragraphs. The clear aqueous dispersion was diluted with acetonitrile to disrupt the micelles and release free paclitaxel. Drug entrapment efficiency (EE) was calculated using the following formula:

$$EE = \frac{\text{Weight of drug in micelles}}{\text{Weight of feeding drug}} \times 100$$

2.3. Micelles characterization

2.3.1. Micelle size determination

The micelle size (hydrodynamic diameter) was measured by dynamic light scattering (DLS) using Nicomp 380 ZLS (Agilent Technologies, Santa Clara, CA). The micelle suspensions were diluted with deionized distilled water until the concentration provided a light scattering intensity 300 kHz. Three runs were made for each sample.

2.3.2. Zeta-potential measurement

Zeta-potential of the micelle formulations was measured by the Nicomp 380 ZLS. The micelle suspensions were diluted with distilled water to have a significant intensity within the limits required by the instrument. For each sample, zeta potential measurement was repeated three times.

2.3.3. Differential scanning calorimetry (DSC)

To confirm the entrapment of paclitaxel inside the micelles, DSC studies were performed. The thermal properties of lyophilized paclitaxel loaded micelles, paclitaxel alone, physical mixture of polymer and paclitaxel were examined using DSC (TA instruments, DE, USA). About 4–7 mg of samples were placed in aluminum pan and crimped. The thermograms covered the range from 0 to 250 °C at a heating rate of 10 °C/min.

2.3.4. Nuclear magnetic resonance spectroscopic studies (NMR)

In order to confirm the DSC results, NMR studies were accomplished. The ^1H NMR spectra were recorded on a JEOL Eclipse NMR spectrometer operating at 400 MHz (JEOL USA, Inc., MA). NMR spectra of paclitaxel in $CDCL_3$, $PEG–DSPE_{5000}$ in D_2O, and lyophilized micelles containing paclitaxel in D_2O and $CDCL_3$ were obtained.

2.3.5. Stability of paclitaxel-loaded micelles

Paclitaxel loaded micelles were evaluated for physical stability in double distilled water at 25 °C. Time-dependent changes in mean diameters of micelles and scattering intensities of micellar solutions were monitored by DLS. Also, the chemical stability was evaluated by examining the changes in the peak shape, retention time and presence of any degradation peak in the chromatograms of micellar paclitaxel dispersion.

2.4. Drug release studies in lung simulated fluid

To mimic the release of paclitaxel from the PEG–lipid micelles in the actual lung environment, lung simulated fluid containing infasurf as natural lung surfactant was used [27]. The composition of simulated lung fluid containing 0.02% infasurf is shown in

Table 1
The composition of simulated lung fluid containing 0.02% infasurf.

Component	Concentration (g/l)
$MgCl_2 \cdot 6H_20$	0.212
NaCl	6.415
$CaCl_2 \cdot 2H_2O$	0.255
Na_2SO_4	0.079
Na_2HPO_4	0.148
$NaHCO_3$	2.703
Sodium tartrate	0.199
Trisodium citrate dihydrate	0.180
Sodium lactate	0.175

Table 2
Drug release kinetics based on the value of diffusion exponent (n) obtained from Peppas model.

Diffusion exponent (n)	Overall solute diffusion mechanism
0.45	Fickian diffusion
$0.45 < n < 0.89$	Anomalous (non-Fickian diffusion)
0.89	Case-2-transport
$n > 0.89$	Super case-2-transport

Table 1. Release studies were performed using the procedure reported by Kwon et al. [28]. Briefly, PEG–lipid micelles containing 250 μg of paclitaxel was suspended with 2.5 ml of distilled water and loaded into the dialysis bag with molecular weight cutoff of 10,000 Da. The dialysis bag was then placed in 2.0 L of lung simulated fluid to maintain the sink conditions. Samples of 100 μl were withdrawn from the dialysis bag at various sampling time intervals and then replaced with 100 μl of distilled water. The sampling time intervals were 0, 0.08, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, and 24 h. The amount of drug in each sample was then determined by HPLC analysis. In order to elucidate the drug-release mechanism, release exponent (n) values were estimated by fitting the dissolution data to the following Peppas model:

$$\log \frac{M_t}{M_\infty} = \log K + n \log t$$

where M_t/M_∞ is the fractional drug released, t is the release time, K is the constant, and n is the release exponent indicative of the release mechanism [29]. The n value is used to characterize different release mechanisms as given in Table 2.

2.5. Paclitaxel tissue distribution and pharmacokinetics

2.5.1. Animals

Male Sprague–Dawley rats weighing 300–400 g were acquired from Harlan Laboratories (Houston, TX). All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Louisiana at Monroe and all surgical and treatment procedures were consistent with the IACUC policies and procedures. Rats were maintained on a 12-h light/dark cycle before the study and were allowed free access to food and water.

2.5.2. Tissue distribution studies

All the formulations were freshly prepared. Twenty-seven rats were divided into three groups. Group A was intratracheally administered with paclitaxel loaded micelles; group B was intravenously administered with paclitaxel loaded micelles; and group C was intratracheally administered with taxol prepared in the laboratory. It was prepared with same composition as that present in the commercially available injectable Taxol® (Bristol-Myers Squibb, NY) to serve as control. Taxol contained 6 mg/ml of paclitaxel, 527 mg/ml of purified Cremophor EL (polyoxyethylated castor oil) and 49.7% (v/v) dehydrated alcohol. All rats were administered with paclitaxel formulations at 2 mg/kg dose. This dose was selected because available *in vivo* animal studies have employed intratracheal doses of paclitaxel in the range from 1.2 mg/kg [30] to 5 mg/kg [4]. Thus, in the current study, an intermediate dose was selected to achieve maximum therapeutic benefits with minimal dosage requirements. Each group was further divided into three subgroups that correspond to tissue sampling points after 1, 6, and 12 h following administration ($n = 3$/time point). Tissues collected were blood, lungs, liver, kidney, heart, and spleen. The collected tissues were blotted dry with paper towel, washed thrice in ice-cold saline, weighed and then kept in glass vials. The tissues were stored in −20 °C until analysis.

On the day of experiment, rats were anesthetized by an intraperitoneal injection of 1.2 g/kg urethane. Intratracheal administration of paclitaxel formulations was achieved by intratracheal instillation because dose can be applied directly into the lungs with minimum drug loss during application. The formulations were instilled into rat lung using Microsprayer® (Model IA-1B; Penn Century Inc., Philadelphia, Pennsylvania, USA). The methodology for this technique has been described previously by Hussain et al. [31] and Bai et al. [32]. Intravenous doses were administered via the femoral vein cannulation.

2.5.3. Pharmacokinetics studies

To investigate paclitaxel pharmacokinetics from different formulations, rats were divided into three groups, as described earlier. Each group was administered with 2 mg/kg single dose ($n = 3$ per group). Following the intratracheal and intravenous administrations of paclitaxel formulations, serial blood samples were collected from femoral artery at 0 (before administration), 0.5, 1, 2, 4, 6, 8, 10, and 12 h. At each time point, 200 μl of blood was collected in a heparinized microcentrifuge tube, kept in ice until the plasma was separated by centrifugation (5000 rpm for 10 min), and stored at −20 °C until further analysis by HPLC.

2.6. HPLC analysis of paclitaxel in plasma and tissues

2.6.1. Chromatographic conditions

An isocratic Waters HPLC system (Waters, Milford, MA, USA) was used. The system was equipped with a UV detector set at a wavelength of 227 nm. Data acquisition was achieved by Water's Empower chromatography data software package. The mobile phase used for the separation of paclitaxel in plasma and tissue homogenate samples consisted of acetonitrile and water (53:47, v/v) delivered at 1.0 ml/min flow rate. The separation was performed at room temperature on Phenomenex C18 column (250 × 4.6 mm, 5 μm; Torrance, CA). All samples were analyzed in duplicate.

Under these chromatographic conditions, the total run time was 13 min with a retention time of 11.1 min for paclitaxel. Standard curves for paclitaxel in plasma and tissue homogenates were prepared in the ranges of 50–5000 ng/ml and 0.25–120 μg/g, respectively. The validation of method included determination of precision and accuracy, where three quality control samples of different concentration were prepared separately. The method precision had relative standard deviation (RSD) below 0.78% for inter-day and 0.85% for intra-day which complies with acceptance criteria proposed [33]. The method accuracy also has proved to satisfy the proposed limits with obtained values within the range of 99.5% and 100.5%.

2.6.2. Extraction of paclitaxel from plasma and tissue homogenate samples

Simple one-step protein precipitation with acetonitrile was used for sample preparation. Tissues were homogenized in saline

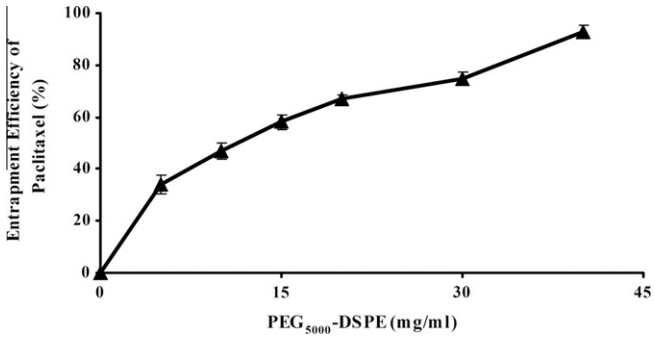

Fig. 1. Effect of PEG_{5000}–DSPE concentration on the encapsulation efficiency of paclitaxel in PEG–lipid micelles.

in the ratio of 1:1 (v/v). Paclitaxel was extracted from plasma and tissue homogenate samples by precipitation with acetonitrile in 1:1 and 1:2 ratios (v/v), respectively. Samples were then vortexed for 1.0 min followed by centrifugation for 10 min at 10,000 rpm. The supernatant was transferred to insert vials from which 20 μl was injected onto the HPLC column. Samples with concentrations higher than the calibration range limit were appropriately diluted to fit within the working calibration curve.

2.7. Short-term repeated dose toxicity study of PEG_{5000}–DSPE

To test the suitability of the polymer as a drug-delivery vehicle to the lungs, short-term repeated dose toxicity studies were conducted. Rats were divided into two groups; both groups were administered intratracheally with saline (Control, $n = 3$), or paclitaxel-free PEG–lipid micelles ($n = 3$). The dose of PEG_{5000}–DSPE micelles given to the rats was 100 mg/kg body weight administered in 200 μl (equivalent to 2 mg/kg dose of paclitaxel loaded PEG–lipid micelles) instilled into the trachea of the rats for 6 consecutive days. On the seventh day, rats were killed and lungs were harvested. The extraction of the bronchoalveolar lavage fluid (BALF) from harvested lungs was done following similar procedures reported by Hussain et al. [31] and Bai et al. [32]. Briefly, mid-level incision was made in the thoracic cavity of anesthetized rats to expose the respiratory apparatus. The abdominal aorta was dissected and the lungs were surgically removed after exsanguinations. Wet lung weights were recorded and then the lungs were lavaged by instilling 3 ml aliquot of normal saline into the trachea.

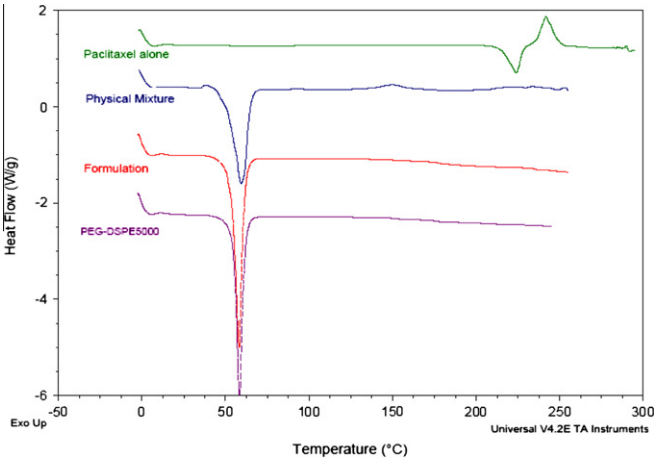

Fig. 2. DSC thermograms of paclitaxel alone, physical mixture, formulation (PEG_{5000}–DSPE micelles containing paclitaxel), and PEG_{5000}–DSPE polymer alone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The instilled saline was allowed to be left in the lungs for 30 s, withdrawn, re-instilled for an additional 30 s and then withdrawn again. Collected BALF was then centrifuged at 10,000 rpm for 10 min, and the obtained supernatant was used for analysis of the injury markers, NAG and ALP. Both enzyme concentrations were determined by using commercial assay kits. Enzyme activities are expressed in IU/l.

2.8. Data analysis

The area under the plasma concentration vs. time curve ($AUC_{0\rightarrow12}$) was calculated by the trapezoidal method. All values are expressed as the mean ± SEM. One-way ANOVA was used to compare the data. When the differences in the means were significant, post hoc pair wise comparisons were conducted using Newman–Keuls multiple comparison (GraphPad Prism, version 3.03, GraphPad Software, San Diego, CA). Differences in p-values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Micelles characterization

Paclitaxel was successfully incorporated in PEG–lipid micelles of PEG_{5000}–DSPE by solvent evaporation method. PEG_{5000} series was used because longer PEG chain provides large corona around the encapsulated drug, thus acting as protective barrier against the biological components [30]. As stated earlier, PEG_{5000}–DSPE has two segments; the hydrophilic PEG and the hydrophobic DSPE segments [19,34]. On evaporation of organic solvent, transparent matrix of drug–polymer mixture is formed in which drug molecules are entangled with the polymer chains. On addition of water, the PEG part associates with the water molecules through hydrogen bonding to make the corona, while the DSPE segments along with hydrophobic paclitaxel are removed from the aqueous environment and form the core of the micelles. This confers the core–shell structure on the micelle. This is especially beneficial for local delivery to the lung because the corona-forming PEG chain block provides a steric protection from non-specific uptake by phagocytic system and allows for prolonged residence in the lung [22], whereas DSPE, which forms the micellar core, has been reported to be degraded by mammalian secreted phospholipase A2 (PLA2), thus conferring biodegradability [35]. Additionally, the reported critical micelle concentration (CMC) value of PEG_{5000}–DSPE is 6.4×10^{-6} M which is very low and implies the stability of the system upon encountering the significant dilutions in the physiological fluids [19,36,37].

The entrapment efficiency of paclitaxel in the polymer increases with increasing the polymer concentration while keeping the amount of paclitaxel fixed. The entrapment efficiency of paclitaxel in PEG_{5000}–DSPE polymer at ratio of 1:40 was more than 95% (Fig. 1); thus, this ratio was selected for subsequent experiments. High lipid-to-drug ratio results in sufficient PEG_{5000}–DSPE micelles to disperse the paclitaxel molecules inside the micellar core. In addition, more polymer chains provide more entrapment sites leading to increased solubilization and thus incorporation of paclitaxel.

The average micelle size was about 5.0 ± 0.7 nm with rather narrow size distribution. It has been reported previously that particles of less than 260 nm can escape phagocytosis by macrophages [38]. Due to their small size, the chance of the PEG–lipid micelles to undergo phagocytosis in the alveoli is much lower than micron-sized particles. The small size of the micelles plays an important role because during nebulization by a microsprayer, the drug-bearing nanoparticles in an aqueous colloidal dispersion are more

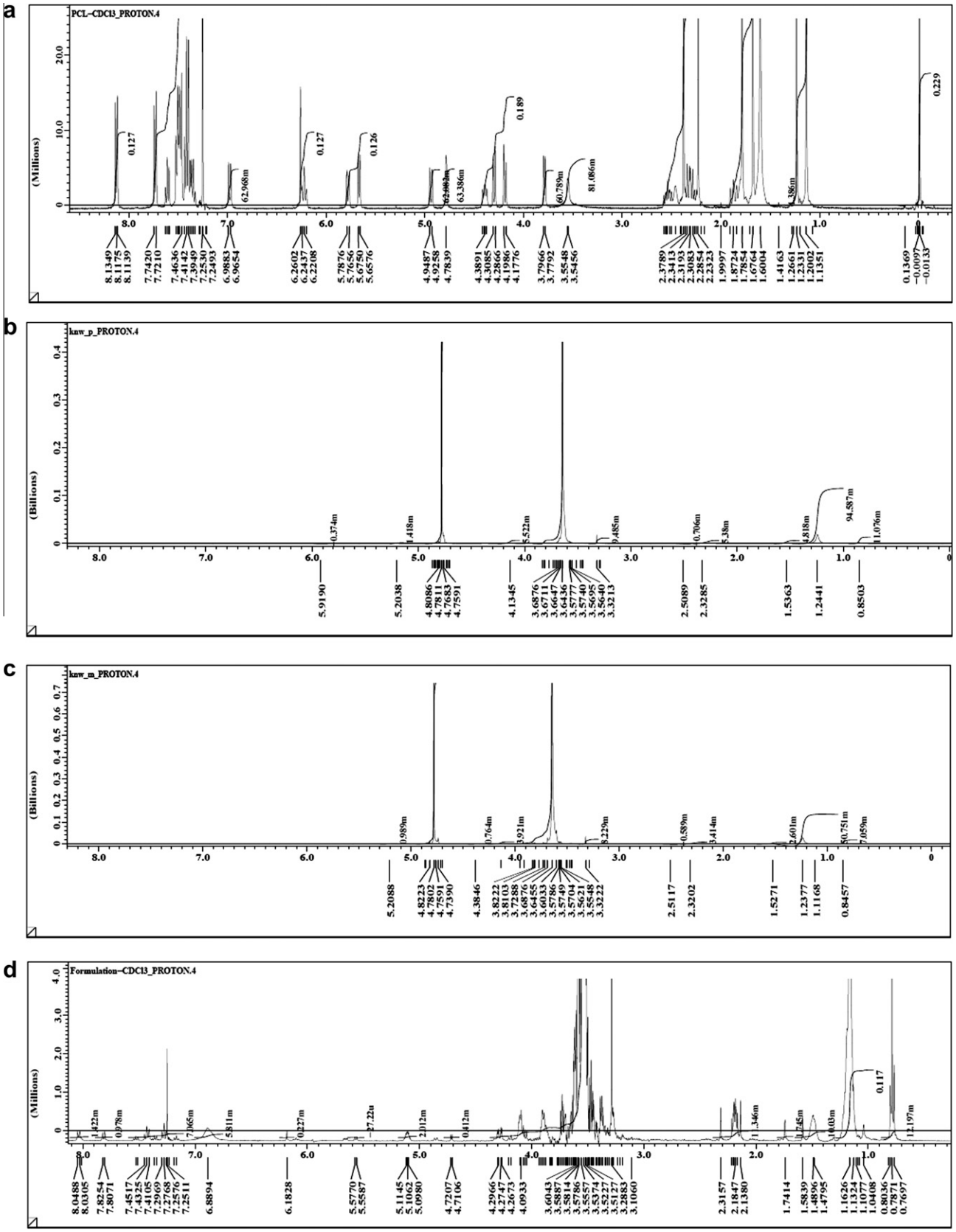

Fig. 3. ^{1}H NMR spectra of: (a) paclitaxel in $CDCL_3$, (b) PEG_{5000}–DSPE in D_2O, (c) PEG–lipid micelles of paclitaxel in D_2O, (d) PEG–lipid micelles of paclitaxel in $CDCL_3$.

easily incorporated into the "respirable percentage" of aerosolized droplets. Therefore, more nanoparticles can be enveloped into the aerosol droplets and delivered to deep lung [39,40]. Moreover, ultra small particles with geometric diameters less than 100 nm

have showed an obstinate residency in the lungs. Once deposited, these nanoparticles may stay in the lung lining fluid until their dissolution, evading phagocytosis and mucociliary clearance. This aids in increasing the residence time of micelles in the lung [17,41,42].

The zeta potential value gives an indication of the potential stability of the colloidal system. Dispersions with large negative or positive zeta potential tend to have better stability against aggregation. The zeta potential of PEG_{5000}–DSPE micelles was −34 mV, confirming the stability of formulation. Negative charge also adds to the efficiency of PEG_{5000}–DSPE as drug carrier because it has been reported previously that negatively charged entities are arrested in the lungs more efficiently than the positively charged molecules [43].

DSC analysis is the technique used to know the nature of drug inside the polymer matrix, which may exist as molecular dispersion or in crystallized form [44]. Fig. 2 illustrates the DSC thermograms of (from top to bottom) paclitaxel alone, physical mixture of paclitaxel and PEG_{5000}–DSPE mixture, lyophilized paclitaxel micelles, and PEG_{5000}–DSPE. Paclitaxel had melting point of 213 °C, whereas PEG_{5000}–DSPE had melting endotherm at 59 °C. The melting endotherm peak of paclitaxel of the physical mixture, prepared with same paclitaxel to polymer ratio as that of paclitaxel loaded micelles, could not be detected. Instead, an exotherm was seen at around 150 °C (Fig. 2). Several studies on the thermal analysis of paclitaxel–polymer physical mixtures reported a glass transition temperature around 150 °C. The observed exotherm in our study may be attributed to the formation of a eutectic mixture between paclitaxel and PEG_{5000}–DSPE whereby two events take place simultaneously, glass transition and recrystallization. The recrystallization dominates the glass transition and results in an exotherm which was seen at 150 °C. In the lyophilized nanoparticles, the exotherm is absent because the drug is molecularly dispersed inside the core of the micelles. Similar DSC thermograms of paclitaxel–polymer nanoparticles were reported in the literature [45,46]. This thermal behavior ascribed to the presence of paclitaxel in a molecularly dispersed state inside the polymer matrix [18,44].

Encapsulation of paclitaxel inside the micelles cores was demonstrated by ^{1}H NMR measurements. The ^{1}H NMR spectra of paclitaxel in $CDCL_3$, PEG_{5000}–DSPE in D_2O and paclitaxel-loaded micelle in D_2O and in $CDCL_3$ are shown in Fig. 3. Paclitaxel resonance peaks were observed with prominent peaks corresponding to methyl protons (1.14 ppm), methine protons (4.39 ppm), and aromatic protons (7.4 ppm) (Fig. 3a). When PEG_{5000}–DSPE is dissolved in D_2O, the micelles shells consisting of PEG blocks showed clear ^{1}H NMR resonance signals as can be seen in the spectrum of Fig. 3b at 4.78 ppm. However, in paclitaxel containing PEG–lipid micelles dissolved in D_2O (Fig. 3c), peaks corresponding to PEG_{5000}–DSPE only were seen, while the peaks corresponding to paclitaxel such as methyl protons at 1.14 ppm, methine protons at 4.39 ppm and aromatic protons at 7.4 ppm were not observed. Examination of ^{1}H NMR spectra of formulation in $CDCL_3$ (Fig. 3d) showed both paclitaxel and polymer peaks, which clearly indicated that in the organic solvent $CDCL_3$, core–shell structure of micelles was broken, thus releasing paclitaxel. These results clearly demonstrated that paclitaxel was constrained within the core of the micelles, which resulted in the insufficient motion of paclitaxel protons in the core, causing the disappearance of paclitaxel peaks. These results strongly demonstrated the core–shell structure of micelles.

Release of paclitaxel loaded micelles was investigated in the simulated surfactant lung fluid in order to closely mimic the behavior of micelles in the actual lung environment. Fig. 4 illustrates that paclitaxel was slowly released from the micellar core to the external lung fluid, with only 22% of the drug released in the first hour of incubation. The drug release was slow with 90% of drug released at 8 h, and the release was almost constant thereafter until the last time point at 24 h. Initial slow release was observed as contrary to other sustained release formulation in which initial high burst release of drug resulted in substantial loss of drug reservoir [47]. This signifies that paclitaxel molecules were not present on the outer surface, i.e. shell of micelles but instead, it was confined inside the core. It has been stated previously that the main mechanism of drug release from the micelle is diffusion of drug from the core to the surrounding environment [48]. Since the drug release was quite slow, it indicates that the core of the micelle is stiff and glassy [19,48], which confers less mobility to the drug as compared to mobile cores. The glassy core results from the two long-chain fatty acyls of the DSPE segment of PEG_{5000}–DSPE, which provide greater micelle stability [48]. On the other hand, the *in vitro* release profile of taxol was quite contrary with almost 94.5% of drug being released into the external medium in 1 h. Furthermore, in order to determine the mechanism of drug release from the polymer matrix, release exponent values were estimated as described in previous sections. The value of n obtained by fitting release data into Peppas model was equal to 0.7, which was indicative of anomalous drug release mechanism [29]. This implies that the drug release is dependent on drug diffusion through the polymer matrix and the polymer relaxation. Thus, it can be suggested that the mechanism that led to the release of paclitaxel from micelles was an anomalous diffusion with constant release rate adequate for a sustained release dosage form [29].

In addition, stability studies demonstrated that paclitaxel loaded micelles suspended in water did not show any changes in the particle size after 3 months when stored at room temperature. There was no cloudiness observed in the micellar dispersion. Furthermore, the HPLC chromatograms of the micellar paclitaxel did not show any changes in drug peak areas with lack of any degradation peaks which implied the stability of the drug within the micelle.

3.2. Paclitaxel tissue distribution and pharmacokinetics

Table 2 lists the AUC_{0-12h} of paclitaxel in different tissues following its administration intratracheally and intravenously as PEG–lipid micelles and intratracheally as taxol in rats. Intratracheal administration of paclitaxel PEG–lipid micelles resulted in significantly higher AUC of paclitaxel in rat lung with about 45-fold increase when compared with its AUC following intravenous administration of the same formulation. Moreover, as shown in Fig. 5a following intratracheal administration of the PEG–lipid micelles, paclitaxel lung concentration at 1 h was

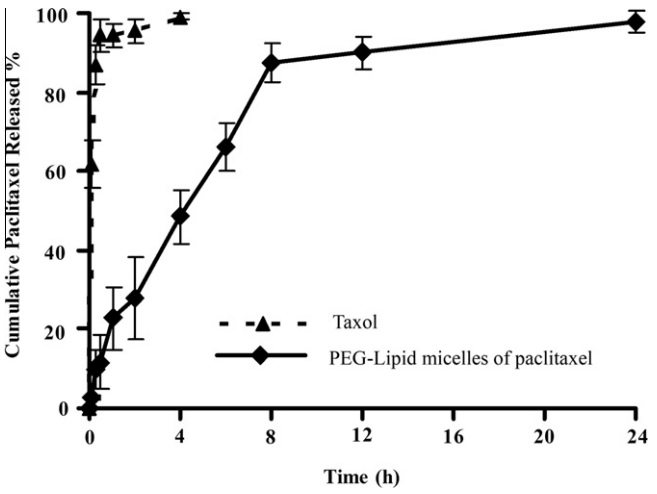

Fig. 4. *In vitro* release of paclitaxel from taxol (- - -) and paclitaxel containing PEG–lipid micelles (–) in lung simulated fluid as a function of time.

128 ± 10.0 μg/g, and decreased to 59.3 ± 3.6 μg/g at 12 h, which is still about 70-fold higher than its lung concentration following intravenous administration of the same formulation (0.813 ± 0.009 μg/g at 12 h). Another parameter estimated was targeting efficiency (T_e) to the lung which was calculated using the formula [9]:

$$T_e = \frac{AUC_{0-12}(\text{target tissue})}{\sum_{i=0} AUC_{0-12}(\text{non-target tissue})}$$

The targeting efficiency to lungs through the pulmonary route was 6.57, which is 132-fold higher than the intravenous route with T_e value of about 0.05 as shown in Table 3. Another important finding was the significant differences in distribution pattern of paclitaxel formulation in liver, kidney, heart, and spleen after the intratracheal and intravenous delivery. Intravenous administration (Fig. 5b) resulted in high drug concentrations in liver, kidney, and spleen as compared to the intratracheal route (Fig. 5a), whereas in the latter route, PEG–lipid micelles were retained in the lung minimizing rapid systemic absorption and subsequent redistribution to other tissues. Lower levels in non-targeted tissues avoid unnecessary side effects. From the aforementioned observations, the delivery of PEG–lipid micelles of paclitaxel as an aerosol provides the following advantages: (1) localized and specific delivery into the lungs protects the drug from other biological barriers like first pass metabolism, and gastrointestinal tract poor absorption, leading to high drug concentrations in lungs; (2) the sustained concentrations of drug up to 12 h indicate the slow diffusion of drug from the micelle core and also the slow degradation of polymer by lung enzymes as observed in the *in vitro* release studies; (3) due to highly hydrated shell and small size, the micelles escaped the clearance by alveolar macrophage system, which in turn prolonged the residence time of the drug carrier in the lungs.

In another set of experiments, paclitaxel PEG–lipid micelles and taxol were delivered intratracheally where comparisons between tissue distributions patterns of these two formulations were made. With taxol (Fig. 5c), initial high drug concentration in lungs (121 ± 12.6 μg/g) in the first hour was observed followed by rapid decline in drug levels down to 3.1 ± 0.9 μg/g at 12 h. On the other hand, though polymeric micellar paclitaxel had similar drug levels as that of taxol in the first hour (128 ± 10.0 μg/g), but at the 12 h much higher paclitaxel concentrations were found in the lungs (59.33 ± 3.6 μg/g) as compared to taxol at 12 h. The AUC values of paclitaxel from the PEG–lipid micelles in the lungs were 3-fold higher than taxol which had an AUC mean value of 317 ± 28 μg h/g (Table 3). In addition, targeting efficiency of micelles to the lungs was nearly 7-fold higher than taxol. These results demonstrated the role of the PEG–lipid micelles as sustained release depots from which paclitaxel is slowly released over time. However, in the case of taxol, free drug is not constrained in the lung for longer time due to rapid absorption in the systemic circulation, which has significantly decreased paclitaxel concentration in rat's lung and elevated levels in blood and other non-targeted tissues (Fig. 5c).

Pharmacokinetic profiles of paclitaxel from PEG–lipid micelles after intravenous and intratracheal administrations were compared to have a better understanding of drug disposition by the two routes. Significantly higher ($p < 0.0001$) plasma levels of paclitaxel were observed following the intravenous dose when compared with the intratracheal route (Fig. 6a). Most of the intravenously administered formulation was accumulated in liver and spleen over time due to rapid uptake by the reticuloendothelial system macrophages and extensive tissue distribution of paclitaxel [49,50]. On contrary, in case of intratracheally administered micelles, paclitaxel plasma maximum concentration (C_{max}) was as low as 73 ± 11.5 ng/ml. This implies the low systemic exposure of paclitaxel resulting from localization of chemotherapy to the lungs, which in turn avoid the unwanted side effects to other tissues.

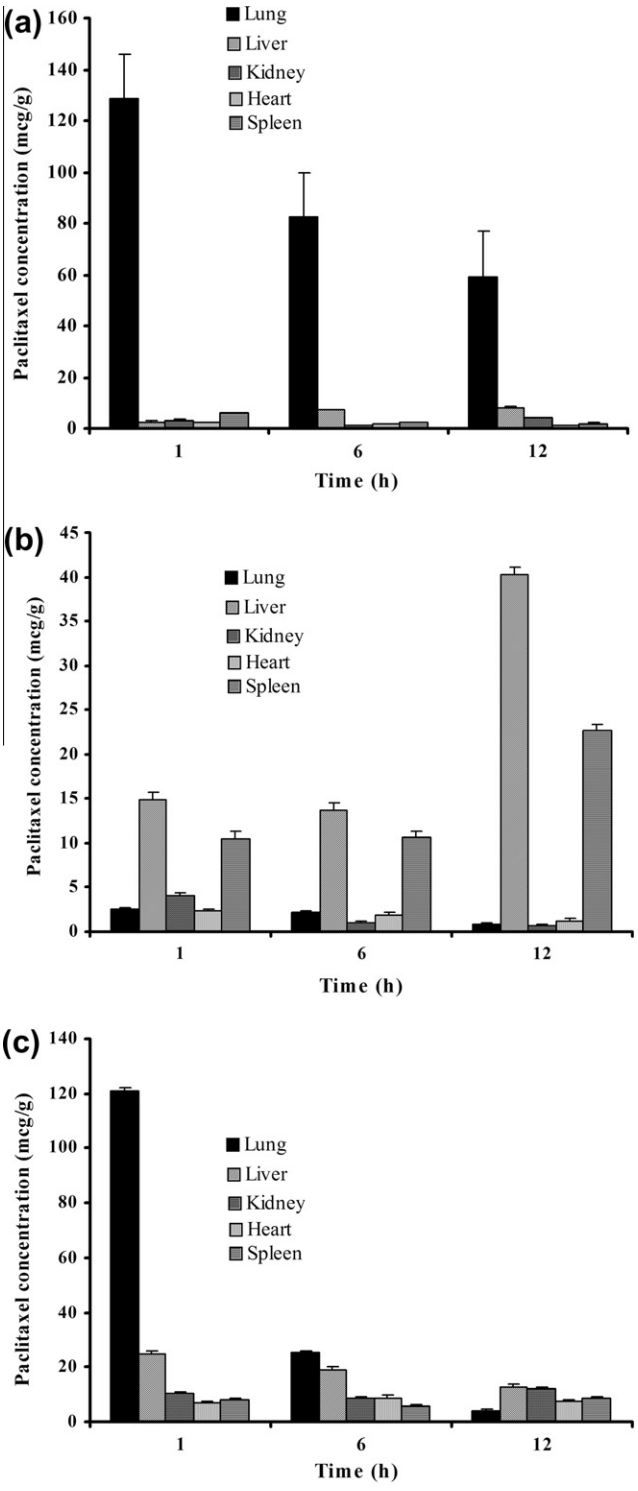

Fig. 5. Tissue distribution of paclitaxel in three groups: (a) intratracheally administered PEG–lipid micelles of paclitaxel, (b) intravenously administered PEG–lipid micelles of paclitaxel and (c) intratracheally administered taxol in Sprague–Dawley rats ($n = 3$) at paclitaxel dose of 2 mg/kg ($p < 0.0001$).

Furthermore, plasma pharmacokinetics of paclitaxel from the PEG–lipid micelles and taxol administered through same intratracheal route were compared (Fig. 6b). Following administration

Table 3
AUC_{0-12} (μg h/g) values of paclitaxel in various tissues and targeting efficiency (T_e) to lungs in three groups of Sprague–Dawley rats ($n = 3$) at a paclitaxel dose of 2 mg/kg.

Tissue	Intratracheal polymeric micellar paclitaxel	Intravenous polymeric micellar paclitaxel	Intratracheal taxol
Lung	955.3 ± 52.5	21.1 ± 8.9	317.0 ± 27.5
Liver	70.8 ± 13.7	225.6 ± 15.9	204.9 ± 18.5
Spleen	32.5 ± 12.3	154.0 ± 19.5	78.3 ± 16.9
Kidney	22.1 ± 4.9	57.7 ± 14.8	23.4 ± 9.6
Heart	20.0 ± 3.2	23.3 ± 6.4	75.5 ± 15.9
T_e to lungs	6.57	0.05	0.82

All AUC values were calculated using mean paclitaxel concentrations of three different rats at each sampling time point by trapezoidal rule ($p < 0.0001$) ± SEM.

of taxol, paclitaxel C_{max} was 554 ± 19.3 ng/ml at 30 min, which was 8-fold higher than its concentration following the PEG–lipid micelles dose (73 ± 11.5 ng/ml at 6 h. This result could be explained by the ability of the PEG–lipid micelles to retain the encapsulated paclitaxel in the lung, while free paclitaxel, having rapid accessibility to the general circulation, quickly redistributed to other tissues, especially to liver and spleen (Table 3).

3.3. Short-term repeated dose toxicity study of PEG_{5000}–DSPE polymer

BALF analysis was carried out to detect the levels of lung cells injury markers ALP and NAG. ALP is a lysosomal enzyme indicative of tissue damage, while NAG is an enzyme secreted by alveolar macrophages during phagocytosis of particulate material. Increased levels of these enzymes indicate lung toxicity [51,52]. It was seen that repeated treatment for 6 consecutive days did not show any significant difference in ALP and NAG levels when compared with saline-treated group ($p > 0.1$). The ALP levels in the BALF were 45.3 ± 1.8 IU/l as compared to saline-treated group which had ALP values of 43.5 ± 1.5 IU/l. Similarly, NAG levels were 0.049 ± 0.013 U/l, and saline-treated group had NAG values of 0.040 ± 0.018 U/l. These results indicate that the polymer PEG_{5000}–DSPE did not elicit any sort of inflammation in lung tissues even when administered for 6 consecutive days. This study apprehended the safety and suitability of this polymer for use as drug-delivery vehicle for lungs.

4. Conclusions

In the present work, we were successful in encapsulating paclitaxel in the PEG_{5000}–DSPE micelles with entrapment efficiency >95% in nanoparticle size range (5 nm). The *in vitro* release studies indicated a sustained release potential of these PEG–lipid micelles. The ability of a drug carrier system to keep the therapeutic agent at or near the desired pharmacological site of action is imperative to provide selective and prolonged activity in the lung and reduce systemic toxicity. This study provides evidence that PEG–lipid micelles made from PEG–phospholipds can favorably alter distribution of paclitaxel in body when locally administered in the lungs with high drug level attained which is desirable in the treatment of lung cancer. We found that altered pharmacokinetics of paclitaxel through encapsulation in PEG–lipid micelles significantly decreases the exposure of cytotoxic paclitaxel to non-targeted organs which thereby lowers the risk of systemic toxicity that is otherwise encountered via intravenously delivered chemotherapeutics. The toxicity studies on PEG_{5000}–DSPE indicated the safety of this drug carrier as pulmonary delivery platform.

In vivo studies to investigating the efficacy of paclitaxel micellar formulations delivered via the pulmonary route in lung tumor-bearing mice are currently in progress.

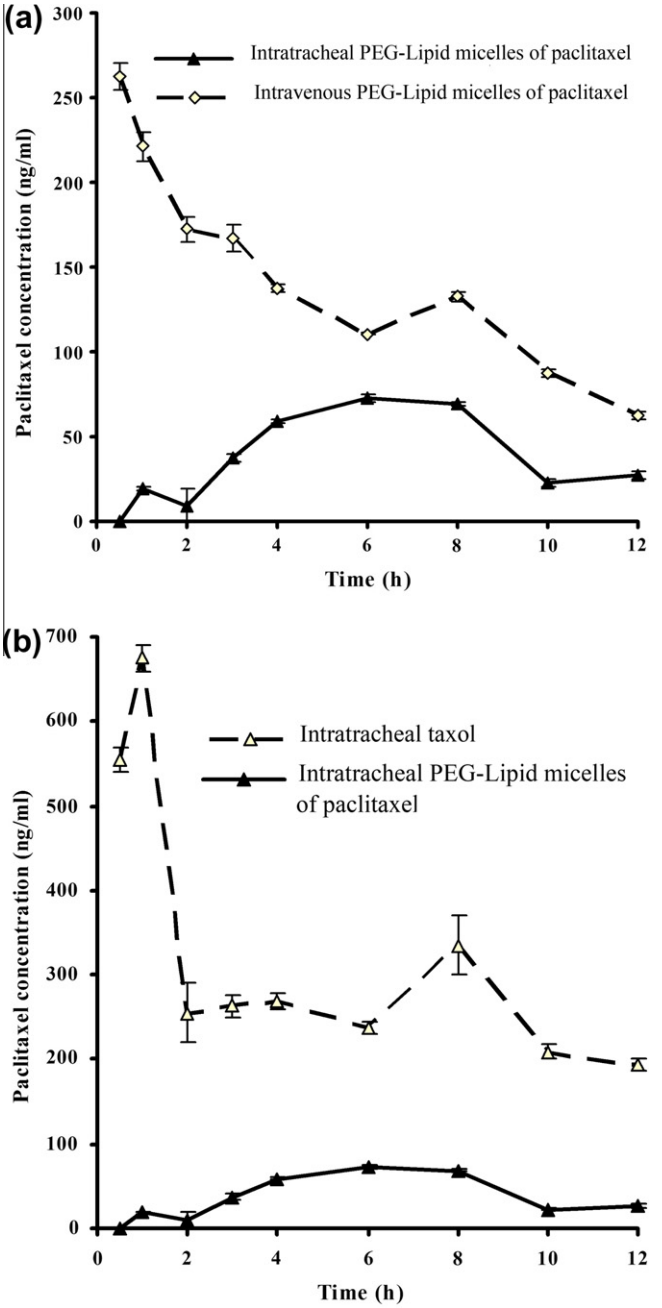

Fig. 6. Plasma concentration–time profile of: (a) intratracheally administered PEG–lipid micelles of paclitaxel vs. intravenously administered PEG–lipid micelles of paclitaxel (b) intratracheally administered PEG–lipid micelles of paclitaxel vs. intratracheally administered PEG–lipid micelles of paclitaxel in Sprague–Dawley rats ($n = 3$) at paclitaxel dose of 2 mg/kg ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Acknowledgment

Authors would like to thank ONY Inc. of Amherst, New York for providing infasurf free samples to carry out *in vitro* release experiments.

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