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SHORT COMMUNICATION

Pharmacokinetics, efficacy and toxicity of different pegylated liposomal doxorubicin formulations in preclinical models: is a conventional bioequivalence approach sufficient to ensure therapeutic equivalence of pegylated liposomal doxorubicin products?

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Abstract
Purpose To examine whether a conventional bioequivalence approach is sufficient to ensure the therapeutic equivalence of liposomal products, the pharmacokinetics, efficacy and toxicity of different formulation variants of the marketed Doxil®/Caelyx® product, pegylated liposomal doxorubicin (PLD), were evaluated in several preclinical models.
Methods Six different variants of the marketed PLD formulation were prepared by incorporating minor changes in the composition and liposome size of the original formulation. The pharmacokinetics of 5 formulations were evaluated in albino mice following i.v. administration at 6 mg/kg. Selected variants along with Doxil®/Caelyx® (formulation 1, Doxil-control) were tested for antitumor activity in the MDA-MB-231 xenograft mouse model following 3 repeated administrations at 2 mg/kg or 3 mg/kg (once weekly for 3 weeks) and/or toxicity in Cynomolgus monkeys following 6 repeated administrations at 2.5 or 4.0 mg/kg. Formulations 1–4 were tested for antitumor activity and formulations 1, 2, 6 and 7 were evaluated in a monkey toxicity study. The toxicokinetics of total doxorubicin was determined after the first and last dose in the monkey toxicity study.
Results In the albino mouse, formulations 2 and 3 had plasma pharmacokinetic profiles similar to Doxil-control (formulation 1). Although these three formulations had similar pharmacokinetic profiles, formulation 2 showed significantly ($P < 0.05$) longer survival time and better efficacy (reduced tumor volume) over other formulations tested for antitumor activity at the 3 mg/kg dose. In monkeys, formulation 2 gave systemic exposure of doxorubicin approximately the same as formulation 1; however, multifocal degeneration of renal cortical tubules and hypocellularity of the bone marrow were observed with formulation 2 but not with formulation 1 (Doxil-control). Formulations 6 and 7 gave lower exposure to doxorubicin compared to Doxil-control, but were associated with higher severity and frequency of toxic effects (hematological effects, elevated liver enzymes). It was concluded that plasma pharmacokinetics and systemic exposure of doxorubicin did not correlate well with the antitumor activity and toxicity profiles for PLD products. Hence, a conventional bioequivalence approach is not appropriate for establishing therapeutic equivalence of generic PLD products. A carefully designed clinical study evaluating clinical safety, efficacy and pharmacokinetics should be considered for establishing the therapeutic equivalency of generic versions of Doxil®.

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Keywords Pegylated liposomal doxorubicin (PLD) · Bioequivalence · Generics · Pharmacokinetics · Antitumor activity · Toxicity

Introduction

Doxorubicin is widely used chemotherapeutic agent in the treatment of a broad range of cancers. The clinical usefulness of doxorubicin is limited due to cumulative, dose-related, progressive myocardial damage, which may lead to congestive heart failure (CHF) and death. The estimated percentage of doxorubicin patients who develop CHF at a cumulative dose of 400 mg/m^2 is 5%, increasing to as much as 16% at 500 mg/m^2 and as high as 48% at 700 mg/m^2 [1]. Additionally, there are acute adverse reactions associated with doxorubicin administration including nausea, vomiting and bone marrow depression, which may also limit the dose of doxorubicin that can be administered [2].

In general, liposome-encapsulated doxorubicin formulations offer distinct advantages over conventional doxorubicin in reducing the cardiac toxicity and increasing the tolerability and efficacy. The nature of the liposomal encapsulation has a significant effect on liposomal circulation time in the blood stream, biodistribution, release of free drug, overall efficacy and safety. The toxicity profiles of marketed pegylated liposomal doxorubicin (PLD) (Doxil®/Caelyx®), liposomal doxorubicin (Myocet®) and conventional doxorubicin (free form) have been reviewed [3]. Doxil® and Caelyx® are two trade names of the same PLD formulation and will be referred as Doxil throughout the article. The most significant advantage of PLD over conventional (non-pegylated) liposomal product is its much longer circulation half-life, which results in greater uptake by tumor tissue by allowing repeated passages through blood vessels that feed the tumors [4]. Due to the leaky vasculature of tumor vessels, PLD preferentially distributes to tumors over normal tissue [5]. The incidence rate of CHF has been shown to be lower with Doxil® in comparison with conventional doxorubicin [6].

PLD consists of mainly unilamellar liposomes with a coating of methoxypolyethylene glycol (mPEG) which is attached to 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) present in the inner and outer surface of the bilayer. Doxorubicin is stably entrapped in the aqueous core of the liposomes using an ammonium sulfate gradient. Modifications in lipid composition, method of drug loading or particle size of PLD could result in significant changes in the pharmacokinetics, tissue distribution, efficacy and toxicity of the product. It has been shown previously that two PLD formulation variants that had the same lipid composition and drug/lipid ratio but different particle size (75 vs. 100 nm) and internal ammonium sulfate concentration had similar plasma pharmacokinetic profiles but different tumor distribution and antitumor efficacy profiles in mouse models [7]. It was noted by Cui et al. that minor changes in formulation would lead to significantly altered behavior of liposomal drugs. Based on his results, he questioned the value of plasma exposure in predicting efficacy and toxicity. In this report, we present efficacy and toxicity data for different PLD products (variant formulations) that have similar pharmacokinetic profiles in animal models. Our data confirm that the conventional method of bioequivalence measuring plasma systemic exposure is not a suitable method for determining whether two PLD products have equivalently safety and efficacy.

Materials and methods

Drug formulations and chemicals

Fully hydrogenated soy phosphatidylcholine (HSPC) was purchased from Lipoid K.G. (Ludwigshafen, Germany), distearoylphosphatidylcholine (DSPC) and 1,2-dibehenoyl-sn-glycero-3-phosphocholine (DBPC) from Avanti Polar Lipids (Alabaster, AL). *N*-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine, sodium salt (mPEG-DSPE) was obtained from Sygena, LTD. (Liestal, Switzerland). α-methoxy-ω-2,3-di(arachidoyloxy)propylcarbamate poly(ethylene oxide) (mPEG-DS) was synthesized in house. Cholesterol was purchased from Croda, Inc. (Edison, NJ). Dehydrated ethanol, USP, was obtained from Quantum Chemical (Cincinnati, OH). Doxorubicin hydrochloride was purchased from Meiji Seika Kaishan Ltd (Tokyo, Japan). Sterile water for injection, USP, was purchased from McGaw, Inc. (Irvine, CA). L-histidine was purchased from Spectrum Chemical (Gardena, CA). Sucrose, NF, was obtained from Pfanstiehl Lab (Waukegan, IL). Ammonium sulfate was supplied by J. T. Baker (Phillipsburgh, NJ), sucrose octasulfate ammonium salt was obtained from Toronto Research Chemicals, Inc. (ON, Canada) and dextran sulfate ammonium salt (DSAS) by Dextran Products Limited (ON, Canada).

The process for making Doxil-control and its formulation variants is as follows: the lipid components were dissolved in ethanol at elevated temperature (60–65°C) and added to a drug loading solution (e.g. ammonium sulfate, dextran sulfate, ammonium salt or sucrose octylsulfate, ammonium salt). Upon hydration, liposomes were formed spontaneously and were subjected to high-pressure extrusion through polycarbonate membranes (Whatman Nuclepore, 0.05, 0.1, and 0.2 μm pore size), resulting in liposomes with a narrow particle size distribution. Following extrusion, the drug loading solution external to the liposomes was replaced with a 10% sucrose solution using diafiltration (Hollow fiber

ultrafiltration cartridges from A/G Technology). Doxorubicin hydrochloride was then added to the liposomal suspension and the mixture was rapidly heated within 10 min to 60–65°C for ~1 h to increase the permeability of the bilayer. Doxorubicin was loaded into the liposomes during this step. An ion-exchange chromatography step was employed to remove non-encapsulated drug if drug encapsulation was less than 90%. The drug-loaded liposomes were then diluted to a target concentration of approximately 2 mg/ml and sterile filtered. In total, 7 formulations (Doxil-control and 6 PLD variant formulations) were prepared, and the products were tested for doxorubicin concentration by HPLC, drug encapsulation efficiency, particle size by dynamic laser light scattering (Coulter N4SD model particle sizer), pH and total phosphorous content. Drug encapsulation efficiency was accomplished using open gel filtration chromatography using disposable excellulose GF-5 desalting columns (Pierce, 102 mm × 8 mm, 2 ml gel volume). Drug concentrations of the free doxorubicin fraction and the liposomal doxorubicin fraction are measured spectrophotometrically at 480 nm. Drug encapsulation is then calculated by dividing the free doxorubicin fraction over the free doxorubicin plus liposomal doxorubicin fraction. The composition of these formulations and their characteristics are presented in Table 1.

Pharmacokinetics in mice

All mice were handled as per Johnson & Johnson Institutional Animal Care and Use Committee (J&J IACUC) guidelines and the study protocol was designed as per J&J IACUC approved guidelines. The pharmacokinetic parameters of doxorubicin were characterized in male mice following a single 6 mg/kg intravenous administration of 5 different liposomal formulations of doxorubicin (Doxil-control and 4 variant formulations). Ninety non-fasted male CD-1 mice (~24 to 34 g at dose administration) procured from Charles River (Portage, MI) were assigned to 5 groups based on their weight (18 mice per formulation, maximum 2 bleeds per mouse, $n = 3$ mice per time point). Formulations 1–5 were diluted using a diluent (10 mM histidine/10% sucrose) to achieve a 1.2 mg/ml concentration (5 ml/kg). Blood samples (~0.25 ml) were collected via retro-orbital sinus (under light anesthesia using CO_2/O_2) puncture into tubes containing EDTA K_2 at predose (0.0) and again at 0.17, 0.5, 1, 4, 8, 24, 30, 48, 72 and

Table 1 The composition of liposomal formulations and its characteristics

Formulation #	Potency (mg/ml)	% encapsulation	Particle Size (nm)	pH	Phosphorus (mg/ml)
Formulation 1 (Doxil-control)	2.09	97	102	6.5	0.42
Lipid composition: HSPC:CHOL:mPEG-DSPE (56.4: 38.3: 5.3); Internal buffer: 250 mM ammonium sulfate; Total lipid: 21.9 mM					
Formulation 2	1.97	97	118	6.6	0.47
Lipid composition: DSPC:CHOL:mPEG-DSPE (56.4: 38.3: 5.3); Internal buffer: DSAS 100 mg/ml; Total lipid: 24.5 mM					
Formulation 3	2.04	97	121	6.5	0.40
Lipid composition: DSPC:CHOL:mPEG-DSPE (56.4: 38.3: 5.3); Internal buffer: Sucrose octasulfate; Total lipid: 20.9 mM					
Formulation 4	1.96	94	125	6.4	0.36
Lipid composition: DBPC:CHOL:mPEG-DS (56.4: 38.3: 5.3); Internal buffer: 250 mM ammonium sulfate; Total lipid: 18.9 mM					
Formulation 5	1.54	89	107	6.5	0.31
Lipid composition: DBPC:CHOL:mPEG-DS (56.4: 38.3: 5.3); Internal buffer: DSAS (100 mg/ml); Total lipid: 16.2 mM					
Formulation 6:	1.90	98	196	6.5	0.40
Lipid composition:; HSPC:CHOL:mPEG-DSPE (52.7: 38.3: 9); Internal buffer: 250 mM ammonium sulfate; Total lipid: 20.3 mM					
Formulation 7	1.94	95	336	6.5	0.71
Lipid composition: HSPC:CHOL:mPEG-DSPE (52.7: 38.3: 9); Internal buffer: 250 mM ammonium sulfate; Total lipid: 26.7 mM					

CHOL cholesterol, *DSAS* dextran sulfate ammonium salt, *HSPC* hydrogenated soy phosphatidyl choline, *DBPC* 1,2-dibehenoyl-sn-glycero-3-phosphocholine, *DSPC* distearoylphosphatidylcholine, *mPEG-DSPE N*-(carboyl-methoxypolyehtylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphatidylethanolamin, sodium salt, *mPEG-DS* α-methoxy-ω-2,3-di(arachidoyloxy)propylcarbamate poly(ethylene oxide), total lipid concentrations were estimated from the total phosphorus values

96 h postdose. Within each formulation group, animals were divided into six subsets (3 mice/subset). The first subset was bled once and the remaining subsets were bled twice. Predose (0.0) samples were collected from the first 3 mice/group. The second set was bled at 0.17 and 24 h, the third set was bled at 0.5 and 30 h, the fourth set was bled at 1 and 48 h, the fifth set was bled at 4 and 72 h and the sixth set was bled at 8 and 96 h postdose. Additionally, a satellite group of 2 mice/dose group was dosed with their respective formulation and considered as satellite animals in the event of accidental death. Plasma was harvested by centrifugation and frozen at −20°C or lower. All samples were processed within 2 h of collection. Plasma was analyzed for total doxorubicin concentrations using a liquid chromatographic–triple quadrupole mass spectrometric (LC-MS/MS) assay procedure with a lower limit of quantification of 100 ng/ml. After spiking daunorubicin (Internal standard (IS), 5 ng) into 50 μl of plasma sample, the analytes were extracted with acetonitrile and separated on a C18 column (Waters Xbridge, 50 × 4.6 mm, 3.5 μm) interfaced with a triple quadrupole tandem mass spectrometer using positive electrospray ionization. Quantification of doxorubicin was conducted by multiple reaction monitoring (MRM) of the transitions of *m/z* 544.2 –> 397.1 for doxorubicin and *m/z* 528.3 –> 321.3 for IS. The assay exhibited a linear range from 100 to 10,000 ng/ml. After successful completion of qualified assay validation, the method was applied in the pharmacokinetic sample analyses.

Antitumor efficacy in human breast (MDA-MB-231) tumor xenografts

Female athymic nu/nu homozygous mice (Harlan Laboratories, Indianapolis, IN), approximately 5–6 weeks old (body weight range: 23–25 g), were used in the study. Animals were maintained in isolator cages on a 12-h light-and-dark cycle. Food and water were available ad libitum. MDA-MB-231 human breast adenocarcinoma cells suspended in Hank's Balanced Salt Solution (HBSS) at 10×10^7cells/ml was injected subcutaneously in 100 μl volumes to yield an inoculum of 10×10^6 cells. The mean tumor size at time of treatment initiation (day 0) was approximately 175 mm^3. Ten animals were assigned to each treatment group. Based on the pharmacokinetic systemic exposure (AUC_τ) in mouse, only formulations 1–4 were evaluated for antitumor efficacy. Formulations were administered intravenously (i.v.) into the lateral tail veins of mice. Doxorubicin dose was 2 or 3 mg/kg given once weekly for 3 weeks. Tumors were measured in three dimensions twice weekly up to Day 59. Tumor volume was calculated using the formula: $V = ½ \times D_1 \times D_2 \times D_3$, where D_{1-3} are perpendicular diameters measured in millimeters (mm). Animal body weights were measured twice a week to assess drug toxicity. Clinical observations included behavior or activity within the cage, and signs of pain or distress. All abnormal clinical observations were recorded during the study. A standard survival analysis was performed to evaluate group differences based on time to reach tumor volumes of 500% (vs. day 0 tumor volume) using Survival/Reliability profile in SAS JMP 6.0.

Repeated dose toxicity evaluation in Cynomolgus monkeys

A 23-week toxicity study in monkeys was conducted to further characterize the differences among formulations. Twenty-eight adult male Cynomolgus monkeys (Gaoyao Kangda Laboratory Animal Science & Technology Co. Ltd., China), approximately 4.0–7.0 years old (body weights in the range of 3.8–6.5 kg at start of dosing) were randomly assigned to 7 groups with 4 animals/group. Based on the results of the pharmacokinetics and efficacy studies performed in mice described above, formulations 1 (Doxil-control) and 2 were selected for the toxicity evaluation. In addition, the toxicity of two other formulation variants (formulations 6 and 7) were evaluated to establish the exposure and safety relationship in monkeys. Formulations 6 and 7 are two variants of Doxil-control with 9 mol % mPEG-DSPE. Animals were administered drug by peripheral vein at 2.5 mg/kg (formulation 1, formulation 2) or 4 mg/kg (formulations 1, 2, 6 and 7) for 6 doses, every 4 weeks for 23 weeks. Two groups of animals ($n = 2$ each per group) each received lipid control formulations corresponding to placebo formulations (1 and 6) were served as vehicle controls. Assessments made included toxicokinetics, mortality, clinical observations, body weight, food consumption, hematology, clinical chemistry (including measurement of Troponin I levels), urinalysis, organ weights and gross and microscopic examinations. The statistical significant difference between the treatment(s) was assessed by two-sided Dunnett's test for pairwise comparison with vehicle group. All tests were performed at a significance level of 5% or less.

Toxicokinetics

After the first (Day 1) and sixth dose (dose given once every 4 weeks), blood samples were collected from the monkeys at predose (0), 0.25, 1, 4, 8, 24, 48, 96 and 120 h postdose into tubes containing EDTA. Blood samples were collected from monkeys in the placebo group at 0 (predose), 1 and 4 h postdose and were retained for analysis of doxorubicin. Following blood sample collection, the samples were centrifuged and the plasma fraction was collected and stored frozen at −20°C (or lower). Plasma samples were analyzed for total doxorubicin concentrations using a

liquid chromatographic–triple quadrupole mass spectrometric (LC-MS/MS) procedure similar to that employed in the mouse plasma doxorubicin analyses. Toxicokinetic analysis was performed after the first and last dose of the repeated dose toxicity study. Statistical analysis was performed on individual $AUC_{(0-\infty)}$ data (log transformed) using statistical software SAS 9.1.4 (SAS, NC) at 95% confidence intervals. A mixed effects model was used to estimate the differences due to formulation, dose and their interaction (first dose vs. last dose, time as a covariate). Formulation 1 was compared with formulation 2 or 6 or 7, across the doses and entire experiment duration.

Data analysis

Pharmacokinetic/toxicokinetic parameters of doxorubicin were computed from the plasma concentration–time data from individual (monkey) or mean (mouse) animal data employing non-compartmental methods using WinNonlin Version 4.0.1a (Pharsight, Palo Alto, CA). Pharmacokinetic analysis was performed to determine the 96 h area under the plasma concentration versus time curve extrapolated to infinity ($AUC_{0-96\ h}$ and $AUC_{0-\infty}$), maximum plasma concentration (C_{max}), terminal half-life ($t_{½}$), plasma clearance (CL) and the apparent volume of distribution at steady state (Vd_{ss}) for all formulations. Two separate statistical analyses were carried out on individual $AUC_{(0-\infty)}$ data (log transformed) for the purpose of formulation comparisons.

Results

Pharmacokinetics in CD-1 mouse

The mean plasma concentration time profile of doxorubicin following IV administration for each formulation over 0–96 h was obtained from sparse sample approach and the time course of the mean plasma levels with 5 PLD formulations are shown in Fig. 1. The pharmacokinetic parameters obtained from mean plasma concentration time profiles are summarized in Table 2. The mean volume of distribution ranged from 48.4 to 58.5 ml/kg, which is significantly lower than the volume of total body water in mice (725 ml/kg) [9], indicating limited distribution outside of the plasma. Relatively rapid plasma clearance of doxorubicin was observed with formulations 4 and 5. As PLDs are given intra-venously and expected to remain in circulation for prolonged periods of time, $AUC_{0-96\ h}$ parameter was considered as a reliable parameter to differentiate formulation sustainability in the circulation. Due to sparse sampling study design, descriptive statistics

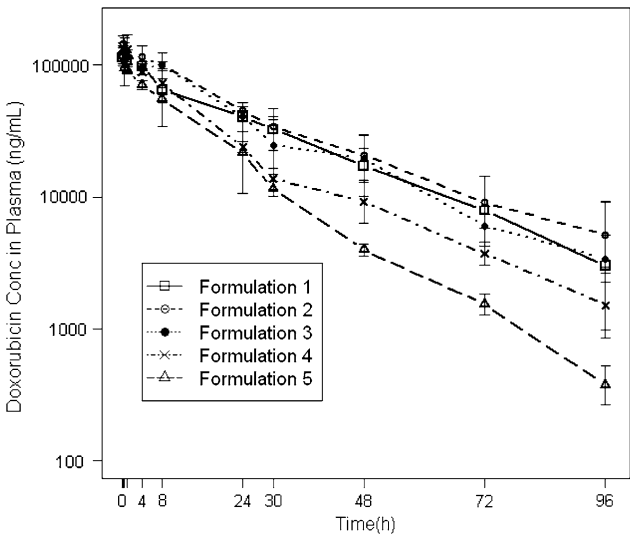

Fig. 1 Pharmacokinetic profiles of total doxorubicin following single i.v. administration of different PLD formulations to mice. (Each data point is mean ± SD of $n = 3$ values)

could not be obtained for the pharmacokinetic parameters in mice and statistical significance cannot be tested for the parameters derived from non-compartmental model analysis (NCA). For statistical comparison of AUC, an alternative approach by Holder et al. [8] was used to measure the standard error for $AUC_{0-96\ h}$ parameter in sparse sampling study designs. The mean AUC estimates obtained by this approach were in agreement with corresponding values obtained from NCA (Table 2). The results of the confidence intervals on differences between the mean of log 2 AUC of two formulations show that formulation 1 is not significantly different to formulation 2, 3 and 4 at 5% level. Only formulation 5 is significantly different from formulation 1 (Doxil-control) and considered pharmacokinetically not equivalent to Doxil-control. To substantiate pharmacokinetic equivalency, selected formulations were further evaluated in Cynomolgus monkeys (see below).

Antitumor efficacy in human breast (MDA-MB-231) tumor xenografts

The antitumor activity of the Doxil-control and its three formulation variants (2, 3 and 4) was evaluated at 2 or 3 mg/kg given once weekly for 3 weeks to mice with MDA-MB-231 human breast adenocarcinoma xenografts. The average tumor volume at the time of first treatment was approximately 175 mm^3. A relative % change in mean tumor volume is presented in Figs. 2 and 3. Tumor volume for untreated control was 1,215% of the initial treatment volume on Day 35. On Day 35, mean tumor volume with formulations 1–4 was 371, 331, 340 and 543% at the 2 mg/kg dose

Table 2 Pharmacokinetic parameters (Doxil-control) of total doxorubicin following single i.v. administration of different pegylated liposomal doxorubicin formulations to male mice

Formulations #	1[a]	2	3	4	5
Pharmacokinetic parameters by non-compartmental analysis[b]					
C_{max} (μg/ml)	115	158	141	149	131
AUC_{0-96} (μg h/ml)	2,700	3,330	2,950	2,080	1,540
$AUC_{0-\infty}$ (μg h/ml)	2,780	3,500	3,040	2,120	1,550
$t_{1/2}$ (h)	19.1	24.0	19.0	18.4	14.1
CL (ml/h kg)	2.16	1.71	1.97	2.83	3.87
Vd (ml/kg)	57.3	48.8	48.4	54.6	58.5
$AUC_{0-96\ h}$ (μg h/ml) determination by Partial AUC method					
Estimate	2,687	3,315	2,933	2,066	1,531
Standard error	303	122	157	81.0	99.0
95% CI estimate[c] (Diff log 2 scale)		−0.09 to 0.70	−0.28 to 0.53	−0.78 to 0.03	−1.27 to −0.35

[a] Doxil-control

[b] Parameters calculated based on sparse data derived from different animals at various time points and descriptive statistics are not available for the parameters

[c] At 95% confidence level interval (CI) on the difference between the mean log 2 AUC of formulations 1–2 or 3 or 4 or 5, only formulation 5 is significantly different from formulation 1. Each of the 95% confidence intervals includes 0. No adjustment was applied to the confidence levels to account for multiple comparisons

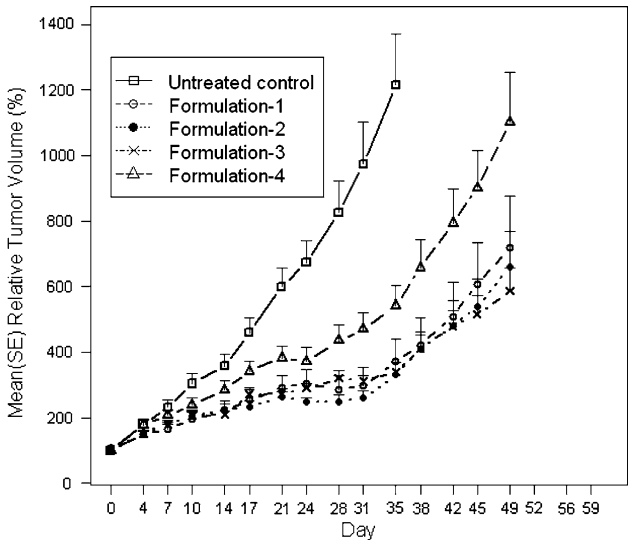

Fig. 2 Efficacy (changes in % mean tumor volumes) of different pegylated liposomal products in established human breast (MDA-MB-231) tumor xenografts after i.v. administration (once weekly for 3 weeks) to mice at 2 mg/kg dose. (Each data point is mean ± SE of $n = 10$ observations.)

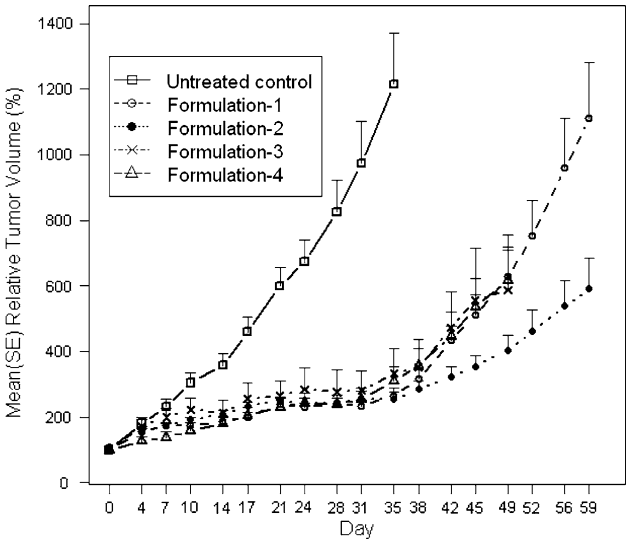

Fig. 3 Efficacy (changes in % mean tumor volumes) of different pegylated liposomal products in established human breast (MDA-MB-231) tumor xenografts after i.v. administration (once weekly for 3 weeks) to mice at 3 mg/kg dose. (Each data point is mean ± SE of $n = 10$ observations.)

and was 265, 253, 333 and 310% at the 3 mg/kg dose, respectively. On Day 49, the corresponding tumor volumes after formulation 1–4 treatments increased to 720, 661, 586 and 1,101% at the 2 mg/kg dose and to 628, 404, 587 and 615% at the 3 mg/kg dose, respectively. Two groups that were treated at the 3 mg/kg dose level were observed until Day 59; the mean tumor volume of 1,110% for formulation 3 and 592% for formulation 2. No significant mean group body weight loss was observed in any of the treatment groups. Survival analysis on time to 500% relative tumor volume is showed in Table 3. And the results indicate that there was a significant differences ($P < 0.05$) between untreated control group and groups treated with Doxil-control (formulation 1) at both dose levels (2 and 3 mg/kg dose). At the 2 mg/kg dose level, Doxil-control had similar survival time to those of formulations 2 and 3 but significantly longer than

Table 3 Survival analysis on time to 500% relative tumor volume for different pegylated liposomal doxorubicin formulations in human breast (MDA-MB-231) tumor xenografts

Formulation	Dose (mg/kg)	Dosing days	Mean survival (days)	Std. error	Median survival (days)	Lower (95%)	Upper (95%)
Untreated control	0	0, 7, 14	21.43	0.367	20.09	19.32	20.10
1[a]	2	0, 7, 14	39.85	0.737	42.69	34.28	48.70
2	2	0, 7, 14	46.32	0.698	47.85	43.00	50.67
3	2	0, 7, 14	30.20	0.685	42.26	41.61	42.26
4	2	0, 7, 14	44.43	0.456	30.02	29.95	30.19
1[a]	3	0, 7, 14	53.89	0.498	46.23	41.46	46.23
2	3	0, 7, 14	49.46	0.466	59.68	52.29	59.68
3	3	0, 7, 14	46.18	1.154	55.98	52.52	60.06
4	3	0, 7, 14	42.60	0.771	48.37	39.04	48.99

$n = 10$ animals were assigned per group

[a] Doxil-control

Table 4 Mean (±SD, $n = 4$) toxicokinetic parameters of total doxorubicin following single and six repeated (once every 4 weeks) i.v. administration of different pegylated liposomal formulations to male Cynomolgus monkeys

Formulation	Dose (mg/kg)	t_{max} (h)	C_{max} (μg/ml)	$AUC_{(0–24\ h)}$ (μg h/ml)	$AUC_{(0–\infty)}$ (μg h/ml)	$t_{1/2}$ (h)	CL (ml/h kg)
After 1st injection							
Formulation 1	2.5	0.625 ± 0.433	66.8 ± 10.8	4,150 ± 954	5,920 ± 1,930	63.1 ± 12.6	0.452 ± 0.124
Formulation 1	4.0	0.438 ± 0.375	136 ± 13.4	8,440 ± 1,540	12,700 ± 3,190	78.1 ± 8.41	0.327 ± 0.066
Formulation 2	2.5	0.813 ± 0.375	72.9 ± 3.57	4,510 ± 891	5,840 ± 1,880	55.1 ± 12.2	0.455 ± 0.114
Formulation 2	4.0	0.625 ± 0.433	125 ± 4.97	8,320 ± 551	12,000 ± 1,870	69.2 ± 16.4	0.340 ± 0.062
Formulation 6	4.0	1.56 ± 1.70	112 ± 10.6	4,870 ± 1,050	6,570 ± 1,690	64.7 ± 7.93	0.641 ± 0.178
Formulation 7	4.0	22.0 ± 18.9	49.6 ± 19.5	2,560 ± 162	3,200 ± 98.6	45.1 ± 15.8	1.33 ± 0.381
After 6th injection							
Formulation 1	2.5	2.56 ± 3.64	82.1 ± 10.1	4,490 ± 367	6,720 ± 1,590	71.2 ± 14.6	0.386 ± 0.076
Formulation 1	4.0	0.750 ± 0.433	135 ± 19.0	7,290 ± 1,080	9,920 ± 198	61.1 ± 16.6	0.353 ± 0.864
Formulation 2	2.5	0.500 ± 0.433	81.8 ± 18.6	4,500 ± 1,470	6,230 ± 2,130	65.1 ± 2.81	0.585 ± 0.177
Formulation 2	4.0	4.50 ± 4.04	113 ± 28.8	5,820 ± 497	7,820 ± 976	60.4 ± 22.2	0.366 ± 0.063
Formulation 6	4.0	0.750 ± 0.433	106 ± 10.9	4,370 ± 1,830	5,690 ± 2,970	53.5 ± 19.5	0.906 ± 0.599
Formulation 7	4.0	0.80 ± 0.40	33.1 ± 5.97	1,900 ± 508	2,180 ± 168	61.6 ± 7.33	1.84 ± 0.139

formulation 4. At the 3 mg/kg dose level, Doxil-control had significantly shorter survival time than formulation 2 but similar to formulations 3 and 4.

Repeated dose toxicity study in Cynomolgus monkeys

Toxicokinetics

The pharmacokinetic parameters after single and six repeated (dosing once every 4 weeks, for a total 6 doses) intravenous doses of PLD formulations in monkeys are shown in Table 4. Following single and six repeated (dosing once every 4 weeks, for a total 6 doses) intravenous doses of formulation 1 (Doxil-control) and formulation 2 (at doses equivalent to doxorubicin 2.5 or 4.0 mg/kg) to male monkeys, there was a dose-related increase in systemic exposure (expressed as AUC) to doxorubicin with both formulations. Pharmacokinetic profiles after the first dose are presented in Fig. 4, and the pharmacokinetic profiles as well as rank order remained similar after the repeated doses. At the 2.5 mg/kg dose, systemic exposure to doxorubicin was slightly higher after the sixth dose than after the first dose whereas at 4.0 mg/kg systemic exposure was slightly decreased after six repeated doses. The plasma elimination half-life of doxorubicin following repeated administrations of formulation 1 or formulation 2 was in the range of 60–70 h. The results of statistical analysis suggested that there was no statistically significant difference between formulations 1 and 2 at both doses after the 1st and the 6th administrations. Overall plasma pharmacokinetic profiles of

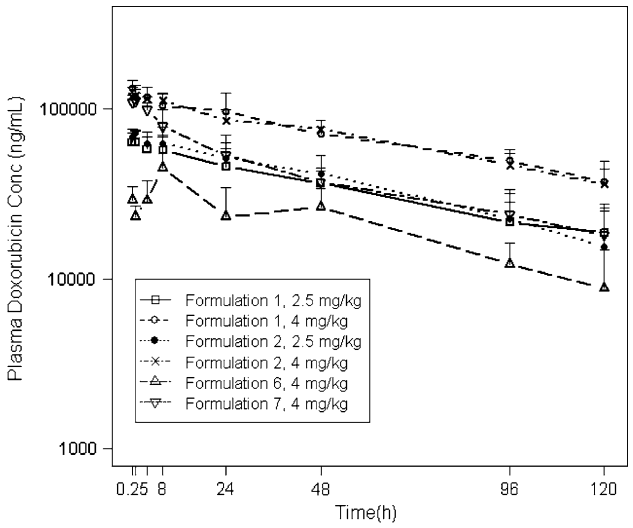

Fig. 4 Mean (±SD) total doxorubicin plasma concentrations (ng/ml) in male Cynomolgus monkeys ($n = 4$) following a single i.v. administration of different liposomal doxorubicin formulations

doxorubicin from formulation 1 and formulation 2 were similar.

The $AUC_{(0-\infty)}$ for formulations 6 and 7 at 4.0 mg/kg dose was statistically significant compared to formulation 1 after the 1st and the 6th administrations. Formulation 6 and formulation 7 gave approximately 75 and 50% lower systemic exposure to doxorubicin compared to formulation 1 (Doxil-control). Following six repeated doses at 4 mg/kg, slight decreases in systemic exposure to doxorubicin was observed with formulation 6 and formulation 7 compared to corresponding values after the first dose. Plasma elimination half-life of doxorubicin after six repeated administrations of formulations 6 and 7 was ~62 and 54 h, respectively.

Toxicity

Following six repeated intravenous doses, there were no compound-related changes in ECG (Electrocardiogram), coagulation or urinalysis parameters during the study with any formulations. At 2.5 mg/kg of formulation 1 (Doxil-control), scurf, abrasion and skin thickening at injection site were observed. At 4 mg/kg of formulation 1, clinical signs of hair loss were also observed. In the 2.5 and 4 mg/kg of formulation 2 dosed groups, a high incidence of anorexia, hair loss, scurf, abrasion and increased skin thickness at injection site were observed in a dose-related manner. At 4 mg/kg of formulation 7, scurf and thickening of skin at injection site were the only clinical signs in this group. At 4 mg/kg of formulation 6, a high incidence of anorexia, hair loss, scurf, abrasion and thickening of skin at injection sites were noted.

Compound-related decreases in mean body weight were noted in all formulation-treated groups at all dose levels during the study. At Week 23, the mean body weights were 0.93×, 0.76×, 0.82×, 0.74×, 0.87× and 0.80× of placebo controls for formulation 1 (2.5, 4.0 mg/kg), formulation 2 (2.5, 4.0 mg/kg), formulation 6 (4.0 mg/kg) and formulation 7 (4.0 mg/kg), respectively. Correspondingly, decreased body weight gains were seen in all doxorubicin-treated groups. Decreased food consumption was sporadically noted in the 4 mg/kg group treated with formulation 1 starting from Week 14; groups treated with formulation 2 at 2.5 and 4 mg/kg starting from Week 11; and the group treated with formulation 6 (4 mg/kg) starting from Week 13 until or toward to the end of dosing (Week 22).

Hematology, clinical chemistry and histopathology results following repeated dosing to monkeys are presented in Table 5. There were slight to moderate decreases in red blood cells (RBC), hematocrit (HCT) and hemoglobin (HGB) levels in all treated groups at 4 mg/kg dose. For groups treated with formulation 1 and formulation 2, decreased RBC parameters were only noted on Day 143, while for groups treated with formulations 6 and 7, the decreases started from Day 31 until Day 143. In addition, in monkeys treated with formulation 7 at 4 mg/kg dose, there were moderate decreases in platelets (PLT), white blood cells, lymphocytes, mid cells total count (MID) and reticulocytes (RC) values, indicating a depression of hematopoiesis, which correlated with bone marrow hypocellularity in this group. Slight to marked increases in aspartate transaminase (AST) levels were noted in groups treated with formulation 2 (4 mg/kg, 2.78–3.21×), formulation 6 (4 mg/kg, 2.79–3.75×) and formulation 7 (4 mg/kg, 5.57–8.91×), and increased total bilirubin (TB) and creatinine phosphokinase (CK) were seen in groups treated with formulation 6 (2.11–2.54× for TB and 1.92–3.36× for CK) and formulation 7 (2.30–3.71× for TB and 1.17–2.10× for CK) from Days 31 to 143. Increased alanine transaminase (ALT) level was noted in formulation 6 from Day 31 (2.25×) to Day 143 (2.58×). Formulation 2 at 4 mg/kg resulted in a moderate increase in Troponin I levels at Week 21. Formulation 7 at 4 mg/kg caused slight to marked increases in Troponin I levels from Weeks 5 to 21.

There were no formulation/compound-related organ weight changes or microscopic findings on the cardiovascular system. Microscopic changes that could possibly be formulation/compound-related involved in the bone marrow, skeletal muscle and kidney. Bone marrow hypocellularity was more pronounced in monkeys treated with formulation 7 at 4 mg/kg; a lower incidence of bone marrow hypocellularity was seen in monkeys treated with either formulation 2 or formulation 6. In the kidneys, multifocal degeneration of renal cortical tubules was noted in monkeys treated with formulations 2 and 7. There was a low incidence of minimal, skeletal muscle myofiber degeneration in monkeys treated with formulation 1 at

Table 5 Toxicological evaluation of different pegylated liposomal formulations following six repeated (once in 4 weeks for 6 months) i.v. dosing to male Cynomolgus monkeys

Formulations	Placebo[a]	Formulation 1	Formulation 1	Formulation 2	Formulation 2	Formulation 6	Formulation 7
Weekly dose (mg/kg)		2.5	4.0	2.5	4.0	4.0	4.0
No. of Animals[b]	M:4	M:4	M:4	M:4	M:4	M:4	M:4
Hematology[c] [historical mean ± SD]							
LYM (%) [38.61 ± 8.03]	25.5	–	–	–	–	–	0.78×
MID (%) [9.95 ± 1.98]	8.5	–	–	–	–	–	0.73×
RBC (10^6/μl) [5.02 ± 0.38]	5.07	–	0.85×	–	0.86×	0.80×	0.76×
RC (%) [0.83 ± 0.16]	0.8	–	–	–	–	–	0.88×
PLT (10^9/l) [210 ± 50.4]	170.3	–	–	–	–	–	0.90×
WBC (10^9/l) [15.95 ± 4.45]	11.3	–	–	–	–	–	0.89×
Serum chemistry[c] [historical mean ± SD]							
ALT (U/l) [44.57 ± 17.6]	100.3	–	–	–	–	2.58×	–
AST (U/l) [41.86 ± 11.7]	46.3	–	–	–	3.21×	3.75×	8.91×
CK (U/l) [354 ± 173]	226.5	–	–	–	–	3.36×	2.10×
TB (g/l) [11.44 ± 4.21]	9.8	–	–	–	–	2.54×	3.71×
Histopathology							
Bone marrow hypocellularity	–	–	–	–	2/4	1/4	4/4
Kidney: multi-focal, degeneration of renal cortical tubules	–	–	–	2/4	1/4	–	2/4
Skeletal muscle degeneration	–	–	2/4	–	–	–	–
Cardiac Troponin I (cTnI) analysis W21[d]	–	–	–	–	5.0×	–	2.4×

[a] There were no differences between two placebo groups that received lipid control of formulations 1 and 6 without doxorubicin, both groups are combined for the purpose of interpretation

[b] Number of animals with the observations/total animals of the group

[c] At end of dosing period. For placebo group, group means are shown. For treated groups, multiples of control/baseline is shown. Statistical significance is based on actual data (not on the multiples of control/baseline)

[d] Compared to pretest values

– No noteworthy findings

4 mg/kg dose. There were no renal effects observed with formulation 1 (Doxil-control, 2.5 and 4.0 mg/kg).

Formulations 6 and 7 gave lower systemic exposure to doxorubicin compared to formulation 1 (Doxil-control); however, a greater severity and frequency of toxic effects were observed with formulations 6 and 7 compared to formulation 1. Formulation 2 produced systemic exposure to doxorubicin similar to formulation 1 (Doxil-control); however, multi-focal degeneration of renal cortical tubules was mainly observed with formulation 2 but not with formulation 1 (Doxil-control). Therefore, plasma systemic exposure to doxorubicin from different liposomal formulations did not correlate well with the toxicity profiles of these formulations.

Discussion

The objectives of this study were to examine the effects of liposome composition on plasma drugs levels and its relationship to drug efficacy and safety. In this study, we designed various PLD formulations with minor changes in lipid composition or internal buffer or vesicle diameter to the commercial formulation (Doxil®). The study demonstrated that minor alterations in the formulation could induce significant differences in toxicity and antitumor efficacy, which did not correlate with plasma levels of doxorubicin. Our data confirm that the conventional method of demonstrating bioequivalence is not a suitable method for determining whether two liposomal doxorubicin products are equivalently safe and effective. We showed this by designing product variants to have a similar pharmacokinetic profile to Doxil-control and then tested them in animal models to compare their biological effects. As shown in Fig. 1 except for formulation 5, the plasma concentration time profiles for formulations 2, 3 and 4 are comparable (within the range of experimental variability) to Doxil-control profile indicating that the formulations are pharmacokinetically equivalent. Since formulation 5 gave the lowest systemic exposure among the test PLDs, we

excluded formulation 5 in the efficacy study. Statistical analyses of $AUC_{(0–96\ h)}$ further confirms (Table 2.) that systemic exposure obtained with formulations 2, 3 or 4 is not statistically different from Doxil-control, whereas systemic exposure with formulation 5 is statistically different from Doxil-control. Based on these observations, the efficacy profiles of these formulations (1, 2, 3 and 4) were expected to be the same with no statistical difference when they were tested at the same dose level. As shown in Fig. 2, formulation 4 had lower antitumor activity based on survival analysis compared to Doxil-control ($P < 0.00083$) when both the formulations tested at same dose level. When the dose was increased from 2 to 3 mg/kg, substantial efficacy benefit was observed with formulation 2 but not with formulation 1 (Doxil-control) or formulations 3 or 4 as shown in Fig. 3. These studies show that the pharmacokinetic profiles of PLD formulations do not necessarily correspond to their in vivo activity.

Formulation 2, which showed similar pharmacokinetics as formulation 1 (Doxil-control), and two other formulation variants (formulation 6 and 7) that gave lower systemic exposure than formulation 1 were tested for toxicity in Cynomolgus monkeys. The study found differences in toxicities among the formulations that were not correlated with the level of systemic exposure. Although formulations 6 and 7 gave lower systemic exposure to doxorubicin compared to formulation 1 (Doxil-control), they caused a greater severity and frequency of toxic effects than formulation 1. These formulations (6 and 7) showed greater hematological toxicities and elevated liver enzymes compared to formulation 1 (Doxil-control). Bone marrow depression was more pronounced in monkeys treated with formulation 6 than in the other groups. Formulation 2 gave systemic exposure to doxorubicin similar to formulation 1 (Doxil-control); however, multi-focal degeneration of renal cortical tubules was mainly observed with formulation 2 but not with formulation 1 (Doxil-control). Furthermore, hypocellularity of the bone marrow was seen in monkeys treated with formulation 2 at 4 mg/kg, but not in monkeys treated with formulation 1 at the equivalent dose level. Therefore, plasma systemic exposure to doxorubicin from different liposomal formulations did not correlate well with the toxicity profiles of these formulations.

All formulations (1–5) that were screened in the mouse pharmacokinetic and tumor efficacy studies had similar particle size (range: 102–125 nm) with varied lipid or internal buffer composition (Table 1). With the exception of formulation 5, plasma pharmacokinetics of 1–4 was similar; however, differences between the efficacy profiles between the formulations (e.g. formulation 1 vs. 4 at 2 mg/kg or formulation 1 vs. 2 at 3 mg/kg) cannot be explained based on the pharmacokinetics. Formulation 3 had similar lipid composition as formulation 2 with varied internal buffer composition but the in vivo tumor efficacy profile of formulation 3 differs from formulation 2. Formulations 6 and 7 had significantly larger particle size than Doxil-control and were not studied in the efficacy study. The mean particle diameter for formulation 6 was 196 nm whereas in formulation 7 it was 336 nm. To ensure full coverage of the larger liposome surface by the hydrophilic coating, the proportion of mPEG-DSPE in the bilayer for formulations 6 and 7 was increased from 5 to 9 mol % based on calculations by Lasic [10] and Torchilin et al. [11]. Given the similarity in their lipid composition to Doxil-control, the lower doxorubicin systemic exposure of formulation 6 and 7 is most likely due to a faster clearance of liposomes from the circulation, and not a dramatic increase in the leakage rate of the drug from the vesicles. In spite of the lower systemic exposure, greater severity and frequency of toxic effects were observed with these formulations. The enhanced toxicity could be a result of differences in tissue distribution or drug release rate from the liposomes between the two formulations and Doxil-control. Although we have not conducted distribution kinetic studies, it appears lipid composition, internal buffer composition and the particle size play an important role in the pharmacokinetics, distribution and disposition behavior of PLDs in vivo. A previous study [7] compared two pegylated liposomal formulations (PLD-75 and PLD-100) that had the same lipid composition and drug/lipid ratio but differences in vesicle diameters and internal ammonium sulfate concentration. Despite having similar pharmacokinetics for PLD-75 and PLD-100, PLD-75 was more efficacious than PLD-100 administered at the same doxorubicin level in a tumor xenograft model. These authors demonstrated the efficacy of 8 mg/kg PLD-75 was comparable to that of 12 mg/kg PLD-100, and treatments with 12 mg/kg PLD-75 and 16 mg/kg PLD-100 were equivalently efficacious. In addition, there were significant differences in how the two formulations accumulated in tumor and other tissues of the mice and in their acute toxicity. Although plasma exposure was similar for PLD-75 and PLD-100 formulations, tumor exposure with PLD-100 was 1.7 fold higher than with PLD-75. It is interesting to note that the formulation that had a higher drug level in the tumor (PLD-100) was actually less efficacious. The authors concluded that the toxicity and efficacy of liposomal drugs cannot be predicted through the measurement of total drug levels in the plasma. Our results from the present study corroborate the published findings of a lack of correlation of biological effects with pharmacokinetics for different versions of PLD formulations.

Liposomal products are complex, multi-component systems, and drug product quality is controlled by many physicochemical characteristics which together, define their biological performance. Physicochemical factors

identified by the FDA [12] that could potentially affect drug product quality include the composition of the lipid bilayer, net charge, mean particle size and distribution, the degree of lamellarity, the percentage of drug encapsulation, the drug to lipid ratio, in vitro release, and in vivo integrity of the liposomes. Although physicochemical sameness might in theory be sufficient to indicate that two products will perform equivalently in the clinic, conventional analytical methods are usually not adequate to demonstrate that two liposomal doxorubicin products are in fact physicochemically the same. The fundamental shortcoming of traditional analytical methodologies is that, with a few exceptions, they measure only the average value of the attribute involved; these tests generally do not provide information on individual liposome within the total population of liposomes present in a particular sample. Although mean liposome physicochemical parameters may be the same, the distributions around the means may be very different, and these differences could affect the clinical performance of the product. For example, we have generated data to show that a distributional difference in % pegylation covering the liposome surface does influence the biological performance, i.e., pharmacokinetics of a PLD product (data on file). We evaluated Doxil-control which had 5.3 mol % mPEG-DSPE in the bilayer and a variant formulation produced by mixing doxorubicin liposomes containing 1 mol % mPEG-DSPE, and liposomes containing 9 mol % mPEG-DSPE in a proportion such that the resulting product had a mean mPEG-DSPE content of 5.3 mol %. Both formulations assayed to contain approximately 5 mol % mPEG-DSPE; there are currently no analytical methods that can determine pegylation coverage of a single liposome in a particular population of liposomes or vial of product. We tested the pharmacokinetics of both formulations in mice and showed that the profiles are different (data not shown). This example clearly demonstrates that physicochemical sameness (e.g. mPEG-DSPE concentration) using the currently available methodologies is not sufficient to ensure bioequivalence.

Our data show that minor changes in formulation can lead to significant alteration in the biological behavior of liposomal drugs. Furthermore, plasma pharmacokinetic equivalence does not ensure equivalent toxicity and efficacy of liposomal doxorubicin products. These observations point to the need to regard different liposomal formulations encapsulating the same drug as different chemical entities, worthy of careful evaluation beyond simple pharmacokinetic based clinical bioequivalence. Recognizing the challenges with development of generic products for advanced drug delivery systems, FDA has stated in the Critical Path Initiative for Generic drugs [13] that plasma concentrations may not be related to the concentration of drugs at specific target tissues (e.g. tumors) for liposomal drugs, and work is needed on novel methods to determine therapeutic equivalence for liposomal products. In the absence of an established method, a careful evaluation is required for establishing safety and therapeutic equivalency of a generic pegylated liposomal doxorubicin product to the original innovator product. Along with pharmacokinetic studies, appropriate clinical studies in refractory ovarian patients with cancer are necessary to determine whether a generic version of PLD is equivalent to Doxil®. In addition, a comparative clinical study of resting left ventricular ejection fractions should be considered to assess the generic product's risk of myocardial damage.

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