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Optimizing Liposomes for Delivery of Chemotherapeutic Agents to Solid Tumors

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I. Introduction

There are many potential barriers to the effective delivery of a drug in its active form to solid tumors. Most small-molecule chemotherapeutic agents have a large volume of distribution on i.v. administration (Speth et al., 1988; Chabner and Longo, 1996). The result of this is often a narrow therapeutic index due to a high level of toxicity in healthy tissues. Through encapsulation of drugs in a macromolecular carrier, such as a liposome, the volume of distribution is significantly reduced and the concentration of drug in the tumor is increased (see *IIF. Comparison of Pharmacokinetic Parameters for Different Liposomal Formulations* and *III. Accumulation of Liposomal Drugs in Tumors*). This results in a decrease in the amount and types of nonspecific toxicities and an increase in the amount of drug that can be effectively delivered to the tumor (Papahadjopoulos and Gabizon, 1995; Gabizon and Martin, 1997; Martin, 1998). Under optimal conditions, the drug is carried within the liposomal aqueous space while in the circulation but leaks at a sufficient rate to become bioavailable on arrival at the tumor. The liposome protects the drug from metabolism and inactivation in the plasma, and due to size limitations in the transport of large molecules or carriers across healthy endothelium, the drug accumulates to a reduced extent in healthy tissues (Mayer et al., 1989; Working et al., 1994). However, discontinuities in the endothelium of the tumor vasculature have been shown to result in an increased extravasation of large carriers and, in combination with an impaired lymphatics, an increased accumulation of liposomal drug at the tumor (see *III. Accumulation of Liposomal Drugs in Tumors*; Huang et al., 1993; Yuan et al., 1994, 1995; Hobbs et al., 1998). All of these factors have contributed to the increased therapeutic index observed with liposomal formulations of some chemotherapeutic agents (Papahadjopoulos et al., 1991; Gabizon, 1994; Martin, 1998).

A diagram depicting both a conventional liposome (CL)[3] and a sterically stabilized liposome (SSL) is shown in Fig. 1. The two types of liposomes share a lipid membrane that is relatively impermeable to both amphipathic and highly water-soluble molecules at physiological temperatures (37°C). This feature is important for the maintenance of stable liposome drug formulations, both during storage and in plasma (see *VII. Stability in Plasma and Storage*). Liposomes composed of a comparably more-fluid membrane are being used as a rapid-

[3] CL, conventional liposome; SSL, sterically stabilized liposome; ara-C, 1-β-D-arabinofuranosylcytosine; AUC, area under the curve for concentration versus time; ABV, doxorubicin/bleomycin/vincristine; BV, bleomycin/vincristine; Chol, cholesterol; DOX, doxorubicin; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DPPE, 1,2-dipalmitoyl-3-*sn*-phosphatidylethanolamine; DPPG, 1,2-dipalmitoyl-3-*sn*-phosphatidylglycerol; DSPA, 1,2-dipalmitoyl-3-*sn*-phosphatidic acid; DSPC, 1,2-distearoyl-3-*sn*-phosphatidylcholine; DSPG, 1,2-distearoyl-3-*sn*-phosphatidylglycerol; eggPC, phosphatidylcholine derived from egg yolk; H-F, hand and foot; HSPC, hydrogenated soy phosphatidylcholine; ILS, increased life span; L-DOX, liposomal doxorubicin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEG, polyethylene glycol; PEG-DSPE, *N*-(polyethylene glycol)distearoylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; RES, reticuloendothelial system; SSL DOX, sterically stabilized liposomal doxorubicin; VCR, vincristine; CSF, colony-stimulating factor; POPC, 1-palmitoyl, 2-oleoyl-3-*sn*-phosphatidylcholine.

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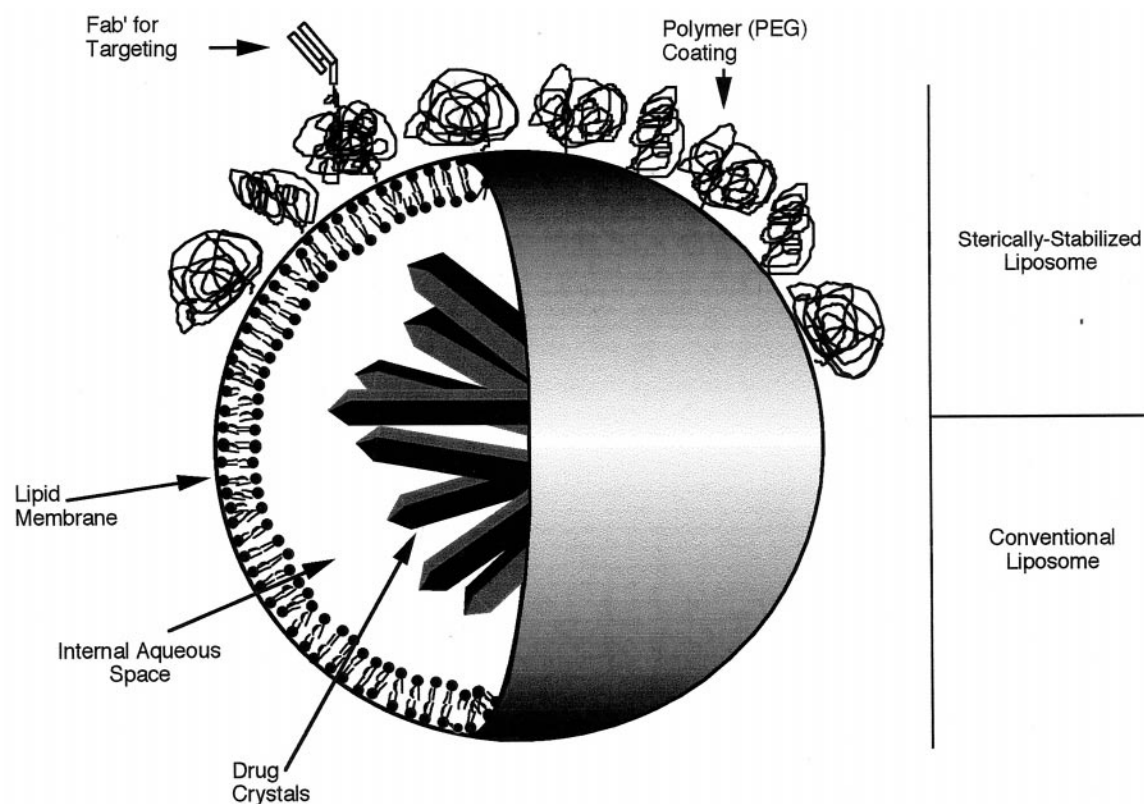

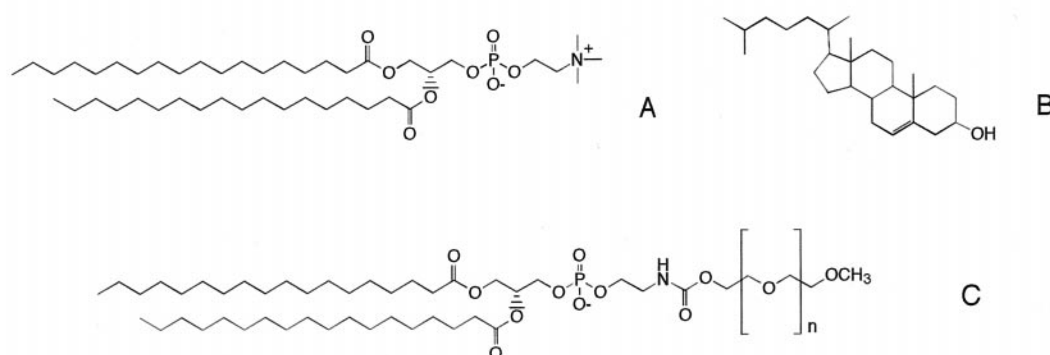

FIG. 1. Diagram of a drug-loaded liposome both with (SSL) and without (CL) a PEG coating. The liposome contains a lipid membrane that encapsulates an internal aqueous space used to entrap chemotherapeutic drugs. DOX can be encapsulated at concentrations exceeding its aqueous solubility, forming drug crystals in the liposome interior. Alternatively, some drugs can be carried within the lipid bilayer. Further modifications of the surface through covalent attachment of targeting ligands such as Fab′ fragments can result in liposomes that are specifically endocytosed by cancer cells expressing a receptor for that ligand (e.g., the HER2 receptor found on certain breast cancer tumors). The structures of the three most commonly used lipids that compose the lipid bilayer are also given. DSPC (A) or an equivalent, HSPC, is the primary phospholipid component, whereas Chol (B) is the neutral lipid component. PEG-DSPE (C) is incorporated at concentrations of 4 to 6 mol% in SSL formulations.

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release system for doxorubicin (DOX) and are described to a limited extent in this review. A liposome also has an internal aqueous space, which can be used to entrap a variety of chemotherapeutic drugs or diagnostic dyes. We discuss in *VII. Stability in Plasma and Storage* how different drugs are efficiently loaded into this space. The

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two types of liposomes differ in the presence of the polymer coating [most commonly, polyethylene glycol (PEG)] on the surface of the SSLs but not CLs. This coating provides steric stabilization to the liposome, which is thought to limit binding of serum opsonins as well as direct interactions with cells, most importantly, of the reticuloendothelial system (RES; Allen et al., 1991, 1994; Lasic et al., 1991). The result is enhanced circulation times and increased localization in the tumor (Papahadjopoulos et al., 1991, 1995; Gabizon and Martin, 1997).

Steric stabilization refers to the colloidal stability (Lasic and Needham, 1995; Lasic and Papahadjopoulos, 1996) conferred on the liposome by a variety of hydrophilic polymers or hydrophilic glycolipids (Allen and Chonn, 1987; Papahadjopoulos et al., 1991; Woodle and Lasic, 1992; Allen, 1994; Torchilin et al., 1995; Zalipsky et al., 1996), the best studied of which are PEG and the ganglioside GM_1. An important finding was that SSLs also show a prolonged lifetime in the circulation (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Klibanov et al., 1990; Allen et al., 1991; Papahadjopoulos et al., 1991). SSLs then typically refer to any liposomes containing PEG-PE, GM_1, or another of these glycolipids or polymers that has a relatively long half-life in the general circulation. The term "conventional liposomes" has a much broader definition and refers to liposomes composed of a variety of different lipid compositions, but typically the most commonly used of these compositions are very high in phosphatidylcholine (PC) and cholesterol (Chol). The pharmacokinetics and tissue distribution of CLs depend on properties such as size, surface charge, and membrane packing. These factors are discussed in more detail in *II. Pharmacokinetics and Biodistribution of Liposomes and Liposomal Drugs*. However, to perform a careful comparison, we limit this discussion to formulations optimized for increased residence in the circulation, accumulation in tumors, and stability in the plasma. For SSLs, we consider liposomes containing 4 to 6 mol% PEG-DSPE, ~30 mol% Chol, and the remainder hydrogenated soy phosphatidylcholine (HSPC) or distearoylphosphatidylcholine (DSPC; Fig. 1). The size of the carrier is usually 60 to 120 nm. For CLs, the optimized formulations are composed of DSPC and Chol in either a 55:45 or 66:33 M ratio or phosphatidylcholine derived from egg yolk (eggPC)/Chol (3:2) and have a similar average size distribution.

The choice of drug for delivery via liposomes is essential to the success of this approach. Broad generalizations as to the usefulness of a certain liposome composition for the delivery of all chemotherapeutic drugs or as to the superiority of liposomal formulation for all classes of drugs is extremely dangerous considering the present limitations in liposome technology. To be effective as a carrier, a liposome must be able to efficiently balance stability in the circulation with the ability to make the drug bioavailable at the tumor. In choosing a drug, there are several criteria to consider. The drug must have sufficient activity against the chosen tumor; a drug such as DOX with a relatively broad activity against a variety of different tumor models is an ideal choice in this regard (Young et al., 1981; Doroshaw, 1996). Second, the drug must be efficiently loaded into the liposomal carrier. Ammonium sulfate and pH gradients have been used for remote loading of a variety of amphipathic basic amines, resulting in encapsulation efficiencies of ~100% (Madden et al., 1990; Lasic et al., 1992a; Haran et al., 1993; Cullis et al., 1997). Finally, the drug must be compatible with the carrier; it must be stably transported in the circulation but still released at the tumor. A wide array of different drugs have been encapsulated in liposomes for the treatment of cancer (Fig. 2; Heath et al., 1983; Papahadjopoulos et al., 1991; Allen et al., 1992; Vaage et al., 1993b; Burke and Gao, 1994; Sharma et al., 1995; Jones et al., 1997; Working, 1998). The listed examples illustrate a diversity of different classes of chemotherapeutic drugs, with distinct chemical stabilities, solubility and membrane partitioning properties, modes of action, and modes of drug resistance.

Barenholz and coworkers (Barenholz and Cohen, 1995; Barenholz, 1998) classified these drugs into one of three classes depending on their hydrophobic properties measured as octanol-to-water partition coefficient (K_p): 1) highly hydrophilic drugs such as *N*-(phosphonoacetyl)-L-aspartate, 2) hydrophobic drugs such as paclitaxel, and 3) amphipathic drugs such as DOX, which represent many current chemotherapeutic agents. Liposomal formulations of highly hydrophilic drugs can be limited by the bioavailability of these drugs at the tumor site, which may be prohibitively low due to their extremely low membrane permeability and, therefore, low drug release once the carrier has reached the tumor. Drugs such as 1-β-D-arabinofuranosylcytosine (ara-C) or methotrexate, which are taken by tumor cells using membrane transporters (Plageman et al., 1978; Wiley et al., 1982; Westerhof et al., 1991, 1995; Antony, 1992), may be useful members of this class of drugs, assuming they can be released from the liposome in adequate quantities (Heath et al., 1983; Matthay et al., 1989; Allen et al., 1992). Future improvements in the design of carriers that are destabilized and release the drugs specifically at the tumor site may make their utilization more feasible, as discussed in *VIII. Bioavailability of Encapsulated Drug*. Highly hydrophobic drugs tend to associate mainly with the bilayer compartment of the liposome; this leads to lower entrapment stability due to faster redistribution of the drug to plasma components. However, liposomes may be used with this class of drugs simply as the means to formulate them for i.v. administration rather than using liposome encapsulation to achieve enhanced tumor delivery of the drugs. For example, paclitaxel has formulated into liposomes (Sharma et al., 1995, 1997) but may be equally suitable when formulated as a microemulsion (Wheeler et al., 1994). Liposomes have also used to solubilize and

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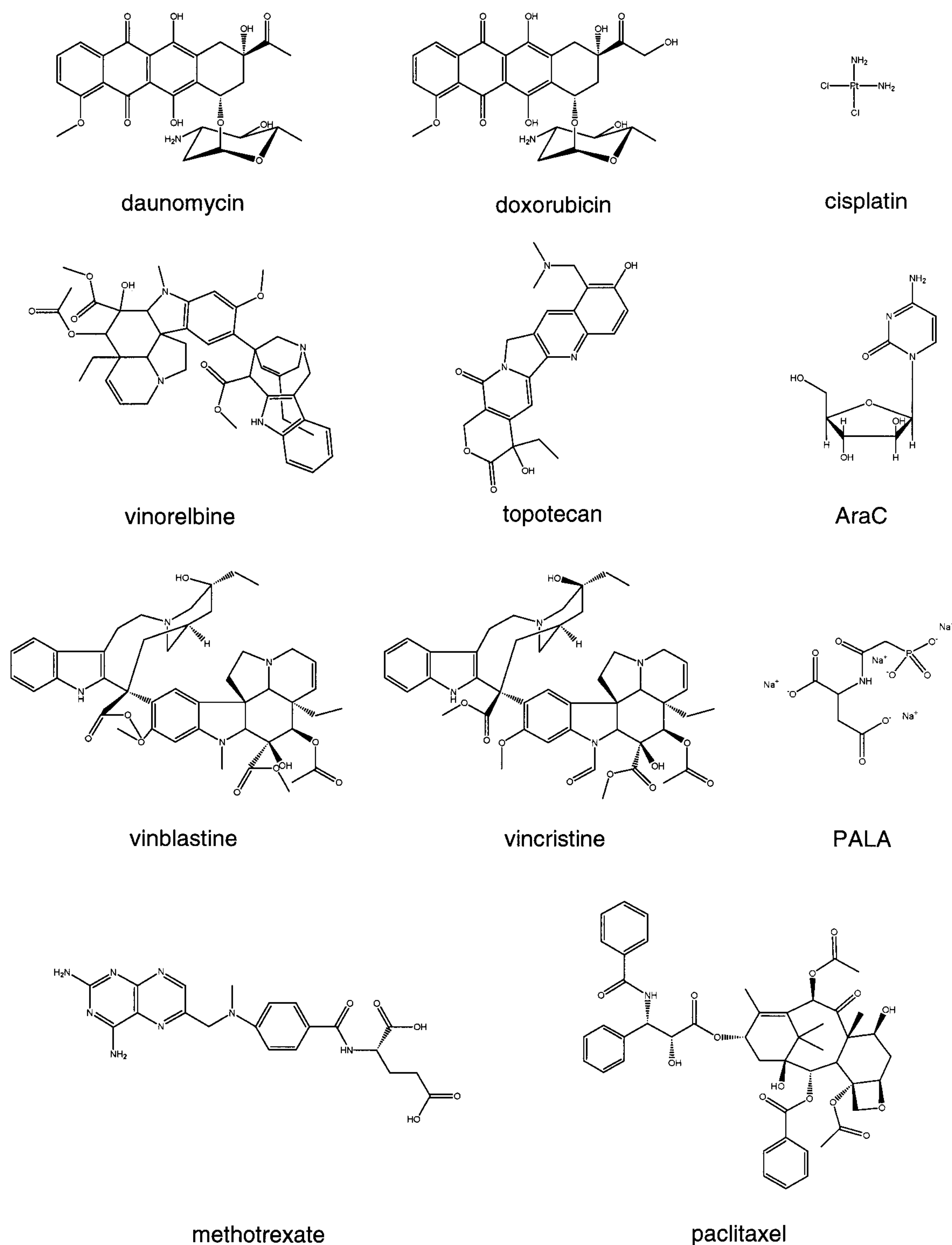

FIG. 2. Structures of a few chemotherapeutic drugs that have been used with liposomes either in vitro or in vivo. These drugs operate via a variety of different mechanisms; some have different mechanisms of drug resistance and varying physical characteristics that make them more or less compatible for encapsulation in liposomes. At the present time, only formulations of anthracyclines (daunorubicin and DOX) have been sufficiently developed for use in the clinic, although a liposomal VCR formulation is presently under study in clinical trials.

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administer hydrophobic photosensitizers for use in photodynamic therapy (Allison et al., 1990; Reddi, 1997).

Considering the present state of liposome technology, amphipathic drugs appear to be the most suitable for liposomal carriers; these drugs include anthracyclines, such as DOX and daunorubicin, and *Vinca* alkaloids, such as vincristine (VCR), vinblastine, and vinorelbine (Fig. 2). With this class of drugs, it is possible to tune the drug-release rates to maintain the stability of the formulation in the plasma, yet allow the drug to be released at the tumor site. This is in large part due to the development of gradient-based loading techniques leading to

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stable liposomal drug formulations (Nichols and Deamer, 1976; Mayer et al., 1985; Madden et al., 1990; Haran et al., 1993). Indeed, the first liposomal oncology drugs approved for medical use in liposomal form are of the anthracyclines daunorubicin (DaunoXome; Nexstar Pharmaceuticals, Boulder, CO) and DOX [Doxil; Alza Corporation, Palo Alto, CA (CAELYX in Europe)]. DaunoXome is formulated as a CL (DSPC/Chol), whereas Doxil is an SSL formulation (hydrogenated soy PC/Chol/PEG-DSPE; Table 1). Another CL formulation (eggPC/Chol) of DOX (Harris et al., 1998), as well as formulations of other amphipathic drugs, such as VCR (Embree et al., 1998) or cisplatin (Newman et al., 1999), is in preclinical or clinical trials or under Food and Drug Administration consideration for commercial release.

In the remaining sections of this review, we attempt to show how optimization of the balance of circulation lifetimes, drug-induced toxicities, accumulation in tumors, and drug release rates from liposomes results in the most clinically effective formulations. This is accomplished through adjustments of both the pharmacological and physical properties of the liposome, including the injected dose, liposome size, presence of steric stabilization, and lipid composition of the carrier.

II. Pharmacokinetics and Biodistribution of Liposomes and Liposomal Drug

For free DOX, the volume of distribution has been estimated at 25 l/kg, suggesting a significant uptake by tissues (Speth et al., 1988). This large volume of distribution, when combined with the relatively rapid clearance rate from the circulation, results in low drug levels in the tumor and significant toxicity to normal tissues. Liposomes can alter both the tissue distribution and the rate of clearance of a drug by causing the drug to take on the pharmacokinetic parameters of the carrier. Pharmacokinetic parameters of the liposomes depend on physicochemical attributes of the liposomes, such as size, surface charge, membrane lipid packing, and steric stabilization, as well as on the administered dose and route of administration. The pharmacokinetics of both CLs and SSLs have been extensively reviewed (Hwang, 1987; Allen et al., 1995; Allen and Stuart, 1999).

Both slow-release CLs and SSLs have a volume of distribution for DOX not significantly different from the total blood volume (see Table 3), indicating the drug is generally confined to the systemic circulation. However, after i.v. administration, CLs have saturable, nonlinear kinetics, whereas SSLs have nonsaturable, log-linear kinetics (Hwang, 1987; Allen et al., 1995). The dose-dependent kinetics for CLs result in relatively rapid clearance rates for liposomes at low doses and complicates the calculations of clinical dosages. Clearance of CLs has been suggested by Allen et al. (1995a) to be due to both a high-affinity, low-capacity system, likely the macrophages of the RES, and a low-affinity, high-capacity system. Steric stabilization slows uptake by the high-affinity, low-capacity system, resulting in dose-independent kinetics. The potential mechanisms responsible for the reduced clearance and dose-independent pharmacokinetics of SSLs are described in more detail in *IIE. Effect of Steric Stabilization on Pharmacokinetic Parameters*.

Liposomes are cleared from the circulation by macrophages of the RES, in particular those of the liver and spleen (Gregoriadis, 1976; Weinstein, 1984; Senior, 1987). Opsonization by serum proteins such as the complement C3b fragment, β_2-glycoprotein I, and the Fc portion of IgG molecules is thought to play a critical role in the recognition and subsequent clearance by RES macrophages (Senior, 1987; Patel, 1992; Devine et al., 1994; Chonn et al., 1995; Devine and Marjan, 1997). The success of a liposome-based approach for drug delivery to sites other than those making up the RES is one that limits the uptake of liposomes by macrophages, either directly by preventing the interaction of liposomes with receptors on the macrophage surface or indirectly by decreasing the binding of serum opsonins. Many studies have concentrated on understanding the mechanisms responsible for regulation of these interactions. These factors are often intricately intertwined, making it impossible to construct sweeping assumptions based on any one factor.

A. Effect of Liposome Size on Pharmacokinetic Parameters

The first aspect of a liposome that affects its disposition is size. Liposomes of a defined size are readily prepared by extrusion of lipid suspensions through filters containing pores of a similar size (Olson et al., 1979; Szoka et al., 1980). Liposomes prepared through this method are slightly larger (20–50%) than the average pore size of the filter. The general trend for liposomes of similar composition is that increasing size translates into more rapid up-

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TABLE 1
Commercial liposome formulations of anthracyclines

Drug	Manufacturer	Active Ingredient	Size	Lipid Composition	Drug/Lipid
			nm		*w/w*
Doxil (CAELYX)[a]	Alza Corporation[b]	DOX	100	HSPC/Chol/PEG-DSPE (56:39:5)	0.125:1
DaunoXome	Nexstar Pharmaceuticals	Daunorubicin	45	DSPC/Chol (2:1)	0.079:1
EVACET (TLC D-99)[c]	The Liposome Company, Inc.	DOX	150	eggPC/chol (55:45)	0.250:1

[a] Doxil (DOX) is known as CAELYX in Europe.
[b] Originally developed by Sequus Pharmaceuticals, Inc.
[c] EVACET was previously known as TLC D-99.

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take by the RES (Abra and Hunt, 1981; Hwang, 1987; Senior, 1987). However, although the trend remains the same, the clearance of liposomes is affected to differing extents depending on the composition. For example, DSPC/Chol (3:2) liposomes extruded through 400-nm filters are cleared 7.5 times as fast as liposomes extruded through 200-nm filters, which in turn are cleared 5 times as fast as small unilamellar vesicles (Senior et al., 1985). The inclusion of PEG-DSPE in the liposome composition results in clearance rates that are relatively insensitive to size in the range of 80 to 250 nm (Allen et al., 1989; Liu et al., 1992; Woodle et al., 1992). Now, a 2-fold increase in size from 100 to 200 nm results in only a 54% increase in clearance (Fig. 3; Woodle et al., 1992). A similar dependence of liposome clearance on size was observed for DSPC liposomes stabilized with small quantities of *N*-glutaryl-phosphatidylethanolamines (Ahl et al., 1997). These liposomes also showed an increased plasma area under the curve (AUC) compared with DSPC/Chol controls, similar to PEG-DSPE-stabilized liposomes. The authors suggested that the aggregation of nonstabilized neutral liposomes may result in an increase in the effective size and, thus, clearance from the circulation via a size-dependent mechanism. Although the dependence of liposome clearance rates on size is relatively less for these two stabilized formulations than for that with CLs, it nevertheless highlights the importance of optimization of liposome size in drug delivery systems not aimed at the RES. For neutral CLs, the window for optimal behavior is considerably narrower, and these data suggest that liposomes should be small enough (preferably <100 nm) but still maintain reasonable drug encapsulation efficiencies.

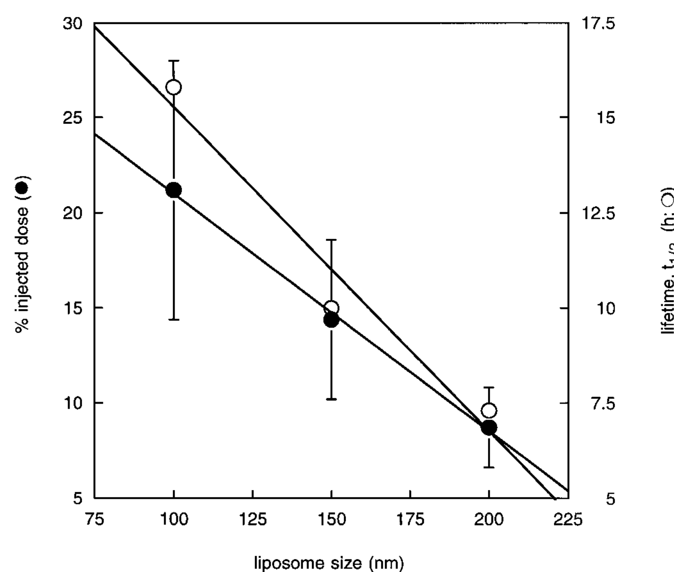

FIG. 3. Effect of liposome size on plasma levels of PEG-DSPE/PC/Chol (0.15:1.85:1 mol/mol/mol). Female adult Sprague-Dawley rats were injected with ^{67}Ga-desferoxamine-loaded liposomes, and blood was drawn at prescribed time points, when ^{67}Ga levels were determined with a gamma counter. From these data, the half-life in the circulation was determined by fitting the data to a single exponential curve (○), and the 24-h time point was recorded (●). The liposomes were prepared by extrusion through polycarbonate filters of defined size as described by Olson et al. (1979), and their size distribution was determined by dynamic light scattering. PC in these liposomes refers to partially hydrogenated egg PC. This figure was adapted from Woodle et al. (1992).

B. Effect of Lipid Dose on Pharmacokinetic Parameters

The administered dose can also play a significant role in the circulation lifetime of a carrier. CLs are removed from the circulation in a dose-dependent manner, indicating a saturation of the mechanisms responsible for their uptake (Gregoriadis and Senior, 1980; Abra and Hunt, 1981; Senior et al., 1985; Hwang, 1987). Circulation lifetimes typically increase as a function of increasing lipid dose. This effect is likely due to a decreased phagocytic capacity of RES macrophages after the ingestion of high lipid doses or to a saturation of plasma factors that bind to circulating liposomes and result in their opsonization. The fact that liposomes composed of high-phase transition lipids, such as SM/Chol or DSPC/Chol, can more readily saturate RES uptake may indicate that these difficult-to-metabolize lipids saturate metabolic pathways responsible for their destruction (Senior et al., 1985; Hwang, 1987). Alternatively, liposomes have been shown to bind serum proteins in a manner inversely proportional to their blood clearance rates (Chonn et al., 1992; Semple and Chonn, 1996; Semple et al., 1996), giving rise to the hypothesis that the depletion of plasma opsonins at high lipid doses results in an increase in blood circulation half-lives ($T_{1/2}$; Harashima et al., 1993; Oja et al., 1996).

RES blockade can also be achieved by delivering cytotoxic drugs such as DOX or dichloromethylene diphosphonate to RES macrophages (Bally et al., 1990b; Parr et al., 1993; Qian et al., 1994; Buiting et al., 1996). Parr et al. (1997) recently considered the effect of dose on DOX-loaded liposomes. In these experiments, the presence of DOX resulted in a ~1.5- to 2-fold increase in the plasma levels of liposomal lipid at higher doses for SSL DOX compared to CL DOX. In DOX-loaded liposomes, a 10-fold increase in plasma levels of liposomal lipid observed at lower lipid doses (<1 μmol lipid/20–22 g mouse) was reduced to a 3-fold increase at higher doses (>2 μmol lipid/mouse). It should be noted it is at these lower doses that SSL preparations are routinely used. Thus, with SSL DOX, long circulation does not necessarily come at the expense of RES toxicity. The possible implications of the use of dose escalation, and the resulting RES toxicity, simply to achieve long circulation times are described in more detail in *VI. Toxicology of Liposomal Chemotherapy*. This indicates that RES blockade is in part due to the drug and not solely to a saturation of plasma opsonins or inability to metabolize liposomal lipid components.

Steric stabilization with PEG-DSPE offers a unique advantage to liposome delivery in that clearance kinetics become dose independent (Allen and Hansen, 1991; Huang et al., 1992; Woodle et al., 1992). The data in Fig. 4 illustrate the relative effect of liposome dose on

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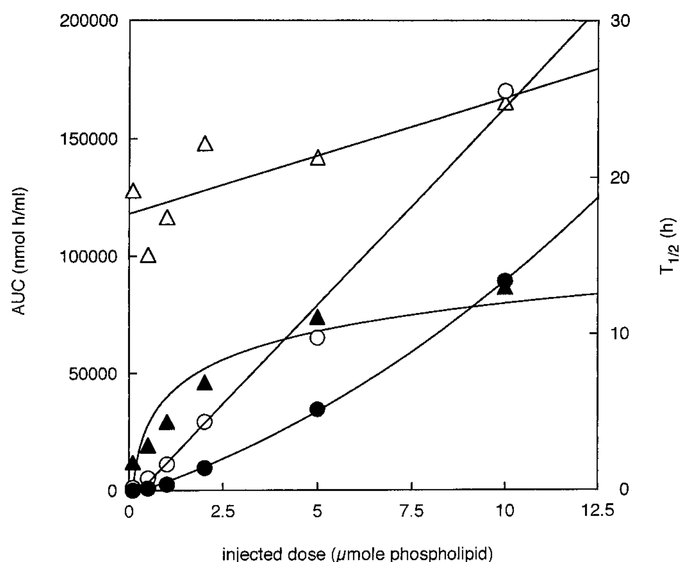

FIG. 4. Dependence of circulation $T_{1/2}$ values (▲, △) and plasma AUCs (●, ○) on administered dose. Female ICR mice (three per group) were given a single bolus tail-vein injection of liposomes containing ^{125}I-tyraminylinulin and 0.1 to 10 µmol of phospholipid. Liposomes were composed of either eggPC/Chol (2:1 mol/mol) or SM/eggPC/Chol/PEG-DSPE (1:1:1:0.2 mol/mol/mol). This figure was adapted from Allen and Hansen (1991).

clearance of both an SSL formulation (SM/eggPC/Chol/PEG-DSPE, 1:1:1:0.2) and a CL formulation (eggPC/Chol, 2:1). For the SSLs, the plasma AUC increases linearly, whereas the $T_{1/2}$ remains relatively unchanged. This dose independence was recently shown to extend down to concentrations of lipid as low as 1 µmol/kg in rabbits (Utkhede and Tilcock, 1998). In stark contrast, the plasma AUC for CLs increases slowly at low doses (<2.5 µmol phospholipid/23–27 g mouse) and then increases exponentially with increasing lipid dose. A look at the circulation $T_{1/2}$ of the conventional formulation shows a leveling off of the $T_{1/2}$, indicating a saturation of the mechanism responsible for their clearance. Although the CLs used in this particular study used a fluid-phase phospholipid component, eggPC, similar pharmacokinetics have been seen with DSPC/Chol and SM/Chol liposomes (Beaumier et al., 1983; Hwang, 1987; Chow et al., 1989). In one of these studies (Beaumier et al., 1983), liposome levels in the liver were shown to saturate at the same dose where plasma clearance rates leveled off, consistent with RES saturation being responsible for increased plasma levels at high lipid doses.

C. Effect of Liposome Charge on Pharmacokinetic Parameters

The effect of liposome surface charge on liposome clearance kinetics is an increasingly misused predictive factor of circulation lifetimes. Early studies have shown that the presence of negatively charged lipids in liposomes, including phosphatidic acid (PA), phosphatidylserine (PS), and phosphatidylglycerol (PG), results in rapid uptake by the RES (Senior et al., 1985; Senior, 1987). However, this relationship between the presence of charged lipids and circulation lifetimes is extremely complex and cannot be readily explained with simple models in which the presence of an anionic lipid necessitates increased clearance from the circulation. Indeed, it now appears that each lipid must be analyzed separately and in the context of similar liposomes with respect to size, membrane packing constraints, and surface charge density.

A more careful characterization of the effect of surface charge on liposome clearance in mice was conducted using liposomes containing different anionic phospholipids (Gabizon and Papahadjopoulos, 1992). In these experiments, anionic lipids were added to fluid eggPC/Chol liposomes in a 1:10:5 ratio (anionic lipid/eggPC/Chol). Although liposomes containing PG, PA, and PS (PS > PA > PG) were cleared more rapidly than neutral liposomes, the inclusion of other anionic lipids such as the ganglioside GM_1 or phosphatidylinositol (PI) resulted in longer circulation lifetimes. Later, this second group of anionic lipids was shown to include PEG-PE conjugates (Papahadjopoulos et al., 1991; Woodle et al., 1992). The new model then divided negatively charged lipids into those with and those without a sterically shielded negative charge. Those with a sterically hindered charge were cleared more slowly, whereas those without were cleared more rapidly than neutral liposomes of a similar composition. This too may have proved to be too simple of a model.

The last statement concerning a similar composition is extremely important, and the effect of the phase transition of the lipid is intricately interrelated with the effect of charge. 1,2-dipalmitoyl-3-*sn*-phosphatidylglycerol (DPPG)/DSPC/Chol liposomes were previously shown to be cleared more rapidly than DSPC/Chol liposomes in mice (Fig. 5; Lasic et al., 1991; Woodle et al., 1992), and in a separate study, eggPG/DSPC/Chol liposomes were cleared more rapidly than DPPG/DSPC/Chol liposomes (Gabizon et al., 1990). However, DSPC has a gel-to-liquid crystalline phase transition (T_m) of 55°C, whereas the T_m value of DPPG is 41.1°C (Table 2; Boggs et al., 1989). Thus, the replacement of some of the DSPC with DPPG does not necessarily result in liposomes with similar permeability and membrane packing characteristics. Recently, DOX-loaded 1,2-distearoyl-3-*sn*-phosphatidylglycerol (DSPG)/HSPC/Chol liposomes, in which the source of PG was distearoylphosphatidylglycerol (T_m = 53.0; Table 2), were shown to have plasma levels of DOX at 24 h that were greater than twice those of HSPC/Chol liposomes (Gabizon et al., 1996). In addition, the requirement for a high phase transition anionic lipid component may also be necessary for PI, where hydrogenated soy PI is most commonly used as the source of PI in long-circulating liposomes (Gabizon and Papahadjopoulos, 1988; Gabizon et al., 1990). Thus, from these few cases, it appears that that the dependence of long circulation is more related to

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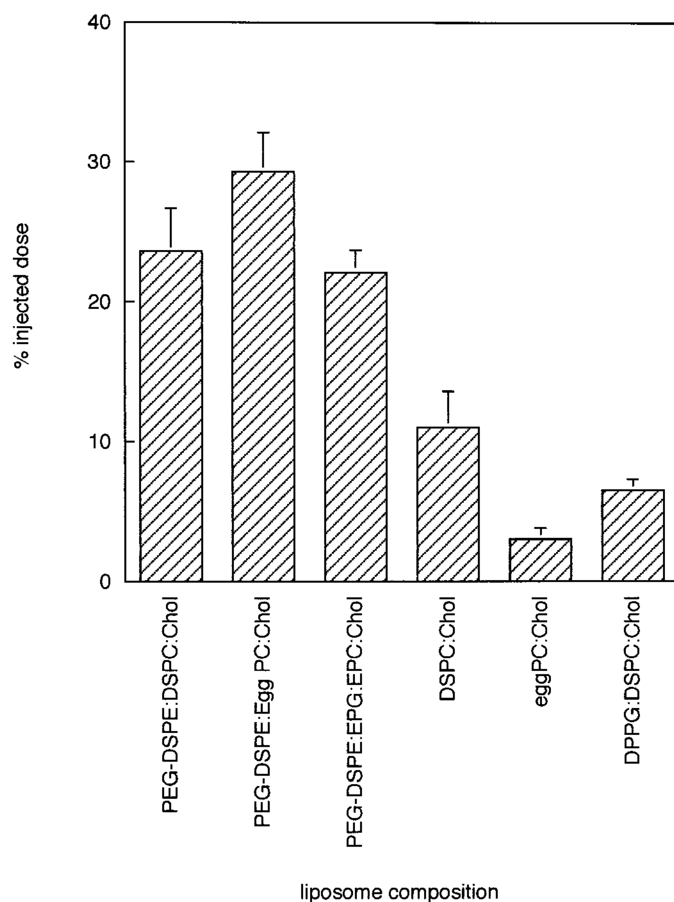

FIG. 5. Effect of steric stabilization and lipid composition on plasma levels of liposomes. Extruded liposomes (70–100 nm) loaded with ^{67}Ga-deferoxamine were injected by i.v. administration into female Swiss–Webster mice at a dose of 1 μmol of phospholipid per mouse. Blood levels of ^{67}Ga were determined by gamma counting 24 h after injection. Lipid molar ratios were 1:10:5 except for eggPC/Chol and DSPC/Chol, both at 10:5, and PEG-DSPE/EPG/eggPC/Chol at 1:3:7:5. This figure was adapted from Woodle et al. (1992) and Lasic et al. (1991).

membrane packing and permeability considerations, and that the inclusion of high-phase transition anionic lipids into solid liposomes can actually increase circulation lifetimes.

However, as was stated previously, all cases must be considered individually. In one study with another anionic phospholipid, PA, liposomes composed of DSPC/1,2-distearoyl-3-*sn*-phosphatidic acid (DSPA)/Chol (3:1:4) were cleared ~10 times faster than DSPC/Chol liposomes (1:1; Senior, 1987). DSPA has a T_m value comparable to those of DSPG and DSPC at 58°C, and so for DSPA at least, liposome charge appears to become more important than membrane packing in the determination of rates of uptake. Of course, DSPA was introduced at 25% of the total phospholipid content and has two negative charges per molecule. In the previous example, DSPG was incorporated at only 10% of the total phospholipid and has only one negative charge, consequently adding an additional layer of complexity involving surface charge density. Janoff and coworkers have indeed shown a steep dependence of blood clearance

TABLE 2
Primary gel-to-liquid crystalline phase transitions of different phospholipids

Phosphatidylcholine	Acyl Chain Length, No. of unsaturations	T_m	Reference
		°C	
DSPC	18:0, 18:0	55	Goodwin et al. (1982)
HSPC	16–18 (mixture)[a]	52	Horowitz et al. (1992)
DPPC	16:0, 16:0	42	Papahadjopoulos et al. (1973b)
POPC	16:0, 18:1	−7	Scherer and Seelig (1989)
SLPC	18:0, 18:2	−16.7	Sanchez-Migallon and Aranda (1996)
DOPC	18:1, 18:1	−21	Barton and Gunstone (1975)
eggPC	Mixture[b]	−2.5	Bach et al. (1982)
DSPG	18:0, 18:0	53.0	Surewicz and Epand (1986)
DPPG	16:0, 16:0	41.1	Boggs et al. (1989)
eggPG	Mixture	37	Vincent et al. (1991)
DSPA	18:0, 18:0	58	Krill et al. (1992)

An excellent database containing easily searchable physical properties of numerous lipids can be found at http://www.lipidat.chemistry.ohio-state.edu.

DOPC, dioloeylphosphatidylcholine; eggPG, egg phosphatidylglycerol; POPC, 1-palmitoyl, 2-oleoyl phosphatidylcholine; SLPC, 1-stearoyl, 2-linoleoyl phosphatidylcholine.

[a] Approximately 18% of the acyl chains are 16 carbons, and 82% are 18 carbons. All unsaturations have been reduced with a hydrogenation reaction.

[b] Contains ~34% of 16:0, ~1% of 16:1, ~10.5% of 18:0, ~31% of 18:1, ~17.7% of 18:2, ~3% of 20:4, and ~1.7% of other.

rates on the mol% of the negatively charged component *N*-glutaryl-dipalmitoylphosphatidylethanolamine (*N*-glutaryl-DPPE) in DSPC liposomes (Ahl et al., 1997). The normalized AUC in plasma was greatest at 10 mol% *N*-glutaryl-DPPE and rapidly declined both below and above this value. The authors suggested the small amounts of negatively charged lipids stabilize neutral liposomes against an aggregation-dependent uptake mechanism. All of these examples point to the reality that different liposomes, and even comparable liposomes with different phospholipid headgroups of similar charge, may have very different mechanisms responsible for their uptake (Daemen et al., 1997).

An even more intriguing question is raised based on these analyses. Have CLs really been optimized? Are small DSPC/Chol liposomes really the most efficient liposomal carriers in the absence of steric stabilization? At least three studies have suggested that the inclusion of small amounts (10 mol%) of certain negatively charged lipids such as DSPG or *N*-acylated phosphatidylethanolamines (Park et al., 1992; Gabizon et al., 1996; Ahl et al., 1997) actually increase circulation $T_{1/2}$ even further. Additional studies will be needed to elucidate the exact nature of this stabilizing effect and determine more carefully the dependence of this stabilization on the structure of the stabilizing lipid and such parameters as membrane packing. Whether these liposomes would offer any improvements over SSLs remains to be seen, but at least one study has suggested that some DOX-loaded anionic liposome formulations demonstrate an efficacy similar to that of SSL DOX (Gabizon et al., 1996).

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D. Effect of Membrane Packing Constraints on Pharmacokinetic Parameters

The effect of bilayer fluidity and the relative nature of the lipid components can have a considerable impact on the clearance from the circulation of both the liposome and the associated drug. These effects can either be direct effects, such as inhibition of penetration and thus binding of serum proteins (Papahadjopoulos et al., 1973b), or indirect effects, such as stabilization of the drug formulation to reduce the rate of drug leakage (*VII. Stability in Plasma and Storage*). The presence of Chol probably has one of the most important roles in the maintenance of membrane bilayer stability and long circulation times in vivo (Gregoriadis and Davis, 1979; Senior and Gregoriadis, 1982; Senior, 1987). In the absence of Chol, CLs are destabilized by HDL particles (Chobanian et al., 1979; Damen et al., 1980) and upon release, their components can be readily eliminated from the circulation. For liposomes with and without Chol, clearance rates were shown to negatively correlate with increased stability in plasma (Senior and Gregoriadis, 1982). The presence of steric stabilization makes the need for Chol less apparent for empty liposomes, but for drug-loaded liposomes, Chol is necessary for maintenance of the drug in the liposomal interior. The phospholipid component also plays a prominent role in the maintenance of high plasma levels of liposomes. DSPC/Chol and SM/Chol liposomes have higher $T_{1/2}$ values in the circulation compared with more fluid liposomes containing eggPC or even 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC; Gregoriadis and Senior, 1980; Senior, 1987). This is presumably due to the decreased affinity of these liposomes for serum opsonins required for their uptake. To be most effective, the PC component must have a phase transition that is significantly above 37°C. An inspection of the gel-to-liquid-crystalline phase transitions (T_m) of a variety of different PC molecules (Table 2) shows the T_m value for eggPC is below 37°C, whereas DPPC has a T_m value of only a few degrees above (with a pretransition at 37°C). However, both DSPC and HSPC have a T_m value that is ~15–17°C higher than 37°C. Thus, at 37°C, HSPC- and DSPC-containing liposomes have a considerably more rigid membrane bilayer that resists penetration of serum opsonins than do eggPC- or DPPC-containing formulations. It is no surprise, then, that these liposomes tend to be the most stable in the circulation and display the longest circulation lifetimes.

Sphingomyelin (SM) has an added effect on circulation lifetimes. SM/Chol and SM/DSPC/Chol liposomes were both shown to have longer circulation lifetimes than DSPC/Chol (Hwang, 1987; Allen et al., 1991), indicating an additional stabilizing effect of SM. SM can form intermolecular hydrogen bonds with neighboring Chol molecules (Schmidt et al., 1977; Sankaram and Thompson, 1990), resulting in greater stability and a decreased ability of plasma proteins to insert into liposomal membranes.

The rate of elimination of a liposomal drug from the circulation is also dependent on the rate of drug leakage from the carrier. Because drugs considered for liposome encapsulation often have circulation times significantly shorter than the liposomal carrier, premature release can lead to an apparent increase in the rate of elimination of the liposomal drug from the circulation. In DOX-loaded SSLs (SSL DOX) with HSPC, DPPC, or eggPC as the phospholipid component of the formulation, a correlation was observed among the phase transition (T_m) of the phospholipid component, the stability of the formulation in in vitro plasma stability tests, and plasma levels of the drug in vivo (Fig. 6 and Table 2; Gabizon et al., 1993). Liposomes containing high-phase transition lipids formed more stable formulations, which were better able to retain their drug and showed apparent increases in drug circulation lifetimes. A similar result was seen by Mayer and coworkers using CLs and DOX (Bally et al., 1990b). A more detailed explanation of the different factors responsible for maintaining a stable formulation of different drugs in the plasma is given in *VII. Stability in Plasma and Storage*.

The conclusions that can be drawn from these data differ for CLs and SSLs. For CLs, a membrane composed

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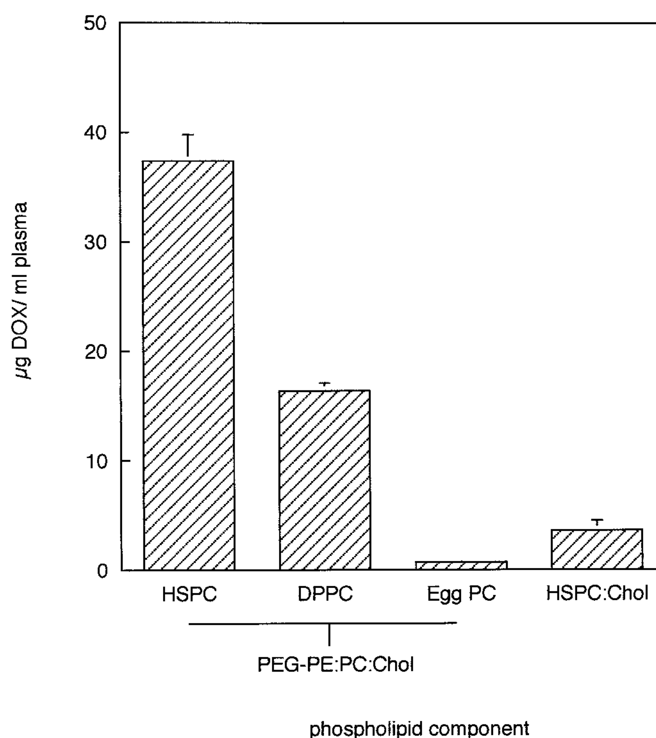

FIG. 6. Dependence of plasma drug levels on liposome composition. SSL DOX lipid formulations containing different species of phosphatidylcholine were injected into Sabra female mice (four per group) at a dose of 10 mg/kg DOX. The molar ratio of PEG-DSPE/PC/Chol was kept constant at 0.75:9.25:8 for each formulation, whereas the species of the PC component was varied. The different PCs used were HSPC, DPPC, and eggPC. HSPC/Chol (10:8) liposomes were used as a CL control. DOX measurements were taken 24 h after drug administration. This figure was adapted from Gabizon et al. (1993).

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of Chol and high-phase transition phospholipids appears to be imperative for maintaining long circulation times and subsequent delivery of high levels of liposomes to solid tumors (see *III. Accumulation of Liposomal Drugs in Tumors*). SSLs are more pliable and can be used with fluid-phase lipids to obtain long circulation times and high tumor levels of liposomes. For both types of liposomes, the lipid composition of the liposome membrane is essential in maintaining a stable encapsulation of the drug while in the circulation. For most amphipathic drugs that are either weak acids or weak bases (the majority of classic chemotherapeutic agents), this is of considerable importance because these drugs will more rapidly leak from the carrier while in the circulation, unless high-phase transition lipids are used.

E. Effect of Steric Stabilization on Pharmacokinetic Parameters

Original attempts to mimic the surface of red blood cells by including the sterically hindered GM_1 or PI in liposome preparations led to the development of long-circulating liposomes (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Gabizon et al., 1990). Later, PEG-DSPE was substituted for GM_1 or PI (Klibanov et al., 1990; Allen et al., 1991; Papahadjopoulos et al., 1991). A common misconception is that the attachment of PEG to the surface of a liposome prevents liposome uptake by the RES; rather, it simply reduces the rate of uptake. One of the most significant advantages of SSLs is the nonsaturable, log-linear pharmacokinetics, as described perviously. SSLs likely resist uptake by the high-affinity, low-capacity RES macrophages, resulting in increased circulation lifetimes (Allen et al., 1995a). Like CLs, the primary site of accumulation for SSLs is also the spleen and liver (Huang et al., 1992). However, the rate of accumulation in these tissues is considerably slower than that for CLs. Plasma levels of PEG-DSPE containing liposomes are increased 2- to 2.5-fold over DSPC/Chol (2:1) CLs and 7- to 10-fold over eggPC/Chol (2:1) liposomes in mice (Fig. 5; Lasic et al., 1991; Woodle et al., 1992). As mentioned earlier, CLs containing anionic lipids or those containing unsaturated lipid components, such as eggPC, are removed more readily from the circulation than those containing high-phase transition phospholipids (SM/Chol or DSPC/Chol). However, in the presence of PEG-DSPE, liposomes containing some charged lipids or low-phase transition phospholipids are found in plasma after 24 h at similar levels to those containing neutral high-phase transition phospholipids (Fig. 5). The presence of steric stabilization thus allows for the rate of clearance to be relatively independent of the remaining lipid composition for "empty" liposomes (Lasic et al., 1991; Woodle et al., 1992).

This is not inclusive of all phospholipid components. Both GM_1 and PEG-DSPE were unable to prevent liposomes containing PS from being cleared rapidly from the circulation (Allen et al., 1988, 1991), indicating that some membrane components may confer a very powerful propensity for a type of liposome being recognized and taken up by the RES. In addition, although the lipids composing the majority of the liposome may not have a direct effect on the removal of the liposomal carrier itself, they may have an indirect effect on clearance of the encapsulated drug. As previously described, when amphipathic drugs such as DOX are loaded into these liposomes, the rate of leakage from the liposome can become the rate-limiting step for clearance of the drug from the circulation if liposomes are not optimized to prevent leakage. The dose independence of liposome clearance, reduced recognition and clearance of liposomes by the RES, and flexibility in lipid compositions that can provide considerable advantages for SSLs that make them more desirable for an assortment of different applications.

The mechanism by which steric stabilization of liposomes increases their longevity in the circulation has been extensively discussed (Lasic et al., 1991, 1992; Needham et al., 1992a, 1999; Allen, 1994; Lasic and Martin, 1995). The basic concept of this discussion has been that a flexible-chained hydrophilic polymer or a glycolipid, such as PEG or GM_1, which occupies the space immediately adjacent to the liposome surface ("periliposomal layer"), tends to exclude other macromolecules from this space. Consequently, access and binding of blood plasma opsonins to the liposome surface are hindered, and thereby interactions of RES macrophages with such liposomes are inhibited. The exclusion of extraneous macromolecules from the periliposomal layer creates steric hindrance ("steric stabilization effect"), which is manifested as increased interbilayer repulsive forces and results in an increased interbilayer separation of PEG-decorated bilayers compared with unmodified ones (Lasic et al., 1992b; Needham et al., 1992b). Ganglioside GM_1 has less effect on the interbilayer separation compared with PEG. Measurements of streptavidin-induced agglutination rate of biotinylated SSLs demonstrated that the steric barrier decreased with decreasing PEG chain length and at higher PEG lengths (PEG M_r = 1900 and 5000) was significantly greater than that produced by GM_1 (Mori et al., 1991).

Several studies have argued that the presence of PEG or GM_1 does in fact lead to both a decreased extent and rate of binding of plasma proteins to liposomes (Senior et al., 1991; Chonn et al., 1992; Blume and Cevc, 1993; Semple and Chonn, 1996), although direct experimental evidence is not abundantly clear for PEG-DSPE-containing liposomes. In two earlier studies in which decreased protein binding or opsonic activity was shown for SSLs, the incubation of PEG-stabilized liposomes with plasma components was completed in 2 to 15 min (Allen et al., 1994; Semple and Chonn, 1996). Another study showed similar findings in vivo after a 2-min incubation but approximately equivalent levels of bound protein for a CL and a PEG-stabilized liposome formulation after 30

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min (Harvie et al., 1996). Chonn et al. (1992) have previously shown a correlation of protein-binding levels in CLs and GM_1-containing liposomes to in vivo circulation lifetimes, which is consistent with the observations seen with SSLs. Thus, decreased binding of serum opsonins by GM_1-containing liposomes can result in decreased opsonin activation, as has been shown for complement factor C3 (Chonn et al., 1992), or reduced uptake by macrophages (Wassef et al., 1991; Alving and Wassef, 1992). Finally, reduced clearance may be partially due to steric hinderance for the binding of liposome-bound opsonins to their receptors on RES macrophages (Klibanov et al., 1991; Mori et al., 1991; Allen, 1994). Allen and coworkers have suggested that the elimination of SSLs may occur via the same mechanism as CLs, after a slow removal of PEG-DSPE from the membrane (Allen et al., 1991; Allen, 1994). Blume and Cevc (1993) suggested that SSLs may simply require longer to bind the opsonins necessary for uptake by the RES. The net effect of these phenomena is that by sterically hindering the approach to the liposome surface by large molecules or cells, liposomes can attain longer circulation lifetimes, allowing them greater time to accumulate in tumors.

F. Comparison of Pharmacokinetic Parameters for Different Liposomal Formulations

SSL DOX has a long half-life in the circulation compared with free DOX (Table 3). Pharmacokinetic data for free DOX are best described by a biexponential fit with a rapid distribution phase and a slow terminal elimination phase. The majority of free DOX is eliminated in the initial rapid phase. With SSL DOX, a major portion of the plasma AUC is attributed to the prolonged terminal phase (Papahadjopoulos and Gabizon, 1995; Gabizon and Martin, 1997). Half-lives in rats were found to be between 22 and 23.6 h (Mayhew et al., 1992; Working and Dayan, 1996), whereas those in humans approximated 45 h (Gabizon et al., 1994). For conventional formulations in humans, the terminal $T_{1/2}$ is significantly shorter: from 6.7 to 25 h for TLC D-99 and from 2.8 to 8.3 h for DaunoXome. Although the $T_{1/2}$ was found to be independent of dose for SSL DOX, it increased with increasing dose for both DaunoXome and TLC D-99. Recently, the dose-independence of SSL DOX at very high concentrations of drug, 60 mg/m^2, was called into question when it was reported that a decreased rate of clearance was observed at these higher doses (Martin, 1998). This may result from a drug-induced toxicity to macrophages responsible for their elimination, similar to the effect described by others with CLs (Bally et al., 1990b; Parr et al., 1997). The increased $T_{1/2}$ values of liposomal drugs relative to free drugs were also consistent with a decreased rate of clearance of the liposomal carriers. DOX is cleared 560 times slower in humans when encapsulated in SSLs, and daunorubicin is cleared 35 to 56 times slower with DaunoXome, a CL formulation, compared with the free drugs. The stable encapsulation of drug within the liposome, combined with the large size of the carrier, likely prevents filtration and removal of the drug by the kidneys.

It should be emphasized that the AUCs that are typically referred to in this discussion are based on drug that is for the most part nonbioavailable. It is entrapped inside the carrier and unable to elicit any response. Thus, the term "AUC," which is commonly used by those in the liposome field of study, for liposomal drugs may appear a little misleading to some because it does not truly represent the pool of bioavailable drug in the plasma or in the tumor. Several studies have shown for DOX that >95% of the drug remains liposome associated in the plasma (Gabizon et al., 1994; Martin, 1998). A technical limitation in the ability to accurately measure the rate at which drug is released from the carrier has also prevented us from expressing true AUC measurements for bioavailable drug in the tumor. The AUC measurements typically being referred to are of total

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TABLE 3
Pharmacokinetic parameters of SSL DOX in various animals

Animal Model	Formulation	Dose	$T_{1/2}$	AUC	V_d	CL
		mg/kg	*h*	*mg * l/h*	*ml*	*ml/h*
Rats[a]	SSL DOX	1.0	T_1 = 1.8 T_2 = 23.6	683	13	0.4
	Free DOX	0.9	T_1 = 0.16 T_2 = 29.1	11.1	1,014	24.3
Male Sprague-Dawley rats[b]	HSPC/Chol/PEG-DSPE (37:20:3)	6.0	T_1 = 2.8 T_2 = 22	2,769	18	0.5
	Free epirubicin	6.0	0.23	15	3,700	111
Rabbits[a]	SSL DOX	1.0	T_1 = 0.5 T_2 = 21.3	368	176	6
	Free DOX	1.0	T_1 = 0.03 T_2 = 4.07	1	13,651	2,536
Dogs[a,c]	SSL DOX	1.5	T_1 = 0.20 T_2 = 25.9	656	596	15.5
	SSL DOX	0.5	27 ± 5	304 ± 118	1000 ± 100	25.2 ± 7.2

T_1, half-life associated with the first exponent of elimination; T_2, half-life associated with the second exponent of elimination; V_d, volume of distribution; CL, total plasma clearance.
[a] Working and Dayan (1996).
[b] Mayhew et al. (1992).
[c] Gabizon et al. (1993).

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drug, including both liposome and free drug. Depending on the lipid composition used and the rate of clearance in the particular organ or compartment being studied, these relative amounts may vary significantly.

The increased $T_{1/2}$ values of liposomal drugs translate into an increased AUC for Doxil in plasma compared with the free drug (Tables 3 and 4). In rats, the AUC for Doxil is >60 times that of free DOX, and this increase is elevated to a 368-fold increase in rabbits (Working and Dayan, 1996). In humans, the AUC is increased by 250 to 600 times in the case of Doxil over free DOX, 20 to 30 times in the case of TLC D-99, and ~60-fold in the case of DaunoXome (Table 4; Conley et al., 1993; Cowens et al., 1993; Gabizon et al., 1994). The smaller plasma AUCs for DaunoXome and TLC D-99 relative to Doxil may reflect both the shorter circulation times of the carriers, as a result of the lack of steric stabilization, and their increased rate of drug leakage. Even doses greater than three times those used for DOX were unable to provide comparable plasma levels of the relevant anthracycline (609 mg/*h/liter for 25 mg/m^2 DOX and 375.3 mg*h/liter for 80 mg/m^2 daunorubicin). For TLC D-99, plasma AUCs reached their maximum value at 50.5 ± 44.9 mg*h/liter, 10-fold lower than that for Doxil. Mayer and coworkers recently demonstrated they could obtain plasma AUCs for DOX in mice from 27 to 57% of those obtained with an SSL formulation by encapsulating DOX in DSPC/Chol liposomes and injecting them at the relatively high doses of 20 mg/kg DOX and 100 mg/kg lipid (Parr et al., 1997; Bally et al., 1998; Mayer et al., 1998). Assuming that a stable formulation can be prepared with SSLs (this is not always the case as will be seen with liposomal VCR), the data suggest that plasma drug levels, and thus the total AUC, will always be greater with SSL formulations. Although there is little debate on this particular point, there is a significant amount of debate over whether higher plasma levels necessitate a more favorable clinical outcome. This question is a complex one, and a considerable part of the remainder of this review focuses on how and whether this question can be answered.

The volume of distribution for free DOX is high in all species examined, indicating a wide tissue distribution. The small molecular size and amphipathic nature of the free drug allow it to rapidly distribute to both healthy and diseased tissues. However, when administered in liposomal form, the volume of distribution was reduced >60-fold to values approximating the plasma volume, suggesting that both DaunoXome and Doxil are restricted to the central compartment (Gabizon et al., 1993; Tables 3 and 4). The relatively large size of liposomal carriers (45–150 nm) prevents them from passing through the 2-nm pores found in the endothelium of blood vessels in most healthy tissues or even the 6-nm pores found in postcapillary venules (Seymour, 1992). In addition to the size of the carrier, the stability of the

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TABLE 4
Pharmacokinetic parameters of CL and SSL DOX in humans

Formulation	Dose	$T_{1/2}$	AUC	V_d	Cl	Reference
	mg/m²	*h*	*mg * h/l*	*l*	*l/h*	
SSL DOX	25	3.2 (0.2–5.4) 45.2 (20.8–59.1)	609 (227–887)	4.1 (3.0–6.5)	0.08 (0.05–0.21)	Gabizon et al. (1994)
	50	1.4 (0.2–7.3) 45.9 (29.3–74.0)	902 (335–2497)	5.9 (2.3–10.1)	0.09 (0.03–0.24)	Gabizon et al. (1994)
Free DOX	25	0.07 (0.05–0.09) 8.7 (3.6–13.3)	1.0 (0.7–1.3)	254 (126–393)	45.3 (39.7–48.6)	Gabizon et al. (1994)
	50	0.06 (0.06–0.08) 10.4 (5.4–26.8)	3.5 (2.6–6.0)	365 (131–501)	25.3 (13.3–35.2)	Gabizon et al. (1994)
SSL DOX	20	5.6 56.6	577	4.7	0.07	Working and Dayan (1996)
TLC D-99	20	0.71 ± 0.37 8.2 ± 6.2	30.4 ± 32.5	21.4 ± 14.0	23.5 ± 15.6	Cowens et al. (1993)
	25	0.29 ± 0.09 6.68 ± 2.94	19.7 ± 17.7	18.8 ± 10.7	23.3 ± 15.7	
	30	0.37 ± 0.16 25.0 ± 22.5	50.5 ± 44.9	8.2 ± 3.0	9.0 ± 7.8	
	90	0.45 ± 0.60 13.5 ± 6.6	14.1 ± 16.6	14.6 ± 7.8	21.8 ± 15.5	
TLC D-99	30	0.2 ± 0.1 25.1 ± 35.5	30.4 ± 25.8	7.1 ± 4.0	9.8 ± 10.7	Conley et al. (1993)
DSPC/Chol (2:1)	10	2.8	16.9	3.75	0.942	Gill et al. (1995)
(daunorubicin)	20	3.8	57.2	4.1	0.858	
	40	4.0	120.1	3.7	0.630	
	60	8.3	301.1	2.9	0.402	
	80	5.2	375.3	2.9	0.396	
Free daunorubicin	80	0.77	10.33	1055	13.38	Forssen and Ross (1994)
DSPC:Chol (55:45) (VCR)	2.0	1.95 ± 1.67 22.5 ± 9.85	6.5 ± 0.6	4.35 ± 0.84		Embree et al. (1998)
	2.8	7.2 ± 4.8 99.3 ± 17.2	40.2 ± 17.2	2.26 ± 0.47		

Errors are expressed as either a range (in parentheses) or as a S.D., depending on the source of the data. For expression of half-lives of elimination, the top value is the half-life associated with the first exponent of elimination, and the bottom value is the half-life associated with the second exponent of elimination.

$T_{1/2}$, half-life elimination of drug from the circulation; V_d, volume of distribution; Cl, total plasma clearance.

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formulation can also have an effect on the volume of distribution. If drug leaks from the liposome before leaving the circulation, then the free drug can readily redistribute to healthy tissues. A comparison of Doxil or DaunoXome, both of which contain high-phase transition phospholipids (DSPC or HSPC), with TLC D-99, which contains the highly unsaturated eggPC, shows a significantly higher volume of distribution (4- to 5-fold at 25 mg/m^2) for the latter (Table 4). This indicates that DOX was released more rapidly from the carrier with TLC D-99 and has distributed more extensively into normal tissues than for more stable preparations with lower leakage rates. The consequences of this are an alteration in the toxicity profile and lower tumor levels of the drug. A more thorough review of the factors that contribute to the stability of a formulation in the plasma is given in *VII. Stability in Plasma and Storage*.

G. Tissue Distribution of Conventional and Sterically Stabilized Liposomes

Due in part to the size of the carrier, L-DOX has an altered tissue distribution compared with free DOX (Tables 5 and 6). Free DOX has a wide distribution, accumulating in most tissues to a significant extent. L-DOX preferentially accumulates in areas containing a discontinuous microvasculature, such as tumors, or in organs containing the macrophages of the RES, such as liver and spleen. This altered distribution reduces the concentration of drug at potential sites of toxicity, such as the heart. A comparison of the biodistribution of free drug and that encapsulated in both CLs and SSLs is given in Tables 5 and 6. When DOX levels are reported as peak levels of drug in various tissues, a significant increase in DOX is found in healthy tissues, such as kidneys, heart, and lung when administered as free DOX compared with both CL and SSL DOX (Table 5). L-DOX shows increased levels in blood, liver, spleen, and tumor (Table 5). In the liver, SSLs were found almost exclusively in Kupffer cells and rarely in the more abundant hepatocytes (Huang et al., 1992; Litzinger et al., 1994). This is consistent with the role of Kupffer cells in removing liposomes from the circulation and suggests less damage to liver tissue than if delivered to parenchymal cells. However, in addition to macrophages, other investigators have demonstrated a significant uptake of liposomes by a low-affinity, high-capacity system involving hepatocytes in a manner dependent on both the size of the liposomes and the presence of PEG-DSPE (Scherphof et al., 1994). The nature of this discrepancy is unclear but may involve problems in detection of liposomes in hepatocytes by some methods. From these data, it appears as though liposomes preferentially accumulate in tumor and tissues of the RES, whereas free DOX distributes more uniformly between the various tissues.

Although peak drug levels indicate L-DOX reaches healthy tissues to a reduced extent, when tissue drug

TABLE 5
Tissue distribution of DOX in animals treated with L-DOX

Animal Model	Liposome Formulation	Time	Dose[a]	Plasma	Spleen	Liver	Skin	Heart	Lung	Kidney
		h	*mg/kg*	*μg/ml*			*μg/g*			
Nude mice (N87 tumor implants)[b]	HSPC/Chol/PEG-PE (92.5:70:7.5)	72	10	12.89		15.84 ± 1.58	5.36 ± 0.35	2.37 ± 0.46		
Rats[c]	HSPC/Chol/PEG-PE (56.4:38.3:5.3)	24	1		7.18 ± 1.41	1.27 ± 0.11	0.46 ± 1.1	1.67 ± 0.55	1.59 ± 0.33	1.70 ± 0.11
	Free DOX	0.5	1		2.56 ± 0.33	1.67 ± 0.16	0.46 ± 0.06	2.21 ± 0.17	2.71 ± 0.49	3.61 ± 0.40
Female CD1 mice[d]	eggPC/Chol (55:45) 200 nm	5	20	14.3 ± 3.8	995.1 ± 284.7	134 ± 20.2		4.1 ± 2.2	16.5 ± 4.1	17.3 ± 6.2
	DSPC/Chol (55:45) 230 nm	5	20	27.4 ± 3.2	832.2 ± 64.8	137.5 ± 10.7		2.4 ± 1.2	11.4 ± 3.4	13.1 ± 2.1
	Free DOX	5	20	0.09 ± 0.04	39.0 ± 8.3	25.8 ± 3.7		15.5 ± 3.4	29.9 ± 5.8	35.5 ± 7.9
Female BDF1 mice[e]	DSPC/Chol (55:45) (100 nm)	C_{max}	10	140.5 ± 5.3	259.8 ± 7.8	68.7 ± 3.7		7.8 ± 1.5		30.9 ± 0.6
	Free DOX	C_{max}	7.5	2.7 ± 0.3	13.5 ± 1.4	59.5 ± 2.4		35.1 ± 1.8		98.5 ± 6.4

C_{max} is the peak concentration of drug achieved, and it varied as a function of time.
[a] Dose is given in mg/kg DOX.
[b] Gabizon et al. (1997).
[c] Working and Dayan (1996).
[d] Mayer et al. (1989).
[e] Krishna and Mayer (1997).
Errors are provided where available.

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TABLE 6

Tissue AUC values after i.v. administration of various liposomal and free drugs

Animal Model	Liposome Formulation	Dose[a]	Plasma	Tumor	Spleen	Liver	Heart	Lung	Kidney
		mg/kg	*μg · h/ml*			*μg · h/g*			
BALB/c mice with C26 colon carcinoma[b,d]	DSPC/Chol/GM_1 (2:1: 0.2)	[c]		662.2	1974	1299	83.25	141.5	241.8
	DSPC/Chol (2:1)	[c]		256.2	3348	1757	31.98	28.3	40.48
Female BDF1 mice[e]	DSPC/Chol (55:45)	10	1095.2	470.6	8923.8	2444.5	67		1265.6
	Free DOX	7.5	1.3	73.8	496.9	874.9	820.4		1258.1
BALB/c mice with colon C26 carcinoma[f]	PEG-PE/DSPC/Chol (6:47:47)	5	809.5	169.6	320.3	309.4	41.9	91.4	132.0
	DSPC/Chol (1:1)	5	342.8	50.1	365.5	341.4	41.2	67.5	72.4
	Free DOX	5	1	18.1	178.1	168.8	63.2	106.4	146.1
BALB/c mice with P1798 lymphosarcoma[g]	DSPC/Chol (2:1) (62 nm)	20	2275.6	2470.5	3596.2	693.6	265.1	685.0	1237.2
	Free daunorubicin	20	10	245.1	2213.4	335.5	249.9	612.2	976.8
BDF_1 mice[h] (mitoxantrone)	DSPC/Chol	10	1970		12630	4832		178	751
	DSPC/Chol/PEG-PE	10	4863		7242	4302		218	697

AUC was calculated from either 0–96 h,[d] 1–24 h,[f] by the trapezoidal rule,[e, h] or 0–48 h.[g]

[a] Dose is given in mg/kg DOX.

[b] Data were collected using encapsulated ^{67}Ga as the tracer. The tissue distribution is expressed as percent of injected dose per gram of tissue versus time (h).

[c] 10–15 μmol of phospholipid/kg.

[d] Huang et al. (1992). Data were collected using encapsulated ^{67}Ga as the tracer. The tissue distribution is expressed as percent of injected dose per gram of tissue versus time (h) after injection.

[e] Krishna and Mayer (1997).

[f] Unezaki et al. (1995).

[g] Forssen et al. (1992).

[h] Chang et al. (1997).

levels are reported as the AUC, even in healthy tissues, tissue drug levels approach those for free DOX (Table 6). DOX delivered via CLs accumulates to a reduced extent in non-RES tissues compared with delivery by SSLs. This is most likely a result of the reduced circulation lifetimes of CLs. The AUC for tumors is still between 2.5- and 10-fold greater than that for free DOX. A more detailed comparison of the extents of accumulation in tumors is given in *IIIB. Rate and Extent of Accumulation in Tumors*. In one of the earliest comparisons of SSLs (GM_1) versus CLs (Huang et al., 1992), liposome levels in tumor (followed with encapsulated ^{67}Ga) were greater than twice those of DSPC/Chol liposomes (Table 6). The levels in spleen and liver for DSPC/Chol liposomes were 1.35 to 1.7 times those of the GM_1-containing formulation, reflecting their more rapid uptake by the RES. In the three healthy non-RES tissues measured (heart, lung, and kidneys), liposome levels were significantly greater for the SSL formulation, suggesting that longer circulation also leads to higher AUCs for liposomes in healthy tissues. The few comparative studies with L-DOX or mitoxantrone showed either insignificant differences or slightly elevated AUCs in healthy tissues for SSLs relative to the CL formulation (Table 6; Unezaki et al., 1995; Chang et al., 1997). In all cases, the total AUC was either comparable or decreased for the liposomal form compared with the free drug. Considering that the overall exposure for these tissues is approaching equivalent amounts for free and encapsulated drug, it might be reasonable to expect the level of toxicity in these tissues to be similar. However, some acute toxicities are dependent on peak levels of the drug, and liposomal drugs accumulate in these tissues at a much slower rate than the free drug. In addition, the liposomal drug is not completely bioavailable, and thus the effective concentration of the drug in these tissues is considerably reduced. In one study, a histological section of cardiac muscle showed accumulation of liposomes only within blood vessels between muscle fibers and not within the muscle itself, indicating that liposomes were unable to extravasate in the heart to areas where they may do considerable damage (Working et al., 1994). This is discussed in greater detail in *VI. Toxicology of Liposomal Chemotherapy*.

H. Metabolism and Elimination of Liposomal Doxorubicin

Anthracyclines are metabolized in human plasma to a variety of both active and inactive metabolites (Takanashi and Bachur, 1976; Fig. 7). The reduction in DOX by an aldo-ketoreductase results in the formation of the most prominent metabolite, doxorubicinol (**II**), in plasma, bile, and urine (Takanashi and Bachur, 1976). A two-electron reduction in DOX with subsequent elimination of the sugar results in the inactive metabolite, a 7-deoxyaglycone (**V**: Takanashi and Bachur, 1976; Doroshaw, 1996). In several studies, researchers looked for the presence of DOX metabolites in plasma and urine after the administration of L-DOX (Gabizon et al., 1991, 1994; Northfelt et al., 1996). Although several of the more common metabolites (doxorubicinol and glucoronide and sulfate derivatives of 4-dimethyl,7-deoxyaglycones) were observed in urine, they were at diminished levels (2.5%) compared with the administration of free DOX (11%; Gabizon et al., 1994). In two separate studies (Gabizon et al., 1994; Northfelt et al., 1996), doxorubicinol (**II**) was not observed in plasma at any time after the administration of SSL DOX. This is not surprising considering that the liposomal membrane protects its contents from inactivation by plasma enzymes. The importance of a stable formulation is essential in

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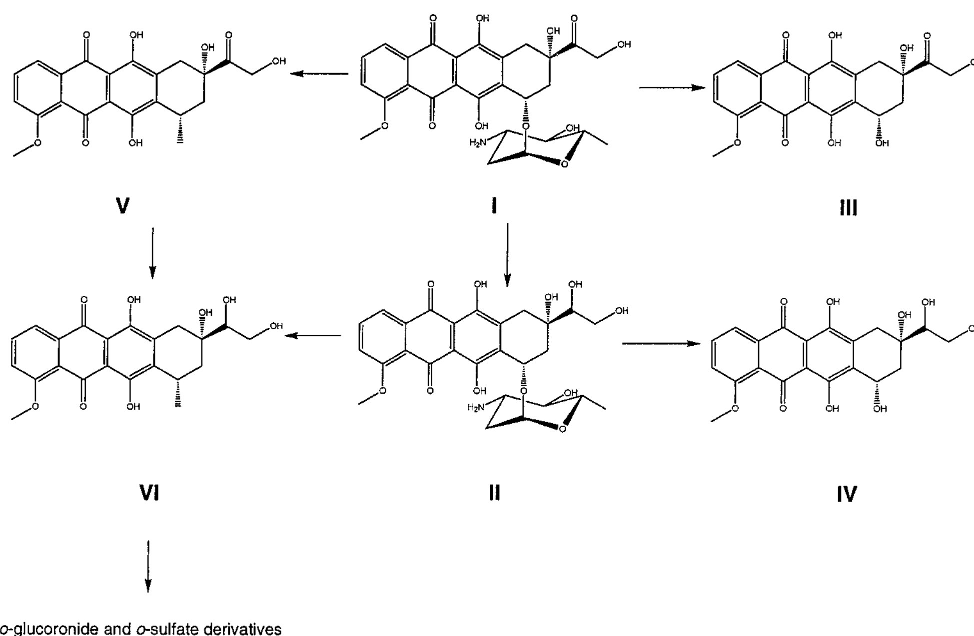

FIG. 7. Metabolism of DOX in vivo. DOX (**I**) can be converted to either inactive (deoxyaglycones; **III** or **V**) or active (doxorubicinol; **II**) metabolites in the circulation. Initially, DOX is converted to doxorubicinol (**II**), DOX aglycone (**III**), or deoxydoxorubicin aglycone (**V**), although the preferred pathway is for the metabolism to **II**. Doxorubicinol (**II**) is the primary metabolite found in both plasma and urine. Doxorubicinol can be further metabolized to doxorubicinol aglycone (**IV**) or deoxydoxorubicinol aglycone (**VI**), with metabolism to **VI** being the preferred pathway. Finally, **VI** can be converted to more polar metabolites such as *o*-sulfate or *o*-glucoronide derivatives. A notable advantage of liposomes is that they are able to protect their contents from metabolism and inactivation in the circulation, thus allowing higher levels of the parent compound to arrive at the tumor site. This figure was modified from Takanashi and Bachur (1976).

maintaining this advantage. In formulations containing unsaturated phospholipids, DOX leaks rapidly from the liposome and doxorubicinol was detected in plasma at times as short as 30 min (Gabizon et al., 1991; Embree et al., 1993). Although small amounts of DOX metabolites have been observed in tumor and tumor exudates (Gabizon et al., 1994; Siegal et al., 1995), its protection from inactivation by plasma enzymes almost certainly increases the percentage of drug that arrives in the active form at the tumor site.

L-DOX is eliminated in the urine at a much slower rate than free DOX (Vaage et al., 1998). In a mouse model, free DOX was found in urine samples as readily as 15 min and up to 48 h after the administration of the drug. SSL DOX was not detected in the urine until almost 1 h and could still be detected up to 5 days after drug administration. This is consistent with a controlled-release mechanism for the liposome-encapsulated drug, where the drug is released from its carrier at a very slow rate.

An understanding of the mechanisms responsible for maintaining high circulating levels of drug in the plasma is essential to design carriers that remain in the circulation sufficiently long to have a high probability of accumulating in tumors. Nevertheless, long circulation time is only one aspect of liposomes that results in their preferential antitumor activity. If liposomes were unable to preferentially accumulate in tumors, they would be useful only as a controlled-release type of therapy. This is increasingly available through mechanical means, and thus there is minor clinical importance for the development of liposomes for this purpose. However, the fact that liposomes do accumulate preferentially in tumors allows them to be passively targeted and gives rise to substantial increases in antitumor efficacy. In *III. Accumulation of Liposomal Drugs in Tumors*, we review the various mechanisms responsible for the uptake of liposomes into tumors and how they may be exploited for further increasing drug delivery to tumors in the future.

III. Accumulation of Liposomal Drugs in Tumors

A. Mechanistic Rationale for Liposome Accumulation in Tumors: Enhanced Permeability and Retention Effect Phenomenon

The accumulation of liposomes or large macromolecules in tumors is a result of a "leaky" microvasculature and an impaired lymphatics supporting the tumor area (Matsumura and Maeda, 1986, 1989; Huang et al., 1992; Seymour, 1992; Yuan et al., 1994; Jain, 1996). This effect is often referred to as the enhanced permeability and retention effect ("EPR phenomenon"; Matsumura and Maeda, 1986, 1989). With gold-labeled liposomes, both extravasation and transcytosis of liposomes in Kaposi's sarcoma-like dermal lesions were demonstrated (Huang et al., 1993). The principal pathway for the movement of liposomes into the tumor interstitium is via extravasation through the discontinuous endothelium of the tumor microvasculature, and transcytosis is thought to be a relatively minor pathway. Once in the

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tumors, nontargeted liposomes are localized in the interstitium surrounding the tumor cells (Huang et al., 1992; Yuan et al., 1994). Liposomes were not seen within tumor cells, although they were observed in resident tumor macrophages. The limited distribution of liposomes within the tumor interstitium results from a high interstitial pressure and a large interstitial space compared with normal tissues (Jain, 1989, 1990). Large tumors are more difficult to treat than small ones, in part because of the resulting increase in interstitial pressure, which prevents access of drugs to the necrotic core (Jain, 1990). Recently, liposomes were shown to penetrate the tumor more uniformly after the addition of an internalizing anti-HER2 Fab′ fragment to the liposome surface (Papahadjopoulos et al., 1999) or by combining liposomal delivery with local hyperthermia. The extravasation and accumulation of liposomes into tumors are depicted in Fig. 8. Targeting to endocytic pathways may also increase the bioavailability of some drugs by degrading the liposomal carrier in the late endosome

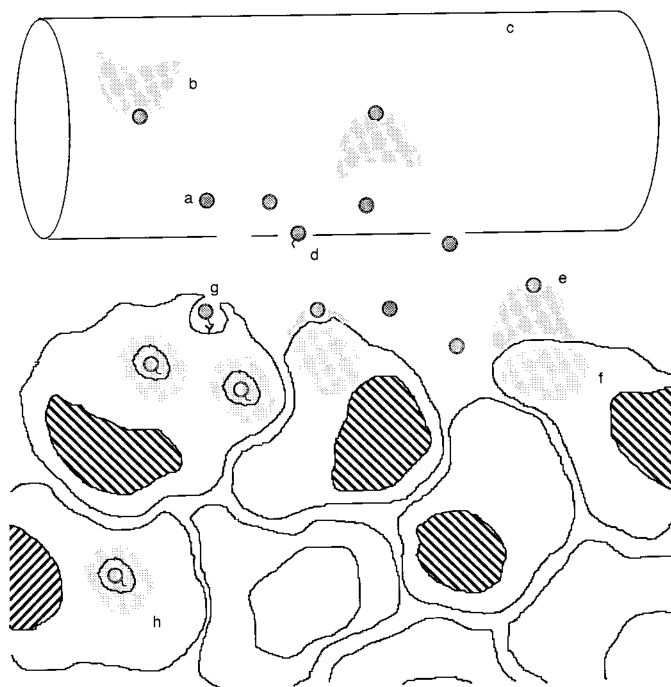

FIG. 8. Scheme showing accumulation of liposomes and drug in tumors. a, slow-release liposomes are able to carry some drugs securely in the circulation. b, rapid-release liposomes leak their drug, to a greater extent in the circulation. The drug is then free to diffuse and take on the pharmacokinetics of the free drug. c, the continuous endothelium of healthy tissues prevents leakage of liposomes into these tissues. d, however, the tumor vasculature is discontinuous with gaps ranging from 100 to 780 nm, allowing liposomes to extravasate and reach the tumor interstitium. e, nontargeted liposomes accumulate in the tumor interstitium, where they eventually leak their drug. f, DOX can then enter nearby tumor cells and accumulate in the nucleus where it elicits its cytotoxic effects. g, targeted liposomes also accumulate in tumors, but on entering the tumor area are endocytosed by tumor cells. This likely results in breakdown of the carrier in the lysosome and an increased delivery of DOX to the nucleus. h, in addition, HER2-targeted liposomes have been shown to distribute within the tumor, increasing the access of DOX to tumor cells deep within the tumor mass. Hyperthermia in combination with SSL DOX was shown to result in a similar distribution (not drawn to scale).

or lysosome. These advances are discussed in more detail in *VIII. Bioavailability of Encapsulated Drug*.

The rate of accumulation and subsequent removal of liposomal drugs are affected by a variety of factors. The absence of functioning lymphatics, in combination with a high interstitial pressure, results in the trapping of liposomes within the tumor area (Yuan et al., 1994). The result is a relatively slow rate of elimination from the tumor. Several reports suggest that the observed elimination of L-DOX from the tumor is more likely due to the release of free drug from the carrier and its subsequent metabolism and diffusion from the tumor. In a brain tumor model, 7-deoxyaglycone metabolites were observed at 96 and 120 h after injection, when tumor drug levels were starting to decrease (Siegal et al., 1995). In a separate experiment, tumor levels of DOX and a nonexchangeable lipid marker, [^{3}H]cholesteryl hexadecyl ether, were measured at identical times (Goren et al., 1996). Although the lipid marker continued to accumulate over the entire time course, 100 h, to a maximum of ~6% of the injected dose/g of tumor, DOX levels reached a maximum after 24 h of ~5% of the injected dose/g tumor and then slowly decreased. This suggests that after the trapping of the liposomal carrier in the tumor area, DOX is made bioavailable and attains its own separate rate of elimination.

1. Effect of Microvasculature Physiology. Liposomes are able to enter tumors due to a discontinuous tumor microvasculature, where pore sizes vary between 100 to 780 nm in size (Yuan et al., 1995; Hobbs et al., 1998). The junctions in the vascular endothelium of healthy tissues vary depending on the type of tissue (Seymour, 1992). In most tissues, including connective tissue and tissues of the muscle, heart, brain, and lung, intercellular tight junctions result in openings of <2 nm. These openings can approach 6 nm in postcapillary venules and are considerably smaller than the size of liposomal carriers (65–125 nm; Seymour, 1992; Lum and Malik, 1994). Organs or tissues with discontinuous endothelium, such as the fenestrated endothelium of the kidney glomerulus or the sinusoidal endothelium of the liver and spleen, can have junctions ranging from 40 to 60 nm for the former and up to 150 nm for the latter (Seymour, 1992). Most liposome formulations are larger than the threshold required for glomerular filtration. As described in *II. Pharmacokinetics and Biodistribution of Liposomes and Liposomal Drug*, it is the macrophages residing in the liver and spleen that are responsible for the removal of liposomes from the circulation and, thus, the other two major sites of accumulation. However, unlike in tumor tissue, where they become effectively trapped, if liposomes are able to avoid uptake by macrophages, then they are free to pass in and out of the liver and spleen. The selective accumulation in tumors is thus made possible by the impervious nature of the endothelium of most healthy tissues.

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Vascular permeability in tumors is heterogeneous with respect to tumor type, location of the vessel within the tumor, and the tumor microenvironment (Yuan et al., 1994; Fukumura et al., 1997; Hobbs et al., 1998). The enhanced permeability and retention effect has been described for a variety of large macromolecules and drug carriers (Matsumura and Maeda, 1986, 1989; Maeda, 1991; Huang et al., 1992; Seymour, 1992; Yuan et al., 1994). Permeability and angiogenesis are also dependent on various growth factors and the microenvironment from which those growth factors are released or act on (Collins et al., 1993; Roberts and Palade, 1995; Dellian et al., 1996). The best studied of these is vascular permeability factor, also known as vascular endothelial growth factor. Vascular permeability factor also is an angiogenic factor; it helps to recruit new blood vessels to support the tumor (Folkman, 1985; Collins et al., 1993; Roberts and Palade, 1995). Basic fibroblast growth factor was shown in another study to increase vascular permeability, although its effects may be secondary to those of vascular endothelial growth factor (Dellian et al., 1996). The availability of these growth factors in different tumor environments will affect the accumulation of large macromolecules and liposomal carriers in tumors.

2. Blood-Brain Barrier. The blood-brain barrier represents a formidable barrier for drug delivery to the central nervous system. Tight junctions, the lack of fenestrations, and a low transcellular pinocytic index severely limit the accumulation of macromolecules in the brain (Levin et al., 1980; Seymour, 1992). Surprisingly, several groups have been able to show that even tumors located in the brain have a "leaky" microvasculature, although pore sizes are significantly smaller (100–380 nm) than those seen with tumors located elsewhere in the body (200–780 nm; Siegal et al., 1995; Hobbs et al., 1998). In a brain tumor model, Gabizon and coworkers were able to show high levels of SSL DOX accumulation in the tumor (Siegal et al., 1995). Fischer rats injected with SSL DOX at a dose of 6 mg/kg showed maximal accumulation at 48 h of 10 to 11 μg DOX/g of tumor tissue. This was 15-fold higher than the levels observed after the administration of an identical dose of free DOX (0.8 μg/g at 4 h), which were not different from levels found in the normal brain (contralateral hemisphere). There was no accumulation of SSL DOX in the contralateral hemisphere, and in brain tissue immediately adjacent to the tumor, levels were <2 μg/g tissue up to 70 h after injection but gradually increased to a maximum of 4 μg/g at 120 h. These results suggest that even in the tightly regulated central nervous system, a high tumor vascular permeability can be exploited for carrier-mediated drug delivery.

B. Rate and Extent of Accumulation in Tumors

The rate and extent of drug accumulation in tumors vary depending on dose, formulation, and tumor type (Table 7; Gabizon et al., 1996; Goren et al., 1996; Harasym et al., 1997; Parr et al., 1997). Drug accumulation in tumor is most often measured at single time points, commonly either 24 or 48 h. In some studies, the free drug is measured at shorter times, such as 1 h, due to its earlier peak of accumulation. Data comparing the degree of drug accumulation in tumors for free and liposomal drug at single time points are given in Table 7. Peak DOX levels are 3- to 15-fold greater in tumors when delivered via liposomes compared with the free drug. A comparison of CL versus SSL DOX showed an approximately equivalent accumulation in three tumor models when administered at high doses (20–55 mg/kg; Mayer et al., 1997; Parr et al., 1997; Table 7). When administered at lower doses (5–10 mg/kg), SSLs accumulated to a greater extent in tumors (Huang et al., 1992; Unezaki et al., 1995; Gabizon et al., 1996). The similar accumulation in the first study is probably an underestimate due to the taking of 24 h as the only or final time point, whereas significant tumor accumulation with SSL DOX occurs after this time. The effect of both dose and formulation is indirectly a result of their effect on liposome circulation lifetimes and on formulation stability. As described in *II. Pharmacokinetics and Biodistribution of Liposomes and Liposomal Drug*, the kinetics of SSL clearance are log linear and dose independent, which allow for significant concentrations of the liposomal drug to be in the circulation, even at low doses (Allen et al., 1995a). CLs, on the other hand, display saturable dose-dependent kinetics that result in rapid clearance of the liposomal drug at lower doses but a much slower rate of clearance, and thus higher blood levels at higher doses (Hwang, 1987; Allen et al., 1995a). It is postulated that at these lower doses, the large differences in circulation lifetimes between SSLs and CLs would result in a larger reservoir of liposomes available to enter the tumor in the case of SSLs. However, at higher doses, as saturation of the mechanisms responsible for liposome clearance occurs, the extent of these differences should be reduced. Using a variety of different lipid compositions with varying circulation lifetimes, a good correlation was observed between increasing lifetimes and high liposome levels in tumors (Gabizon and Papahadjopoulos, 1988). This relationship may prove to be overly simplistic. As factors such as drug-induced RES blockade and high liposome dose bring clearance rates of differing formulations closer together, poorly understood effects of liposome composition and physical properties (e.g., size) on rates of extravasation may begin to become important. Recent evidence suggests that CL formulations may accumulate in tumors at a more rapid rate than SSLs, but due to lower circulation lifetimes, they give rise to lower overall extents of accumulation (Gabizon et al., 1996; Mayer et al., 1998).

Gabizon et al. (1996) were the first to show that liposomes with decreased circulation lifetimes may accumulate in tumors at a more rapid rate. Later, Mayer and

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TABLE 7
Accumulation of liposomes in tumors

Animal and Tumor Model	Formulation	Dose	Time	Accumulation
		mg/kg	*h*	*μg/g*
CD2F$_1$ mice (P-1798 lymphosarcoma)[a]	DaunoXome	20	24	39.57 ± 15.38
	Free daunorubicin	20	24	2.02 ± 1.42
B6C3F1 mice (MA16C mammary adenocarcinoma)[a]	DaunoXome	20	24	19.98 ± 8.77
	Free daunorubicin	20	24	8.41 ± 1.11
Fischer rats (fibrous histiocytoma)[b]	Doxil	6	48	10.92
	Free DOX	6	4	0.8
Female C3H/HeJ mice (FSa-R fibrosarcoma)[c]	DSPC/Chol (55:45), 100 nm	20	24	16.3 ± 2.3
	DSPC/Chol/PEG-DSPE (50:45:5)	20	24	15.7 ± 1.7
	Free DOX	20	1	7.8 ± 3.1
Female C3H/HeJ mice (FSa-N fibrosarcoma)[c]	DSPC/Chol (55:45), 100 nm	20	24	23.9 ± 2.7
	DSPC/Chol/PEG-DSPE (50:45:5)	20	24	32.1 ± 4.7
	Free DOX	20	1	12.8 ± 1.2
BDF1 mice (Lewis lung carcinoma)[d]	DSPC/Chol (55:45), 100 nm	55	168	143.8 ± 18.2
	DSPC/Chol/PEG-DSPE (50:45:5)	55	168	133.0 ± 11.9
Shionogi mice (SC115 mammary carcinoma)[e]	TLC D-99	13	24	10.2 ± 3.6
		6.5	24	5.5 ± 1.1
		6.5	24	1.9 ± 0.08
BALB/c mice (M109 carcinoma)[f]	Doxil	5	48	6.5 ± 4
		10	48	16 ± 17.3
		20	48	42 ± 39.3
	Free DOX	5	3	3.6 ± 2.1
		10	3	5.2 ± 2.1
BALB/c female mice (J6456 ascites)[g]	PEG-DSPE/HSPC/Chol	10	24	5.9 ± 3.4
	DSPG/HSPC/Chol	10	24	4.2 ± 2.95
	HSPI/HSPC/Chol	10	24	5.3 ± 1.4
	HSPC/Chol	10	24	1.8 ± 0.13
	Free DOX	10	24	<0.05
BALB/c female mice (M-109 carcinoma)[g]	PEG-DSPE/HSPC/Chol	10	24	17.4 ± 8.8
	DSPG/HSPC/Chol	10	24	17.4 ± 6.1
	HSPC/Chol	10	24	19.7 ± 5.0
	Free DOX	10	24	2.2 ± 0.6
		mg/m²		
Humans (various carcinomas)[h]	Doxil	25	3–7 days	0.44
		50	3–7 days	0.69
	Free DOX	25	4–24	0.051
Humans (Kaposi's sarcoma lesions, biopsy specimens)[i]	DaunoXome	20	24	1.07
		40	24	1.06
Humans (Kaposi's sarcoma lesions, biopsy specimens)[j]	Doxil	10	72	2.06 ± 0.42
		20	72	1.61 ± 0.80
		40	72	7.71 ± 2.72
	Free DOX	10	72	0.18 ± 0.07
		20	72	0.31 ± 0.16
		40	72	0.82 ± 0.18

The composition, size, and drug/lipid ratio for daunorubicin and DOX are listed in Table 1. When available, the time of analysis was the time of maximal accumulation.
[a] Forssen et al. (1992).
[b] Siegal et al. (1995).
[c] Mayer et al. (1997).
[d] Parr et al. (1997).
[e] Mayer et al. (1990a).
[f] Gabizon et al. (1997).
[g] Gabizon et al. (1996).
[h] Gabizon et al. (1994).
[i] Forssen and Ross (1994).
[j] Northfelt et al. (1996).

coworkers proposed the use of a factor termed "tumor accumulation efficiency" (T_e), defined as the AUC of the drug in the tumor divided by the AUC in the plasma, to determine the efficiency of extravasation for a given liposome formulation (Mayer et al., 1997, 1998; Parr et al., 1997). In several different tumor models, the T_e value was 1.5- to 3-fold higher for DSPC/Chol formulations than the sterically stabilized equivalent (Mayer et al., 1997, 1998; Parr et al., 1997). The effect of steric stabilization on the rate of tumor accumulation is still controversial; at least one study that used videomicroscopy to follow fluorescently labeled liposomes showed a higher permeability for SSLs (Yuan et al., 1994). In another study, CL DSPC/Chol (55:45) liposomes delivered at 20 mg/kg to two fibrosarcoma models showed elevated drug levels in the tumor for CLs at 4 h but for SSLs at 24 h (Mayer et al., 1997). The initially increased rate of accumulation may be due to a higher permeability of the tumor microvasculature to CLs, but the later increase in SSL accumulation likely reflects the disappearing pool of CLs in the circulation, relative to SSLs.

The role of long-circulating properties and tissue uptake rate on the expected efficacy of a liposomal drug after i.v. bolus administration may be explored using a two-compartment open pharmacokinetic model (Scheme 1; Welling, 1986).

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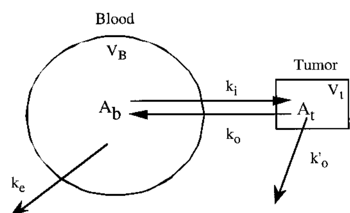

SCHEME 1.

The amount of liposomes in the blood compartment (A_b) and in the selected tissue [e.g., tumor (A_t)] is governed by the following set of equations and boundary conditions:

$$dA_b/dt = -(k_e + k_i)A_b + k_oA_t \quad (1)$$

$$dA_t/dt = k_iA_b - k_oA_t \quad (2)$$

$$A_b(0) = A_0; \quad A_b(\infty) = 0 \quad (3)$$

$$A_t(0) = A_t(\infty) = 0 \quad (4)$$

This set of linear differential equations with constant coefficients allows simple analytical solution resulting in somewhat cumbersome formulas for $A_b(t)$ and $A_t(t)$ (Welling, 1986). Assuming that drug efficacy correlates with the tissue exposure to the liposomes, which, in turn, is characterized by AUC, we need only calculate AUC for blood and tumor:

$$AUC_b = 1/V_b \int_0^\infty A_b dt \quad (5)$$

$$AUC_t = 1/V_t \int_0^\infty A_t dt \; AUC_t = \quad (6)$$

where V_b and V_t are physical volumes of the blood and tumor compartments, respectively.

After integration of right and left parts of eqs. 1 and 2 from zero to infinity and applying boundary conditions, we obtain:

$$(k_e + k_i) \cdot V_b \cdot AUC_b - k_o \cdot V_t \cdot AUC_t = A_0 \quad (7)$$

$$k_i \cdot V_b \cdot AUC_b - k_o \cdot V_t \cdot AUC_t = 0 \quad (8)$$

and, finally:

$$AUC_t = A_0 \cdot k_i/(k_e \cdot k_o \cdot V_t) \quad (9)$$

$$T_e = (AUC_t)/(AUC_b) = (k_i \cdot V_b)/(k_o \cdot V_t) \quad (10)$$

In these equations, "blood elimination" first order rate constant k_e includes all processes that lead to the removal of the carrier from the blood, including excretion, phagocytic clearance, and distribution into organs and tissues other than the tumor. Interestingly, T_e, perhaps deceptively termed "tumor accumulation efficiency" (Mayer et al., 1997, 1998), does not include liposome longevity in the circulation (characterized by k_e) as a factor and is determined essentially by the liposome uptake rate into the tumor. On the contrary, tumor AUC correlates not only with the liposome uptake (uptake rate constant k_i) but also with the liposome longevity in circulation (blood elimination constant k_e). These equations point out the importance of another process, often neglected: the rate of liposome elimination from the tumor (k_o). Liposome elimination from the tumor, which appears to be slower than tumor uptake and blood elimination, is poorly understood and awaits adequate experimental investigation.

The above simple model of liposome pharmacokinetic behavior can be modified for the case in which liposomes are not recycled from the tumor into the blood but instead are metabolized in situ. In this case, the effects of liposome uptake rate by the tumor and circulation longevity on the tumor AUC are similar to the previous model:

$$AUC_t = A_0k_i/(k_e + k_i)/(k_o'V_t) \quad (11)$$

and the expression for T_e is exactly the same as eq. 10.

Thus, within the framework of these models, circulation longevity of liposomes is not a factor in tumor accumulation efficiency, T_e, but rather is an important factor in the determination of overall tumor exposure to the drug.

In light of this controversy, a complicated question remains. If the permeability is increased for CLs, what is the overall significance of a 1.5- to 3-fold increase in permeability, taking into consideration the difference in circulation lifetimes? As expected, when the T_e was calculated for free DOX at 24 h after injection, the result was a 2.8-fold increase over that for an HSPC/Chol formulation (Gabizon et al., 1996). This is not surprising considering the small molecular size of the free drug and its rapid redistribution into tissues. However, the extent of accumulation in the tumor is greater for the liposomal formulation, as is the tumor AUC calculated at extended times. The overall tumor AUC appears to be a more relevant indicator of the effectiveness of a drug delivery system in liposomes where drug release from the liposome is similar. Mayer et al. (1998) showed two examples where the tumor AUC was slightly greater for CL formulations. One of these examples compared SM/Chol versus SM/Chol/PEG-PE formulations of VCR, where the concentrations of PEG-DSPE used were shown to result in a destabilization of the formulation and, consequently, lower amounts of drug available for delivery to the tumor (Webb et al., 1995). A second example was completed with DOX-loaded liposomes (Mayer et al., 1997). An important consideration when considering at total accumulation in tumors is the duration in which the AUC was calculated. The tumor AUC levels for all the examples given were calculated over the time period of 0 to 24 h. Other work, with substantially lower doses of SSL DOX, has shown that maximum tumor accumu-

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lation does not occur until 48 h or longer in some cases and that a substantial portion of the tumor drug level-versus-time curve exists after 24 h in all cases (Papahadjopoulos et al., 1991; Vaage et al., 1994a; Siegal et al., 1995; Gabizon et al., 1996, 1997; Goren et al., 1996). In addition, the peak time of accumulation is conceivably even later considering the large doses (20 mg/kg) used in these studies. Thus, the tumor AUC levels reported by Mayer and coworkers are likely biased in favor of CLs by limiting the calculation to the first 24 h. Finally, although it is theoretically possible to increase circulation lifetimes to a sufficiently high level to achieve an advantage from the proposed increased rate of extravasation, the conditions required to do so may not be pharmacologically relevant. The doses required to obtain the needed circulation lifetimes (20–55 mg/kg) are 4 to 20 times larger than those being used in current studies with SSL DOX and will likely result in substantial toxicity if administered in multiple doses (see *VI. Toxicology of Liposomal Chemotherapy*).

Although differences in the rates of extravasation may not be sufficiently great to obtain a true advantage for CLs over SSLs, it is nevertheless an important parameter to keep in mind when designing drug delivery systems. Liposome size may be another important parameter that affects accumulation in tumors. Several studies have shown that the permeability of large macromolecules is independent of size as long as the translocating molecule is much smaller than the pore size in the endothelium of the tumor microvasculature (Yuan et al., 1995; Hobbs et al., 1998). However, even small liposomes (100 nm) are between 13 and 100% of the average pore size found in tumor endothelium, and liposomes (100 nm) have already been shown to have a reduced permeability compared with fluorescently labeled BSA molecules. Although a study with carefully sized liposomes has yet to be completed, theoretically these data suggest that even small changes in liposome size (50 nm) may significantly affect the rate of accumulation in tumors. A DSPC/Chol (2:1) formulation containing daunorubicin shows maximal accumulation in a lymphosarcoma solid tumor mouse model at 8 h with a subsequent elimination rate similar to free daunorubicin (Forssen et al., 1992). This initial rate of drug accumulation in this study is far more rapid than that seen with other CL or SSL DOX formulations (Gabizon et al., 1997; Mayer et al., 1997) and may be due to the smaller size (50 nm compared with 100 nm for the DOX-loaded liposomes), although differences in tumor type or drug-leakage rates cannot be ruled out. In situ fluorescence measurements for an identical small formulation showed a slower rate of accumulation, similar to that of other larger CL formulations (Forssen et al., 1996), thus complicating this interpretation. A carefully designed study that considered the effect of liposome size (50-nm increments) on tumor accumulation rates or vascular permeability would help test this hypothesis.

C. Hyperthermia and Vascular Permeability Factors for Increasing Vascular Permeability

Differences in vascular permeability have been exploited in two different ways to increase accumulation of liposomes in diseased tissues. Permeability of the tumor microvasculature was increased with the use of local hyperthermia, resulting in increased tumor levels of SSL DOX (Huang et al., 1994; van Bree et al., 1996). Hyperthermia can also result in increased rates of drug release from specially engineered thermosensitive liposomes (see *VIIIC. Hyperthermia and Thermosensitive Liposomes*; Gaber et al., 1995, 1996). In a second example, substance P was used in an inflammation model to increase vascular permeability and, thus, the extents of liposome accumulation (Rosenecker et al., 1996; Zhang et al., 1998). In both of these examples, increased accumulation in the diseased tissue resulted from a direct effect on the tumor microvasculature. It may prove advantageous in future studies to increase accumulation in tumors by altering liposome surface properties or vascular permeability directly to promote extravasation.

D. Sterically Stabilized versus Rapid-Release Conventional Liposome Carriers

In addition to using liposomes as slow-release liposomal carriers, such as SSL DOX, they can be used as rapid-release systems, such as TLC D-99. The low-phase transition phospholipid component, eggPC, of rapid-release liposomal carriers allows the drug to leak more quickly from the liposome, at least partially while in the circulation (Bally et al., 1990b; Gabizon et al., 1993). Although slow-release liposomal carriers accumulate in tumors on a time scale similar to or greater than the release of DOX from the carrier, rapid-release systems can release their drug to a greater extent before reaching the tumor, where it can diffuse into the tumor as the free drug. It should be emphasized that “slow” and “rapid” are relative terms, and the magnitude of the leakage rates will ultimately be determined by the physicochemical properties of both the drug and the carrier. The release of DOX in the plasma for TLC D-99 can be demonstrated by considering the drug/lipid ratio, which drops from 0.29 to <0.05 μg DOX/μg lipid in 24 h (Harasym et al., 1997). Approximately 58% of the drug was released from the carrier within the first hour. The differences in tumor accumulation show that the drug slowly accumulates in tumors when delivered via slow-release liposomal carriers (Papahadjopoulos et al., 1991; Bally et al., 1994; Gabizon et al., 1996, 1997), likely reflecting delivery of the intact liposome-encapsulated drug. In rapid-release CL liposomal carriers, DOX accumulated rapidly in the tumor and levels remained constant for up to 72 h at levels 2- to 3-fold greater than that achieved with free DOX (Harasym et al., 1997). Due to the increased leakage of DOX in the circulation, the drug presumably reaches normal tissues at a faster rate as

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well (Mayer et al., 1989), although levels at very early times were not measured. When rates of lipid and DOX accumulation were compared, DOX delivered via rapid-release carriers reached peak levels by 1 h, whereas lipid levels did not peak until 48 h. With slow-release carriers, the initial rate of tumor accumulation is similar for lipid and drug (Bally et al., 1994; Goren et al., 1996), but at later times, tumor drug levels decrease upon drug release from the carrier and subsequent metabolism and redistribution (Goren et al., 1996). Drug can thus accumulate in tumor via several different mechanisms, although the primary mechanism for delivery by slow-release liposomal carriers is via extravasation of liposome plus drug through a discontinuous microvasculature. Studies of the effects of various factors (e.g., size, charge, tumor microenvironment, regulation by growth factors) on the permeability and distribution of liposomes within the tumor will help engineer liposomes for more effective delivery of their contents in the tumor. With the exception of one study (Yuan et al., 1994), few studies have considered the movement of liposomes through the tumor interstitium. The long distances and high interstitial pressure make this an obstacle that may prove as important as the permeability of the tumor vasculature.

IV. Efficacy of Liposomal Drugs in Animal Tumor Models

SSL DOX has been examined for antitumor efficacy in a variety of different tumor models, including a human lung tumor xenograft (Williams et al., 1993), human pancreatic carcinoma xenograft (Vaage et al., 1997), mouse lymphoma (Gabizon et al., 1996, 1997, 1998), rat brain sarcoma (Siegal et al., 1995; Gabizon et al., 1997), mouse colon carcinoma (Papahadjopoulos et al., 1991; Huang et al., 1992; Mayhew et al., 1992), prostatic tumor xenografts (Vaage et al., 1994a; Working et al., 1994), mouse mammary carcinomas (Mayer et al., 1990a; Forssen et al., 1992; Vaage et al., 1992), ovarian carcinoma xenograft (Vaage et al., 1993a), and an HER2-overexpressing human breast carcinoma xenograft (Park et al., 1997). The combination of a broad activity of DOX to a wide assortment of different cancers and the common mechanistic rationale for liposomal accumulation in solid tumors (see *IIIA. Mechanistic Rationale for Liposome Accumulation in Tumors: Enhanced Permeability and Retention Effect Phenomenon*) results in a drug formulation with substantial antitumor efficacy compared with the free drug and relatively independent of the type or location of the tumor. Even in a brain tumor model where the blood-brain barrier is thought to severely limit drug accumulation, SSL DOX showed a significant increase in mean survival times compared with free DOX (189% compared with 126%; Siegal et al., 1995; Gabizon et al., 1997). Efficacy results in a variety of different liposome formulations and tumor models are listed in Table 8. There are possible exceptions to these observations that may result in different liposome formulations being more suitable for the treatment of different cancers, depending on circumstances. These potential exceptions are described later.

In critical evaluation of previous studies using liposomal anthracyclines, one must be careful of comparisons drawn between CL and SSL formulations. All too often, the CL formulation being referred to is of a suboptimal formulation, containing either unsaturated lipids that allow the drug to leak rapidly from the carrier or negatively charged lipids that facilitate their clearance from the circulation. Under exceedingly complex conditions, such as in vivo drug delivery, where a variety of factors can influence pharmacokinetics, stability, extravasation into tumors, and clinical efficacy, it is best for comparisons to be drawn where a minimum number of variables, ideally only one, are altered at any one time.

A. Comparison of Efficacy for Sterically Stabilized and Conventional Liposomes

There are few studies that directly compare small neutral CLs and SSLs in therapeutic efficacy studies (Huang et al., 1992; Unezaki et al., 1995; Gabizon et al., 1996; Chang et al., 1997; Mayer et al., 1997; Parr et al., 1997). With the exception of one study that targets splenic and liver tumors (Chang et al., 1997), these comparisons are usually divided into one of two experimental designs. We examine the results and relevance of each of these experimental designs individually. In the first design, liposomes are injected at a dose of ≤10 mg/kg DOX and show significant improvement in therapeutic efficacy for SSL DOX compared with both free DOX and CL L-DOX (L-DOX; Table 8; Huang et al., 1992; Unezaki et al., 1995; Gabizon et al., 1996). Using a BALB/c mouse C26 colon carcinoma model, an increased life span (ILS) of 48.3% for SSL DOX was observed compared with 5.1% for DSPC/Chol liposomes and −4.2% for free DOX when administered as a single dose at 10 mg/kg (Huang et al., 1992). When SSL DOX or SSL-epirubicin was injected at either a single dose of 10 mg/kg DOX or three weekly doses of 6 or 9 mg/kg DOX, tumors regressed to a nonmeasurable size over time, whereas free drug administered at an identical dose and schedule only slightly delayed tumor growth compared with controls. This was similar to the first efficacy study completed with a Stealth® liposomal drug formulation showing increased therapeutic efficacy of SSL-epirubicin compared with free epirubicin (Papahadjopoulos et al., 1991). Epirubicin is a DOX analog that shows markedly reduced cardiac toxicity compared with DOX. In this study, both an increase in life span and an inhibition of tumor growth were noted with three weekly injections of SSL-epirubicin (6 mg/kg epirubicin), whereas free epirubicin had only minimal effects. Unfortunately, due to the significantly decreased ILS of the CL formulation, neither study examined the effect of CL DOX or

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TABLE 8
Efficacy studies in animals

Animal Model	Formulation	Dose	ILS[a]	*p*
		mg/kg	*%*	
Fischer rats (fibrous histiocytoma)[e]	Doxil	8	65	<.0003
	Free DOX	8	35	<.05
	Doxil	5 × 3	89	<.00001
	Free DOX	5 × 3	23	<.05
$CD2F_1$ mice (P-1798 lymphosarcoma)[f]	DaunoXome	20	15	<.05
	Free daunorubicin	20	54	<.05
$B6C3F_1$ mice (MA16C mammary adenocarcinoma)[f]	DaunoXome	20	>217	<.05
	DaunoXome	2	100	<.05
	Free daunorubicin	20	94	
Nude mice (ovarian HEY cancer)[g]	Doxil	9	40	<.001
	Free DOX	9	−50	
Female BALB/c mice (C26 colon carcinoma)[h]	PEG-DSPE/HSPC/Chol (DOX)	10	48.3	
	DSPC/Chol (2:1)	10	5.1	NS
	Free DOX	10	−4.2	NS
BALB/c mice (J6456 lymphoma)	Doxil	10 (i.v.)	116	<.0001
	Free DOX	10 (i.v.)	21	<.0001
	Doxil	10 (i.p.)	60.5	<.0001
	Free DOX	10 (i.p.)	60.5	<.0001
Female BALB/c mice (C26 colon carcinoma)[j]	PEG-DSPE/HSPC/Chol (DOX)	10	−7	
	DSPC/Chol (1:1)	10	21	
	Free DOX	10	114	
BALB/c mice (M-109 carcinoma)[k]	PEG-DSPE/DSPC/Chol (DOX)	10	168	<.05
	PEG-DSPC/DPPC/Chol	10	118	
	DSPG/HSPC/Chol	10	168	<.1
	HSPC/Chol	10	94	
	Free DOX	10	97	
BALB/c mice (J6456 lymphoma)[k]	PEG-DSPE/DSPC/Chol (DOX)	15	144	<.01
	DSPG/HSPC/Chol	15	197	<.01
	Free DOX	15	19	
BALB/c mice (C26 colon carcinoma)[l]	PEG-DSPC/DSPC/Chol (epirubicin)	6 × 3	148[b]	
	Free epirubicin	6 × 3	20	
BDF1 mice (P388 lymphoma)[m]	DSPC/Chol (55:45) (VCR)	3	38	<.05
	SM/Chol (55:45)	3	214	<.05
	Free VCR	3	>445	<.05
B6D2F1 mice (P388 leukemia)[n] (i.p.)	PEG-DSPE/DSPC/Chol (VCR)	2	199	<.01[c]
	Free VCR	2	79	<.01[c]
BDF1 mice (L1210 tumors)[o]	DSPC/Chol (mitoxanthrone)	20	189	
	DMPC/Chol	20	>590	
	Free mitoxantrone	10[d]	98	
B6D2F1 mice (L1210 leukemia)[p] (i.v.)	PEG-DSPE/DSPC/Chol (ara-c)	50	138	
	PEG-DSPE/DSPC/Chol/SM	50	197	
	PG/DSPC/Chol	50	160	
	Free ara-C (24-h infusion)	50	90	

The composition, size, and drug/lipid ratio for DaunoXome and Doxil are listed in Table 1.
[a] ILS, mean survival: 100 * treated/control − 100.
[b] ILS was calculated only from mean survival time of mice dying before day 120 (there were 6 of 10 long-term survivors for >120 days).
[c] Between-treatment groups at same dose level; other *p* values are between treatment and control groups.
[d] The mean survival time for free mitoxantrone.
[e] Siegal et al. (1995).
[f] Forssen et al. (1992).
[g] Vaage et al. (1994b).
[h] Huang et al. (1992).
[i] Cabanes et al. (1998).
[j] Unezaki et al. (1995).
[k] Gabizon et al. (1996).
[l] Mayhew et al. (1992).
[m] Webb et al. (1995).
[n] Allen et al. (1995b).
[o] Lim et al. (1997).
[p] Allen et al. (1992).

CL-epirubicin on tumor growth. At the concentrations of DOX (6–9 mg/kg) and liposomes (50–75 mg/kg phospholipid) used in these studies, clearance rates are likely too rapid to observe a significant therapeutic result for CLs.

Two additional studies completed with a comparable experimental design showed similar results to those observed earlier (Unezaki et al., 1995; Gabizon et al., 1996). In an almost identical design (including the tumor and animal model), Unezaki et al. (1995) showed a 6-fold increase in the percent ILS for SSLs compared with CLs (Table 8). This correlated to a >3-fold decrease in tumor AUC levels for DOX when encapsulated in CLs (Table 6). Gabizon et al. (1996) completed one of the most thorough and careful studies of the effect of lipid composition on therapeutic efficacy. In this study, five different liposome compositions were evaluated for therapeutic efficacy and tumor accumulation in two different tumor models (J6456 lymphoma and M-109 carcinoma, a murine lung metastasis model). The number of variables was kept to a minimum to allow for careful com-

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parisons, and even rates of accumulation in tumors were measured in some instances, rather than simply measuring DOX levels at single time points. At a single dose of 10 mg/kg, a linear correlation between circulation lifetimes and antitumor efficacy could not be found. Liposomes with similar but still reduced lifetimes (HSPI/HSPC/Chol, DSPG/HSPC/Chol, GM_1/HSPC/Chol) compared with PEG-DSPE/HSPC/Chol liposomes had a similar therapeutic efficacy. In one example, DSPG/DSPC/Chol liposomes were shown to have plasma levels at 24 h, approximately half that seen for PEG-DSPE/HSPC/Chol liposomes but an identical degree of accumulation in tumor and percent ILS. This was not shown to be an artifact of the type of tumor investigated, in that both the J6456 lymphoma and M-106 carcinoma gave similar results. Interestingly, the one liposome composition that showed significantly less efficacy was HSPC/Chol. Plasma levels were between one fourth and one half of the closest formulation and this translated into a reduced percent ILS (168 versus 94%) in both tumor models. For the conditions examined in this study, SSLs certainly showed a greater accumulation in tumors and an increase in therapeutic efficacy compared with neutral CLs (HSPC/Chol). However, the far more interesting result, obtained with other negatively charged formulations, demonstrated that longer circulation times do not necessitate greater tumor accumulation and efficacy.

In a second experimental design, liposomes were injected as a single dose of 20 to 55 mg/kg DOX and showed approximately equivalent therapeutic efficacy for SSL DOX and CL DOX (Table 8; Mayer et al., 1997; Parr et al., 1997). At these high doses, the circulation lifetimes for the CL formulation are markedly elevated due to a toxic effect on RES macrophages, and initial rates of tumor accumulation appear to be greater for CLs than for SSL. The rates of tumor accumulation favor the SSL formulation at later times, likely due to the more rapidly disappearing pool of CLs. In both of these studies, tumor growth rates were slowed to comparable extents with SSL DOX and CL DOX. There was no significant difference in either study. However, a serious question is raised in one of these studies due to the significant activity of free DOX (Mayer et al., 1997). This may be due to the nature of the tumor investigated: either methylcholanthrene-induced or “spontaneously” arising fibrosarcoma. In any case, there is no significant difference between free and liposome encapsulated DOX with one tumor, and free DOX actually appears more efficacious in the second tumor investigated. This stands in stark contrast to most other studies with L-DOX in which a significant therapeutic advantage is gained by liposome encapsulation. In the other study, L-DOX was used at a concentration (55 mg/kg), 5.5-fold greater than that used with previous studies with SSL DOX (Parr et al., 1997). This raises serious toxicological concerns, which are addressed in *VI. Toxicology of Liposomal Chemotherapy*. Even at these concentrations, tumor growth continued rapidly after a short delay. It is unlikely that the animals would tolerate multiple injections of the drug at these doses. The authors appear to make logical arguments as to why RES blockade and an increased permeability of the tumor microvasculature to CLs could give rise to similar therapeutic efficacy. However, the experiments in these two reports appear to be unconvincing because of the unrealistically high doses of drug being administered, and a more careful set of experiments on the effect of dose on tumor accumulation, toxicity, and therapeutic efficacy in already established tumor models may prove to be more persuasive. In addition, even at the elevated doses used in these studies, little information is given concerning the types and degree of severity of different toxicities. This appears to be an especially important concern considering the doses under investigation.

B. Model Dependency of Results

There are a number of characteristics of animal and tumor models, and the study design in general, that may influence the observed results for a given formulation in efficacy studies; these include such factors as the initial size of the tumor before the start of treatment, the growth rate of the tumor, the route of administration, the frequency of injection, and the tumor microenvironment. Investigators should be aware of how these factors potentially influence the observed efficacy of a particular liposomal drug, as well as comparisons between different formulations. Each of these characteristics is examined in detail here.

1. Initial Size of Tumor. The size of the tumor is an important determinant in its ability to be treated. As mentioned in *III. Accumulation of Liposomal Drugs in Tumors*, the tumor microvasculature varies depending on the size of the tumor. Vascular permeability has been shown to increase with increasing tumor size, and some very small lesions (<1–2 mm) appear to be avascular (Folkman, 1971, 1990; Blasberg et al., 1981; Zhang et al., 1992). Thus, some extremely small tumors may not be particularly amenable to treatment with liposomal drugs that require extravasation for activity. In other instances, small tumors may coopt already existing blood vessels (Holmgren et al., 1995; Pezzella et al., 1997; Holash et al., 1999). A more relevant problem occurs as tumors increase to very large sizes; the necrotic regions in the interior of large tumors have a reduced vascular density and an increased interstitial pressure compared with the surface of the tumor (Jain, 1987, 1990; Jain and Baxter, 1988; Baxter and Jain, 1990). The result is a reduced access of liposome-associated drug, which enters the tumor via extravasation, to certain areas of the tumor. Experiments can be effectively biased toward a favorable therapeutic outcome by choosing to start the drug administration at early times when the tumor size is small (<0.1 cm^3). Several studies

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have shown a difference in the SSL DOX or CL DOX to free DOX efficacy depending on the day of treatment relative to tumor inoculation (Huang et al., 1992; Vaage et al., 1992; Cabanes et al., 1998). Of course, this applies to treatment with free drug as well; tumors treated early after tumor inoculation can be considerably easier to treat than those whose for which treatment was delayed (Huang et al., 1992). Recently, most experiments completed in our laboratory have used 0.20 to 0.25 cm^3 as the size at which treatments are begun.

2. Rapidly Growing versus Slowly Growing Tumors. Certain tumor models may give ambiguous results with long-circulating liposomes due to their rapid doubling times (Allen et al., 1992; Papahadjopoulos and Gabizon, 1995). Both L1210 and P388 leukemias in mice fit into this category. In these two models, the cells divide more rapidly than liposomes can distribute to tumors and release their contents. With fast growing tumors, liposomes that accumulate in tumors or release their contents more rapidly may be more efficacious. Thus, although DPPC/Chol or PEG-DSPE/DPPC/Chol liposomes may release their contents too rapidly to be effective against slower growing tumors, they may show greater efficacy than the slow-releasing HSPC/Chol liposomes in these tumor models. Allen et al. (1992) have shown that formulations with increased release rates of encapsulated ara-C were more efficacious in the treatment of mice injected with rapidly growing L1210 leukemia cells (Table 8). In addition, if CLs do accumulate more rapidly in tumors, they may have an advantage over SSLs, even if the long-term accumulation is not as great. As mentioned previously, the rate of liposome accumulation in tumors remains a point of controversy and must be more thoroughly studied. Of course, the rates of tumor drug accumulation and drug release rates from liposomes must be of a similar magnitude to be the most effective in the delivery of bioavailable drug to tumors. If liposomes release most of the drug before reaching the tumor or are taken up so rapidly by the RES that they cannot accumulate in tumors to a significant extent, then the effective concentration of bioavailable drug in the tumor will still be less than that for long-circulating liposomes. Most solid tumors targeted in animal studies grow at a sufficiently slow rate, as to be compatible with long-circulating liposomes.

The difference in tumor doubling times between human and animal tumor models may also play a role in the effectiveness of a particular type of liposomal treatment. The animal tumor models described in many of these studies have doubling times on the order of days to a few weeks. Most human tumors have doubling times on the order of weeks to months, a substantial increase compared with that seen in animals. In slowly growing tumors, small differences in the rate liposomes accumulate in tumors, or the rate at which the drug becomes bioavailable (i.e., is released from the carrier) will have less impact on efficacy than in rapidly growing tumors, where the overall flux of bioavailable drug through the tumor is more likely to determine treatment success. The move from animals to humans should favor SSLs, where liposomes continue to accumulate in tumors for days after administration.

3. Route of Administration. The route of administration is another important variable when considering the relative therapeutic enhancement provided by liposomes in the treatment of cancer. The i.v. route is the commonly used route of administration for liposomal drugs due primarily to its ability to reach distant sites of metastasis. Because the vasculature of even tumor metastasis requires angiogenesis and increased vascular permeability to obtain the nutrients required for its rapid growth, delivery via the i.v. route allows the drug to be simultaneously targeted to all sites of primary growth or metastases. Delivery via other routes may reduce the amount of drug that effectively reaches the tumor and thus decrease the efficacy of the drug. In a mouse J6456 lymphoma model, SSL DOX injected i.v. was shown to increase the ILS from 121 to 215 ($p <$ .0001) compared with free DOX at 10 mg/kg (Table 8; Cabanes et al., 1998). When administered by i.p. injection, the life spans were identical (ILS = 60.5%) for free and L-DOX, showing that a considerable difference in effectiveness does exist depending on the site of administration.

4. Frequency of Injection. The frequency of drug injection is also likely to have an effect on the therapeutic response. CLs require high doses of both lipid (>100 mg/kg) and drug (>20 mg/kg) to obtain comparable tumor levels of drug to SSL DOX (see *IIIB. Rate and Extent of Accumulation in Tumors*; Mayer et al., 1997; Parr et al., 1997). At these high doses, repeated injections may not be possible due to nonspecific toxicities. In addition to the toxicities associated with the drug, Allen et al. (1984) have shown that multiple injections of free liposomes at high doses also cause significant toxicity to liver and spleen (see *VIA. Tolerability of Liposome Components*). The dose independence of SSL DOX allows liposomes to be administered at low doses on a schedule that varies from once a week to once every 4 weeks. This is likely necessary to keep tumor drug levels high and thus maintain a greater efficacy. If CLs are found to have similar efficacy at a single high dose, then the next step will be to show that this similarity in efficacy can be maintained after multiple injections without compromising the reduced toxicity of the drug.

5. Environment of Tumor. The site of tumor implantation is also important in determination of the relative efficacy of a liposomal drug formulation. Tumors vary in permeability, vascular density, and response to local permeability or growth factors depending on the microenvironment of the tumor (Dellian et al., 1996; Fukumura et al., 1997; Hobbs et al., 1998). Tumors implanted s.c. have different properties from those implanted in the liver or in the brain. Tumors found in the liver and

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spleen may be more susceptible to drug delivered by CLs than tumors in other areas of the body, due to the ability of CLs to localize rapidly and preferentially in these organs. Drug released from macrophages may kill neighboring tumor cells through the bystander effect (Storm et al., 1988). This underscores the question as to why more studies have not been completed with liver metastatic models. Early studies completed with DOX loaded in PG- or PS-containing liposomes demonstrated an enhanced activity toward liver metastasis of colon carcinomas (Mayhew et al., 1987) or lymphomas (Gabizon et al., 1993) compared with free DOX. These, or similar, liposome formulations were ineffective against a variety of cancers located elsewhere in the body (Gabizon et al., 1990). The close proximity of liver metastasis to Kupffer cells responsible for liposomal drug uptake may make tumor models more sensitive to treatment with liposomal drug therapy and alter the characteristics necessary for effective treatment.

Allen et al. (1992) showed that mice injected i.v. with L1210 leukemia cells were treated more effectively after the encapsulation of ara-C in liposomes than by a 24-h infusion of free ara-C, presumably because liposomes can more efficiently deliver the drug to liver or splenic tumors. In the same study, liposomes that released ara-C at a faster rate were shown to have the greatest activity (Table 8). This same group used liposomal VCR to show that depending on the route of tumor implantation for L1210 leukemic cells, drugs may benefit from either a more rapid or a slower release from the liposomal carrier (Allen et al., 1995b). These authors concluded that peritoneal or s.c. tumors may be more amenable to slow-release systems, whereas intravascular cancers are better treated with rapid-release systems. Another rather elegant study used liposomal mitoxantrone encapsulated in different liposome formulations to show that liposomes that rapidly release their contents may be more efficacious than slow-release formulations in two such tumor models (Lim et al., 1997). L1210 and P388 cells seed preferentially in the liver and spleen when injected i.v. (Lim et al., 1997). In this study, mitoxantrone delivered via DMPC/Chol liposomes (rapid-release liposomes) were considerably more effective than when delivered via DSPC/Chol liposomes (Table 8; >590 versus 189% ILS), suggesting the rapid leakage of drug from the former carrier may facilitate the cytoxic activity of the drug in this model. In addition, CLs (DSPC/Chol) were shown to have a similar percent ILS compared with SSL-mitoxantrone at both 10 and 20 mg/kg (Table 8; Chang et al., 1997). Although SSL-mitoxantrone may distribute to the tumors themselves to a greater extent, the high concentration of mitoxantrone in the liver and spleen, due to its rapid uptake by RES macrophages in these organs, may provide higher overall concentrations of bioavailable drug after its release from these macrophages. Unlike DOX, mitoxantrone was unable to increase the circulation lifetime by poisoning RES macrophages. The studies suggest that distribution to the liver and spleen may favor a good therapeutic response with CLs programmed to release the encapsulated drug at a faster rate than experienced with solid DSPC/Chol or PEG-DSPE/DSPC/Chol liposomes. Although these experiments show that proximity of tumors to RES macrophages may alter the characteristics needed to engineer effective liposomal drug delivery vehicles for their treatment, it should be noted that both L1210 and P388 tumors are fast growing tumors that likely favor rapid drug release for the reasons mentioned earlier. It would be interesting for the authors to repeat these experiments with slower growing tumors to see whether a more general relationship truly exists between tumor environment and drug-release characteristics.

C. Efficacy with Nonanthracyclines

There have been several in vivo therapeutic efficacy studies completed with drugs other than anthracyclines. SSL-cisplatin (SPI-77) is presently being developed by Alza Corporation (Palo Alto, CA; formerly Sequus Pharmaceuticals, Inc., Menlo Park, CA). Although details are limited, SSL-cisplatin proved more efficacious than free cisplatin in both Lewis lung carcinoma and C26 colon carcinoma tumor models (Working et al., 1998; Newman et al., 1999), delaying tumor growth in one model by >30 days compared with 3.7 for free cisplatin. In another study, SSLs were used to deliver ara-C in a murine L1210/C2 leukemia model (Allen et al., 1992). Encapsulation in SSLs increased the percent ILS from 90 to 197%. The liposomes were shown to act as a slow-release depot for drug, and the increased therapeutic efficacy was thought to result primarily from this effect and not preferential accumulation in tumors. These liposomes were not optimized for long circulation and high tumor accumulation because the liposomes were prepared by reversed-phase evaporation followed by extrusion through 0.4-μm-pore filters, giving liposomes in excess of 400 nm.

Several studies have been completed with liposomal paclitaxel (Sharma et al., 1995, 1996, 1997). In two of these studies, liposomal paclitaxel showed significant activity against human ovarian tumor xenografts, inhibiting tumor growth (Sharma et al., 1995, 1997). The liposomes in these studies incorporated paclitaxel into liposomes at low drug/lipid ratios (1:33), much lower than that used for DOX (see Table 1), likely a result of the drug being carried in the liposomal membrane and not encapsulated within its internal aqueous space, as is DOX. This is consistent with the physicochemical properties of the drugs, where hydrophobic drugs such as paclitaxel would be expected to reside in the hydrophobic membrane core and amphipathic drugs, such as DOX, can be loaded by remote-loading techniques (see *VIIA1. Drug-Loading Methods*) to high concentrations in the encapsulated aqueous space. The pharmacokinetic pa-

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rameter associated with the drug were similar for liposomal paclitaxel and paclitaxel formulated with Cremophor EL, suggesting that liposomes in this formulation are simply acting as a drug-solubilizing agent (Sharma et al., 1997), and the drug rapidly redistributes to other hydrophobic sites after administration. The high toxicity of the Cremophor EL vehicle makes delivery by liposomal solubilization therapeutically beneficial, due to the low toxicity of the liposomal carrier (see *VIA. Tolerability of Liposome Components*). However, because this formulation acts as an extremely rapid-release liposomal carrier, it differs from slow-release systems, which selectively accumulate in tumors and release their contents on a more compatible time scale. A lipophilic cisplatin derivative, *cis*-bis-neodecanoato-*trans*-*R*,*R*-1,2-diaminocylcohexaneplatinum(II), has also shown some promise when incorporated into liposomal membranes (Mori et al., 1996). This prodrug reverts to the active drug after hydrolysis.

Finally, liposomal VCR has been the most thoroughly studied nonanthracycline liposome formulation in vivo. VCR is an alkaloid derived from *Vinca rosea* that has been used clinically for the treatment of various types of cancer (Carter and Livingston, 1976). Like other *Vinca* alkaloids, VCR exerts its antitumor activity by inhibiting cell division via interactions with tubulin (Owellen et al., 1976). The major dose-limiting toxicities of VCR is a peripheral neurotoxicity (Rowinsky and Donehower, 1996). VCR exhibits low solubility in aqueous solution at physiological pH and relatively high permeability to membranes. Due to physicochemical similarities with DOX, methods of drug loading in liposomes developed for DOX could be efficiently used for VCR; this is discussed in more detail in *VIIA1. Drug-Loading Methods*.

For CL formulations, the exchange of DSPC ($T_m >$ 37°C) for eggPC ($T_m \ll$ 37°C) increased the circulation lifetime of VCR by >100% (Mayer et al., 1990b, 1993). Despite this advance, no significant difference in the toxicity profile were observed compared with free VCR in dogs, although a moderate reduction in toxicity could be observed in mice (Kanter et al., 1994). However, the drawback of liposomal drug retention was still to be overcome because 85 to 90% of encapsulated VCR leaked from DSPC/Chol liposomes within 24 h of i.v. administration (Boman et al., 1994). Webb et al. (1995) showed that drug retention was increased after the substitution of SM for DSPC, and this translated into a significant improvement in efficacy in BDF1 mice bearing P388 tumors (214 versus 38% ILS for SM- and DSPC-containing liposomes, respectively). This demonstrates the importance of maintaining a stable formulation for the effective use of liposomes as a drug delivery system and that a particular lipid composition is not necessarily the best for all drugs, even if both are amphipathic in nature. Each drug must be considered individually.

SSL formulations of VCR in which liposomes were coated with GM_1 showed highly efficient cures of mice with P388 leukemia (Boman et al., 1994). When PEG was used as the stabilizing agent, liposomal VCR showed efficient antitumoral activity in s.c. and i.p. solid tumors but did not improve efficacy on rapidly growing i.v. disseminated leukemias (Allen et al., 1995b). This study also did not find a significant difference in the LD_{50} of SSLs and free VCR in mice, with both having an LD_{50} of ~2.5 mg/kg (Allen et al., 1995b). In this study, pharmacokinetic studies comparing SL- and CL-VCRs were performed using EPG/HSPC/Chol liposomes as a CL formulation. Although similar in surface charge to the SSL formulation, the presence of the exposed negative charge in EPG results in a relatively rapid clearance of the CL formulation from the circulation, essentially accentuating the differences between CLs and SSLs. Other studies have used a similar CL control with studies of L-DOX delivery (Mayhew et al., 1992; Vaage et al., 1992; Williams et al., 1993; Sakakibara et al., 1996). In our opinion, the use of small neutral CL formulations as a control when comparing SSL and CL formulations may be more accurate and informative as to the extent of the differences between optimized formulations of both SSLs and CLs. Finally, combination therapy, using SSL VCR and DOX, gave highly efficient stop growth and disappearance of mammary carcinoma MC2 bearing mice at doses for which no toxic systemic side effects could be detected (three weekly injections of 1.3 and 6 mg/kg for liposomal VCR and DOX, respectively; Vaage et al., 1993b).

D. Multidrug Resistance

Multidrug resistance can severely limit the effectiveness of some types of chemotherapy. Although drug resistance can take on many forms, one of the most common comes in the form of the multidrug resistance transporter, a membrane-spanning ATPase located in the plasma membrane and responsible for the efflux of positively charged amphipathic drugs from the cell (Endicott and Ling, 1989; Pastan and Gottesman, 1991; Gottesman and Pastan, 1993). Overexpression of P-glycoprotein (Pgp170) in tumor cells can lead to a marked decrease in sensitivity to drugs such as DOX. The delivery of L-DOX has resulted in the effective treatment of a number of chemotherapy refractory cancers both in animal models and in the clinic (Treat et al., 1990; Vaage et al., 1994b; Muggia et al., 1997; Northfelt et al., 1997). This has raised some questions as to whether L-DOX is able to sensitize tumor cells and thus partially reverse multidrug resistance.

Results in cell culture suggested that drug resistance could be partially reversed by treatment with L-DOX, although these cells were still significantly less sensitive than non-drug-resistant cell lines (Richardson and Ryman, 1982; Thierry et al., 1989; Rahman et al., 1992). The mechanism responsible for liposome-mediated partial reversal of drug resistance is not well understood. Several of the formulations used in these studies contain negatively charged phospholipid components, such as

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phosphatidylserine (Fan et al., 1990) or cardiolipin (Thierry et al., 1989; Oudard et al., 1991; Rahman et al., 1992), which may act to directly regulate the P-glycoprotein transporter. Alternatively, they may act to provide sustained high levels of drug to the resistant cells over long periods of time (Allen, 1998), or if endocytosed, they may deliver the drug internally where it doesn't immediately reach the P-glycoprotein transporters located in the plasma membrane (Mickisch et al., 1992). Apart from cell sensitization, liposomal drug delivery may help overcome a broader range of drug resistance due to favorable pharmacokinetics. Thus, the increased response rates in these refractory patients may have to do with the increased concentration of drug that accumulates in the tumor after treatment with L-DOX. In any event, although the fact that L-DOX appears to be more effective against refractory patients is an encouraging observation, the likelihood that DOX delivered via liposomes will completely reverse multidrug resistance is low. Thus, to more effectively treat patients resistant to a particular type of chemotherapy, it will be important to combine L-DOX with other presently used chemotherapeutic agents or develop additional liposomal chemotherapeutic agents, with nonoverlapping mechanisms of drug resistance.

V. Clinical Efficacy of Liposomal Anthracyclines

There are three forms of L-DOX or daunorubicin being manufactured by different pharmaceutical companies. The properties of these formulations are given in Table 1. Doxil and DaunoXome have been approved for the treatment of AIDS-related Kaposi's sarcoma and are being evaluated in clinical trials for the treatment of a variety of cancers (Eckardt et al., 1994; Gill et al., 1995; Muggia et al., 1997; Ranson et al., 1997; Martin, 1998; Northfelt et al., 1998; Schmidt et al., 1998). The Liposome Company, Inc. has recently completed several large phase II and phase III clinical trials using EVACET (also known as TLC D-99) for the treatment of metastatic breast cancer and is now awaiting approval for the drug by the Food and Drug Administration (Harris et al., 1998; Swenson et al., 1998; Valero et al., 1999). The data obtained from trials thus far suggest that all three liposomal drugs offer a significant therapeutic benefit compared with the free drug and often compared with current chemotherapy combinations indicated for the studied form of cancer. Liposomal drugs can be therapeutically beneficial based on their ability to decrease nonspecific toxicities associated with the drug, a process referred to as toxicity buffering, or by being more efficacious against a specific type of cancer, increasing the response frequency, average time to relapse, or response duration. DaunoXome and Doxil have been shown to offer similar or greater efficacy, and decreased levels of most toxicities (see *VI. Toxicology of Liposomal Chemotherapy*; Table 9) compared with free DOX and standard chemotherapy regimens (Gabizon et al., 1994; Gill et al., 1995, 1996; Muggia et al., 1997; Ranson et al., 1997; Martin, 1998; Northfelt et al., 1998; Stewart et al., 1998). EVACET was shown to decrease most toxicities and has a similar response frequency to DOX alone (Batist et al., 1998; Harris et al., 1998).

This review focuses primarily on PC/Chol CLs and SSLs. There have been several earlier clinical studies with alternative formulations, mostly containing small quantities of negatively charged lipids, but due to their unproven clinical utility they are not discussed further in this review. The reader is referred to the following references for information on these studies (Gabizon et al., 1989, 1991; Treat et al., 1990; Rahman et al., 1992; Gabizon, 1998). In addition, efficacy can be dependent on a variety of different patient characteristics; these include such characteristics as sex, age, prior chemotherapy treatments, degree of disease severity, presence of metastatic disease, and overall performance status. The reader is encouraged to return to the original citations to obtain information concerning these characteristics because a detailed evaluation of the complete clinical findings is beyond the scope of this review.

A. AIDS-Related Kaposi's Sarcoma

Kaposi's sarcoma is the most common neoplasm associated with AIDS (Northfelt, 1994). It is characterized by painful and disfiguring cutaneous lesions that can also have tumor-associated lymphedema. Some patients have visceral involvement, including gastrointestinal and pulmonary nodules. Single-agent standard chemotherapy is relatively ineffective. Until recently, combination regimens, including bleomycin/VCR (BV) or DOX/bleomycin/VCR (ABV), were most commonly used as a front-line defense (Gill et al., 1990, 1994). Both liposomal daunorubicin and SSL DOX have shown significant activity against Kaposi's sarcoma in a number of phase II and III clinical trials (Presant et al., 1993; Simpson et al., 1993; Gill et al., 1995, 1996; Harrison et al., 1995; Girard et al., 1996; Amantea et al., 1997; Coukell and Spencer, 1997; Northfelt et al., 1997, 1998; Stewart et al., 1998). Response rates have varied from 25 to 73.5% depending on patient characteristics and trial design. Many of the results from these trials are listed in Table 9.

Recently, Doxil (20 mg/m^2) was shown to compare very favorably with either the ABV (20 mg/m^2:10 mg/m^2:1 mg) or BV (15 IU/m^2:2 mg) regimens (Northfelt et al., 1998; Stewart et al., 1998). The overall response rate in the ABV comparison was 45.9% for Doxil and 24.8% for the ABV arm (Northfelt et al., 1998). Doxil showed an overall response rate of 58.7% compared with 23.3% for the BV arm (Stewart et al., 1998). In both of these studies, the duration of response was similar for both arms of the study. In addition to the superiority in response rate achieved with Doxil, both studies reported a significant decrease in certain toxicities and greater

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TABLE 9
Phase II and III clinical studies with L-DOX

Cancer[a]	Liposome Formulation[b]	Dose	Schedule	Total Responses	Complete Response	Response Duration
		mg/m²		%		*days*
Kaposi's sarcoma (n = 16)[e]	Doxil	20	×3 wk	75		98
Kaposi's sarcoma (n = 24)[f] (patients failed standard chemotherapy)	DaunoXome	40	×2 wk	54.2	8.3	84
Kaposi's sarcoma (n = 22)[g]	DaunoXome	50–60	×2 wk	55	5	
Kaposi's sarcoma (n = 34)[h]	Doxil	20	×3 wk	73.5	5.8	63
Kaposi's sarcoma (n = 29)[i]	Free DOX	20	×2 wk	48	3	105
Kaposi's sarcoma (n = 116)[j]	DaunoXome	40	×2 wk	25	2.5	175
	ABV	10, 15 U, 1 mg	×2 wk	28	0.9	168
Kaposi's sarcoma (n = 30)[k]	DaunoXome	40	×2 wk	73		153
Kaposi's sarcoma (n = 29)[l]	DaunoXome	40	×2 wk	45		70
Kaposi's sarcoma (n = 53)[m] (patients failed standard chemotherapy)	Doxil	20	×3 wk	36	1.8	128
Ovarian carcinoma (n = 35)[c, n]	Doxil	40–50	×3 wk	25.7	2.9	180
Metastatic breast carcinoma (n = 64)[o]	Doxil	45–60	3–4 wk	31	6.3	270
Kaposi's sarcoma (n = 40)[p]	Doxil	20	×3 wk	70		
Kaposi's sarcoma (n = 121)[q]	Doxil	20	×3 wk	58.7	5.8	160.4
	BV	15 IU/m², 2 mg	×3 wk	23.3	0.8	156.7
Kaposi's sarcoma (n = 133)[r]	Doxil	20	×2 wk	45.9	0.8	90
	ABV	20, 10, 1 mg	×2 wk	24.8		92
Metastatic breast carcinoma (n = 69)[s]	TLC D-99	75	×3 wk	33	3	
	Free DOX	75	×3 wk	28	1	
Metastatic breast carcinoma (n = 69)[t]	TLC D-99/ CPA/5-FU[e]	60, 500, 500	×3 wk[d]	68	5	

CPA, cyclophosphamide; 5-FU, 5-fluorouracil.
[a] Values are number of accessable patients.
[b] The composition, size, and drug/lipid ratio for DaunoXome and Doxil are listed in Table 1.
[c] Patients failed to respond to platinum- and paclitaxel-based regimens.
[d] Both CPA and TLC D-99 were administered on day 1, and 5-FU was administered on days 1 and 8.
[e] Simpson et al. (1993).
[f] Presant et al. (1993).
[g] Gill et al. (1995).
[h] Harrison et al. (1995).
[i] Gill et al. (1991).
[j] Gill et al. (1996).
[k] Girard et al. (1996).
[l] Uthayakumar et al. (1996).
[m] Northfelt et al. (1997).
[n] Muggia et al. (1997).
[o] Ranson et al. (1997).
[p] Amantea et al. (1997).
[q] Stewart et al. (1998).
[r] Northfelt et al. (1998).
[s] Harris et al. (1998).
[t] Valero et al. (1999).

patient compliance with the liposomal drug. Similar to patients receiving daunorubicin (Gill et al., 1996), patients receiving Doxil developed more opportunistic infections than those receiving the standard chemotherapy regimens (Stewart et al., 1998). The toxicological advantages are described in more detail in *VI. Toxicology of Liposomal Chemotherapy*.

DaunoXome was also compared with the ABV regimen in a large randomized trial (232 patients; Gill et al., 1996). DaunoXome (40 mg/m^2) was found to have an overall response rate of 25 compared with 28% for the ABV arm (10 mg/m^2:15 U:1 mg) and an almost identical response duration (175 versus 168 days). Although DaunoXome did not appear to have any advantage over the ABV regimen in terms of response rate, patients receiving DaunoXome experienced less alopecia (8 versus 36%) and neuropathy (13 versus 41%) but a slightly greater incidence of opportunistic infections. Other differences in toxicities observed were not statistically significant.

An indirect comparison of Doxil and DaunoXome suggests that Doxil is significantly more active against Kaposi's sarcoma than DaunoXome. Although DaunoXome is comparable in response rate to the ABV regimen, Doxil shows a considerable increase in response rate compared with both ABV and BV regimens. The response duration was shorter for the Doxil study completed by Stewart et al. (1998). However, the duration of response was likely underestimated due to the inclusion of stable disease as an endpoint for response and the restriction in this study of a maximum number of six cycles of drug therapy (Bennett et al., 1998). Toxicities also appeared to favor Doxil over DaunoXome. This is not surprising, considering the DaunoXome dose was

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twice that of Doxil (40 versus 20 mg/m^2) and injections (every 2 weeks versus every 3 weeks) of daunorubicin were given at a higher frequency. In the treatment of Kaposi's sarcoma at least, SSLs appear to be far more efficient drug-delivery vehicles than CLs. Although DOX and DaunoXome are very similar in mechanism of action, pharmacokinetic parameters, and toxicological profiles, the difference in the encapsulated drug precludes us from making any definitive statements concerning the superiority of SSLs based on these observations. In addition, the weak immune system and increased susceptibility to opportunistic infections of AIDS patients prevent escalation of the daunorubicin dose to dosages that may allow for longer circulation lifetimes of CLs.

B. Treatment of Breast and Ovarian Carcinomas

L-DOX was suggested to have greater activity against breast and ovarian cancers, which are typically only moderately sensitive to DOX, due to the enhanced tumor accumulation of the drug. In patients with advanced ovarian cancer, who were refractory toward paclitaxel- and platinum-based regimens, Doxil was shown to have a response rate of 25.7% and a response duration of 180 days (Table 9; Muggia et al., 1997). This favorable response rate was significantly greater than that for free DOX in similar patients (<10%; Young et al., 1981), and there were fewer problems with patient compliance due to reduced toxicities (Alberts and Garcia, 1997).

Two studies have been completed with SSL DOX (Doxil) and CL DOX (TLC D-99) in metastatic breast cancer patients (Table 9; Ranson et al., 1997; Harris et al., 1998). Response rates were similar in the two studies (31% for Doxil and 33% for TLC D-99). The response rate of free DOX was 29% in the randomized phase III study comparing TLC D-99 and free DOX (both at 75 mg/m^2). This response rate for free DOX was similar to that described previously in similar patients (Young et al., 1981). It will be interesting to do a more detailed comparison of Doxil and TLC D-99 when the full results from the clinical trials with TLC D-99 are published. Pharmacokinetic and tumor accumulation considerations would theoretically favor Doxil. However, TLC D-99 is administered at a dose of 75 mg/m^2 compared with 45 mg/m^2 for Doxil (both are administered every 3 weeks). Thus, the higher dose and increased rate of drug release from the carrier at the tumor may contribute to its similar activity. The principal reason for the decreased dose in the case of Doxil is the high incidence of hand and foot (H-F) syndrome associated with Doxil at elevated doses (60 mg/m^2). In both studies with ovarian and breast cancers, H-F syndrome was dose limiting (Muggia et al., 1997; Ranson et al., 1997). H-F syndrome is characterized by dermal lesions on both the palms of the hand and soles of the feet and is also found in patients receiving prolonged infusions of some chemotherapeutic agents. This toxicity is described in more detail in *VI. Toxicology of Liposomal Chemotherapy*. If the severity of H-F syndrome can be controlled by means other than dose reduction, then the administration of a similar dose will almost certainly give rise to an enhanced therapeutic effect. Mucositis, another significant toxicity in patients treated with Doxil, causing dose modification in some patients (Muggia et al., 1997; Ranson et al., 1997). The plasma AUC of TLC D-99 at elevated doses (Cowens et al., 1993; Embree et al., 1993) is far below the plasma AUC of Doxil at 25 or 50 mg/kg DOX (Table 4; Gabizon et al., 1994). These results emphasize the fact that the long circulating property of SSLs is not the only factor responsible for increased levels of efficacy with liposomal drugs. A mechanism by which DOX is at least partially released from the liposome in the circulation, avoiding the high peak levels of drug responsible for some types of toxicities, is most likely responsible for the increased therapeutic effect of TLC D-99. Thus, the altered toxicity profile of TLC D-99 allows the dose to be escalated to a point at which the efficacy is comparable to Doxil. The long-term effects of this dose escalation on the cumulative cardiotoxicity of DOX, compared with Doxil, are unknown. However, recent results did show a significant reduction in the cardiotoxicity when TLC D-99 was compared with free DOX (Batist et al., 1998). The reader is also referred to other reviews on the clinical activity of both Doxil (Gabizon, 1994, 1998; Coukell and Spencer, 1997; Muggia, 1997; Martin, 1998) and DaunoXome (Forssen and Ross, 1994; Schmidt et al., 1998) for additional analysis of the clinical data.

VI. Toxicology of Liposomal Chemotherapy

A. Tolerability of Liposome Components

"Empty" CLs or SSLs are usually considered nontoxic unless administered at very high doses (Storm et al., 1993; Working and Dayan, 1996). This is one of the characteristics that makes them attractive as a drug delivery vehicle and is not surprising because they are typically composed of natural lipids and small amounts of well-tolerated synthetic stabilizers (PEG-DSPE). At very high doses (multiple injections at a dose of ≥100 mg/kg lipid), liposomes have been shown to result in an impairment of RES function, hepatomegaly, granulomas, and splenomegaly (Allen et al., 1984, 1987; Allen and Smuckler, 1985; Storm et al., 1993). However, these effects are usually considered irrelevant due to the dose-limiting effects of the encapsulated drug. PEG is considered nontoxic at the degree of polymerization (1900–5000 Da) used to prepare SSLs and is excreted unmetabolized in the urine (Carpenter et al., 1971). Toxicity studies completed with PEG-DSPE micelles at a concentration 30-fold greater than that applied in a standard dose of SSL DOX demonstrated no deaths or clinical signs of toxicity (Working and Dayan, 1996). In addition, the various types of toxicities observed with SSL DOX treatment in animals are consistent with

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those for free DOX, although at significantly reduced levels (Working and Dayan, 1996). Although for SSL DOX these results suggest that the lipid components have little if any effect on the overall toxicity profile, the picture for CLs is less clear. SSL DOX is typically administered at a total lipid dose of 8 to 54 mg/kg (1–6 mg/kg DOX) in rats or mice, with subsequent injections occurring between 3 days and 4 weeks. However, using CL a number of recent studies have used a single bolus injection of 100 to 300 mg/kg total lipid and 20 to 55 mg/kg DOX (Mayer et al., 1989, 1997; Parr et al., 1997). One of these studies showed minimal improvements in therapeutic efficacy over free DOX (Mayer et al., 1997). If multiple injections are to be used, then the toxicity of the lipid component may eventually become important; this will require further study.

In addition, an increasing lipid dose has been shown to deplete plasma of various proteins (Senior, 1987; Oja et al., 1996). Both the quantities and types of protein bound to liposomes are dependent on the lipid composition (Senior, 1987; Oja et al., 1996). Although the identity and significance of all the depleted proteins are unclear, it is possible that their loss will result in a disruption of normal homeostasis. Although toxicities related specifically to high lipid doses might prove to be relevant in some situations, in most instances the toxicity of the encapsulated drug is considered to be far more limiting.

B. Toxicities Associated with Free Drug

For the treatment of cancer, liposomal drug delivery has primarily involved the use of anthracyclines such as DOX or daunorubicin (Gabizon, 1994; Papahadjopoulos and Gabizon, 1995; Martin, 1998) or *Vinca* alkaloids such as VCR (Vaage et al., 1993b; Allen et al., 1995b; Webb et al., 1995). A few studies have chosen alternative drugs such as paclitaxel (Sharma et al., 1996, 1997), ara-C (Allen et al., 1992), methotrexate (Matthay et al., 1989; Jones and Hudson, 1993), or cisplatin derivatives (Perez-Soler et al., 1990; Mori et al., 1996), but preclinical toxicological results with these drugs are limited at the present time. Because the toxicity profile of a liposomal drug is primarily dependent on the encapsulated drug rather than the lipids, it is first important to understand the toxicities associated with the free drug. With free DOX, myelosuppression is considered dose limiting (Legha et al., 1987; Speth et al., 1988; Doroshaw, 1996). However, the therapy-limiting toxicity is considered to be cardiotoxicity and results after a high cumulative dose of the drug (Von Hoff et al., 1979; Doroshaw, 1996). High peak levels of DOX in the plasma have been shown to correlate with an increased risk of cardiac toxicity. When delivered by bolus injection, the most commonly used total cumulative dose of DOX is 450 to 500 mg/m^2, where the risk of cardiac toxicity is between 1 and 10% (Doroshaw, 1996). There is significant evidence that DOX delivered by continuous infusion displays similar efficacy but reduced toxicity, with cumulative doses up to 1100 mg/m^2 delivered without signs of cardiac toxicity (Legha et al., 1987; Hortobagyi et al., 1989). Although drug-induced congestive heart failure is the most significant concern due to its very poor prognosis, it is lethal in 60% of patients (Von Hoff et al., 1979); there are several other important toxicities associated with anthracyclines.

DOX-induced myelosuppression and alopecia are also delayed toxicities but are independent of the rate of drug administration (Speth et al., 1988; Doroshaw, 1996). Recent work has shown that myelosuppression can be partially combated with the use of colony-stimulating factors (granulocyte-CSF and granulocyte-macrophage-CSF), which stimulate activation and proliferation of hematopoietic cells (Vose and Armitage, 1995; Petros and Peters, 1996; Henry, 1997; Lieschke et al., 1997; Nemunaitis, 1997; Clemons et al., 1998). These growth factors are currently being used to allow dose intensification of conventional chemotherapy by effectively reducing one of the most common dose-limiting toxicities. Another toxicity associated with anthracyclines is a severe necrosis of the skin adjacent to the site of injection due to injection related drug extravasation (Von Hoff et al., 1979; Doroshaw, 1996). The resulting lesions are difficult to treat, and extreme care should be taken to prevent infection at these sites. Nausea, vomiting, and mucositis, including gastrointestinal toxicity and stomatitis, are additional toxicities resulting from chemotherapy with anthracyclines (Speth et al., 1988; Doroshaw, 1996). Finally, H-F syndrome, which is characterized by severe dermal lesions on the soles of the feet and the palms of the hand, is seen in patients receiving long-term continuous infusions of free DOX (Lokich and Moore, 1984; Vogelzang and Ratain, 1985). As discussed in *Effect of Liposome Encapsulation on Toxicity Profile*, although encapsulation of anthracyclines in liposomes does not alter the types of toxicities observed, the severity can be significantly reduced due to the resulting alterations in the pharmacokinetics and tissue distribution of the drug. In addition to these alterations, toxicity buffering is also a result of the relatively slow release of the DOX from the liposome, giving rise to relatively low peak levels of the free drug in the circulation.

C. Effect of Liposome Encapsulation on Toxicity Profile

1. Cardiotoxicity. The toxicity profile for L-DOX is altered due to the changes in pharmacokinetics described previously in *II. Pharmacokinetics and Biodistribution of Liposomes and Liposomal Drug*. Drug encapsulation in either SSLs or CLs eliminated or significantly reduced the amount of cardiotoxicity compared with the free drug (Table 10; Olson et al., 1982; Herman et al., 1983; Balazsovits et al., 1989; Working and Dayan, 1996; Working et al., 1999). This is thought to be due to the inability of liposomes to cross the endothelial cell barrier in the heart and the low bioavailabil-

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TABLE 10
Toxicities associated with free and L-DOX

Toxicity	Effect of Liposome Encapsulation
Cardiac toxicity	Reduced or not observed
Myelosuppression	Reduced (to a greater extent with SSLs)
Mucositis	Slightly increased with Doxil
Alopecia	Reduced or not observed
Severe local tissue necrosis after drug extravasation	Reduced
Nausea and vomiting	Reduced or not observed
H-F syndrome	Observed with Doxil or with continuous infusion of free DOX

ity of the free drug due to its encapsulation in liposomes (Gabizon, 1994, 1997). Indeed, in tissue sections of cardiac muscle, liposomes are found exclusively in the blood vessels and not in the muscle fibers (Working et al., 1994), suggesting that most of the drug is not bioavailable in the myocardium. A comparison of cardiotoxicity in beagle dogs showed a higher incidence of cardiomyopathy and vacuolization of cardiac muscle fibers when administered as the free drug compared with TLC D-99, confirming a protective effect of liposomal encapsulation on cardiotoxicity (Kanter et al., 1993). A similar cardioprotective effect was seen with SSL DOX in both rabbits and beagle dogs (Working and Dayan, 1996; Working et al., 1999). Compared with the considerable damage observed to the myocardium of beagle dogs treated with free DOX (cumulative dose of 10 mg/kg), no histiological indication of cardiotoxicity was seen when treated with the same cumulative dose of SSL DOX (Working et al., 1999).

Clinical data are limited due to the relatively low cumulative dose of DOX delivered in most studies. In one phase I study, four patients received a total cumulative dose of >500 mg/m^2. There was no clinical cardiotoxicity or decrease in the resting left ventricular ejection fraction in any of these patients (Casper et al., 1997). In a recent study with TLC D-99 for the treatment of metastatic breast cancer (75 mg/m^2 every 3 weeks), 16% of patients on TLC D-99 had a cardiac event (either a problem with the electrocardiogram or a decrease in left ventricular ejection fraction), but there were no instances of congestive heart failure, compared with 25% of patients with cardiac events and three instances of congestive heart failure for patients receiving free DOX (Batist et al., 1998). Although significant compared with free DOX, this reduction in cardiotoxicity is not as great as expected for stable liposomes that show reduced drug leakage in the plasma. Patients with a cumulative dose of liposomal daunorubicin between 600 and 1000 mg/m^2, including one patient with a cumulative dose of >1000 mg/m^2, showed no evidence of cardiotoxicity (Gill et al., 1995). Recently, endomyocardial biopsy was used to demonstrate significantly less DOX-induced cardiac damage in patients receiving SSL DOX (cumulative doses of 440–840 mg/m^2) compared with two earlier studies with free DOX (cumulative doses of 174–671 mg/m^2; Berry et al., 1998). In another study, patients receiving 40 to 50 mg/m^2 Doxil every 3 to 4 weeks for the treatment of refractory ovarian cancer showed no decrease in the left ventricular ejection fraction (Muggia et al., 1997); this included nine patients who received >550 mg/m^2. A similar response was seen with breast cancer patients treated with Doxil (Ranson et al., 1997), with only 1 of 71 patients showing a decrease in the ejection fraction of >10%. It should be noted that the average administered dose in this final study was only 179 mg/m^2 (45–399 mg/m^2), which is well below the recommended maximum cumulative dose for free DOX. From these limited studies, it appears that Doxil, TLC D-99, and DaunoXome are significantly less cardiotoxic than the free drugs. However, the data available thus far are unable to indicate conclusively a benefit for one liposomal formulation over another. A more thorough study of the cardiotoxicity at higher cumulative doses is needed to establish a new recommended cumulative dose for liposomal formulations.

2. Vesicant Properties. The vesicant effect seen with free DOX is also markedly reduced by encapsulation in eggPC/Chol- (Balazsovits et al., 1989) or HSPC- (Gabizon et al., 1993; Oussoren et al., 1998) containing liposomes. Mice injected s.c. with free DOX showed severe necrosis, acanthosis, edema, and inflammatory infiltration when free DOX was injected, whereas only a mild edema and inflammatory infiltration were observed with SSL DOX (Gabizon et al., 1993). This suggests liposomes are able to effectively protect the skin from vesicant damage due to DOX until the carrier can be drained from the injection site by the lymph and the blood.

3. Myelosuppression. The toxicity of DOX-loaded liposomes is extremely sensitive to the rate of drug leakage from the liposome in the circulation, and thus the lipid composition (Mayer et al., 1989; Bally et al., 1990a; Oussoren et al., 1998). Liposomes composed of Chol and high-phase transition phospholipids such as DSPC or SM have rigid membranes and retain the drug well in the circulation. However, when DSPC is replaced by the more fluid eggPC, DOX is able to more readily transverse the membrane and be released into the general circulation (Hwang, 1987; Mayer et al., 1989; Gabizon et al., 1993). The free drug is generally considered responsible for most types of toxicity. Indeed, the calculated LD_{50} decreased almost 3-fold from 161 to 57 mg/kg DOX when going from DSPC/Chol (55:45) to eggPC/Chol (55:45) liposomes (Mayer et al., 1989). It should be noted, however, that the LD_{50} for eggPC/Chol (55:45) liposomes was still greater than two times that for free DOX, indicating that even encapsulation in "leaky" liposomes provides some degree of toxicity buffering. The effect of DOX on myelosuppression was also greater when encapsulated in eggPC/Chol liposomes compared with in

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DSPC/Chol liposomes (Bally et al., 1990a). The decrease and recovery in the number of bone marrow cells (90% reduction on day 3) and in spleen weight were similar for free DOX and DOX-loaded eggPC/Chol liposomes (100 nm) administered at a dose of 20 mg/kg. DOX-loaded DSPC/Chol liposomes depressed the number of bone marrow cells by only 40% and were around normal levels on day 7. The effect on spleen weight was less severe, causing a 23% reduction on day 1 that returned to normal by day 14, compared with a 50% reduction for free DOX or DOX-loaded eggPC/Chol liposomes. An unusual finding was that DOX-loaded DSPC/Chol liposomes caused extended reductions in peripheral white blood cell counts (leukopenia) that were ~50% below initial values on day 14, a time at which mice treated with eggPC/Chol L-DOX had nearly returned to normal. From this study, eggPC/Chol L-DOX showed little improvement over free DOX in terms of myelosuppression. A similar study in dogs showed that free drug and TLC D-99 resulted in similar levels of myelosuppression (Kanter et al., 1993). This translated into the clinic where neutropenia and leukopenia were the most common adverse effects for both DaunoXome and TLC D-99 (Conley et al., 1993; Gill et al., 1996) and was dose limiting for TLC D-99 (Conley et al., 1993; Cowens et al., 1993; Casper et al., 1997). Myelosuppression could be partially controlled by the addition of colony-stimulating factors for bone marrow support (Casper et al., 1997).

4. Nausea, Vomiting, and Alopecia. Alopecia, mucositis, nausea, and vomiting were observed at all doses (75–105 mg/m^2) of TLC D-99 (Casper et al., 1997) but were less severe than free DOX when compared at a dose of 75 mg/m^2 (Harris et al., 1998). Although grade 3 and 4 alopecia, nausea, and vomiting were observed for DaunoXome delivered at 40 mg/m^2 every 2 weeks, it was usually <2 to 3%. Alopecia, nausea, and vomiting was rare in patients treated with Doxil at a concentration of 45 to 60 mg/m^2 (Muggia et al., 1997; Ranson et al., 1997). Myelosuppression was mild, occurring in most cycles at a grade of 2 or less (Ranson et al., 1997). Toxicity benefits were also observed in patients treated for AIDS-related Kaposi's sarcoma, where the combination of free Adriamycin, bleomycin, and VCR produced more instances and greater severity of most toxicities than Doxil (Northfelt et al., 1998).

5. Hand and Foot Syndrome (Palmar-Plantar Erythrodysesthesia Syndrome). One of the most significant toxicities for SSL DOX is a condition consisting of dermal lesions referred to as H-F syndrome) (Gordon et al., 1995; Uziely et al., 1995; Amantea et al., 1999). This same condition was previously described in patients receiving long continuous infusions of 5-fluorouracil, DOX, or vinorelbine (Lokich and Moore, 1984; Vogelzang and Ratain, 1985; Hoff et al., 1998) but is not observed in patients receiving chemotherapy by bolus injection. This particular toxicity, also called palmar-plantar erythrodysesthesia syndrome, is most likely a toxic effect of DOX on the rapidly dividing keratinocytes. Histological sections of the affected areas showed significant hyperkeratosis and parakeratosis in the stratum corneum of the epidermis (Gordon et al., 1995). Interruption of chemotherapy results in desquamation and reepithelialization of the affected areas and was complete within 3 to 7 weeks after the discontinuation of treatment (Gordon et al., 1995; Uziely et al., 1995). Interestingly, when free DOX is delivered via continuous infusion, the reversal of this syndrome was complete within 1 to 2 weeks after the discontinuation of treatment (Vogelzang and Ratain, 1985). There has been little success in reversing this toxicity without discontinuation of treatment. Discontinuation of treatment often leads to relapse of the cancer (Uziely et al., 1995). One promising strategy for the treatment of H-F syndrome without interruption of treatment involves the use of the strong reductant DHM3, which converts anthracyclines to the inactive 7-deoxyaglycone (Averbuch et al., 1985, 1986, 1988; Dorr, 1990). This agent was studied because of its capacity to reduce the vesicant effect observed with free DOX at the site of injection, but it may be useful in treating this syndrome as well. In other studies, the oral administration of pyridoxine (vitamin B_6) was shown to reduce the severity of H-F syndrome, resulting in fewer delays or discontinuations of treatment (Vukelja et al., 1989, 1993; Fabian et al., 1990; Vail et al., 1998). Topical dimethyl sulfoxide may be another method of reducing skin toxicity resulting from treatment with SSL DOX. It was previously shown to reduce vesicant damage in patients administered free DOX (Olver et al., 1988; Bertelli et al., 1995). The use of peripheral vasoconstrictors, such as ergotamine, may also potentially reduce the severity of H-F syndrome in patients treated with SSL DOX. Ergotamine is presently used in the treatment of migraine headaches (Perrin, 1995; Silberstein, 1997), but its ability to constrict blood vessels in peripheral tissues may restrict blood flow, and thus the accumulation of SSL DOX in the skin of the hands and feet. The choice and subsequent modification of the dose intensity appear to be the current anecdotal strategy for reducing the seriousness of this toxicity (Uziely et al., 1995; Ranson et al., 1997). The further study and use of compounds such as DHM3, pyridoxine, ergotamine, and dimethyl sulfoxide in combination with SSL DOX will potentially allow for dose intensification and increased antitumor efficacy.

It is possible that liposome-mediated drug delivery results in a slow-release mechanism of DOX into the tissues that mimics the effect seen with continuous infusion chemotherapy. Drug delivered by bolus injection may saturate mechanisms responsible for its uptake by the skin, where a majority of the drug can be removed from the circulation by alternative pathways: a wide tissue distribution and rapid excretion in the bile. A continuous infusion of free drug or slow release of DOX from SSL DOX results in lower peak levels of the drug in

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the circulation and possibly increased uptake by the skin. Alternatively, several studies have shown that long-circulating liposomes accumulate to a limited degree in skin (Gabizon et al., 1990, 1997), where slow release in the near vicinity of keratinocytes can give rise to their toxicity. Although H-F syndrome is a serious concern, the dosage and treatment schedule can be adjusted to minimize this toxicity and still maintain a high antitumor efficacy compared with standard chemotherapy regimens (45 mg/m^2 every 3 weeks; Ranson et al., 1997).

6. Mucositis. Mucositis was also slightly increased in patients treated with SSL DOX (Gabizon et al., 1994; Uziely et al., 1995; Alberts and Garcia, 1997). Like H-F syndrome, mucositis is increased by prolonged infusion of free DOX, so its increased incidence is not surprising (Alberts and Garcia, 1997). Stomatitis was dose limiting at single doses of >70 mg/m^2 in one study (Uziely et al., 1995), but at the doses used presently (45 mg/m^2 every 3 weeks), it is mild. Although most toxicities are greatly reduced with SSL DOX, those toxicities normally associated with prolonged infusions of the free drug seem to manifest themselves in SSL DOX, most likely as a result of the long circulation lifetimes.

7. Reticuloendothelial System Impairment and Opportunistic Infections. Despite claims to the contrary (Mayer et al., 1998), several studies have shown that both SSL and CL DOX can impair the phagocytic activity of liver macrophages (Kupffer cells), as well as significantly deplete their total numbers in rats, with the use of clinically relevant doses of L-DOX (Allen et al., 1984; Daemen et al., 1995, 1997). Mayer et al. (1998) referenced a dose of liposomal DOX (2 mg/kg) relevant in some studies with SSL DOX but <10-fold the dose of CL DOX used in studies from their laboratory (Mayer et al., 1997; Parr et al., 1997). Thus, at clinically relevant concentrations of CL DOX, macrophage toxicity and depletion do appear to be serious concerns. This is not surprising because the primary route of clearance for liposomal DOX of either form is via splenic or liver macrophages (Hwang, 1987; Senior, 1987), whereas free DOX is primarily excreted in the bile (Benjamin et al., 1974; Speth et al., 1988). Thus, their preferential accumulation at these sites might be expected to result in a toxic effect. RES impairment is a serious concern, especially in immunocompromised patients, where it is the first-line defense against bacterial or fungal infections. In addition to the increased susceptibility to infection (Qian et al., 1994), macrophage toxicity has been shown to result in a decreased ability to fight metastatic growth (Levy and Wheelock, 1974; Roh et al., 1992; Heuff et al., 1993). Although in theory this is a logical concern, there are at least two studies that suggest that L-DOX may enhance the efficacy in the treatment of liver metastatic cells due to its increased localization in the liver (Gabizon et al., 1983; Mayhew et al., 1987).

Male Wag/Rij or female R-strain albino rats treated with DOX-loaded DSPC/Chol (55:45) liposomes at a dose of 5 mg/kg DOX showed a reduced ability to phagocytose unloaded liposomes (70% after a single injection or 90% after three injections) or *Klebsiella pneumoniae* bacteria (Daemen et al., 1995). A decrease in the total number of macrophages by 56 or 85% for rats treated with two or three injections of L-DOX, respectively, was also observed. Placebo liposomes (25 μmol lipid/kg) or free DOX (5 mg/kg) had no measurable effect on phagocytic activity, indicating this toxicity was carrier dependent. The dose of L-DOX used to produce a therapeutic effect with DSPC/Chol liposomes has been from 20 to 55 mg/kg DOX in rats (Mayer et al., 1997; Parr et al., 1997), or 4- to 11-fold greater than the dose used in these studies (and 10-fold greater than the dose quoted by Mayer et al., 1998). The effects at 20 to 55 mg/kg DOX would be expected to be even greater and thus significantly inhibit RES function in both liver and spleen. The saturable pharmacokinetics, shown in *II. Pharmacokinetics and Biodistribution of Liposomes and Liposomal Drug* to be responsible for increased circulation lifetimes of high concentrations of CLs, results from a partial toxicity to RES macrophages. In these studies, liposomes of identical composition and size, but lacking DOX, had more rapid clearance rates, indicating the toxicity was due to DOX (Mayer et al., 1998).

Two studies have been carried out with SSL DOX (Daemen et al., 1997; Storm et al., 1998). The first study used doses and schedules similar to those chosen for the study with CL DOX mentioned earlier (Daemen et al., 1997). Male Wag/Rij rats received a single or two or three injections, at 3-day intervals, of 5 mg/kg SSL DOX. The maximum tolerated dose for SSL DOX in humans is 60 mg/m^2 DOX at 4-week intervals for patients with Kaposi's sarcoma (Uziely et al., 1995; Coukell and Spencer, 1997), which translates to ~1.5 mg/kg DOX in rats.

It should be noted that the liposomes used in the studies with a CL formulation were ~200 nm, almost twice that of optimized formulations (Daemen et al., 1995). The lipid composition of a second study, eggPC/Chol/PEG-DSPE (55:45:5), raises questions about the bioavailability of the drug due to the source of phosphatidylcholine chosen (Daemen et al., 1997). Although these studies suggest macrophage toxicity may be a serious concern, due to the size dependence of clearance for CLs (Hwang, 1987; Senior, 1987) and the increased bioavailability of DOX from egg PC-containing SSL DOX (Gabizon et al., 1993), a study comparing the effects of optimized L-DOX formulations on splenic and liver macrophages toxicity is warranted.

Clinically, CL daunorubicin (DaunoXome) is only indicated for advanced HIV-related Kaposi's sarcoma due to the short time until observance of opportunistic infections in treated patients compared with controls (145 versus 371 days; White, 1997). In a second case, SSL DOX was responsible for one case of fatal hepatic failure

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in an AIDS patient with impaired liver function (Hengge et al., 1993). Both of these cases suggest that toxicity to liver macrophages impairs bacterial or viral clearance. Although immunocompromised patients represent a special class of patients, not necessarily representative of the majority of cancer patients, the injected dose used in these studies was several fold-less than the dose needed to obtain the desired RES blockade effect required by Mayer et al. (1998). At these levels, even healthy patients may experience liver toxicity. Although this, of course, is speculative, it does suggest the need for additional toxicity studies in animals, specifically looking at the effect of L-DOX administration on RES and liver function. These studies may be more informative if formulations were optimized for size and composition of both CL and SSL DOX and at a range of relevant doses.

D. Final Comparisons of Conventional and Sterically Stabilized Liposomes

An additional problem when using CLs is the difficulty in predicting the effects of drug encapsulation on the different types of toxicity in humans based on earlier animal studies. As was mentioned previously, one of the most important advantages of steric stabilization is the dose independence provided by this particular carrier. Differences in serum opsonins between species, and thus rates of uptake by various tissues, may be more radically affected by CLs. Differences in pharmacokinetic parameters and toxicity profiles may differ not only between different animal species but also between different strains of the same animal model. Scid and immunodeficient nude mice are commonly used in antitumor efficacy studies. Scid mice have less efficient scavenging systems than normal mice and deficient DNA repair mechanisms. The high concentrations of L-DOX required to maintain long circulation times with CLs may prove especially toxic to these strains of mice and preclude their use. Even with SSLs, the dose must be scaled down significantly (1–2 mg/kg) to prevent significant drug-induced toxicities (Williams et al., 1993). This is possible with the dose-independent pharmacokinetics of SSL DOX but may not be possible with CL L-DOX.

The toxicology studies reviewed here show that liposome encapsulation offers significant protection against many common toxicities of anticancer drugs (Table 10). The degree of protection is higher when the liposomes leak their contents less readily. H-F syndrome and mucositis appear to be the most significant obstacle preventing dose escalation of the long-circulating Doxil. In animals, the dose of DOX being used in CLs (20 mg/kg) is between 3 and 20 times that being used with SSLs (1–6 mg/kg). Although some nonspecific toxicities may be slightly reduced for CL DOX (DSPC/Chol) compared with SSL DOX, DOX is unlikely to be tolerated at even three times the dose of SSL DOX in humans. The resulting increased levels of toxicity observed at the doses required to obtain long circulation will likely prevent their use in multidose regimens at these concentrations. It is unknown whether the drug-induced RES blockade required to obtain long circulation times will be maintained at schedules requiring lower doses and multiple injections.

In any event, independent of the liposome formulation, entrapment of DOX inside liposomes significantly alters the toxicity profile of DOX. This altered profile makes the liposomal drugs more tolerable, preventing patients from leaving treatments due to unbearable toxicities. Another related issue is the increased quality of life. Although alopecia, nausea, and vomiting are severe with many standard chemotherapeutic agents, they are rare or significantly reduced among patients treated with L-DOX. The toxicity buffering provided by liposomes is a considerable improvement in itself over standard chemotherapy.

VII. Stability in Plasma and Storage

The stability of drug-loaded liposomes over time is an important concern in pharmaceutical formulations. Stability can refer to several different aspects of a liposomal drug formulation: chemical stability of both drug and lipid components, colloidal stability, and drug retention. For applications of liposomes where specific delivery of liposome-associated drug to solid tumors is desired, liposomes must substantially retain their contents while in the circulation (Senior, 1987). In other applications, such as the delivery of photosensitizers to tumors in photodynamic therapy, liposome-associated photosensitizers immediately redistribute to other hydrophobic sites, such as plasma lipoproteins in the circulation, which in turn accumulate in tumors (Allison et al., 1990; Reddi, 1997). Various factors can affect the relative stabilities of such preparations in the presence of plasma. This plasma-induced destabilization is exquisitely sensitive to the lipid composition of the liposome. To be more attractive for pharmaceutical development, liposomal drug formulations also must be stable during prolonged storage. Liposomes have either been stored preloaded with DOX, as is the case for PEG-coated liposomes, or as "empty" liposomes that are loaded by a pH gradient immediately before injection (Madden et al., 1990; Haran et al., 1993; Lasic et al., 1995; Cullis et al., 1997). Compositions containing more fluid lipid components, such as eggPC, require remote-loading just before injection, due to a high level of leakage during storage.

A. Physical Stability of Liposomal Drug Formulations

For amphipathic drugs that can readily cross membranes, there are a variety of factors that can influence the stability of a liposomal formulation. The presence of Chol and saturated phospholipids appear to be the most important factors for reducing membrane permeability of these drugs (Bally et al., 1990b; Gabizon et al., 1993). Other factors, such as the drug-loading method, which

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can result in internal concentrations of the drug exceeding the aqueous solubility of the free drug, also act to stabilize the formulation.

Cholesterol appears to be especially important in stabilization of liposomes to the effects of plasma components such as HDL (Mayhew et al., 1979; Allen and Cleland, 1980). In addition, several early studies have indicated that Chol was essential for controlling the permeability properties of membranes to ions or small molecules (Papahadjopoulos et al., 1972, 1973a,b). The presence of Chol in a 1:1 ratio with PG and PC was shown to reduce the amount of ara-C leakage observed in the presence of serum from 88 to 28% after 24 h in one study (Mayhew et al., 1979). HDL has been shown to destabilize pure PC liposomes by catalyzing the net exchange of PC from liposomes to HDL particles (Scherphof et al., 1978; Chobanian et al., 1979; Damen et al., 1980). The addition of Chol to liposome formulations results in an increase in plasma stability, inhibiting transfer of lipid components to plasma lipoproteins (Allen, 1981; Damen et al., 1981).

In vitro stability studies using human plasma or serum have some inherent limitations. Plasma is often isolated in the presence of calcium chelators to prevent blood coagulation and results in some uncertainty because calcium can often modulate interactions of proteins with membrane surfaces and, with some formulations, interact with membranes directly, causing destabilization. Although plasma can be isolated in the presence of heparin, heparin may also affect protein interactions with membranes. In addition, there is considerable interpatient variability in the levels of plasma proteins and lipoproteins, adding another level of complexity to these in vitro studies. The most relevant studies of liposome stability are completed in vivo, simultaneously monitoring the concentrations of both the encapsulated drug and a nonexchangeable lipid marker. With chemotherapeutic drugs, such as DOX, that are removed rapidly from the circulation, the drug/lipid ratio becomes an excellent measure of the stability of the formulation. Measurement of free and L-DOX after cation exchange or size-exclusion chromatography is not reflective of liposome stability because the free drug is rapidly removed from the circulation, resulting in an underestimation of the amount of drug leakage.

The in vivo leakage of DOX from DSPC/Chol (55:45) and eggPC/Chol (55:45) was measured in mice by following the clearance of a lipid label [^{3}H]cholesterylhexadecyl ether and DOX from the circulation (Bally et al., 1990b; Mayer et al., 1998). Although the DSPC/Chol formulation proved relatively stable, releasing <10% of the encapsulated DOX in 24 h, the eggPC/Chol formulation released almost 50% of its DOX within 1 h and ~70% by 4 h. A PEG-DSPE/HSPC/Chol DOX formulation appears to have even greater stability with little apparent leakage in the first 24 h and <10% leakage up to 72 h after injection (Gabizon et al., 1993). It should be noted that in the first study, DOX was loaded into liposomes by the pH gradient method of Mayer, Cullis, and coworkers, whereas in the second study, DOX was loaded according to the ammonium sulfate method. These loading methods are discussed in more detail in *VIIA1. Drug-Loading Methods*. However, differences in the loading methods, including a more rapid dissipation of the pH gradient, in the case of first method, and the formation of a stable drug-sulfate gel in the liposome interior of liposomes loaded using the ammonium sulfate method (Lasic et al., 1992a, 1995), may result in greater stability for liposomes loaded with DOX via the second method (Frézard, 1994; Frézard et al., 1994). Because the kinetics of tumor accumulation are more rapid than the rate of DOX release from liposomes loaded via either method, it is not known whether a further increase in stability is desirable or may simply act to limit the bioavailability of the drug in the tumor. Also, for amphipathic compounds such as DOX the choice of a saturated phospholipid component, such as DSPC or HSPC, is essential in maintaining a stable formulation in the circulation. The substitution of SM for phosphatidylcholine may also increase the liposome stability of some drug formulations (Parr et al., 1994; Webb et al., 1995). Intermolecular hydrogen bonding between the Chol hydroxyl group and the neighboring amide nitrogen of SM gives rise to a tightly packed bilayer that likely resists drug permeation (Schmidt et al., 1977; Smaby et al., 1996).

1. Drug-Loading Methods. A diagram depicting the ammonium sulfate remote-loading procedure is given in Fig. 9 (Haran et al., 1993; Lasic et al., 1995). Lipids are typically hydrated to form suspensions in high concentrations of ammonium sulfate (250 mM) and subsequently extruded to the desired size. Unencapsulated ammonium sulfate is removed (for example using a size-exclusion column), and the drug is added to the liposomes. Although ammonia can freely pass through membranes in its neutral form, sulfate is trapped in the liposomal lumen. When ammonia moves out of the liposome going with the concentration gradient, a hydrogen ion is left behind and a self-sustaining pH gradient is formed; DOX moves in its neutral form in the opposite direction and becomes protonated, eventually forming an insoluble salt with the entrapped sulfate anions. The resulting gel helps stabilize the drug in the interior. The cooling that occurs after the loading step, which is performed at 55–60°C, also likely plays a role in solidifying the drug precipitate, and thus increasing the stability of the formulation. A pH gradient strategy for loading weak bases was reported initially by Nichols and Deamer (1976) and later used extensively by Cullis and coworkers, with a pH gradient to drive the accumulation of drugs into liposomes (Mayer et al., 1985; Madden et al., 1990; Harrigan et al., 1993; Cullis et al., 1997). Weak acids can be loaded in an analogous manner using calcium acetate or reverse pH gradients (Clerc and Baren-

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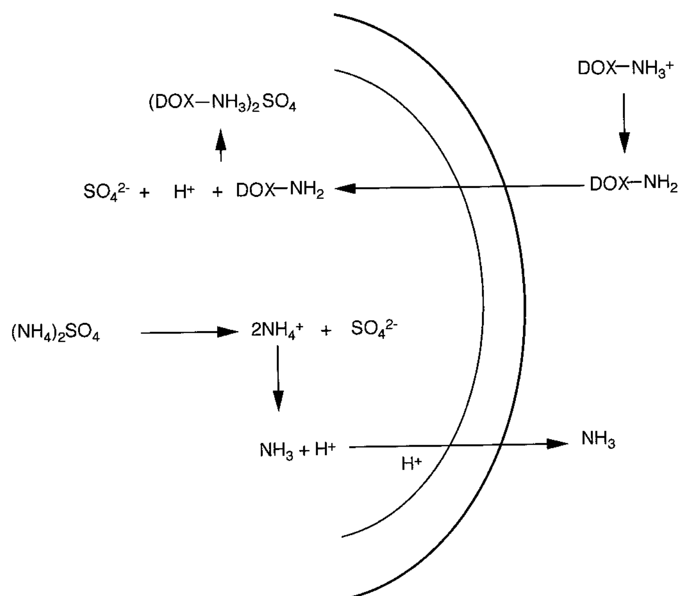

FIG. 9. Ammonium sulfate-loading procedure for weak bases. Liposomes are first prepared in the presence of ammonium sulfate (250 mM). On removal of the exterior ammonium sulfate on a size-exclusion column, DOX is added to the extraliposomal media. Ammonium sulfate can dissociate to two ammonium cations and one sulfate anion. Ammonia (NH_3) is free to cross the liposomal membrane, giving rise to a pH gradient across the membrane. DOX in its uncharged form can then cross the liposome membrane and form an insoluble gel under acidic conditions with the remaining sulfate anion on cooling, effectively trapping it in the liposomal interior. The concentration of DOX in the liposomal lumen can reach concentrations in excess of the aqueous solubility of DOX. This loading procedure can be applied to a variety of weak bases, such as those composing the anthracyclines, *Vinca* alkaloidsm or camphothecins. However, the stability of the complex formed with sulfate and thus the gel in the liposomal lumen may help determine the overall stability of the formulation.

holz, 1995; Cullis et al., 1997). These gradients also help stabilize formulations and reduce leakage during storage and while in the circulation. The small difference between DSPC/Chol (55:45) and HSPC/Chol/PEG-DSPE (92.5:70:7.5) in the observed amount of in vivo DOX leakage is possibly due to differences in the remote loading procedure (Gabizon et al., 1993; Mayer et al., 1998). Most studies with SSLs typically use ammonium sulfate gradients to entrap amphiphatic basic amines such as DOX, whereas studies using CLs prefer the pH gradient method (Madden et al., 1990; Haran et al., 1993; Lasic et al., 1995; Cullis et al., 1997).

2. Physical Stability of Liposome Formulations with Nonanthracyclines. An excellent review recently described the relationship of drug structure and physical properties of the liposomal membrane to drug-loading efficiencies and the stability of liposome-drug formulations (Barenholz, 1998). The drugs described thus far are considered membrane active, meaning they are amphipathic in nature and able to insert into and transverse both artificial and biological membranes. Other drugs, such as *N*-(phosphonoacetyl)-L-aspartate, have a more polar character and are unable to freely transverse membranes (Heath and Brown, 1989). Stable liposome formulations with such drugs can include monounsaturated or polyunsaturated phospholipid components that were previously considered undesirable with drugs such as DOX or VCR. However, although it becomes easier to prepare stable formulations with highly water-soluble drugs, it also becomes more difficult to release the drug from the carrier at the tumor site where it can elicit its desired response. This aspect in investigated in more detail in *VIII. Accumulation of Liposomal Drugs in Tumors*. An additional concern with highly water-soluble drugs is how to entrap them at very high efficiencies in liposomal carriers. Methods for entrapping amphipathic drugs using a pH gradient or ammonium sulfate gradient are dependent on the partition coefficient of the drug between the aqueous phase and the liposomal membrane (Lasic et al., 1995; Cullis et al., 1997). Amphipathic drugs that partition to a greater extent in the liposomal membrane are more readily entrapped in the liposomal interior. Highly water-soluble drugs are likely to be loaded only by passive encapsulation, which is limited by the entrapped volume, and with 100-nm vesicles rarely exceeds 95% of the total volume (Szoka and Papahadjopoulos, 1978; Mayer et al., 1985).

It should be emphasized that results obtained with DOX cannot necessarily be extrapolated to other drugs, even those similar in chemical structure. For example, VCR-loaded liposomes loaded by the pH gradient method are significantly less stable in the circulation, losing ~85% of the encapsulated drug in a 24-h period (Mayer et al., 1998). Apparent rates of elimination of VCR and ara-C are also affected by membrane stability (Mayhew et al., 1979; Webb et al., 1995). When SM and Chol are included in these formulations to reduce leakage of the drug, the drug shows higher plasma levels and reduced rates of elimination from the circulation.

The presence of PEG-DSPE may decrease the stability of some liposomal drug formulations (Webb et al., 1995, 1998). SM/Chol liposomes containing entrapped VCR were shown to rapidly leak VCR in the circulation in the presence of PEG-DSPE. We have observed reduced loading of vinorelbine, another *Vinca* alkaloid, at high mol% value of PEG-DSPE (5–6 mol%; Kirpotin et al., 1999a). However, reducing the concentration of PEG-DSPE to 3 mol% significantly increased the loading efficiency. Thus, the stability dilemma observed with some drugs in the presence of PEG-DSPE may be overcome by simply reducing the concentration of PEG-DSPE, but the effect of reducing the concentration of PEG-DSPE has on the pharmacokinetics of the liposome remains to be seen. A simple increase in the molecular weight of PEG used from 2000 to 5000 may result in a similar degree of protection to that observed with the lower molecular weight PEG at a higher mol% value. Alternatively, Mayer and coworkers suggested the stability problem is a consequence of the negative charge found at the membrane interface with PEG-DSPE (Webb et al., 1998). The exchange of PEG-DSPE for a neutral PEG-ceramide con-

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jugate resulted in greater stability of liposomal VCR preparation.

3. Drug/Lipid Ratio. An optimal drug/lipid ratio is known to be important in the development of a stable formulation. The drug/lipid ratio should be as high as possible to maximize the payload of drug reaching the tumor without compromising stability. The maximum amount of drug loaded per liposome is dependent on the method used for drug loading, the size of the liposome, and the presence of trapping components such as acidic lipids to which the drug can bind. Because the latter two factors are traditionally associated with negative effects on pharmacokinetic parameters, the drug-loading method is the most readily adjustable. For passive encapsulation of daunorubicin in CLs, the concentration achieved was 0.079 μg drug/μg lipid. For remote loading via a simple pH gradient, the most effective concentration reached was 0.250 μg drug/μg lipid, and for remote-loading using an ammonium sulfate gradient, it was 0.125 μg drug/μg lipid (Table 1). Drug/lipid ratios that are too high can also form less stable formulations, presumably due to the dissipation of the pH gradient during drug loading (Mayer et al., 1990c, 1993). These results emphasize the care needed in optimization of drug-loading methods to prepare stable liposomes and at the same time maximize encapsulation efficiencies.

4. Osmolarity Effects. Several studies have investigated the role of osmolarity on the development of stable liposomal drug formulations (Allen et al., 1992, 1995b; Mui et al., 1993, 1994). Allen et al. (1992) showed that entrapped ara-C was released more rapidly when entrapped under hyperosmotic conditions, and its release was characterized by initially rapid kinetics, followed by a slower second rate of leakage. This is consistent with the work of Madden and coworkers, who showed that osmotic lysis results in only partial release of liposomal contents and that after resealing of the liposome membrane, the liposomal lumen remains hyperosmotic (Mui et al., 1993, 1994). VCR-loaded SSLs loaded using an ammonium citrate gradient were relatively stable (leakage $T_{1/2}$ = 84 h) when loaded under iso-osmotic conditions (125 mM ammonium citrate; Allen et al., 1995). In contrast, 90% of the encapsulated VCR was released after 24 h from DSPC/Chol liposomes loaded via the pH gradient method under hyperosmotic conditions (400 mM sodium citrate; Boman et al., 1994). A fine balance may exist between the osmotic stability of the liposome, residual pH gradients after loading, and the formation of drug precipitates in the liposomal lumen. For the pH gradient drug-loading method, a high buffer capacity is typically required in the intravesicular medium to maintain a reasonable pH gradient and obtain high amounts of drug loading (Mayer et al., 1990c; Boman et al., 1993; Cullis et al., 1997). High concentrations of DOX form gel-like precipitates with low osmotic activity (Lasic et al., 1992a; Haran et al., 1993). For example, DOX loaded into PEG-DSPE/DSPC/Chol or DSPC/Chol liposomes, using either the pH (400 mM sodium citrate) or ammonium sulfate (250 mM ammonium sulfate) gradient method, is stably encapsulated in the presence of plasma. However, other drugs that form less stable complexes or gels may still have a considerable osmotic gradient, after the drug-loading process, that can increase further during drug loading (Boman et al., 1993). For instance, both daunorubicin and VCR have a considerably greater aqueous solubility than DOX (Madden et al., 1990), and both leak at a faster rate than DOX (Boman et al., 1993; Haran et al., 1993; Mayer et al., 1993). Other factors, such as the pK_a of titratable groups on the drug, a more rapid dissipation of the pH gradient, and the ability of the soluble form of the drug to partition more readily into the liposome membrane, as opposed to drug precipitates or crystals, also likely play a role in the decreased stability of such formulations relative to DOX (Madden et al., 1990; Mayer et al., 1993; Cullis et al., 1997).

5. Stabilizing against Aggregation. Although more solid CLs composed of DSPC and Chol leak drug very slowly, they are difficult to work with due to increased flocculation and aggregation over time (Crommelin, 1984; Gamon et al., 1989; Barenholz et al., 1993). Early preparations were often stabilized with small quantities of negatively charged lipids such as PG to prevent aggregation from occurring during storage (Gabizon et al., 1983, 1986, 1989). However, as was previously discussed, the presence of certain anionic phospholipids increases the rate of clearance from the circulation (Hwang, 1987; Senior, 1987). The presence of PEG on the surface provides a steric barrier that prevents liposome aggregation. PEG-coated liposomes are stable with respect to both size and drug-encapsulation over the period of many months to years when stored below the phase transition of the PC component (Haran et al., 1993; Lasic and Needham, 1995).

B. Chemical Stability of Drugs and Lipid Components

Thus far, we have been primarily concerned with the physical stability of liposomal drug formulations, either in storage or in the circulation. However, another important concern is the chemical stability of both the drug and lipid components (Barenholz et al., 1993). Are the drugs and lipid components compatible with the remote loading techniques used? If ligand-mediated targeting results in endocytosis of the liposome, is the drug stable in the low pH environment of late endosomes and lysosomes or in the presence of degradative enzymes present in these structures? These are important questions that must be answered when designing a liposomal drug delivery system. When ara-C-loaded liposomes were targeted to cells in vitro, the uptake and delivery to the lysosome resulted in degradation of the drug (Huang et al., 1993). In contrast, DOX is relatively stable and able to escape the harsh conditions of the lysosome intact (Barenholz et al., 1993).

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Many drugs and lipids are susceptible to base hydrolysis. A calcium-acetate gradient has been used to load amphipathic weak acids into liposomes (Clerc and Barenholz, 1995). This method presumably generates a very high internal pH. When using this method, the stability of the drug must be considered. DOX, paclitaxel, topotecan, and other drugs can be hydrolyzed under basic conditions (Ringel and Horwitz, 1987; Barenholz et al., 1993; Burke et al., 1993; Chabner and Longo, 1996). The lactone ring of topotecan is readily hydrolyzed at even neutral pH, giving rise to serious stability concerns under basic conditions (Burke et al., 1993; Subramanian and Muller, 1995), although entrapment in liposomes with an acidic interior has been shown to stabilize topotecan formulations (Burke and Gao, 1994). Finally, the fatty acid esters are sensitive to both acid and base hydrolysis giving rise to membrane-destabilizing lysolipids under certain conditions (Barenholz et al., 1993; Zuidam et al., 1995). It is wise to analyze the lipid components of a newly developed liposome formulation by thin-layer chromatography or HPLC to be confident in the chemical stability of the lipids used.

Lipid peroxidation is another important concern for unsaturated lipid components. Lipid peroxidation can be initiated by a variety of different factors and can lead to the formation of membrane-destabilizing secondary oxidation products such as 4-hydroxynonenal and malondialdehyde (Frankel, 1987a,b; Barenholz et al., 1993). Phospholipids containing diunsaturated fatty acyl chains such as linoleic, linolenic, or arachidonic acid are particularly susceptible to lipid peroxidation due to the ready abstraction of hydrogen radicals from doubly allylic carbons (Frankel, 1980, 1985). Linolenate- and arachidonate-containing phospholipids are the most likely to form complex secondary oxidation products that are particularly damaging to membranes (Frankel, 1987a,b). This brings up an important point concerning the use of unsaturated lipids. There may be liposomal drug delivery scenarios in which a more fluid membrane is preferred. When the use of unsaturated lipids is required, it is the gel-to-liquid crystalline phase transition (T_m) that is often an excellent predictor of bilayer fluidity. Table 2 gives the primary phase transitions for several different phosphatidylcholines. Increasing the acyl chain length gives rise to a higher T_m whereas increasing the number of unsaturations decreases the T_m. Thus, a lipid component with the desired T_m can be found by balancing the acyl chain length and the number of unsaturations found in a particular phospholipid component. eggPC is a widely used fluid phase lipid component that is in the liquid crystalline state at physiological temperatures. Unfortunately, it also contains a high proportion of fatty acyl groups with multiple unsaturations (18% with two olefins and 3% with four olefins), making it particularly susceptible to oxidation. As can be seen from Table 2, POPC has a comparable T_m value with only one olefin in one of the two acyl chains. eggPC was originally used because it was readily available and relatively inexpensive. It is now being used for mostly historic reasons, because most investigators prefer to continue using what is familiar to them in the literature. However, improvements in organic synthetic methods for phospholipids have led to the increased availability of synthetic lipids such as POPC and resulted in a cost that is comparable to the natural product. Combined with an increased chemical stability, POPC becomes a far more appropriate candidate for use as the unsaturated lipid component of a liposome formulation than eggPC.

The stability of a liposomal formulation is dependent on many physical and chemical factors, ranging from the individual drug and lipid components to the stable encapsulation of the drug within the carrier. A rigorous undertaking is necessary in developing any new liposomal drug formulation to ensure these stability considerations are addressed. In *VIII. Bioavailability of Encapsulated Drug*, we discuss how to balance stability in the circulation with release from the carrier on reaching the tumor.

VIII. Bioavailability of Encapsulated Drug

It is important to emphasize that most of the work described thus far has been concerned with drugs considered to be membrane active. They are amphipathic in nature and able to transverse the bilayer at a rate dependent on the physical properties of the membrane, as well as any ionic or pH gradients across the membrane (Madden et al., 1990; Lasic et al., 1995; Cullis et al., 1997). Other drugs, such as ara-C, are more water soluble and after a slow release from the carrier (Allen et al., 1992) can be taken up by specific transporters located in the plasma membrane of tumor cells, such as the nucleoside transporter (Plageman et al., 1978; Wiley et al., 1982) or the reduced folate carrier (Westerhof et al., 1991, 1995; Antony, 1992). The bioavailability of such compounds is dependent on how readily they are able to escape their liposomal carrier. We define bioavailability in the case of liposomal carriers as the amount of free drug that is able to escape the confines of the carrier and is thus available for redistribution to neighboring tissues and tumor. A fine balance is required to prevent premature leakage in the circulation, and thus nonspecific toxicities, but still allow for release of the drug on reaching the tumor. For DOX-loaded slow-release liposomes (PEG-DSPE/HSPC/Chol or DSPC/Chol), the drug is thought to leak very slowly and thus be similar to a slow infusion of the drug specifically near the cancerous cells (Horowitz et al., 1992; Vaage et al., 1998). Using scanning confocal fluorescence microscopy to look at s.c. implants of a prostate carcinoma xenograft, DOX delivered via SSL DOX was shown to reside immediately adjacent to tumor capillaries and venules at early times (1 h; Vaage et al., 1998). At 24 h,

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DOX had leaked from the liposome and was found within the tumor in a pattern indicating diffusion away from the capillaries and venules. Free DOX was found deep within the tumor at 1 h but was nearly undetectable at 24 h. This is likely due to both elimination and metabolism of the drug, as well as fluorescence quenching after intercalation of the drug into nucleic acids (Gigli et al., 1988). These results indicate that DOX does become bioavailable on reaching the tumor, where it slowly and continuously bombards the nearby cancer cells with low levels of the cytotoxic agent. Thus far, most detailed studies have used anthracyclines for delivery studies. Although the antitumor cytotoxicity of drugs such as anthracyclines and ara-C are less dependent on peak levels of the drug, cytotoxicity of other drugs may show a considerably greater dependence on peak levels of the drug, and hence the rate at which the drug is released from its carrier. Consequently, the selection of drugs with these properties or the selective increase in the rate of release at the tumor site will be very important in designing an effective carrier.

A. Release of Doxorubicin in Tumor

The mechanisms responsible for liposome breakdown and drug release in tumors have not been well elucidated. Several potential mechanisms have been proposed, but all are highly speculative and little direct evidence has been provided, primarily due to technical difficulties associated with monitoring drug release in vivo. Some of the properties of the tumor microenvironment believed to play a role in liposome destabilization include the slightly acidic pH found in interstitial fluids surrounding tumors, lipases released from dying tumor cells, inflammatory cells present in response to tumor release factors, enzymes, and oxidizing agents (Martin, 1998). In addition, phagocytic cells residing in tumors could metabolize liposomes and release free DOX, killing neighboring tumor cells via the bystander effect (Storm et al., 1988). The effect of local interstitial media on DOX leakage from SSL DOX was investigated in an in vitro study (Gabizon, 1995). Although leakage in plasma was relatively slow ($T_{1/2} \sim 100$ h), liposomes incubated in the presence of fluid obtained from pleural malignant effusions leaked DOX at a significantly elevated rate. Another investigator suggested that ammonium sulfate used to remote loaded DOX could also catalyze liposome breakdown, although a logical rationale for its mechanism was not provided (Lasic, 1993). With SSLs, a certain amount of PEG-DSPE can be released from the liposome over time, allowing liposomes to undergo more interactions with neighboring cells and or plasma components. Finally, it may be possible that DOX passively crosses the liposome membrane and that as the DOX-sulfate gel is gradually destabilized by loss of more and more drug, the drug release is accelerated. Finding methods to selectively destabilize liposomal drug formulations in the tumor area is a major challenge to the liposome field, which if overcome could lead to substantial increases in drug bioavailability at the tumor site and subsequent increased efficacy.

The release of DOX from eggPC/Chol liposomes is rapid compared with liposomes composed of HSPC/Chol or DSPC/Chol (Bally et al., 1990b; Gabizon et al., 1993). eggPC/Chol liposomes release a significant portion of their drug before reaching the tumor and thus act as a rapid-release system (Harasym et al., 1997), in contrast to the more stable formulations that act as slow-release systems and are the focus of this review.

B. Active Targeting of Liposomes

Although clearly more beneficial than the use of free DOX, one disadvantage of SSL DOX or L-DOX is that cancer cells deep within the tumor are not readily reached with high concentrations of drug and are given an opportunity to select for drug-resistant cells. One strategy for increasing drug bioavailability and distribution within the tumor has been to target liposomes to internalizing receptors. Liposomes have been targeted to cells via small molecules (Lee and Low, 1994, 1995), sugar molecules (Spanjer and Scherphof, 1983; Banerjee et al., 1996), serum proteins (Afzelius et al., 1989; Brown and Silvius, 1990; Lundberg et al., 1993), and antibodies (Heath et al., 1983; Debs et al., 1987; Matthay et al., 1989; Maruyama et al., 1990a; Allen et al., 1995c; Lopes de Menezes et al., 1998) or antibody fragments (Park et al., 1995; Kirpotin et al., 1997b). Recently, HER2-targeted immunoliposomes were shown to distribute within solid tumors and not simply in the extracellular space surrounding the tumor blood vessels (Kirpotin et al., 1997a, 1999a; Park et al., 1997). Release of the drug within the tumor itself presumably increases the bioavailability of the drug to the more-difficult-to-reach cells within the solid tumor mass. Indeed, this property is most likely responsible for the increased therapeutic effect observed with these carriers, as there was no overall increase in liposome localization to the tumor (Fig. 10).

Active targeting of pharmaceuticals is often perceived as a means of getting increased amounts of drug into the diseased site. However, the passive trapping of liposomes due to a discontinuous tumor microvasculature, the lack of a functioning lymphatics, and a high interstitial pressure result in a rate-limiting accumulation of liposomal drug in solid tumors. It is unlikely that active targeting to cell surface proteins of solid tumors that are not internalized will offer a significant therapeutic benefit. When anti-HER2-targeted immunoliposomes are prepared with an antibody that is not internalized, there was no increase in therapeutic efficacy compared with nontargeted liposomes (Goren et al., 1996). Similar to the results seen with the internalizing anti-HER2 Fab′ fragment (Kirpotin et al., 1998; Park et al., 1998a,b), there was no increase in tumor levels of the targeted liposomes compared with nontargeted liposomes (Goren

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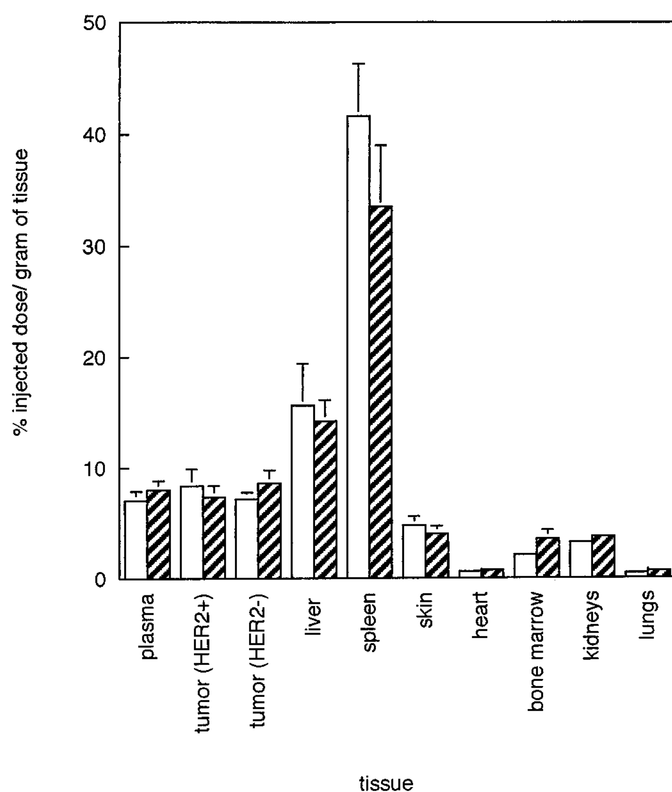

FIG. 10. Tissue distribution of anti-HER2 targeted SSLs (□) and nontargeted SSLs (rhbox) in nude mice with breast cancer xenografts. Xenografts were derived from both HER2-overexpressing (BT-474) and HER2-negative (MCF-7) cell lines. The biodistribution was determined at 24 h by following ^{67}Ga-labeled liposomes after injection of 1 μmol of phospholipid via the tail vein in nude mice. This figure was adapted from Kirpotin et al. (1998).

et al., 1996). Vingerhoeds et al. (1996) also failed to show increased efficacy of noninternalizing immunoliposomes targeted against the OA3 antigen present on 90% of human ovarian carcinomas. Some investigators have even suggested that cell surface binding by itself may serve to limit the distribution of liposomes within the tumor (Weinstein et al., 1987; Jain, 1989). Allen et al. (1995c) showed that SSL DOX was more effective than sterically stabilized immunoliposomal DOX targeted against a carbohydrate epitope on an ovarian cancer cell line grown s.c. in nude mice. The authors suggested the reduced activity may be due in part to the binding-site barrier. However, the circulation $T_{1/2}$ values of the immunoliposomes in this study were significantly shorter than those for the nontargeted SSL, and there was no evidence presented showing that these liposomes were internalized, giving rise to two alternative explanations for the reduced activity. Furthermore, this study used whole antibodies for targeting. In our studies with the anti-HER2 antibody, antibody fragments were used: either Fab′ or single chain FV fragments (Fig. 11; Park et al., 1995, 1998a,b; Kirpotin et al., 1998; Papahadjopoulos et al., 1999). In addition to the advantages associated with reduced immunogenicity of antibody fragments, the reduced avidity of the fragments for their cell surface targets may serve to reduce the binding-site barrier, allowing a deeper penetration of the carrier within the tumor. A deeper penetration of antibody fragments compared with full antibodies has been previously attributed to both the reduced size of the molecule and a reduced avidity for its target (Fujimori et al., 1989; Yokota et al., 1992). This, of course, is speculation, and additional studies must be completed to determine more precisely the mechanisms responsible for regulation of the tumor penetration of targeted liposomes.

Allen and coworkers have also been successful in targeting liposomes to a lung metastatic cancer model, where cancer cells travel through the blood and localize in the lung as small tumor colonies (0.5 mm; Ahmad et al., 1993; Allen et al., 1995c). An increased localization to tumor-bearing lungs was seen with targeted immunoliposomes compared with nontargeted SSL, and this correlated with a significant decrease in the tumor burden of mice treated with immunoliposomes (Ahmad et al., 1993). Cancer cells in this metastatic model differ greatly compared with the solid tumors described earlier due to their small size and the greater accessibility of liposomes to their receptor. This same group has also been successful in targeting liposomes against hematological cancers, such as B cell malignancies (Allen et al., 1995c; Lopes de Menezes et al., 1998), where the tumor cells are also more available for binding to targeted liposomes. Huang and coworkers have targeted the pulmonary endothelium using antibodies directed against the lung endothelial protein thrombomodulin (Maruyama et al., 1990a,b; Mori et al., 1993, 1995). This type of organ-specific targeting allows liposome-associated drug to be delivered near the site of tumors located in the lung, where on their disassociation from the carrier they can act on neighboring tumor cells (Mori et al., 1995). The greater accessibility of the receptors in each of these approaches offers a significant advantage for targeted therapies compared with the treatment of solid tumors.

The choice of targeting ligand is important when designing targeted liposomes. The ligand should be relatively specific for cancer cells, especially in contrast to cells readily accessible in the general circulation, where many passes may occur before extravasation into tumors. Second, as mentioned, the epitope bound should result in internalization of the liposome. Binding to a receptor that is known to be endocytosed does not necessitate endocytosis, especially in the case of antibodies or antibody fragments (Goren et al., 1996). Ligands, such as folate, for internalized receptors usually induce endocytosis, but binding of a protein or peptide to an unrelated part of the receptor may simply constrain the carrier on the membrane surface. An additional problem with the attachment of targeting molecules to the surface of liposomes is that they may increase liposome clearance by tissues other than the tumor. For instance, early studies indicated antibody-targeted liposomes are

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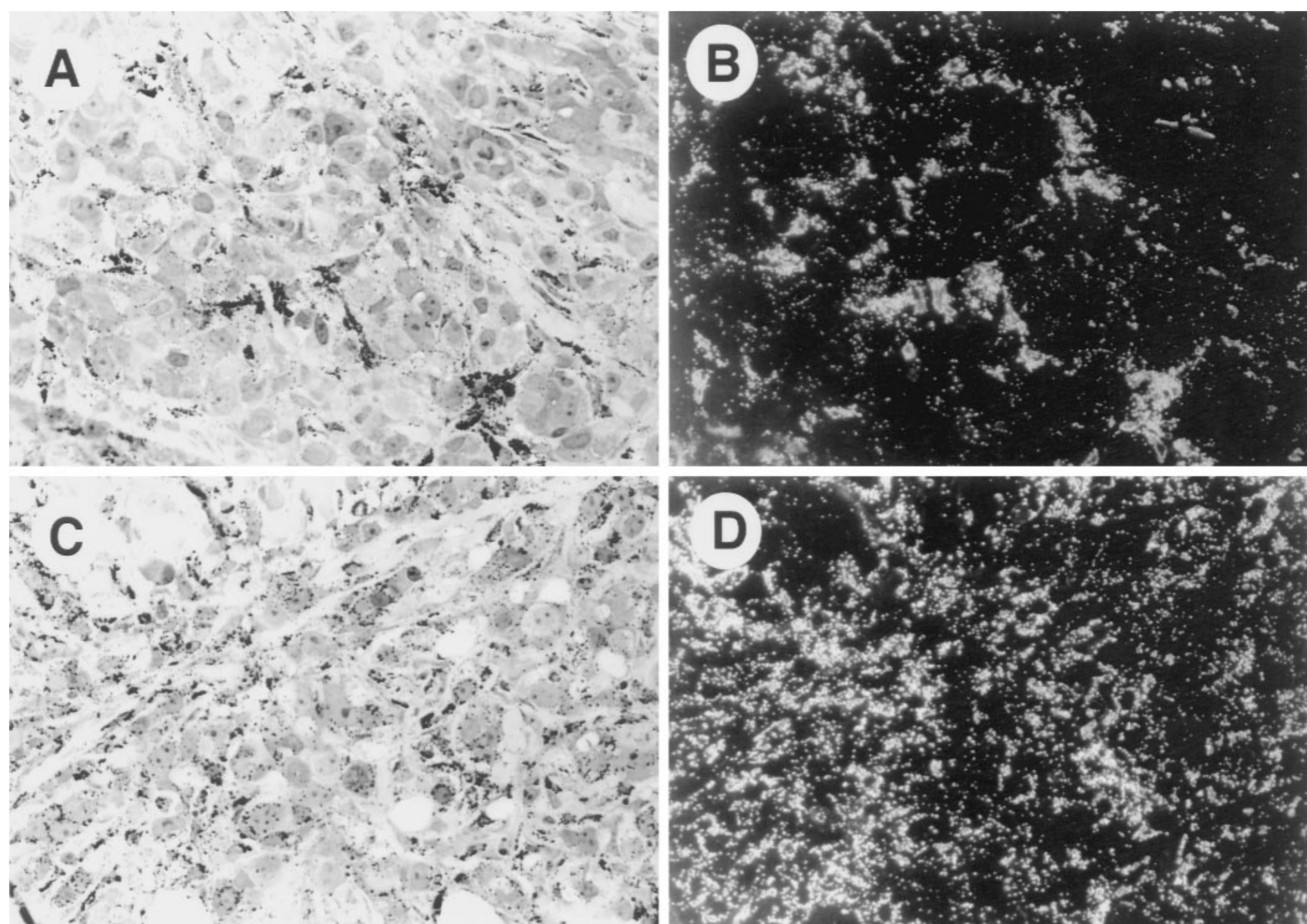

FIG. 11. Effect of tumor cell targeting on the localization of SSLs in HER2-overexpressing human breast cancer xenografts (BT-474). A and B, nontargeted SSLs. C and D, anti-HER2 SSL. Liposomes labeled with entrapped colloidal gold were injected i.v. into the mice with s.c. xenograft tumors (200–300 mm^3 in size) at the dose of 5 mmol of phospholipid/animal. Twenty-four hours later, the tumors were harvested, and liposomes were visualized on tumor sections by the silver enhancement method. Liposomes appear as bright dots on the dark field images (B and D) or as black grains on the matching bright-field images counterstained with light H&E (A and C). Nontargeted liposomes are concentrated in the interstitial spaces within the cells with characteristic macrophage morphology. HER2-targeted SSL are more uniformly dispersed throughout the tumor tissue and often found within tumor cells.

rapidly removed from the circulation by macrophages of the RES (Debs et al., 1987). This was likely due to recognition of the Fc portion of the antibody by Fc receptors located on the surface of macrophages (Aragnol and Leserman, 1986; Raghavan and Bjorkman, 1996) or by recognition of noncompatible portions of the antibody by antibodies of the humoral immune system. The recent endeavors with Fab′ targeted liposomes do not contain the Fc region of the antibody and are prepared from humanized versions of the antibody (Park et al., 1995; Kirpotin et al., 1997b). Indeed, these immunoliposomes have a nearly identical tissue distribution as that of nontargeted SSL DOX (Fig. 10). A more in-depth description of these immunoliposomes and the various properties necessary for their optimization is given in several recent reviews (Kirpotin et al., 1997a, 1998; Park et al., 1997). There also are many reviews describing the methods for preparing and applying other targeted liposomes (Allen and Moase, 1996; Allen et al., 1997, 1998; Forssen and Willis, 1998; Park et al., 1998a,b). The most relevant aspect of targeted liposomes is that targeting to internalizing receptors can potentially increase the bioavailability of the drug. It can accomplish this by altering the intratumoral distribution of the liposome and thus increasing the percentage of cells exposed to the drug. This effect has only been observed for HER2-targeted immunoliposomes and may very well differ depending on the targeting ligand.

Liposomes targeted to internalizing receptors have shown considerably greater tumor cell cytotoxicity both in vitro and in vivo (Heath et al., 1983; Huang et al., 1983; Berinstein et al., 1987; Matthay et al., 1989; Lee and Low, 1995; Park et al., 1995, 1997; Lopes de Menezes et al., 1998). This may be due in part to an increased bioavailability after transport of the liposomes to lysosomes, where degradative enzymes can breakdown the liposomal membrane and release the drug. Many studies have demonstrated degradation of both lipid and either encapsulated or bound protein after internalization by macrophages (Dijkstra et al., 1984;

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Storm et al., 1988; Derksen et al., 1988). Increased release of DOX from liposomes was observed after uptake by peritoneal macrophages, and collected supernatants were shown to have considerable growth-inhibitory activity (Storm et al., 1988). The degradation rate was dependent on the lipid composition of the liposomes, with liposomes containing high-phase transition phospholipids (slow release) being degraded more slowly than those containing low-phase transition phospholipids (rapid release; Storm et al., 1988). However, intracellular processing may vary depending on the cell type and may be significantly different in tumor cells compared with phagocytes such as macrophages. For example, two studies have shown that T cells are able to process liposome-delivered drugs more rapidly than B cells (Machy et al., 1982; Lopes de Menezes et al., 1998). Nevertheless, several other cell types, such as fibroblasts, endothelial cells, and tumor cells, have demonstrated processing of liposomes or their components after internalization (Straubinger et al., 1983; Jett et al., 1985; Trubetskaya et al., 1988; Chu et al., 1990). Internalization of liposomal drugs has also been suggested to increase efficacy by limiting diffusion of the drug away from the cancer cells (Allen et al., 1998). This is especially a concern in the turbulent environment of the general circulation or peritoneal cavity (Allen and Moase, 1996). Both of these factors likely play a role in the increased efficacy observed with actively targeted liposomes. Regardless of the mechanism, targeting to internalizing receptors appears to increase the growth-inhibitory effects of some liposomal drugs.

Methotrexate or other reduced folates are good candidates for delivery via this kind of targeted approach. Due to the relatively low pK^a of the 2′ carboxyl group, methotrexate and its derivatives are not readily protonated and thus cannot passively transverse artificial or biological membranes. The result of this is relatively stable liposome formulations of reduced folates such as methotrexate, which are significantly less likely than even anthracyclines to leak prematurely in the circulation and cause nonspecific toxicities. These reduced folates enter the cell by reduced folate carriers located in the plasma membrane (and membranes of endosomes and lysosomes) of certain cells (Kamen et al., 1991; Westerhof et al., 1995). Reduced folate carriers are upregulated in a variety of different tumor models, as is expected considering the rapid growth rate of cancerous cells (Westerhof et al., 1991; Weitman et al., 1992a; Ross et al., 1994). After the delivery to late endosome and lysosomes where the carrier is degraded and methotrexate is released, methotrexate can be transported by the reduced folate carrier into the cytosol, where it can elicit its cytotoxic action on folate-requiring enzymes (Weitman et al., 1992b; Antony, 1996). A number of in vitro studies with methotrexate or methotrexate-γ-aspartate have shown a marked dependence of cytotoxicity on targeting to endocytic pathways (Heath et al., 1983; Matthay et al., 1986, 1989; Bernstein et al., 1987; Straubinger et al., 1988; Singh et al., 1989). An additional advantage of the development of a liposomal formulation of a drug such as methotrexate is that the modes of drug resistance to methotrexate and anthracyclines are markedly different. Consequently, a combination of targeted liposomal methotrexate with targeted or nontargeted DOX may provide an even greater chance for long-term survival. Of course, this is a technical advantage of the use of antifolates with targeted liposomes. The first and most obvious consideration is that the type of cancer is sensitive to antifolates.

C. Hyperthermia and Thermosensitive Liposomes

Hyperthermia has also been used to increase the bioavailability of liposomal drugs in the tumor area. In addition to simply increasing the amount of liposomes that enter the tumor area (see *IIIC. Hyperthermia and Vascular Permeability Factors for Increasing Vascular Permeability*), hyperthermia makes the distribution of liposomes within the tumor more uniform, increasing the bioavailability of the released drug to cells within the tumor (Kirpotin et al., 1999b). This is similar to the effect seen with HER2-targeted immunoliposomes. Hyperthermia can also be used to increase drug bioavailability via a second mechanism. Liposomes can be rendered thermosensitive by replacing some of the DSPC lipid component with DPPC, resulting in an increased leakage of the encapsulated material (Yatvin et al., 1978; Gaber et al., 1995, 1996; Wu et al., 1997) when heated to 42°C. This effect was found to be dependent on the presence of plasma proteins. At 37°C these liposomes are stable and do not release DOX. However, heating to 42°C for 30 min results in a release of >60% of the encapsulated DOX (Gaber et al., 1995). The combination of hyperthermia and L-DOX appears to be a very promising strategy for the treatment of cancer due to its ability to enhance three important characteristics of liposomal drug delivery: tumor accumulation, intratumoral distribution, and bioavailability. One study has already demonstrated increased therapeutic efficacy for DOX-loaded thermosensitive liposomes used in conjunction with hyperthermia (Huang et al., 1994). Different regimens and treatment schedules are currently being investigated for their effect on efficacy and tolerability. The strategy used here illustrates another important aspect of the optimization of liposomal drug delivery. Currently, it is difficult to resolve even the complex relationships existing between various liposome properties (size, charge, permeability characteristics) and pharmacological factors (dose, route of administration) regulating liposome delivery in vivo. Although these relationships have been the primary focus of this review, the future holds a need for elucidation of the complex processes responsible in vivo for regulating tumor permeability and the movement of liposomes within the tumor after extravasation. The ability to manipulate

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these processes will undoubtedly provide a greater avenue for increasing drug bioavailability in vivo for difficult-to-treat solid tumors.

A diagram depicting the accumulation and distribution of liposomes in tumors is given in Fig. 8. After extravasation through large pores in the tumor microvasculature, liposomes accumulate in the tumor interstitium. Here they can release their encapsulated drug slowly, where it can be taken up by neighboring tumor cells. Targeted liposomes can also obtain a deeper tissue distribution after endocytosis or transcytosis of the carrier and thus expose a greater area of the tumor to the drug. In addition, liposomes targeted to endocytic pathways are destabilized by lysosomal enzymes, releasing the drug within the tumor cells, where it can act on intracellular targets.

D. Problems with Highly Hydrophilic Drugs and Bioavailability

As mentioned in *VII. Stability in Plasma and Storage*, it is relatively easy to prepare stable liposome formulations with polar drugs that are unable to permeate membranes. However, the usefulness of these liposomes is more limited due to present limitations in the ability to make these drugs bioavailable at the tumor site. Increased delivery of highly hydrophilic drugs (Chu and Szoka, 1992) or oligonucleotides (Woodle et al., 1997) to the site of action is not sufficient in itself to obtain an enhanced therapeutic effect. On arrival, the drug must both be released by the carrier and be taken up by the cells of interest. Drugs that can be are recognized and transported by plasma membrane transporters, such as ara-C and methotrexate (Plageman et al., 1978; Wiley et al., 1982; Kamen et al., 1991), may be useful if they can be released from the carrier (Allen et al., 1992). In the case of methotrexate, liposome targeting and internalization likely give rise to increased drug release, and thus greater cytotoxicity (Heath et al., 1983; Matthay et al., 1989). After internalization, the drug can be subsequently transported by an internal anion transporter into the cytosol (Kamen et al., 1991).

Several approaches are being studied to improve the bioavailability of this class of drugs. pH sensitive liposomes composed of unsaturated phosphatidylethanolamines and mildly acidic amphiphiles have been the most thoroughly studied (Straubinger et al., 1985; Chu et al., 1990; Litzinger and Huang, 1992). The problem with this approach is that these formulations are readily stabilized by plasma components, which insert into the membrane bilayer and reduce the liposome's sensitivity to pH (Liu and Huang, 1989, 1990; D. C. Drummond and D.-L. Daleke, unpublished observations). Recently, two approaches have been attempted to induce acid-mediated leakage of water-soluble content markers. The first is the development of pH-sensitive lipid-anchored copolymers (Meyer et al., 1998b). Incorporation of these polymers into eggPC/Chol liposomes was shown to result in substantial leakage of a water-soluble content marker (pyranine), when the pH was lowered below 5.5. Unlike other pH-sensitive liposomes, release of the marker is not due to fusion but rather to a collapse of the polymer at the phase transition and subsequent collapse of the liposomes or resulting local defects in the membrane that allow for contents leakage. The second approach is the design of cleaveable PEG-DSPE conjugates (Kirpotin et al., 1996). PEG-DSPE is known to stabilize 1,2-dideoyl-3-*sn*-phosphatidylethanolamine containing membranes and prevent fusion of liposomes (Holland et al., 1996a,b; Basanez et al., 1997). Release of PEG from the surface with a sulfhydryl- or an acid-sensitive trigger gives a fusion-competent liposome, capable of releasing its contents. Programming release of PEG-lipid conjugates from the liposome surface through adjustment of the acyl chain composition has been another mechanism for release of the stabilizing polymer (Holland et al., 1996b; Webb et al., 1998). Some groups have even attempted to destabilize liposomes using enzymes that can cleave peptides or sugars from the liposome surface (Pinnaduwage and Huang, 1988; Pak et al., 1997) or by using pH-sensitive peptides (Parente et al., 1988, 1990). Finally, water-soluble polyanions such as oligonucleotides have been complexed with cationic lipids and then delivered effectively to the nucleus of target cells (Zelphati and Szoka, 1996; Meyer et al., 1998a). Although some progress has been made with these systems in vitro and in cell culture, they are still a considerable way from being useful in an in vivo application.

If this class of drugs is to be used in vivo, it will undoubtedly be in the context of SSLs. To be readily released from the liposome, highly water-soluble compounds will likely require the use of fluid phase liposomes. Although low-phase transition lipids such as 1,2-dideoyl-3-*sn*-phosphatidylethanolamine, eggPC, or POPC can be incorporated into SSLs and still remain long circulating, CLs containing these lipids are rapidly removed from the circulation (see *IID. Effect of Membrane Packing Constraints on Pharmacokinetic Parameters*). Thus, steric stabilization provides more flexibility for the type of drug class that can be delivered to tumors with liposomes.

IX. Conclusions

A. Sterically Stabilized versus Rapid-Release Conventional Liposomal Formulations

In theory, slow-release systems that effectively deliver their drug to tumors and release the drug in the near vicinity of tumor cells are more advantageous, and thus should be more therapeutically efficacious, than a rapid-release system where the drug is released from the carrier to a significant extent while in the circulation. When used at equivalent doses, there are no known instances where DOX-loaded eggPC/Chol liposomes (TLC D-99) were shown to be more efficacious than SSL DOX. How-

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ever, by definition, efficacy is not dependent on dose, and at present TLC D-99 can be administered at higher doses than Doxil due to the dose-limiting toxicity of H-F syndrome. In the treatment of patients with metastatic breast cancer, TLC D-99 was shown to have an almost identical response rate as reported for Doxil (Ranson et al., 1997; Harris et al., 1998) but had to be delivered at a dose 70% greater than used for Doxil (75 versus 45 mg/m^2 every 3 weeks). Although both formulations are undoubtedly better than free DOX due to decreased toxicities, better patient compliance, and an increased quality of life, drug delivered via sterically stabilized slow-release systems offers two significant advantages. First, because a comparable therapeutic response requires higher doses of DOX to be administered in the case of TLC D-99, cumulative toxicities such as cardiotoxicity are likely to be higher. In addition, due to significant leakage of the drug in the central compartment, compared with the tumor for DOX, more bioavailable drug likely reaches the heart and other healthy tissues. Initially, the large improvements over free DOX will likely make these differences seem minor in comparison. Nevertheless, as L-DOX becomes more widely accepted and replaces free DOX in treatment regimens, these differences in the new higher limits placed on cumulative doses of L-DOX will become important.

A second advantage of slow-release liposomes is that they are more amenable to active targeting of solid tumors. Because eggPC/Chol liposomes release a large proportion of their contents before reaching the tumor, a significantly reduced advantage would be gained by targeting than would be expected for slow-release liposomes. The use of hyperthermia to increase extravasation of liposomes would also benefit more using slow-release systems, where the increased uptake and distribution of drug-loaded liposomes in the tumor would result in a greater increase in overall tumor drug levels. If the drug is released to a greater extent in the circulation, then the drug takes on the pharmacokinetics of the free drug and would not benefit as substantially from hyperthermia, which alters further the pharmacokinetics of the carrier. If hyperthermia were used in conjunction with thermosensitive liposomes to trigger the release of contents, then heat would be administered after accumulation of the drug in the tumor and would in effect reversibly trigger the transformation of liposomes from a slow-release to a very rapid-release system. In addition, the rapid-release liposomes have reduced circulation lifetimes compared with slow-release liposomes, especially SSLs. Liposomes with longer circulation lifetimes would be expected to benefit more from hyperthermia, which has its effect on increasing passive targeting. Future improvements in liposome design by preparing triggerable liposomes that are slow-release systems whereas in the plasma but revert to rapid-release systems on reaching the tumor will presumably result in the most efficacious formulation.

Finally, efficacy results have not shown thus far a favorable response rate for TLC D-99 over Doxil, and potential remedies are presently under consideration for reducing the severity of H-F syndrome. If these are effective, then dose escalation of Doxil will undoubtedly provide a greater therapeutic response.

B. Conventional and Sterically Stabilized Slow-Release Systems

Small, neutral, and solid CLs for drug delivery appear to be limited in their potential usefulness as a drug delivery vehicle if compared with SSL formulations at a similar dose. The dose-independent clearance kinetics of PEG-modified liposomes provides these carriers with a unique ability to remain in the circulation long enough for a therapeutically relevant concentration of drug to accumulate in tumors but at low enough concentrations to avoid certain nonspecific toxicities. However, as mentioned, H-F syndrome limits the dose of SSL DOX that can be administered and thus CLs (DSPC/Chol) may be administered at a dose high enough to give rise to similar tumor concentrations of drug. One potential problem with CL formulations is that increased circulation times and high intratumoral drug concentrations are dependent on drug-induced toxicity to RES macrophages. This presents a problem that has not been adequately addressed: susceptibility to opportunistic infections. In addition, the requirement of long circulation times on drug-induced toxicity would depend heavily on the drug that is formulated. Some drugs such as mitoxantrone are unable to enhance circulation lifetimes by this approach. For delivery of liposomal drug to solid tumors, slow-release CLs are also limited to the delivery of amphipathic drugs that are able to freely transverse the bilayer. SSLs offer the potential advantage of being able to be modified to increase the bioavailability of a variety of drugs whereas maintaining their long circulation times. Thus, CLs appear to be more limited in applicability to very specific conditions, whereas SSLs appear to be more flexible.

The development of SSLs has shifted the focus of liposome research away from improved CL formulations for long circulation. The question of whether CLs have been truly optimized was raised in *IIC. Effect of Liposome Charge on Pharmacokinetic Parameters*. There is at least some evidence to suggest they have not been. If this is the case, then a careful study of the rate of accumulation in tumors, formulation stability, toxicity, and efficacy will have to be completed to determine whether they can be effectively used as carriers of antineoplastic drugs in vivo. This new generation of CLs may provide a plausible alternative to SSLs if carefully optimized.

C. Visions for Future

Liposomal drugs have been suggested to be the long-awaited "magic bullet" cancer therapy due to their abil-

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ity to accumulate selectively in the tumor (Matsumura and Maeda, 1986; Maeda and Matsumura, 1989). However, the problem remains that not all cancers and not all patients respond to the "bullet" equivalently. The drug being delivered by liposomes plays an important role in the response achieved. Multidrug resistance has led to significant obstacles in the ability of standard chemotherapy regimens to cure cancer (Chapman and Powis, 1993; Chabner and Longo, 1996). Many studies have shown that combinations of chemotherapeutic agents with nonoverlapping mechanisms of drug resistance may provide a greater opportunity for treating cancer more effectively. Liposomal drugs have the advantage of continually bombarding the cancer cells with low doses of standard chemotherapy and possible overwhelming drug transporters responsible for pumping drugs such as anthracyclines out of the cell (Richardson and Ryman, 1982; Thierry et al., 1989; Rahman et al., 1992). Even with this possibility in mind, it is unlikely that tumors resistant to DOX will be completely eradicated using only L-DOX. One prominent member of the liposome field of study has continually and quite understandably raised the important question, "When are we ever going to get out of the A's" (Szoka, 1998), referring, of course, to the four most studied liposomal drugs: ara-C, anthracyclines, aminoglycosides, and amphotericin B (a potent antifungal agent). It is an excellent question and one that deserves a considerable amount of thought. Liposomes provide an efficient vehicle for delivery of anticancer agents to tumors, but it will almost certainly be necessary to use combinations of different drugs to provide the most effective treatment. Several studies have addressed this concern (Vaage et al., 1993b; Fonseca et al., 1995; Mitsuyasu et al., 1997; Valero et al., 1999). In one study, combination therapy with low doses of both SSL DOX and SSL-VCR was shown to be more effective in the treatment of MC2 mammary tumors than higher doses of either liposomal drug alone (Vaage et al., 1993b). Other studies have reported the combination of L-DOX with other free drugs (Fonseca et al., 1995; Mitsuyasu et al., 1997; Valero et al., 1999). Additional studies that attempt to encapsulate drugs with nonoverlapping modes of drug resistance and significant activity against a particular form of cancer in liposomes or combine free drugs with nonoverlapping modes of drug resistance with presently developed liposomal drugs are needed and may result in more effective drug regimens for the treatment of a variety of difficult-to-treat cancers.

What liposomal drugs should be developed next? This is a difficult question and is dependent on several different variables; including the type of cancer and its response to a particular drug, the stability of the liposome formulation in the circulation, the ability to make the drug bioavailable at the tumor site, and the mode of drug resistance. There is no one correct answer, and investigators are encouraged to be both creative and thorough in their selection and development of other drug formulations. Although methods for liposome targeting using tumor-specific ligands, for increasing extravasation of liposomes into tumors (hyperthermia) and for increasing the bioavailability of the drug selectively at the tumor site, will in all probability increase the overall therapeutic index of a drug such as L-DOX, there is no doubt an important need to develop liposomal formulations of other drugs. These attempts are currently being made in our laboratory (Kirpotin et al., 1999a) and those of others (Allen et al., 1995b; Chang et al., 1997; Colbern et al., 1998; Embree et al., 1998; Gelmon et al., 1999; Newman et al., 1999; Vaage et al., 1999) with an assortment of well-studied chemotherapeutic agents.

Increasing the efficiency of L-DOX by altering its accumulation in tumor or its distribution within tumors or by increasing its bioavailability selectively within the tumor are important strategies being investigated by many in the field. We have concentrated on two approaches to achieve these goals: local hyperthermia and specific targeting to tumor cell-specific epitopes that internalize on binding. The encouraging preclinical studies with HER2-targeted immunoliposomes are in part a result of the long circulation lifetimes provided by steric stabilization combined with the increased bioavailability resulting from endocytosis of the targeted carrier. Anti-HER2 immunoliposomes are presently being considered (HER2 overexpressing) for the treatment of aggressive forms of breast cancer in clinical trials. In addition, Allen and coworkers have recently shown promising preclinical results with SSL immunoliposomes targeted to B cell malignancies using an anti-CD19 antibody (Lopes de Menezes et al., 1998).

The field of liposomal chemotherapy brings together a broad arena of scientific disciplines, including such varied practices as membrane biophysics, chemistry, biochemistry, cell biology, pharmaceutical technology, tumor physiology, toxicology, and clinical oncology. To be successful, scientific groups in the liposome field need to operate at the interface of these various disciplines and take as many of these practices into consideration when rationally designing a drug-carrier system, including the liposomes described in this review. It is hoped that this review will serve as a focal point from which future improvements in liposome technology can be made and, at the same time, as a reminder of how far we have come. The development of additional liposomal drug formulations should be aided by the advancements of the past, many of which are described in this review, but with the realization that no one formulation is ideal for all classes or even subclasses of drugs. The ability to be creative and to adapt what we have learned thus far will determine the success of this field in the future.

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This review was one of several projects that Dr. Papahadjopoulos was working on at the time of his sudden death in September 1998. Dr. Papahadjopoulos was a pioneer in the field of liposome-mediated drug delivery and an integral part of many major breakthroughs in the liposome field. His contributions started with basic membrane biophysics, leading to an understanding of the basic principles that would allow us to use these artificially prepared membranes as drug carriers, and evolved into the clinic with the development of Doxil, a liposome-based chemotherapeutic. Those contributions intervening are far too numerous to list here, and the full impact of his career on the oncology and liposome fields awaits time's trials. His dedication to the treatment of cancer and, more specifically, breast cancer ensured us all of a greater meaning for our research and for our lives. He remains an inspiration to all of us who remain in his laboratory (The Liposome Research Laboratory) at California Pacific Medical Center and, we are sure, to many others in the field as well.

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