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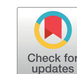
Doxebo (doxorubicin-free Doxil-like liposomes) is safe to use as a pre-treatment to prevent infusion reactions to PEGylated nanodrugs



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ABSTRACT

The increasing use in the last decade of PEGylated nanodrugs such as Doxil® has seen a rise in the number of associated occurrences of hypersensitivity reactions (HSRs). These reactions (also called infusion reactions or IR), can range from harmless symptoms to life-threatening reactions. Current means to prevent IR include the prophylactic use of antihistamines and steroids, but they cannot ensure total prevention. We previously showed that an intravenous injection of doxorubicin-free Doxil-like PEGylated nano-liposomes (Doxebo) prior to Doxil treatment suppresses Doxil-induced complement activation-related pseudoallergy (CARPA) in pigs, a model of human hypersensitivity reactions to Doxil. However, in order to use Doxebo to prevent Doxil-induced IR, we have to prove its safety and that it does not affect Doxil's performance. Here we show that Doxebo itself does not have toxic effects on the host or tumor, and it does not interfere with Doxil's antitumor activity in mice. Blood, microscopic and macroscopic organ evaluation of rats after repeated administration confirm the lack of intrinsic adverse effect of Doxebo. Likewise, the repeated injection of Doxebo before Doxil did not impact Doxil's pharmacokinetics in plasma and therefore does not cause accelerated blood clearance (ABC). Taken together with our previous publications, these data suggest that the injection of Doxebo prior to Doxil administration can help protect against Doxil-induced IR without adversely affecting treatment efficacy and safety.

1. Introduction

Liposomal doxorubicin (Doxil®) is the first FDA-approved nanodrug used for the treatment of ovarian cancer, Kaposi's sarcoma, multiple myeloma and more recently was approved in Europe for the treatment of metastatic breast cancer. Since its approval by the FDA in 1995 as the first nanomedicine, Janssen stated that up until December 2017 it has been used for the treatment of over 600.000 patients, providing unique protection against doxorubicin-induced cardiomyopathy and other side effects, leading to substantial improvement of life quality following chemotherapy [1]. But the "price" of this beneficial effect is a substantially increased occurrence and severity of two side effects; palmar-plantar erythrodysesthesia (PPE) and complement (C) activation-related infusion reactions (IR) [2–6].

Infusion reactions are well known phenomenon for many drugs and more so for nano-drugs. The underlying causes are in most cases unclear (Szebeni et al. [7] and references listed therein). The symptoms of IR are often harmless (flushing, shortness of breath, headaches, chills…) and usually arise during the first drug infusion. Their prevalence varies greatly from < 1% to > 80% of patients treated depending of the drug administered [7]. The current measures taken to reduce their occurrence include premedication with steroids, anti-histaminic and/or anti-inflammatory drugs, reducing the speed of infusion or treatment interruption. These actions are globally effective and IR

Abbreviations: ABC, accelerated blood clearance; ALT, alanine amino-transferase; AST, aspartate amino-transferase; AUC, area under the curve; BW, body weight; C, complement; CARPA, complement activation-related pseudoallergy; HSR, hypersensitivity reactions; HSPC, hydrogenated soy phosphatidylcholine; IR, infusion reactions; I.V., intravenous; LR, lissamine-rhodamine; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PEG, polyethylene glycol; PK, pharmacokinetics; PL, phospholipid; PLD, PEGylated liposomal doxorubicin; PPE, palmar-plantar erythrodysesthesia; S.C., subcutaneous; SD, standard deviation; RT, room temperature; RTV, relative tumor volume; TX, thromboxan

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resolves without consequences in most cases. Nevertheless, despite systematic premedication, some patients experience life-threatening reactions in occasional cases. These infusion reactions have been observed in patients treated with liposomal drugs, but also with micellar drugs, antibodies, proteins and enzymes to which polyethylene glycol (PEG) moieties are covalently attached and therefore are referred to as "PEGylated" drugs [5,8]. The PEGylation of biologicals provides a "steric" shielding from opsonization and protection from quick uptake by the reticuloendothelial system (RES), resulting in a much longer circulating time in plasma compared to the non-PEGylated drug [9,10]. But these advantages seem to come at a cost and in the last decades there have been increasing reports of anti-PEG antibodies (IgM and IgG) found in the blood of individuals after PEGylated drug administration [11–13], but also in individuals who were never treated with a PEGylated drug [13–16]. These pre-existing anti-PEG antibodies seem to be related to the occurrence of IR upon the first Doxil treatment.

IgG and IgM are well-known mediators of two types of HSR (according to Gell and Coombs classification [17]): type II HSR, that are mainly mediated by IgG, and type III HSRs that are induced by IgM-/IgG-drug complexes (type I are IgE-dependent and type IV are induced by T-helper cells and therefore less relevant in the case of PEGylated drugs).

The wide diversity of the drugs affected and the potential life-threatening character of the reaction clearly puts to high stakes the need for early detection and prevention of the risk of drug-induced IR, not only for the existing drugs, but also for the ones in developmental stages.

Recently, in an effort to prevent IR in general, and Doxil-induced IR, in particular, we pretreated pigs with a small dose of drug-free Doxil, called Doxebo, immediately before Doxil therapy. This pretreatment was not reactogenic and reduced or eliminated IR to subsequent Doxil treatments in a model of porcine IR known as C-Activation Related Pseudoallergy (CARPA) [18].

The goals of the present studies were to address questions that are critical for the possible clinical application of Doxebo, namely, the intrinsic toxicity of Doxebo on one hand, and on the other hand its possible interference with Doxil's antitumor therapeutic efficacy (directly or through accelerated plasma clearance) and its pharmacokinetic profile.

The effect of Doxebo alone on tumors was assessed in a model of lymphoblastic leukemia (mice bearing tumors from CCRF-CEM line), and to address the question of the possible interference with Doxil's therapeutic efficacy, we compared the anticancer activity of Doxil, with and without Doxebo pre-treatment in a model relevant to Doxil therapy: mice bearing MDA-MB-231 cells, a breast cancer model. To assess Doxebo's intrinsic toxicity, toxicity studies (repeated injections) were conducted in rats. Finally, to explore the potential effect of Doxebo on Doxil clearance (ABC phenomenon), we checked the pharmacokinetic profiles of PEGylated liposomal doxorubicin (PLD) in mice pretreated or not with repeated injections of Doxebo. The results obtained suggest that Doxebo is safe, that it has no negative effect on Doxil's efficacy, and that the joint use of Doxebo and Doxil is not contraindicated.

It should be noted that, due to the worldwide shortage of the original drug Doxil® and its European counterpart Caelyx® at the time these studies were conducted, some experiments (namely the pharmacokinetic and macrophage activity studies in mice) were performed with Dox-NP® (a Doxil-like PLD for research applications, prepared by LipoCure Ltd. and distributed by Avanti Polar lipids). The physicochemical parameters of Dox-NP were identical to that of the original Doxil® [19,20]. Other experiments (the animal models of tumors and the *in vitro* experiments) were performed with Lipodox® (Sun Pharmaceuticals Industries, India), the formulation allowed temporarily by the FDA in order to replace Doxil before a generic was approved. Recently a retrospective objective clinical comparison showed a great similarity in the clinical performance of Doxil and Lipodox [21].

2. Materials and methods

2.1. Drugs and chemicals

Lipodox was purchased from Sun Pharmaceutical Industries Ltd., India and Dox-NP was from Avanti Polar Lipids (cat. #300112). Sterile saline for injection and doxorubicin hydrochloride powder were from TEVA Pharmaceuticals (Petach Tikva, Israel). Cholesterol, hydrogenated soy phosphatidylcholine (HSPC) and DSPE-PEG2000 were purchased from Lipoid KG (Ludwigshafen, Germany). All lipids had a purity of ≥97%. L-histidine of US grade was from Merck KGaA (Darmstadt, Germany). Sucrose, analytical grade was purchased from Bio-Lab Ltd. (Jerusalem, Israel). Anti-PEG monoclonal antibodies (AGP4, AGP6, 3.3-biotin, and 15-2b-biotin) were developed by S. Roffler's lab. Streptavidin-HRP was purchased from Jackson ImmunoResearch and ABTS and skim milk from Sigma Israel.

2.2. Liposomes preparation and characterization

Drug free (empty) PEGylated liposomes (Doxebo) were prepared as described previously [20]. Briefly, the freeze-dried lipid mixture composed of hydrogenated soybean phosphatidylcholine (HSPC), cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(methoxy(polyethyleneglycol)-2000 Da) sodium salt (DSPE-PEG2K), at 56.6:38.1:5.3 mol ratio or 3:1:1 weight ratio were hydrated (in 10 mM sucrose-histidine buffer pH 6.5) and vortexed for 2–3 min at 60 °C to form multilamellar vesicles (MLVs). The MLVs were downsized through 400, 100, 80 and 50 nm-pore size polycarbonate filters using a 10-ml extruder barrel from Northern Lipids (Vancouver, British Columbia, Canada). The phospholipid concentration of Doxebo (16 mg/ml) was determined by a modified Bartlett procedure as described earlier [22,23]. Liposomes size was measured by dynamic light scattering (NanoZetasizer, Malvern) and the *Z*-average of the preparation was found to be 82 nm with a polydispersity index (PDI) of 0.03.

Liposomes labelled with Lissamine-Rhodamine-PE (LRPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt), Avanti Polar Lipids, #810150) composed of E-PG (L-α-phosphatidylglycerol from egg, LIPOID), E-PC (L-α-phosphatidylcholine from egg, Avanti Polar Lipids) and LRPE at 1:9:0.05 mol ratio were prepared in two steps, first by preparing liposomes of E-PG/E-PC, then by incorprating the LRPE by post-insertion. The preparation of E-PG/E-PC started by evaporating the chloroform of the E-PG by nitrogen stream, then adding E-PC to the dried E-PG. The dried lipids were then dissolved in ethanol (approximately 26.5 µl ethanol/mg lipids), mixing the ethanolic lipidic solution for 15 min at 37 °C, which is above these lipids phase transition temperature range (below 0 °C). Dextrose 5% (Teva) was added to obtain a suspension at approximately 3.4 mg/ml lipids, which was stirred at 37 °C for 30 additional minutes. The MLVs obtained were downsized in two steps, the first one using 400 nm pore size polycarbonate filters, and the second one through 200 nm polycarbonate filters. Extrusion was performed using a 100 ml extruder barrel from Northern Lipids at 40 °C and the liposomes then were filtered for sterility through a 0.2 µm filter. The liposomes were labelled fluorescently by post insertion of LRPE at 7 µg/µmol lipids. The liposomes were incubated with the label for 10 min at 37 °C while stirring. Liposomes size was measured by dynamic light scattering (NanoZetasizer, Malvern) and the *Z*-average of the preparation was found to be 235 nm with a polydispersity index (PDI) of 0.26.

The absence of free fluorophore was assessed by loading the labelled liposomes on a column of Sepharose-4B and running with 5% Dextrose followed by a wash with 20% isopropanol. It was determined that the above fluorescent liposomal formulation contained practically no free LRPE.

2.3. Anti-PEG antibodies affinity to PLD

Anti-PEG antibody binding to PLD was assessed by anti-PEG sandwich ELISA as described previously [24,25]. In brief, Maxisorp (Nunc) plates were coated with 250 ng antibody/well (AGP4 or AGP6 mouse anti-PEG IgM) in 50 mM carbonate buffer, pH 8.0, and incubated at 37 °C for 2 h then 4 °C overnight. Plates were washed 3 times with PBS then blocked with 200 µl 5% skim milk in PBS for 2 h at room temperature (RT). After 3 additional washes in PBS, PLD samples diluted to 1 µg/ml (in 2% skim milk in PBS) then serially diluted 1:6 were added to each well (50 µl/well) and incubated for 2 h at RT. Wells were washed 3× with PBS-Tween-20 (0.05%) then 2× with PBS (for 15–2 detection) or 3× with PBS (3.3 detection). The detection antibody (biotinylated anti-PEG 3.3-biotin or biotinylated anti-methoxyPEG 15–2-biotin) at 5 µg/ml was added (50 µl/well) and incubated for 1 h at RT. After washes (identical to previous washes), 50 µl/well streptavidin-HRP (Jackson Immunoresearch, 1 µg/ml in 2% skim milk/PBS) were added for 1 h at RT. Wells were again washed (6× PBS-Tween then 2× PBS for 15–2-biotin detection or 3× PBS-Tween then 2× PBS for 3.3-biotin detection) and 100 µl/well of freshly prepared ABTS substrate (Sigma, 500 mg/l) containing 0.01% H_2O_2 were added for 30 min in the dark at RT. Absorbance was read at 405 nm.

2.4. Cell culture

MDA-MB-231 cells (ATCC) were cultured in Leibowitz's L-15 media (Hi Media Laboratories Pvt. Ltd., India) supplemented with 10% Fetal Bovine Serum (FBS, Biosera). The cells growing at log phase were harvested by trypsinization and washed with Leibowitz's L-15 media. CCRF-REM cells (ATCC) were cultured and expanded in RPMI1640 media (Life Technologies Pvt. Ltd., India) supplemented with 10% FBS. Cell viability was estimated by Trypan blue exclusion test. Finally, cells were adjusted to 25×10^6 cells/ml suspension before injection into mice.

2.5. Mouse model of human lymphoblastic leukemia

A volume of 200 µl of cell suspension of CCRF-CEM containing approximately 7.5×10^6 cells was subcutaneously injected into the flank region of female athymic nude Fox^{n-1} nu/nu mice (Charles River Laboratory, India) aged 6–10 weeks. When tumor reached a sufficient size, 30 mice were injected I.V. once a week for three consecutive weeks with saline 5 ml/kg ($n = 7$), Doxebo 5 ml/kg equivalent to 80 mg phospholipids/kg ($n = 7$) or Lipodox 8 mg/kg equivalent to 64 mg phospholipids/kg ($n = 8$).

2.6. Mouse model of human breast cancer

MDA-MB-231 cells were subcutaneously injected into the flank region of donor female athymic nude Fox^{n-1} nu/nu mice (10×10^6 /200 µl/mouse). Animals were monitored for tumor growth and the mice sacrificed when solid tumors reached ~400–500 mm^3. The solid tumor from donor animal were isolated aseptically, fragmented to ~30 mg and subcutaneously implanted to experimental animals (same mouse line as donors) using a trocar. When tumor reached a sufficient size, the mice were allocated into six groups who received one of the following treatments: I.V.: saline 4 ml/kg ($n = 7$), Lipodox (8 mg/kg) equivalent to 64 mg phospholipids/kg, Doxebo 4 ml/kg ($n = 7$) equivalent to 64 mg phospholipids/kg, or Doxebo 4 ml/kg followed 1 h later by an injection of Lipodox (8 mg/kg).

2.7. Animals follow up and tumors measurement

The first day of administration was designated day 0. For the mice xenografts models and rats toxicity study, cage side observation to check morbidity and mortality were performed daily; body weight and tumor size (if relevant) were recorded twice a week. The tumors' dimensions were measured with a digital Vernier Caliper and Mean Tumor Volume (MTV) was determined by using the formula $MTV = length \times width^2 \times 0.5$.

2.8. Mouse Pharmacokinetics study

BALB/c male mice aged 8–10 weeks were injected I.V. with either Doxebo at 1 mg/kg followed 2 h later by an injection of Dox-NP at 10 mg/kg, or Dox-NP alone at 10 mg/kg. Half of the mice were sacrificed as described below after the first treatment. The other half was injected exactly one week later so each mouse was injected twice with the same treatment (either Doxebo alone or Doxebo + Dox-NP) for two weeks. They were then sacrificed according to the protocol that follows at the same time-points. At $T = 1$ h, 6 h, 24 h and 48 h post Dox-NP injection (week 1 or 2), 3 mice in each group were humanely sacrificed by CO_2 inhalation and the blood, collected immediately following death from retro-orbital sinus in K_3EDTA tubes, was centrifuged at 2600 ×g for 10 min to collect plasma. The plasma samples were kept frozen until analysis. For doxorubicin quantification, acidified isopropanol (0.075 N HCl in 90% isopropanol) was added to dilute plasma samples at 1:10 and after vortex, the fluorescence was measured using a BioTek Synergy™ 4 Hybrid Microplate Reader (λ_{exc} 485 nm and λ_{em} 620 nm). To correct for non-specific background fluorescence, the samples were analyzed using a standard curve obtained by diluting increasing concentrations of Dox-NP in the plasma of naïve mice as described previously [26].

2.9. Macrophage Activity Test

Twenty BALB/c male mice aged 9–10 weeks were injected I.V. with either Doxebo at 1 mg/kg followed 2 h later by an injection of Lipodox at 10 mg/kg ($n = 10$), or with Lipodox only at 10 mg/kg ($n = 10$). The following week, the mice were injected so each mouse received the same treatment (either Doxebo alone or Doxebo + Dox-NP) for two consecutive weeks. Twenty-four hours after the second Lipodox injection, the mice were injected I.V. with Lissamine-Rhodamine labelled liposomes (200 µl). Each treatment group (with or without Doxebo pretreatment) was divided into 2 subgroups of 5 mice. At each of the time points ($T = 10$, 30, 60, 120 and 240 min after the fluorescently labelled liposomes were injected), a blood sample of maximum 100 µl was taken from each subgroup alternatively from the facial vein. One subgroup was sampled at 10, 60 and 240 min and the other at 30 and 120 min.

Upon collection, the blood samples were centrifuged at 2600g for 10 min to precipitate the red blood cells. The plasma was collected and immediately frozen pending analysis. The plasma fluorescence was measured by diluting the plasma samples 4 times in acidified isopropanol and reading (λ_{exc} 565 nm and λ_{em} 585 nm) the fluorescence with a BioTek Synergy™ 4 Hybrid Microplate Reader. A standard curve with increasing concentrations of LRPE labelled liposomes in the plasma of naïve mice was prepared. To correct for non-specific background fluorescence, the fluorescence of the plasma of naive mice diluted 4 times in acidified isopropanol was subtracted from the values obtained.

2.10. Toxicity study upon repeated injection in rats

The study was performed in GLP (Good Laboratory Practice) conditions. Sprague-Dawley rats (16 males and 16 females) were injected once a week for 6 weeks with Doxebo 2 ml/kg (equivalent to 32 mg phospholipids/kg). The animals were observed on a daily basis for signs of physiological or behavioral changes and weighed 3 times a week. Rats were sacrificed after the last injection by carbon dioxide inhalation. Blood was collected from the vena cava into K_3EDTA and hematology, clinical chemistry and coagulation parameters were analyzed. Gross (macroscopic) observations were recorded, the organs were

collected, weighed and prepared for pathological microscopic examination.

2.11. Statistical analysis

For *in vivo* mice studies and the toxicity study in rats, values were expressed as the mean ± standard deviation (SD). Tumor volumes and body weight data were compared using Bonferroni multiple comparison for the CCRF model and Dunnett's multiple range test for the MDA-MB-231 model. Survival data was analyzed using the “Kaplan Meier” method. All analyses were done with either IBM SPSS 23 Statistics software or GraphPad Prism 8; $P < .05$ was the threshold for statistical significance.

2.12. Ethics statement

All *in vivo* experiments were conducted in strict accordance with protocols approved by the animal ethical committees of the different facilities where the experiments took place. The mice xenografts experiments were conducted at Dabur Research Foundation (India) with the approval of the Institutional Animal Ethics Committee (IAEC, India). Toxicity studies on rats were conducted at MPI Research, Inc. (Mattawan, MI, U.S.A) and the mice studies were approved by the Animal Ethical Care Committee of the Hebrew University of Jerusalem, Israel (ethics approval 22,406).

3. Results

3.1. Doxebo and Lipodox display similar binding to anti-PEG antibodies

This experiment serves two objectives, firstly to demonstrate that Doxebo can bind efficiently anti-PEG IgG and anti-PEG IgM antibodies, secondly to compare the binding of these antibodies to Doxebo and Doxil. The formation of antibody-liposome complexes (anti-PEG IgM-liposomes but also to a lesser extent anti-PEG IgG-liposomes) can activate the cascade of proteins of the complement system [27,28], leading to the clinical manifestations of IR. Doxebo liposomes have been prepared with similar mPEG-HSPC content as Doxil and Lipodox preparations, leading to a similar mole percentage of PEG on its surface. But the presence of doxorubicin crystals in the liposomes modifies the spheroid shape to the coffee bean shape characteristic of Doxil [29]. Therefore, it was important to study if this change of liposome shape affect antibodies binding to the liposome surface. In these studies we compared the binding avidity of anti-PEG antibodies to the different liposomal formulations by determining their EC50 (Fig. 1A-D). We performed an anti-PEG sandwich ELISA as described previously by Cheng and Roffler [24,25] using two different anti-PEG IgM (AGP4 or AGP6) as capture antibodies. For detection we used either an IgG directed against the terminal methoxy moiety of PEG (15-2b) or against the PEG backbone (3.3), all developed by Roffler et al. [30,31]. Firstly, it was found that the PLD and Doxebo bind antibodies efficiently. Secondly the binding curves of Doxebo and Lipodox were found to be very similar (not statistically different) for 3 out of 4 combinations tested (Fig. 1E). For these three combinations of antibodies, it seems that the difference in liposome shape does not affect antibodies binding. The statistical difference in EC50 values obtained with the combination AGP6 and 15-2b could be explained by the fact that these antibodies are more selective than AGP4 and 3.3, and that therefore a smaller fraction of the antibodies could bind the liposomes. AGP6 recognizes the backbone of PEG but binds a smaller epitope than APG4 and 15-2b binds the methoxy extremity of PEG and is therefore limited to one antibody per PEG molecule—if the conformation of the PEG (mushroom or brush) and the steric hindrance allow the antibody to reach its epitope. This experiment demonstrates that the binding of anti-PEG antibodies to Doxil and Doxebo and similar, therefore it is reasonable to assume that *in vivo* Doxebo will be able to bind in a similar fashion various kinds of anti-PEG antibodies (IgG and IgM) and cause their fast clearance.

3.2. Doxebo has no antitumor effect by itself, and no impact on the therapeutic efficacy of PLD

To evaluate the effect of Doxebo alone on tumor growth, we compared the relative tumor volume (RTV) of mice xenografted with CCRF-CEM cells (a model of human lymphoblastic leukemia) and injected with either Doxebo, saline or Lipodox (the reference treatment). The RTV is used as a measure of the therapeutic efficacy of anticancer drugs (in our case, Lipodox treatment). These results are presented in Fig. 2A. We also assessed whether pretreatment with Doxebo affects the efficacy of anticancer treatment by comparing the RTV of MDA-MB-231 tumor bearing mice (a breast cancer model) treated with either Doxebo alone, saline, Lipodox alone or a combination treatment consisting of Doxebo followed 1 h later by Lipodox (Fig. 2B). In both models the results clearly show that Doxebo did not exhibit any antitumor effect by itself as shown by the very similar RTV observed in mice injected with Doxebo and saline in the CCRF-CEM model (Fig. 2A), and in MDA-MB-231 tumor-bearing animals (Fig. 2B). Similarly, the injection of Doxebo before Lipodox did not alter its antitumor efficacy, as seen in Fig. 2B.

3.3. Lack of systemic toxicity of Doxebo in mice with leukemia and breast cancer xenografts

After showing that Doxebo has no pro- or antitumoral effects, we assessed its general toxicity by measuring the body weigh variation of mice bearing tumors (models described in Fig. 2) and injected with Doxebo, saline or Lipodox (or Doxebo followed by Lipodox for the MDA-MB-231 model). As expected from an anticancer drug, the cytotoxic effect of Lipodox is translated into significant loss of body weight starting after the first injection and lasting for the duration of the treatment (21 days). Doxebo alone, just as saline, triggered no such effect as seen in Fig. 3A where the body weight gain curve for mice injected with saline and Doxebo are increasing similarly. This effect is also observed in the MDA-MB-231 bearing mice (Fig. 2B) where the body weight variation curves of Doxebo and saline injected mice are hardly distinguishable. Likewise, the administration of Doxebo before Lipodox did not affect the toxic effect of Lipodox alone (Fig. 3B) as shown by similar body weight loss in the two groups. In both models, the administration of Doxebo has no effect on body weight compared to saline, suggesting that Doxebo has no intrinsic systemic toxicity in this regimen.

3.4. Doxebo has no impact on the survival of mice with leukemia or breast cancer

The effect of Doxebo on the survival profile of mice bearing the previously described CCRF-CEM (Fig. 4A) and MDA-MB-231 (Fig. 4B) tumors was also examined. Consistently with the previous findings, Doxebo did not affect the survival of mice in both leukemia and breast cancer models as compared to the saline injected mice. In the leukemia model (Fig. 4A), the survival of mice injected with saline was 21 *vs.* 24 days for the mice injected with Doxebo; and in the breast cancer model it stood at 32 days for both the Doxebo and saline group.

Taken together, these data suggest that Doxebo had no impact on the host mice or on the tumors, and that it does not alter the anti-tumor efficacy of Doxil *in vivo*.

3.5. Doxebo injections prior to Lipodox do not trigger accelerated plasma clearance

To determine whether Doxebo alters the pharmacokinetic profile of liposomal doxorubicin after repeated injections (a phenomenon known as accelerated blood clearance, abbreviated as ABC), the levels of

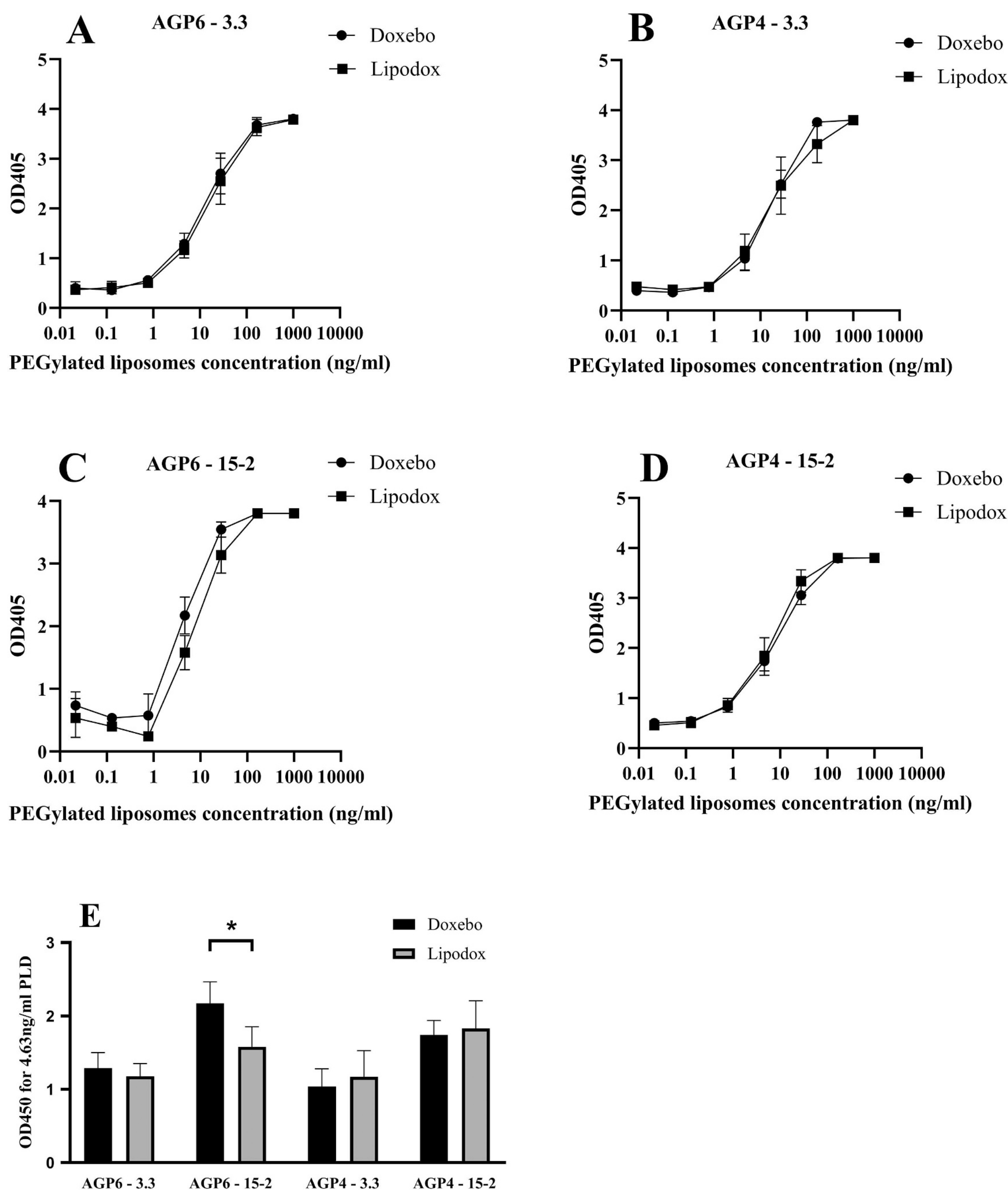

Fig. 1. Detection of PEGylated liposomal formulations using sandwich ELISA. The plates were coated with two kinds of anti-PEG IgM as capture antibodies: AGP6 (1A and C) or AGP4 (1B and D) to capture increasing concentrations of Doxebo (circles) and Lipodox (squares). The detection was performed using IgG either directed against the PEG backbone (3.3, Fig. 1A and B) or against the terminal methoxy moiety of PEG (15-2b, Fig. 1C and D). The values of OD closest to EC50 were compared statistically using *t*-test with Welch's correction (Fig. 1E). The half-maximum effective concentration (EC_{50}) represents the concentration of liposomes that generates 50% of the maximum signal on the standard curve. The means absorbance values (405 nm) of 5 repeated measures are shown ± SD. * $P < .01$.

doxorubicin were quantified in the plasma of mice injected once a week for 2 consecutive weeks with either Dox-NP alone ("Dox-NP" group), or with Doxebo as pre-treatment followed 2 h later by Dox-NP injection ("Doxebo + Dox-NP" group). As in the tumor models, Doxebo was injected at 1/10th of the Dox-NP dose, which is the dose that effectively provided tachyphylaxis in pigs.

Fig. 5 shows the blood clearance of doxorubicin in mice after the administration of Dox-NP with or without Doxebo pre-treatment for repeated injections. As expected from the stealth character of Dox-NP which is identical to Doxil, the clearance rate of doxorubicin was slow with > 30% of the injected doxorubicin still remaining in the blood after 48 h, and with no significant differences among the groups on the first week of the experiment. On the second week, both groups had a significant increase in the plasma levels of doxorubicin (seen as an increase in the Area Under the Curve - AUC), which is in line with the very long circulation time of Dox-NP in the plasma and its accumulation upon the second injection (for Dox-NP treatment $P < .05$ and for Doxebo + Dox-NP $P < .01$ between week 1 and 2). However, and contrary to what happens usually with the ABC phenomenon, the mice injected with Doxebo had a slightly higher AUC (slower clearance) compared to the mice without pre-treatment. The increase is not statistically significant between the two treatments for each week (when comparing the AUC) but this trend is repeated at each injection and is more pronounced on the second week. These results are in accordance

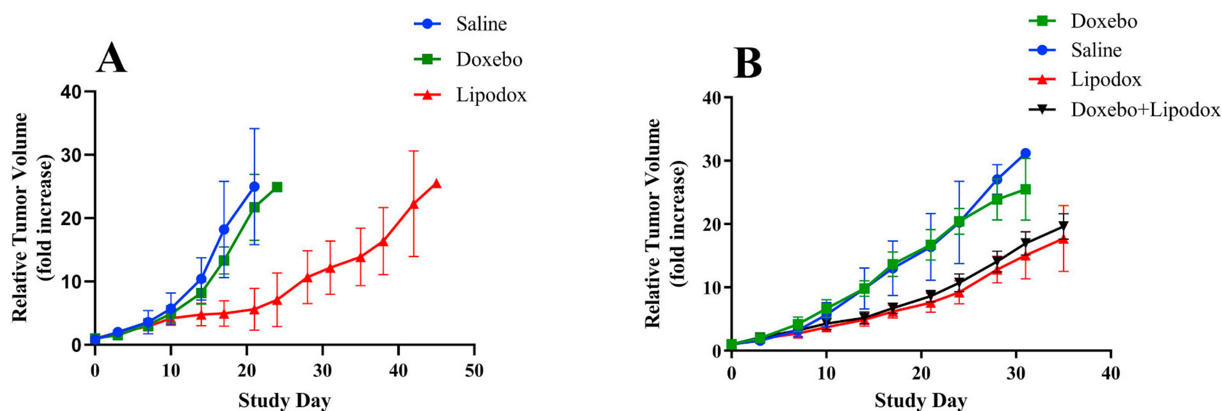

Fig. 2. Lack of effect of Doxebo alone or in combination with Lipodox on tumor growth in mice models of lymphoblastic leukemia and breast cancer. Mice received subcutaneous injections of CCRF-CEM (A) or MDA-MB-231 cells (B) to induce lymphoblastic leukemia and breast cancer, respectively. After tumors reached sufficient size, mice were treated I.V. once a week for 3 weeks. Fig. 2A: the mice were injected with either saline at 5 ml/kg, Doxebo at 5 ml/kg or Lipodox at 8 mg/kg dox (equivalent to 4 ml/kg). Fig. 2B: the mice were administered with either saline or Doxebo at 5 ml/kg, Lipodox at 8 mg/kg dox or with an injection of Doxebo (at 5 ml/kg) followed 1 h later by administration of Lipodox at 8 mg/kg dox. The PL contents of Doxebo and Lipodox were 65 and 52 mg/kg, respectively. Tumor volumes were calculated from tumors measurements for each mouse twice a week and the relative tumor volume (RTV) was calculated from initial mean tumor volume in each group. RTV is represented in fold-increase.

with previous studies [32] which show that the injection of PLD causes a partial blockade of the RES. Since the RES is responsible for the majority of liposomes clearance, this results in a reduction of liver uptake and a prolongation of liposome circulation time.

3.6. The macrophage clearance activity in the mice pre-treated with Doxebo is unchanged

The PEGylation of nanoparticles and biomaterials reduces dramatically their uptake by the mononuclear phagocyte system (MPS), increasing their circulation time in the blood compared to non-PEGylated formulations [33–35]. Macrophages play a key role in the innate immune system, since they are in the first line of the body defense with their capacity to identify and clear foreign material in the blood within a matter of minutes. Biodistribution studies showed that after injection in the bloodstream, nanoparticles accumulate extensively in the liver [36]. The residents macrophages of the liver, the Kuppfer cells, have been thought to be mostly responsible for nanoparticles uptake, but recent studies demonstrate that other cells are also involved in this process [37–39]. In addition it was recently proposed that liposome-induced immunosuppression and tumor growth is mediated by macrophage activity in mice [40].

The slight difference we observed in the PK experiment between the plasma doxorubicin values of mice injected with Dox-NP only or with the Doxebo pre-treatment may also be due to a difference in macrophage clearance efficacy in the mice. For these two reasons we performed a macrophage clearance test 24 h after the liposomal doxorubicin injection on the second week of treatment. This tests consists of injecting to the mice non-PEGylated, negatively charged ~250 nm liposomes labelled with Lissamine-Rhodamine attached to the amino group of the lipid phosphatidylethanolamine (LRPE).

The LRPE is part of the liposomal membrane, and because the fluorophore is part of the LRPE headgroup it is retained in the membrane. The dense hydrophilic PEG layer of the liposomes, which act like a steric barrier, prolongs their circulating time by reducing and modifying the proteins corona [41,42], thus decreasing opsonization and slowing uptake by the MPS. On the contrary, negatively charged, non-PEGylated liposomes of varying size (average 200 nm) are quickly uptaken by macrophages. A decrease in the clearance rate of the plasma fluorescence in the plasma in this test indicates impairment of the macrophages activity. We see in our study (Fig. 6) that the rate of decrease in plasma fluorescence is identical for mice injected with Lipodox alone or after the Doxebo pre-treatment (2 h before). This indicates that the macrophages particle-clearance capacity was not affected and that the slight increase in the AUC of Doxebo injected group during the PK experiment (Fig. 5) was not due to a decrease in macrophage activity.

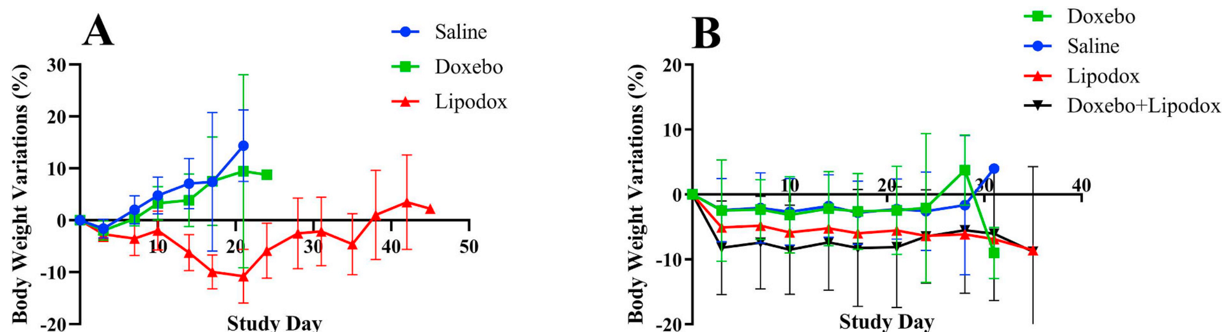

Fig. 3. Lack of intrinsic toxicity of Doxebo in mouse tumor models. Mice received subcutaneous injections of CCRF-CEM (A) or MDA-MB-231 cells (B) to induce lymphoblastic leukemia and breast cancer, respectively. After tumors reached sufficient size, mice were treated I.V. once a week for 3 weeks. The mice were injected with either saline at 5 ml/kg, Doxebo at 5 ml/kg, Lipodox at 8 mg/kg dox (equivalent to 4 ml/kg) or (Fig. 3B) with an injection of Doxebo (at 5 ml/kg) followed 1 h later by administration of Lipodox at 8 mg/kg dox. Results are expressed as mean ± SD.

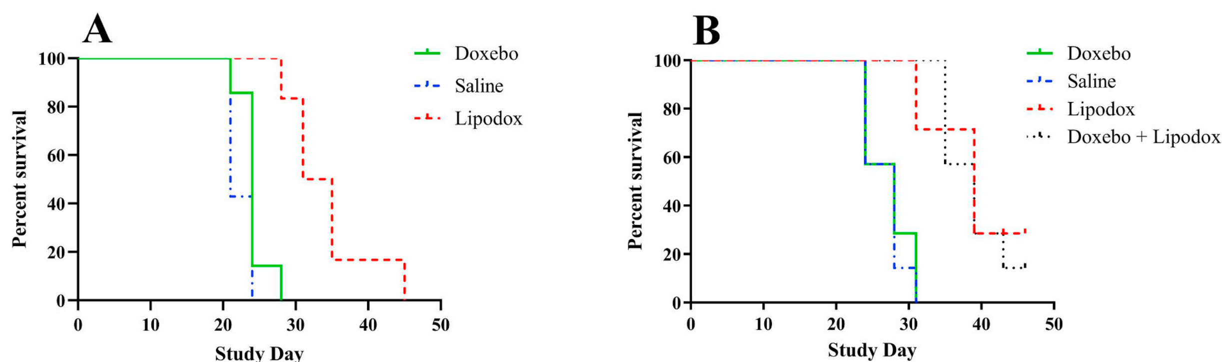

Fig. 4. Doxebo showed no effect on the survival of mice bearing tumors derived from CCRF-CEM line (lymphoblastic leukemia) (A) and MDA-MB-231 line (breast cancer) (B). After the respective tumors reached sufficient size, mice were injected I.V. once a week for 3 weeks with either saline at 5 ml/kg, Doxebo at 5 ml/kg, Lipodox at 8 mg/kg dox (equivalent to 4 ml/kg) or (Fig. 4B) with an injection of Doxebo (at 5 ml/kg) followed 1 h later by administration of Lipodox at 8 mg/kg dox. Survival curves were generated according to Kaplan-Maier estimate.

3.7. Toxicology study in rats

To further evaluate the potential toxic effects of Doxebo, a cohort of 20 Sprague-Dawley rats (10 males and 10 females) received repeated injections of Doxebo (6 injections total) at 2 ml/kg IV (equivalent to 25 mg/kg PL). The rats were sacrificed at the end of the experiment and their blood and organs were collected. Hematology (complete blood count) as well as clinical chemistry and coagulation parameters were analyzed from the blood collected at the time of sacrifice, and the organs were weighed to calculate the ratio organ/body weight and to compare it to historical reference data from the same rats and category of age. The results are detailed in the supplementary data (Table S1-S5) but the most common blood tests (Table 1) and organ/body weight (BW) ratio (Table 2) are summarized below.

The data summarized in Tables 1 and 2 as well as the supplementary Tables S1–S5 detailing the results of the blood and urine analysis show that all the parameters (from hematology and blood chemistry analysis as well as the organ weight ratios and urine analysis) are within the normal limits of values both for males and females.

Gross pathology examination (macroscopic observations) was performed on the rats at the time of their sacrifice and did not reveal any abnormalities. Only one occurrence of a minimal hepatodiaphragmatic nodule, a developmental anomaly that can occur sporadically in rats, was observed in one female. The microscopic observations revealed few occurence of benign cardiomyopathy-related findings (minimal myofiber degeneration/necrosis and/or minimal mononuclear cell infiltration), but since cardiomyopathy is a common background findings in SD rats [43], they don't have any significance in term of toxicity.

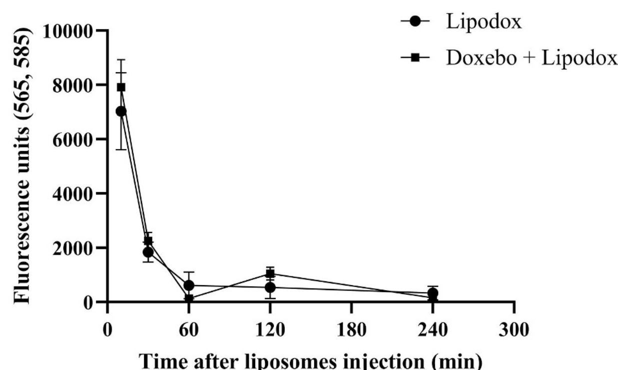

Fig. 6. Mice were injected I.V. once a week for 2 consecutive weeks with either Lipodox at 10 ml/kg ("Lipodox"), or with a combination of Doxebo at 0.5 ml/kg followed 2 h later by Lipodox at 10 ml/kg ("Doxebo + Lipodox"). Twenty-four hours after the Lipodox injection, the mice were injected with liposomes labelled with LRPE. At different time-points after the LRPE-liposomes injection, blood samples were collected and LRPE fluorescence was quantified from the plasma. The data represent the mean ± S.D. of triplicate aliquots from four or five mice.

4. Discussion

The use of Doxebo to provide tachyphylaxis to prevent

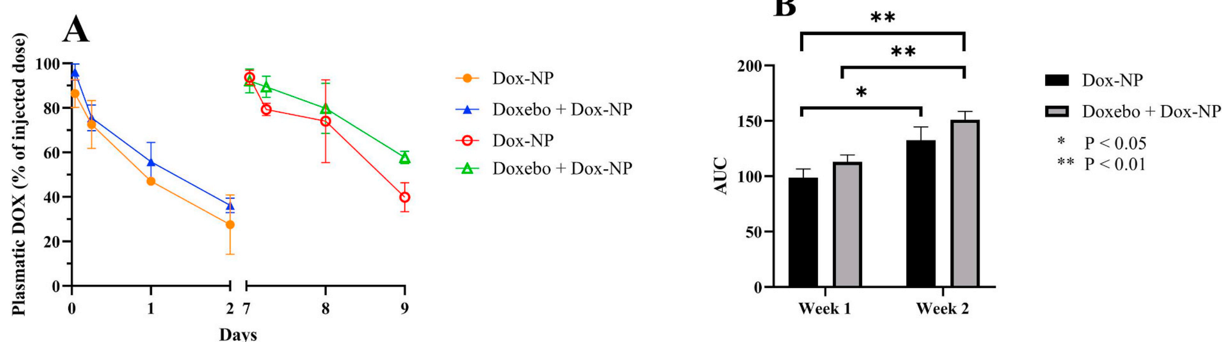

Fig. 5. Doxorubicin plasma levels following single or repeated injection of Dox-NP with or without pre-treatment with Doxebo. A: BALB/c mice were injected I.V. once a week with Dox-NP at 10 ml/kg ("Dox-NP") either for one week (single injection, black circles) or for 2 consecutive weeks (empty circles). Half of the mice (labelled "Doxebo + Dox-NP") received 2 h before each Dox-NP injection a bolus of Doxebo at 0.5 ml/kg (6.7 mg PL): black triangles represent the mice who were sacrificed after the first treatment and empty triangles the mice who received two treatments. Mice (n = 3 mice from each group for each time-point) were sacrificed at the indicated time-point after the Dox-NP injection and the level of doxorubicin in their plasma was quantified. B: Comparison of the AUC of the curves from A for statistical comparison (t-test with Welch's correction). Values are mean ± SD.

Table 1
Summary of Hematology and blood chemistry after repeated Doxebo injection to SD rats.

Test (Unit)	Males (*n* = 10)		Females (n = 10)	
	Mean (± SD)	Normal range*	Mean (± SD)	Normal range*
Erythrocytes,10^6/µl	8.54 (± 0.52)	7.32–9.37	7.75 (± 0.38)	7.17–9.35
Hematocrit, %	52.35 (± 3.23)	45.2–59.7	47.90 (± 2.33)	43.3–57.6
Hemoglobin, g/dl	16.00 (± 0.91)	14.3–17.9	14.89 (± 0.64)	13.8–17.6
Leukocytes, 10^3/µl	13.50 (± 2.78)	7.8–19.4	10.90 (± 3.00)	6.1–17.1
Neutrophils, 10^3/µl	2.28 (± 0.98)	0.71–2.63	1.30 (± 0.60)	0.41–2.08
Lymphocytes, 10^3/µl	10.53 (± 2.04)	6.37–17.22	9.07 (± 2.59)	4.89–14.32
Monocytes, 10^3/µl	0.35 (± 0.14)	0.11–0.61	0.22 (± 0.11)	0.09–0.45
Eosinophils, 10^3/µl	0.09 (± 0.03)	0.05–0.19	0.13 (± 0.05)	0.03–0.17
Basophils, 10^3/µl	0.07 (± 0.02)	0.03–0.15	0.05 (± 0.01)	0.03–0.11
Platelets, 10^3/µl	1101.90 (± 132.34)	791–1401	1090.20 (± 107.65)	670–1471
Triglyceride, mg/dl	66.8 (± 22.5)	28–136	46.6 (± 11.2)	25–79
Cholesterol, mg/dl	73.3 (± 13.9)	39–96	78.2 (± 11.2)	48–104
Alkaline Phosphatase l(U/L)	138.1 (± 27.8)	156 (91–269)	81.9 (± 12.7)	92 (51–174)
Total Bilirubin (mg/dl)	0.11 (± 0.03)	0.1 (0.1–0.2)	0.13 (± 0.05)	0.1 (0.1–0.2)
AST (U/l)	69.0 (± 5.9)	67 (52–94)	65.7 (± 4.2)	63 (49–96)
ALT (U/l)	30.2 (± 6.0)	31 (21–48)	25.1 (± 4.4)	27 (18–48)
Albumin (g/dl)	3.44 (± 0.2)	3.4 (3.1–3.7)	3.75 (± 0.2)	3.8 (3.4–4.4)
Total Protein (g/dl)	6.76 (± 0.4)	6.4 (5.9–7.1)	7.12 (± 0.4)	7.1 (6.2–8.1)
Urea Nitrogen (mg/dl)	16.4 (± 2.0)	14 (10 – 20)	15.3 (± 2.9)	15 (11 – 22)
Creatinine (mg/dl)	0.42 (± 0.04)	0.4 (0.3–0.5)	0.40 (± 0.05)	0.4 (0.3–0.5)
Triglyceride, mg/dl	66.8 (± 22.5)	57 (28–136)	46.6 (± 11.2)	41 (25–79)
Cholesterol, mg/dl	73.3 (± 13.9)	62 (39–96)	78.2 (± 11.2)	73 (48–104)
Glucose (mg/dl)	298.3 (± 56.0)	263 (147–454)	266.8 (± 76.5)	220 (103–371)

ALT - Alanine Amino-transferase, AST - Aspartate Amino-transferase, MCH - Mean Corpuscular Hemoglobin, MCHC - Mean Corpuscular Hemoglobin Concentration, MCV - Mean Corpuscular Volume.

* Data from Historical data of MPI Research Inc., for 10–17 week old rats.

hypersensitive reactions has already been tested in a pig model of CARPA [18]. The reason for developing a pre-treatment based on empty liposomes, instead of simply injecting a low dose of Doxil, is that the presence of doxorubicin in the liposomes significantly increases their reactogenicity both *in vitro* (in human serum) and *in vivo* (in pigs) [4]. In fact, a low dose of Doxil (0.06 mg/kg PL) in pigs is followed by strong CARPA reaction, while the same dose of Doxebo does not trigger immune reaction, or much weaker [18]. For these reasons we believe that the use of Doxebo only can circumvent adverse effects in patients.

But the effect of Doxebo on the efficacy and pharmacokinetics of PLD have never been studied before. In addition, it is also the first time that its toxicity (besides the immunogenic effect) and side effects upon single or repeated injections are evaluated. Our studies in two different tumor models in mice clearly showed that Doxebo did not affect the tumor growth, with results similar to saline. These studies were conducted in immunocompromised athymic Foxn-1 nu/nu (nude) mice since they are by far the most common and best characterized mice model to assess the efficacy of anticancer drugs. These mice lack a functional thymus, which results in a greatly reduced number of T-cells, but they compensate with an increased activity of macrophages and natural killer (NK) cells [44–46]. Lately there has been a controversy around the use of immunocompromised mice models when testing PEGylated liposomal nanoparticles. Some studies pointed toward an immunosuppressive effect of PEGylated liposomes, mediated through macrophages, that could override the antitumor efficacy of the drug payload, resulting in enhanced tumor growth [40,47]. In spite of this controversial issue, we decided to use human cancer models in nude mice because immunocompetent mice models are not that well established. However, to make sure we do not affect macrophages activity, we tested the effect of Doxebo on macrophage clearance activity and we found that this activity was unaltered by Doxebo.

Repeated injections of Doxebo also did not produce any signs of toxicity despite the relatively high dose. All macroscopic and microscopic observations of the rats who received 6 injections of Doxebo were within the normal range, including hematology parameters and organ weight. In addition, the total dose of PL injected (52 mg/kg/week or 312 mg/kg total) did not cause a noticeable increase in the plasma values of triglycerides and cholesterol, that were still in the average values accepted for animal of similar strain and age (see Table 1).

In addition to IR, another possible consequence of the injection of PEGylated liposomes in the bloodstream is the ABC phenomenon, characterized by accelerated clearance of the Doxil liposomes from the plasma upon repeated injections. The protective properties of the PEG corona on the surface of liposomes have been extensively demonstrated

Table 2
Summary of body weight and organ weight ratio from SD rats after repeated Doxebo injection.

Endpoint	Males (n = 10)		Females (n = 10)	
	Mean (± SD)	Normal range**	Mean (± SD)	Normal range**
Body weight (g)	472 (± 22)	397–729	265 (± 13)	193–393
Liver/BW (%)	3.24 (± 0.23)	2.36–3.82	3.32 (± 0.26)	2.30–4.03
Heart/BW (%)	0.36 (± 0.03)	0.26–0.44	0.38 (± 0.03)	0.28–0.52
Kidneys/BW (%)	0.72 (± 0.08)	0.52–0.96	0.75 (± 0.04)	0.53–0.94
Spleen/BW (%)	0.19 (± 0.03)	0.11–0.31	0.22 (± 0.02)	0.13–0.27
Brain /BW (%)	0.43 (± 0.03)	0.29–0.53	0.72 (± 0.03)	0.50–0.91
Gonads/BW (%)	0.73 (± 0.05)	0.46–0.95	0.04 (± 0.01)	0.01–0.07

** Organ weight ratio from Historical data of MPI for SD Rats in 13-week studies.

[48] and used for the development of Doxil to extend its plasma half-life by evading the fast recognition and phagocytosis by the mononuclear phagocyte system. But it has been shown that when PEGylated liposomes are repeatedly injected at an interval varying from 5 days to 3 weeks, the second dose loses its long-circulating properties [49] and tends to accumulate in the liver and the spleen [49,50]. The ABC phenomenon is extensively documented in rats [49,51–54], but also in other animals (dogs [55,56], rhesus monkeys [49], rabbits and guinea pigs [57]), and especially in mice injected at similar PL concentrations (5 μmol/kg) [50,58–60]. It was shown that the occurrence and intensity of ABC depend on several characteristics of the liposomes of the first (induction) injection, including the size of the nanoparticle, lipid composition and total phospholipid dose injected. According to several studies, the intensity of ABC seems to have an inverse relationship with the lipid dose during the first injection of liposomes [52,61]: the higher the lipid dose, the lower the resulting ABC phenomenon at subsequent injections. In our PK experiment in mice, the dose of Doxebo used for the first injection (0.5 ml/kg, equivalent to 6.8 μmol/kg) was calculated based on the dose used previously to provide tachyphylaxis effects in the pig model of CARPA [18] (*i.e.* a tenth of the dose of Doxil Human Equivalent Dose –HED— the dose that was administered to the pigs). Empty liposomes of similar size and composition to Doxebo elicited ABC in rats after a week when first injected at a dose of 5 μmol/kg [54], which is comparable to the dose we injected. However, the injection of the same liposomes with encapsulated doxorubicin (Doxil), at similar doses did not elicit ABC [54,61]. Only when the dose of Doxil was much lower (0.2 μmol/kg PL for rats or 0.4 μmol/kg PL in mice) did the animals develop ABC [55,62].

It is now well-accepted that the ABC phenomenon is triggered by the presence of anti-PEG antibodies (IgM as well as IgG to a smaller extent), either pre-existing or elicited following the first liposomes injection [58,63], and that the injection of Doxil prevents ABC. One hypothesis is that ABC is prevented because Doxil kills the B cells responsible for the synthesis of anti-PEG antibodies [61,64]. But pre-existing anti-PEG antibodies have also been found in the serum of naïve individuals [14,65], *i.e.* prior to any treatment with PEGylated drugs, but also in the serum of naïve pigs (Kozma et al., in review). The presence of such antibodies decreases the efficacy of the PEGylated drugs and increases the risk for IR [13,56,66]. Interestingly, no ABC has been reported in patients treated with PLD (either Doxil, the European Caelyx or Lipodox). But results from previous experiments on ABC showed that at this dose of PL, the injection of empty liposomes similar to Doxebo caused ABC in mice 5 days afterward [58], so we wondered if in our study the combination of Doxebo with Lipodox would have an impact on the pharmacokinetics profile on the second Lipodox injection. In our case, the injection of Lipodox 2 h after Doxebo did not result in a fast clearance of Doxil the following week, and it even seemed to slightly increase the plasma AUC of Lipodox. We showed that this increase in the AUC is not due to a change in the macrophages activity, and this result could be explained in 2 ways. First, because the macrophages are not the only cells responsible for PEGylated liposomes clearance. It is known that other cells also play a key role in their clearance, namely endothelial cells lining the sinusoids of the liver and the spleen. But another possible explanation for this difference in AUC could be the presence of pre-existing anti-PEG antibodies. Several studies demonstrate the presence of pre-existing antibodies in different species: in humans, but also in pigs as mentioned previously. The source of the pre-existing anti-PEG antibodies in the latter is probably food, which contains increasing amount of PEGylated components (PEG is used as a binding agent and lubricant for dry tabs in the food industry). Therefore, it is possible that mice also have pre-existing anti-PEG antibodies; however, to the best of our knowledge no one checked for their presence in rodents.

In conclusion, we demonstrated in this study that pre-treatment with Doxebo at a dose efficient for tachyphylaxis, followed by the regular dose of Doxil not only was safe and did not alter the therapeutic efficacy of Doxil, but it also did not elicit the ABC phenomenon. It is therefore a further important step toward the evaluation of Doxebo as a mean to avoid IR before treatment with PLD.

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Declaration of competing interests

Yechezkel Barenholz and Janos Szebeni are co-inventors on US Patent 9,078,812 B2, July 13, 2015 titled: “Particular drug carriers as desensitizing agents”, owned by Yissum, the technology transfer office of the Hebrew University of Jerusalem, and Semmelweis University, Budapest, that was not licensed.

The other authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2019.06.007.

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Supplementary Data

Endpoint	Males		Females	
	Mean (± SD) *n = 10*	Normal values*, Median (Range)	Mean (± SD) *n = 10*	Normal values*, Median (Range)
Alkaline Phosphatase (U/l)	138.1 (± 27.8)	156 (91 - 269)	81.9 (± 12.7)	92 (51 - 174)
Total Bilirubin (mg/dl)	0.11 (± 0.03)	0.1 (0.1 - 0.2)	0.13 (± 0.05)	0.1 (0.1 - 0.2)
Aspartate Amino-transferase (AST) (U/l)	69.0 (± 5.9)	67 (52 - 94)	65.7 (± 4.2)	63 (49 - 96)
Alanine Amino-transferase (ALT) (U/l)	30.2 (± 6.0)	31 (21 - 48)	25.1 (± 4.4)	27 (18 - 48)
Albumin (g/dl)	3.44 (± 0.2)	3.4 (3.1 - 3.7)	3.75 (± 0.2)	3.8 (3.4 - 4.4)
Total Protein (g/dl)	6.76 (± 0.4)	6.4 (5.9 - 7.1)	7.12 (± 0.4)	7.1 (6.2 - 8.1)
Urea Nitrogen (mg/dl)	16.4 (± 2.0)	14 (10 - 20)	15.3 (± 2.9)	15 (11 - 22)
Creatinine (mg/dl)	0.42 (± 0.04)	0.4 (0.3 - 0.5)	0.40 (± 0.05)	0.4 (0.3 - 0.5)
Triglyceride, mg/dl	66.8 (± 22.5)	57 (28 - 136)	46.6 (± 11.2)	41 (25 - 79)
Cholesterol, mg/dl	73.3 (± 13.9)	62 (39 - 96)	78.2 (± 11.2)	73 (48 - 104)
Glucose (mg/dl)	298.3 (± 56.0)	263 (147 - 454)	266.8 (± 76.5)	220 (103 - 371)
Sodium (mEq/l)	143.6 (± 1.5)	144 (140 - 148)	142.0 (± 1.8)	142 (138 - 145)
Potassium (mEq/l)	8.36 (± 1.50)	8.7 (5.7 - 11.3)	8.28 (± 1.40)	9.2 (6.0 – 12.7)
Chloride (mEq/l)	100.0 (± 1.3)	99 (96 - 103)	100.5 (± 1.3)	100 (97 - 103)
Calcium (mEq/l)	12.40 (± 0.54)	12.4 (11.2 - 13.7)	12.19 (± 0.44)	12.5 (11.3 - 13.8)
Phosphorus (mEq/l)	11.24 (± 0.14)	12.2 (9.5 - 14.4)	9.86 (± 1.10)	11.4 (8.8 - 14.1)
Globulin (g/dl)	3.32 (± 0.27)	3.1 (2.6 - 3.6)	3.37 (± 0.26)	3.3 (2.7 - 3.8)
Albumin/Globulin Ratio	1.04 (± 0.05)	1.1 (0.9 - 1.3)	1.12 (± 0.09)	1.2 (1.0 - 1.4)

* Data from Historical data of MPI Research Inc, for 10-17 week old rats

Table S1: Summary of Clinical Chemistry values after repeated Doxebo injection to SD Rats. The values obtained are expressed as Mean (± SD) while the historical data are a range of 95% central values (n ≥ 350 for males and n ≥ 367 for females).

	Males		Females	
Test (Unit)	Mean (± SD) *n = 10*	Normal values*, Median (Range)	Mean (± SD) *n = 10*	Normal values*, Median (Range)
Leukocytes, 10^3/µl	13.50 (± 2.78)	12.7 (7.8 - 19.4)	10.90 (± 3.00)	9.8 (6.1 - 17.1)
Erythrocytes,10^6/µl	8.54 (± 0.52)	8.44 (7.32 - 9.37)	7.75 (± 0.38)	8.18 (7.17 - 9.35)
Hemoglobin, g/dl	16.00 (± 0.91)	16.2 (14.3 - 17.9)	14.89 (± 0.64)	15.8 (13.8 - 17.6)
Hematocrit, %	52.35 (± 3.23)	52.5 (45.2 - 59.7)	47.90 (± 2.33)	49.9 (43.3 - 57.6)
MCV, fL	61.30 (± 1.44)	62.4 (56.9 - 69.3)	61.87 (± 2.12)	61.1 (56.5 - 66.7)
MCH, pg	18.76 (± 0.24)	19.2 (17.5 - 20.8)	19.23 (± 0.45)	19.2 (17.8 - 20.8)
MCHC, g/dl	30.61 (± 0.41)	30.8 (28.7 - 32.5)	31.09 (± 0.53)	31.5 (29.4 - 33.9)
Platelets, 10^3/µl	1101.90 (± 132.34)	1071 (791 - 1401)	1090.20 (± 107.65)	1048 (670 - 1471)
Absolute Reticulocytes 10^3/µl	203.70 (± 21.06)	195.9 (118.5 - 308.5)	209.38 (± 28.36)	176.6 (102.4 -327.4)
Neutrophils, 10^3/µl	2.28 (± 0.98)	1.34 (0.71 - 2.63)	1.30 (± 0.60)	0.92 (0.41 - 2.08)
Lymphocytes, 10^3/µl	10.53 (± 2.04)	10.71 (6.37 - 17.22)	9.07 (± 2.59)	8.45 (4.89 - 14.32)
Monocytes, 10^3/µl	0.35 (± 0.14)	0.28 (0.11 - 0.61)	0.22 (± 0.11)	0.21 (0.09 - 45)
Eosinophils, 10^3/µl	0.09 (± 0.03)	0.10 (0.05 - 0.19)	0.13 (± 0.05)	0.08 (0.03 - 0.17)
Basophils, 10^3/µl	0.07 (± 0.02)	0.08 (0.03 - 0.15)	0.05 (± 0.01)	0.06 (0.03 - 0.11)
Other Cells 10^3/µl	0.150 (± 0.084)	0.14 (0.05 - 0.33)	0.134 (± 0.053)	0.11 (0.05 - 0.31)

* Data from Historical data of MPI Research Inc, for 10-17 week old rats

Table S2: Summary of Hematology data after repeated Doxebo injection to SD Rats. The values obtained are expressed as Mean (± SD) while the historical data are a range of 95% central values (n ≥ 347 for males and n ≥ 367 for females). MCH - Mean Corpuscular Hemoglobin, MCHC - Mean Corpuscular Hemoglobin Concentration, MCV - Mean Corpuscular Volume.

Endpoint	Males		Females	
	Mean (± SD) *n = 10*	Normal values*, Median (Range)	Mean (± SD) *n = 10*	Normal values*, Median (Range)
APTT - (sec)	17.93 (± 1.16)	17.0 (11.8 - 21.4)	14.11 (± 2.55)	15.6 (10.7 - 19.6)
Prothrombin time (sec)	15.71 (± 0.35)	15.6 (14.5 – 17.0)	15.33 (± 0.68)	15.4 (14.1 – 17.0)
Fibrinogen (mg/dl)	334.3 (± 22.9)	327 (227 - 402)	275.0 (± 23.6)	268 (222 - 329)

* Data from Historical data of MPI Research Inc, for 10-17 week old rats

Table S3: Summary of Blood Coagulation Values after repeated Doxebo injection to SD Rats. The values obtained are expressed as Mean (± SD) while the historical data are a range of 95% central values (n ≥ 343 for males and n ≥ 348 for females). APTT - Activated Partial Thromboplastin Time (sec)

	Males		Females	
Endpoint	Mean (± SD) *n = 10*	Normal values*, Median (Range)	Mean (± SD) *n = 10*	Normal values*, Median (Range)
Volume	8.85 (± 5.88)	7.0 (0.5 - 23.0)	7.80 (± 2.87)	3.5 (0.2 – 15.0)
Specific gravity	1.0522 (± 0.0174)	1.050 (1.015 - 1.099)	1.0373 (± 0.0113)	1.051 (1.019 - 1.099)
pH	7.95 (± 0.55)	7.5 (6.0 - 8.5)	7.55 (± 0.60)	7.0 (6.0 - 8.5)

* Data from Historical data of MPI Research Inc, for 10-17 week old rats

Table S4: Summary of urine analysis values after repeated Doxebo injection to SD Rats. The values obtained are expressed as Mean (± SD) while the historical data are a range of 95% central values ($n \geq 340$ for males and $n \geq 316$ for females).

	Males		Females	
Endpoint	Mean (± SD), n=10	Normal values*, Mean (Range)	Mean (± SD) n=10	Normal values*, Mean (Range)
Body Weight (BW), g	472 (± 22)	533.3 (397 -729)	265 (± 13)	291.6 (193 - 393)
Brain/BW, %	0.4348 (± 0.0322)	0.4058 (0.2866 -0.5349)	0.7186 (± 0.0259)	0.6822 (0.4987 - 0.9088)
Adrenal gland/BW, %	0.0150 (± 0.0030)	0.0128 (0.0047 - 0.0489)	0.0303 (± 0.0061)	0.0253 (0.0121 - 0.0415)
Epididymes/BW, %	0.2662 (± 0.0236)	0.2748 (0.2034 - 0.3863)	N/A	N/A
Heart/BW, %	0.3635 (± 0.0261)	0.3290 (0.2613 - 0.4368)	0.3795 (± 0.0263)	0.3739 (0.2806 0.52)
Kidneys/BW, %	0.7162 (± 0.0785)	0.7210 (0.5228 - 0.9654)	0.74745 (± 0.0396)	0.7269 (0.5321 0.9447)
Liver/BW, %	3.2373 (± 0.2311)	2.9702 (2.3601 - 3.8205)	3.3176 (± 0.2598)	2.9879 (2.3003 - 4.0330)
Ovaries/BW, %	N/A	N/A	0.0359 (± 0.0042)	0.0318 (0.0136 -0.0664)
Pituitary gland/BW, %	0.0034 (± 0.0005)	0.0030 (0.0022 - 0.0068)	0.0072 (± 0.0010)	0.0073 (0.0033 - 0.0117)
Prostate gland/BW, %	0.2778 (± 0.0381)	0.3074 (0.1474 - 0.4712)	N/A	N/A
Spleen/BW, %	0.1858 (± 0.0316)	0.1562 (0.1055 - 0.3115)	0.2226 (± 0.0207)	0.1912 (0.1343 - 0.2713)
Testes/BW, %	0.7340 (± 0.0504)	0.7070 (0.4581 - 0.9467)	N/A	N/A
Thymus gland/BW, %	0.1015 (± 0.0099)	0.0661 (0.0290 - 0.1347)	0.1788 (± 0.0405)	0.1017 (0.0530 - 0.2473)
Thyroid/parathyroid gland/BW, %	0.0052 (± 0.0012)	0.0055 (0.0030 - 0.0081)	0.0073 (± 0.0017)	0.0080 (0.0048 - 0.0125)
Uterus with cervix/BW, %	N/A	N/A	0.2226 (± 0.0556)	0.2467 (0.1336 - 0.5606)

* Data from Historical data of MPI Research Inc, for 10-17 week old rats

Table S5: Organ weight values relative to Body Weight from SD rats after repeated Doxebo injection. The values obtained during the study are expressed as Mean (± SD) while the historical data are a range of 95% central values (n ≥ 264 for males and n ≥ 258

for females except for pituitary gland, thyroid/parathyroid where n=198 for males and n=209 for females, epididymes n=148, testes n= 264, ovaries n=208 and uterus n=258).