Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1663 (2004) 167–177
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Drug release rate influences the pharmacokinetics, biodistribution, therapeutic activity, and toxicity of pegylated liposomal doxorubicin formulations in murine breast cancer

Gregory J.R. Charrois, Theresa M. Allen*

Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Received 12 January 2004; received in revised form 11 March 2004; accepted 11 March 2004

Available online 12 April 2004

Abstract

The pharmacokinetics (PK), biodistribution (BD), and therapeutic activity of pegylated liposomal doxorubicin formulations with different drug release rates were studied in an orthotopic 4T1 murine mammary carcinoma model. The focus of these experiments was to study the effects of different release rates on the accumulation of liposomal lipid and doxorubicin (DXR) into the tumor and cutaneous tissues of mice (skin and paws). These tissues were chosen because the clinical formulation of pegylated liposomal doxorubicin (Caelyx®/Doxil®) causes mucocutaneous reactions such as palmar-plantar erythrodysesthesia (PPE). Liposomes with different doxorubicin (DXR) leakage rates were prepared by altering liposome fluidity through changing the fatty acyl chain length and/or degree of saturation of the phosphatidylcholine component of the liposome. Liposomes with fast, intermediate, and slow rates of drug release were studied. The plasma PK of the liposomal lipid was similar for all formulations, while the plasma PK of the DXR component was dependent on the liposome formulation. Liposomal lipid accumulated to similar levels in tumor and cutaneous tissues for all three formulations tested, while the liposomes with the slowest rates of DXR release produced the highest DXR concentrations in both cutaneous tissues and in tumor. Liposomes with the fastest drug release rates resulted in low DXR concentrations in cutaneous tissues and tumor. The formulation with intermediate release rates produced unexpected toxicity that was not related to the lipid content of the formulation. The liposomes with the slowest rate of drug leakage had the best therapeutic activity of the formulations tested.

Keywords: Pegylated liposomal doxorubicin; Biodistribution; Pharmacokinetic; Breast cancer; Drug release rate

1. Introduction

Drug release rates, liposome diameter and circulation half-life play an important role in determining the therapeutic activity and toxicity of liposomal drug delivery systems in animal models of cancer [1–3]. Small diameters and long circulation half-lives allow liposomal drugs to accumulate in tissues with increased vascular permeability (e.g., tumor tissue), but only if liposomes retain their drug load over several hours to days will high drug concentrations accumulate in tumor tissues [4]. After liposomes have localized to tumors, the drug must be released, i.e., become bioavailable, in order to exert its biological effects. However, if liposomes release their drug content at a rate that is rapid compared to the rate of tissue accumulation, then their therapeutic activity may be compromised. In this paper, we have examined the effect of different drug release rates on the accumulation of liposomal lipid and liposomal contents (doxorubicin, DXR) in mucocutaneous tissues and tumors in orthotopic murine breast cancer models.

Clinically, the importance of the drug release rate for the pharmacokinetics (PK) and toxicity of liposomal formula-

Abbreviations: CHOL, cholesterol; C_{max}, maximal tissue concentration; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DXR, doxorubicin; FBS, fetal bovine serum; ^{3}H-CHE, ^{3}H-cholesteryl hexadecylether; HBS, HEPES-buffered saline pH 7.4; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSPC, fully hydrogenated soy phosphatidylcholine; MEM, minimal essential medium; mPEG-DSPE, methoxypolyethyeneglycol (M_r 2000)-distearoylphosphatidylethanolamine; PMPC, palmitoyl-myristoyl-phosphatidylcholine; POPC, palmitoyl-oleoylphosphatidylcholine; S.D., standard deviation; SMPC, stearoyl-myristoylphosphatidylcholine

* Corresponding author. Tel.: +1-780-492-5710; fax: +1-780-492-8078.

E-mail address: terry.allen@ualberta.ca (T.M. Allen).

0005-2736/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2004.03.006

tions is highlighted by the differences between Caelyx® (Doxil® in the United States) and Myocet™, two liposomal formulations of doxorubicin (DXR) (reviewed in Ref. [5]). Caelyx® is a STEALTH® liposomal formulation of DXR; its rigid bilayer is composed of fully hydrogenated soy phosphatidylcholine (HSPC), cholesterol (CHOL), and methoxypolyethyene glycol (M_r 2000)-distearoylphosphatidylethanolamine (mPEG-DSPE) at a molar ratio of 55:40:5 [6]. Myocet™ is composed of egg yolk phosphatidylcholine and CHOL at a molar ratio of 55:45 and has a fluid bilayer [7]. The different lipid compositions of these two formulations are primarily responsible for the differences in the PK of the encapsulated DXR. Myocet™ has a much faster rate of drug release than Caelyx®, as evidenced by the much shorter half life (6.7 vs. 45 h) and larger volume of distribution of DXR released from Myocet™ (18.8 vs. 4.1 l) [7,8]. The PK of the encapsulated DXR, in turn, influences the toxicity profile of each formulation. Compared to conventionally administered DXR, both liposome formulations show reduced cardiac toxicity [9,10]. The acute dose-limiting toxicity of the Myocet™ formulation is myelosuppression, similar to that of conventionally administered DXR [7]. Caelyx® has a low incidence of myelosuppression and its dose-limiting toxicities are mucocutaneous reactions such as stomatitis and palmar-plantar erythrodysesthesia (PPE) [11,12]. Clinically, the incidence of PPE is related to the dose intensity of Caelyx® therapy, with an increased incidence in patients receiving >10–12 mg/m^2/week [12,13]. Caelyx®-induced PPE develops primarily on the hands and feet and can lead to severe pain and blistering desquamation [11,12,14]. PPE is hypothesized to develop as DXR-loaded liposomes localize to cutaneous tissues and slowly release DXR, which then damages the basal layers of the skin. Current attempts to reduce the incidence and severity of PPE have not proven effective; using a murine model to develop a better understanding of the mechanisms underlying this toxicity may lead to newer strategies to reduce this painful toxicity [15,16].

Caelyx® has dose-independent, log-linear PK (reviewed in Ref. [17]), small size (100 nm diameter), slow rates of drug release and long circulation times allowing it to extravasate into diseased tissues through the 'enhanced permeability and retention' or EPR effect [18,19]. Consequently, Caelyx®, or liposomes with similar properties, mediate accumulation of encapsulated drugs in solid tumors, which has translated into improved therapeutic activity relative to free (non-encapsulated) drugs [20,21]. However, long-circulating liposomes also accumulate in the cutaneous tissues of experimental animals to a greater extent than formulations with shorter half-lives, supporting the hypothesis that cutaneous accumulation of drug-loaded liposomes underlies the development of PPE [22–25].

Previous work from our laboratory in the 4T1 murine mammary carcinoma model demonstrated that liposomes accumulate in tumor tissue at higher concentrations and at a faster rate than in cutaneous tissues such as skin and paws [26]. Peak skin and paw DXR levels occurred as long as 48 h after peak tumor DXR levels. These findings led to the hypothesis that the DXR concentration in cutaneous tissues, relative to tumor DXR levels, could be reduced if the rate of liposomal drug release was increased. By manipulating drug release rates, it may thus be possible to reduce cutaneous toxicities without affecting the therapeutic activity of pegylated liposomal doxorubicin.

To explore this hypothesis, we prepared pegylated liposomal formulations of DXR with different rates of drug release. Fatty acyl chain length and saturation are important determinants of liposomal drug retention in vivo and in vitro [1,27,28]. Hence, pegylated liposomes with faster, intermediate, and slower rates of DXR leakage were formulated by altering the fatty acyl chain length and degree of saturation of phosphatidylcholine, the bilayer-forming element in the liposomes. The PK, biodistribution (BD), and therapeutic activity of the three formulations was examined in the 4T1 murine mammary carcinoma model.

2. Materials and methods

2.1. Materials

Caelyx®/Doxil® (STEALTH® liposomal doxorubicin), doxorubicin hydrochloride (DXR), and methoxypolyethyleneglycol (M_r 2000)-distearoylphosphatidylethanolamine ($mPEG_{2000}$-DSPE) were from ALZA Corporation (Mountain View, CA). Dimyristoylphosphatidylcholine (DMPC), dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), palmitoyl-myristoylphosphatidylcholine (PMPC), palmitoyl-oleoylphosphatidylcholine (POPC), stearoyl-myristoylphosphatidylcholine (SMPC), and cholesterol (CHOL) were purchased from Avanti Polar Lipids (Alabaster, AL). Sephadex-G50 was from Amersham-Pharmacia Biotech (Baie d'Urfe, PQ, Canada). Minimal essential medium (MEM) was from Sigma Chemical Company (St. Louis, MO) and fetal bovine serum (FBS), penicillin, and streptomycin were from Life Technologies Inc. (Burlington, ON, Canada). Halothane was from MTC Pharmaceuticals (Cambridge, ON, Canada). Sterile, pyrogen-free saline was purchased from Baxter (Toronto, ON, Canada), and sterile saline was supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4 (HEPES-buffered saline; HBS). ^{3}H-Cholesteryl hexadecylether (^{3}H-CHE) was from Perkin Elmer Biosciences (Boston, MA). Solvable and Ultima Gold were from Perkin Elmer Life Sciences (Woodbridge, ON, Canada). All other chemicals were of the highest grade possible.

2.2. Liposome preparation

All liposomal formulations were composed of the bilayer-forming phospholipid phosphatidylcholine, with fatty acyl chains of various lengths and degrees of saturation, in combination with CHOL (2:1 molar ratio) and contained

5 mol% (relative to phospholipid) of mPEG-DSPE. Liposomes were prepared by the thin film hydration technique, and DXR was remote-loaded using an ammonium sulfate gradient as previously described [29,30]. Briefly, dried lipid films were hydrated with 250 mM ammonium sulfate. Using a Lipex Extruder (Northern Lipids, Vancouver, BC, Canada), the lipid mix was extruded through stacked Nuclepore filters to yield small unilamellar vesicles. The temperature of extrusion depended on the phosphatidylcholine component of the mixture. Fluid lipids whose phase transition temperatures are below room temperature (POPC, DOPC) were extruded at room temperature; other lipids were also extruded slightly above their phase transition temperatures (DSPC was extruded at 65 °C, SMPC and PMPC were extruded at 35 °C, and DMPC was extruded at 30 °C). Liposomes were sized by dynamic light scattering using a Brookhaven BI-90 Particle Sizer (Brookhaven Instruments, Holtsville, NY). All liposomes had a mean diameter of 100 ± 20 nm. After sizing, the external buffer was exchanged by passage over a Sephadex G-50 column in sodium acetate buffer (pH 5.5). DXR in 10% sucrose (wt/vol) was then incubated with the liposomes at a 0.2:1 (wt/wt) drug to lipid ratio. Small amounts of residual non-encapsulated DXR were removed by passage over a Sephadex G-50 column equilibrated with HBS, pH 7.4. For PK and BD experiments, the non-exchanged, non-metabolized lipid marker ^{3}H-CHE (2 μCi/μmol lipid) was incorporated into the lipid film. Lipid concentrations were determined by using the specific activity of ^{3}H-CHE, and DXR concentrations were calculated from the absorption at 480 nm in methanol by comparison to a standard curve.

2.3. In vitro release rate of DXR in serum

The in vitro leakage of DXR from liposomes was measured using a dialysis method. Liposomes, at a concentration of 0.5 mM phospholipid, were diluted in 50% adult bovine serum (ABS) in HBS, pH 7.4, placed in a dialysis cassette with a molecular weight cutoff of 10 kDa, and dialyzed against 200 ml of 50% ABS containing penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C [31]. ABS was used instead of human plasma as a source of plasma proteins due to difficulties in obtaining human plasma. The concentration of lipid was selected to approximate the liposome concentration expected in the blood compartment of a 20-g mouse receiving liposomal DXR at a dose of 5–6 mg DXR/kg body weight. At various time points, aliquots were withdrawn from the cassette and stored at 4 °C until analysis. Total DXR concentration, including possible fluorescent metabolites, was measured by fluorescence at λ_{ex} 470, λ_{em} 590 in methanol acidified with 0.075 N HCl, as previously described [21] using a SLM-AMINCO Model 8100 Series 2 Spectrometer (Spectronics Instruments Inc., Rochester, NY). The results were plotted as log percent of initial fluorescence versus time, and the $t_{1/2}$ was calculated using the regression line from the linear portion of the curve; r^2 values for these lines were greater than 0.926 (range 0.926–0.994).

2.4. Tumor implantation

Female BALB/c cr Alt BM mice (6–8 weeks) were purchased from the breeding colony at the University of Alberta's Health Sciences Laboratory Animal Services. Mice were housed under standard conditions and had access to food and water ad libitum. All protocols were approved by the Health Sciences Animal Policy and Welfare Committee, University of Alberta, and are in accordance with the Guide to the Care and Use of Experimental Animals set forth by the Canadian Council on Animal Care.

The 4T1 murine mammary carcinoma, a metastatic, thioguanine-resistant cell line, was a generous gift from Dr. Fred Miller (Barbara Ann Karmanlos Cancer Institute, Detroit, MI) [32]. The cell line was maintained in MEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. Tumors were orthotopically implanted as previously described [33]. Briefly, mice were anesthetized with halothane, the lower abdomen was shaved, and a small incision (6–8 mm) was made adjacent to the mid-line. The right #4 mammary fat pad was exposed, and 10^5 cells in 10-μl media were implanted. The wound was closed with a surgical wound clip that was removed 7 days later.

2.5. Pharmacokinetic and biodistribution studies

In initial experiments, the plasma total DXR concentrations, which included possible fluorescent metabolites, were determined 4 h after injection of various liposomal formulations of DXR in naïve mice. The tested formulations were DSPC:CHOL:mPEG-DSPE, SMPC:CHOL:mPEG-DSPE, POPC:CHOL:mPEG-DSPE, PMPC:CHOL:mPEG-DSPE, DOPC:CHOL:mPEG-DSPE, and DMPC:CHOL:mPEG-DSPE, all at a 2:1:0.1 molar ratio, and Caelyx®. Female BALB/c mice (6–8 weeks) were injected with 6 mg DXR/kg (18 mg/m^2) as liposomal DXR, prepared as above. Four hours after injection, mice were euthanized by halothane anesthesia followed by cervical dislocation. Whole blood was collected via cardiac puncture using a heparinized syringe, and plasma prepared by centrifugation at $3000 \times g$ for 5 min. DXR concentrations were determined as described below.

In other experiments, mice were implanted with the 4T1 murine mammary carcinoma as described above. Ten days after tumor implantation, when the tumors were large enough to excise, mice were injected with 6 mg DXR/kg (18 mg/m^2) liposomal doxorubicin with varying drug release rates (DSPC:CHOL:mPEG-DSPE, POPC:CHOL:mPEG-DSPE, or DOPC:CHOL:mPEG-DSPE at 2:1:0.1 molar ratios). At various time points after injection (1, 12, 24, 48, 72, 168 h), mice (five per group) were euthanized,

and tissue doxorubicin and lipid were determined as described in Section 2.6. The area under the plasma or tissue concentration-versus-time curve (AUC) was calculated using the trapezoidal rule with extrapolation to infinity, and $t_{1/2}$ was calculated using the formula $t_{1/2} = 0.693/k_{elm}$, where k_{elm} is the elimination constant derived from the best-fit line of the data plotted on a semi-log plot. Clearance (CL) was calculated using the formula $CL = dose/AUC_{0-\infty}$ [34]. V_d was estimated by dividing the injected dose by the plasma concentration extrapolated back to time zero using all five data points for DSPC:CHOL:mPEG-DSPE and POPC:CHOL:mPEG-DSPE liposomes and the first three data points for DOPC:CHOL:mPEG-DSPE liposomes.

2.6. Determination of doxorubicin and liposomal lipid

For PK and BD experiments, total doxorubicin and liposomal lipid were quantified using a method similar to the one described by Harasym et al. [35]. Tissue homogenates (10% wt/vol) were prepared in water using a Polytron homogenizer (Brinkman Instruments, Mississauga, ON, Canada). Dorsal skin samples and samples of paws from below the wrist joint were first frozen in liquid nitrogen and pulverized with a pestle and mortar before homogenization. Plasma was prepared as described above. Homogenates (200 μl) or 25% plasma in water (200 μl) was extracted overnight at −25 °C by adding first 100-μl 10% Triton-X 100 and 200-μl water and then 1500-μl acidified isopropanol (0.75 N HCl) [21]. The next day, the tubes were warmed to room temperature, vortexed for 5 min, spun at 15,000 × *g* for 20 min, and stored at −80 °C until analysis. Total DXR was quantified spectrofluorometrically at λ_{ex} 470 nm and λ_{em} 590 nm. To correct for background fluorescence, a standard curve was obtained by spiking tissue extracts derived from mice that had not received drug with DXR. This method was shown to recover on average >90% of DXR-associated fluorescence. Tissue concentrations of DXR were expressed as microequivalents per milliliter of plasma or per gram of tissue, as this assay does not discriminate between doxorubicin and any fluorescent metabolites with similar excitation and emission profiles that may have been present.

Liposomal lipid was quantitated using a method similar to the one described by Harasym et al. [35], using the non-exchangeable and non-metabolizeable lipid marker ^{3}H-CHE at a concentration of 2 μCi/μmol total lipid as a tracer. Briefly, 500 μl of Solvable® was added to 200 μl of homogenate or 50 μl of plasma. The solutions were then digested for 2 h at 60 °C. After the vials had cooled to room temperature, 50 μl of 200 mM EDTA was added, and the samples were bleached overnight with 200 μl of hydrogen peroxide (30% vol/vol). The next day, 100 μl of 1 N HCl and 5 ml of Ultima Gold scintillation fluor were added in sequence, and the samples were counted in a Beckman LS 6500 liquid scintillation counter. Lipid concentrations are expressed as micrograms of total lipid per milliliter of plasma or per gram of tissue. For both liposomal lipid and DXR, values given represent the mean ± S.D. of triplicate samples from five mice.

2.7. Tumor growth delay studies

Mice were implanted with the 4T1 tumor as described above. Four days after tumor implantation, groups of six mice were injected intravenously with liposomal DXR (6 mg DXR/kg, 18 mg/m^2) composed either of DSPC:CHOL:mPEG-DSPE, POPC:CHOL:mPEG-DSPE, or DOPC:CHOL:mPEG-DSPE (2:1:0.1 molar ratio); control mice received 200 μl of sterile saline. Because the 4T1 tumor grows very rapidly, mice were treated 4 days after tumor implantation when tumors were just palpable, instead of after 10 days as in the PK and BD experiments. Tumor growth was measured using calipers, and volume was calculated using the formula $v = 0.4ab^2$ where a and b are perpendicular diameters and $a>b$ [33]. The values given represent the mean tumor volume ± S.D. from two separate experiments.

2.8. Statistics

Statistical comparisons were performed using a multiple-comparison ANOVA assay with a Tukey–Krammer post test with Graph Pad InStat Version 3.01 for Windows 95/NT (GraphPad Software, San Diego CA).

3. Results

3.1. In vitro leakage of DXR from liposomes

The results of in vitro DXR leakage experiments are presented in Table 1. Incorporating phosphatidylcholines with lower phase transition temperature into the liposomal membrane (i.e., lipids with unsaturated and/or shorter fatty acyl chains) increased drug leakage rates compared to liposomes containing lipids with higher phase transition temperatures. Based on the in vitro data, a range of formulations was used for in vivo studies.

3.2. Plasma doxorubicin concentrations 4 h after injection

Based on the data presented in Table 2, three liposome compositions were selected for in vivo testing. To determine in vivo drug retention of those liposome formulations, plasma DXR concentrations were determined in naïve BALB/c mice at 4 h after injection of 6 mg DXR/kg as liposomal DXR. Plasma levels provide a good measure of drug retention, since DXR released in plasma rapidly redistributes to other tissues (greater than 95% of circulating DXR is entrapped within STEALTH® liposomes [8,36]). In our experiments, liposomes were composed of DSPC, SMPC, POPC, DOPC, or DMPC with 50 mol% CHOL

Table 1
Leakage half-lives of DXR from liposomes

Lipid composition	Phase transition temperature of phosphatidylcholine (°C)	Ratio (mol)	n	$t_{1/2}$ (h)
Caelyx (HSPC:CHOL:mPEG)	55	55:40:5	5	118.4 ± 18.8
HSPC:CHOL:mPEG	55	2:1:0.1	5	91.8 ± 11.2
DMPC:CHOL:mPEG	23	2:1:0.1	4	23.0 ± 6.4
POPC:CHOL:mPEG	− 2	2:1:0.1	2	14.6, 11.9
DOPC:CHOL:mPEG	− 20	2:1:0.1	2	14.9, 10.2

Liposomes (0.5 mM phospholipid) were placed inside a dialysis cassette (MW cutoff 10 kD) and incubated at 37 °C in adult bovine serum in HBS, pH 7.4 (50% v/v). At various time points, aliquots were withdrawn, and the DXR was extracted in acidified methanol and measured fluorometrically. Data were then plotted on a semi-log scale as percent of initial fluorescence vs. time. Half-lives were determined from the linear portion of each curve by using regression analysis. Half-lives represent the mean ± S.D. of n experiments; for $n < 3$, the individual half-lives are given for each experiment.

and 5 mol% mPEG-DSPE. The synthetic lipid DSPC was used instead of HSPC as it has a similar phase transition temperature to HSPC; Caelyx® was tested as well. Liposomes that contained phosphatidylcholines with long, saturated fatty acyl chains had a more rigid bilayer, and gave rise to higher plasma DXR concentrations at 4 h after injection than more fluid DXR-loaded liposomes. Based on these results, liposomes with faster (DOPC:CHOL:mPEG-DSPE), intermediate (POPC:CHOL:mPEG-DSPE) and slower (DSPC:CHOL:mPEG-DSPE) rates of drug release were chosen for further study in tumor-bearing mice.

3.3. Pharmacokinetic and biodistribution experiments in tumor-bearing mice

Plasma concentrations of DXR and liposomal lipid, and also drug to liposomal lipid ratios, are presented in Fig. 1 for tumor-bearing mice receiving various formulations of liposomal DXR. Liposomes composed of DSPC:CHOL:mPEG-DSPE (slower release) achieved the highest plasma concentration of DXR (Fig. 1A), which was similar to that we have previously reported for Caelyx® [37]. Liposomes composed of POPC:CHOL:mPEG-DSPE had intermediate release and DOPC:CHOL:mPEG-DSPE liposomes had the fastest release. PK parameters given in Table 3 demonstrate that, for DSPC-containing liposomes, the plasma $t_{1/2}$ for DXR was longest and approximated that of the liposomal lipid. Further, the DSPC liposomes showed the slowest rate of

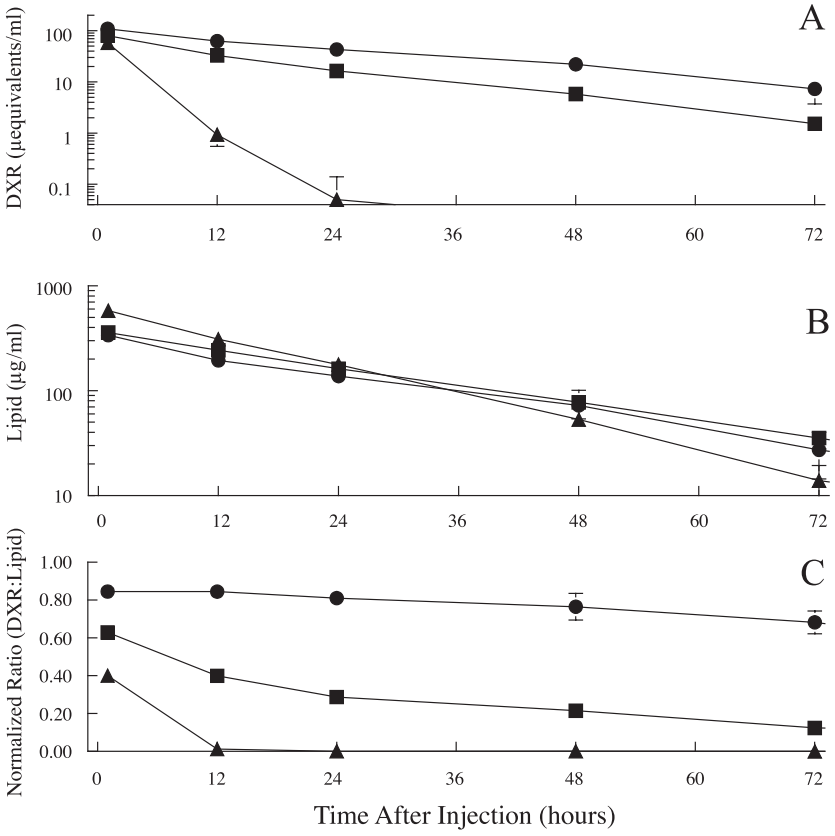

Fig. 1. Plasma concentrations of DXR (panel A), liposomal lipid (panel B), and normalized drug to lipid ratios (panel C). BALB/c mice bearing the 4T1 murine mammary carcinoma were injected with 6 mg/kg liposomal DXR and 30 mg/kg lipid (●, DSPC:CHOL:mPEG-DSPE; ■, POPC:CHOL:mPEG-DSPE; ▲, DOPC:CHOL:mPEG-DSPE at a molar ratio of 2:1:0.1) 10 days after tumor implantation. Drug and lipid were quantified as detailed in text. The results represent the mean ± S.D. from triplicate samples from five mice per time point. Drug to lipid ratios in plasma were normalized to the drug to lipid ratio of the injected liposomes.

DXR clearance among the tested formulations, indicating that the drug is stably retained within this formulation. The V_d's for DXR increased with increasing rates of drug release, and is consistent with faster drug leakage followed by redistribution and metabolism. Although the plasma PK for DXR differed for each formulation, the plasma PK for the liposomal lipid, measured using a lipid label (^{3}H-CHE), were similar for each formulation (Fig. 1B and Table 3), showing comparable values for AUC, $t_{1/2}$, CL, and V_d (Table 3). Drug to lipid ratios decreased as liposome contents were lost and were lower for the more fluid formulations (Fig. 1C). The decrease in drug to lipid ratios paralleled the decrease in plasma concentrations of DXR for each formulation. In other words, for formulations with more rapid release rates, drug-depleted liposomes are present in circulation.

The accumulation of DXR and liposomal lipid into 4T1 mammary tumors is presented in Fig. 2. The most stable liposomes, composed of DSPC:CHOL:mPEG-DSPE, delivered the highest amount of total DXR into tumors, leading to tumor DXR concentrations far exceeding those achieved with POPC:CHOL:mPEG-DSPE or DOPC:CHOL:mPEG-DSPE liposomes (Fig. 2A). This trend is reflected in the respective AUCs (Table 3). The time course for DXR accumulation in tumor was also different for the three formulations. Peak tumor DXR concentration (C_{max}) was reached at 24 h with liposomes composed of DSPC:CHOL:mPEG-DSPE, while C_{max} was reached at 12 h for POPC:CHOL:mPEG and DOPC:CHOL:mPEG-DSPE liposomes (Fig. 2A). The earlier time to peak concentrations for the more leaky formulations may reflect the delivery of some of the released drug to the tumor tissue in the free form. In contrast, the accumulation of liposomal lipid was similar for all three formulations, with tumor concentrations of lipid plateauing by 24 h (Fig. 2B). The lower concentration of drug in the tumor relative to lipid for the more leaky formulations suggests that drug-depleted liposomes were accumulating in tumor. Liposomal lipid (i.e., radiolabelled ^{3}H-CHE) did not appear to be substantially cleared from the tumor over the time course of the experiments. For DXR, gradual clearance from the tumor could be observed over the time course of the experiment. As can be seen from the clearance $t_{1/2}$'s for DXR from tumor for the different liposomal formulations, DSPC:CHOL:mPEG-DSPE was the most stable, followed by the POPC and DOPC formulations (Table 3).

Accumulation of total DXR and lipid into skin and paws is shown in Figs. 3 and 4, respectively. In these tissues, DXR concentrations peaked later and at lower levels than in tumor. Lipid levels in skin and paws continued to increase over several days, and lipid accumulation in these tissues was independent of the formulation used (Table 3). Furthermore, the lipid data for both skin and paws suggest that drug-depleted liposomes continued to accumulate in these

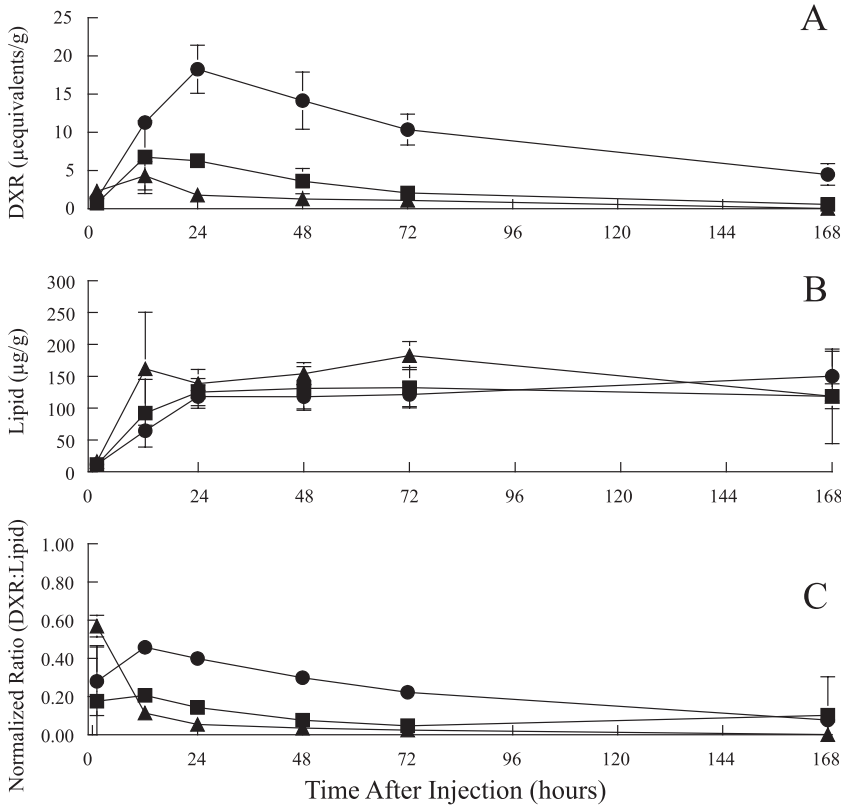

Fig. 2. Tumor concentrations of DXR (panel A), liposomal lipid (panel B) and normalized drug to lipid ratios (panel C). (● DSPC:CHOL:mPEG-DSPE; ■ POPC:CHOL:mPEG-DSPE; ▲ DOPC:CHOL:mPEG-DSPE at a molar ratio of 2:1:0.1). Details as in Fig. 1.

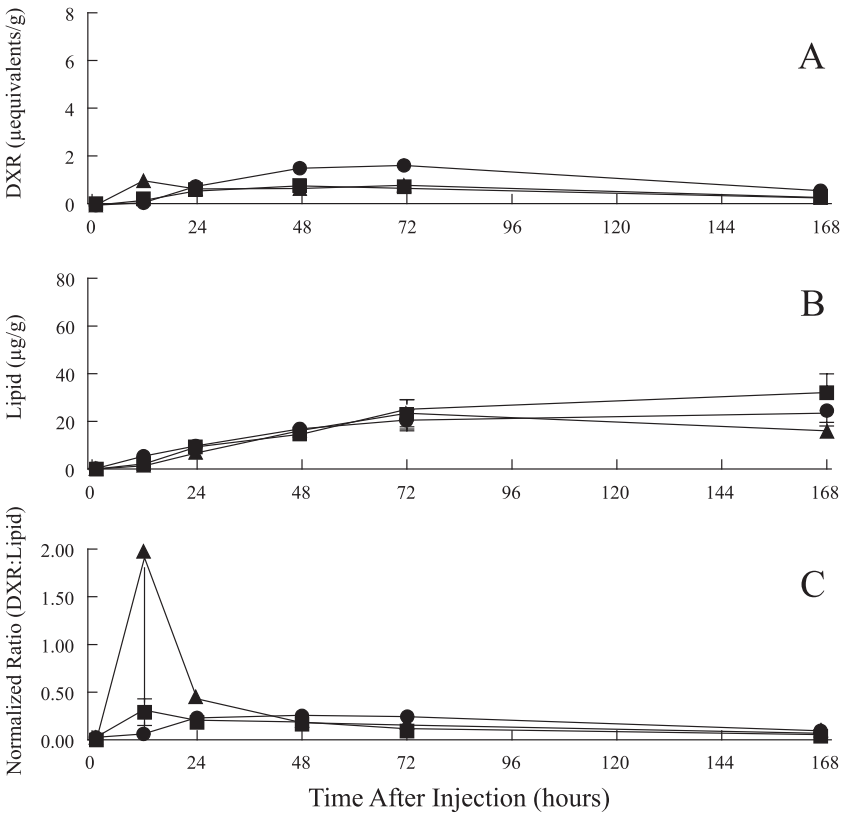

Fig. 3. Skin concentrations of DXR (panel A), liposomal lipid (panel B) and normalized drug to lipid ratios (panel C). (● DSPC:CHOL:mPEG-DSPE; ■ POPC:CHOL:mPEG-DSPE; ▲ DOPC:CHOL:mPEG-DSPE at a molar ratio of 2:1:0.1). Details as in Fig. 1.

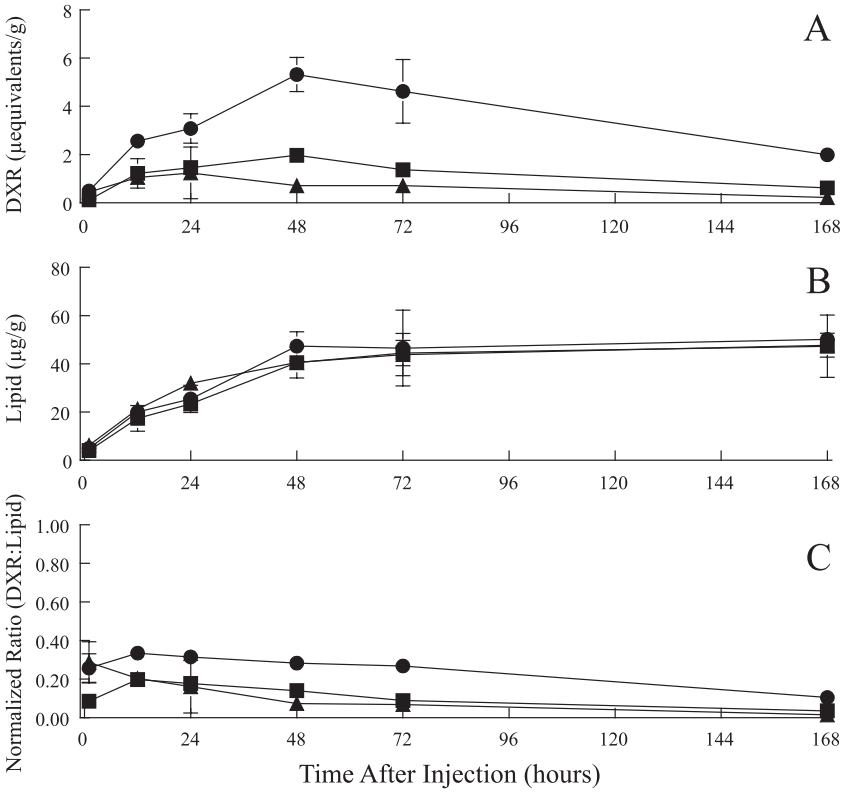

Fig. 4. Paw concentrations of DXR (panel A), liposomal lipid (panel B) and normalized drug to lipid ratios (panel C). (● DSPC:CHOL:mPEG-DSPE; ■ POPC:CHOL:mPEG-DSPE; ▲ DOPC:CHOL:mPEG-DSPE at a molar ratio of 2:1:0.1). Details as in Fig. 1.

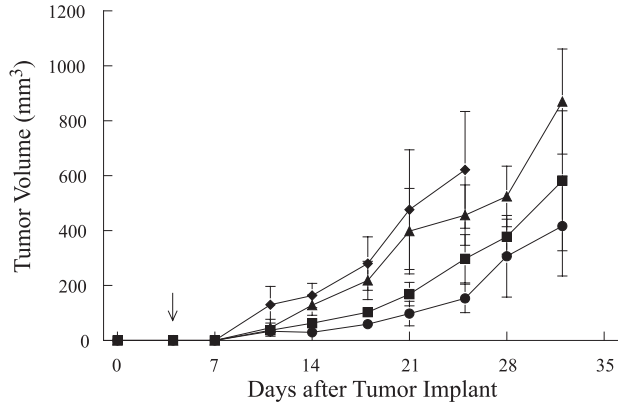

Fig. 5. Therapeutic activity of different formulations of liposomal DXR against the 4T1 murine mammary carcinoma. BALB/c mice were implanted in the #4 mammary fat pad with the 4T1 murine mammary carcinoma. Four days later (arrow), mice were treated with liposomal DXR at a DXR dose of 6 mg/kg. Liposomes (2:1:0.1 mol) were composed of DSPC:CHOL:mPEG (●), POPC:CHOL:mPEG (■), DOPC:CHOL:mPEG (▲) or saline control (♦). Data represent the mean ± S.D. of 5–12 mice from two pooled experiments, except for POPC:CHOL:mPEG liposomes, where data represent the mean ± S.D. of four to six mice from one experiment.

tissues. For each formulation, levels of liposomal lipid in paws were higher than in skin. For liposomes with slow and intermediate drug release rates (DSPC and POPC-containing formulations), this translated into higher DXR concentrations and AUCs in paws than in skin. In contrast to DXR clearance from tumor, the DXR tissue clearance $t_{1/2}$'s for skin and paws were similar for all three formulations (Table 3). Overall, the data presented in Table 3 demonstrate that more solid liposomes delivered more DXR to all tissues of interest, while equal amounts of lipid were delivered by each formulation.

3.4. Tumor growth delay experiments

The results of the pooled experiments comparing the therapeutic efficacy of liposomal formulations with different leakage rates are presented in Fig. 5. Treatment of mice with any of these formulations resulted in delayed tumor growth. The therapeutic effect was inversely correlated with drug release rate. The more solid liposomes containing DSPC appeared to have superior therapeutic activity compared to more fluid formulations with more rapid drug release rates. Fewer mice were analyzed in the POPC:CHOL:mPEG group due to toxicity encountered with this formulation. In one therapeutic experiment, all the mice in this group were euthanized due to severe weight loss within 1 week of treatment. Administration of empty liposomes of the same formulations resulted in no adverse effects, however. Gross post mortem exam (performed by the staff veterinary pathologist, University of Alberta Health Sciences Laboratory Animal Services) revealed blood congestion of organs, consistent with cardiovascular toxicity. In the second therapeutic experiment with this formulation, two mice were euthanized due to severe weight loss: One mouse had myocardial necrosis, and the other had hepatic necrosis. No further experiments were performed with this formulation.

4. Discussion

The experiments in this study used a murine mammary carcinoma model to compare the PK, BD, and therapeutic activity of different formulations of liposomal DXR having different DXR release rates. In vitro DXR leakage experiments were consistent with previously published data for DXR and other drugs, which demonstrated that the fluidity of the liposomal membrane plays an important role in the release of liposomal contents [3,31,36]. The only liposome formulation for which the 4-h plasma DXR concentrations did not parallel the in vitro results was POPC:CHOL:mPEG-DSPE (Tables 1 and 2). These liposomes had a short in vitro leakage $t_{1/2}$ but a higher than expected plasma DXR concentration at 4 h after injection. This discrepancy may be due to the differences in the composition of the adult bovine serum and mouse blood. Other investigators have found discrepancies between in vitro and in vivo leakage results for other liposomal drugs, such as vincristine [38].

Results from the PK and BD experiments demonstrated that the PK of the liposomal carrier were the same for all formulations tested, and that long circulation times for the carrier were independent of whether or not drug was still present in the liposomes. This is consistent with data demonstrating that mPEG-DPSE can impart long circulating times on liposomes composed of fluid as well as on liposomes composed of solid lipids [24,39].

The normalized drug to lipid ratios provided a measure of drug leakage in vivo. In plasma, the decrease in this ratio paralleled the decrease in plasma concentrations of DXR for each formulation, suggesting that empty liposomes or liposomes with low contents levels remained in circulation. Since ^{3}H-CHE is reported to be non-exchangeable and non-metabolizeable, the decrease in lipid:drug ratios for at least

Table 2
Plasma concentrations of total DXR achieved from various formulations of liposomal DXR at 4 h after injection

Formulation	Phase transition temperature of phosphatidylcholine (°C)	Composition (molar ratios)	Plasma concentration (μequivalent/ml)
Caelyx® (HSPC:CHOL:mPEG)	55	55:40:5	97.4 ± 11.4
DSPC:CHOL:mPEG	55	2:1:0.1	91.7 ± 5.8
SMPC:CHOL:mPEG	30	2:1:0.1	80.4 ± 4.1
POPC:CHOL:mPEG	− 2	2:1:0.1	63.5 ± 9.7
PMPC:CHOL:mPEG	27	2:1:0.1	43.2 ± 4.9
DOPC:CHOL:mPEG	− 20	2:1:0.1	18.4 ± 2.7
DMPC:CHOL:mPEG	23	2:1:0.1	14.2 ± 1.6

Mice were injected intravenously with a DXR dose of 6 mg/kg (18 mg/m^2). Values represent the mean ± S.D. from three mice.

the first few hours after injection reflects contents release [40]. Many liposome PK studies use gamma ray-emitting labels such as ^{125}I-tyraminylinulin (^{125}I-TI) and ^{67}Ga-desferoxime as surrogate markers for intact liposomes, while other studies use lipid labels that may not be as stable as ^{3}H-CHE. This is often a matter of convenience since direct measurements of drug concentrations can be difficult and time-consuming [41,42]. As this study shows, PK and BD studies that use a surrogate label and not the pharmacologically active agent should be interpreted with caution.

Our data demonstrate that the accumulation of liposomal lipid (i.e., the carrier) into a given tissue (tumor, skin, or paws) was not dependent on the formulation, whereas the accumulation of DXR in these tissues was formulation-dependent. The observations that tumor tissue accumulated liposomal lipid faster and to a larger extent than skin and paws is consistent with previous data obtained in this model [26]. The finding that liposomal lipid concentrations were higher in paws than in skin is also consistent with previous data from this model and supports a pressure-dependent extravasation of liposomes into the paws of the mice as they walk around the cage, groom, feed, etc. [26].

Since tissue levels of liposomal lipid plateaued, the decrease in the drug-to-lipid ratio in tissues reflects a decrease in tissue DXR concentrations, presumably after it has been released from liposomes. After release, DXR is either cleared from the tissue or metabolized in situ to either fluorescent or non-fluorescent forms; since this study did not differentiate between the parent drug and metabolites, it is not possible to say which process predominated. Hence, data at the longer time points should be interpreted with caution. In skin and paws, the tissue DXR elimination rates (k_{elm}) were of a similar magnitude for all three formulations (Table 3). In tumor, however, the liposomes containing DOPC showed a faster DXR elimination rate than the other two formulations, indicating a faster rate of DXR release for the DOPC-containing liposomes in the tumor-specific microenvironment. This is consistent with the faster DXR release rate these liposomes showed in plasma.

For all liposomal formulations of DXR tested, DXR elimination from tumor and cutaneous tissues was slower than from plasma. DSPC:CHOL:mPEG-DSPE liposomes had the best drug retention and produced the highest total DXR AUC in tumor, as well as in cutaneous tissues. The slow cutaneous clearance of DXR has potential implications for the clinical use of Caelyx®. Cancer therapy is usually administered in repeated cycles; if the dose interval is not long enough to allow for complete elimination of DXR-loaded liposomes from the skin, the drug may accumulate and cutaneous toxicities will be more likely to occur. Recent experimental data in the mouse model used here as well as clinical evidence support this hypothesis, as prolonging the dose interval reduces the incidence and severity of PPE lesions [11].

Unexpectedly, liposomes composed of POPC:CHOL:mPEG-DSPE (intermediate drug release rate) showed a high level of toxicity in therapeutic experiments. These liposomes apparently produced sustained, cardiotoxic concentrations of bioavailable DXR, an assumption that was supported by gross pathological observations. In contrast, neither DSPC:CHOL:mPEG-DSPE nor DOPC:CHOL:mPEG-DSPE liposomes showed gross signs of cardiac toxicity. The slow rate of DXR release from the DSPC:CHOL:mPEG-DSPE formulation and the rapid redistribution of DXR from the DOPC:CHOL:mPEG-DSPE formulation into a large tissue volume of distribution may have prevented the build-up of cardiotoxic drug concentrations in the heart for these formulations. Thus, drug leakage rates are important in determining the toxicity as well as the therapeutic activity of liposomal formulations. Administering

Table 3
Pharmacokinetic parameters for tumor-bearing mice receiving liposomal DXR (6 mg/kg) of either DSPC:CHOL:mPEG (DSPC) POPC:CHOL:mPEG (POPC), or DOPC:CHOL:mPEG (DOPC)

	DSPC		POPC		DOPC	
	DXR	^{3}H-CHE	DXR	^{3}H-CHE	DXR	^{3}H-CHE
Plasma						
k_{elm}	0.0380	0.0339	0.0581	0.0365	0.3266	0.0398
r^2	0.983	0.977	0.971	0.989	0.981	0.974
$t_{1/2}$ (h)	18.2	20.4	11.9	19.0	2.1	17.4
AUC[a]	3020	10600	1410	12200	430	12700
CL[b]	0.0020	0.0028	0.0043	0.0025	0.014	0.0024
V_d[c]	0.060	0.100	0.100	0.075	0.300	0.067
Tumor						
k_{elm}	0.0097	N/A	0.0163	N/A	0.0280	N/A
r^2	0.994	N/A	0.985	N/A	0.968	N/A
$t_{1/2}$ (h)	71.4	N/A	42.5	N/A	24.8	N/A
AUC[d]	1640	20200	432	20100	193	24800
C_{max}[e]	18.2	150.2	6.8	182.5	4.3	132.2
Skin						
k_{elm}	0.0087	N/A	0.0083	N/A	0.0063	N/A
r^2	0.936	N/A	0.996	N/A	0.811	N/A
$t_{1/2}$ (h)	79.6	N/A	83.5	N/A	110	N/A
AUC[d]	183	2300	89	3370	107	2700
C_{max}[e]	1.7	23.3	0.81	23.3	1.0	30.0
Paws						
k_{elm}	0.0084	N/A	0.0092	N/A	0.0115	N/A
r^2	0.997	N/A	0.986	N/A	0.969	N/A
$t_{1/2}$ (h)	82.5	N/A	75.3	N/A	60.2	N/A
AUC[d]	588	7060	200	6560	108	6750
C_{max}[e]	5.3	50.2	2.0	47.7	1.2	47.3

Molar ratios for all formulations were 2:1:0.1. AUCs were calculated using the trapezoidal rule, and plasma and tissue $t_{1/2}$'s were calculated using the formula $t_{1/2} = 0.693/k_{elm}$, where k_{elm} is the elimination constant derived from the plasma or tissue concentration versus time curve; r^2 represents the coefficient of determination for this equation. (For the purposes of AUC calculation, 1 μequivalent is assumed to equal 1 μg of DXR and/or metabolites).

[a] Units for plasma $AUC_{0-\infty}$ are (μg·h/ml).

[b] Units for CL are ml/h/g.

[c] Units for V_d are ml/g.

[d] Units for tissue $AUC_{0-\infty}$ are (μg·h/g).

[e] Units for C_{max} are RFU/g tissue for DXR and μg lipid/g tissue for liposomal lipid.

"empty" POPC:CHOL:mPEG-DSPE liposomes in conjunction with free DXR did not produce this toxicity, again suggesting that the rate of drug release plays an essential role in the development of this toxicity.

The bioavailability of the encapsulated drug is another important concept to consider when interpreting these data. Current thinking regarding the mechanism of action of Caelyx® is that the liposomes accumulate in tumor and slowly release their contents. This released and thus bioavailable drug then is able to exert its therapeutic activity. If the concentration of bioavailable drug does not reach a minimum cytotoxic concentration, then no appreciable therapeutic response will be seen. This problem was encountered with a STEALTH® liposomal formulation of cisplatin (SPI-077), which produced high tumor drug AUCs in experimental models, but failed to produce correspondingly high anti-tumor responses both in animal models and in humans, so that its development was stopped in Phase II clinical trials [43,44]. In the current study, administration of DXR-loaded DSPC:CHOL:mPEG-DSPE liposomes yielded not only a high total DXR concentration in tumor, but also the best therapeutic activity of the formulations tested, suggesting that drug was bioavailable at levels above the minimum therapeutic dose.

In conclusion, our data demonstrate that drug leakage rates play an important role in the therapeutic activity and the toxicity of liposomal drug formulations. Solid liposomes composed of DSPC:CHOL:mPEG-DPSE produced the highest total DXR concentration in all tissues of interest and had the best therapeutic activity of the formulations tested. Even though this formulation had the slowest rate of DXR leakage, it produced cytotoxic concentrations of bioavailable drug in tumor tissue in vivo, resulting in a good therapeutic effect. The large values of cutaneous total DXR concentrations obtained with DSPC:CHOL:mPEG-DPSE liposomes suggest that PPE is a result of skin damage resulting from the skin accumulation of liposomal DXR followed by the sustained release of DXR in the skin. From these results, the mouse appears to be an appropriate model in which to study this phenomenon. In these studies, we were unable to uncouple skin toxicity from therapeutic effect by manipulating the rate of drug release from the liposomes; in other words, leakage rates that led to lower skin and paw levels of DXR also resulted in reduced therapeutic effects.

Acknowledgements

This research was supported by the Canadian Institutes of Health Research (UOP 48092) and ALZA. GJRC is the recipient of a graduate studentship from the Alberta Heritage Foundation for Medical Research. The authors gratefully acknowledge Dr. Dion Brocks (Faculty of Pharmacy, University of Alberta) for helpful discussions, Dr. Richard Uwiera (staff pathologist, University of Alberta Health Sciences Laboratory Animal Services) for performing gross post mortem exams. The authors thank Elaine Moase for technical assistance and for critically reviewing this manuscript. The technical assistance of Dr. Janny Zhang and the University of Alberta Health Sciences Laboratory Animal Services with tumor implantation is also gratefully acknowledged.

References

[1] L.D. Mayer, L.C. Tai, D.S. Ko, D. Masin, R.S. Ginsberg, P.R. Cullis, M.B. Bally, Cancer Res. 49 (1989) 5922–5930.
[2] M.B. Bally, R. Nayar, D. Masin, P.R. Cullis, L.D. Mayer, Cancer Chemother. Pharmacol. 27 (1990) 13–19.
[3] T.M. Allen, T. Mehra, C.B. Hansen, Y.C. Chin, Cancer Res. 52 (1992) 2431–2439.
[4] D.C. Drummond, O. Meyer, K.L. Hong, D.B. Kirpotin, D. Papahadjopoulos, Pharmacol. Rev. 51 (1999) 691–743.
[5] D.N. Waterhouse, P.G. Tardi, L.D. Mayer, M.B. Bally, Drug Safety 24 (2001) 903–920.
[6] P. Working, A.D. Dayan, Human Exp. Toxicol. 15 (1996) 751–785.
[7] J.W. Cowens, P.J. Creaven, W.R. Greco, D.E. Brenner, Y. Tung, M. Osto, F. Pilkiewicz, R. Ginsberg, N. Petrelli, Cancer Res. 53 (1993) 2796–2802.
[8] A. Gabizon, R. Catane, B. Uziely, B. Kaufman, T. Safra, R. Cohen, F. Martin, A. Huang, Y. Barenholz, Cancer Res. 54 (1994) 987–992.
[9] G. Berry, M. Billingham, E. Alderman, P. Richardson, F. Torti, B. Lum, A. Patek, F.J. Martin, Ann. Oncol. 9 (1998) 711–716.
[10] G. Batist, G. Ramakrishnan, C.S. Rao, A. Chandrasekharan, J. Gutheil, T. Guthrie, P. Shah, A. Khojasteh, M.K. Nair, K. Hoelzer, K. Tkaczuk, Y.C. Park, L.W. Lee, J. Clin. Oncol. 19 (2001) 1444–1454.
[11] B. Uziely, S. Jeffers, R. Isacson, K. Kutsch, D. Wei-Tsao, Z. Yehoshua, E. Libson, F.M. Muggia, A. Gabizon, J. Clin. Oncol. 13 (1995) 1777–1785.
[12] M.R. Ranson, J. Carmichael, K. O'Byrne, S. Stewart, D. Smith, A. Howell, J. Clin. Oncol. 15 (1997) 3185–3191.
[13] F. Muggia, J.D. Hainsworth, S. Jeffers, P. Miller, S. Groshen, M. Tan, L. Roman, B. Uziely, L. Muderspach, A. Garcia, A. Burnett, F.A. Greco, C.P. Morrow, L.J. Paradiso, L.-J. Liang, J. Clin. Oncol. 15 (1997) 987–993.
[14] M. Lotem, A. Hubert, O. Lyass, M.A. Goldenhersh, A. Ingber, T. Peretz, A. Gabizon, Arch. Dermatol. 136 (2000) 1474–1480.
[15] A.M. Lopez, L. Wallace, R.T. Dorr, M. Koff, E.M. Hersh, D.S. Alberts, Cancer Chemother. Pharmacol. 44 (1999) 303–306.
[16] K.A. Remlinger, Arch. Dermatol. 139 (2003) 77–81.
[17] T.M. Allen, C.B. Hansen, D.E. Lopes de Menezes, Adv. Drug Deliv. Rev. 16 (1995) 267–284.
[18] H. Hashizume, P. Baluk, S. Morikawa, J.W. McLean, G. Thurston, S. Roberge, R.K. Jain, D.M. McDonald, Am. J. Pathol. 156 (2000) 1363–1380.
[19] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, J. Control. Release 65 (2000) 271–284.
[20] D. Papahadjopoulos, T.M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S.K. Huang, K.D. Lee, M.C. Woodle, D.D. Lasic, C. Redemann, F.J. Martin, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 11460–11464.
[21] A.A. Gabizon, Cancer Res. 52 (1992) 891–896.
[22] A. Gabizon, D. Papahadjopoulos, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 6949.
[23] T.M. Allen, C. Hansen, J. Rutledge, Biochim. Biophys. Acta 981 (1989) 27–35.
[24] T.M. Allen, C.B. Hansen, F. Martin, C. Redemann, A. Yau-Young, Biochim. Biophys. Acta 1066 (1991) 29–36.
[25] A. Gabizon, D. Goren, A.T. Horowitz, D. Tzemach, A. Lossos T. Siegal, Adv. Drug Deliv. Rev. 24 (1997) 337–344.

[26] G.J.R. Charrois, T.M. Allen, Biochim. Biophys. Acta 1609 (2003) 102–108.
[27] T.M. Allen, L.G. Cleland, Biochim. Biophys. Acta 597 (1980) 418–426.
[28] A.A. Gabizon, Y. Barenholz, M. Bialer, Pharm. Res. 10 (1993) 703–708.
[29] L.D. Mayer, M.J. Hope, P.R. Cullis, Biochim. Biophys. Acta 858 (1986) 161–168.
[30] E.M. Bolotin, R. Cohen, L.K. Bar, S.N. Emanuel, D.D. Lasic, Y. Barenholz, J. Liposome Res. 4 (1994) 455–479.
[31] H.J. Lim, D. Masin, T.D. Madden, M.B. Bally, J. Pharmacol. Exp. Ther. 281 (1997) 566–573.
[32] C.J. Aslakson, F.R. Miller, Cancer Res. 52 (1992) 1399–1405.
[33] E. Moase, W. Qi, T. Ishida, Z. Gabos, B.M. Longenecker, G.L. Zimmermann, L. Ding, M. Krantz, T.M. Allen, Biochim. Biophys. Acta 1510 (2001) 43–55.
[34] L. Shargel, A.B.C. Yu, Applied Biopharmaceutics and Pharmacokinetics, 4th ed., McGraw Hill, New York, 1999.
[35] T.O Harasym, P.R. Cullis, M.B. Balley, Cancer Chemother. Pharmacol. 40 (1997) 309–317.
[36] A. Gabizon, R. Shiota, D. Papahadjopoulos, J. Natl. Cancer Inst. 81 (1989) 1484–1488.
[37] G.J.R. Charrois, T.M. Allen, J. Pharm. Exp. Ther. 306 (2003) 1058–1067.
[38] L.D. Mayer, M.B. Bally, H. Loughrey, D. Masin, P.R. Cullis, Cancer Res. 50 (1990) 575–579.
[39] M.C. Woodle, K.K. Matthay, M.S. Newman, J.E. Hidayat, L.R. Collins, C. Redemann, F.J. Martin, D. Papahadjopoulos, Biochim. Biophys. Acta 1105 (1992) 193–200.
[40] J.T. Derksen, H.W. Morselt, G.L. Scherphof, Biochim. Biophys. Acta 931 (1987) 33–40.
[41] E.F. Sommerman, P.H. Pritchard, P.R. Cullis, Biochem. Biophys. Res. Commun. 122 (1984) 319–324.
[42] A. Gabizon, J. Huberty, R.M. Straubinger, D.C. Price, D. Papahadjopoulos, J. Liposome Res. 1 (1989) 123–135.
[43] S. Bandak, D. Goren, A. Horowitz, D. Tzemach, A. Gabizon, Anticancer Drugs 10 (1999) 911–920.
[44] E.S. Kim, C. Lu, F.R. Khuri, M. Tonda, B.S. Glisson, D. Liu, M. Hong, W.K. Hong, R.S. Herbst, Lung Cancer 34 (2001) 427–432.