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RESEARCH PAPER

In Vivo Evaluation of Doxorubicin-Loaded $(PEG)_3$-PLA Nanopolymersomes (PolyDoxSome) Using DMBA-Induced Mammary Carcinoma Rat Model and Comparison with Marketed LipoDox™

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ABSTRACT

Purpose To evaluate *in vivo* doxorubicin-loaded $(PEG)_3$-PLA nanopolymersomes (PolyDoxSome) using 7,12-dimethyl benz[α]anthracene (DMBA)-induced mammary carcinoma rat model compared to marketed formulation LipoDox™.

Methods Sprague Dawley female rats with mean tumor volume of about 2 cm^3 were used for pharmacokinetics, biodistribution, antitumor efficacy and toxicity studies.

Results This study demonstrates that PolyDoxSome has higher AUC (569 *vs.* 4 h*μg/mL), longer plasma circulation half life (21.9 *vs.* 0.49 h), decreased clearance (10.5 *vs.* 1579 mL/h/kg) and volume of distribution (137.7 *vs.* 1091 mL/kg) as compared to free doxorubicin. Tissue distribution profile showed increased doxorubicin concentration in tumor and decreased concentration in heart as compared to free doxorubicin. The toxicity studies as measured from liver function tests, cardiac enzyme assays, hematology test and body weight has demonstrated that it is better tolerated than free doxorubicin. When PolyDoxSome was compared with LipoDox™, it differs in size (171 *vs.* <100 nm), plasma circulation half life (22 *vs.* 35 h), C_{max} (34 *vs.* 67 μg/mL), and AUC (568 *vs.* 2291 h*μg/mL), however PolyDoxSome was comparable on efficacy and toxicity profile of LipoDox™.

Conclusions Results suggest that PolyDoxSome has better *in vivo* profile than free doxorubicin and comparable efficacy and toxicity to LipoDox™.

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INTRODUCTION

Doxorubicin is an anthracycline antibiotic with potent antineoplastic properties effective against a broad spectrum of malignancies in clinical use. It is rapidly and broadly distributed (V_d=20–30 L/kg) and is accumulated in irrigated tissues such as liver, lung, kidney and heart. Accordingly, the clinical use of doxorubicin is hampered by acute and sub acute side effects such as bone marrow suppression, alopecia, nausea, mucositis and most importantly dose limiting irreversible cardio-toxicity. Such side effects are limiting therapeutic activity and preclude adequate dosing in its clinical use (1–3). Hence, pharmaceutical scientists have explored several approaches to improve the therapeutic index of doxorubicin and overcome its side effects to extend its utility by using several drug delivery systems for example, microspheres (4), liposomes (2,5–9), nanoparticles (10–13), micelles (14–19) and polymersomes (20–31). The objective of entrapment of doxorubicin in nanocarriers is to reduce its uptake into non target organs, specifically to the heart compared with free doxorubicin, to extend plasma circulation time and enhance accumulation in tumor (5–7,9,11,12,15,16,26,29,30,32). Liposomal formulations containing doxorubicin, which are PEGylated (Doxil/Caelyx/Lipodox™) and non-PEGylated (Myocet) have been approved clinically and are available in the market. PEGylated formulations demonstrate reduced toxicities and increased accumulation in tumor in comparison to Myocet; however manifest different toxicity profiles, such as mucocutaneous toxicities (palmar plantar erythrodysesthesia

(PPE) and stomatitis) that hinder dose escalation. For example, regarding the breast carcinoma, phase II clinical study of Doxil demonstrated that dose and schedule were modified from 60 to 40 mg/m^2, and from 3-to-4 week interval due to skin toxicity, which may have also accounted for a major drop in response rate (33). These side effects are hypothesized to be associated to small size (<100 nm) and pharmacokinetic parameters of PEGylated liposomal doxorubicin formulations. The pharmacokinetic parameters correlated with mucocutaneous toxicities are C_{max}, plasma circulation half life and AUC. The relevance of C_{max} to stomatitis and half life to PPE is emphasized by significantly greater incidence of severe toxicities in patients with high C_{max} and half life. For example, the dose limiting toxicities shifted from PPE with Doxil/Caelyx to stomatitis with LipoDox™ as the AUC increased (34–39). Hence, by optimizing the doxorubicin release rate, carrier size and surface characteristics, it may be possible to reduce the mucocutaneous toxicities without affecting the therapeutic activity.

Polymersomes as colloidal drug carriers were proposed in 1999 (40) and have received growing attention in past decade, mainly due to their mechanical stability from a thicker bilayer membrane, and plethora of possibilities of synthetic control over physicochemical properties of amphiphilic copolymers chemistry that enables tunable design of polymersomes (21,23,40–43). For this purpose, our group has developed new polymersome carrier system (44) and has used the same for the delivery of amphotericin B (45,46). This carrier system is also tuned and optimized for doxorubicin which showed good response in breast cancer cell line (MCF7) (47).

In the present work, developed PolyDoxSome as doxorubicin-loaded nanopolymersomes is evaluated in DMBA induced mammary carcinoma rat model to assess the pharmacokinetics, biodistribution, efficacy and toxicity profiles along with free doxorubicin and LipoDox™.

MATERIALS AND METHODS

Materials

Amphiphilic block copolymer, $(PEG)_3$-PLA (Mw ~ 17.5 KDa) and PolyDoxSome were prepared in our lab as described in our previous publication (47). LipoDox™ was purchased from a local market which is a product manufactured by Sun Pharma Enterprises Ltd. (Vadodara, India). 7,12-Dimethylbenz[α]anthracene (DMBA) was procured from Sigma (USA). HPLC grade methanol and acetonitrile were obtained from J. T. Baker (USA) and used as received. Elga water is ultra pure water and in house supply purified by Elga Stat, UK. All other chemicals used in this study were analytical grade and used as received.

Preparation of PolyDoxSome

PolyDoxSome was prepared and lyophilized according to method described elsewhere (47). Briefly, 15 mg of amphiphilic block copolymer, $(PEG)_3$-PLA (17.5 KDa), and 4 mg of doxorubicin were weighed and dissolved in 1.5 ml mixture of dimethyl sulphoxide and acetone (1:4 v/v ratio). The organic solution of copolymer and doxorubicin was rapidly injected into 5 ml of aqueous phase (10 mM tris buffer, pH 7.4) to get a polymeric dispersion. The injection process was carried out under magnetic stirring and allowed to equilibrate until the turbidity of the dispersion was stabilized (<20 min). The organic solvents and free doxorubicin of the stabilized suspension were removed through dialysis (4 h) against tris buffer using a dialysis membrane (MWCO: 10,000 Da) and thereafter lyophilized using inulin as lyoprotectant. The characteristics of the formulation used for this study are given in Table I.

Animals and Development of Tumor Model

Female Spargue-Dawley rats of 40 to 45 days old were obtained from Institute animal facility (National Institute of Pharmaceutical Education and Research, NIPER, SAS, Nagar, India). They were kept in a group of 5 in a cage, housed in animal facility in an environmentally conditioned room with respect to light, temperature or air humidity, and fed with standard laboratory food and water *ad libitum*. At 50 to 55 days of age, rats were fed 65 mg/kg body weight of DMBA suspension in soya bean oil to induce mammary carcinoma as breast cancer model that expresses similar histology and biomarker expressions to the human breast cancer within 2–5 months (48–50). Five weeks post administration of DMBA, animals were checked by inspection, touching and palpation once weekly for detection of mammary tumors development. Furthermore, animals were monitored for the body weight throughout the study period. Palpable tumors (multiple tumors in a few animals) were generated approximately 90 days post DMBA administration at breast position. Once DMBA induced rat mammary carcinomas had developed, animals with palpable tumor were divided into treatment groups. All protocols and procedures were approved by Institutional Animal Ethics Committee (IAEC) of National Institute of Pharmaceutical Education and Research (NIPER), S.A.S Nagar, India and experiments were performed as per guideline of IAEC.

Pharmacokinetics and Biodistribution Studies

In order to study the plasma pharmacokinetics and tissue distribution of free doxorubicin, LipoDox™ and PolyDoxSome, animals with mean tumor volume of

Table I Characteristics of the Formulation (PolyDoxSome) Used in this Experiment

Copolymer	Size (nm) ± SD	Loading capacity (% w/w) ± SD	Encapsulation efficiency (% w/w) ± SD	Zeta potential
$(PEG)_3$-PLA	171.3 ± 7.45	10.9 ± 0.49	51.7 ± 2.3	−5.1 mV

approximately 2 cm^3 were divided into 3 treatment groups of 3 animals per treatment group. Individual animals were weighed on the day of dosing and mean ± S.D. weight for the animals was 270 ± 10 g. For drug administration, animals were anesthetized with 90 mg/kg ketamine using intra peritoneal injection and after 10 min, doxorubicin (6 mg/kg) and doxorubicin formulations at equivalent dose namely PolyDoxSome and LipoDox™ were administered by *i.v.* injection via femoral vein. Prior to dosing, free doxorubicin and PolyDoxSome were reconstituted in normal saline at 2 mg/mL of drug equivalent and LipoDox™ was used as obtained (2 mg/mL, 10 mL). Blood samples of 1 mL were collected into 2 mL eppendorf containing heparin at time points of 5, 30, min and 4, 12, 24, 48, 72 and 96 h after the drug injection. Plasma was collected by centrifugation (5000 g × 10 min) of blood samples at 4°C and stored at −65°C for further use. The same animals after blood collection were euthanized and liver, lung, spleen, kidney, heart and tumor were rapidly excised. The collected organs were rinsed in ice cold phosphate buffer saline, weighed and homogenized in phosphate buffer saline (500 mg tissue/mL) to get tissue homogenates and stored at −65°C immediately until further analysis for drug concentration. The samples were carried forward for HPLC analysis using a lab developed analytical method on a reversed phase HPLC system equipped with fluorescence detector.

Efficacy and Toxicity Studies

For *in vivo* efficacy and toxicity profile of free doxorubicin, LipoDox™ and PolyDoxSome, animals with similar tumor volume (approximately 2 cm^3) were divided into 5 treatment groups of 6 animals per treatment group and received three injections of normal saline and blank nanopolymersomes as control groups, doxorubicin (6 mg/kg/dose) and doxorubicin formulations at equivalent dose namely PolyDoxSome and LipoDox™. In order to avoid the overlapping myelosuppression, each injection was put on every 21 days time interval because full regeneration of the activity of myeloid centers in the spleen occurred at about 21 days after drug administration. Animals were monitored for tumor growth, survival, body weight, serum biochemical parameters and hematological toxicity profiles for 70 days from the starting dose. Tumor measurement was performed at the time of first injection and on every week till the sacrifice/death of the animal. Measurement was carried out using vernier caliper in two dimensions and calculated using a volumetric formula (51):

$$V = L \times W^2 \times 0.52;$$

L and W are the length and width of the tumor, respectively.

The initial tumor size was considered as 100% and subsequent measurements at each time point were expressed as a percentage of initial volume of each tumor. All the calculations were done as relative volume, $R_v = V_t / V_o \times 100\%$, where V_t is the mean tumor volume at any subsequent time and V_o is the mean initial tumor volume. In the survival analysis, the animal survival was monitored till the death of the animal or 70 days from the first treatment and median survival time (MST) of treated group (T) and normal saline control groups (C) were determined using Kaplan Meier survival analysis. To analyze the treatment effects on the lifespan, the percentage of increased lifespan (ILS) was calculated as:

$$ILS = (T/C - 1) \times 100\%,$$

where T and C are the median survival time of treated and control rats, respectively.

In comparative toxicological evaluation of PolyDoxSome, animals were assessed for the dynamics of body weight, serum biochemical parameters, and hematological parameters. The body weight was measured prior to dosing, after 24 h of treatment and then on every week till the death/sacrifice of the animal. The body weight prior to dosing was considered as 100% and the results on the subsequent measurements were expressed as a percentage of the initial weight of each animal. For biochemical and hematological assays, blood samples were collected into eppendorf tubes into two parts with or without anticoagulant. Blood without anticoagulant was kept at room temperature for 45 min for clotting, centrifuged at 5000 rpm for 10 min and serum was separated for biochemical analysis. Blood with anticoagulant (potassium ethylenediamine tetracetic acid "EDTA-K_3" at 1 mg/mL of blood) was used for hematological examinations and analysis was carried out on the same day of collection. Cardio-toxicity was assessed using biomarker enzyme assays in which the levels of serum creatine phosphokinase (CPK), lactate dehydrogenase (LDH), and aspartate aminotransferase (AST) were determined. For hepatotoxicity test, levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and bilirubin were

determined by biochemical analyzer. Both types of assays were carried out using commercial kits according to the manufacturer's guideline (ERBA Diagnostics, Manheim, Germany) and the results were expressed in terms of IU/L. For hematology tests, the levels of erythrocyte count (RBC), packed cell volume (PCV), hemoglobin, leukocyte count (WBC), platelet count and differential leukocyte count were determined using an automated analyzer.

Statistics and Data Analysis

All data expressed as means ± S.D. are representative of at least three different experiments. When comparing mean values of variables obtained from experiment, one way analysis of variance (ANOVA, Tukey test) was performed using SAT program and all $p<0.05$ were considered significant. The pharmacokinetic parameters were calculated using Kinetica v5.0 and a standard *i.v.* bolus non-compartmental method was selected for the plasma and tissue distribution pharmacokinetics. Mortality rate (survival analysis) was analyzed using Kaplan Meier survival analysis.

RESULTS

Pharmacokinetics

The plasma concentration-time curves of doxorubicin in rats following *i.v.* administration of a single dose of 6 mg/kg of doxorubicin and equivalent doses of LipoDox™ and PolyDoxSome is shown in Fig. 1. In case of free drug, two phases were observed where first phase accounting >95% of total drug showed $t_{1/2}$ at 30 min and second phase for <5% of total drug showed $t_{1/2}$ at 72 h (Fig. 1). This observation demonstrated that doxorubicin is rapidly cleared from plasma circulation when it is administrated as free doxorubicin; however doxorubicin within the carrier system showed higher drug levels in both formulations (PolyDoxSome and LipoDox™). When comparison was made within the two formulations, residence drug concentration in plasma was more in case of LipoDox™ than PolyDoxSome which might be due to higher surface hydrophilicity of LipoDox™ vesicles leading to longer blood circulation time than PolyDoxSome vesicles. As shown in Table II, clearance value of free doxorubicin was in the order of 1578.9 mL/h/kg, while the apparent volume of distribution was very large (1090.9 mL/kg) compared with LipoDox™ (CL=2.6 mL/h/kg; Vd=90.9 ml/kg) and PolyDoxSome (CL=10.5 ml/h/kg; Vd=137.7 ml/kg). Very high volume of distribution and low AUC of free drug indicates the non specific distribution of drug within tissue compartment and thus leads to higher toxicity which is also supported by toxicity results in the forthcoming section.

Tissue Distribution

In this study, doxorubicin was analyzed in liver, spleen, kidney, heart, lung and tumor at predetermined time intervals (5 min, 30 min, 4, 12, 24, 48, 72 and 96 h) after *i.v.* injection of equivalent doses of free doxorubicin, LipoDox™ and PolyDoxSome. The doxorubicin distribution profile in all organs is shown in Fig. 2.

Following free doxorubicin administration, drug concentration rapidly increased in liver with a maximum at 18.4 µg/g and then decreased by 20-fold at 0.9 µg/g after 12 h. Animals that received LipoDox™ and PolyDoxSome showed sustained doxorubicin concentration and the maximum drug concentration was achieved by PolyDoxSome as 16.7 µg/g at 4 h, while for LipoDox™ it was 14.8 µg/g at 12 h (Fig. 2). In case of free drug, these levels were decreased rapidly after 12 h with time probably due to fast metabolism of free drug present in the blood. In spleen, the major accumulation of the drug was observed for LipoDox™ treated animals which was 4 times higher than PolyDoxSome and 12 times higher than free doxorubicin treated animals. In the spleen, LipoDox™ uptake was 1.4 times

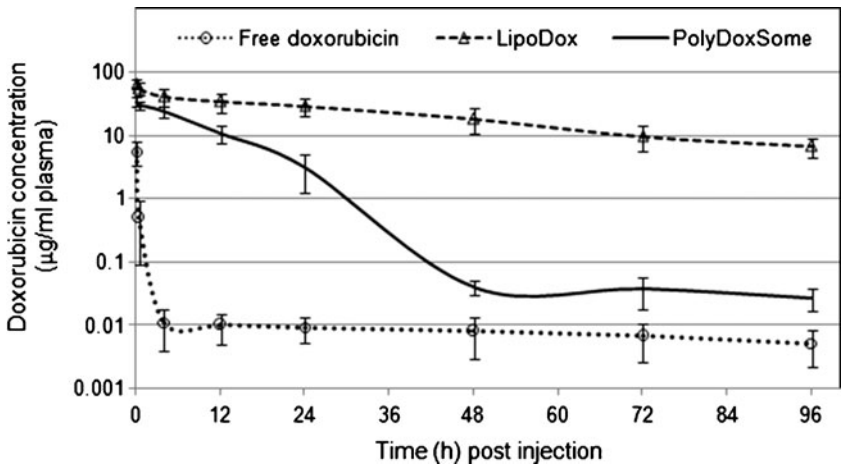

Fig. 1 Plasma concentration-time profile of free doxorubicin, LipoDox™ and PolyDoxSome after a single *i.v.* administration via femoral vein (6 mg/Kg) to DMBA induced mammary carcinoma rat model. Each data points presented are average values of three replicate experiments ($n=3$) and error bars indicate standard deviations.

Table II Pharmacokinetic Parameters of Doxorubicin Administered as the Free Doxorubicin, or Lipodox™ or PolyDoxSome (dose: 6 mg/kg)

Parameters	Free doxorubicin	LipoDox™	PolyDoxSome
C_{max} (μg/ml)	5.5	66.4	33.8
$t_{1/2}$ (h)	0.49/72[a]	34.3	21.9
AUC (μg/ml*h)	3.8	2291.6	568.7
CL (ml/h/kg)	1578.9	2.6	10.5
Vd (ml/kg)	1090.9	90.9	137.7

[a] Elimination phase

Cmax = peak plasma concentration after single dose administration; t1/2 = half life associated with the exponents of distribution phase and, where indicated, of elimination phase; AUC = area under the concentration time curve; Vd = apparent volume of distribution; CL = total plasma clearance

higher than the liver, whereas PolyDoxSome uptake was almost equal in both organs. It is well reported that spleen plays an important role for uptake and clearance of liposomal carriers and that might be the reason of higher residual drug concentration for LipoDox™ in the spleen (52,53).

The same type of observation was also seen in kidney and lungs as shown in Fig. 2. It is also important to mention that even though all four organs showed higher drug levels for both carrier systems, the free drug concentration in plasma may not high as most of the drug molecules are encapsulated within the carrier matrix and are released at a slow rate. Thus, drug related toxicity may be less in PolyDoxSome group than in free drug which is also supported by other toxicity experiments described in the forthcoming sections.

In heart, doxorubicin concentration was about 6-fold higher following a single treatment with free doxorubicin (14.16 μg/g heart at 5 min) in comparison to PolyDoxSome (2.27 μg/g heart at 4 h) and 5-fold higher than LipoDox™ (2.65 μg/g heart at 12 h). It is observed that drug deposition in heart remained higher for free doxorubicin over 96 h after administration which clearly indicates the lower cardiac toxicity level of PolyDoxSome and LipoDox™. On comparing the carrier systems, it was found that doxorubicin deposition from PolyDoxSome remained lower than LipoDox™ at all time points, though this difference was not statistically significant ($p > 0.05$).

The objective of the carrier system is to transport the drug at target site in efficacious way which is the tumor in present case and this system should deliver more drug molecules to tumor site and least to non target organs. Following *i.v.* administration of free doxorubicin, 3.8 μg/g of drug in tumor was observed at 5 min which was reduced to 1.33 μg/g of drug concentration after 4 h. The peak drug concentration in tumor for PolyDoxSome and LipoDox™ was observed at 12 h (16.4 μg/g tissue) and 72 h (19.05 μg/g tissue), respectively. Thus, it is clear that both carriers show comparable drug concentrations; however the highest concentration was first achieved for PolyDoxSome, which may also be a better sign from formulation view point. The difference in tumor concentration between the PolyDoxSome and free doxorubicin is 4.3-fold ($p < 0.001$) and higher drug level was maintained for several hours following injection. There was no significant difference observed for doxorubicin tumor deposition when administered as LipoDox™ and PolyDoxSome ($p > 0.05$). The increased doxorubicin deposition in tumor following LipoDox™ or PolyDoxSome administration compared to free doxorubicin can be explained by increased plasma circulation half life of both carriers, which allowed a greater proportion of carriers to extravasate into tumor by EPR effect (54). Overall observation demonstrated that doxorubicin concentration reached peak concentration in these organs between 5 min to 30 min for free doxorubicin, 4 to 12 h for PolyDoxSome, and 12 to 24 h for LipoDox™.

In tissue distribution studies, the $AUC_{(0-\infty)}$ of doxorubicin following administration of free doxorubicin, LipoDox™, and PolyDoxSome were also calculated using trapezoidal rule (Fig. 3).

It is clearly seen from the Fig. 3 that doxorubicin has higher distribution in tumor for both the carriers when administered as PolyDoxSome (9.5-fold) or LipoDox™ (9.3-fold) than free doxorubicin and comparable between both the carrier systems.

Efficacy and Toxicity Studies

Efficacy and Survival

Antitumor efficacy was evaluated in DMBA induced mammary carcinoma rat model following administration of three doses of 6 mg/kg equivalent doses of doxorubicin as free drug, LipoDox™ and PolyDoxSome using normal saline and blank nanopolymersomes as control. An improved efficacy of both carriers was observed at equivalent dose of doxorubicin compared with free doxorubicin from tumor suppression (Fig. 4) and survival plot (Fig. 5). However, there was no difference in case of normal saline and blank nanopolymersomes.

The re-growth of tumor was noticed after 2 weeks following single dose administration of free doxorubicin, whereas the suppression of tumor growth was maintained for LipoDox™ and PolyDoxSome starting from the first dose. This can be explained due to the high drug exposure of tumor from sustained release nanoformulations and extended plasma circulation time that keeps on feeding adequate dose of doxorubicin to tumor tissues.

From Kaplan Meier survival curve (Fig. 5) and survival time (Table III) shown for control groups and treatment groups, enhanced survival time for tumor bearing rats was observed for PolyDoxSome and LipoDox™ indicating improved efficacy and a reduction of side effects.

Median survival time for five treatment groups was determined and was found to be 70 days for PolyDoxSome and LipoDox™, 47 days for free doxorubicin, and 45 days for

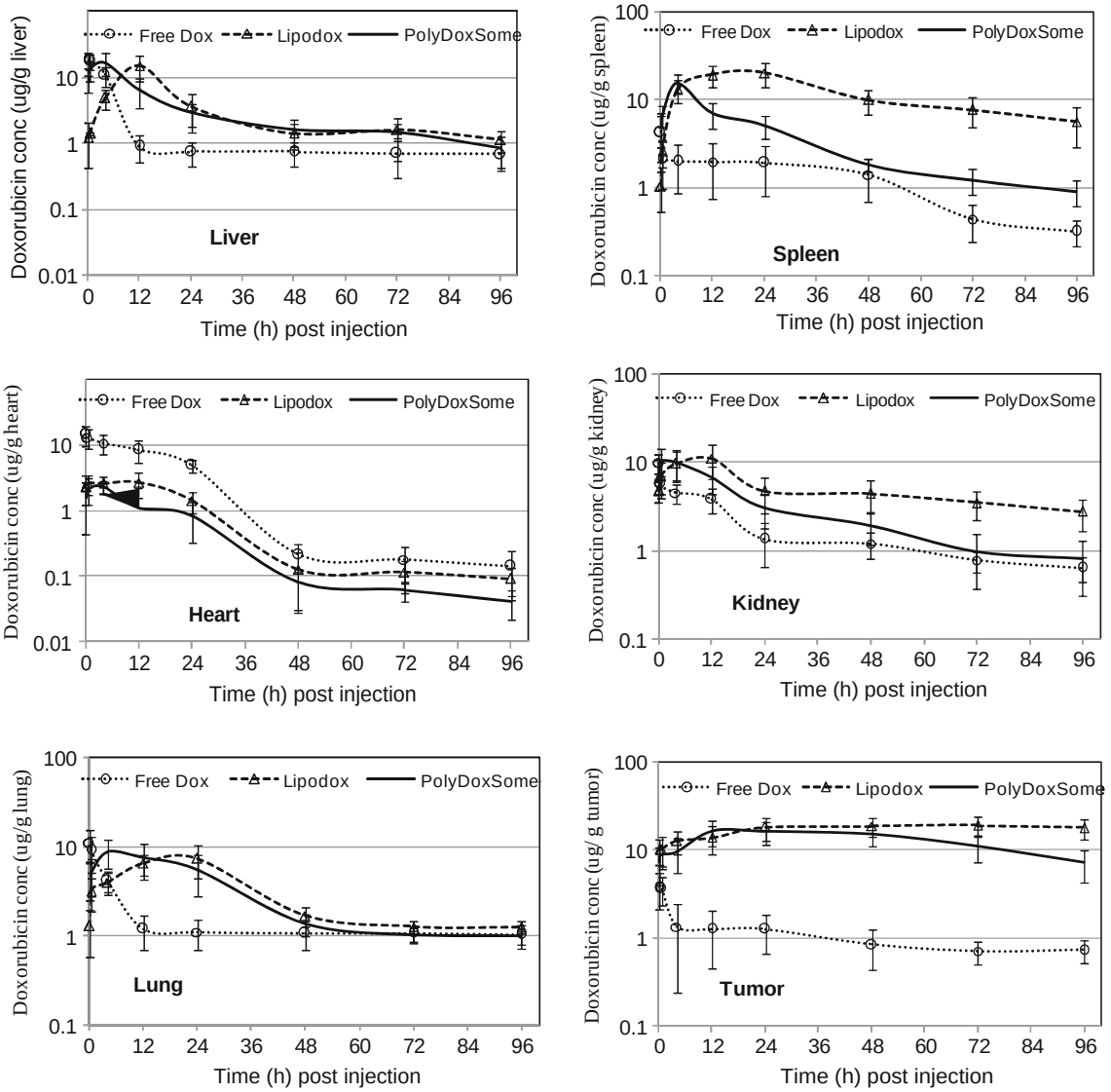

Fig. 2 Tissue deposition of free doxorubicin, LipoDox™ and PolyDoxSome at different time points after a single *i.v.* injection of 6 mg/kg doxorubicin equivalent dose via femoral vein to DMBA induced mammary carcinoma rat model. Each data points presented are average values of three replicate experiments (n=3) and error bars indicate standard deviations. Each data points presented are average values of three replicate experiments (n=3) and error bars indicate standard deviations.

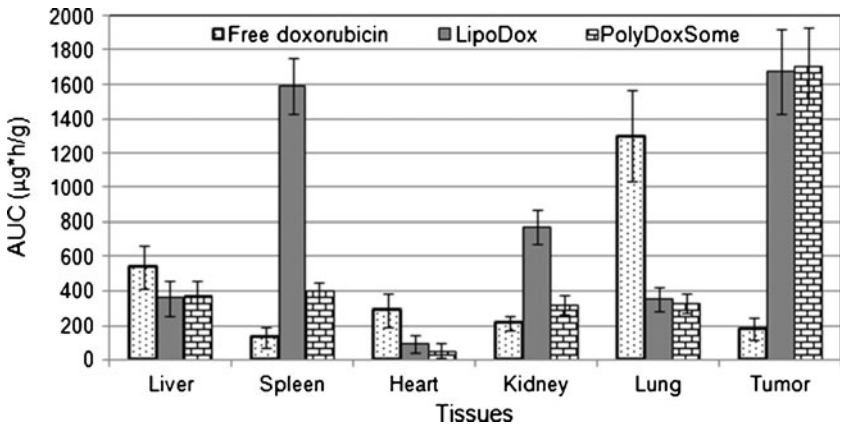

Fig. 3 The area under curve (AUC) of doxorubicin in various tissues following a single i.v. injection (6 mg/kg doxorubicin equivalent) of free doxorubicin, LipoDox™, and PolyDoxSome to DMBA induced mammary carcinoma rat model.

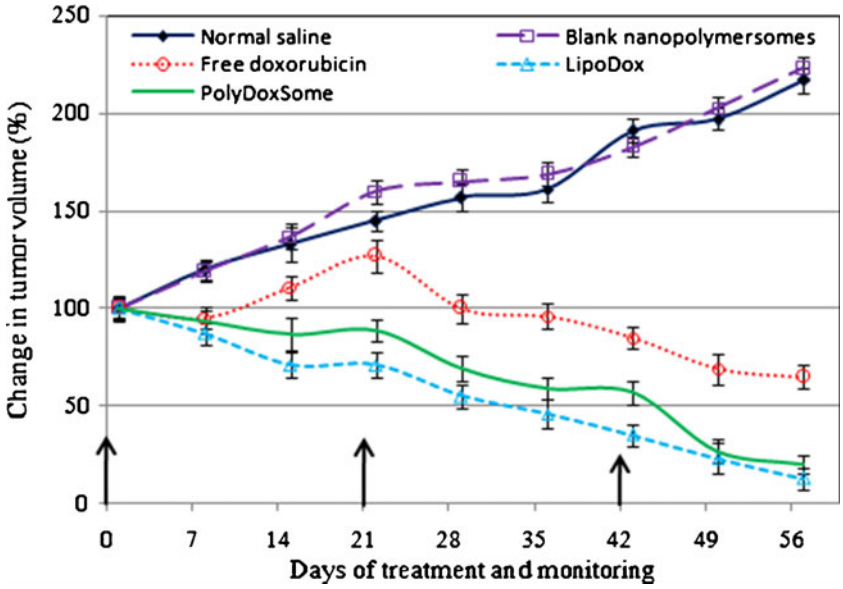

Fig. 4 Plot of tumor volume change (in percent of initial volume) in days for rats treated with saline and blank nanopolymersomes as control, free doxorubicin, LipoDox™ and PolyDoxSome at 6 mg/kg per dose for three doses. The arrow indicates the injection days. Each data points presented are average values of three to six replicate experiments (n=3–6) and error bars indicate standard deviations.

normal saline and nanopolymersomes (Table III). In comparison with control groups, the increase in median survival time was 56% for PolyDoxSome and LipoDox™ and 5% for free doxorubicin.

Body Weight

The dynamics of body weight change was monitored as indicator of toxicity profile of treatment regimen. The rats treated with free doxorubicin showed about 20% loss in body weight just after 24 h of first injection, whereas the rats treated with LipoDox™ did not show any body weight loss in each dosing. The rats treated with PolyDoxSome recovered their body weight after the second injection with slight loss of body weight (~7.5% loss) after the first dose. In addition, no pronounced body weight change was observed in the control groups receiving normal saline and blank nanopolymersomes of the same formulation till 42 days, thereafter a declining trend was observed which can be explained by the progression of disease (Fig. 6). The decreasing trend in body weight at initial time points and a recovery after that is the indication of no dose related toxicity during the treatment on the tested dose.

Cardiac and Hepatic Function

To evaluate the cardio-toxicity of the prepared formulation, tumor bearing S.D. female rats were administered separately with normal saline and blank nanopolymersomes as control groups, and free doxorubicin, LipoDox™ and PolyDoxSome as treatment groups containing a total cumulative dose of 18 mg/kg doxorubicin. The serum creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) levels are well characterized cardiac biomarkers for cellular

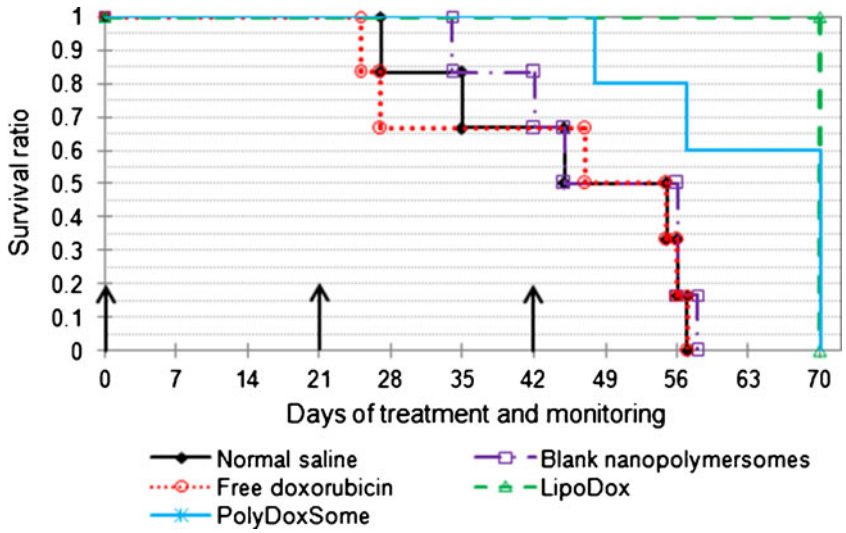

Fig. 5 Kaplan Meier survival plot showing ratio of surviving animals in days after treatment with normal saline and blank nanopolymersomes (controls); and doxorubicin (6 mg/kg/dose) as free doxorubicin, LipoDox™ and PolyDoxSome. Injection was repeated three times at interval of 21 days and monitored for 70 days. The arrows indicate the injection days.

Table III Antitumor Activity in DMBA Induced Mammary Carcinoma Rat Model

Treatment group	MST	ILS (%)	*p* vs. Normal saline
Normal saline	45	–	–
Blank nanopolymersomes	45	–	–
Free doxorubicin	47	5%	0.825
LipoDox™	70	56%	0.0005
PolyDoxSome	70	56%	0.01

MST median survival time; *ILS* increase-in-life span. Data presented are from survival analysis of group of 6 animals ($n=6$). Statistical analysis of median survival times were expressed using pair wise multiple comparison procedure (Holm-Sidak method) *vs.* normal saline. Overall significance level $\alpha=0.05$

damage in a variety of cardiac disease models. Analysis was conducted 14 days after the final treatment to distinguish persistent cardio-toxic effects from acute (<72 h) changes. The relative increment in value of CPK was observed in the serum of free doxorubicin treated rats as compared with untreated and LipoDox™/PolyDoxSome treated groups. Level of LDH were also increased in rats treated with free doxorubicin and was statistically significant ($p<0.05$) from control groups (saline and blank nanopolymersomes) and treatment groups (LipoDox™ and PolyDoxSome) (Table IV). However, no significant difference was observed on comparison of both treatment groups.

It is well know that doxorubicin is metabolized by liver and eliminated by bile. Hence, hepato-toxicity requires dose adjustment according to serum bilirubin concentration or liver enzyme levels. Liver function tests showed significant increase in alkaline phosphatase (ALP) values in all treatment groups compared with normal saline or blank nanopolymersomes, especially considerable increment was observed for free doxorubicin treated groups. Alanine aminotransferase (ALT) value obtained from free doxorubicin treated groups was significantly higher than that of other treatment groups. At the same time, aspartate aminotransferase (AST) values showed only slight fluctuations in all treatment groups but remained within the physiological norm though the value for free doxorubicin treatment groups was relatively higher than normal saline control, blank nanopolymersomes, PolyDoxSome and LipoDox™ treated groups. Bilirubin was significantly increased for free doxorubicin treatment groups as 6-fold and 7-fold compared to LipoDox™ and PolyDoxSome, respectively (Table IV).

Hematological Parameters

The hematological parameters were influenced by administration of free doxorubicin as it is observed from RBC count, WBC count and hemoglobin level as compared with control and PolyDoxSome treated groups (Table V).

DISCUSSION

After administration, drug carriers are distributed in the blood, tissues and degrade or excrete from the body by well defined mechanisms. Pharmacokinetic studies of any formulation suggest the fate of carrier system in the blood compartment. In this study, PolyDoxSome treated rats shows 150 fold increase in AUC ($p<0.001$) along with a half life of 21.9 h in plasma concentration time profile, as compared to free doxorubicin ($t_{1/2}$; 0.49 h. $p<0.001$)). The clearance and

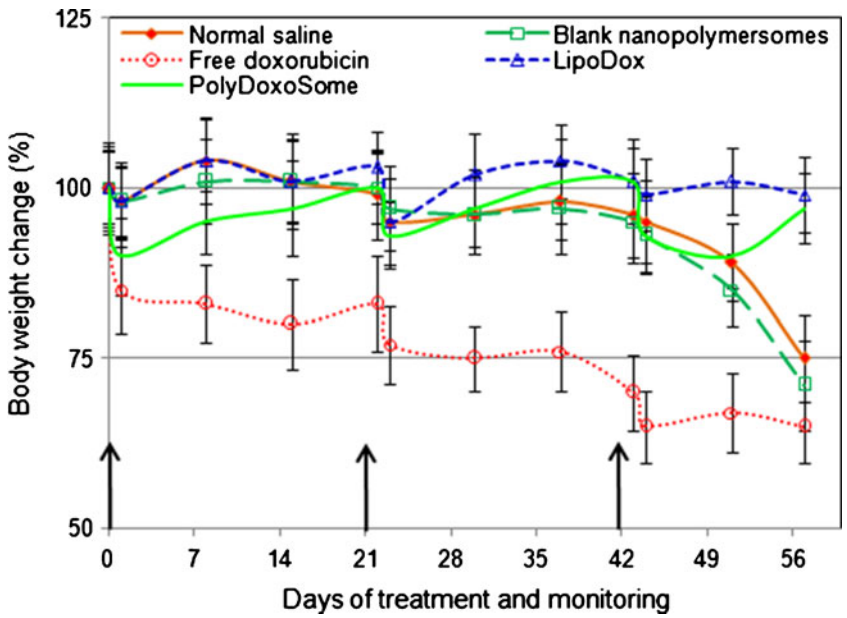

Fig. 6 Plot of body weight change (in percent of initial body weight) of rats treated with three doses of doxorubicin (6 mg/kg/dose) as free doxorubicin, LipoDox™, PolyDoxSome, and blank nanopolymersomes and normal saline as control groups. Each data points presented are average values of three to six replicate experiments ($n=3$–6) and error bars indicate standard deviations. The arrows indicate the injection days.

Table IV Cardiac and Liver Function Enzyme Levels

Treatment group	LDH (IU/L)	CPK (IU/L)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Bilirubin[$]
Normal saline	285 ± 94	273 ± 68	143 ± 4.6	83 ± 2.7	180 ± 29	0.3 ± 0.01
Blank nanopl.	259 ± 40	326 ± 56	151 ± 25	85 ± 9.6	135 ± 38	0.4 ± 0.01
Free dox.	474 ± 33*	814 ± 96**	199 ± 44*	141 ± 63*	698 ± 28**	8 ± 7.0**
Lipodox™	348 ± 91	683 ± 51	149 ± 69	94 ± 27	415 ± 26**	1.27 ± 1.3
PolyDoxSome	328 ± 104	492 ± 38	166 ± 128	89 ± 20	460 ± 32**	1.1 ± 1.2

$ mg/dL; *CPK* creatine phosphokinase; *LDH* lactate dehydrogenase; *ALT* alanine aminotransferase; *AST* aspartate aminotransferase; *ALP* alkaline phosphatase. Statistical analysis of enzyme levels were expressed using one way ANOVA-Tukey test *vs*. normal saline treated animals. *$p < 0.01$ and **$p < 0.001$ *vs*. normal saline group. Data presented are the average values of three replicate experiments ($n=3$) followed by the standard deviation

apparent volume of distribution of PolyDoxSome is significantly decreased and comparable to that of LipoDox™. The highest plasma AUC, as obtained by LipoDox™, may not always be advantageous to the patients because it shows a dose related toxicity in the form of stomatitis due to increased AUC and smaller stealth particles. In this case, it is also true that prolonging plasma stability may lead to stomatitis and tolerable dose may show reduced efficacy. In this regard, PolyDoxSome having larger mean diameter (171 *vs*. <100 nm), shorter plasma circulation half life (22 *vs*. 35 h), lower C_{max} (34 *vs*. 67 μg/ml), and lower AUC (568 *vs*. 2291 h*μg/ml) compared with LipoDox™ could be better approach in reducing or avoiding mucocutaneous toxicities such as PPE or stomatitis, which may be associated due to small size and altered pharmacokinetic parameters. The same aspect can be explored clinically for the products to find out the possible clinical advantage of PolyDoxSome or LipoDox™ thereof.

Distribution of drug in various tissues is responsible for efficacy and toxicity profile of the molecule. Doxorubicin with a molecular weight of 543 Da is known to accumulate in highly perfused organs, preferentially in the heart, and thus cardiac toxicity of doxorubicin is a dose limiting toxicity. The objective of entrapment of doxorubicin in nanopolymersomes is to reduce its uptake into non target organs, specifically to the heart compared with free doxorubicin, to extend plasma circulation time and enhance accumulation in tumor tissues. Over all, the higher doxorubicin concentration was observed in all tested organs of formulation treated animals except heart while this order was reverse for free drug treated animals. It is important to note that drug molecules encapsulated in carrier system are not exposed to the actual tissue environment and thus, release of the drug from the carriers is slow in both cases while free drug molecules are fully available to the exposed environment. It is observed that the accumulation of drug in encapsulated form is mostly due to the carrier system, not due to free drug. Liver plays a critical role to eliminate all types of free and encapsulated drug molecules. In case of spleen and kidney, doxorubicin levels were higher for LipoDox™ than PolyDoxSome; however were comparable in heart, liver and lung. It is interesting to note that PolyDoxSome treated animal show higher doxorubicin levels in tumor in comparison to all other tested organs which shows the accumulation of drug in the tumor tissues. LipoDox™ treated animals show equal drug levels in tumor and spleen tissues, half levels in kidney and quarter levels in liver and lung. Free doxorubicin treated animals have higher AUC in heart, liver and lung while less AUC in spleen, kidney and tumor. Specifically, higher free drug levels in heart for free doxorubicin treated animals than LipoDox™ (3-fold) and PolyDoxSome (5.8-fold) attribute to cardiac toxicity which is also supported by toxicity data. Consistent with this, it was also shown that doxorubicin has mainly distributed to liver and lung that can contribute to its fast metabolism. In the present scenario, PolyDoxSome may serve as a better drug carrier due to its highest level in tumor tissue and lowest level in the heart, which is clinically desired for better efficacy and

Table V Hematological Parameters after Multiple Injections of PolyDoxSome and Controls

Parameters	Saline	Blank nanopl.	Free Dox	Lipodox™	PolyDoxSome
RBCs ($10^6/\mu l$)	4.9 ± 0.56	5.1 ± 0.97	4.1 ± 1.0	4.9 ± 0.73	5.4 ± 0.30
WBCs ($10^3/\mu l$)	4.9 ± 0.30	5.7 ± 3.2	3.5 ± 1.05*	5.3 ± 0.45	3.9 ± 1.60
Platelets ($10^5/\mu l$)	3.02 ± 0.55	2.58 ± 0.25	2.4 ± 0.38	2.52 ± 0.6	2.25 ± 0.30
Hemoglobin (g/dl)	13.6 ± 0.57	13.6 ± 1.44	10.6 ± 2.7*	14.2 ± 0.25	12.8 ± 0.28
PCV (%)	39.3 ± 2.08	42.0 ± 4.35	33.0 ± 7.8*	42.6 ± 0.57	40.0 ± 1.73

Data presented are the average values of three animals ($n=3$) followed by the standard deviation. Statistical analysis of values was expressed using one way ANOVA-Tukey test *vs*. normal saline groups. *$p < 0.01$ *vs*. saline group

minimized toxicity. Thus, results indicated that a limited potential for cardiac damage and increased tumor exposure of doxorubicin can be obtained when administered as PolyDoxSome compared to free doxorubicin.

PolyDoxSome has been found to be as effective as LipoDox™ in suppressing tumor growth and prolonging survival. The tumors of animals receiving normal saline or blank nanopolymersomes grew rapidly and ulcerated before end of the study. Whereas administration of both LipoDox™ and PolyDoxSome resulted in significant suppression of tumor and marked difference in tumor volume was observed after the second dosing ($p<0.05$ *vs.* free doxorubicin). Data on survival analysis and increase-in-life span (ILS) indicated that PolyDoxSome and LipoDox™ were more effective than control groups and free doxorubicin treatment group ($p<0.01$), and there is no significant difference between carrier systems ($p>0.138$ by log-rank test). There was no significant improvement in survival with free doxorubicin treatment in comparison to control groups ($p>0.825$). Such observation can be explained with selective and higher accumulation of doxorubicin in tumor tissue of this model from PolyDoxSome and LipoDox™ than free doxorubicin, which in turn was attributed to extended circulation time of both carriers. In control groups, tumors became larger, ulcerated, and some of the rats became weaker and were euthanized or died during study period.

A significant body weight loss with no recovery was observed in free doxorubicin treated rats however no weight loss was observed in the treatment group. The maintenance of body weight in PolyDoxSome and LipoDox™ treated groups demonstrated no toxicity of both carriers at the tested drug concentrations. The major dose limiting toxicity of doxorubicin in its clinical use is cardio-toxicity because of its ability to produce free radicals upon increased cumulative dose. Unlike other organs, which have adequate supply of special enzymes to destroy free radicals, heart is vulnerable to injury by doxorubicin reactive oxygen species due to the presence of more oxygen (for highly oxidative metabolism) and iron (non enzymatic mechanism for free radical formation through reaction of doxorubicin and iron(III)) or less developed antioxidant defense mechanisms (55,56). The CPK and LDH levels were not considerably higher in LipoDox™ or PolyDoxSome treatment groups when compared to the control group. This is attributed to less doxorubicin accumulation in heart as shown in tissue distribution studies suggesting the cardio protective efficiency of nanopolymersomes. This can also be explained by decreased distribution of liposomes or nanopolymersomes to the myocardium, presumably as a result of tight junctions of myocardium capillaries preventing the extravasation of liposomes or nanopolymersomes and their plasma stability and low or no leakage of free doxorubicin. In the hematological study, throughout the monitoring period, no clinically significant change in hematological parameters was observed in formulation treated group and fluctuations observed during the study remained within physiological range for all experiments except for free doxorubicin, which has values less than the reference values in all parameters except platelet count. These results further indicate that formulation treated group are safe in comparison to free drug treated groups.

CONCLUSIONS

PolyDoxSome treatment results in an increased AUC of doxorubicin equivalents and a longer plasma circulation time, whereas clearance and volume of distribution are significantly decreased when compared with free doxorubicin administration, however it remained comparable with marketed LipoDox™ formulation. Considerable doxorubicin concentrations were found in the tumor from PolyDoxSome compared with free doxorubicin, whereas its concentration in the heart is much less than the concentration obtained from free doxorubicin demonstrating significant therapeutic advantage of PolyDoxSome over free doxorubicin. On the other hand, when PolyDoxSome is compared with LipoDox™, apart from its composition, being a polymeric carrier, it differs from LipoDox™ in having larger mean diameter (171 *vs.* 100 nm), shorter plasma half life (21 *vs.* 35 h), lower Cmax (34 *vs.* 66 μg/ml), and lower AUC (568 *vs.* 2291 h*μg/ml). This composition and pharmacokinetic parameters differences could drop mucocutaneous adverse events associated with LipoDox™. In general, the preliminary findings of this study justify its potential as alternative doxorubicin carrier system to liposomal formulations and may be clinically beneficial to proceed for further clinical trials.

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