

## Review

# Liposomal Doxorubicin

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Doxorubicin is a potent antineoplastic agent with activity against numerous human cancers. Encapsulation of doxorubicin inside a liposome alters bioavailability, biodistribution and thus its biological activity significantly. The physical properties of the liposome (size, lipid components and lipid dose) play a major role in determining drug retention and pharmacokinetics. The therapeutic benefits of liposomal doxorubicin will therefore depend on these physical characteristics. Here we review the toxicity and efficacy of liposomal doxorubicin determined for various liposome compositions (size, lipid composition and drug-to-lipid ratio). These physical properties can be independently varied using the transmembrane pH gradient-dependent drug encapsulation procedure. The results show that the toxicity of the formulation is related to drug retention in the circulation. The antitumor activity is more sensitive to the size of the liposomes. By optimizing these parameters, liposomal doxorubicin formulations can be optimized for improved therapeutic activity.

*Keywords:* anticancer agents, doxorubicin, efficacy, liposomes, toxicity

## INTRODUCTION

Doxorubicin is one of the most commonly used anticancer drugs. Its antitumor efficacy is primarily attributed to direct interactions with DNA (Frederick et al., 1990) or DNA topoisomerase (D'Arpa and Liu, 1989). The total lifetime dose of the drug is limited to 550 mg/m<sup>2</sup> to prevent chronic irreversible cardiotoxicity. Early studies found that encapsulation of doxorubicin inside liposomes decreases the cardiotoxicity associated with the free form of the drug

(Forssen and Tokes, 1979; Rahman et al., 1980) while maintaining anticancer potency. Subsequent studies have shown that the acute and chronic toxicities associated with the use of free drug are reduced when the drug is in the liposomal form (Gabizon et al., 1982; Olson et al., 1982). As well as a general reduction in toxicity, the liposomal form is at least equipotent to free drug in treating tumor bearing animals (Gabizon et al., 1982; Olson et al., 1982; Gabizon et al., 1985; Storm et al., 1987; Balazsovits et al., 1989; Mayer et al., 1990). Since the liposomal

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form of the drug is less toxic, increased drug dosages can be administered, resulting in enhanced efficacy and an increase in the therapeutic index.

A variety of liposomal doxorubicin formulations have been used to assess the therapeutic value of these systems in animal models. Unfortunately, these formulations are difficult to compare due to the wide variation in the degree of lamellarity, size, lipid composition, drug-to-lipid ratio, and trapping efficiency of these systems. These variations are often associated with the traditional "passive" entrapment procedure. In this technique, the lipid and drug are codispersed in an aqueous buffer, thus achieving entrapment while the liposomes are being formed. When liposomal doxorubicin is prepared in this way poor drug retention is observed, although changes in lipid composition can improve drug entrapment and retention. The presence of negatively charged lipids in a liposome formulation, for example, increases the association of doxorubicin with the vesicles. However, it is also well established that the presence of negatively charged lipids results in the rapid clearance of liposomes from the circulation (Senior *et al.*, 1985). Therefore, the resulting changes in biological behavior cannot be correlated to a specific physical characteristic of the liposomal system, since changes in physical properties of the liposome influence other properties.

The inability to separately manipulate the properties of liposomal doxorubicin preparations limited attempts to select optimized formulations. The generation of an optimized system requires a process for generating well-characterized formulations with well defined physical properties; such as vesicle size, drug-to-lipid ratio, lipid composition and drug retention. Such preparations are required for relating characteristics that alter the *in vivo* fate and efficacy of the liposomal drug carrier. Factors that are known to play important roles in liposome pharmacokinetics and biodistribution are vesicle size, lipid composition, drug content and dose (Mayer *et al.*, 1989). A procedure for preparing liposomal doxorubicin, which avoids the problems associated with passive entrapment, uses a pH gradient system to load doxorubicin into liposomes (Mayer *et al.*, 1986). This method has been extremely useful in identifying variables that

reduce the toxicity and enhance the efficacy of liposomal doxorubicin (Bally *et al.*, 1990).

## GENERATING LIPOSOMAL DOXORUBICIN

When liposomes are prepared with a transmembrane pH gradient (inside acidic), well characterized liposomal doxorubicin preparations exhibiting a wide range of vesicle size, lipid composition and drug-to-lipid ratios can be obtained. A brief overview of the generation of such systems is discussed below.

### Liposome Preparation

Lipids are co-dissolved in an organic solvent such as chloroform, which is subsequently removed by high vacuum evaporation until a solvent free lipid film is produced. Lipid films are then rehydrated with buffer at temperatures above the highest lipid transition temperature for the formulation. The resulting multilamellar vesicles (MLVs) are heterogeneous in size (500 nm to several microns in diameter) with small aqueous trapped volumes ( $\leq 1 \mu\text{l}/\mu\text{mol}$  lipid). The trapped volumes can be increased to approximately  $5 \mu\text{l}/\mu\text{mol}$  lipid by freezing and thawing the sample (Mayer *et al.*, 1985). Liposomes can be generated with very specific size distributions if high pressure extrusion is used. Extrusion generates liposomes with defined sizes according to the filter pore size used (Hope *et al.*, 1993).

### Generating Transmembrane pH Gradients and Doxorubicin Loading

It is straightforward to generate a transmembrane pH gradient in liposomes. The content of the vesicle interior aqueous compartment is determined by the solution used during the lipid hydration step. If an acidic buffer is used, the pH of the extravascular compartment can be adjusted to pH 7 or higher by the addition of base or by exchanging external buffer with another buffer using column chromatography. In

this way a transmembrane pH gradient can be generated and subsequently used to encapsulate doxorubicin, as indicated below.

To measure transmembrane pH gradients, weak bases such as methylamine are used (Harrigan et al., 1992). Methylamine can cross lipid membranes by transmembrane diffusion when in the neutral form. The neutral form is highly membrane permeable, while the charged or protonated form is highly impermeable (Rottenberg, 1989). When methylamine is added to liposomes displaying a transmembrane pH gradient, equilibrium is established when the perme-

able (neutral) form is at equal concentrations on both sides of the membrane. This occurs when  $[\text{MeAm}]_{\text{in}}/[\text{MeAm}]_{\text{out}} = [\text{H}^+]_{\text{in}}/[\text{H}^+]_{\text{out}}$  for situations in which  $[\text{H}^+]_{\text{in}}, [\text{H}^+]_{\text{out}} \gg K_d$ , which are readily achieved, as the  $K_d$  for most amino containing weak bases is in the range of  $10^{-9}$  (see Figure 1). Since both methylamine and doxorubicin are lipophilic weak bases, doxorubicin and other weak bases can also be loaded into liposomes in a similar fashion (Mayer et al., 1986). This has proven to be true for a large variety of drugs (Madden et al., 1990), and the resulting loading properties are impressive. For a pH gradient of 3 units, for

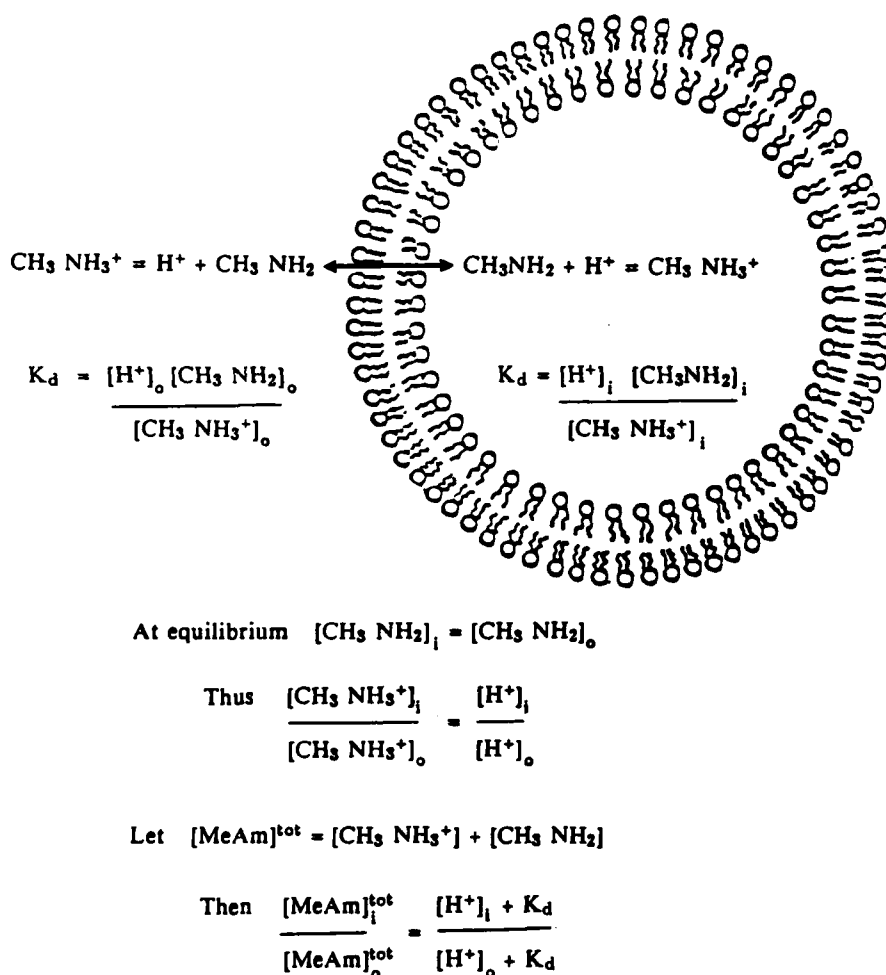


FIGURE 1 Influence of a pH gradient on the transbilayer distribution of methylamine in a large unilamellar vesicle (LUV).  $K_d$  is the dissociation constant.  $[\text{MeAm}]^{\text{tot}}$  represents the total concentration of methylamine, both the charged ( $\text{CH}_3\text{NH}_3^+$ ), and uncharged ( $\text{CH}_3\text{NH}_2$ ) forms. (Reprinted with permission from Cullis, P.R., Bally, M.B., Madden, T.D., Mayer, L.D. and Hope, M.J. (1991) in Trends in Biotechnology).

example, an interior/exterior concentration gradient of 1000 or more is achievable for many lipophilic weak bases (including doxorubicin). This uptake is associated with activation energies that are sensitive to the lipid composition of the liposome. Doxorubicin uptake into EPC liposomes exhibits an activation energy of 28 Kcal/mol while EPC/Chol (55:45, mol/mol) exhibits an activation energy of 38 Kcal/mol (Harrigan *et al.*, 1993).

This uptake process proceeds by a mechanism where the drug crosses the membrane bilayer in the neutral (unprotonated) form. Upon reaching the acidic interior of the liposome, the amine function on the drug is protonated. This protonated form is effectively impermeable to the bilayer, resulting in a sequestering of the drug inside the liposome (Mayer *et al.*, 1986, 1990a). Due to the high lipid/water partition coefficient of doxorubicin, more than 95% of encapsulated doxorubicin is partitioned into the inner monolayer, presumably near the lipid/water interface (Harrigan *et al.*, 1993). Analysis of doxorubicin under these conditions indicates no detectable drug breakdown.

The benefits of the pH gradient-dependent encapsulation procedure are numerous (Table I). A wide range of drug-to-lipid ratios can be achieved with trapping efficiencies over 95% (assuming a high interior buffering capacity, as a proton is consumed for every doxorubicin that is accumulated). Liposomes containing 0–50% cholesterol, saturated or unsaturated acyl chains, charged or neutral lipids can all be loaded with doxorubicin to achieve equal drug-to-lipid ratios and trapping efficiencies. The trapping efficiency is independent of liposome size assuming that adequate interior buffering capacity is available.

The drug-to-lipid ratios achieved (0.25:1) using the pH gradient are approximately 3–10 times higher than can be achieved by other procedures. Equally important, the transmembrane pH gradient also enhances the retention of doxorubicin within the liposomes. In EPC/Chol (55:45, mol/mol) liposomes, for example, pH gradient encapsulation results in a 5-fold increase in the proportion of doxorubicin retained after 12 hours of dialysis compared to passive entrapment (Mayer *et al.*, 1990c). The pH gradient loading procedure also allows doxorubicin to be encapsulated into preformed vesicles immediately prior to use. The high trapping efficiencies (>95%) make the removal of free drug unnecessary. 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 the storage of liposomal doxorubicin.

## FACTORS INFLUENCING DRUG RETENTION AND CIRCULATION LIFETIME

### Lipid Composition

The degree of doxorubicin retention in the liposome is sensitive to the lipid composition. For *in vivo* preparations, the absence of cholesterol can result in lipoprotein-induced vesicle destabilization and concomitant release of entrapped agents (Scherphof *et al.*, 1978; Kirby *et al.*, 1980). Increasing acyl chain length and degree of saturation enhances the retention of doxorubicin within circulating liposomes (Mayer *et al.* 1990c). Increased drug retention results in en-

TABLE I Liposome Characteristics Suitable for pH Gradient-Dependent Doxorubicin Encapsulation

Liposome property	Range producing $\geq 95\%$ trapping efficiency
Cholesterol content	0–45 mol %
Anionic lipid content	Phosphatidylserine, phosphatidylglycerol or cardiolipin to 20 mol %
Phospholipid acyl chains	Saturated or unsaturated to C-20 in length
Specialty lipids	GM <sub>1</sub> or PEG-PE to 10 mol %
Liposome size	50 nm (SUVs) to 2 $\mu$ m (MLVs)
Drug-to-lipid ratio	To 0.2:1, w/w for liposomes < 100 nm To 0.3:1, w/w for liposomes $\geq 100$ nm

hanced circulation lifetimes. For example, when doxorubicin loaded liposomes (DSPC/Chol, 100 mg/kg dose) are administered i.v. to mice, nearly 80% of the injected dose remains in circulation after 24 hours, whereas for empty liposomes with the same formulation, only 25% of the injected dose is present in the circulation at 24 hours (Bally et al., 1990a). These results imply that doxorubicin-containing liposomes have increased circulation longevity over empty liposomes. Administration of a cytotoxic agent such as doxorubicin encapsulated in liposomes likely results in direct toxicity to the cells responsible for the clearance of the liposome carrier.

The blood clearance and distribution properties of liposomal formulations are also dependent on lipid composition. Vesicles containing certain acidic phospholipids such as phosphatidylserine are rapidly removed from circulation by phagocytic cells of the RES that reside primarily in the liver and spleen (Baumier and Hwang, 1983; Schroit et al., 1985). The addition of the polymer, poly (ethylene glycol) modified lipids or the glycolipid  $G_{M1}$ , can dramatically increase liposome blood residence times and decrease RES uptake (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988). The presence of 5–7 mol%  $G_{M1}$  can increase circulating levels of liposomes 3–10 fold. Liposomes containing these lipids are commonly referred to as “sterically stabilized.” Liposomes which exhibit extended circulation lifetimes have more opportunity to cross the endothelium of small blood vessels, extravasating into extracellular spaces (Vaage et al., 1993). This results in an enhanced accumulation within tumor tissue (Gabizon and Papahadjopoulos, 1988). When these liposomes contain doxorubicin, increased antitumor effects are reported (Huang et al., 1992; Vaage et al., 1993).

### Vesicle Size and Lipid Dose

Liposome circulation longevity is also regulated by vesicle size and liposome dose. Decreasing the size of uncharged vesicles from approximately 1  $\mu\text{m}$  to less than 50 nm can increase the circulating levels of liposomes by 10-fold or more (Baumier and Hwang,

1983). Up to 90% of large liposomes administered i.v., at low lipid doses, accumulate in the liver and spleen within minutes (Hwang, 1987). The circulation lifetime can be substantially increased by increasing the lipid dose (Abra and Hunt, 1981). The relationship between lipid dose, liver uptake and clearance from circulation was studied by Baumier et al. (1983). Liposomal biodistribution was measured 23 hours after i.v. administration of SM/Chol (2:1, mol/mol) liposomes. When the liposome dose was increased from 6 to 120 mg/kg, the percentage of the dose accumulating in the liver decreased from 60 to 20%. The dose remaining in the blood leveled off at 40%. For doses of 120 to 320 mg/kg, the liver value plateaus at 20%, suggesting liver saturation. Factors which increase blood residence times for liposomes result in a decreased proportion of vesicles accumulating in the RES.

## BIOLOGICAL ACTIVITY OF LIPOSOMAL DOXORUBICIN

### Evaluation of Toxicity

An initial evaluation of drug toxicity can be achieved via dose-response survival and weight loss data. For doxorubicin, this response occurs over a narrow dose range (Mayer et al., 1989), therefore toxicity is often measured by the mortality rate  $LD_{50}$  (50% lethal dose). The encapsulation of doxorubicin inside liposomes composed of EPC/cholesterol (55/45, mol/mol) increases the  $LD_{50}$  from 23 mg/kg for free drug to 57 mg/kg (Table II). If the cholesterol content is decreased, the toxicity of the formulation increases. The enhanced toxicity may result from interactions between lipoproteins and cholesterol-poor liposomes resulting in doxorubicin leakage from the vesicles (Hwang, 1987). If DSPC is substituted for EPC in the liposomal formulation, doxorubicin leakage is reduced resulting in a dramatic increase in the  $LD_{50}$  to 161 mg/kg (Mayer et al., 1990c). The higher the phase transition temperature of the main lipid com-

TABLE II The Effect of Lipid Composition on the Toxicity of Liposomal Doxorubicin<sup>a</sup>

Preparation	Mean vesicle diameter $\pm$ S.D.	LD <sub>50</sub> (mg/kg)
Free	—	23
EPC/Chol (55:45) <sup>b</sup>	160 $\pm$ 43	57
EPC/Chol (67:33)	163 $\pm$ 49	53
EPC/Chol (85:15)	166 $\pm$ 49	44
EPC	158 $\pm$ 37	38
DSPC/Chol (55:45)	175 $\pm$ 41	161
EPC/EPG/Chol (27.5:27.5:45)	180 $\pm$ 51	55

<sup>a</sup>Doxorubicin to lipid ratios for all samples were 0.27  $\pm$  0.04:1 (wt/wt).

<sup>b</sup>The numbers in parentheses reflect molar ratios of lipid components. (reproduced from Mayer *et al.* 1990c)

ponent, the less toxic the formulation (Horowitz *et al.*, 1992). The drug-to-lipid ratio also plays an important role in determining the toxicity of liposomal doxorubicin. If the same doxorubicin dose is given, decreasing the drug-to-lipid ratio of an EPC/cholesterol doxorubicin system from 0.28:1 (wt/wt) to 0.038:1 (wt/wt) decreases the LD<sub>50</sub> from 57 mg/kg to 39 mg/kg. This may be due to an increased circulation time for the higher lipid doses, thus increasing liposomal doxorubicin exposure to blood components, resulting in enhanced drug leakage and concomitant toxicity.

Myelosuppression and cardiotoxicity are the major dose limiting toxicities associated with the clinical use of doxorubicin. Many studies have shown that the encapsulation of doxorubicin inside liposomes reduces cardiotoxic effects (Rahman *et al.*, 1980; Olson *et al.*, 1982) by decreasing the accumulation of free drug within the heart (Gabizon *et al.*, 1982). When doxorubicin is given i.v. at a dose of 20 mg/kg, heart associated doxorubicin levels decrease from 15.5  $\mu$ g/g tissue for free drug to 4.1  $\mu$ g/g for EPC/cholesterol liposomes (55:45, mol/mol) at 5 hours after injection (Mayer *et al.*, 1990c). If "leakier" cholesterol-free liposomal systems are used, doxorubicin levels in the heart increase, while substituting DSPC for EPC decreases cardiac levels of doxorubicin to 2.4  $\mu$ g/g tissue. Decreasing the drug-to-lipid ratio of the formulation 10-fold, enhances the doxorubicin levels in the heart from 4.1  $\mu$ g/g to 7.3  $\mu$ g/g. The doxorubicin levels found in the heart parallel the leakage char-

acteristics of the liposomes in circulation (Harashima *et al.*, 1992). Therefore, retention of doxorubicin within the liposome, by increasing cholesterol content, membrane lipid chain length and saturation, lowers the accumulation of doxorubicin in cardiac tissue.

Phase I clinical trials with the TLC D-99 formulation have revealed that myelosuppression is the acute dose-limiting toxicity of liposomal doxorubicin (Gabizon *et al.*, 1986; Treat *et al.*, 1988), and that other toxicities are substantially reduced in comparison to the free drug. The extent of liposomal association with bone marrow is related to liposome size. Marrow associated doxorubicin is 10–20 times higher when doxorubicin is encapsulated in large liposomes ( $\approx$ 1  $\mu$ m) compared to small liposomes ( $\approx$ 100 nm) (Bally *et al.*, 1990b).

## ANTITUMOR ACTIVITY

For anticancer agents, the therapeutic index is based on toxicity and antitumor efficacy. Therefore in order for liposomal doxorubicin to be an improvement over free drug it must result in increased antitumor activity at the maximum tolerated dose. This may be accomplished by decreasing drug toxicity while maintaining antitumor potency. This would result in higher tolerated drug doses and lead to an increased therapeutic index. Alternatively, increased antitumor potency improves therapy in the absence of toxicity. Ideally, a liposomal doxorubicin preparation would display im-



proved antitumor potency as well as reduced toxicity. The therapeutic value of various liposomal formulations can be compared by evaluating the increase in life span (ILS) value (average survival time of treated group/average survival time of control group). The antitumor potency of liposomal doxorubicin formulations is determined as the ratio of average survival time for liposomal/free (L/F) drug treatment groups at equal drug doses. If the ratio is greater than 1, then the liposomal form of the drug shows increased potency.

Vesicle lipid composition has little effect on antitumor potency, while it was previously shown to have a large effect on drug toxicity. Liposomes composed of EPC, EPC/cholesterol (55:45, mol/mol) and DSPC/cholesterol (55:45, mol/mol) were prepared at a drug dose of 20 mg/kg and found to have similar efficacy as free drug. Altering the drug-to-lipid ratio 10-fold also had little impact on the antitumor potency (Mayer et al., 1989). In a murine mammary tumor study, however, EPC/cholesterol (55:45, mol/mol) liposomes containing doxorubicin (drug-to-lipid ratio of 0.27:1 and 170 nm in size) displayed enhanced antitumor efficacy compared to free doxorubicin when given at a dose of 13 mg/kg. Tumor associated doxorubicin levels were enhanced when given in the liposomal form. This resulted in a de-

crease in tumor growth rate and tumor burden (Mayer et al., 1990b). The maximum therapeutic efficacy, however, is primarily dependent on drug-to-lipid ratios, lipid compositions and vesicle size. Dependence on these variables results from the differing toxicities and the corresponding maximum tolerated doses obtained for these preparations. For example, the maximum drug dose that can be administered, using pure EPC entrapped doxorubicin preparations, is similar to that of free drug (20 mg/kg) with little improved efficacy (Table III). In the case of DSPC/cholesterol formulations, the decreased toxicity of the formulation allows for elevated doses of 30 mg/kg, resulting in % ILS values of 220 (Mayer et al., 1989).

The antitumor activity of liposomal doxorubicin formulations were shown to be very sensitive to vesicle size. Large ( $\geq 1000$  nm) vesicles composed of EPC/cholesterol are less potent than free drug at doses of 20 mg/kg and below. Decreasing the size of the liposomal doxorubicin formulation to 100 nm significantly increases the antitumor potency of encapsulated doxorubicin. By decreasing the vesicle size from approximately 1000 nm to 100 nm, the L/F value increased from 0.67 to 1.94 and the % ILS from 65 to 375 (Mayer et al., 1989). If the doxorubicin dosage is increased to 30 mg/kg in the 100 nm system, the ILS value was further increased to 465%.

TABLE III. Influence of Lipid Composition on L1210 Antitumor Activity of Liposomal Doxorubicin

Preparation	Drug dosage (mg/kg)		Survival time (days)		%ILS <sup>b</sup>	L/F <sup>c</sup>
	Dox <sup>a</sup>	Lipid	60 Days	Median		
Saline	—	—	0/58	10	—	—
Free Dox	10	—	0/46	15	55	
	20	—	2/42	24.5	145	
	25	—	0/6	19	90	
	30	—	0/6	15	50	1.00
EPC	10	29	0/6	15	50	1.00
	20	58	0/6	25.5	155	1.04
	30	88	0/6	7.5	-25.0	N/A
DSPC/Chol (55:45 mol %)	10	42	0/6	17	70	1.13
	20	85	1/6	30.5	205	1.27
	30	128	2/6	32	220	N/A
	50	212	0/6	23.5	135	N/A

<sup>a</sup>Doxorubicin

<sup>b</sup>Percentage increase in life span, taken as median survival of treated/median survival of control.

<sup>c</sup>Ratio of median survival time of liposomal treated animals versus animals treated with the equivalent dose of free doxorubicin. (Mayer et al. 1989)

TABLE IV. Liposomal Doxorubicin Phase I and II Clinical Trials

Group	Liposome Composition	Encapsulation Procedure	Results
Rahman, A. <i>et al.</i> (1990)	CL/EPC/Chol/SA	Passive	Phase I clinical cardiotoxicity not seen in cumulative doses up to 400 mg/m <sup>2</sup> .
Treat, J. <i>et al.</i> (1990)	CL/EPC/Chol/SA	Passive	Phase II advanced breast cancer. 60–75 mg/m <sup>2</sup> dose every 3 weeks. Partial regression 9/20, total regression 5/20.
Lohri, A. <i>et al.</i> (1991)	EPC/Chol (TLC D-99)	pH gradient	Phase I/II found the MTD at 75 mg/m <sup>2</sup> . Good circulating levels over 24 hours.
Batist, G. <i>et al.</i> (1992)	TLC D-99	pH gradient	Phase II metastatic breast cancer. 9/17 patients showed major responses, no cardiotoxicity but dose-limiting neutropenia observed.
Conley, B.A. <i>et al.</i> (1993)	EPC/Chol	pH gradient	Phase I study determined MTD when given weekly for 3 weeks (30 mg/m <sup>2</sup> /week).
Cowens, J.W. <i>et al.</i> (1993)	TLC D-99	pH gradient	Phase I study determined MTD as 90 mg/m <sup>2</sup> every 3 weeks or 25 mg/m <sup>2</sup> /day for 3 days.
Mazanet, R. <i>et al.</i> (1993)	TLC D-99 plus G-CSF	pH gradient	Phase I with G-CSF given 3 days after liposomes. Less nausea and no cardiotoxicity.
Northfelt, D.W. <i>et al.</i> (1993) & Northfelt, D.W. (1994)	HSPC/Chol/PEG-PE/cetocopherol (Doxil)	ammonium sulphate	Brief neutropenia, no cardiotoxicity. AIDS-Kaposi's sarcoma patients treated with single doses to 40 mg/m <sup>2</sup> . Doxil delivers 10–20× more drug to lesions than skin, and 5–11× more drug then free.
O'Day, S.J. <i>et al.</i> (1994)	TLC D-99 and G-CSF	pH gradient	Partial responders 3/10 and 1/2 for non small and small cell lung cancers respectively. G-CSF increased MTD by 1.5–2 times.
Fonseca, G. <i>et al.</i> (1994)	TLC D-99 5-fluorouracil cyclophosphamide	pH gradient	Phase II metastatic breast carcinoma. Doses of 60 mg/m <sup>2</sup> , 10/17 partial responders with no cardiac dysfunction.
Nexstar Pharmaceuticals	DSPC/Chol (Daunoxome)	pH gradient	First-line treatment for advanced Kaposi's sarcoma. Effective against breast ca and other solid tumors



These results clearly illustrate the importance of vesicle size on the antitumor activity of liposomal doxorubicin.

Liposomal doxorubicin preparations containing PEG-PE or  $G_{M1}$ , resulting in extended circulation lifetimes, can also result in improved therapy. When mice bearing colon carcinoma were injected with sterically stabilized liposomal doxorubicin, the area under the curve value was 2–3-fold higher in the tumor than for non-sterically stabilized liposomal doxorubicin. When these mice were injected with three weekly doses of liposomal doxorubicin, tumor regression was observed. The mice given sterically stabilized liposomal doxorubicin (9 mg/kg, i.v. once a week for 3 weeks), were cured of the tumor at day 80 while the free doxorubicin group had no survivors (Huang et al., 1992). In nude mice carrying human ovarian carcinoma, mice injected with sterically stabilized liposomal doxorubicin (9 mg/kg), were free of tumor 70 days after implantation (Vaage et al., 1993). These results suggest that liposomes displaying increased circulation lifetimes and increased tumor localization are likely to increase the therapeutic index of liposomal doxorubicin.

## CLINICAL STUDIES

There are numerous liposomal doxorubicin phase I and II clinical trials in progress (Table IV). In some of these studies, the liposomal doxorubicin is prepared by the passive entrapment method (Rahman et al., 1990). Numerous phase I and II trials are also underway using EPC/cholesterol liposomes with a pH gradient doxorubicin loading procedure (TLC D-99)(Lohri et al., 1991; Batist et al., 1992; Cowens et al. 1993). These liposomes have trapping efficiencies in excess of 98%. Results from these studies show considerable efficacy without any detectable cardiotoxicity. The dose limiting toxicity of this formulation in the clinic appears to be leucopenia. The TLC D-99 formulation has also been used in clinical trials together with granulocyte colony stimulating

factor (G-CSF)(Mazanet et al., 1993; O'Day et al. 1994). The inclusion of G-CSF in the treatments has not only decreased the level of neutropenia usually observed, but also increased the maximum tolerated dose of liposomal doxorubicin by approximately 2 fold to 150 mg/m<sup>2</sup> (O'Day et al. 1994).

Initial studies with TLC D-99 attempted to increase the efficacy of doxorubicin by allowing administration of a higher cumulative dose due to decreased toxicity (Cowens et al. 1993). This toxicity is prevented because the liposomes direct the drug away from sites which have tight capillary junctions such as the heart muscle and the gastrointestinal tract. They are instead accumulated within organs rich in cells of the reticuloendothelial system. For this reason, the TLC D-99 formulation has been shown to be highly effective against metastatic breast cancer in Phase II clinical trials (Fonseca et al. 1994, Batista et al. 1992). This is likely due to the enhanced accumulation of the liposomal drug in the lung, bone marrow, liver, and lymph nodes.

Clinical trials are also underway for sterically stabilized liposomal doxorubicin formulations (Martin and Gabizon, 1992; Northfelt et al. 1993; Thommes et al. 1994). These liposome formulations display an enhanced circulation lifetime due to the inclusion of polyethyleneglycol linked lipids. These lipids have been shown to prolong the blood circulation time of liposomes and diminish their uptake by cells of the reticuloendothelial system (Woodle et al. 1990, Klibanov et al. 1990, Blume and Cevc 1990, Papahadjopoulos et al. 1991). Due to the enhanced circulation longevity, they are more readily available to extravasate across the leaky capillary endothelia associated with malignant tumors (Papahadjopoulos et al. 1991). It is unlikely that particles the size of liposomes could diffuse and penetrate within the tumor mass itself, but the extravasated liposomes surrounding the tumor cells provide a local depot for drug release. These formulations deliver doxorubicin to lesions in AIDS-Kaposi's sarcoma patients at levels 5–11 fold higher than free drug. This is likely due to the fact that the histopathology of Kaposi's sarcoma reveals proliferation of abnormal vascular structures with increased

permeability. Tumors of this type accumulate more of the liposomal drug when the blood circulation time is increased.

The results from the clinical trials outlined in Table IV suggest that encapsulation of doxorubicin within a liposome decreases its cardiotoxicity and increases its antitumor potency compared to free drug. For the TLC D-99 formulation, due to its increased uptake by cells of the RES, an increased efficacy is seen clinically in metastatic breast cancer due to accumulation within sites of metastatic disease. In contrast, the sterically stabilized Doxil formulation allows increased circulation longevity by avoidance of cells of the RES. Therefore the drug is more able to accumulate at sites of tumor growth due to extravasation across the highly permeable endothelium of the newly vascularized tumor.

## SUMMARY

As detailed in this review, the physical properties of a liposomal doxorubicin preparation (vesicle size, lipid composition, and drug-to-lipid ratio) play a large role in its bioavailability, biodistribution and biological activity. The therapeutic benefits of liposomal doxorubicin are dependent on these same physical properties, which are best studied when doxorubicin is loaded into liposomes using a pH gradient. The results show that the toxicity of a formulation is related to its drug retention properties *in vivo*. Sterically stabilized liposomes display increased circulation lifetimes as well as enhanced antitumor activity. The methods developed for the preparation of liposomal doxorubicin will ultimately lead to the development of other liposome based anticancer pharmaceuticals.

## ABBREVIATIONS

EPC egg phosphatidylcholine  
 Chol cholesterol  
 EPG egg phosphatidylglycerol  
 DSPC distearoyl phosphatidylcholine

MeAm methylamine  
 SM sphingomyelin  
 LDX liposomal doxorubicin  
 MTD maximum tolerable dose  
 G-CSF granulocyte colony stimulating factor  
 CL cardiolipin  
 SA stearylamine  
 HSPC hydrogenated soybean phosphatidylcholine

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