6. PRECLINICAL AND CLINICAL ACTIVITY OF LIPOSOMAL DOXORUBICIN

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A number of studies have shown that the encapsulation of doxorubicin within liposome can significantly improve its therapeutic index. Variables such as lipid composition, liposome size, and drug-to-lipid ratios have been investigated for their role in doxorubicin toxicity and therapeutic activity. In order to examine these variables, pH-gradient encapsulation was utilized in order to achieve very high drug-to-lipid ratios and to develop systems which retained the drug for extended periods of time. Studies have shown that lipids with higher phase-transition tempetatures produce liposomes with better drug retention capabilities, preventing drug toxicity. Doxorubicin toxicity also appean to be significantly reduced by increasing the drug-t&lipid ratio. Liposome size appears to be the most significant factor in determining antitumor activity of Iiposomal doxorubicin. Smaller liposomes are better able to deliver their encapsulated drug to the site of tumor growth. Clinical trials with liposomal doxorubicin are clearly reflecting laboratory findings. Very promising results are becoming evident as to the ability of liposomal encapsulation to decrease toxic side effects of doxorubicin while maintaining its therapeutic activity.

INTRODUCTION

Doxorubicin is an anthracycline antibiotic which displays potent antineoplastic activity against a wide spectrum of human and animal solid tumors and leukemias (Carter, 1975; Young et al., 1981). Despite its therapeutic benefits, it displays a wide variety of acute toxicities including myelosuppression and gastrointestinal toxicity as well as a cumulative dose-limiting cardiotoxicity (Minlow et al., 1975). Cardiac damage is cumulative and irreversible, where doses greater than approximately 550 mg/m^2 can result in the development of congestive heart failure in humans (Gottheb et al., 1973; Ugoretx, 1976). In preclinical models, additional indicators of toxicity include weight loss (Herman et al., 1983; Gabizon et al., 1986), alopecia (Herman et al., 1983), urinary albumin concentration (van Hoesel et al., 1984; Gabixon et al., 1986). and dermal necrosis as a result of extravasation (Forssen and Tokes, 1983a).

Liposomal encapsulation has been employed as one approach to reducing toxicities associated with conventional doxorubicin administration. The underlying premise of this approach is that the liposomal drug delivery vehicle will alter the biodistribution of the drug in such a way as to reduce uptake of the drug by susceptible tissues while maintaining or enhancing therapeutic activity of the drug.

A significant number of studies have indicated that encapsulation of doxorubicin within liposomes can significantly improve its therapeutic index. Reports from as early as the late 1970's indicated that encapsulation of doxorubicin could significantly reduce its cardiotoxicity (Forssen and Tokes, 1979; Rahman et al., 1980; Forssen and Tokes, 1981). Other early work indicated that other toxicities associated with doxorubicin administration were decreased by liposomal encapsulation of the drug (Gabizon et al., 1982; Olson et al., 1982; Forssen and Tokes, 1983a; Herman *et al.,* 1983; van Hoesel et *al.,* 1984; Rahman *et al., 1985;* Gahizon *et al.,* 1986; Balasovits *et al., 1989).*

This chapter will focus on the parameters which affect in vivo behaviour of liposomal doxorubicin. Variables such as lipid composition, liposome size, and drug-to-lipid ratio can play an important role in doxorubicin toxicity and therapeutic activity. The use of pH-gradient encapsulation procedures to elucidate the effects of these parameters on both drug toxicity and efficacy will be discussed as well as the results from early clinical trials employing the use of doxorubicin entrapped in these and other liposomal systems.

DRUG LOADING OF DOXORUBICIN INTO LIPOSOMES

Previous work has demonstrated that the leakage of "passively" entrapped doxorubicin (in the absence of a pH-gradient) is relatively rapid, resulting in 50% drug leakage after approximately lh at 37°C (Mayer et al., 1985a). For this reason, it was necessary to develop a means of increasing drug retention. Early studies employed the use of K^+ ion gradients to drive the uptake of doxorubicin (Mayer et al., 1985a) and other hydrophobic drugs (Nichols and Deamer, 1976; Bally et al., 1985; Mayer et al., 1985b). This K+ diffusion potential induced by valinomycin also significantly improved liposomal drug retention. The improved uptake and retention may be attributed to the transbilayer pH gradient induced in response to the K^+ diffusion potential. The next line of investigation focused on the direct use of pH-gradients rather than K^+ ion-gradients to accumulate doxorubicin within liposomes. It is well known that the distribution of weak bases and acids across biological membranes is strongly influenced by the presence of pH-gradients (Chappel and Crofts, 1966; Crofts, 1967).

Many biological compounds or pharmaceutical agents (such as doxorubicin) have proton accepting (basic) groups. The dissociation constant (K_a) for a weak base can be described as follows:

$$K_a = [H^+][D]/[DH^+] \quad (1)$$

where $[H^+]$ is the hydrogen ion concentration, [D] is the concentration of the neutral form of the weak base and $[DH^+]$ is the concentration of the protonated drug. The pK_a is the negative log of K_a. According to the Henderson-Hasselbach equation:

$$pH = pK_a + \log ([D]/[DH^+]) \quad (2)$$

It may be expected then, that pH-gradients can be utilized to drive the net accumulation of weak bases into liposomal systems. Assuming that K_a is the same on both sides of the liposomal membrane, then:

$$K_a = [H^+]_i[D]_i/[DH^+]_i = [H^+]_o[D]_o/[DH^+]_o \quad (3)$$

where the subscripts i and o refer to the inside and outside of the liposome. Since it is well accepted that the neutral form of molecules is by far the most membrane permeable species (Rottenberg, 1979; Addanki et al., 1986), at equilibrium, the concentration of the neutral species will be the same on both sides of the membrane. Therefore, if $pK_a \gg pH_o \gg pH_i$:

$$[DH^+]_i/[DH^+]_o = [H^+]_i/[H^+]_o \quad (4)$$

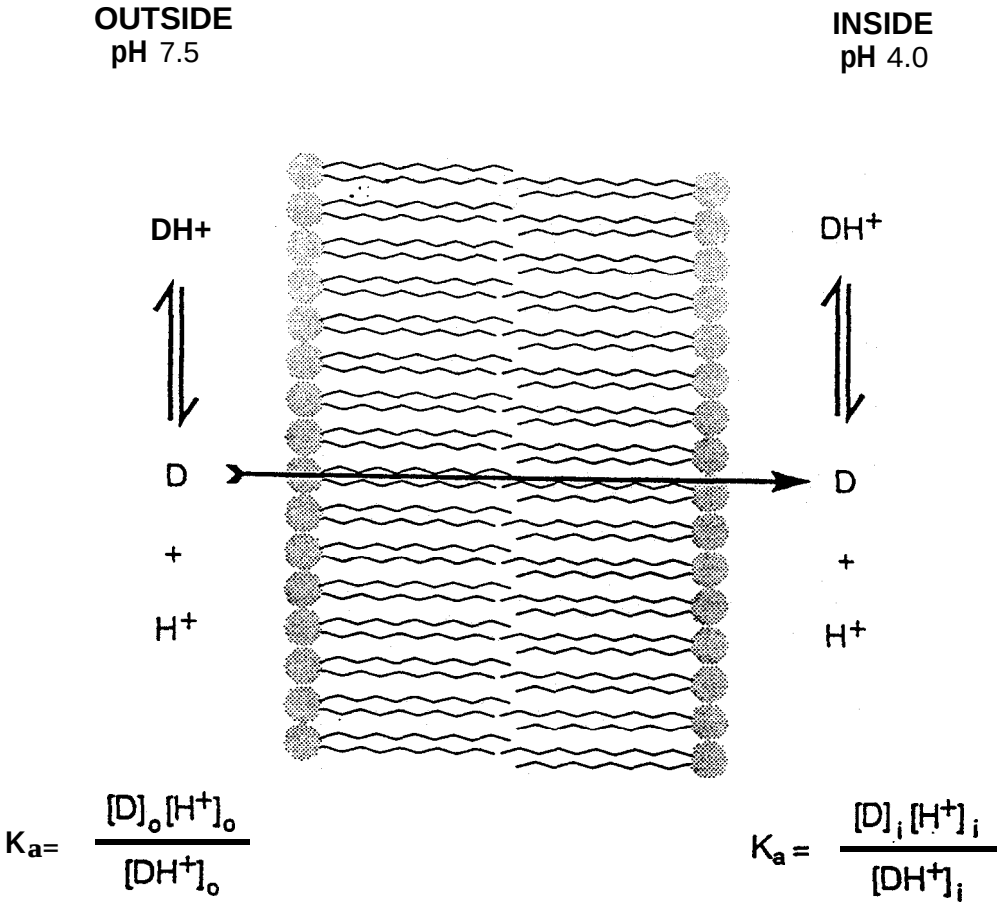
The uptake of doxorubicin into large unilamellar vesicles (LUVs) exhibiting a transbilayer pH-gradient is diagrammed in Figure 1.

It has been shown that doxorubicin will rapidly accumulate into both egg-yolk phosphatidylcholine (eggPC) and eggPC-cholestero1 (1:1) LUVs in response to a pH-gradient (Mayer et al., 1986) at 37°C. Later studies demonstrated the importance of temperature on the rate and efficiency of doxorubicin encapsulation into liposomes containing cholesterol. With a pH-gradient of 3.5 units (inside acidic) there is only a 30% trapping efficiency after 90 min. at 21°C. At **37°C,** virtually 100% of the drug can be loaded within 90 mm., and when the temperature is increased to **60°C,** the same entrapment efficiency can be achieved within 2 mm (Figure 2). Therefore, standard procedures for drug loading now employ liposomal incubation with doxorubicin at 60°C for 10 min. to maximize drug encapsulation efficiency (Mayer et al., 1989).

CHARACTERIZATION OF pH GRADIENT-BASED LIPOSOMAL SYSTEMS CON-INNING DOXORUBICIN

Using the pH-gradient loading procedure for liposomal encapsulation of doxorubicin, the effects of liposome size, drug-to-lipid ratio, and lipid composition have been studied (Mayer et **al.,** 1989). These comparisons were possible due to the fact that greater than 98% trapping efficiencies can be achieved for liposomes ranging in diameter from 0.1 to 1.4 **μm,** for drug-to-lipid ratios ranging from 0.03:1 (wt:wt) to 0.3:1 (wtwt) and for vesicles with varying lipid compositions (Table 1).

In addition to benefits for drug entrapment, it has been observed that the retention of liposomal doxorubicin is much improved following pH-gradient loading as compared to "passively" loaded systems (Mayer et **al.,** 1989). Between 20-50% of passively loaded doxorubicin is released fiom eggPC-cholesterol(55:45; mol:mol) liposomes by lh at 3'7°C (Gabizon et al., 1982; Mayer et **al.,** 1985a). The same systems which have been pH-gradient loaded release less than 5% of the drug by 24h under the same conditions. These studies also demonstrated



At equilibrium, if:

$$[D]_o = [D]_i$$

Then:

$$\frac{[DH^+]_i}{[DH^+]_o} = \frac{[H^+]_i}{[H^+]_o}$$

Figure 1. Redistribution **of weak bases in response to transmembrane pH gradients where D represents the drug/weak base of interest.**

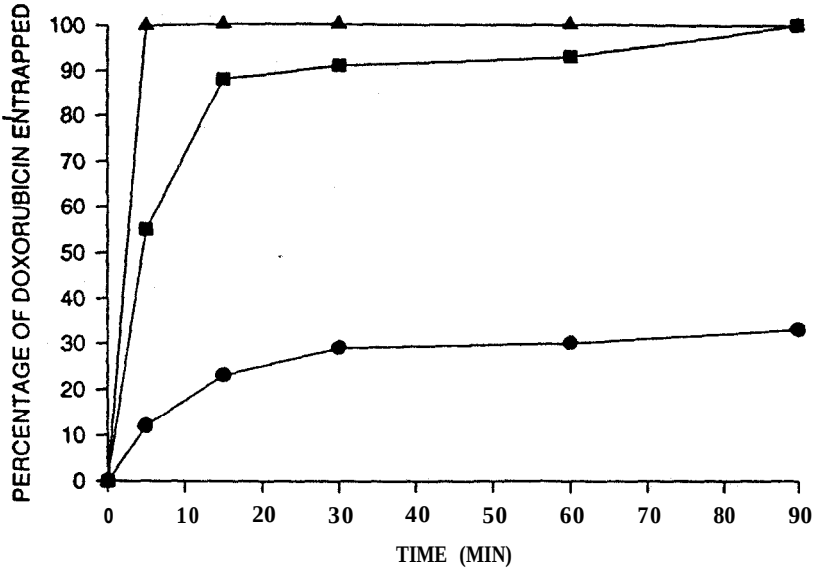


Figure 2. Effect of incubation temperature on ΔpH-dependent doxorubicin uptake in eggPC-cholesterol (55:45; mol:mol) vesicles. Vesicles were prepared in 300 mM citric acid (pH 4.0) and extruded through 200 nm pore size polycarbonate filters Prior to doxorubicin addition, the external vesicle medium was brought to pH 7.8 with sodium hydroxide. Doxorubicin (3.0 mg/mL) was added to liposomes (11.0 mg lipid/mL) equilibrated at 21 (●), 37 (■), and 60°C (▲).

that decreasing the cholesterol content of eggPC-cholesterol vesicles as well as incorporating the negatively charged lipid phosphatidylglycerol (PG), results in enhanced drug leakage.

This procedure for "active" entrapment of liposomal doxorubicin is clearly of advantage due to the high trapping efficiencies which can he achieved and the enhanced drug retention properties. Because the technique is also independent of lipid composition, vesicle size, and drug-to-lipid ratio it is well suited for the generation of new liposomal formulations aimed at improving the specificity of doxorubicin activity.

BIOLOGICAL ACTIVITY OF LIPOSOMAL DOXORUBICIN TOXICITY

Early studies utilized dose response weight loss and srvival curves in order to compare the toxicity of various liposomal doxorubicin formulations. In this manner, the influence of lipid composition, liposome size, and drug-to-lipid ratio on the toxicity of liposomal doxorubicin has previously been examined (Mayer et al., 1989).

Table 1 The effect of lipid composition, liposome size, and drug-to-lipid ratio on pH-gradient driven uptake of doxorubicin in liposomes

Lipid Compostition (molar ratio)	*Vesicle Size mean±SD (nm)*	*Drug-to-Lipid Ratio (wt:wt)*	*Drug Trapping Efficiency (%)*
EPC	**158±37**	**0.29:1**	**>99.0**
EPC/Chol (85:15)	**166±49**	**0.28:l**	**99.0**
EPC/Chol (67:33)	**163±49**	**0.31:1**	**>99.0**
EPC/Chol (55:45)	**106±31**	**0.25:1**	**98.8**
EPC/Chol (55:45)	**160143**	**0.29: 1**	**>99.0**
EPC/Chol (55:45)	**1400±400**	**0.28:1**	**299.0**
EPC/Chol (55:45)	**160±48**	**0.038: 1**	**>99.0**
DSPC/Chol (55:45)	**773±140**	**0.28:1**	**98.7**
DSPC/Chol (55:45)	**175±41**	**0.28:1**	**>98.9**

Adapted from Mayer et al., 1989

For free doxorubicin, an LD_{50} value over 14 days of 23 mg/kg is determined for CD-l mice. For liposomal doxorubicin with a drug-to-lipid ratio of approximately 0.3:1 (wt:wt), encapsulation of the drug with eggPC-cholesterol vesicles (55:45; mokmol) increases the LD_{50} value to 57 mg/kg. Decreasing the cholesterol content of eggPC vesicles progressively decreases the LD_{50} for the drug and exchanging the eggPC molecule with the saturated distearoylPC molecule dramatically increases the LD_{50} value to 161 mg/kg. These toxicity results reflect the ability of various liposome compositions to retain the drug suggesting that the extent of toxicity observed for liposomal doxorubicin is related to in vivo stability of the liposomes (Senior and Gregoriadis, 1984).

Vesicle size appears to have a more modest effect on liposomal doxorubicin toxicity (Mayer et al., 1989). When the size of eggPC-cholestero1 vesicles (55:45; mol:mol) is decreased from a diameter of 1.4 μm to approximately 0.1 μm, the LD_{50} value over 14 days decreases from 60 mg/kg to 45 mg/kg. A similar increase in toxicity is seen as the size of distearoylPC-cholestero1 (55:45; mokmol) vesicles is decreased. The reason for this increased toxicity is likely due to the fact that the smaller liposomes remain in the circulation for extended periods of time (Senior, 1987).

Another parameter that plays a role in determining toxicity of liposomal doxorubicin is the drug-to-lipid ratio. LD_{50} studies in mice indicate that increasing the drug-to-lipid ratio of liposomal doxorubicin significantly decreases toxicity (Table 2). Since lower drug-to-lipid ratios require injection of a higher lipid dose, one may conclude that the lipid is causing the increase in toxicity. This was found to not be the case since an identical dose of empty liposomes resulted in no deaths (Mayer et al., 1989). Consequently, it would appear that liposomal doxorubicin formulations exhibiting high drug-to-lipid ratios are inherently less toxic.

Table 2 The effect of drug-to-lipid ratio on the LD, values of doxorubicin encapsulated within EPC/Chol vesicles in CD1 mice

Drug-to-Lipid Ratio (wt:wt)	LD_{50} *(mg/kg body weight)*
0.28:1	**57**
0.072:1	45
0.038:l	39

Adapted from Mayer et al., 1989

Since the dose-limiting toxicity associated with doxorubicin use is its cardiotoxicity, it is extremely important to examine the effects of liposomal encapsulation on this parameter. Experimental results have demonstrated the liposomal encapsulation of doxorubicin significantly reduces drug uptake by the heart in Wistar rats (Harashima et al., 1993). It is expected that by decreasing the amount of cardiac exposure to the drug, adverse side effects may be decreased or eliminated. Further, it has been suggested that the release of doxorubicin from liposomes is the rate limiting step in the accumulation of doxorubicin in the heart tissue (Harashima et al., 1992). One would, therefore, expect the extent of cardiotoxicity to decrease when liposomal systems with better drug retention capabilites are used as delivery vehicles.

In vitro studies on chick heart cells have been performed to determine the effectiveness of liposomal encapsulation on reducing the cardiotoxicity associated with doxorubicin (Olson at al., 1982). In these studies **cultured cells from embryonic chicken hearts were incubated with various drug preparations before determining cell beating rates at various time points. Liposomal doxorubicin was found to be much less toxic than free drug in terms of reduced myocardiaI beating rates.**

Myocardial damage has also been assessed by other means (Rahman et al., 1980; Forssen and Tokes, 1981; Herman et al., 1983; Balazsovits et al., 1989). Studies have shown a decrease in doxorubicin accumulation in mouse heart tissue when encapsulated within liposomes compared to free drug (Rahman et al., 1980). Vacuolization and myofibrillar loss has been observed in the hearts of dogs following chronic injection of free doxorubicin while liposomal encapsulation prevents these cardiac **lesions (Herman et al., 1983; Banter et al., 1993). Similar results have been observed in rats (van Hoesel et al., 1984). Finally, histological analysis of mouse heart tissue following a single i.v. injection of free or liposomal doxorubicin 'reveals that liposomal doxorubicin is much less cardiotoxic (Balazsovits et al., 1989). Cellular necrosis is evident after injection of free drug but no Iiposomal drug when administered at a drug dosage of 25 mg/kg.**

The measurement of serum enzymes has also been used as a marker for damage to organs such as the heart and liver. The latter is especiaIly relevant given that liposomes accumulate to a significant level in the liver. Creatine phosphokinase

(CPK) levels are used to determine evidence of cardiomyopathy while alkaline phosphatase (AP) levels reveal any hepatic damage (Everett and Harrison, 1983). Lactate dehydrogenase (LDH) and glutamate oxaloacetic aminotransferase (COT) can act as a marker for damage to either tissue. When doxorubicin is administered to CD-1 mice at a drug dosage of 20 mg/kg, free drug results in a 2-3-fold increase in the serum levels of CPK, LDH, and COT which persist over 10 days. When the drug is encapsulated within eggPC-cholestero1 vesicles (55:45; mol:mol; 160 nm diameter) there is a slight increase in LDH and COT levels which return to normal levels by day 10. Administration of doxorubicin within distearoylPC-cholestero1 vesicles (55:45; mol:mol; 160 nm diameter) results in normal enzyme levels. All doxorubicin preparations had no effect on AP levels. It is therefore evident that liposomal encapsulation dramatically decreases cardiotoxicity and does not appear to promote hepatotoxicity. This is particularly evident for more stable liposomal preparations.

Another important toxicity associated with doxorubicin usage is it myelosuppressive activity. Thii toxicity can be monitored by the measurement of bone marrow nucleated cells, changes in spleen weight, and peripheral white blood cell counts. When doxorubicin is administered at a dosage of 20 mg/kg to DBA/2J mice, by day 3 free drug reduces bone marrow cellularity by 90% which returns to normal by day 7 (Bally et al., 1990b). Encapsulation of the same dosage of doxorubicin into either eggPC-cholestero1 (55:45; mol:mol) or distearoylPC-cholestero1 (55:45; mol:mol) vesicles sized through 1.0 μm pores appears to prolong the reduction in bone marrow cellularity. However, encapsulation of doxorubicin inside small (0.1 μm diameter) distearoylPC-cholesterol (55:45; mol:mol) vesicles significantly reduces the initial decay in bone marrow cellularity On examination of the Iiposomal drug accumulation in the bone marrow nucleated cells, both lipid and drug accumulate to a much higher extent in the bone marrow when the liposomes are of larger size.

Changes in spleen weight correlate well with the decrease in bone marrow cellularity. DistearoylPC-cholesterol vesicles sized through 0.1 μm pores display the smallest decrease in spleen weight when used to encapsulate doxorubicin (Bally et al., 1990b). Free doxorubicin decreases peripheral white blood cell counts to 46% of normal on day 3 and recovers to normal by day 14 (Sally et al., 1990b). Liposomal encapsulation of the drug, however, enhances the initial decrease in peripheral white blood cell count and prolongs the recovery to normal levels by approximately 21 days. This liposomal doxorubicin-induced decrease in peripheral white blood cells does not reflect the results seen in the bone marrow or spleen. It may in fact indicate toxic effects on other lymphopoietic organs such as the thymus or direct effects on circulating cells.

The local toxicity of liposomal doxorubicin has also been evaluated using a subcutaneous model. Studies have reported that encapsulation within eggPC-cholesterol (55:45; mol:mol) systems dramaticaIIy reduces the vesicant properties of the drug (Balazsovits et al., 1989). When 0.5 mg of either free or liposomal drug is injected S.C. in DBA/2J mice, the free drug results in immediate erythema and edema which progresses to ulceration. The liposomal drug, in contrast, does not progress to ulceration. The mild erythema and edema seen in the liposomal injections completely resolves by 3 weeks following administration.

PHARMACOKINETICS OF LIPOSOMAL DOXORUBICIN

The circulation longevity of liposomal doxorubicin is affected by two factors: the ability of the liposomal carrier to retain the drug and the circulation time of the lipesomes themselves. It is also evident that liposomal encapsulation substantially improves drug circulation times especially for small vesicles of 0.12 μm diameter. Plasma doxorubicin levels are cleared extremely rapidly when free drug is administered but when encapsulated within 120 nm dipalmitoylPC-cholesterol vesicles, plasma drug levels are approximately 500-fold higher at time points beyond 4h following i.v. administration.

It has been shown that doxorubicin remains within the more highly stable distearoylPC-cholestero1 liposomes than either dipalmitoylPC-cholesterol or eggPC-cholesterol vesicles (Bally et al., 1990). Liposomal doxorubicin formulations prepared at a drug-to-lipid ratio of approximately 0.2:1 (wt:wt) with 0.1 μm liposomes composed of distearoylPC-cholestero1 (55:45; mol:mol) showed 100% drug retention 24h following i.v. administration in mice. By comparison, dipalmitoylPC-cholesterol systems displayed a drug retention of 73% at 24h and eggPC-cholestero1 systems showed a 20% drug retention at the same time point. The in vivo drug-to-lipid ratios for eggPC-cholestero1 and dipalmitoylPC-cholesterol liposomal doxorubicin can be Seen in Figure 3. Here we see, at 24h following i.v. administration, a 4-fold increase in drug-to-lipid ratio for 100 nm vesicles composed of dipalmitoylPC-cholestero1 (55:45; mol:mol) as compared to eggPC-cholestero1 liposomes (55:45; mol:mol) .

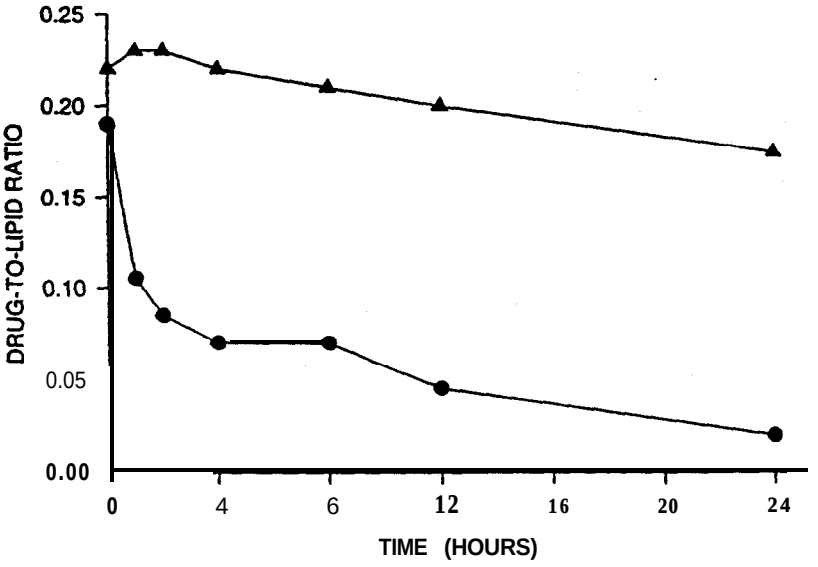


Figure 3. Drug-to-lipid ratio determined in the plasma of mice treated with 0.12 μm dipalmitoylPC-cholesterol (A) or 0.12 μm eggPC-cholestero1 (●) liposomal doxorubicin.

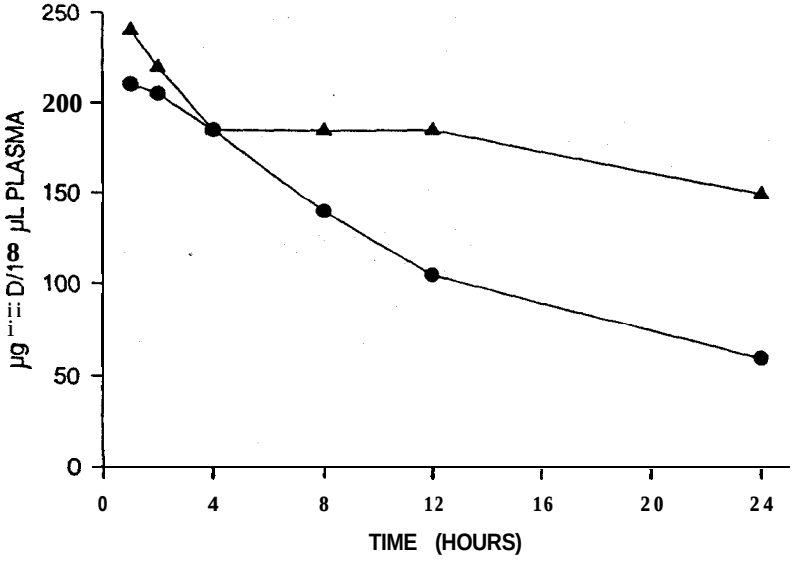


Figure 4. Liposomes (•) and liposomes loaded with doxorubicin (A) employing ΔpH driven uptake were injected into DBA/2J mice at a doxorubicin dose of 20 mg/kg or a lipid dose of 100 mg/kg. DipalmitoylPC-cholesterol vesicles had a *mean* diameter of 0.12 µm.

The circulation time of the liposomal carrier also influences the pbarmacokinetic profile of liposomal doxorubicin. The effects of lipid composition on circulation times have been investigated Bally et al., 1990a). The results indicate that the incorporation of saturated lipids into tbe liposomal membrane increases lipid circulation time for doxorubicin-containing systems but not for empty liposomes. For distearoylPC-cholestero1 vesicles (55:45; mol:mol) sized through 100 nm pores, there is approximately 80% of both drug and lipid remaining in the circulation 24 h post injection. In contrast, approximately 20% of doxorubicincontaining eggPC-cholesterol vesicles (55:45; mol:mol) remains in the circulation at the same time point

Liposome circulation time has been shown to be significantly increased by the encapsulation of doxorubicin as compared to empty liposomes (Bally et al., 1990a). DipalmitoylPC-cholesterol vesicles (55:45; mol:mol) display increased blood residence times when they contain doxorubicin compared to empty liposomes. For vesicles with 120 nm diameter, at a drug-to-lipid ratio of 0.2:1 (wt:wt), drug loaded liposomes display a 2-fold increase in plasma lipid concentration at 24h following i.v. injection in DBA/2J mice (Figure 4). It should also be noted that smaller vesicles (0.12 µm diameter) display much longer circulation times than larger vesicles (1.2 µm diameter). Regardless of the size of the liposomal carrier, doxorubicin entrapment results in a 2 to IO-fold increase in plasma lipid concentration at time points beyond 8h after i.v. administration to DBA/2J mice.

The ability of doxorubicin to improve iiposome circulation longevity appears to be specific for encapsulated doxorubicin since pre-treatment with free doxorubicin 4 of 24h prior to injection of empty liposomes had no effect on lipid clearance rates (Bally et al., 1990a). 'However, pre-treatment with an identical dose of liposomal doxorubicin results in increased circulation times for a subsequent injection of empty liposomes (Bally et al., 1990a Parr et al., 1993). This effect has been suggested to occur due to impairment of RES function by liposomal doxorubicin.

The biodiitribution of liposomal doxorubicin has been examined in humans during a Phase I clinical trial. Imaging studies on 19 patients revealed that liposomes were cleared mainly by the liver and spleen but also to a smaller extent by the bone marrow (Gabizon et al., 1991). This is consistent with previous results (Gregoriadis, 1988; Hwang and Beaumier, 1988). Further, in patients with hepatoma formation, sites of tumor growth revealed no liposome accumulation, suggesting that liposomes are solely accumulated within cells of the RES.

ANTITUMOR ACTIVITY OF LIPOSOMAL DOXORUBICIN

Liposomal encapsulation of doxorubicin is of significant benefit due to its prevention of many of the toxicities of free drug. It is also necessary to determine how this encapsulation within liposomes will affect its therapeutic activity. Numerous studies have been performed in this regard both examining the in vitro and in vivo cytotoxicity of various tumor models.

In vitro studies have demonstrated either similar or improved antitumor efficacy when doxorubicin is encapsulated within liposomes. In vitro studies using primary cell cultures of J-6456 lymphoma cells showed liposomal doxorubicin to be equally effective to free drug in inhibiting cell growth (Gabizon et al., 1982). Similar results were obtained for multilamellar vesicles composed of eggPC, eggPC-cholesterol, and eggPC-cholesteroI with either PS or PG.

There is also evidence that liposomal doxorubicin can actually enhance antitumor activity over free drug. Investigations have shown enhanced tumor uptake and efficacy in various animal models (Gabizon et al., 1990; Mayer et al., 1990; Gabizon, 1992; Huang et al., 199%; 1992b; Ahmad et al., 1993; Gabizon et al., 1994; Huang et al., 1994). Studies employing tissue cultures of Kaposi's sarcoma cells support this fact (Masood et al., 1993). Analyis of cellular cytotoxicity thymine incorporation, and cellular drug uptake were performed following incubation with drug for various time periods. Doxorubicin encapsulated within CL-eggPC-cholsterol (2:10:6.8; mol:mol:mol) at a drug-to-lipid ratio of approximately 0.12:1 displayed a 38-fold increase in cytotoxicity over free drug. Studies of thymine incorporation demonstrated a significant decrease in cell proliferation when incubated with liposomal drug. Cellular uptake of free doxorubicin peaked at 1h while liposomal drug continued to be taken up over 4h. There was approximately a 5-fold higher uptake of doxorubicin when encapsulated within liposomes.

Several in vivo studies have examined the effects of liposomal doxorubicin on antitumor activity against various tumor models. These include liver metastasis of mouse colon carcinoma (Mayhew et al., 1987), P388 ascitic leukemia, disseminated gross leukemia, and advanced mammary carcinoma (Rahman et al., 1986), Sarcoma 180 and Lewis lung carcinoma (Forssen and Tokes, 1983b), liver metastases of J-6456 lymphoma cells (Gabizon et al., 1985), IgM immunocytoma cells (Storm et al., 1987), the SC115 murine mammary tumor (Mayer et al., 1990). L1210 lymphocytic leukemia (Mayer et al., 1989), i.m. tumor implants of J-6456 lymphoma (Gabizon, 1992), KLN-205 squamous cell carcinoma of the lung (Ahmad et al., 1993), C-26 colon carcinoma (Huang et al., 1992b; 1994). These studies all conclude that the encapsulation of doxorubicin within liposomes significantly improves therapeutic activity.

The means by which liposomal doxorubicin gains access to tumor cells is unquestionably of interest_ In vitro studies employing the human ovarian carcinoma cell line (OV-1063) indicate that cytotoxicity is mediated by the release of doxorubicin from the liposomes and not be direct cellular uptake of the liposomal doxorubicin (Horowitz et at., 1992). This conclusion was supported by the fact that cytotoxicity was decreased when doxorubicin was encapsulated within Iiposomes containing lipids with high phase-tmnsition temperatures. Further, in the presence of resin beads which bind free doxorubicin but not liposomal doxorubicin, the cytotoxicity of both drug preparations was almost completely inhibited

Since liposomes accumulate to a high degree in the liver, several researchers have examined the effects of hposomal anticancer drugs on liver metastases. Studies have revealed that weekly i.v. injections of 10 mg/kg liposomal doxorubicin (passively entrapped) significantly increases the survival time of mice bearing CT38LD or CT26 liver metastases over the same dose of free drug (Mayhew et al., 1987). Similar results were seen with liver metastases of J-6456 lymphoma cells (Gabizon et al., 1985). In this work, tumor cell death was enhanced by approximately 100-fold when doxorubicin was encapsulated within liposomes. These studies also employed the use of passive entrapment procedures.

Studies employing immunoglobulin solid immunocytoma-bearing Lou/M Wsl rats demonstrated that the lipid composition of liposomes can significantly affect the antitumor activity of liposomal doxorubicin (Storm et al., 1987). When doxorubicin is encapsulated within vesicles composed of eggPC-PS-cholesterol the antitumor activity is much higher than when distearoylPC-PS-cholesterol vesicles are used The reasons for this decrease in therapeutic activity are thought to be due to the fact that when liposomes are composed of lipids with higher phase transition temperatures, they are degraded much more slowly so the drug is less able to gain access to the tumor cells. Further studies on the correlation between solid tumor drug uptake and tumor regression have been performed using the SC115 Shionogi mouse mammary tumor (Mayer et al., 1990), as well as the J-6456 and C-26 colon carcinomas (Gabizon, 1992; Huang et al., 1992b; 1994). The mammary tumor experiments utilized liposomal doxorubicin prepared by pH-gradient driven drug encapsulation whereas the colon carcinoma studies used a related NH_4SO_4 entrapment procedure. The results with mammary tumors indicated that liposomal

encapsulation significantly improved tumor uptake of doxorubicin which correlated well with improved therapeutic activity. Liposomal encapsulation enabled much higher doxorubicin dosages to be administered due to decreased toxicity; when liposomal drug was administered at 13 mg/kg, tumors showed approximately 10-fold decreases in tumor weight Studies with the J-6456 lymphoma and the C-26 colon carcinoma in mice indicated that a further improvement in cytotoxicity is evident when doxorubicin is encapsulated within liposomes with extended circulation life-times.

Since numerous studies support enhanced antitumor activity of doxorubicin when encapsulated within liposomes, it is important to determine which liposomal parameters are important in this regard. Studies have been performed investigating the effects of lipid composition and vesicle size on pH-gradient loaded liposomal doxorubicin (Mayer et al., 1989). Vesicles of 180 nm diameter were prepared using eggPC-cholesterol (55:45; mol:mol) , eggPC, distearoylPG-cholesterol (55:45; mol:mol), and eggPC-eggPG-cholesterol (27.5:27.5:45, mol:mol:mol) and doxorubicin was loaded to achieve a drug-to-lipid ratio of 0.3:1 (wt:wt). All vesicle preparations exhibited similar efficacy to that of free drug against L1210 lymphocytic leukemia in mice when administered at doses of 20 mg/kg or lower. Contrary to previous studies (Storm et al., 1987), this suggests that lipid composition has a small effect on antitumor activity of liposomal doxorubicin. However, studies have demonstrated that when doxorubicin is encapsulated within sterically stabilized liposomes containing either G_{M1} ganglioside, phosphatidylethanolamine derivatized with poly(ethylene glycol) , or hydrogenated phosphatidylmositol (HPI) , there is a marked improvement in therapeutic activity over liposomal systems with shorter circulation times (Gabizon, 1992; Huang et al., 1992b; Huang et al., 1994). Alterations in liposome size also dramatically influence the efficacy of liposomal doxorubicin. When liposomal drug is administered i.v. within eggPC-cholestero1 (55;45; mokmol) vesicles with mean diameters of 1110 nm, at 20 mg/kg there is decreased therapeutic activity as compared to free drug. In contrast, when the same drug dosage is administered within liposomes of 100 nm diameter, there is a much higher efficacy than for the same dosage of free drug. A similar size dependency is also seen for vesicles composed of distearoylPC-cholestero1 (55:45; mol:mol) .

CLINICAL TRIALS

Due to the success of llposomal encapsulation of doxorubicin in preventing toxicity while maintaining or improving therapeutic activity, formulations of liposomal doxorubicin have now entered clinical trials.

Early trials employed intraperitoneally administered liposomal doxorubicin in patients with advanced ovarian cancer (Delgrado et al., 1989). Previous work has demonstrated that intraperitoneal free doxorubicin can be extremely effective against ovarian cancer (Ozols et al., 1979). However, i.p. doxorubicin can result

in significant chemical peritonitis. Therefore, liposomal encapsulation of the drug could be of distinct advantage. Liposomal doxorubicin formulations were prepared by passive entrapment of drug into vesicles composed of cardiolipin, PC, cholesterol, and stearylamine. Intraperitoneal administration resulted in no myelosuppression, abnormalities of liver function tests, or alopecia. Also, accidental extravasation of drug in two patients results in no evidence of toxicity. Of the 15 patients studied, only one experienced chemically induced peritonitis after a body temperature elevation to 39.5%. It is suggested that this temperature elevation induced leakage of the drug from the liposomes, resulting in the chemical peritonitis. In terms Of therapy, three out of four patients with minimal ovarian disease at the onset of peritoneal therapy demonstrated a partial response to the treatment. This is in agreement with other phase II clinical trials (Markman et al., 1984; Ozols et al., 1984; ten Bokkel Huinink et al., 1985) demonstrating therapeutic response only in patients with small residual disease after induction chemotherapy. The majority of patients who did not respond to intraperitoneal administration of liposomal doxorubicin had bulky disease prior to therapy.

Another phase I clinical study specifically evaluated the toxicity of i.v. liposomal doxorubicin when administered to patients with various tumor types (Rahman *et al.,* 1990). Doxorubicin was encapsulated in liposomes composed of cardiolipin, PC, cholesterol, and stearylamine. This investigation revealed that nausea and vomiting as well as other gastrointestinal toxicities were mild or absent and evidence of stomatitis was absent These toxicities have been shown to be present in more severe form with the administration of free drug (O'Bryan et al., 1973; Tan et al., 1973; Benjamin et aL, 1974; Gottlieb, 1975; O'Bryan et al., 1977). Granulocytopenia, however, was present after administration of liposomal doxorubicin and appeared to be dose limiting resulting in a maximum tolerated dose (MTD) of 90 mg/m^2. However, more severe granulocytopenia would be expected at similar doses of free drug. Similar decreases in nausea, vomiting, and stomatitis have been observed with the TLC D-99 preparation of liposomal doxorubicin (Cowens et al., 1993).

Altered toxicity was not as pronounced when liposomal doxorubicin was administered on a weekly basis (Conley et al., 1993). In this phase I clinical trial, toxicities of liposomal doxorubicin were judged to be essentially identical to free drug. Apparent toxicities included leukopenia, thrombocytopenia, nausea, vomiting, fever, alopecia, diarrhea, fatigue, stomatitis, and infection. An MTD of 30 mg/m^2 per week x 3 was observed. Reasons for differences in buffering of toxicity between weekly and three weekly dosing are unknown.

In attempts to prevent the dose limiting granulocytopenia associated with both free and liposomal doxorubicin, recent Phase I clinical studies have examined the effect of simultaneous administration with granulocyte colony stimulating factor (G-CSF) (O'Day et al., 1994). The administration of G-CSF was able to increase the dose limiting toxicity level of liposomal doxorubicin from 90 mg/m^2 to 165 mg/m^2. Phase II clinical trials with G-CSF are currently under investigation.

Cardiotoxicity of doxorubicin is associated with cumulative doses of drug in excess of 550 mg/m^2 (L.enaz and Page, 1976). Of the 20 patients with advanced breast cancer, 12 received cumulative doses of liposornal doxorubicin in excess of

400 mg/m^2 and were evaluated with radionucleotide ventriculograms. In 5 patients who had received cumulative doses in excess of 500 mg/m^2, endomyocardial biopsies were performed. Of these biopsies, only one demonstrated Billingham grade 1 changes with mild loss of myofibrils and dilatation of the sarcoplasmic reticulum involving less than 5% of cardiac myocytes. This one patient had received a cumulative dose of approximately 750 $mg/m.^2$ This is clearly less toxic than administration of free doxorubicin where histologic changes in the endomyocardium can be observed for cumulative doses as low as 180 mg/m^2 (Druck et al., 1984).

Recent Phase II studies of liposomal doxorubicin (TLC D-99) in patients with metastatic breast cancer have revealed promising results (Batist et al., 1992). In these studies, 17 patients with disease in liver, lung, lymph nodes, skin and bone were evaluated after treatment with liposomal doxorubicin administered at a dose of 75 mg/m^2. Nine of these patients showed major responses in visceral and soft tissue sites of disease. Toxicity was seen to be much reduced from that expected with the administration of free drug. It was concluded that liposomal doxorubicin is highly effective against metastatic breast cancer and is well tolerated.

SUMMARY

It is clearly evident that liposomal encapsulation of doxorubicin significantly reduces toxicities associated with the drug while maintaining or improving therapeutic activity. Since this form of the drug is less toxic than free drug, higher dosages can be administered, allowing for a further enhancement in therapeutic activity.

Toxicities of liposomal doxorubicin appear to be determined by drug retention within the vesicles. This stability is primarily related to the lipid composition of the vesicles. Lipids with higher phase-transition temperatures produce liposomes with higher drug retention capacities, reducing toxic side effects of the drug. Also of interest is that doxorubicin toxicity is reduced by increasing the drug-to-lipid ratio of liposomal doxorubicin preparations. The reasons for this effect are presently not well understood. Higher drug-to-lipid ratios can be achieved by using a pH-gradient to load doxorubicin into liposomes.

The antitumor activity of liposomal doxorubicin is mainly dependent on liposome size. In vivo studies have demonstrated that when doxorubicin is encapsulated within liposomes of 100 nm diameter, the therapeutic activity is substantially improved over that of free drug. Smaller liposomes appear to be able to enhance the accessibility of the encapsulated drug to the tumor.

Although clinical trials using liposomal doxorubicin are still in their early stages, they appear to be demonstrating promising results in terms of decreasing toxic side effects while maintaining therapeutic activity. As various parameters of liposomal doxorubicin preparations are examined further improvements in toxicity protection and antitumor activity will likely be revealed.

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