0022-3565/97/2803-1319$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 280, No. 3
Printed in U.S.A.

JPET 280:1319–1327, 1997

Accumulation of Liposomal Lipid and Encapsulated Doxorubicin in Murine Lewis Lung Carcinoma: The Lack of Beneficial Effects by Coating Liposomes with Poly(ethylene glycol)[1]

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Accepted for publication November 19, 1996

ABSTRACT

The efficiency of drug accumulation in tumors was measured after intravenous administration of doxorubicin encapsulated in distearoyl phosphatidylcholine/cholesterol liposomes prepared in the presence or absence of 5 mol % polyethylene glycol-modified phosphatidylethanolamine (PEG-PE). These liposomal formulations of doxorubicin were administered at the maximum tolerated dose in female BDF-1 mice bearing subcutaneously established Lewis Lung carcinoma. The parameters used to determine tumor targeting efficiency (T_e) included area under the doxorubicin plasma (AUC_P) and tumor (AUC_T) concentration-time curves. Extended time-course studies evaluating lipid and drug levels in plasma and tumors during 7 days after administration indicated that the T_e (AUC_T/AUC_P) was greater for liposomes that did not contain PEG-PE. The AUC_P after administration of free doxorubicin, doxorubicin encapsulated in distearoyl phosphatidylcholine/cholesterol liposomes and doxorubicin encapsulated in distearoyl phosphatidylcholine/cholesterol/PEG-PE-stabilized liposomes were 0.087 μmol·ml^{-1}·h, 50 μmol·ml^{-1}·h and 78 μmol·ml^{-1}·h, respectively. Maximum drug levels achieved in the tumors were similar for both liposomal doxorubicin formulations, 140 μg (250 nmol)/g tumor; however, this level was achieved faster when the liposomes did not contain PEG-PE. Maximum levels measured after administration of free drug were less than 5 μg/g tumor, and these were achieved within 15 min. The results suggest that some of the benefits associated with the use of PEG-modified liposomes, such as increased blood levels and enhanced circulation lifetime, may be of little advantage in terms of maximizing liposomal drug accumulation in sites of tumor growth.

Strategies designed to maximize the antitumor activity of chemotherapeutic agents must contend with the fact that tumors consist of heterogeneous cell populations that are 1) in various stages of the cell cycle, 2) proliferating at different rates, 3) growing in different tissues and 4) capable of adapting rapidly to the therapeutic stresses exerted on them. In practical terms this means that chemotherapy typically involves the use of multiple drugs that exert antitumor activity *via* different mechanisms (DeVita, 1989). Another general principle for the use of antineoplastic agents concerns maximizing dose intensity (Livingston, 1994). Tumor cells must be exposed to the highest levels of drug for the longest periods if maximum therapeutic effects are to be achieved (Lin, 1994). The advantage of anticancer drug carrier technology is based on carrier characteristics that give rise to increased drug exposure in sites of tumor growth.

Examples of how liposome drug carrier technology can improve the pharmacodynamic behavior of an anticancer drug is provided by the anthracycline doxorubicin (Gabizon *et al.*, 1982; Mayer *et al.*, 1994) as well as the vinca alkaloid vincristine (Mayer *et al.*, 1990; Webb *et al.*, 1995). Pharmacodynamic studies that characterize the mechanisms whereby liposomes improve the therapeutic profile of anticancer drugs have defined two areas of interest. First, there is good evidence from preclinical studies that the reduced toxicity of liposomal formulations is a consequence of reduced drug accumulation in healthy tissue, such as cardiac tissue in the case of doxorubicin (Gabizon *et al.*, 1982; Herman *et al.*,

Received for publication July 23, 1996.
[1] These studies were supported by grants from the Medical Research Council of Canada (M.B.B.) and from Inex Pharmaceuticals Corporation (P.R.C.). M.J.P. was supported by a Medical Research Council studentship.
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ABBREVIATIONS: DSPC, distearoyl phosphatidylcholine; Chol, cholesterol; PEG-PE, poly(ethylene glycol)-modified phosphatidylethanolamine; LLC, Lewis Lung carcinoma; DOX, doxorubicin; RES, reticuloendothelial system; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; T_e, targeting efficiency; AUC, area under the curve; C_{Tmax}, peak tumor drug concentration levels; MTD, maximum tolerated dose.

1983; Balazsovits *et al.*, 1989). Second, it is strongly believed that therapeutic activity arises as a consequence of liposome-mediated increases in drug circulation lifetimes and improved drug delivery to tumor sites (Gabizon, 1992; Mayer *et al.*, 1994).

Liposome-mediated increases in doxorubicin delivery to tumors is often directly correlated with factors that lead to enhanced liposome drug levels in the circulation as well as increased circulation lifetime. There are several ways in which these two parameters can be enhanced including: 1) increasing drug retention in systemically administered liposomes (Mayer *et al.*, 1994), 2) increasing the lipid dose (Abra and Hunt, 1981) and 3) using liposome compositions that have a reduced propensity to be recognized and removed by phagocytic cells of the RES (Blume and Cevc, 1990; Allen *et al.*, 1991, 1992; Gabizon *et al.*, 1994a). All three approaches may be expected to facilitate increased accumulation of drug within sites of tumor growth. The studies summarized here evaluate the relationship between circulation lifetime/plasma drug concentration and tumor drug accumulation. LLC bearing mice were given (i.v.) a single dose (equivalent to the maximum tolerated drug dose) of free doxorubicin, doxorubicin encapsulated in DSPC/Chol (55:45) liposomes and doxorubicin encapsulated in DSPC/Chol/PEG-PE (50:45:5) liposomes. The latter exhibit a reduced propensity to localize in the cells of the RES and exhibit longer circulation lifetimes. It is demonstrated that incorporation of PEG lipids in the liposomal doxorubicin formulation did not lead to improved tumor delivery or enhanced therapeutic activity under these conditions.

Materials and Methods

Preparation of liposomes and doxorubicin loading. The production of 100-nm LUVs was carried out as described previously (Hope *et al.*, 1985). Dry lipid mixtures composed of DSPC/Chol (55:45 mol/mol) or DSPC/Chol/PEG-PE (55:45:5) each with trace amounts of [^{3}H]cholesteryl hexadecyl ether (NEN/Dupont, Boston, MA) as a nonmetabolizable and nonexchangeable liposome marker (Derksen *et al.*, 1987) were dissolved in chloroform. This mixture was reduced to a minimum volume under a stream of nitrogen gas in a warm water bath (50°C). To avoid precipitation of cholesterol the mixture was quickly placed under high vacuum and dried for a further 4 h. This procedure results in a homogeneous expanded lipid foam with a significant total surface area which facilitates complete lipid hydration. Hydrating the dried lipid in 300 mM citrate buffer (pH 4.0) followed by vigorous vortexing, warming and five freeze-thaw cycles produced MLVs. The MLVs were then extruded ten times through two stacked 100-nm pore size polycarbonate filters (Costar/Nucleopore, Toronto, Ontario, Canada) with an extrusion device (Lipex Biomembranes, Vancouver, British Columbia, Canada) equilibrated at 65°C. The resulting LUVs had a mean diameter of 100 ± 15 nm, as determined by quasielastic light scattering on a NICOMP model 270 submicron particle sizer operating at a wavelength of 632.8 nm. No differences in liposome size were observed between systems prepared with and without PEG-modified lipids. The lipid concentration of each liposome preparation was determined by the Fiske and Subbarow phosphorous assay (Fiske and Subbarow, 1925), in which the colored product was measured spectrophotometrically at 815 nm with a Shimadzu UV-visible recording spectrophotometer. This measurement was used to derive a specific activity for the radiolabeled liposomes (dpm/μmol total lipid); thereafter, liposomal lipid concentrations were estimated by scintillation counting by use of a Beckman LS 3801 instrument. PicoFluor 40 scintillation fluid (Packard, Mississauga, Ontario, Canada) was used as a high-efficiency scintillation cocktail. DSPC was from Avanti Polar Lipids, Alabaster, AL, Chol and other chemicals were from Sigma Chemical Co. (St. Louis, MO) and PEG-PE was synthesized as described previously (Parr *et al.*, 1994).

Doxorubicin was encapsulated by the transmembrane pH gradient loading procedure (Mayer *et al.*, 1986). To establish a pH gradient across the LUVs, the resultant LUVs were dialyzed (12,000–14,000 molecular weight cut off, Spectrapor; Spectrum Medical Industries Inc., Los Angeles) against 150 mM $NaHCO_3$ buffer, pH 7.5, for several hours to remove the external citrate and raise the external pH to 7.5. Aliquots of LUVs and doxorubicin (Adria Laboratories, Columbus, OH) dissolved in saline were preheated to 65°C and then combined in a 0.2-mol drug-to-lipid ratio. These samples were incubated for an additional 10 min. at 65°C, which resulted in greater than 95% trapping efficiency. "Empty" liposomes were prepared in parallel with saline as a replacement for doxorubicin.

Animal and tumor models. All mice used were 20- to 22-g female BDF-1 mice (Charles River, St.-Constant, Quebec, Canada). The LLC was obtained from the National Cancer Institute Tumor Repository (Bethesda, Maryland) as a frozen tumor fragment from stock number G50132. Solid tumor tissue was processed by mechanical and enzymatic (Dispase/Collagenase/DNase) digestion to generate single-cell suspensions which were used for experiments. For these studies tumors were established from cells obtained from tumors passaged two to five times from the original stock. For each passage, 3×10^5 cells in a volume of 50 μl were injected s.c. in each of the mouse flanks (bilateral tumors). Tumors were left to grow to an estimated size (based on two-dimensional caliper measurements as described below) of 0.2 to 0.4 g before initiation of pharmacology or therapeutic studies. At this time the time required for the tumor size to double was approximately 3 days. All drug and liposome injections were injected i.v. through the lateral tail vein in a volume of 200 μl. At various times after injection the mice were anesthetized by i.p. administration of ketamine/xylazine (155 mg/kg, 18 mg/kg, MTC Pharmaceuticals, Cambridge, Ontario, Canada). Blood was collected *via* cardiac puncture, placed in microtainer tubes with EDTA (Becton Dickinson, *via* VWR Scientific, Edmonton, Alberta, Canada) and centrifuged at 1500 × *g* for 10 min to isolate plasma. Tissues were carefully removed, washed, blotted to remove attached blood, weighed and homogenized with a Polytron to a 20% (liver, tumor) and 10% (spleen) homogenate (w/v) in saline.

Assays for liposomal lipid and doxorubicin. To determine lipid levels, 100 μl plasma and 200 μl tissue homogenate were solubilized with 500 μl Solvable (NEN/Dupont) for 2 h at 60°C. The samples were cooled and treated overnight with 200 μl H_2O_2. Five milliliters of scintillation fluid was added and samples were counted to determine [^{3}H]cholesteryl hexadecyl ether. To determine doxorubicin levels, 100 μl plasma and 200 μl tissue homogenate were diluted with dH_2O up to 800 μl, and 100 μl each of 10% sodium dodecyl sulfate and 10 mM H_2SO_4 were added. These samples were vortexed and 2 ml of chloroform/isopropyl alcohol (1:1 v/v) was added before additional mixing. The resulting samples were frozen overnight, thawed and centrifuged for 10 min at 1000 × *g*. The organic phase (lower) was removed and the amount of associated doxorubicin fluorescent equivalents was measured with a Perkin-Elmer fluorimeter (excitation/emission at 500:550 nm). Doxorubicin standards (0–20 nmol) were prepared for each set of assays after mixing appropriate volumes of the standard with tissue homogenates derived from organs isolated from untreated mice. Because this assay does not discriminate between doxorubicin and its fluorescent metabolites, doxorubicin levels are referred to as doxorubicin fluorescent equivalents. Previous studies from this laboratory have shown that the doxorubicin extraction efficiency for this assay is greater than 90% for serum samples and between 70 and 90% for tissue samples. All tissue drug and lipid levels were corrected for drug and lipid in the plasma compartment by use of published plasma volume correction factors (Bally *et al.*, 1993).

Acute toxicity evaluation. Tumor-free mice were used to test the doxorubicin-mediated acute toxicity and to establish the MTD for both free and liposomal drug formulations. Previous work from our laboratory indicated that in BDF-1 mice free doxorubicin has a MTD of between 20 and 25 mg/kg, whereas conventional liposomal doxorubicin has a MTD greater than 55 mg/kg. Therefore, 0.66 μmol doxorubicin-HCl (MW of doxorubicin is 543.54) dissolved in saline as the free drug and 2.00 μmol doxorubicin-HCl entrapped within 10 μmol lipid (LUV) was administered i.v. per mouse. For an additional comparison, 0.66 μmol DOX entrapped within 3.3 μmol lipid was evaluated. Toxicity was measured qualitatively by evaluating mean body weight loss and survival up to 40 days after treatment. These studies were done in accordance to Canadian Council on Animal Care (CCAC) Guidelines; it should be noted that only 1 of 25 animals used in this experiment died as a consequence of drug-related toxicity, and no animals had to be terminated as a result of unacceptable suffering. The CCAC does not permit formal LD_{10} or LD_{50} studies where death is used as an end-point, so the MTD was approximated with a small number of animals and a limited number of drug doses.

Plasma elimination and tumor accumulation. To demonstrate the influence of lipid dose on elimination of liposomal lipid from the blood compartment, a dose titration of liposomal lipid was completed up to the amount of lipid required to deliver the MTD of associated drug. For the dose titration, increasing doses of empty and drug loaded (0.2 drug/lipid ratio) liposomes were administered i.v. in tumor-free mice. The mice were sacrificed 24 h after injection, and the levels of liposomal lipid in plasma and selected tissues were determined as described above.

Additional plasma elimination and tissue distribution studies were completed in BDF-1 mice bearing Lewis Lung tumors. All mice receiving liposomal doxorubicin were given 2 μmol doxorubicin/10 μmol total lipid (approximately 55 mg doxorubicin/kg) and sacrificed at 1, 4, 24, 48, 96 and 168 h. Free doxorubicin-treated mice were given 0.66 μmol doxorubicin i.v. (approximately 18 mg drug/kg) and sacrificed at 15 min, 1, 4, 24, 48 and 72 h. Lipid and drug levels in plasma and tissues were determined as described.

Measurement of tumor size. Animals (groups of 5) bearing 0.2- and 0.4-g tumors were treated with free and the liposomal doxorubicin formulations at doses of 0.66 (18 mg/kg) and 2.00 (55 mg/kg) μmol drug per mouse, respectively. Tumor size was determined at various times after a single drug dose with use of a caliper to estimate length and width. Tumor mass (g) was calculated by the following formula:

$$\frac{(a) \times (b)^2}{2}$$

where a = length and b = width measurements in centimeters. Mice were terminated on the nearest whole day when tumor mass equaled or exceeded 1.5 g or when tumors became ulcerated. The study was repeated two times, and the data were combined to give a total n of 10 per treatment group.

Statistical analysis. Differences between results obtained after administration of the two liposomal formulations of doxorubicin and free drug were determined by an analysis of variance. Comparisons were made for various common time points incorporating all sets of collected data for that time point by the Post Hoc Comparison of Means, Scheffé test (Milliken and Johnson, 1984). Mean AUCs were calculated on the basis of mean values obtained for individual time points, where means were derived from at least four animals. Because individual animals were required to generate each data point and sequential sampling over time was not possible in the murine models used here, it was not feasible to assess whether differences in mean AUC were statistically different.

Results

Estimation of maximum tolerated doses. Dose ranging studies in tumor-free BDF-1 mice indicated that the MTD for free and liposomal doxorubicin was approximately 18 mg/kg and 55 mg/kg, respectively. Both liposomal formulations of doxorubicin were tolerated up to 55 mg/kg; however, there was significant loss of body weight at this high dose (table 1). A nadir equivalent to almost a 25% decrease in mean body weight was observed between days 8 and 10 after drug administration. Recovery of normal body weight was achieved by day 18, and all mice survived the 55 mg/kg drug dose with the exception of one that died in the group treated with doxorubicin encapsulated in the liposomes containing PEG-PE. On day 40 surviving animals were sacrificed, blood was collected and a necropsy was performed. Blood hematocrit and peripheral leukocyte counts were normal and there were no signs of gross pathological abnormalities in any of the major organs. Free drug-treated animals (18 mg/kg) exhibited a mean body weight loss of 10 to 12% (observed from day 4 through to day 10). Weight loss data observed after a similar drug dose (18 mg/kg) given in either liposomal formulation showed reduced nadir weight loss and much faster recovery to normal body weight (day 7) consistent with the well-established liposome-mediated reduction in doxorubicin toxicity (Balazsovits *et al.*, 1989; Gabizon *et al.*, 1994b). Based on weight loss toxicity and long-term survival (40 day), doses of 18 mg/kg (0.66 μmol/mouse) for free and 55 mg/kg (2 μmol/mouse) for liposomal doxorubicin were used for the plasma elimination and tumor accumulation studies summarized below.

Influence of dose escalation on plasma liposomal lipid levels. Previous studies have shown that the circulating blood levels of liposomal lipid increase as the injected dose of liposomes increase (Abra and Hunt, 1981; Mauk and Gamble, 1979). In PEG-PE-containing liposomes dose-independent pharmacokinetic characteristics were observed (Allen and Hansen, 1991; Huang *et al.*, 1992a), whereas lower doses of liposomes prepared in the absence of PEG-modified lipids were cleared from the circulation more rapidly than higher doses (Mauk and Gamble, 1979). These effects are illustrated in figure 1, which shows the liposomal lipid levels present in the plasma (24 h after administration) as a function of the total lipid dose. These results illustrate two important attributes of drug-loaded liposomes and PEG-PE-containing liposomes. First, the addition of PEG-modified lipids greatly improved the circulating level of liposomal lipid

TABLE 1

Toxicity/weight loss in response to the maximum tolerated dose for free and liposomal doxorubicin

Female BDF-1 mice (20–22 g) were given various doses of free or liposomally encapsulated doxorubicin delivered i.v. in 200 μl volume *via* the lateral tail vein. Weight loss (mean for four mice per group) was recorded daily over several weeks.

Drug Dose	Formulation	Nadir % Weight Loss (day)	Estimated MTD[a]
μmol/mouse			*mg/kg*
0.66	free drug	12 (5)	≈20
2.0	DSPC/Chol	23 (9)	≈60
2.0	DSPC/Chol/PEG-PE	25 (9)	≈60
0.66	DSPC/Chol	8 (3)	
0.66	DSPC/Chol/PEG-PE	7 (5)	

[a] These studies were performed in accordance to Canadian Council on Animal Care Guidelines which does not permit formal LD_{10} or LD_{50} studies where death is used as an endpoint, so the MTD was approximated with a small number of animals and a limited number of drug doses. It should be noted that only 1 of 25 animals used in this experiment died as a consequence of drug-related toxicity and no animals had to be terminated as a result of unacceptable suffering.

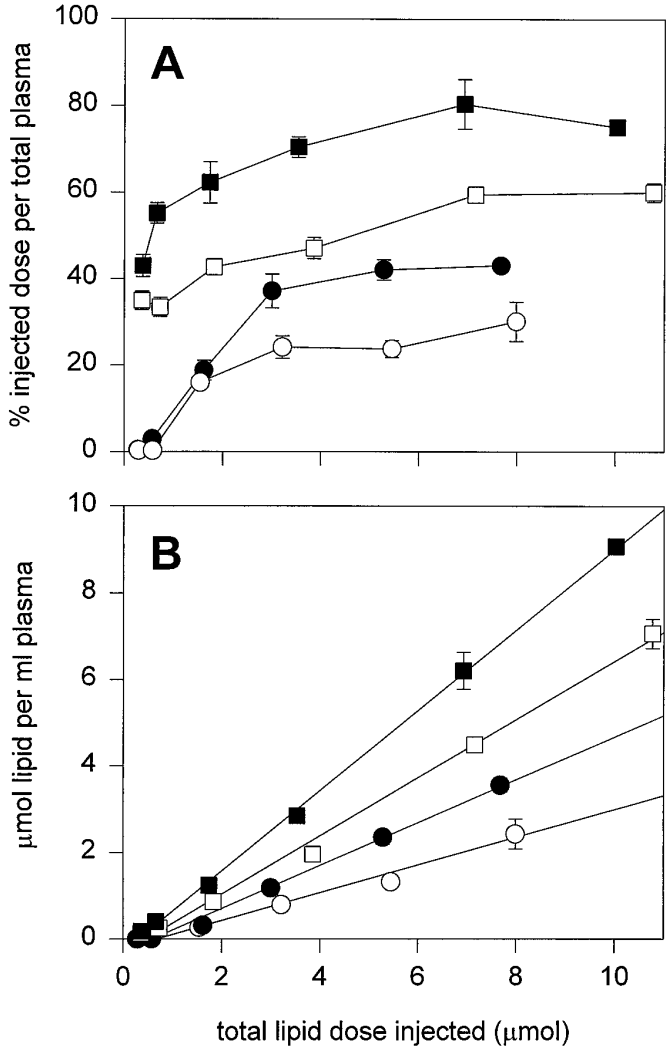

Fig. 1. Dose titration of the liposomal carrier. Various doses of "empty" or drug-loaded (0.2 drug/lipid ratio) liposomes were administered i.v. in a volume of 200 μl. Female BDF1 mice were used and the levels of lipid in the plasma were determined at 24 h as described under "Materials and Methods." The results shown represent the mean of at least four animals ± S.E.M. per group. If the error bars are not visible they are contained within the space of the symbol. (A) Plasma recovery at 24 h expressed as percent injected dose per total plasma; ○, DSPC/Chol; ●, DSPC/Chol + DOX; □, DSPC/Chol/PEG-PE; ■, DSPC/Chol/PEG-PE + DOX. (B) Same results as in A but expressed as lipid concentration (μmole lipid/ml plasma).

achieved at 24 h for both the empty and doxorubicin-loaded liposomes. At lipid doses less than 1 μmol lipid per mouse, typically 40% of the injected dose was present in the plasma 24 h after administration of DSPC/Chol/PEG-PE liposomes, whereas less than 5% of the injected dose was observed in plasma after administration of DSPC/Chol liposomes (fig.1A). As the lipid dose increased the differences between DSPC/Chol/PEG-PE and DSPC/Chol liposomes were still substantial, but these differences (significant at $P < .005$ for the 2 μmol lipid per mouse dose) were reduced from 10-fold (observed below the 1 μmol lipid per mouse dose) to less than 3-fold (observed above the 2 μmol lipid per mouse dose).

Plotting these data as a function of micromoles of lipid per milliliter of plasma (shown in fig. 1B) demonstrates that a linear relationship exists between the lipid dose administered and the levels of lipid in the circulation at 24 h, regardless of the liposomal formulation used. Correlation coefficients (r^2) were 0.89 ($P = .05$) and 0.97 ($P = .01$) for empty and loaded DSPC/Chol liposomes and 0.98 ($P = .01$) and 0.99 ($P = .01$) for the empty and loaded DSPC/Chol/PEG-PE liposomes, respectively. The second important attribute defined by the data presented in figure 1 is that entrapped doxorubicin significantly increases the plasma blood levels obtained 24 h after i.v. administration of DSPC/Chol/PEG-PE liposomes or DSPC/Chol liposomes. This typically resulted in a 1.5- to 1.7-fold increase in circulating levels of liposomal lipid measured at 24 h when comparing doxorubicin-loaded liposomes to liposomes without encapsulated drug.

Analysis of drug elimination from plasma and tumor accumulation in BDF-1 mice bearing Lewis Lung tumors. A comprehensive examination of drug and liposome circulation lifetimes after i.v. administration was completed in BDF-1 mice bearing Lewis Lung tumors and is summarized in figure 2. Figure 2A shows elimination of the liposomal carriers from the blood compartment for the tumor-bearing mice. At 24 h and later, the dominant factor dictating enhanced circulating blood levels was the presence of entrapped doxorubicin. The drug-loaded liposomes, for both DSPC/Chol/PEG-PE liposomes and DSPC/Chol liposomes, were consistently at much higher concentrations in the blood than their respective empty systems and resulted in 3- to 10-fold increases (significant at $P < .001$ for the 1-, 2-, 4- and 7-day time points) in the plasma concentrations of liposomal lipid. It should be noted that for both doxorubicin-loaded liposomal carriers there was an approximately equivalent reduction in liposome uptake by the liver (data not shown). The plasma liposomal lipid levels obtained after 24 h were significantly greater ($P < .05$ for the 1-, 2- and 4-day time points) for the PEG liposomes.

The differences in blood levels achieved after administration of liposomal preparations were less than those observed in tumor-free animals (refer to fig. 1, 8–10 μmol/mouse dose). This is an important consideration related to tumor-induced increases in liposome elimination from the circulation. For the DSPC/Chol liposomes, nearly half of the injected dose was eliminated from the circulation within 1 h. Further, the blood levels obtained at 24 h were between 2- and 4-fold lower in tumor-bearing mice than in tumor-free mice. For the conventional liposomes, greater then 90% of the circulating lipid was lost because of the presence of tumor could be accounted for through increased liposome uptake by the liver, spleen and solid tumors (data not shown). For the PEG-PE-containing systems only 75% could be accounted for in increased uptake in these three tissues.

The plasma concentrations of doxorubicin measured after injection of free and liposomal drugs are shown in figure 2B. Measurements made 1 and 2 days after injection indicate drug levels of greater than 0.5 μmol of drug per ml plasma (equivalent to 25% of the injected drug) for both DSPC/Chol/PEG-PE liposomes or DSPC/Chol liposomes. Drug levels were significantly higher ($P < .01$ for all time points except day 7) for samples obtained from mice given the DSPC/Chol/PEG-PE liposomal formulation in comparison with DSPC/Chol liposomes. An estimation of the mean doxorubicin AUC for the blood compartment indicated a marginal 1.5-fold increase in AUC when the drug was given encapsulated in DSPC/Chol/PEG-PE liposomes (AUC = 78 $\mu mol \cdot ml^{-1} \cdot h$) *ver-*

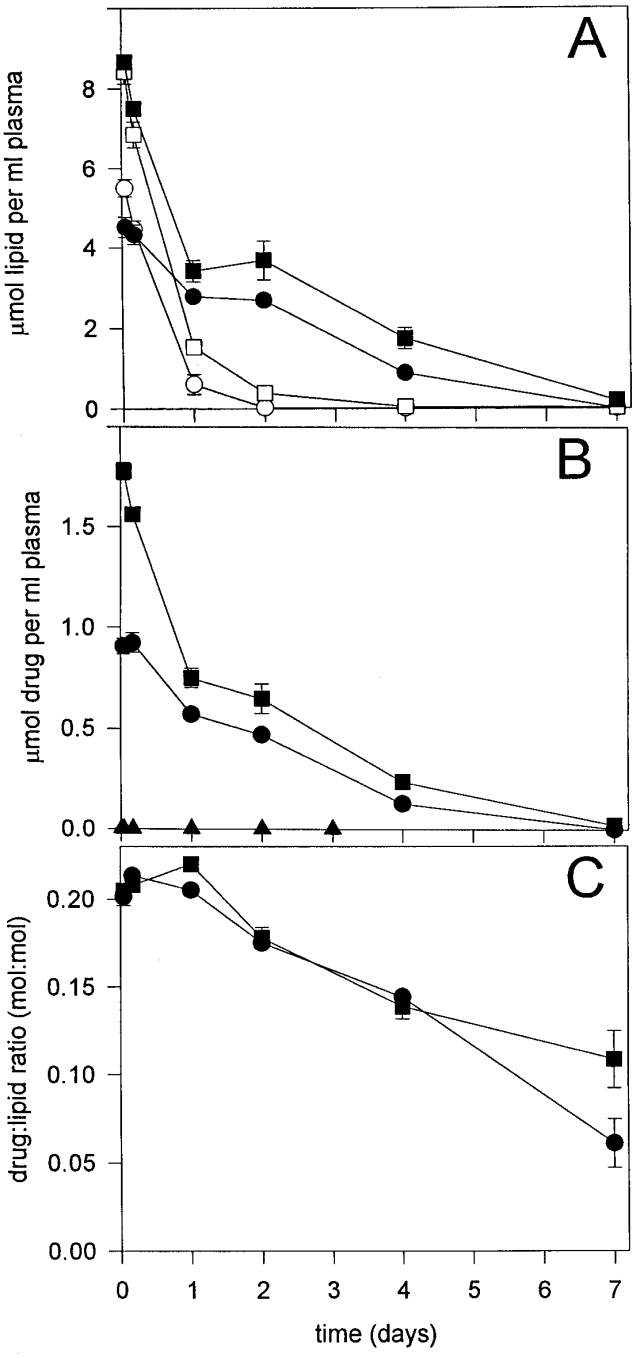

Fig. 2. Pharmacokinetic analysis of liposome clearance in LLC bearing BDF1 mice. Mice were injected (i.v.) with 10 μmol total lipid per mouse of either DSPC/Chol or DSPC/Chol/PEG-PE liposomes with or without entrapped doxorubicin (2 μmol drug). Mice were sacrificed at 1, 4, 24 h, 2, 4 and 7 days, and lipid and drug plasma concentrations were determined. Results shown represent the mean of four animals ± S.E.M. per group. If the error bars are not visible they are contained within the space of the symbol. (A) Liposomal lipid plasma concentrations: ○, DSPC/Chol; ●, DSPC/Chol + DOX; □, DSPC/Chol/PEG-PE; ■, DSPC/Chol/PEG-PE + DOX. (B) Drug plasma concentrations: ▲, 0.66 μmol free DOX; ●, 2 μmol DOX in DSPC/Chol; ■, 2 μmol DOX in DSPC/Chol/PEG-PE. (C) Drug-to-lipid ratio: ●, DSPC/Chol; ■, DSPC/Chol/PEG-PE liposomes.

sus DSPC/Chol liposomes (50 $\mu mol \cdot ml^{-1} \cdot h$). This is in contrast to the remarkable differences in plasma drug levels observed when comparing the liposomal formulations to free drug (given at the MTD). In the absence of a carrier, plasma doxorubicin levels fall below detectable limits within 4 h. Assuming that the plasma volume of a 20- to 22-g mouse is 1 ml (Bally *et al.*, 1993) and an injected drug dose of 0.66 μmol per animal, it was estimated that greater than 99% of the injected drug was eliminated from the plasma compartment within 15 min after injection. The AUC for free drug was estimated to be 87 $nmol \cdot ml^{-1} \cdot h$, which is approximately 600 and 900 fold less than that obtained for doxorubicin given in DSPC/Chol liposomes or DSPC/Chol/PEG-PE liposomes, respectively.

Drug retention by liposomes in the blood compartment can be estimated by calculating the drug-to-lipid ratio in plasma over time. Figure 2C shows that DSPC/Chol and DSPC/Chol/PEG-PE liposomes retain encapsulated drug equally well, with a half-life for drug release in excess of 5 days. There was no measurable change in the drug-to-lipid ratio over the initial 24-h period after i.v. injection. After 2 and 4 days in the circulation, the drug-to-lipid ratio was approximately 90% and 70% of the value measured before injection, respectively. The doxorubicin leakage rate after 24 h was estimated to be 0.75 nmol drug/μmol lipid/h.

To ascertain whether DSPC/Chol/PEG-PE liposomes exhibited a greater propensity to accumulate in tumors, we compared drug and liposomal lipid levels in Lewis Lung tumors during 7 days after i.v. administration. These data, shown in figure 3, indicate that tumor uptake was similar for both drug-loaded and "empty" liposomal carriers during the initial 24 h after i.v. administration. In the absence of encapsulated drug DSPC/Chol liposomes reached a peak level in tumor tissue of 0.6 μmol lipid/g tissue. This value was achieved 24 h after administration. For "empty" DSPC/Chol/PEG-PE liposomes, the peak lipid concentration was achieved 48 h after administration and a value of 1.0 μmol lipid/g tissue was observed. The gradual decline in peak values was attributed to continued tumor growth. The maximum amount of liposomal lipid delivered per tumor was equivalent to approximately 5% and 8% of the injected lipid dose for DSPC/Chol and DSPC/Chol/PEG-PE liposomes, respectively. At 2, 4 and 7 days the level of liposomal lipid measured in the tumor was significantly ($P < .01$) greater for the DSPC/Chol/PEG-PE liposomes than for the DSPC/Chol liposomes.

For the drug-loaded systems, tumor levels of liposomal lipid (micromoles lipid per gram) increased during the 7-day time course. This apparent increase in liposome delivery was primarily a consequence of doxorubicin-mediated regression of the solid tumors. Although a maximum lipid concentration cannot be estimated from the data in figure 3, levels of liposomal lipid achieved in the tumor exceeded 2.5 μmol lipid/g. There was not a significant difference (except on day 2 where a $P < .05$ was obtained) between the DSPC/Chol and DSPC/Chol/PEG-PE liposomes when the carriers had encapsulated doxorubicin. The results shown in figure 3B indicate that for both liposomal drug formulations, peak solid tumor concentrations of drug (C_{Tmax}) were achieved by day 4 and this level was maintained through day 7. The combined effects of tumor regression and drug release from liposomes within the tumor lead to this plateau effect. Tumor drug levels after administration of doxorubicin encapsulated in DSPC/Chol liposomes were higher (significantly different at

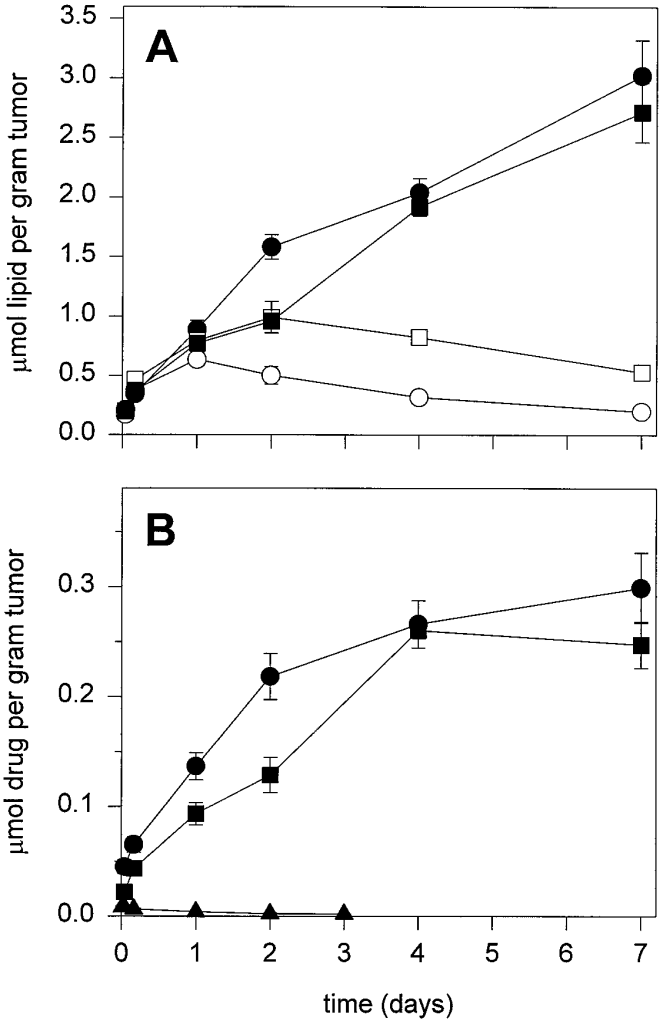

Fig. 3. Tumor loading of liposomal lipid and doxorubicin after i.v. administration of either DSPC/Chol or DSPC/Chol/PEG-PE liposomes with or without entrapped doxorubicin (2 μmol drug per injection). The lipid dose was 10 μmol total lipid per mouse. When free drug was given i.v. at the MTD a dose of 0.66 μmol per mouse was administered. Mice were sacrificed at 1, 4, 24 h, 2, 4 and 7 days, and lipid and drug plasma concentrations were determined. Results shown represent the mean of four animals ± S.E.M. per group. If the error bars are not visible they are contained within the space of the symbol. (A) Liposome accumulation in the Lewis Lung solid tumor: ○, DSPC/Chol; ●, DSPC/Chol + DOX; □, DSPC/Chol/PEG-PE; ■, DSPC/Chol/PEG-PE + DOX. (B) Drug accumulation. ▲, free DOX; ●, DOX in DSPC/Chol; ■, DOX in DSPC/Chol/PEG-PE.

$P < .05$ for the 1-, 4-, 24- and 48-h time points) than that delivered *via* PEG liposomes.

With the mean AUC (micromoles of doxorubicin per gram of tissue-time curve, calculated from data integrated from 0 time through to day 7) as an estimate of tumor drug exposure, DSPC/Chol liposomes (AUC_T of 38 $\mu mol \cdot g^{-1} \cdot h$) delivered slightly more doxorubicin to tumors than DSPC/Chol/PEG-PE liposomes (AUC_T of 31 $\mu mol \cdot g^{-1} \cdot h$). The peak level of drug obtained in tumors was approximately 250 nmol/g, and this represents approximately 140 μg equivalents of doxorubicin per g tumor. In contrast, after administration of free doxorubicin, peak drug levels were achieved within 15 min, and these levels (10 nmol/g) were 25-fold lower than those obtained after administration of the liposomal formulations. In animals receiving liposomal doxorubicin there was a progressive decline in the drug-to-lipid ratios measured within the tumor. For DSPC/Chol liposomes the calculated drug-to-lipid ratios (mol/mol) dropped from 0.2 at the earliest time points down to 0.13 and 0.10 at 4 and 7 days, respectively. Similar results were obtained in tumors from animals given DSPC/Chol/PEG-PE liposomal doxorubicin, where ratios of 0.14 and 0.09 were observed at 4 and 7 days, respectively. These tumor drug-to-lipid ratios were similar to those measured in the circulation (fig. 2C). Based on these changes in drug-to-lipid ratio, it can be estimated that the rate of drug release from liposomes within the tumor is between 0.60 and 0.65 nmol drug/μmol lipid/h.

Inhibition of tumor growth. To provide a complete pharmacodynamic assessment of free doxorubicin and both liposomal doxorubicin formulations, the antitumor activity of the drug given at the MTD was determined. Tumor size was measured after a single bolus injection of drug when the tumors were 0.2 to 0.4 g. These data are shown in figure 4.

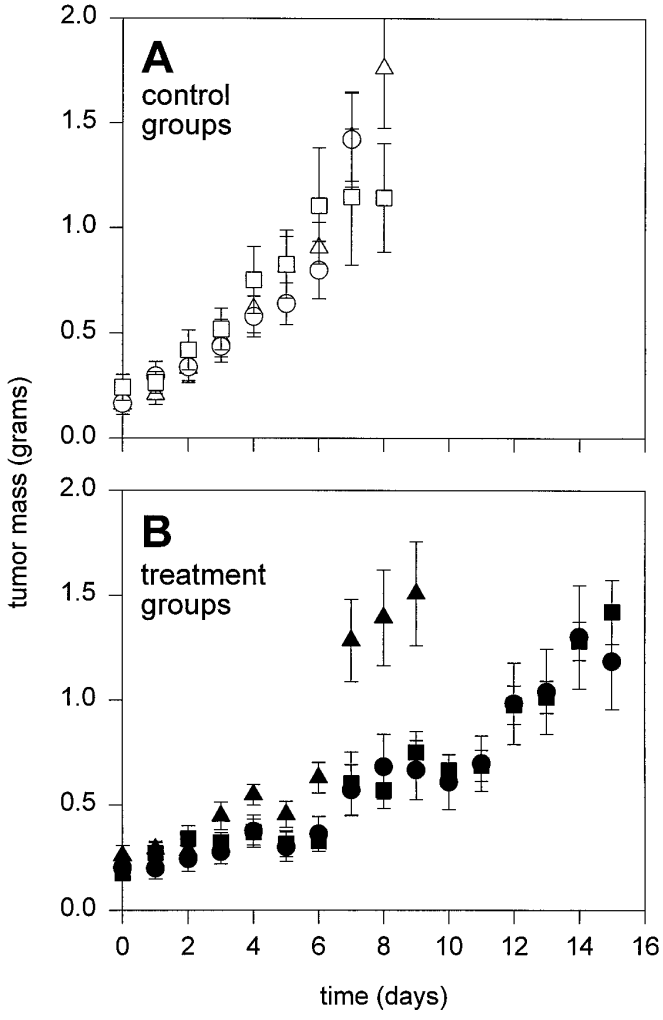

Fig. 4. Tumor growth inhibition. Tumor-bearing mice were given various treatments and tumor mass was estimated daily by use of caliper measurements. (A) Control groups: △, normal control; ○, 10 μmol empty DSPC/Chol; □, 10 μmol empty DSPC/Chol/PEG-PE. (B) Treatment groups: ▲, 0.66 μmol free DOX; ●, 2 μmol DOX in 10 μmol DSPC/Chol; ■, 2 μmol DOX in 10 μmol DSPC/Chol/PEG-PE. Results shown represent the mean of 10 animals ± S.E.M. with the experiment being repeated two times with groups of five mice.

Tumors in the control group grew rapidly, reaching more than 1.5 g within 8 days (22 days after tumor cell inoculation). The administration of empty DSPC/Chol or DSPC/Chol/PEG-PE liposomes (10 μmol total lipid) had no significant effect on tumor growth. Treatment with free drug resulted in a slight (not significant) delay in tumor growth, where the time required for the tumor to double in size increased from 3 days in control animals to 4 to 6 days in free drug-treated animals. After this time point the tumors progressed rapidly to achieve a tumor mass of 1.5 g by day 9 (similar to control animals). DSPC/Chol and DSPC/Chol/PEG-PE liposomal doxorubicins were more effective than free drug, increasing the tumor doubling time to approximately 7 days in treated groups; however, tumors continued to grow and within 16 days after treatment the tumor mass approached 1.5 g. These results indicated that there is no difference between the two liposomal systems in terms of therapeutic activity.

Discussion

The results presented here indicate that at higher drug dose levels, PEG incorporation into the liposomal drug carrier does not result in improved doxorubicin delivery to Lewis Lung solid tumors. This contrasts with previous studies which indicate that liposomes with surface-associated PEG have increased microvascular permeability compared with similar liposomes prepared in the absence of PEG-modified lipids (Wu *et al.*, 1993). When these liposomes are given at the maximum tolerated dose with a cytotoxic drug, such as doxorubicin, our results suggest little difference in either the kinetics or total amount of drug accumulation. These discrepancies in results will provide the focus of this discussion.

In an attempt to define biological attributes that are important for maximizing drug delivery to tumors after i.v. administration of liposomes it has been suggested that the efficiency of passive delivery will correlate with exposure levels around sites of extravasation. If the mechanism of extravasation is through gaps in the endothelial layer (Yuan *et al.*, 1994), rather than *via* vesicular transport or leukocyte-mediated extravasation, and if transport of the liposomal drug carriers is based solely on diffusion through pores in the blood vessels, then tumor accumulation should increase when the concentration of circulating liposomes increases. Assuming that the rate of passive diffusion of DSPC/Chol and DSPC/Chol/PEG-PE liposomes are similar then a 1.5-fold increase in AUC_P, obtained for the PEG-containing systems, should have effected an increase in tumor drug levels. The results shown in figure 3 do not support this. More specifically, we believe that the movement of drug from the plasma compartment to the tumor site can be described with a drug-targeting efficiency parameter, T_e, relating the AUC in the circulation to the tumor AUC ($T_e = AUC_T/AUC_P$). With this parameter, DSPC/Chol liposomes gave a T_e value of 0.76 which was almost a factor of 2 higher than that for the PEG-PE-containing liposomes (T_e value of 0.40).

It can be concluded that, under the conditions in which drug is given intravenously at the MTD, there are certainly no distinct benefits associated with the use of PEG-modified liposomes. Three specific observations may help account for this discrepancy with current literature: 1) encapsulated doxorubicin-mediated increases in the circulation lifetime of both DSPC/Chol and DSPC/Chol/PEG-PE liposomes; 2) the presence of established LLC increased liposome elimination rates; and 3) differences in circulation lifetimes were not substantial when the liposomal drug formulations were administered at the MTD. Regarding the influence of entrapped doxorubicin on the elimination behavior of the associated liposomal carrier, this effect has been characterized previously (Bally *et al.*, 1990; Parr *et al.*, 1993; Daemen *et al.*, 1995). It is thought to be a consequence of the toxic activity of the encapsulated drug on the phagocytic cells of the RES that are responsible for elimination of liposomes after parenteral administration. Importantly, this effect has also been observed for liposomes with encapsulated vincristine (Parr, 1995) and cisplatin (Bally, unpublished observations) and therefore is not unique to the cytotoxic activity of doxorubicin. As noted here (see figs.1 and 2) and elsewhere (Parr *et al.*, 1993) this effect on the RES is not diminished through use of liposomes prepared with PEG-modified lipids. Coating liposomes with PEG may reduce delivery of liposomes to the RES; however, sufficient delivery is achieved to engender a significant enhancement in circulation lifetime in comparison with PEG-coated liposomes prepared in the absence of a cytotoxic drug.

Increased liposome elimination in animals bearing small, but well-established, tumors has been observed previously for animals bearing a subcutaneous S180 tumor model (Oku *et al.*, 1992). Metastatic solid tumors such as the LLC may shed large amounts of cells and other debris (Butler and Gullino, 1975; Glaves, 1983), and it can be suggested that the release of this material into the circulation may subsequently lead to stimulation of the RES (Thomas *et al.*, 1995). In addition, solid tumors can either directly or indirectly stimulate the release of tumor necrosis factor-α or other lymphokines such as interleukin-2 (Thomas *et al.*, 1995; Nagarkatti *et al.*, 1990;). Such molecules are implicated in vascular leak syndrome (Fujita *et al.*, 1991; Deehan *et al.*, 1994). Although no evidence for increased tissue plasma volumes was found, a slightly increased liver size and greatly increased spleen size was observed, an effect which has also been noted for cytokine induced vascular leak system (Fujita *et al.*, 1991). Regardless of the factors mediating enhanced liposome elimination, the effect results in increased elimination of both DSPC/Chol and DSPC/Chol/PEG-PE liposomes, reducing the recovery of lipid in the circulation at 24 h by a factor of 2 or more. Increased elimination rates could be accounted for, in part, by enhanced liposome uptake in liver and spleen as well as tumor accumulation. We observed no changes in normal tissue blood or plasma volume.

The third observation of interest is a consequence of both doxorubicin-induced increases and tumor-mediated decreases in liposome circulation lifetime which, in combination, resulted in circulating blood levels for DSPC/Chol/PEG-PE liposomes that were only marginally better than DSPC/Chol liposomes. Specifically a 1.5-fold increase in mean AUC was achieved with the PEG-coated liposomes in comparison with DSPC/Chol liposomes. The peak drug concentrations ($C_{T_{max}}$) achieved within the tumors must also be considered. These were achieved at approximately the same time for both liposomal formulations (day 2 for DSPC/Chol liposomes and day 4 for DSPC/Chol/PEG-PE liposomes) and the $C_{T_{max}}$ values obtained were approximately 250 nmol

doxorubicin or 140 μg doxorubicin equivalents per g tumor. This is far higher than achieved in previous studies, and dramatically illustrates the effect of dosing at the MTD. Previous tumor drug loading values are of the order of 20 nmol/g solid tumor (Mayer *et al.*, 1990; Gabizon, 1992; Maruyama *et al.*, 1993) to 40 nmol/g (Ning *et al.*, 1994).

We believe that there is considerable evidence that the mechanism mediating increased drug delivery to solid tumor involves extravasation of the intact liposome to the tumor site followed by slow release of drug. Thus the antitumor effect may be attributed to drug released from liposomes that localize in the tumor as opposed to systemic release of drug (Mayer *et al.*, 1994). In fact, studies have shown that intact liposomes can be found within the interstitial space between tumor cells (Gabizon, 1992; Huang *et al.*, 1992b). The lipid and drug data presented here are consistent with this mode of doxorubicin delivery to sites of tumor growth.

Differences in liposome-engendered increases in drug delivery to the LLC were not substantial when the formulation was changed from DSPC/Chol to DSPC/Chol/PEG-PE. Therefore, it is not surprising that the antitumor activity of the two liposomal formulations were not significantly different. Although the liposomal drug formulations used did achieve a significant reduction in growth rate, it can be suggested that the poor efficacy results were caused by the fact that drug within the tumor is not freely bioavailable. Studies (not shown) measuring the doxorubicin concentrations necessary for 95% inhibition of growth (IC_{95}) of LLC cells in culture suggest that concentrations in excess of 900 nM are required for efficient cell kill. We have achieved overall drug concentrations of 250 nmol/g tumor and it can be suggested that the drug concentrations within the tumor, if released from the liposomes, will be in excess of that required to achieve maximum cytotoxic effects. However, calculated rates of drug release from liposomes in the tumor (0.60–0.65 nmol drug/μmol lipid/h, which corresponds to a half-time for release of more than 5 days) may not be adequate for inhibition or elimination of the tumor cells. The development of techniques which result in increased rates of drug release combined with the techniques for achieving efficient and substantial liposomal drug delivery to tumors could be of great therapeutic advantage. One example of this approach involves the use of thermosensitive liposomes that can be induced to release drug by hyperthermia (Maruyama *et al.*, 1993; Ning *et al.*, 1994; Huang *et al.*, 1994).

In summary, the results of this work establish that increasing the liposomal carrier dose up to the MTD for encapsulated doxorubicin increases plasma drug and lipid concentrations. Extended circulation lifetimes and high levels of tumor-associated drug can be achieved. Although inclusion of PEG-PE in the liposomal drug formulation does increase circulating blood levels, this does not result in improved tumor delivery of the drug. Given the extremely high levels of drug delivery achieved, it is proposed that techniques leading to the triggered release of liposome contents may lead to more significant improvements in the therapeutic activity of conventional liposomal drug carriers. The benefits associated with use of PEG-coated liposomes will likely be restricted to formulations that provide effective therapy with low lipid doses.

Acknowledgments

We are also grateful for the useful editorial comments and critical evaluation provided by Troy Harasym, Dr. L.D. Mayer and Dr. D. Reimer.

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