Journal of Liposome Research 1(4) 463–480, (1990)

Strategies for Optimizing Liposomal Doxorubicin

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Liposome encapsulation of doxorubicin can dramatically alter its biological activity, resulting in decreased toxicity and equivalent or increased antitumor potency. Since the physical characteristics of the liposome carrier system (size, lipid composition, and lipid dose) can have profound effects on the pharmacologic properties of vesicles administered intravenously, it may be expected that the therapeutic activity of liposomal doxorubicin will be sensitive to these properties. To determine the influence of these variables on the toxicity and efficacy properties of liposomal doxorubicin, transmembrane pH gradient-dependent active encapsulation techniques have been utilized to generate liposomal doxorubicin preparations in which the vesicle size, lipid composition, and drug to lipid ratio can be independently varied. These studies indicate that the toxicity of liposomal doxorubicin is related to the stability of the preparation in the circulation. This property is dictated primarily by vesicle lipid composition, although the drug to lipid ratio can also exert an influence. In contrast, the antitumor activity of liposomal doxorubicin appears most sensitive to the size of the vesicle system. Specifically, antitumor drug potency increases as the vesicle size is decreased. These studies demonstrate that manipulating the physical characteristics of liposomal anticancer pharmaceuticals can lead to preparations with optimized therapeutic activity.

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INTRODUCTION

A substantial body of literature indicates that liposome encapsulation of doxorubicin can significantly enhance the therapeutic index of this antineoplastic agent. The first reports describing the benefits of liposomal doxorubicin appeared over 10 years ago (1–3). These studies demonstrated that liposomal doxorubicin preparations display decreased drug-induced cardiotoxicity, which is a clinically relevant dose-limiting side effect for doxorubicin. Subsequent investigations by numerous laboratories using widely differing liposomal doxorubicin systems showed that acute and chronic toxicities typically associated with administration of free drug (in addition to cardiotoxicity) were also reduced (4–11). Furthermore, these investigations have not revealed significant new vesicle-mediated toxicities following administration of doxorubicin in liposomal form. In addition to toxicity evaluations, various antitumor efficacy studies have shown that liposomal doxorubicin preparations are generally equipotent to free drug in treating tumor-bearing animals (4,5,9,12–18). Due to the decreased toxicity of liposomal systems, increased drug dosages can be administered, resulting in enhanced efficacy and an increase in the therapeutic index of doxorubicin.

Table 1 presents a representative survey of the various liposomal doxorubicin preparations that have been used for therapeutic assessment in animal models as described above. These vesicle systems display wide variations in the degree of lamellarity, size, lipid composition, drug to lipid ratio, and trapping efficiency. The difficulties associated with controlling these parameters arise from the nature of the drug-trapping procedures traditionally used to generate liposomal doxorubicin systems. This is due to the fact that inclusion of negatively charged lipids in the membrane dramatically increases the association of doxorubicin with the vesicles. More important, alterations in one physical characteristic of the liposomal carrier are often accompanied by changes in one or more of the other properties. Changes in lipid composition, for example, often result in altered vesicle size distributions and entrapped drug to lipid ratios. As a consequence, differences in the biological behavior observed between different formulations often cannot be attributed directly to any specific physical characteristic of the liposomal system.

Alterations in the physical characteristics of liposomal doxorubicin effectively yield numerous drug "analogs" that may exhibit widely differing biological activity; and the inability to manipulate individual properties of liposomal doxorubicin preparations limits attempts to select optimized formulations. A program for optimizing liposomal doxorubicin therefore requires a versatile process for generating well-characterized formulations with the desired physical properties. These preparations should then be utilized in studies that focus on

identifying characteristics that significantly affect the in vivo fate of the liposomal drug carrier, since these properties will ultimately dicatate drug disposition. The basis for such an approach can be found in numerous reports investigating the influence of vesicle size, lipid composition, and lipid dose on the pharmacokinetic and biodistribution behavior of intravenously administered liposomes. It is instructive to review these relationships and examine their potential implications for the therapeutic activity of liposomal doxorubicin.

Lipid composition plays an important role in determining the integrity of liposomal drug preparations in vivo. For example, the absence of cholesterol can result in lipoprotein-induced vesicle destabilization and concomitant release of entrapped agents (19–21). Also, increasing the degree of acyl chain saturation and chain length of component lipids increases the retention of encapsulated materials in circulating liposomes. Lipid composition also affects the blood clearance and distribution properties of liposomes. For example, vesicles containing certain acidic phospholipids such as phosphatidylserine are rapidly removed from the circulation by cells of the reticuloendothelial system (RES) that reside primarily in the liver and spleen (22–24), whereas inclusion of the glycolipid GM_1 can dramatically increase liposome blood residence times (25,26). The longevity of liposomes in the circulation is also dependent on the vesicle size and lipid dose. Results from several laboratories have shown that decreasing the size of uncharged vesicle systems from approximately 1 μm to less than 50 nm can increase the liposome content in blood by 10-fold or more over 24 hr after intravenous (iv) injection (22,23). This is accompanied by a decrease in vesicle accumulation in the RES. Such increased circulation times appear to enhance accumulation of the liposomes in tumor tissue (26) and can also lead to elevated drug levels in the blood for extended periods of time. In contrast, as much as 90% of large liposomes administered iv at low lipid doses can be sequestered within minutes by the liver and spleen (23,27). Increasing the lipid dose also substantially increases the circulation lifetime of liposomes (28–30). This relationship is observed until the amount of administered lipid is sufficient to saturate the RES, at which point further increases in the lipid dose have little effect on relative liposome clearance.

Some previous studies have addressed the effects of vesicle size and lipid composition on the antitumor activity of liposomal doxorubicin (2,4,13,17). Although these investigations revealed changes in drug efficacy when the physical properties of liposomal doxorubicin systems are altered, interpreting cause and effect relationships is complicated by the inability to vary such physical characteristics independently. In this context, we have developed procedures for preparing liposomal doxorubicin formulations that circumvent the problems associated with passive trapping techniques. Here we describe this technology and demonstrate its usefulness in identifying variables that reduce the toxicity and enhance the efficacy of liposomal doxorubicin.

Table 1. Characteristics of Liposome-Encapsulated Doxorubicin Preparations

Ref.	Liposome type	Size (nm)	Composition	Ratio	Drug / lipid (mol:mol)	Trapping efficiency (%)	Biological activity
13	SUV	135 ± 70	PS/PC/C	3:7:10	1:18.6	25	More effective than free drug against metastatic tumor
4	MLV	N.D.	PC/C	1:1	1:33	14	MLV systems as effective against J.6457 tumor as free drug in single-dose regimen
	MLV	N.D.	CL/PC/C	1:4:5	1:21.2	62	
	MLV	N.D.	CL/PC	1:4	1:14.8	58	
	MLV	N.D.	PS/PC/C	3:7:10	1:23	42	
	SUV	N.D.	PC/C	1:1	1:14	15	
	SUV	N.D.	CL/C	5:2:5	1:18	90	
	SUV	N.D.	CL/PC/C	1:4:5	1:26.7	45	
	SUV	N.D.	PS/PC/C	3:7:10	1:44.2	22	
18	SUV	N.D.	PC/C	7:2	1:130	7	More effective than free drug against Ehrlich solid tumor
	SUV	N.D.	PC/C/DCP	7:2:1	1:37	26	
	SUV	N.D.	PC/C/SA	7:2:1	1:225	4	
2	SUV	N.D.	PC/C/PS	10:4:1	1:11.6	55	Negatively charged systems inferior to positively charged systems in cardiotoxicity and efficacy
	SUV	N.D.	PC/C/SA	10:4:3	1:18.4	35	

6	SUV	90 ± 20	CL/PC/C/SA	1:5:3.5:2	1:12.4	55	Significant reduction of chronic cardiotoxicity in dogs
5	LUV	150	PG/PC/C	1:4:5	1:30	50	Less cardiotoxic and equally potent against L1210 tumor
8	SUV	75 ± 27	PC/PG/C	7:3:4	1:14	50	Decreased chronic toxicity compared to free drug
17	LUV	300	PC/PS/C	10:1:4	1:20.4	57	Saturated lipid systems exhibit delayed antitumor activity
	LUV	730	DPPC/DPPG/C	10:1:10	1:43.2	27	

PC, phosphatidylcholine; PS, phosphatidylserine; C, cholesterol; CL, cardiolipin; DCP, dicetylphosphate; SA, stearylamine; PG, phosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; N.D., not determined.

GENERATION OF LIPOSOMAL DOXORUBICIN PREPARATIONS

Well-characterized liposomal doxorubicin preparations exhibiting a wide range of vesicle sizes, lipid compositions, and drug-to-lipid ratios can be obtained by accumulating the drug into preformed liposomes displaying a transmembrane pH gradient (inside acidic). During the production of the empty liposomes, the vesicle lipid composition and size are selected. Moderate pressure extrusion of liposomes appears most suitable for generating liposomes with a defined size distribution. This technique (31,32), which is a modification of a low-pressure extrusion process first described by Olson et al. (33), involves repeated extrusion of precursor multilamellar vesicles (MLVs) directly through membrane filters with a uniform pore size. The resulting vesicles are homogeneous with respect to size and the mean diameter approximates the filter pore size (Fig. 1). Lipid concentrations as high as 400 mg/ml buffer can be used and the procedure is applicable to virtually any lipid composition that adopts a bilayer structure in aqueous media.

The use of transmembrane ion gradients to encapsulate doxorubicin in liposomes is similar to the response of various probes to membrane potentials and pH gradients (34). In the case of transmembrane pH gradients, these probes redistribute across the membrane according to the relationship:$[probe]_{in}/[probe]_{out} = [H^+]_{in}/[H^+]_{out}$](35,36). Thus for a pH gradient of 3 units (inside acidic), for example, an interior/exterior concentration gradient of 1000 is achieved for the probe molecule. Since most probes of pH gradients are lipophilic cations (as is doxorubicin), it was reasoned and shown that a similar process can result in efficient loading of doxorubicin into liposomes (37). As shown in Figure 2, doxorubicin can be efficiently accumulated into egg phosphatidylcholine (EPC)/cholesterol vesicles displaying an imposed transmembrane pH gradient (inside acidic). The need to incubate the mixture at elevated temperatures to obtain efficient entrapment rapidly reflects the permeability properties of the membrane to doxorubicin. Analysis of this uptake process has indicated that doxorubicin permeates the bilayer in the neutral (unprotonated) form and is sequestered inside the liposome upon reprotonation of the amine function when exposed to the acidic vesicle interior (38). It should be noted that high-performance liquid chromatographic (HPLC) analysis of doxorubicin under these conditions indicates no detectable breakdown of the drug.

There are several striking features of the pH gradient-dependent encapsulation procedure that make it well suited for use in the in vivo delivery of doxorubicin (39). First, a wide range of drug to lipid ratios can be readily achieved in association with trapping efficiencies greater than 95%. Second, doxorubicin uptake is relatively insensitive to the vesicle lipid composition. Liposomes containing 0–50% cholesterol, saturated or unsaturated acyl chain phospholipids, and charged

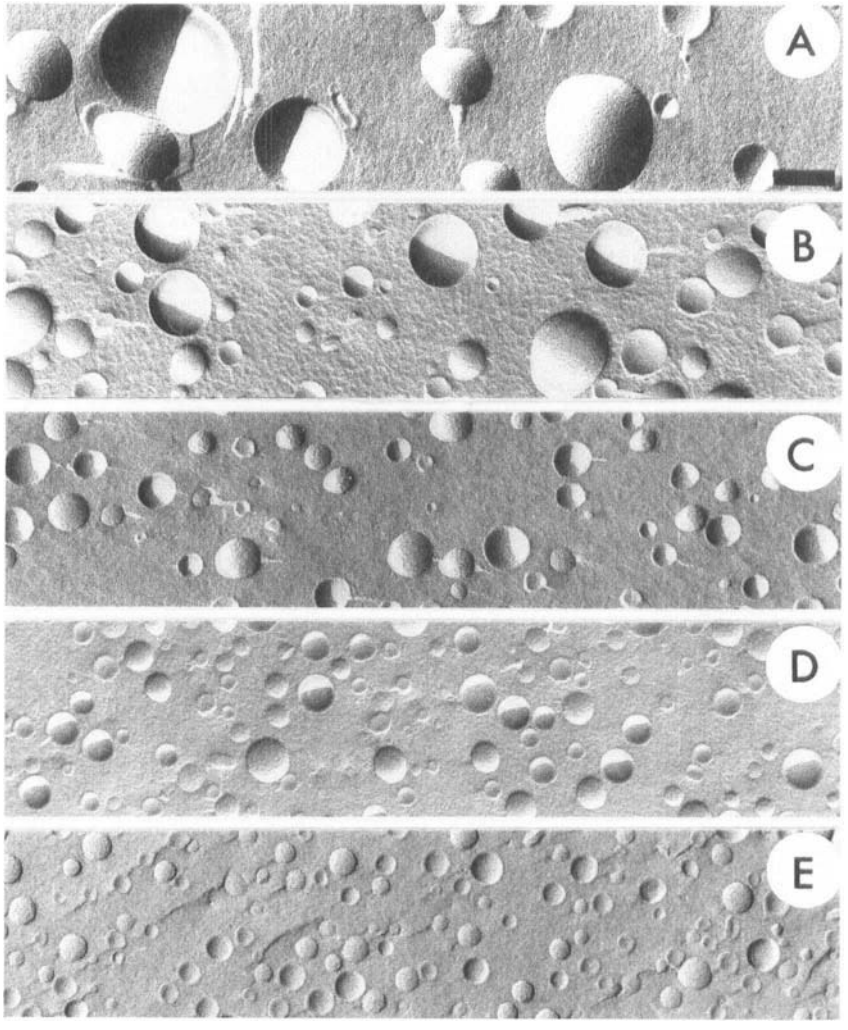

Figure 1. Freeze fracture electron micrographs of EPC frozen and thawed MLVs (100 mg/ml) extruded 10 times through polycarbonate filters with pore sizes of 400 nm (A), 200 nm (B), 100 nm (C), 50 nm (D), and 30 nm (E). All micrographs were obtained under the same magnification; the bar in A represents 150 nm.

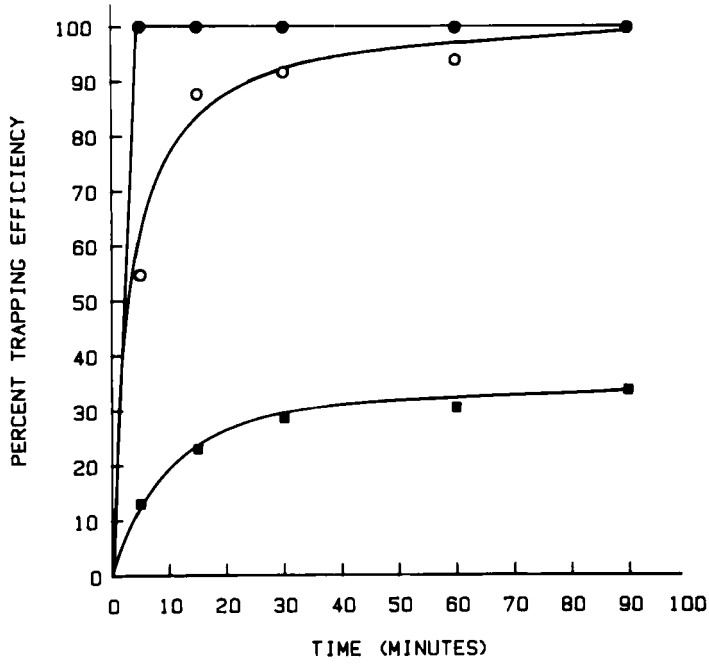

Figure 2. Uptake of doxorubicin (3 mg/ml) into EPC/cholesterol (55:45, mol/mol) liposomes (11 mg/ml) exhibiting a pH gradient (pH 4.0 inside, 7.5 outside) at 21°C (■), 37°C (○), and 60°C (●).

or neutral components can all be loaded with drug at equivalent drug to lipid ratios and trapping efficiencies. Third, entrapment is relatively independent of liposome size. It should be noted, however, that the maximum drug to lipid ratio that can be used while maintaining trapping efficiencies in excess of 95% decreases slightly for vesicle systems ≤ 100 nm. This limit results from the decreased trapped volumes and correspondingly lower buffering capacities obtained for the smaller vesicle systems. Nevertheless, liposomal doxorubicin preparations exhibiting a wide range of vesicle sizes can be generated for drug to lipid ratios as high as 0.25:1. This is 3–10 times higher than can be achieved by previously used entrapment procedures (see Table 1). The drug to lipid ratio is of particular importance since this variable will dictate the lipid dose required for in vivo administration of liposome-encapsulated drugs.

In addition to inducing efficient encapsulation of doxorubicin, the transmembrane pH gradient also enhances drug retention in the liposomes. Figure 3 shows that doxorubicin entrapped in vesicles exhibiting a variety of lipid compositions is retained far longer than for EPC/cholesterol systems in which the drug had been passively trapped. Some degree of variability in the drug release rate from ΔpH-loaded vesicles is observed between vesicles of differing lipid composition. These leakage rates correlate with well-characterized phenomena (40) in which, for

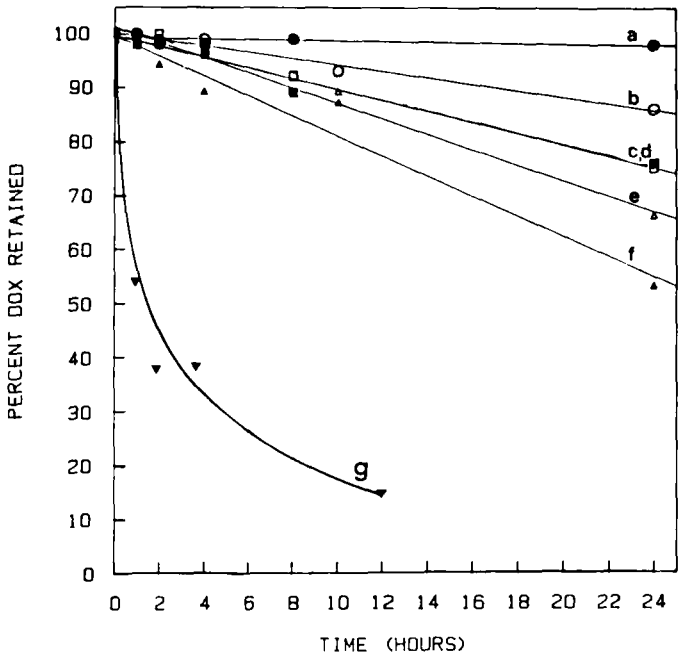

Figure 3. Release of doxorubicin from liposomes under dialysis conditions at 37°C after pH-gradient-dependent encapsulation (a–f) or passive encapsulation (g). The liposomes were composed of EPC/cholesterol at molar rations of 55:45 (a,g), 67:33 (b), and 85:15 (e); pure EPC (f); EPC/EPG/cholesterol at molar ratios of 52.5:2.5:45 (c) and 27.5:27.5:45 (d).

example, membranes that are cholesterol-poor or contain high amounts of acidic lipids exhibit increased leakage of entrapped solutes. In addition, doxorubicin release from liposomes increases significantly upon dissipation of the pH gradient caused either by decreasing the external pH or by addition of proton gradient uncouplers (38).

Additional benefits of the pH-gradient-driven encapsulation procedure arise from features that are pharmaceutically desirable. First, because efficient trapping is accomplished independent of lipid composition, labile lipids can be omitted unless dictated by biological response requirements. Second, the simplicity of the ΔpH active entrapment procedure allows doxurubicin to be encapsulated into preformed vesicles immediately prior to use and the high trapping efficiencies obviate requirements for the removal of free drug. Such a "remote loading" protocol alleviates the possible stability problems related to chemical integrity of the drug and drug retention in the vesicles that may occur during storage of loaded liposomes.

BIOLOGICAL ACTIVITY OF LIPOSOMAL DOXORUBICIN

Evaluation of Toxicity

Dose–response survival and weight loss curves provide a general measure of drug toxicity. For doxorubicin, this response occurs over a very narrow dose range (39). As a consequence, differences in toxicity are readily resolved by determining dose levels that induce a defined mortality rate (e.g., 50% lethality dose; LD_{50}). Table 2 presents the LD_{50} values obtained for doxorubicin administered intravenously to CD-1 mice in free form as well as liposomal doxorubicin preparations of differing lipid compositions. Encapsulating doxorubicin in EPC/cholesterol (55:45, mol/mol) vesicles increases the LD_{50} from 23 mg/kg for free drug to 57 mg/kg. Decreasing the cholesterol content results in increased toxicity, as indicated by an LD_{50} value of 38 mg/kg for pure EPC systems. This trend is presumed to be due to the interaction of lipoproteins with cholesterol-poor liposomes, which results in increased leakage of entrapped contents from the vesicles (25–27). Such an interpretation is corroborated by the observation that doxorubicin plasma clearance rates for EPC formulations approach those for free drug (Fig. 4). Although inclusion of phosphatidylglycerol does not significantly alter the toxicity of liposomal doxorubicin as assessed by LD_{50} values, substituting distearoylphosphatidylcholine (DSPC) for EPC in cholesterol-containing preparations can dramatically increase the LD_{50}. Again, this is consistent with the reduced leakage of doxorubicin from vesicles observed for DSPC/cholesterol systems (17).

The pH-gradient-dependent drug loading technique allows the generation of systems displaying drug to lipid ratios (and corresponding lipid doses) that vary by 10 times or more (39). As shown in Table 3, such variations can significantly affect the toxicity of liposomal doxorubicin preparations. Decreasing the drug to lipid

Table 2. Effect of Lipid Composition on the Toxicity of Liposomal Doxorubicin[a]

Preparation	Mean vesicle diameter ± S.D. (nm)	LD_{50} (mg/kg)
Free	—	23
EPC/Chol (55:45)[b]	160 ± 43	57
EPC/Chol (67:33)	163 ± 49	53
EPC/Chol (85:15)	166 ± 49	44
EPC	158 ± 37	38
DSPC/Chol (55:45)	175 ± 41	161
EPC/EPG/Chol (27.5:27.5:45)	180 ± 51	55

[a]Doxorubicin to lipid ratios for all samples were 0.27 ± 0.04:1 (wt/wt).
[b]The numbers in parentheses reflect molar ratios of lipid components.

ratio of EPC/cholesterol doxorubicin systems from 0.28:1 (wt/wt) to 0.038:1 (wt/wt) decreases the LD_{50} from 57 mg/kg to 39 mg/kg. The increased toxicity observed when the drug to lipid ratio is decreased from 0.28:1 to 0.038:1 is consistent with an increase in the circulation time for systems using high lipid doses. This extended exposure of the vesicles to blood components may be expected to cause increased leakage of doxorubicin from the liposomes. As a result, sensitive tissues may be exposed to elevated levels of free doxorubicin, leading to increased toxicity. This interpretation is consistent with the relationship between vesicle size and liposomal doxorubicin toxicity (Table 4). Although decreasing vesicle size from approximately 1 μm to 200 nm has negligible effects on toxicity,

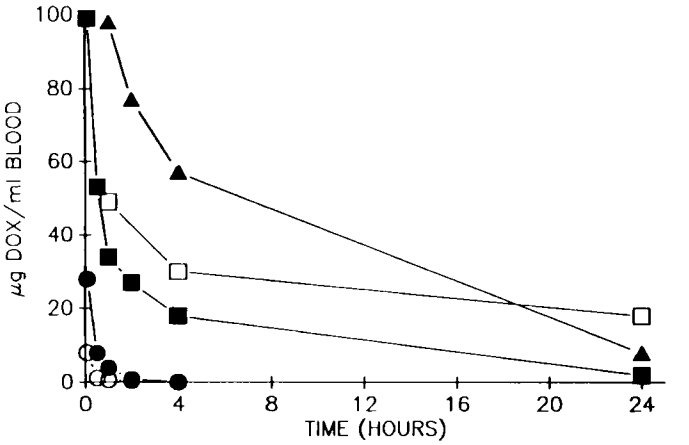

Figure 4. Blood doxorubicin levels observed in mice subsquent to intravenous injection of free or liposomal doxorubicin at a drug dosage of 20 mg/kg. Doxorubicin was encapsulated in EPC (●), 106 nm (▲), and 170 nm (■) EPC/cholesterol, and 180 nm DSPC/cholesterol (□) vesicles employing pH gradient techniques.

Table 3. Effect of Drug to Lipid Ratio on the Toxicity of Doxorubicin Encapsulated in EPC/Cholesterol Vesicles[a]

Dox/total lipid (wt/wt)	LD_{50} (mg/kg body weight)
0.28:1	57
0.072:1	45
0.038:1	39
0.038:1[b]	38

[a]Doxorubicin was encapsulated in EPC/cholesterol (55:45 mol ratio) vesicles.
[b]Doxorubicin was encapsulated in EPC/cholesterol (55:45 mol ratio) vesicles at a drug to lipid ratio of 0.28:1 and subsequently diluted with empty liposomes to achieve a final drug to lipid ratio of 0.038:1.

Table 4. Effect of Vesicle Size on the Toxicity of Liposome-Encapsulated Doxorubicin[a]

Preparation	Mean vesicle diameter ± S.D. (nm)	LD_{50} (mg/kg body weight)
EPC/Chol (55:45)[b]	1400 ± 400	60
EPC/Chol (55:45)	160 ± 43	57
EPC/Chol (55:45)	106 ± 31	45
DSPC/Chol (55:45)	773 ± 140	>200[c]
DSPC/Chol (55:45)	175 ± 41	161

[a]Vesicle-entrapped doxorubicin was prepared at a drug to lipid ratio of 0.27 ± 0.04:1 (wt/wt).
[b]The numbers in parentheses reflect molar ratios of lipid components.
[c]Only 20% mortality was observed for a dosage of 200 mg doxorubicin/kg body weight. Higher dosages could not be administered due to the viscosity of the solution.

further decreasing the vesicle size to 100 nm results in a 21% decrease in the LD_{50} value for EPC/cholesterol-entrapped doxorubicin. This also may be due to the increased circulation times exhibited by the smaller vesicle systems.

An important organ-specific toxicity observed for doxorubicin is cardiotoxicity, in which irreversible myocardial damage clinically limits the total drug dosage that can be safely administered to 450–550 mg/m^2 (41). Numerous animal studies have demonstrated the ability of liposomes to reduce the cardiotoxic effects of doxorubicin (2–9). The ability of liposomal doxorubicin preparations to diminish cardiotoxic side effects correlates well with observations that doxorubicin accumulation in cardiac tissue is significantly reduced when the drug is administered in liposome-encapsulated form (4,7,9). This is due to the fact that liposomes do not normally accumulate in the heart and the peak levels of free doxorubicin are much decreased when the drug is administered in liposomal form. Given the relationship between heart tissue exposure to doxorubicin and the extent of myocardial damage, quantification of doxorubicin accumulation in the heart can provide a predictive measure for the relative cardiac toxicities of various liposomal doxorubicin formulations. As shown in Table 5, heart-associated doxorubicin levels observed 5 hr after iv administration decrease from 15.5 μg/g for free drug to 4.1 μ/g for doxorubicin encapsulated in 175 nm EPC/cholesterol liposomes (55:45, mol/mol; drug to lipid ratio, 0.28:1). Using a "leakier" cholesterol-free liposomal carrier results in an increase in the amount of doxorubicin found in the heart, whereas substituting the DSPC for EPC in cholesterol-containing systems reduces the cardiac drug levels to 2.4 μg/tissue. Furthermore, decreasing the drug to lipid ratio of EPC/cholesterol preparations by 10 times increases the heart doxorubicin value from 4.1 μg/g to 7.3 μg/g (Table 4). It should be noted that similar trends are observed in full time course studies (39). These doxorubicin

Table 5. Cardiac Tissue Doxorubicin Levels 5 Hr After I.V. Injection in Mice[a]

Sample	μg Doxorubicin/g wet tissue
Free Dox	15.5 ± 3.4
EPC	7.5 ± 0.6
EPC/Chol (55:45)	4.1 ± 2.2
EPC/Chol[b] (55:45)	7.3 ± 2.2
DSPC/Chol (55:45)	2.4 ± 1.2

[a]Liposomal doxorubicin was prepared with vesicle systems ranging in mean diameters from 160 to 230 nm and drug to lipid ratios of 0.27:1 (wt:wt). Doxorubicin i.v. dosage was 20 mg/kg.
[b]Doxorubicin was encapsulated in EPC/Chol vesicles at a drug to lipid ratio of 0.037:1 (wt/wt).

levels observed in heart tissue correlate well with the leakage characteristics of liposomes in the circulation. Conditions that enhance retention of doxorubicin in the liposomes, such as increased cholesterol content and increased membrane lipid saturation, decrease accumulation of the drug in cardiac tissue.

Assessment of Antitumor Activity

The therapeutic index of anticancer agents is based not only on the relative toxicity of the drug but also on its antitumor efficacy. The use of liposomes to improve the activity of doxorubicin for specific applications must therefore result in increased antitumor therapy at the maximum tolerated dosage. This can be accomplished in two ways. First, decreasing drug toxicity while maintaining antitumor potency will result in higher tolerated drug doses and a corresponding increase in the therapeutic index. Second, increased antitumor potency will improve therapy even in the absence of toxicity-buffering effects. An optimized liposomal doxorubicin preparation should ideally exhibit the properties of both decreased drug toxicity and increased antitumor potency. Therefore, the influence of vesicle size, lipid composition, and drug to lipid ratio on antitumor efficacy must be determined and evaluated in conjunction with the toxicity information provided above.

Numerous tumor models (ascitic, solid, and metastatic) have been used to assess the antitumor activity of liposomal doxorubicin (4,5,9,12–18). For the purpose of screening the efficacy of a variety of liposomal doxorubicin formulations, we have selected the L1210 lymphocytic leukemia model. In this ascitic tumor model mice are injected intraperitoneally (ip) with approximately 10^6 L1210 cells and treatment is initiated 24 hr later via iv administration (39). In the absence of any treatment (injection of saline or empty liposomes), animals typically die between days 9 and 11. Therapy provided by doxorubicin administration is manifested by an extension

of the survival time, and the relative activities of various treatments can be evaluated by comparing increase in life span (ILS) values (median survival time of treatment group/median survival time of control group). In addition, the antitumor potency of liposomal doxorubicin preparations is reflected by the ratio of median survival times for liposomal/free (L/F) drug treatment groups at equal drug doses, where an L/F value > 1.0 indicates increased potency over free doxorubicin.

In contrast to trends observed for drug toxicity, the antitumor potency of liposomal doxorubicin is not significantly affected by alterations in the vesicle lipid composition. At drug dosages of 20 mg/kg, doxorubicin encapsulated in EPC, EPC/cholesterol (55:45, mol/mol), DSPC/cholesterol (55:45, mol/mol), and EPC/EPG/cholesterol (27.5:27.5:45, mol/mol) vesicles with a size range of 160–180 nm all exhibit comparable efficacy to free drug as indicated by L/F values between 0.88 and 1.27 (39). Varying the drug to lipid ratio of EPC/cholesterol formulations by 10-fold likewise has little effect on antitumor potency.

Although changes in vesicle lipid composition and drug to lipid ratio do not appear to alter significantly the antitumor potency of liposomal doxorubicin, differences in the maximum therapeutic efficacy are observed for these systems. Such variations stem from the different toxicities and corresponding maximum tolerated doses exhibited by these preparations. As a result, the maximum drug dose that can be administered using pure EPC-entrapped doxorubicin is comparable to that for free drug (20 mg/kg), and little improvement in efficacy is provided. However, the less toxic formulations such as EPC/cholesterol and DSPC/cholesterol can be injected at elevated doses and maximum %ILS values of 190 and 220, respectively, are obtained (39). In comparison, the maximum ILS value that can be achieved for free drug at 20 mg/kg is 145%. This increased antitumor activity therefore represents an increase in the therapeutic index of doxorubicin. Such behavior has been reported by numerous laboratories investigating a wide variety of liposomal doxorubicin formulations (4,5,9,11,12). Of more immediate interest is the use of liposomes to increase not only the maximum tolerated dose of doxorubicin but also the antitumor potency of the drug.

A detailed analysis of the influence of vesicle size on the antitumor activity has been performed using pH gradient-loaded liposomal doxorubicin formulations for treatment of the ascitic L1210 tumor (39). The results demonstrate that large (≥ 1 μm) vesicle systems composed of EPC/cholesterol or DSPC/cholesterol are less potent than free doxorubicin at drug dosages of 20 mg/kg and below as indicated by L/F values that are significantly less than 1.0 (Table 6). Decreasing the vesicle size to the range of 180–230 nm increases the potency of entrapped doxorubicin for both systems and L/F values approaching 1.0 are observed. Decreasing the size of EPC/cholesterol liposomal doxorubicin further to 100 nm results in a significant

Table 6. Influence of Vesicle Size on L1210 Antitumor Activity of Liposomal (PC/Chol; 55:45) Doxorubicin

	Drug dosage (mg/kg)		Survival time (days)			
Preparation (nm)	Dox	Lipid	60 Days	Median	% ILS	L/F
EPC/Chol						
1110[a]	20	61	0.6	16.5	65	0.67[b]
180	20	60	0/18	21.5	115	0.88
100	20	68	3/10	47.5	375	1.94[b]
DSPC/Chol						
770	10	40	0/10	13	30	0.87[b]
	30	120	3/10	25	150	N/A
230	10	42	0.6	17	70	1.13
	30	128	2/6	32	220	N/A

[a]The mean diameter of the vesicle preparation as estimated by QELS techniques.
[b]Difference compared with the same dose of free doxorubicin is significant at $p<0.05$ level.

increase in the antitumor potency of encapsulated doxorubicin. This is manifested not only by an L/F value of 1.94 but also by the occurrence of a 30% long-term (greater than 60 day) survival rate. This preparation also exhibits the attribute of reduced toxicity, resulting in a substantial increase in the therapeutic activity of doxorubicin. A doxorubicin dosage of 30 mg/kg for this formulation results in an ILS value of 465% and a 50% long-term survival rate.

CONCLUDING REMARKS

The toxicity and antitumor efficacy screening studies described here provide insight into the physical properties (vesicle size, lipid composition, and drug to lipid ratio) an optimized liposomal doxorubicin formulation may exhibit. It is apparent that the stability (drug retention) of liposomal doxorubicin in the circulation is a major factor in determining toxicity behavior and is related primarily to lipid composition. Although small (100 nm) liposomal doxorubicin systems are somewhat more toxic than large preparations, this effect is more than offset by a dramatic increase in antitumor activity. This increase in the therapeutic effect of small liposomal doxorubicin preparations may be related to the ability of small liposomes to enhance the accessibility of the drug to the tumor. The studies reviewed here present an example of the use of ΔpH-loaded, presized liposomes to resolve questions regarding structure/function relationships for therapeutic liposomes. Future studies investigating more detailed aspects of toxicology, efficacy, and pharmacology will

likely lead to further refinement and optimization of liposomal doxorubicin. In addition, this information should serve as a model for the development and optimization of other liposome-based anticancer pharmaceuticals.

ACKNOWLEDGMENTS

This work was supported by the National Cancer Institute of Canada and The Liposome Company, Inc. (Princeton, N.J.).

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