Journal of
Nanomedicine & Nanotechnology

Ali et al., J Nanomed Nanotechnol 2016, 7:2
DOI: 10.4172/2157-7439.1000361

Research Article Open Access

Bioequivalence Study of Pegylated Doxorubicin Hydrochloride Liposome (PEGADRIA) and DOXIL® in Ovarian Cancer Patients: Physicochemical Characterization and Pre-clinical studies

Ali SM[1], Sheikh S[1], Ahmad A[1], Ahmad MU[1], Chen P[2], Paithankar M[3], Choudhury K[4], Makadia RD[5], Kumar A[6], Velavan K[7], Satheesh CT[8], Singh JK[9], Mamillapalli G[10], Saptarishi D[3], Kale P[11], Patel R[11], Barkate HV[3] and Ahmad I[1*]

[1]*Jina Pharmaceuticals Inc., 28100 Ashley Circle, Suite 103, Libertyville, IL 60048, USA*
[2]*Nia Life Sciences Inc., 28100 Ashley Circle, Suite 102, Libertyville, IL 60048, USA*
[3]*Intas Pharmaceuticals, Ahmedabad, Gujarat, India*
[4]*Health Point Multispeciality Hospital, Kolkata, West Bengal, India*
[5]*Shree Giriraj Multispeciality Hospital, Rajkot, Gujarat, India*
[6]*Sapthagiri Institute of Medical Science and Research Centre, Bengaluru, Karnataka, India*
[7]*Erode Cancer Centre, Erode, Tamilnadu, India*
[8]*Sri Venkateshwara Hospital, Bengaluru, Karnataka, India*
[9]*S. S Hospital and Research Centre, Patna, Bihar, India*
[10]*City Cancer Centre, Vijayawada, Andhra Pradesh, India*
[11]*Lambda Therapeutic Research Ltd. Ahmedabad, Gujarat, India*

Abstract

Objective: To develop Pegylated Doxorubicin Hydrochloride Liposome (PEGADRIA) and compare its physicochemical properties, preclinical safety and efficacy, clinical pharmacokinetic and safety profiles with those of the reference product, Doxil®.

Methods: PEGADRIA was prepared and the structure morphology, lamellarity, size, shape, drug loading, lipid bilayer and Peg layer thickness were determined. Safety studies were conducted in mice and rats and efficacy study was conducted in a P-388 leukemia mouse model. To evaluate the pharmacokinetic profile of PEGADRIA, a multicenter, open label, balanced, randomized, two-treatment, two-period, two-sequence, single dose cross-over bioequivalence study was conducted with Doxil® in ovarian cancer patients whose disease had progressed or reoccurred after platinum based chemotherapy under fasting conditions. The pharmacokinetic parameters were determined based on the concentration-time profiles of doxorubicin, whereas the concentration was determines using LC-MS/MS methods.

Results: PEGADRIA was similar to Doxil® in terms of general morphology, lamellarity, size, shape, drug loading, lipid bilayer and Peg layer thickness. PEGADRIA and Doxil® showed comparable survival benefit in leukemic mice. The toxicity profiles of PEGADRIA in both mice and rats were comparable to those of Doxil®. Plasma concentrations of doxorubicin from cancer patients were measured to determine the pharmacokinetics profile. The geometric mean ratios (90% confidence intervals) of PEGADRIA /Doxil® for free doxorubicin and encapsulated doxorubicin were similar.

Conclusion: PEGADRIA was found to have similar physicochemical profile compared to Doxil®. In addition, it was safe and bioequivalent to Doxil® in ovarian cancer patients.

Keywords: Doxorubicin; Liposomes; Pegylation; Drug loading; Pharmacokinetics; Bioequivalence

Introduction

Doxorubicin is an anthracycline antibiotic isolated from *Streptomyces peucetius* var. *caesius* and is one of the most widely used chemotherapeutic agents. It is known for its ability to bind DNA and inhibit nucleic acid synthesis [1]. Doxorubicin has been utilized against variety of tumors, leukemias, sarcomas, and breast cancer. However, the therapeutic use of doxorubicin is limited due to its dose-dependent cardiotoxicity leading to congestive heart failure and death in addition to common toxicities such as bone marrow suppression and alopecia as observed with other chemotherapeutic agents [2]. To circumvent the toxicities associated with free doxorubicin, various pegylated and non-pegylated liposomal formulation of doxorubicin such as Doxil®, and Myocet® were developed [3,4]. Doxil® is a formulation that encapsulates doxorubicin in an aqueous compartment of liposome. Liposomal doxorubicin also contains surface-bound methoxypolyethylene glycol (MPEG)-lipid to protect liposomes from detection by the mononuclear phagocyte system and to prolong blood circulation time. Preclinical and clinical studies have shown that encapsulation of doxorubicin in liposomes resulted in reduced cardiotoxic effect and improved therapeutic efficacy compared to free doxorubicin [5-8]. Doxil® is approved for the treatment of AIDs-related Kaposi's sarcoma, recurrent ovarian cancer, and metastatic breast cancer [8-10]. The most challenging part lies in the manufacturing of liposomal doxorubicin having maximum drug loading efficiency.

***Corresponding author:** Imran Ahmad, Jina Pharmaceuticals Inc., 28100 Ashley Circle, Suite 103, Libertyville, IL 60048, USA, Tel: 8475730700; E-mail:Imran@jinapharma.com

Received March 02, 2016; **Accepted** March 16, 2016; **Published** March 23, 2016

Citation: Ali SM, Sheikh S, Ahmad A, Ahmad MU, Chen P, et al. (2016) Bioequivalence Study of Pegylated Doxorubicin Hydrochloride Liposome (PEGADRIA) and DOXIL® in Ovarian Cancer Patients: Physicochemical Characterization and Pre-clinical studies. J Nanomed Nanotechnol 7: 361. doi:10.4172/2157-7439.1000361

The aim of this study was to develop a generic version of Pegylated Liposomal Doxorubicin for Injection (DOXIL®) and compare the physicochemical and physiological properties with the commercially available Doxil®. The pharmacokinetic profile of PEGADRIA was also compared with Doxil® in ovarian cancer patients whose disease was progressed or recurred after platinum based chemotherapy to assess the bioequivalence. In addition, safety was monitored in patients treated with both PEGADRIA and Doxil®.

Materials and Methods

Doxorubicin Hydrochloride was obtained from Synbias Pharma Ltd. (Donetsk, Ukraine). Fully Hydrogenated Soy phosphatidylcholine (HSPC) and N-(carbonylmethoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt (DSPE-MPEG2000) were procured from Lipoid GmbH (Nattermannallee 1, Germany) and Cholesterol was obtained from Dishman Pharmaceuticals and Chemicals, Ltd. (Ahmedabad, India). Ammonium Sulfate, Histidine, and Sucrose were purchased from Merck KGaA (Darmstadt, Germany). Doxil®, a commercially available liposomal doxorubicin formulation was manufactured by Ben Venue Laboratories, Inc. (Bedford, Ohio) for Janssen Products, LP (Horsham, PA). Doxil® contains 2 mg/mL doxorubicin, 3.19 mg/mL of DSPE-MPEG2000, 9.58 mg/mL of HSPC, and 3.19 mg/mL of cholesterol.

Preparation of pegylated liposomal doxorubicin HCl (PEGADRIA)

Pegylated Liposomal Doxorubicin HCl (PEGADRIA) was manufactured similar to Doxil® manufacturing process using an active loading process with an ammonium sulfate gradient [11]. The manufacturing steps include formation of liposomes containing ammonium sulfate, liposome size reduction, creation of ammonium sulfate gradient and active drug loading. The product was filtered through 0.2 μm sterile filter and filled into individual vial having 2 mg/mL doxorubicin concentration.

Physicochemical studies

HPLC method: The HPLC analysis of PEGADRIA and Doxil® were carried out using Agilent 1100/1200 series HPLC system equipped with UV Detector, BDS Hypersil C18 (250 mm x 4.6 mm, 5 μ) column. A mixture of sodium lauryl sulfate in water and acetonitrile (1:1) containing Orthophosphoric acid was used as mobile phase, column oven temperature was set at 50°C and detection by UV was done at 254 nm wavelength. The concentration of hydrogenated soy phosphatidylcholine and cholesterol in the products was assayed on HPLC system at 210 nm wavelength using Waters Symmetry C18 (150 mm x 4.6 mm, 5 μ) and a mixture of methanol, water, and tetrahydrofuran ((90:6:4). DSPE-MPEG2000 concentration was determined on HPLC System equipped with RI detector and Ultrasphere 5 OCTYL (150 mm x 4.6 mm, 5 μ) column using a mixture of methanol and water (95:5) containing ammonium acetate as mobile phase.

***Particle size measurement*:** The particle size measurement was carried out using Nicomp Model 380/ZLS&S Potential/Sub-Micron Particle Sizer (Particle Sizing Systems, New Port Richly, FL, USA). The measurements were carried out at 23°C at a scattering angle of 90°. During the study, liquid viscosity was kept at 0.933 with refractive index of 1.333. The intensity set point was set to 300 kHz.

***Zeta Potential*:** The zeta potential of PEGADRIA and DOXIL® was measured using Nicomp 380 ZLS Particle Sizing System. The zeta potential was measured at 23°C at 14.7 degree scattering angle, 18.9 degree external fiber angle, and 632.8 nm laser wavelength. One mL of test sample was diluted with 9 mL of water for injection.

***Encapsulation efficiency*:** The percent drug encapsulated was determined using size exclusion chromatography. Briefly, 500 μL of PEGADRIA (2 mg/mL) or Doxil® (2 mg/mL) was loaded on Sephadex™ G-25M PD-10 columns separately equilibrated with 5% dextrose solution. The column was then eluted with 5% dextrose solution and small fractions (~300 μL) were collected. Fractions containing the lipids were pooled and volume was measured. For control, 500 μL of PEGADRIA (2 mg/mL) and Doxil® (2 mg/mL) were diluted separately to the measured volume with 5% dextrose solution. The eluted pooled fractions and control were analyzed by HPLC for the drug content.

Morphology, Size, and Drug encapsulation of PEGADRIA and Doxil® were assessed using transmission electron microscopy (TEM) cryo imaging sizing, fraction counting analysis, and internal volume distribution estimation. Electron microscopy was performed using FEI Tecnai T12 electron microscope, operating at 120 keV equipped with an FEI Eagle 4k x 4k CCD camera. Vitreous ice grids were transferred into the electron microscope using a cryostage that maintains the grids at a temperature below -170°C. Images of each grid were acquired at multiple scales to assess the overall distribution of specimen.

***In vitro* drug release**

***In vitro drug release in PBS buffer at various pH and temperature*:** The in vitro release of PEGADRIA and Doxil® were measured in PBS buffers, pH 6.5, using Electro Lab ERB-40A Bottle Rotating Apparatus maintained at various temperatures (37°C, and 47°C). PBS buffers dissolution media were prepared by dissolving 1.79 g of sodium hydrogen phosphate dehydrate, 1.36 g of potassium dihydrogen orthophosphate, and 7.02 g of sodium chloride in 1L of water. The desired pH was adjusted with dilute hydrochloric acid or dilute sodium hydroxide. The test sample was prepared by mixing 0.5 mL of test sample (equivalent to 1 mg of Doxorubicin) in 50 mL of dissolution medium containing 0.5 g of Dowex® 50WX4 (H^+, 200-400 mesh) in Polypropylene tubes. 1 mL of the sample is withdrawn (initial time point) and replaced it with 1 mL of dissolution medium. The sample tubes were placed in Bottle Rotating apparatus and the apparatus were started immediately. At the end of each specified time point the apparatus was stopped and the vessel was undisturbed until the Dowex® resin was settled at the bottom before 1 mL of sample from the medium was withdrawn and replaced with the same volume with fresh dissolution medium equilibrated to a same temperature. Doxorubicin concentration in withdrawn samples at each specified time points were analyzed by HPLC.

***In vitro drug release by sonication*:** The in vitro release of PEGADRIA and Doxil® were measured in PBS buffer (pH 6.5) using SONICS, Vibracell (VC-505) Probe Sonicator maintained at 37°C. PBS buffer was prepared as described above. The sample preparation was done by mixing 2.5 mL of sample (equivalent to 5 mg of Doxorubicin) with 250 mL of dissolution medium containing 2.5 g of Dowex® 50WX4 (H^+, 200-400 mesh) in a glass beaker. 1 mL of the sample was withdrawn (initial time point) and replaced with 1 mL of dissolution medium. The vessel was immediately kept in the apparatus and the sonication was started. At the end of each specified time point 1 mL of sample from the medium was withdrawn and replaced with the same volume with fresh dissolution medium equilibrated to a same temperature. Doxorubicin concentration in withdrawn samples at each specified time points were analyzed by HPLC.

***In Vitro drug release in human plasma*:** The in vitro release of PEGADRIA and Doxil® were measured in 50% Human Plasma using Electro Lab ERB-40A Bottle Rotating Apparatus maintained at 37°C. The dissolution medium was prepared by mixing human plasma and water (50:50) followed by centrifugation at 5000 rps for 10 minutes and filtration. The test sample was prepared by mixing 0.5 mL of test sample (equivalent to 1 mg of Doxorubicin) in 50 mL of dissolution medium in a Polypropylene tube. 1 mL of the sample was withdrawn (initial time point) and replaced with 1 mL of dissolution medium. The sample tubes were placed in Bottle Rotating apparatus and the apparatus were started immediately. At the end of each specified time point the apparatus was stopped and the vessel was undisturbed until the Dowex® resin was settled at the bottom before 1 mL of sample from the medium was withdrawn and replaced with the same volume with fresh dissolution medium equilibrated to a same temperature. Doxorubicin concentration in withdrawn samples at each specified time points were analyzed by HPLC.

Pre-clinical studies

All animals were purchased from Harlan Laboratories (Indianapolis, IN) and were handled as per SOPs of Nia Life Sciences (Libertyville, IL). CD2F1 mice at 8-9 weeks of age were used in efficacy study, ICR (CD-1) mice at 6-7 weeks of age and Sprague-Dawley rats at 6-7 weeks of age were used in toxicity studies. All animals were housed in temperature and humidity-controlled rooms with a 12-h light/dark cycle and were offered feed and water ad libitum. The guidelines for the Care and Use of Laboratory Animals and Standard Operating Procedures for animal well-being were followed.

***Tumor model and antitumor activity*:** Anti-tumor activity was evaluated in mouse leukemia (P388) model. To establish P388 mouse leukemia model, 0.2 mL of P388 cell suspension was i.p. injected to lower left quadrant of abdomen of each CD2F1 mouse (equivalent to 1×10^6 cells/mouse) on Day 0. On Days 1, 3, 5 and 7 each mouse received an i.v. injection (via lateral tail vein) of 5% dextrose (non-treatment control) or Doxil® or PEGADRIA at a dose level of 3 mg/kg. Injection volume (10 mL/kg) was based on individual mouse body weight. There were 7 animals in each group. Survival time of each mouse was used for antitumor activity evaluation.

Animal toxicity studies: Toxicity studies (28-day) were conducted in male and female mice and rats, respectively. Mice (5 males and 5 females in each group) received 3 i.v. injections (via tail vein) at a dose of 6 mg/kg on days 1, 8 and 15. Rats (6 males and 6 females in each group) received an i.v. injection at dose levels of 0.5, 1.0 and 1.5 mg/kg on days 1 and 8. The mortality, clinical signs of toxicity and body weight were recorded over 28 days. The organ weight, hematology, blood chemistry along with gross necropsy, histopathology were evaluated on day 29.

Clinical studies

To characterize the pharmacokinetic profile of Pegylated Liposomal Doxorubicin HCl (PEGADRIA), a multicenter, open label, balanced, randomized, two-treatment, two-period, two-sequence, single dose cross-over bioequivalence study of PEGADRIA, 2 mg/mL (test) was conducted with that of Doxil® 2 mg/ mL (reference) in ovarian cancer patients whose disease had progressed or reoccurred after platinum based chemotherapy under fasting conditions. The study was conducted according to the current version of the Declaration of Helsinki (Fortaleza, 2013) and in compliance to the current ICMR Guidelines for Biomedical Research on Human Patients, Schedule Y (amended version 2005) of Drug and Cosmetics Act, ICH GCP Guidelines and other applicable regulatory guidelines. The protocol and the Informed Consent Forms (ICF) were approved by the Independent ethics Committee (IEC) and Institutional Review Board (IRB).

A total of 29 patients were dosed in this study. The patients were randomized using SAS statistical software (SAS Institute Inc., USA; Version 9.3 or higher). As per the randomization sequence, patients were dosed with either the test or the reference product on day 1 or day 29. These patients were hospitalized for at least 11 hours before administration of the drug in both periods and were remained in the clinical facility for at least 24 hours after administration of the drug in both the periods. Following the overnight fast of at least 10 hours, patients were administered with the drug product and no food was allowed for at least 4 hours post-dose. Prophylactic medicines such as Granisetron Injection 2 mg (Antiemetic) and Dexamethasone Injection 8 mg (to avoid hypersensitivity reaction) were given to all patients before drug administration in both the periods.

The dose of Doxorubicin HCl for individual patient was calculated according to body surface area as calculated by Dubois formula. The calculated dose were diluted in 250 mL of 5% dextrose injection USP prior to the administration of both test and reference drugs for doses up to 90 mg. Doses which exceeds 90 mg were diluted in 500 mL of 5% dextrose injection USP. Patients were randomized to receive intravenous infusion of test or reference product of doxorubicin HCl injection 2 mg/mL (50 mg/m^2) by IV infusion after dilution over 1 hour (±5 minutes) as per randomization schedule. The drug was administered at a rate of 1 mg/min for first 10 minutes to minimize the risk of infusion reaction. If no infusion related reactions occurred, rate of infusion was increased uniformly to complete administration over 1 hour (±5 minutes).

A total of 24 blood samples (Post-dose samples of 4 mL each & 1 pre-dose sample of 6 mL) were collected in each period before start of infusion (Pre-dose) (within 60 minutes prior to dosing), during infusion (0.083, 0.167, 0.333, 0.500 and 0.750 hours), end of infusion (1.000 hour) and after end of infusion (1.25, 1.50, 2.00, 3.00, 4.00, 6.00, 8.00, 12.0, 16.0, 24.0, 48.0, 96.0, 120.0, 168.000, 216.0, 264.0 and 336.0 hours). The post-dose samples were collected +2 minutes of the scheduled time. The ambulatory samples scheduled at and after 48 hours were collected with an allowable deviation of 2 hours. The collected blood samples were centrifuged at 3000 ± 100 rcf or 4300 ± 100 rpm for 5 minutes at or below 10°C to separate plasma. The blood samples from each patient were kept in ice cold water bath before centrifugation and during separation. 1.5 mL of the separated plasma were transferred to pre-labeled polypropylene tube containing 300 µL of Glycerol. For pre-dose sample, 2.3 mL of separated plasma were transferred to pre-labelled polypropylene tube containing 460 µl of Glycerol. The tubes were vortexed briefly. Three aliquots were prepared from the buffered plasma. The first aliquot was used for analysis of free Doxorubicin and second aliquot was used for analysis of liposome encapsulated Doxorubicin and third lot was use as back up lot for both. All samples were stored upright at -55°C or colder until analysis.

***Bioanalytical method*:** The concentration of free Doxorubicin and liposome encapsulated doxorubicin from plasma samples were quantified using two separate LC-MS/MS methods, which were validated according to international guidelines. The analyses of patient's samples were done using a calibration curve with quality control samples, distributed throughout the batch. The details of the preparation of the calibration curve and quality control samples, analytical run organization and the analytical run criteria were followed during analysis.

***Pharmacokinetic analysis*:** The primary and secondary pharmacokinetic parameters were calculated from drug-concentration profile by non-compartmental model of Win-Nonlin Professional software, Version No. 5.3 or higher (Pharsight Corporation, USA). The maximum blood concentration (C_{max}) and the time to reach the peak concentration (T_{max}) were calculated from blood concentration vs. time profile of individual patients. The area under the plasma concentration vs. time curve from time zero to the last measurable concentration (AUC_{0-t}) was calculated by linear trapezoidal method. The area under the plasma concentration vs. time curve from time zero to infinity was calculated by dividing the last measurable concentration (C_t) by terminal elimination rate constant λ_z. The residual area in percentage was calculated by the formula ($AUC_{0-\infty}$ — AUC_{0-t})/ $AUC_{0-\infty}$ *100. The terminal half-life ($T_{1/2}$) = 0.693/ λ_z, volume of distribution (V_d = Dose/ λ_z * $AUC_{0-\infty}$), and total body clearance (CL = Dose/ $AUC_{0-\infty}$) were also calculated. Statistical analysis was performed on the data obtained from patients completing both periods. Descriptive statistics of primary and secondary pharmacokinetic parameters were computed for free Doxorubicin and liposome encapsulated Doxorubicin. The In-transformed pharmacokinetic parameters C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ were subjected to Analysis of Variance (ANOVA) for Doxorubicin.

Based on the statistical results of 90% confidence intervals for the ratio of geometric least mean for In-transformed pharmacokinetic parameters C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ conclusion were drawn whether the test formulation is bioequivalent to the reference formulation. Bioequivalence were concluded if the In-transformed 90% confidence interval falls within the acceptance range of 80-125% for pharmacokinetic parameters C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ of free Doxorubicin and liposome encapsulated Doxorubicin.

Safety and tolerability assessment: The safety and tolerability were assessed by evaluating changes from the baseline in the assessment of physical examination, clinical laboratory tests, vital sign measurements, and infusion of reference/test drug. Adverse events were assessed for severity and their relationship to the infused drug. ECG evaluations, estimation of hematology, blood chemistry, urinalysis, and hepatitis screening were also done.

Results and Discussion

Pegylated Doxorubicin Hydrochloride Liposome (PEGADRIA) was prepared similar to Doxil® by active loading process under the influence of ammonium sulfate gradient [11]. In active loading process, the drug was loaded after the liposomes are formed and the drug molecules gather inside the preformed liposomes by active transport mechanism. The loading efficiency of such active loading process was nearly 100%. The prepared PEGADRIA formulation was characterized and compared with the commercially available Doxil® product. The concentrations of drug and excipients, related substances in the drug product and Doxil® were assayed by HPLC. The concentrations of drug in both the test and the reference product were nearly 100% (Table 1). The Particle Size distribution of PEGADRIA and Doxil® were determined by dynamic light scattering method and the results showed similar mean particle size and 10%, 50% and 90% distribution for both the products. The zeta potential is influenced by the surface charge of the liposome primarily contributed by negatively charged phosphodiester moiety of DSPE-MPEG2000. The results demonstrated that the zeta potential analysis for both PEGADRIA and Doxil® are at -25.75 mV and -24.89 mV respectively. The encapsulation efficiency of Doxorubicin in PEGADRIA and Doxil® were 102.9 % and 97.9% respectively. The physicochemical characteristic comparison for both PEGADRIA and Doxil® are presented in Table 1. It was observed that both PEGADRIA and Doxil® had very similar physicochemical properties.

TESTS	Doxil®	PEGADRIA
pH	6.5	6.5
Assay	101.10%	101.40%
Cholesterol	96.10%	96.10%
HSPC	98.30%	98.80%
DSPE-MPEG2000	93.30%	93.20%
Encapsulation Efficiency	97.90%	102.90%
Particle Size (Mean diameter & % Distribution)	67.0 nm, 45.3 (D10), 64.4 (D50), 91.8 (D90)	61.2 nm, 45.1 (D10), 59.7 (D50), 79.2 (D90)
Zeta Potential (mV)	-24.89	-25.75

Table 1: Physicochemical, characterization of PEGADRIA and Doxil®.

Morphology, Size, and drug encapsulation were also assessed using transmission electron microscopy (TEM). The particles in PEGADRIA and Doxil® were almost entirely unilamellar (~99%) with no observed multivesicular particles. The doxorubicin sulfate crystal observed to be encapsulated by the liposomes and appeared as a dense round particle in the center of the liposome when viewed head-on. The particles were entirely loaded with the drug (~95-99%) and no structures that resemble free drug product were seen in the samples. The bilayer thickness in test and reference product appeared to be ~7 nm in width with about 3-5 nm PEG layer thickness. The diameter ranges of liposomes in test batches were in the range 30-90 nm while particles ranging from 25-110 nm were found in the reference product. All samples had similar mean area diameters (AED) of 51-56 nm and mean circularities of 0.84-0.87. The morphology and electron microscopy data is shown in Table 2 and Figure 1.

To characterize the physical state of the lipid bilayer and encapsulated doxorubicin, in vitro release experiments were performed under range of physiological conditions. To evaluate the effect of temperature on the lipid bilayer, the in vitro release experiments were carried out in PBS buffer, pH 6.5 at 37°C, and 47°C. Both the test product and the reference product showed similar in vitro release profile. The results confirmed that doxorubicin was slowly released from liposomes during in vitro incubation in PBS at pH 6.5 at 37°C but as expected it was found to be temperature dependent. The difference in release as a function of temperature depends on how rigid the lipid bilayer is which in turn depends on the transition temperature of the lipid composition. Both PEGADRIA and Doxil® showed similar temperature dependent pattern in the release of doxorubicin (Figure 2). In both cases, the release of doxorubicin was about 90% at 47°C after 24 hours.

PEGADRIA and Doxil® products were also evaluated to determine the effect of low-frequency ultrasound. It has been shown that the low frequency ultrasound can cause pore-like defects in the liposome membrane through which the drug is rapidly released and the pore-like effects in the membrane reseal once the low frequency ultrasound irradiation is stopped (Shroeder, Langmuir 2007). The results showed similar release profile for both PEGADRIA and Doxil® on exposure to low frequency ultrasound radiation (20 kHz) in PBS buffer, pH 6.5 at 37°C. In both cases about 40% doxorubicin was released within 30 minutes and about 90% after 4 hours of exposure to irradiation (Figure 3). Both PEGADRIA and Doxil® were also evaluated for in vitro release of doxorubicin in 50% human plasma in water at 37°C. The results show that both PEGADRIA and Doxil® are stable with almost no release after incubating for 24 hours.

The anti-leukemic efficacy of PEGADRIA and Doxil® was evaluated in male CD2F1 mice. Compare to the median survival of 12 days from

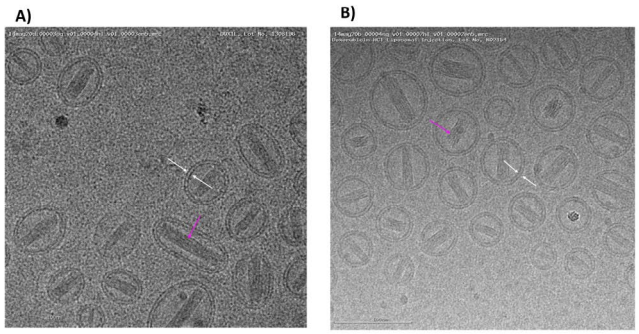

Figure 1: Transmission Electron Microscopy Study of A) Doxil® B) PEGADRIA. Higher magnification (110,000x) of the field in view. Striated Doxorubicin crystal (magenta arrow), structure that resembles a liposome bilayer (white arrows). Scale bar: 100 nm.

	Doxil®	PEGADRIA
Unilamellar	99.7%	99.0%
AED (Mean)	51.4 nm ± 12.0 nm	55.8 nm ± 13.66
Circularity (Mean)	0.84 ± 0.06	0.87 ± 0.07
Loaded	98.4%	98.8%
Bilayer thickness	~7 nm	~7 nm
PEG Layer Thickness	3-5 nm	3-5 nm
AED: area diameter		

Table 2: Morphology of PEGADRIA and Doxil® by Electron Microscopy Studies.

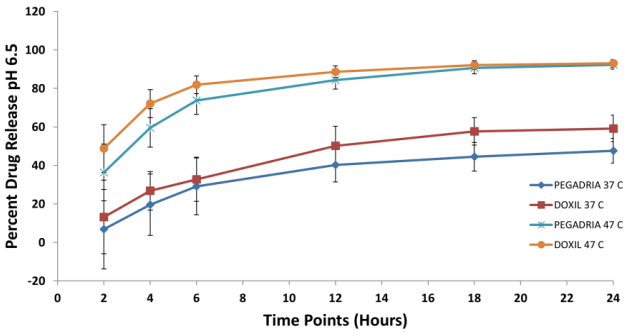

Figure 2: *In vitro* Release study of PEGADRIA and Doxil® in PBS buffer, pH 6.5 various temperatures.

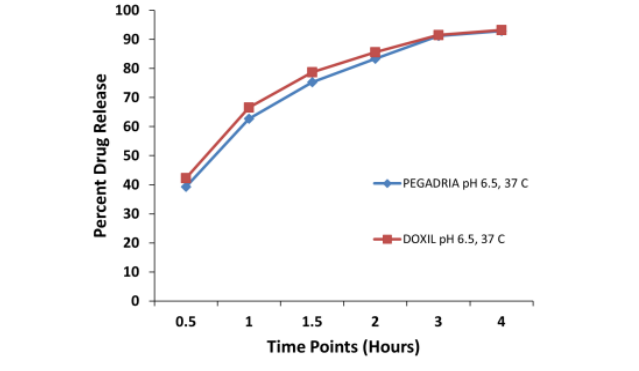

Figure 3: *In vitro* Release study of PEGADRIA and Doxil® when exposed in Low Frequency (20 kHz) Ultrasound energy.

non-treatment control group (5% dextrose), treatment with PEGADRIA or Doxil® resulted in significant increase in survival of P388-bearing leukemic mice. The median survival of PEGADRIA group was 27 days and Doxil® group was 29, respectively (Table 3). There was no statistical difference in survival between the two groups. The toxicity profiles of PEGADRIA in both mice and rats were comparable to those of Doxil®. There were no mortality, no clinical signs of toxicity in animals treated with either PEGADRIA or Doxil®. All animals were bright, alert and responsive following dose-administration and thereafter. The body weight changes from Day 1 to Day 29 were comparable between those treated with PEGADRIA and Doxil® in both mice and rats (Table 4). At the end of the study necropsy results showed that all organ weights were within the normal limits for animals treated with PEGADRIA and Doxil®. There were no abnormal changes in Complete Blood Count and Blood Chemistry values for both male and female animals treated with Doxil® or PEGADRIA. There were no histopathological changes or generic toxicity in kidneys, liver, heart, spleen and lungs in mice treated with either PEGADRIA or Doxil®. Age and environmental inflammatory responses in lungs, livers and kidneys were observed in some animals in both Doxil® and PEGADRIA. However, those inflammatory responses were judged to be within normal ranges. In rats, mild toxic effects were observed only in cardiac myocytes of some of the animals treated with Doxil® or PEGADRIA. No other toxic effects in kidneys, liver, spleen and lungs were observed in any of the animals.

Clinical pharmacokinetics and bioequivalence study was conducted in ovarian cancer patients. The study was evaluated based on 29 patients who complied with all the inclusion and exclusion criteria. Plasma concentrations of Doxorubicin from human patients were measured to determine the pharmacokinetics profile. The detailed pharmacokinetic parameters of the test and reference formulation on a single dosing are shown in Tables 5A and 6A. It is to be noted that pharmacokinetic properties including C_{max}, AUC, T_{max}, CL, V_d and $t_{1/2}$ for test and reference product were in the same range as and similar.

To evaluate bioequivalence, the 90% confidence interval (CIs) for the test/reference ratio of long-transformed C_{max}, AUC_{0-t}, and $AUC_{0-\alpha}$ are shown in Tables 5B and 6B. The geometric mean ratios (90% CI) of PEGADRIA /Doxil® for free Doxorubicin were 84.64-119.97% for C_{max}, 87.96-108.71% for AUC_{0-t}; and 94.41-112.21% for $AUC_{0-\infty}$. The 90% CI for encapsulated doxorubicin were 95.56-103.68% for C_{max}, 89.65-104.32% for AUC_{0-t}, and 92.09-105.83% for $AUC_{0-\infty}$. These values indicated that PEGADRIA is bioequivalent to Doxil® with respect to C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ of free doxorubicin and liposome encapsulated doxorubicin under fasting condition in ovarian cancer patients. The 90% CI were completely contained within the predefined bioequivalence FDA accepted criteria of 80-125% for the primary endpoint of C_{max} and AUC.

There were a total of 60 adverse events (AEs) and 03 pre-dose AEs during the conduct of study. Thirty-four (34) were reported after receipt of Doxil® in 14 (51.85%) patients whereas, and twenty-six (26) AEs were reported in 11 (39.29%) of patients after receipt of PEGADRIA. One (01) death and one (01) other serious adverse event were reported

Group	Median Survival (day)	Increased Survival (%T/C)
Control	12	100
Doxil®	29	259 ± 23
PEGADRIA	27	241 ± 33
%T/C: (100 × Survival of Treatment / Survival of Control); Data represent Mean ± SD, (N = 7)		

Table 3: Efficacy of PEGADRIA and Doxil® in P388-bearing leukemic CD2F1 mice. Median survival and increased survival.

	Dose (mg/kg)	Gender	Changes in Body Weight (%BW_{29}/BW_1)	
			Doxil®	PEGADRIA
Mice (N = 5)	6	Male	109.6 ± 5.1	112.1 ± 7.0
		Female	117.2 ± 3.0	116.7 ± 11.9
Rats (N = 6)	0.5	Male	151.7 ± 6.1	146.8 ± 8.1
		Female	140.9 ± 7.7	142.2 ± 9.9
	1.0	Male	147.2 ± 6.1	137.8 ± 6.5
		Female	137.8 ± 4.5	137.2 ± 5.3
	1.5	Male	146.3 ± 3.4	134.7 ± 10.9
		Female	136.8 ± 5.5	141.6 ± 7.7

%BW_{29}/BW_1 = 100 × BW_{29}/BW_1. BW_1: Body weight on day 1; BW_{29}: Body weight on day 29; Data represent mean ± SD.

Table 4: Toxicity of PEGADRIA and Doxil® in ICR (CD-1) mice and Sprague-Dawley rats. Changes in body weight of mice and rats.

A. Pharmacokinetics parameters

Parameters (Units)	Free Doxorubicin (N = 23) Mean ± SD (Un-transformed data)	
	PEGADRIA	DOXIL®
T_{max} (h)*	16.00 (4.0-48.00)	24.00 (0.950-72.00)
C_{max} (ng/ml)	750.5 ± 422.6	687.5 ± 388.9
AUC_{0-t} (ng.h/ml)	83282 ± 36407	83253 ± 33591
$AUC_{0-\infty}$ (ng.h/ml)	91793 ± 37710	90447 ± 36597
λ_z (1 /h)	0.009 ± 0.004	0.010 ± 0.004
$t_{½}$ (h)	85.17 ± 27.7	80.18 ± 28.09
AUC_% Extrap_Obs (%)	7.27 ± 5.63	6.37 ± 4.59
V_d (L)	118.19 ± 75.83	114.61 ± 75.25
Cl (L/ h)	0.958 ± 0.476	0.988 ± 0.577

*T_{max} is represented in median value

B. Relative bioequivalence

Parameters	Geometric Least Squares Mean		ISCV (%)	T/R Ratio (%)	90% CI
	PEGADRIA	DOXIL®			
lnC_{max}	608.44	603.78	34.7	100.8	84.64 - 119.97
$lnAUC_{0-t}$	74011.5	75687.3	20.7	97.8	87.96 - 108.71
$lnAUC_{0-\infty}$	85215.8	81868.1	17.8	104.1	93.78 - 115.53

ISCV: Intra subject coefficient of variation; CI: Confidence interval

Table 5: Pharmacokinetic parameters and Relative Bioequivalence of free doxorubicin for PEGADRIA and DOXIL®.

A. Pharmacokinetic parameters

Parameters (Units)	Encapsulated Doxorubicin (N = 23) Mean ± SD (Un-transformed data)	
	PEGADRIA	DOXIL
T_{max} (h)*	2.00 (1.08 - 8.00)	2.00 (0.97-8.00)
C_{max} (ng/ml)	38171.4 ± 4980.7	38307.7 ± 6638.1
AUC_{0-t} (ng.h/ml)	3282967 ± 1059002	3300315 ± 804859
$AUC_{0-\infty}$ (ng.h/ml)	3573474± 1201583	3503506 ± 969677
λ_z (1 /h)	0.011± 0.0043	0.012 ± 0.0060
$t_{½}$ (h)	73.06 ± 23.68	68.07 ± 29.92
AUC_% Extrap_Obs (%)	4.74 ± 3.96	5.03 ± 4.53
V_d (L)	2.28 ± 0.71	2.12 ± 0.69
Cl (L/ h)	0.023 ± 0.009	0.023 ± 0.008

*T_{max} is represented in median value

B. Relative bioequivalence

Parameters	Geometric Least Squares Mean		ISCV (%)	T/R Ratio (%)	90% CI
	PEGADRIA	DOXIL®			
lnC_{max}	38412.8	38591.5	7.9	99.5	95.56-103.68
$lnAUC_{0-t}$	3253252.7	3364131.8	14,7	96.7	89.65-104.32
$lnAUC_{0-\infty}$	3523579.7	3569206.4	13.1	92.09	105.83

ISCV: Intra subject coefficient of variation; CI: Confidence interval

Table 6: Pharmacokinetic parameters and relative bioequivalence of liposome encapsulated doxorubicin for PEGADRIA and DOXIL®

during the conduct of study. The causality assessment was judged as unlikely for the death and other serious adverse event. Overall, data from the study demonstrated that both PEGADRIA and the Doxil® were well tolerated.

Conclusion

Pegylated Doxorubicin Hydrochloride Liposome (PEGADRIA) demonstrated similarity with Doxil® in physicochemical, pre-clinical efficacy and safety profiles. It also showed bioequivalence in patients with ovarian cancer to the marketed Doxil® product. Hence, PEGADRIA can be successfully developed as a generic product of Doxil®.

Acknowledgements

The authors would like to acknowledge Nanoimaging Services Inc. San Diego, USA for electron microscopy data.

References

1. Myers CE, Chabner BA (1990) In cancer Chemotherapy. Lippincott, Philadelphia, USA.
2. Abraham SA, Waterhouse DN, Mayer LD, Cullis PR, Madden TD, et al. (2005) The liposomal formulation of doxorubicin. Methods Enzymol 391: 71-97.
3. Gabizon AA, Lyass O, Berry GJ, Wildgust M (2004) Cardiac safety of pegylated liposomal doxorubicin (Doxil®/Caelyx) demonstrated by endomyocardial biopsy in patients with advanced malignancies. Cancer Invest 22: 663-669.
4. Leonard RCF, Williams S, Tulpule A, Levine AM, Oliveros S (2009) Improving the therapeutic index of anthracycline chemotherapy: focus on liposomal doxorubicin (Myocet®). The Breast 18: 218-224.
5. Soloman R, Gabizon AA (2008) Clinical pharmacology of liposomal anthracyclines: focus on pegylated liposomal Doxorubicin. Clin Lymphoma Myeloma 8: 21-32.
6. Alberts DS, Muggia FM, Carmichael J, Winer EP, Jahanzeb M (2004) Efficacy and safety of liposomal anthracyclines in phase I/II clinical trials. Seminars in Oncology 31: 53-90.
7. Torchilin VP1 (2005) Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 4: 145-160.
8. Gabizon AA, Shmeeda H, Zalipsky S (2006) Pros and cons of the liposome platform in cancer drug targeting. J Liposome Res 16: 175-183.
9. Gabizon A, Shmeeda H, Barenholz Y (2003) Pharmacokinetics of pegylated liposomal Doxorubicin: review of animal and human studies. Clin Pharmacokinet 42: 419-436.
10. (2015) Doxil® (Doxorubicin hydrochloride liposome injection). TTY Biopharm Company Limited, Taoyuan, Taiwan.
11. Martin FJ (2005) DOXIL, the development of pegylated liposomal Doxorubicin. Burgess DJ, Taylor and Francis, Boca Raton, Florida, USA.