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THE USE OF TRANSMEMBRANE pH GRADIENT-DRIVEN DRUG ENCAPSULATION IN THE PHARMACODYNAMIC EVALUATION OF LIPOSOMAL DOXORUBICIN

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ABSTRACT

The toxicity and efficacy properties of doxorubicin entrapped inside liposomes are sensitive to the physical characteristics of the vesicle carrier system. Studies addressing such relationships must use preparation procedures with the ability to independently vary vesicle size, lipid composition and drug to lipid ratio while maintaining high trapping efficiencies. The transmembrane pH gradient-driven encapsulation technique allows such liposomal doxorubicin formulations to be prepared. Pharmacokinetic, toxicology and antitumour studies with these systems have revealed several important relationships between liposome physical properties and biological activity. The acute toxicity of liposomal doxorubicin is related primarily to the ability of the liposomes to retain doxorubicin after administration. Including cholesterol and increasing the degree of acyl chain saturation of the phospholipid component in the liposomes significantly decreases drug leakage in the blood, reduces cardiac tissue accumulation of doxorubicin and results in increased LD_{50} values. In contrast, the efficacy of liposomal doxorubicin is most influenced by liposome size. Specifically, liposomes with a diameter of approximately 100 nm or less exhibit enhanced circulation lifetimes and antitumour activity. While these relationships appear to be rather straightforward, there exist anomalies which suggest

that a more thorough evaluation of liposomal doxorubicin pharmacokinetics may be required in order to fully understand its mechanism of action. A key feature in this regard is the ability to differentiate between non-encapsulated and liposome encapsulated doxorubicin pools in the circulation as well as in tumours and normal tissues. This represents a major challenge that must be addressed if significant advances in the design of more effective liposomal doxorubicin formulations are to be achieved.

INTRODUCTION

The sophistication of liposomal systems used to deliver doxorubicin intravenously has increased significantly since their utility in improving the therapeutic index of this drug was first reported (1,2). The vesicle carriers employed for this purpose have progressed from heterogeneous multilamellar vesicle (MLV[1]) or sonicated SUV preparations containing various anionic phospholipids that complex doxorubicin, to homogeneously sized liposomes containing lipid species that impart enhanced biological effects, and in some cases attached antibodies that impart tumour targeting characteristics (1-8). This progression was initially limited by constraints involved in the production of well defined liposomal systems as well as in the ability to effectively entrap and retain doxorubicin inside liposomes. Liposome preparation and encapsulation techniques have improved significantly over the last 10 years and it is now possible to optimize liposomal doxorubicin formulations on the basis of desirable toxicology and efficacy properties. This optimization process is based on selective changes in the physical attributes of the liposomal carrier.

The importance of vesicle size, surface charge and dose in determining the fate of intravenously administered liposomes is well established (see ref. 9 for review). It is therefore not unexpected that manipulations of these parameters can cause significant changes in the pharmacokinetic, toxicity and efficacy behaviour of liposomal doxorubicin. Given the complicated matrix of size, lipid composition and drug to lipid ratio (equivalent to lipid dose) combinations possible for a liposomal preparation of doxorubicin, it is imperative that these properties can be varied independently if their biological influences are to be clearly elucidated. We have found the pH gradient-dependent doxorubicin entrapment technique to be particularly useful in this regard. In this procedure, doxorubicin is sequestered into preformed liposomes displaying a pH gradient (inside acidic) under conditions where trapping

efficiencies approach 100% (4,10). Further, this encapsulation method can be effectively used for a wide variety of liposome types. As a result, liposomal doxorubicin formulations differing only in one property can be generated and their toxicity or efficacy responses in animal models compared.

In this report we describe the use of the pH gradient-driven encapsulation procedure to reveal the influence of vesicle physical properties on the pharmacodynamics of liposomal doxorubicin. The studies presented here demonstrate that the toxicity and efficacy of liposomal doxorubicin are dependent on different formulation characteristics. In addition, the importance of differentiating between liposome-associated and free drug pools in the blood compartment and tissues is addressed.

MATERIALS AND METHODS

Materials

All phoshpolipids were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesterol and other reagent grade chemicals were purchased from Sigma Chemicals (St. Louis, MO). Doxorubicin was purchased from Adria Laboratories (Mississauga, Ont., Canada) and ^{3}H-cholesterylhexadecyl ether was obtained from New England Nuclear (DuPont, Canada). CD-1, BDF1 and DBA/2J mice, 6-8 weeks old, were obtained from Jackson Animal Laboratories.

Methods

Liposome Preparation. Lipid mixtures were prepared in chloroform solution and subsequently dried under a stream of nitrogen gas. The resulting lipid film was placed under high vacuum for a minimum of 2 h. Multilamellar vesicles (100 mg/ml) were formed by hydrating the dried lipid with 300 mM citric acid (pH 4.0). The resulting preparation was frozen and thawed 5 times prior to extrusion 10 times through two stacked polycarbonate filters of the indicated pore size (11). When DSPC or DPPC were employed the sample and extrusion apparatus (Lipex Biomembranes Inc., Vancouver, B.C., Canada) were heated to 5°C above the thermotropic phase transition temperature of the phospholipid prior to extrusion (12). Liposome particle size was determined by quasielastic light scattering (QELS) measurements (employing a Nicomp 370 particle sizer operating at a wavelength of 632.8 nm).

Doxorubicin Encapsulation. Doxorubicin was encapsulated in liposomes in response to transmembrane pH gradients as described previously (4,10). Briefly, the pH of the liposome suspension, initially at pH 4.0, was raised to pH 8.0-8.5 with 0.5 M Na_2CO_3. The liposome preparation was subsequently heated to 60°C for 5 min. and then transferred to a preheated (60°C) vial of doxorubicin, adding enough lipid to achieve the indicated drug to lipid ratio. This mixture was incubated with intermittent mixing for 10 min. at 60°C. Doxorubicin concentration, lipid concentration and encapsulation efficiency were determined as described previously (4). Solutions for injection were prepared with sterile physiological saline such that the specified dose could be delivered in 200 μl.

Quantitation of Liposomal Lipid and Doxorubicin. Liposomal lipid was quantified employing the lipid marker (^{3}H) cholesteryl hexadecyl ether. Previous studies have demonstrated that this lipid label is non-exchangeable and non-metabolizable (13). Doxorubicin was determined employing a fluorescent assay procedure. Plasma or 10% tissue homogenate samples (up to 800 μl) were mixed with 0.1 ml of 10% SDS and 0.1 ml of 10 mN H_2SO_4 and, where necessary, were diluted to a final volume of 1 ml with distilled water. Subsequently 2 ml of isopropanol/chloroform (1:1) was added and the sample was mixed vigorously. The resulting solution was frozen at -70°C, thawed and spun at 500 g for 3-5 min. The organic phase was carefully removed and the fluorescence of this phase was determined (excitation wavelength of 500 nm and emission wavelength 550 nm) employing a Shimadzu RF-540 spectrofluorometer. If required sample volumes were increased or decreased such that the doxorubicin level within the 1 ml aqueous solution fell within the range of the standard curve. The resulting data were converted to doxorubicin fluorescent equivalents derived from a standard curve prepared from doxorubicin. HPLC analysis of selected samples was performed to provide an indication of the amount of fluorescence which was due to non-metabolized doxorubicin.

***In Vivo* Studies.** Doxorubicin and empty or drug loaded liposomes were administered intravenously via injection into a lateral tail vein of the indicated mouse strain. For pharmacokinetic and tissue uptake experiments, 3-5 mice were utilized per experimental point. At the indicated times post injection, the mice were

anaesthetized, blood was collected by heart puncture and tissues were immediately removed, blotted and weighed.

Toxicity of free and liposomal doxorubicin formulations was assessed by dose response weight loss and survival studies. Mice (6-10 per group) were injected i.v. and monitored over 14 days. LD_{50} values and 95% confidence intervals were determined by logistic dose response analysis utilizing generalized linear modelling.

Doxorubicin antitumour activity was assessed employing the L1210 murine ascitic leukaemia model. DBA/2J or BDF1 mice (6-10 per group) were injected i.p. with 1 x 10^5 L1210 cells harvested from the ascites fluid of a previously infected mouse. The indicated drug doses were administered 24h later and animal weights as well as mortality rates were monitored. Increase in life span (ILS) values were calculated from the median survival times. Liposomal/free (L/F) drug therapeutic ratios were calculated by dividing the median survival time obtained for a liposomal doxorubicin formulation by the median survival time obtained for free drug administration at the identical drug dose.

RESULTS

Description of the pH Gradient-Dependent Encapsulation System

The ability to generate well defined formulations under controlled conditions is crucial for the phamacological assessment and optimization of a drug delivery system such as liposomal doxorubicin. Traditional "passive" drug encapsulation procedures, where liposomes are prepared in the presence of the drug and unentrapped material is subsequently removed, are not well suited for such studies. This is due to the fact that alterations in vesicle size or lipid composition often change the trapping efficiency and drug to lipid ratio (which defines the lipid dose) of the resulting systems (3,14). Since doxorubicin is a positively charged amphipathic drug, inclusion of anionic or cationic lipids in the vesicle membrane dramatically alter entrapment and retention characteristics. Consequently, in many early investigations it is difficult to determine unequivocally what effects specific lipid compositions or liposome sizes exert on the toxicity and efficacy of liposome entrapped doxorubicin (2,3,14).

We have utilized "active" drug entrapment procedures extensively to generate liposomal doxorubicin formulations with well defined size, lipid composition and drug

TABLE 1

Liposome Characteristics Suitable for pH Gradient-Dependent Doxorubicin Encapsulation

Liposome Property	Range Yielding $\geq$ 95% Entrapment
Lipid Composition	Cholesterol Content 0 - 45 mol%
	Anionic Lipid (PS,PG,CL) content to 20%
	Phospholipid Acyl Chain Length to C-20
	Specialty Lipids (GM1, Biotinylated PE, PEG-PE, MPB-PE) to 10%
Liposome Size	50 nm (SUVs) to 2 μm (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

to lipid ratio properties. In this encapsulation approach, liposomes exhibiting a transmembrane pH gradient (inside acidic) are incubated with doxorubicin, often at elevated temperatures. The drug's amphipathic and cationic properties enable it to redistribute across the bilayer in response to the pH gradient and accumulate to very high levels in the vesicle interior (10,15). For example, liposomes prepared in 300 mM citrate buffer at pH 4.0 can sequester doxorubicin at levels equimolar to the vesicle lipid (10). Typically, however, drug to lipid ratios $<$ 0.4 (w/w) are employed where trapping efficiencies in excess of 95 % are readily achieved and drug retention is not compromised since the pH gradient remains intact. An interesting adaptation of this technique has been reported by Gabizon, Paphadjopoulos and coworkers where ammonium sulfate is entrapped inside the liposomes and the permeable NH_3 is utilized to establish and maintain the pH gradient (6,7).

One of the most useful characteristics of this drug encapsulation procedure in the context of designing systems for *in vivo* use is that the physical properties of the liposomes can be manipulated independently without significantly altering other drug

entrapment characteristics (e.g. % trapping efficiency or drug to lipid ratio). This is shown in Table 1 where liposomes ranging in size from several microns to approximately 50 nm, containing a variety of neutral, anionic, ganglioside and head group-derivatized lipids, and exhibiting a wide range of drug to lipid ratios can be employed in the pH gradient doxorubicin entrapment procedure. Trapping efficiencies for these systems are in excess of 95%, thus alleviating the need to remove unencapsulated doxorubicin. Also, the drug retention properties for a given lipid composition can be manipulated by changing the buffering capacity of the entrapped aqueous contents (10). Since doxorubicin is accumulated into preformed liposomes, vesicles with specific characteristics can be mixed with the correct amount of drug to yield the desired drug to lipid ratio.

Pharmacodynamic Relationships of pH Gradient-Loaded Liposomal Doxorubicin

The pH gradient-dependent doxorubicin entrapment procedure described above has proven to be very instrumental in elucidating the liposome characteristics that influence the toxicity and efficacy profiles of encapsulated doxorubicin. Although numerous studies have previously described the ability of liposome encapsulation to decrease a wide variety of nonhematological toxicities associated with doxorubicin, most notably cardiotoxicity (1-4), it was unclear to what extent these effects correlated with specific liposome properties.. In contrast, the active pH gradient entrapment procedure has allowed *in vivo* comparisons to be made between liposomal doxorubicin systems differing only in one parameter. Such studies have revealed important relationships between vesicle properties, drug pharmacokinetics and toxicity/efficacy.

Figure 1 presents the LD_{50} values and cardiac drug accumulation levels for free doxorubicin and doxorubicin entrapped in liposomes of varying lipid composition. An inverse relationship is observed between the toxicity of doxorubicin formulations (free and liposomal) and the extent of doxorubicin uptake into cardiac tissue for liposomes composed of phosphatidylcholine and cholesterol (Fig. 1). Since the level of

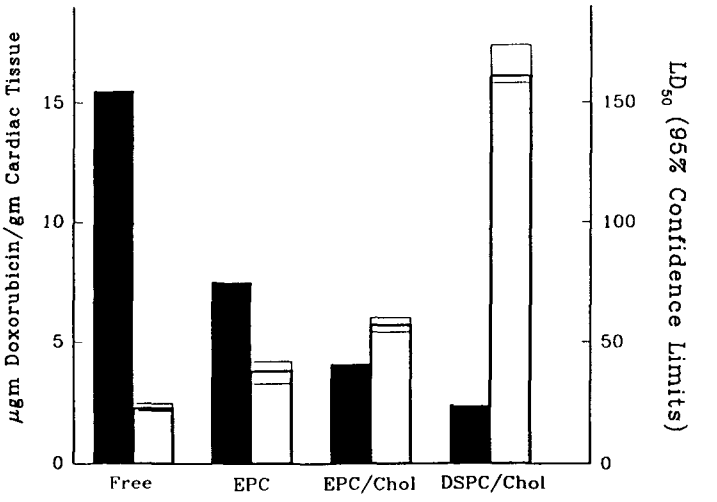

FIG 1: Cardiac accumulation and LD_{50} values of doxorubicin in free and liposome encapsulated form. The different formulations were administered i.v. to CD-1 mice at 20 mg/kg for cardiac accumulation studies. Liposomes were prepared with 45 mol% cholesterol where indicated and all drug to lipid ratios were 0.27 (w/w). Doxorubicin equivalents in cardiac tissue were determined 5 hours after injection. LD_{50} values and confidence limits were determined from 14 day acute toxicity studies.

liposomal lipid accumulation in cardiac tissue is negligible, these results suggest that the toxicity of doxorubicin entrapped inside liposomes may arise from free drug released from the vesicle carrier system after i.v. administration.

It is well established that cholesterol-free liposomes are rapidly destabilized by circulating lipoprotiens, causing rapid release of entrapped contents from liposomes in the circulation (16). This is reflected by the almost 2-fold decrease in cardiac drug uptake and increase in LD_{50} in CD-1 mice from 38 mg/kg to 57 mg/kg when cholesterol is included in EPC liposomes at a 45% molar ratio (Fig. 1). A further decrease in the cardiac uptake and toxicity of liposomal doxorubicin is observed when the saturated acyl chain phosphatidylcholine, DSPC, is substituted for the more fluid EPC. The LD_{50} of the DSPC system is increased to 161 mg/kg while cardiac

TABLE 2

Influence of Vesicle Size on the Antitumour Activity of EPC/CHOL Liposomal Doxorubicin Against L1210 Leukaemia

Preparation	DOX Dose (mg/kg)	%ILS	L/F
EPC/CHOL (55:45)			
1 μm	20	65	0.67[a]
180 nm	20	115	0.88
100 nm	20	375	1.94[a]

a. The %ILS compared with the same dose of free doxorubicin is significantly different at the $P < 0.05$ level.

doxorubicin levels are reduced approximate 2-fold compared to the EPC/CHOL liposomes (Fig. 1). It should be noted that inclusion of the negatively charged phospholipid PG had negligible effects on either cardiac drug uptake or the LD_{50} of PC/CHOL preparations (4). These data are consistent with the fact that DSPC/CHOL membranes are much less permeable to doxorubicin than EPC/CHOL membranes.

Whereas the toxicity of liposomal doxorubicin appears dependent on factors that enhance drug retention inside the liposome, its antitumour activity is relatively insensitive to this parameter. Doxorubicin in free form or entrapped in liposomes varying only in lipid composition (e.g. EPC, EPC/CHOL, DSPC/CHOL) all display similar antitumour potency, even though their plasma drug AUCs vary by orders of magnitude (4). However, the size of the liposomal vehicle significantly affects the therapeutic potency of encapsulated doxorubicin. This is shown in Table 2 where the efficacy of liposomal doxorubicin against the ascitic L1210 leukaemia model is compared for EPC/CHOL vesicles of varying sizes. Decreasing the size of the

TABLE 3

Effect of Liposome Size on DSPC/CHOL (55:45) Liposomal Doxorubicn Plasma and Peritoneal Levels 24h After IV Administration[a]

Liposome Size	μg /ml Plasma		μg /Peritoneum	
	Lipid	DOX	Lipid	DOX
1 μm	5.5	1.2	1.9	0.3
100 nm	142.0	22.7	18.1	2.4

a. Values represent mean levels from three mice after injection of DSPC/CHOL liposomal doxorubicin (0.2, w/w drug to lipid ratio) at a drug dose of 20 mg/kg.

liposomes from approximately 1 μm to 100 nm increases the ILS from 65% to over 300% for the EPC/CHOL systems. This reflects a 3-fold increase in antitumour potency at 20 mg/kg as revealed by the L/F values (Table 2), where a value of 1.0 indicates equal antitumour potency to free doxorubicin administration.

The pharmacological basis for the increase in antitumour activity of liposomal doxorubicin as vesicle size is decreased can be seen in Table 3 where plasma and peritoneal levels of doxorubicin and lipid at 24h post i.v. injection are presented. The dramatic increase in plasma drug and lipid seen for the 100 nm systems at the 24h time point are consistent with the established relationship between vesicle size and liposome clearance from the circulation (9). Large liposomes are rapidly cleared from the blood by phagocytic cells of the RES. Consequently, greater than 90% of the 1 μm liposomes and entrapped doxorubicin are removed from the circulation within 1h of injection. In contrast, greater than 50% of the administered lipid dose remains in

the circulation 24h after administration for the 100 nm DSPC/CHOL liposomal doxorubicin preparation (Table 3). Correspondingly, delivery of liposomal lipid and doxorubicin into the peritoneum (site of the L1210 ascites tumour) increases approximately 10-fold. The decreased accumulation of large liposomes most likely results from a combination of a size dependent decreased ability to extravasate into the peritoneum and the significant reduction in the plasma AUC. Results from studies investigating the influence of GM1 and PEG-derivatized phospholipids on liposome circulation longevity, tumour uptake and antitumour efficacy extend from the observations here and demonstrate that increased circulation residence times of liposomal doxorubicin preparations can translate to enhanced tumour accumulation and therapy (5-8, 17-19).

When correlating pharmacokinetic and therapeutic activities of various liposomal systems, it is important to stress the influence that the encapsulated drug has on the blood clearance properties of the liposomes and their entrapped doxorubicin. This is shown in Figure 2 where the clearance properties of empty and doxorubicin-loaded liposomes administered i.v. at a dose sufficient to administer drug at 20 mg/kg are presented. Panel A demonstrates that empty PC/CHOL liposomes are cleared similarly regardless of the acyl chain composition of the phospholipid. The discrepancy between these data and similar reported studies (17) is most likely due to the fact that the experiments presented here utilize a nonexchangeable, non-metabolizable radiolabelled lipid marker, ^{3}H-cholesterylhexadecyl ether, whereas the previous studies employed a water-soluble radionuclide label that may be expected to leak from liposomes at different rates depending on their membrane permeability properties.

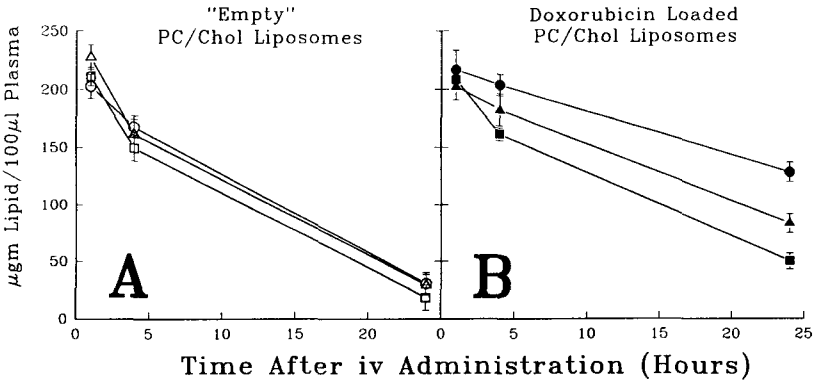

FIG 2: Plasma clearance profiles for empty (Panel A) and doxorubicin loaded (Panel B) liposomes. 100 nm PC/CHOL (55:45) liposomes were loaded with doxorubicin to achieve a final drug to lipid ratio of 0.2 (w/w). The phosphatidylcholine species were DSPC (●,○), DMPC (▵,▲) and EPC (□,■). Liposomes were administered into BDF1 mice at a lipid dose of 100 mg/kg. Liposomal lipid was measured using ^{3}H-cholesterylhexadecyl ether.

In Panel B, liposome clearance from the circulation is seen to decrease significantly for liposomes containing doxorubicin as the degree of acyl chain saturation of the phospholipid component is increased. It should be noted that there was no significant change in the size distribution of these liposomal doxorubicin preparations. These results point out that drugs entrapped in liposomes can themselves influence the pharmacokinetic properties of their delivery vehicle. The results also indicate that the degree to which the entrapped doxorubicin influences the clearance of the liposomes is dependent on the drug leakage properties of the vesicles.

Many recent studies on liposomal doxorubicin have focussed on the use of "stealth" lipids such as GM1 and PEG derivatized PE to enhance the circulation lifetime and tumour accumulation of the liposome carrier (5-8, 17-19). As shown in Figure 3A, the plasma lipid levels after i.v. administration of empty DSPC/CHOL and

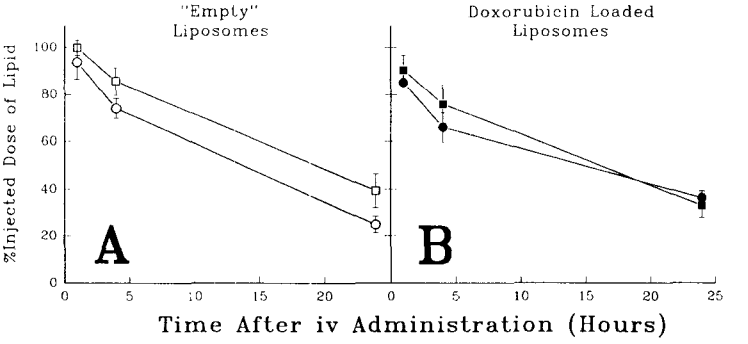

FIG 3: Plasma clearance of empty (Panel A) and doxorubicin loaded (Panel B) 100 nm DSPC/CHOL (○,●) and 100 nm GM1/DSPC/CHOL (□,■) liposomes. Liposomes with doxorubicin were prepared using the pH gradient loading procedure to achieve a final drug to lipid ratio of 0.2 (w/w). The liposomes were administered i.v. into BDF1 mice at a lipid dose of 50 mg/kg (10 mg/kg doxorubicin). Percent injected lipid dose in plasma was calculated using a plasma value of 0.046 ml/gm total body weight.

GM1/DSPC/CHOL liposomes (50 mg/kg lipid dose) are comparable over the first 4h and then diverge to yield approximately 2-fold more liposomal lipid in the circulation for the GM1-containing liposomes at 24h. These results are consistent with those reported previously in studies utilizing an entrapped radionuclide as the liposome marker (17, 18).

In contrast, the plasma clearance properties of doxorubicin-containing DSPC/CHOL and GM1/DSPC/CHOL liposomes administered at a lipid dose of 50 mg/kg (10 mg/kg doxorubicin) are identical over the 24h time course. In this context, it should be noted that studies in our laboratories comparing the therapeutic activity of doxorubicin entrapped in DSPC/CHOL and GM1/DSPC/CHOL liposomes suggest that the therapeutic benefits of utilizing GM1 are rather marginal at doxorubicin doses of 20 mg/kg or more (100 nm liposomes, drug to lipid wt. ratio = 0.2:1). The data presented in Figures 2 and 3 are not meant to imply that lipids such as GM1 may not

have useful therapeutic properties, but rather that special attention must be given to relevant dose selection and analytical techniques for free, entrapped drug and liposomal lipid if meaningful pharmacodynamic information is to be obtained. For example, many studies on the pharmacodynamics of liposomes containing "stealth" lipids have employed lipid doses below 50 mg/kg, yet the lipid dose often required to deliver therapeutically active quantities of cytotoxic drugs such as doxorubicin are often greater than 200 mg/kg.

Figure 4 presents the plasma doxorubicin concentrations and circulating drug to lipid ratios for various 100 nm liposomal doxorubicin formulations exhibiting an initial drug to lipid ratio of 0.2:1 (w/w) after i.v. administration to mice at a drug dose of 20 mg/kg. A wide range of plasma drug clearance kinetics is observed for the PC/CHOL and CL/PC/CHOL preparations (Panel A). For example, the CL-containing formulation is cleared very rapidly from the blood and less than 5% of the administered dose is present in the plasma within 1h of injection. In contrast, greater than 50% of the injected drug dose remains in the plasma at 24h when doxorubicin is encapsulated in DSPC/CHOL liposomes.

The plasma doxorubicin concentrations observed for EPC/CHOL, DMPC/CHOL and DPPC/CHOL are consistent with the relative permeability barriers presented by these membrane lipid compositions. This is demonstrated in Panel B where the circulating drug to lipid ratios for the PC/CHOL systems decreases with decreased membrane rigidity (i.e. DSPC/CHOL > DPPC/CHOL > DMPC/CHOL > EPC/CHOL). Consequently, the differences seen in plasma doxorubicin levels for PC/CHOL liposomes are related to both drug leakage from the vesicle (Fig. 4B) as well as drug-induced alterations in circulation lifetime of the liposomes (Fig 2B). The

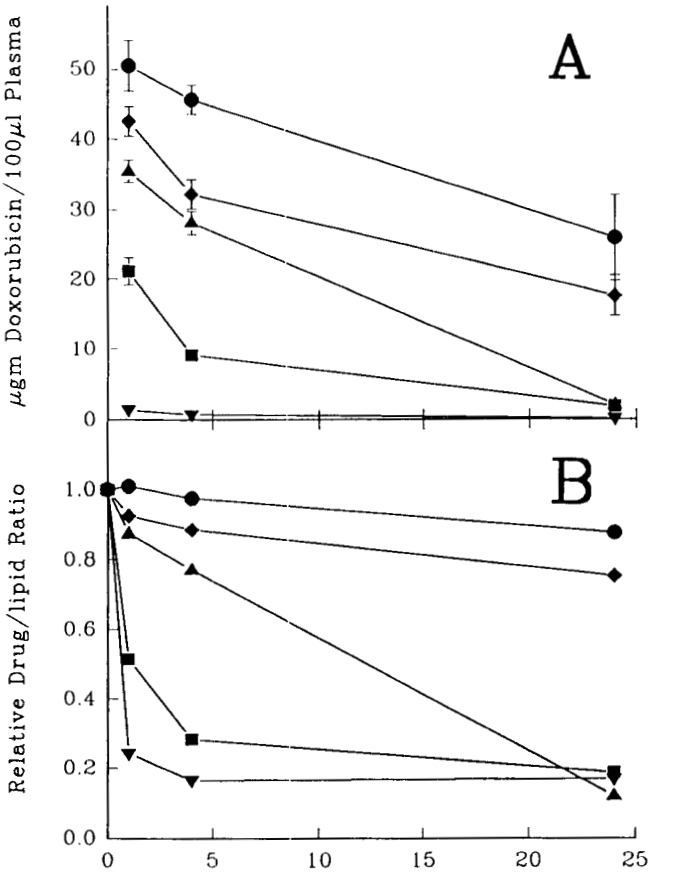

FIG 4: Influence of lipid composition on the circulation lifetime of liposomal doxorubicin formulations prepared using DSPC/CHOL (55:45, ●), DPPC/CHOL (55:45, ◆), DMPC/CHOL (55:45, ▲), EPC/CHOL (55:45, ■) and CL/DPPC/CHOL (10:45:45, ▼). All liposomal formulations were administered i.v. at a drug dose of 20 mg/kg using liposomes exhibiting a drug to lipid ratio of 0.2 (w/w). Doxorubicin equivalents in plasma (Panel A) were measured by fluorescence as described in Materials and Methods. The drug to lipid ratio (Panel B) of liposomal doxorubicin formulations in the circulation were determined by measuring plasma lipid and doxorubicin levels.

rapid clearance of doxorubicin entrapped in CL-containing liposomes appears related primarily to removal of the liposomes from the blood compartment as the change in drug to lipid ratio accounts for only a minor proportion of the cleared doxorubicin.

On the basis of the pharmacokinetic data presented in Figures 2-4 and the apparent correlations between drug leakage and toxicity as well as between circulation longevity/size and efficacy, predicted orders of increasing toxicity and antitumour potency of the cholesterol containing 100 nm liposomal systems would be as follows: Toxicity, DSPC/CHOL (least toxic) < DPPC/CHOL < DMPC/CHOL < EPC/CHOL < CL/DPPC/CHOL (most toxic); Efficacy, CL/DPPC/CHOL (least potent) < EPC/CHOL < DMPC/CHOL < DPPC/CHOL < DSPC/CHOL (most potent). However, the data shown in Table 4 indicate that such generalized relationships are not necessarily appropriate. Specifically, although a correlation exists between liposomes exhibiting dramatic differences in doxorubicin leakage properties (e.g. EPC, EPC/CHOL and DSPC/CHOL) and toxicity (see Figure 1) this is not observed for the DMPC/CHOL liposomal doxorubicin preparation where the LD_{50} is actually 2- to 4-fold lower than free drug even though its doxorubicin retention properties are superior to EPC/CHOL liposomes. It should be noted that the differences in tolerated doses in Table 4 and Figure 1 are related to the different animal strains utilized in the two studies where CD-1 mice are less sensitive to liposomal doxorubicin than the BDF1 mice.

Plasma clearance of doxorubicin encapsulated inside CL/DPPC/CHOL liposomes would suggest that its antitumour potency should be significantly lower than the PC/CHOL systems. This trend is observed at a drug dose of 10 mg/kg, however, at 20 mg/kg this preparation is significantly more potent than free drug. These results

TABLE 4

Influence of Lipid Composition on the Toxicity and L1210 Antitumour Activity of Doxorubicin in BDF1 Mice

Formulation	Toxicity[a] (mg/kg)	Median Life Span in Days (Dose)	%ILS[b]
Control	N.A.[c]	9.0 (N.A.)	N.A.
Free Drug	20 - 25	18.0 (10 mg/kg) 21.0 (20 mg/kg)	100 133
EPC/CHOL (55:45)	35 - 40	21.0 (10 mg/kg) 32.5 (40 mg/kg)	133 261
DMPC/CHOL (55:45)	5 - 10	13.0 (5 mg/kg)	44
DPPC/CHOL (55:45)	60 - 80	17.5 (10 mg/kg)	94
DSPC/CHOL (55:45)	60 - 80	17.0 (10 mg/kg) 52.0 (60 mg/kg	88 477
CL/DPPC/CHOL(10:45:45)	35 - 40	15.0 (10 mg/kg) 42.0 (20 mg/kg)	67 367

a. Acute toxicity dose range where 50% of the animals will die within 14 days after i.v. administration.
b. Percent increase in life span as calculated by dividing the median survival time for treated animals by the median survival time for control animals.
c. Not applicable.

strongly indicate the need to differentiate between free and liposome-associated drug pools after administration of liposomal doxorubicin in order to identify the critical pharmacokinetic parameters responsible for toxicity and efficacy behaviour.

Pharmacokinetics of Free and Bound Drug after Liposomal Doxorubicin Injection

A limited amount of information is available on the contributions made by liposome-bound and free doxorubicin released from the liposomes on overall drug

TABLE 5

Free Doxorubicin and Liposomal Doxorubicin Plasma Levels in Mice Injected with Liposomal Doxorubicin[a]

Time After IV Injection	Total DOX (μg/ml)	Liposomal DOX μg/ml	% of total	Free DOX μg/ml	% of total
2 min	171.6 $\pm$ 7.4	161.6 $\pm$ 8.1	94.1	10.0 $\pm$0.8	5.9
30 min	160.9 $\pm$ 7.0	157.1 $\pm$ 6.8	97.7	3.8 $\pm$0.1	2.3
4 hr	49.7 $\pm$ 5.9	48.8 $\pm$ 5.8	98.3	0.8 $\pm$ 0.1	1.7

a. Values represent the mean and SD doxorubicin plasma levels of three mice at the indicated times after injection of EPC/CHOL liposomal doxorubicin at a drug dose of 20 mg/kg

pharmacokinetics after injection of a liposomal doxorubicin formulation. Given the rapid clearance of doxorubicin in the absence of liposome encapsulation, it is clear that the majority of drug present in the blood upon administration of liposomal doxorubicin must be associated with the circulating liposomes. This has been demonstrated by Gabizon and coworkers (7,20) where the two drug pools in plasma and ascites fluid were separated by ion exchange chromatography. In a similar approach, we have utilized C-18 and carboxylic acid-based solid phase extraction columns to rapidly separate free and liposome-bound doxorubicin from plasma samples (21). As shown in Table 5, the level of free doxorubicin in mouse plasma after i.v. injection of doxorubicin (20 mg/kg) entrapped inside EPC/CHOL liposomes represents 5.9%, 2.3% and 1.7% of the total drug present at 2 min., 30 min. and 4h, respectively, post administration. At these levels, the free drug concentrations are

comparable to that observed after injection of unencapsulated doxorubicin at the 2min. and 30 min. time points and significantly increased at the 4h time point.

Although the analysis of free and liposome-bound doxorubicin levels presented above confirms the assumption that the majority of doxorubicin present in the circulation after administration of liposomal doxorubicin is contained inside the vesicle carrier, attempts to correlate free drug concentrations with toxicity or efficacy activity must be approached with caution. This is due to the technical limitations inherent in chromatographic separation of liposome-associated drug from free or protein-bound doxorubicin. For example, a 1% non-specific crossover of liposome-bound doxorubicin into the free doxorubicn fraction during the separation procedure for the 100 nm DSPC/CHOL formulation would result in erroneous free drug concentrations that are several fold greater than plasma doxorubicin levels observed over 24h after injection of conventional, nonencapsulated drug. The difficulty in accurately evaluating the free drug pool in plasma samples lies in the fact that total drug concentrations can vary by orders of magnitude between doxorubicin administered in nonencapsulated and liposomal forms (22). In practice, we typically observe non-specific crossover levels on the order of 0.1% to 0.5% of the total doxorubicin concentration when studying plasma samples containing liposomal doxorubicin. Nonetheless, these limits can significantly influence the analysis of doxorubicin bioavailability in the circulation for liposomal systems and compromises the ability to elucidate pharmacodynamic relationships between therapeutic behaviour and free or liposome-bound doxorubicin plasma concentration/time curves.

DISCUSSION

Phase I and Phase II clinical trials with two different formulations being developed by pharmaceutical companies are providing promising results on the

toxicological and therapeutic activity of liposome encapsulated doxorubicin (23-25). Data from these studies appear to support predictions based on preclinical investigations, in that non-hematological toxicities such as mucositis, GI toxicity and cardiotoxicity are significantly reduced for liposomal doxorubicin compared to similar doses of conventional free drug while tumour delivery is enhanced (23-25). Further, Phase II data on liposomal doxorubicin in breast cancer patients suggest that these toxicity buffering effects are complemented by significant antitumour activity (24). These early stage clinical investigations imply that liposome technology will play a significant role in the development of improved chemotherapeutic agents for cancer therapy.

Preclinical studies such as those described here have been instrumental in designing, evaluating and optimizing liposomal doxorubicin formulations prior to clinical testing. In this regard, the pH gradient-dependent doxorubicin encapsulation procedure described here has been used to identify vesicle physical properties that influence the pharmacokinetic, toxicity and efficacy properties of liposomal doxorubicin administered intravenously. This process has led to the selection of liposomal doxorubicin properties (diameters approaching 100 nm, cholesterol enriched lipid compositions and high drug to lipid ratios) that would appear desirable for clinical applications. Similar preclinical investigations by other laboratories (6,17,18) have resulted in the development of a small, "sterically stabilized" PEG-PE containing liposome formulation that is also being assessed clinically. These liposomal doxorubicin systems represent the first line of vesicle-based cancer chemotherapeutic agents being developed for commercial applications.

It is likely that further refinements of liposomal doxorubicin systems through surface modifications such as antibody/ligand attachment (8) as well as inclusion of

specific carbohydrate/polymer-derivatized or fusogenic lipids will significantly improve on the tumour specificity and therapeutic index of the current liposomal doxorubicin preparations. However, such directions are being pursued in the face of a limited understanding on the mechanism of liposomal doxorubicin action. It is generally accepted that for liposome entrapped anticancer drugs to exert their therapeutic effects on tumour cells, they must first be released from the vesicle carrier in order to have access to their intracellular target. The manner in which liposomes enhance drug delivery to the tumour target is still unclear, despite a great number of reports on correlations between liposome physical properties and biological activity. Potential mechanisms of action include 1) direct delivery of liposomal contents into tumour cells by fusion with the plasma membrane or tumour endocytosis of the carrier, 2) increased tumour exposure to free drug from a long circulating pool of liposome entrapped drug, 3) increased tumour exposure to free drug from liposomes that have accumulated in the tumour, and 4) macrophage-mediated processing of liposomes (phagocytosis and re-release of free drug) at the tumour site.

To date, most studies on encapsulated agents such as liposomal doxorubicin have been limited to phenomenological relationships primarily due to the fact that the experiments were unable to distinguish liposome encapsulated from unencapsulated drug. Consequently, the fact that tumours display increased drug levels does not *a priori* mean that all of the drug is bioavailable nor does it indicate to what extent the tumour cell-associated drug was derived from a pool of free drug released from liposomes in the circulation or from a liposome pool located in the tumour itself. This is particularly difficult to resolve on the basis of historical studies since tumour accumulating liposomes also tend to display extended circulation lifetimes (17-19).

In addition, it is becoming established that many different liposomal formulations are capable of accumulating in both ascitic and solid tumours. Drug delivery is maximized when the liposomes exhibit enhanced drug retention properties after i.v. administration. For these reasons, it could be argued that the liposome properties which enhance circulation longevity and tumour accumulation (small, non-leaky, "stealth" liposomes) may actually work against the delivery of drugs like doxorubicin in free form to targets within the tumour cells once the drug-containing liposomes extravasate into the tumour site. Regardless, it is almost certain that enhanced tumour accumulation of liposomal anticancer agents is an important feature dictating therapeutic activity. However, strategies for improving antitumour activity must be based on drug bioavailability and pharmacodynamic relationships.

In summary, improvements in the technical aspects of generating different liposomal doxorubicin formulations have created a situation where our ability to generate more sophisticated delivery systems may have surpassed our understanding of the mechanism(s) whereby liposomes enhance the therapeutic potential of encapsulated drugs. Combining the versatility of entrapment procedures such as the pH gradient technique with improved analytical approaches to accurately determine free and liposome-bound pharmacokinetic will expedite the development of ligand targeted or tumour-specific triggered release liposomal preparations that optimize the therapeutic potential of the encapsulated drugs. Finally, pharmacodynamic relationships such as those identified here may eventually lead to improved monitoring of clinical pharmacokinetics for liposomal drugs, a greater understanding of the correlations between preclinical models and human responses to liposomal drug delivery systems and ultimately significantly improved liposomal therapeutic agents.

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FOOTNOTES

[1]Abbreviations: MLV, multilamellar vesicle; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; DOX, doxorubicin; EPC, egg phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; CHOL, cholesterol; CL, cardiolipin; PEG, polyethylene glycol; PE, phosphatidylethanol; PG, phosphatidylglycerol.

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