

Designing therapeutically optimized liposomal anticancer delivery systems: Lessons from conventional liposomes

LAWRENCE D. MAYER,^{a,b} PIETER R. CULLIS^{c,d} AND MARCEL B. BALLY^{a,e}

^aDepartment of Advanced Therapeutics, BC Cancer Agency, 600 W. 10th Ave., Vancouver, B.C. V5Z 4E3, Canada; ^bPharmaceutical Sciences; ^cBiochemistry and ^ePathology Departments, University of British Columbia, Vancouver, B.C.; ^dInex Pharmaceuticals Corp., 100-8900 Glenlyon Parkway, Burnaby, BC V5J5J8

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I. Summary

Recent technological advances in the production, stability and biological (RES avoidance, targeting and intracellular delivery) properties of liposomes have greatly increased the degree of sophistication that can be designed into liposomes in order to improve their therapeutic/toxicity activity profile. Transmembrane ion gradients to control drug retention, surface stabilizing lipids to increase circulation longevity, targeting ligands to increase selectivity for disease sites/cells and fusogenic/internalizing components that increase intracellular delivery of liposome contents all have been employed for various therapeutic applications. However, the use of these technologies for particular disease states has often been based on rather intuitive projections of how such systems may act therapeutically in vivo.

For example, the increased delivery of liposome encapsulated anticancer agents to tumor sites relative to free drug is frequently used as a comparative characteristic to evaluate liposomal formulations. However, the improvements in efficacy obtained with liposomes are often small relative to the increase in disease site drug accumulation. Similarly, increases in liposome circulation lifetimes do not always translate into enhanced disease site localization and/or efficacy. Such examples point out that issues including drug bioavailability, disease site micro-environment and drug pharmacology (concentration vs. time dependence) must be addressed in order to efficiently optimize therapeutic liposomes. Further, the advantages provided by specific design components incorporated into conventional liposomes must be critically assessed in the context of what can be achieved by manipulating basic properties of the conventional liposomes themselves.

II. Liposomal anticancer drugs

Before considering the design attributes of liposomal carriers, it is useful to comment on some of the common rules that govern cancer chemotherapy and to reflect briefly on the rationale(s) for developing liposomal anticancer drugs. We would argue that there are two general reasons for developing a liposomal anticancer drug: (i) because the drug is hydrophobic and difficult or impossible to dissolve in aqueous solutions and thus requires a hydrophobic environment in order to stay in solution/suspension; and (ii) because the liposome can serve as a carrier that will improve drug specificity by increasing delivery to the site of disease and/or decrease delivery to a site where toxicities are manifested. The former is an important, perhaps underdeveloped, role for lipid-based carriers. However, the methods and characterization studies required for development of **liposomes formulated for optimal drug solubilization** should be clearly distinguished from those used in the development of **liposome drug carrier technology**. Differences in the two approaches can be defined primarily through in vivo studies that determine plasma elimination behavior of both drug and liposomal lipid. If the drug dissociates from the liposome immediately following administration then the lipid-based carrier is acting as an excipient for drug solubilization. When drug elimination parameters are dictated by the elimination behavior of the liposomes, then the systems are acting as a true delivery vehicle. This review will focus on the use of liposomes developed as drug carriers. In this regard, the primary consequence of anticancer drug encapsulation is liposome mediated changes in drug elimination and biodistribution. With this in mind, questions regarding why improvements in anticancer drug therapy should be achieved through the use of liposomal drug carrier technology can be addressed.

II.1. Optimal cancer chemotherapy requires the use of several drugs in combination

It is important to recognize that therapeutic responses obtained following administration of anticancer drugs, in free form or associated with a drug carrier, are

dependent on tumor physiology and tumor cell heterogeneity. Ideally, an effective drug must access the target cell populations at levels sufficient to cause cytotoxic effects and should be effective in all microenvironments present within tumors. In humans, strategies designed to maximize the antitumor activity of chemotherapeutic agents must, therefore, contend with a heterogeneous population of proliferating cells that are: (i) in various stages of the cell cycle; (ii) proliferating at different rates; (iii) growing in different tissues and (iv) capable of adapting rapidly to the chemotherapeutic stresses exerted on them. In practical terms this means that chemotherapy typically involves the use of multiple drugs that exert antitumor activity via different mechanisms.^{1,2} Vincristine, for example, is a cell cycle specific agent that acts by destabilizing microtubules and is almost always used in combination with two or three other anticancer drugs. The therapeutic action of vincristine is complemented by drugs such as doxorubicin, an anthracycline that is a DNA intercalator and topoisomerase II inhibitor, as well as cyclophosphamide, a nitrogen mustard pro-drug and strong alkylating agent. The mechanisms of therapeutic action of these drugs are complementary and, importantly, the toxicities of each drug are sufficiently different such that they can be used in combination without aggravation of any one specific target organ toxicity.

There are over 30 cytostatic and cytotoxic drugs commonly employed in the treatment of cancer and these drugs have been used in a variety of combinations that have been refined through years of clinical testing. Interestingly, drug carrier technology has been utilized for a limited number of drugs and there are only a few examples where pre-clinical studies evaluated a liposomal anticancer drug in combination with a second drug, either free or liposomal. Table 1 provides a list of drugs that have been formulated using liposomes. It is important to note that this list includes drugs that have been associated with liposomes in a manner that does not require chemical modification of the drug. We have not included, for

Table 1

Major antineoplastic agents evaluated in a liposomal drug carrier system

Class/Drug	# of different liposomal formulations	Pre-clinical evaluations	Clinical testing
Plant Alkaloids-			
Vincristine	<10	Extensive	Phase II
Vinblastine	<5	Very limited	—
Antibiotics-			
Doxorubicin	>10	Extensive	Approved
Daunorubicin	<5	Extensive	Approved
Antimetabolites-			
Methotrexate	<5	Limited	—
5-Fluorouracil	<5	Limited	—
Cytosine arabinoside	<5	Limited	—
Alkylating Agents			
<i>cis</i> -diamminedichloroplatinum	<5	Limited	—
Other-			
Mitoxantrone	<5	Extensive	Phase II

example, the valuable work completed by Roman Perez-Soler and his associates on lipophilic derivatives of doxorubicin and dach-platinum or the studies from Dr. Schwendener's laboratory on lipophilic derivatives of cytosine arabinoside. We have also not included the extensive work completed on the lipophilic macrophage activator muramyl tripeptide phosphatidylethanolamine or the more recent studies evaluating biopharmaceuticals such as the immune modulator IL-2, plasmid DNA or antisense oligonucleotides. We have restricted discussions to conventional anticancer drugs that exhibit proven therapeutic activity against cancer in humans.

II.2. Maximizing dose intensity

In addition to the necessity of using multiple agents to achieve optimal therapy, another general principle of cancer chemotherapy concerns maximizing dose intensity.³ Tumor cells must be exposed to the highest levels of drug for the longest time periods if maximum therapeutic effects are to be achieved.⁴ The advantage of anticancer drug carrier technology is based on carrier characteristics that give rise to increased drug exposure in sites of tumor growth. An example of how liposome drug carrier technology can improve the pharmacodynamic behavior of an anticancer agent is evident when evaluating previous studies with doxorubicin. Efforts to maximize the dose intensity of this chemotherapeutic agent (in free form) have been limited due to non-specific toxic side effects. For example, doxorubicin is a potent myelosuppressive agent.⁵⁻⁷ Therapeutic doses must, therefore, be limited to schedules and amounts that do not compromise regeneration of blood cells or cells of the immune system. In addition, doxorubicin exhibits a dose limiting cardiotoxicity^{8,9} restricting the total dose to approximately 450 mg/m². Myelosuppression can be counteracted using the hematopoietic growth factor granulocyte-macrophage colony stimulating factor (GM-CSF).¹⁰ Cardiotoxicity on the other hand can be reduced by administering the drug in a liposomally encapsulated form.^{11,12} It has also been shown that the therapeutic activity of the liposomal drug is greater than or equal to free doxorubicin in a variety of pre-clinical and clinical studies.^{13,14}

The pharmacodynamic alterations provided by liposomes appear to be well suited to basic principles of cancer chemotherapy. This review considers the relationship between circulation lifetime/plasma drug concentration and tumor drug accumulation as well as how liposome design is critical if optimal drug exposure at the disease site is to be achieved. The importance of achieving a balance between drug exposure and drug delivery to sites of tumor progression will become apparent.

II.3. Liposomal drug carriers versus drug infusion technology

For many applications, liposomal delivery systems are employed to improve the therapeutic index of encapsulated agents by selectively accumulating in extravascular disease sites. Further, there is an increase in evidence indicating that drug released from liposomes in the circulation does not contribute significantly to

therapeutic activity of liposomal anticancer agents. There is no question that liposomes can provide sustained exposure of therapeutic agents in the blood compartment through controlled release kinetics of encapsulated drugs, however it is difficult to justify development of liposomal drugs using a rationale that involves sustained systemic exposure. This is largely due to significant advances made in the area of drug infusion technology. Compact and cost effective infusion pumps are now widely used and these can provide well controlled systemic drug exposure over several days. We maintain that the most significant advantage for the use of liposome drug carriers arises as a consequence of disease specific changes in vascular permeability that favor accumulation of the intact liposome and associated drug into the site of disease progression. We differentiate this property from the benefits of drug infusion technology, which are primarily concerned with maintenance of circulating blood levels of free drug.

III. The compartmental model of liposome fate in vivo

In vivo studies are usually initiated only after one has developed a formulation that exhibits the necessary chemical and physical stability properties to be considered pharmaceutically viable. Subsequent in vivo analysis must then consider the fact that the liposomal drugs will interact with a number of distinct physiological "compartments" and associated barriers between compartments. For the purpose of discussions here, we will focus on systemic administration and, in particular, on the fate of liposomes injected intravenously (i.v.). After injection, liposomes are exposed to a variety of circulating protein and cellular components that reside within the central blood compartment, many of which can destabilize the liposomes through interactions with the lipid bilayer or initiate biological processes that lead to increased liposome leakage and/or clearance via the RES. To gain access to a disease in an extravascular compartment liposomes must cross the vascular endothelium, the blood vessel lining which is composed primarily of endothelial cells and, in most cases, an underlying basement membrane and associated smooth muscle cells. This vascular barrier represents the greatest obstacle for liposomal drug delivery to extravascular disease sites, however, at the same time it offers properties that can be utilized to differentiate between normal and diseased tissue. Should liposomes traverse this barrier, a second compartment is encountered consisting of the interstitial space and associated fluids and cells. This compartment can vary significantly not only between normal and disease tissues but also among normal tissues in different organs of the body. Within this compartment, the barriers to liposome movement and distribution are varied and include factors such as interstitial volume, interstitial pressure, and the presence (or absence) of a lymphatic system. The final physiological compartment(s) are the cells into which liposomes and/or their encapsulated agents are taken up. This includes intracellular organelles that may be involved in processing of the administered agent or that contain the molecular target through which the drug exerts its therapeutic activity. The critical barrier that must be crossed in order to access this final compartment is the cell membrane. Similar to the vascular endothelium,

crossing this barrier is a significant obstacle to the development of therapeutically optimized liposomal anticancer drugs.

In the following sections we will follow the fate of liposomes as they enter these physiological compartments and pass through the various barriers. We will focus on specific interactions between liposomes and the biological milieu in the various compartments that directly impact on the delivery of encapsulated agents to their therapeutic target. Further, we will highlight where strategies have been employed to augment conventional liposomes (defined as underivatized membrane bilayers composed of naturally-occurring or synthetic lipids) with components that alter these interactions. Importantly, we critically review the impact such manipulations have on meaningful therapeutic endpoints.

III.1. Liposomes in the central blood compartment

When liposomes are injected intravenously they are immediately exposed to a plethora of circulating cells, lipoproteins and soluble factors including proteins, carbohydrates and small ions (Na^+ , Cl^- , Ca^{2+} , Mg^{2+} , etc.). Assuming that liposomes contain sufficient amounts of cholesterol to avoid the bilayer destabilization effects of lipoproteins,^{15,16} the fate of liposomes in this compartment is dictated primarily by interactions between the liposome surface and serum protein components. Two deleterious responses can result when proteins adsorb to liposomes: (i) increased membrane permeability which compromises drug retention in the liposomes; and (ii) recognition and subsequent clearance of liposomes by the RES.

III.1.1. Liposome-protein interactions and membrane permeability

The ability of adsorbed blood proteins to increase liposome permeability properties has been demonstrated by several laboratories.¹⁶⁻¹⁹ Such interactions can be simply modeled by determining the drug release kinetics for liposomes suspended in serum compared to protein-free buffer. An example of this is shown in Figure 1. The leakage of vincristine from DSPC/Chol liposomes is approximately 5-fold faster in the presence of serum. Interestingly, comparison of these results with the release kinetics of vincristine from DSPC/Chol liposomes after i.v. administration (as determined by monitoring changes in the circulating drug-to-lipid ratio) reveals that drug leakage is further increased in vivo (Figure 1). These differences are not simply due to the presence of a "tissue sink" into which the released vincristine is absorbed since increased dilutions or extended dialysis times in the presence of serum do not increase in vitro drug release rates (L. Mayer, unpublished observations). Consequently, we believe that in vivo drug retention properties as well as comparisons of drug release kinetics for different liposomes cannot always be predicted simply on the basis of in vitro data.

In addition to increasing the permeability of liposome bilayers in the blood, protein adsorption can also lead to increased susceptibility to transmembrane stresses caused by ion gradients or high levels of encapsulated drugs. The high concentrations of buffer components and/or drug entrapped in liposomes often result in significant osmotic gradients across the liposome membrane when exposed

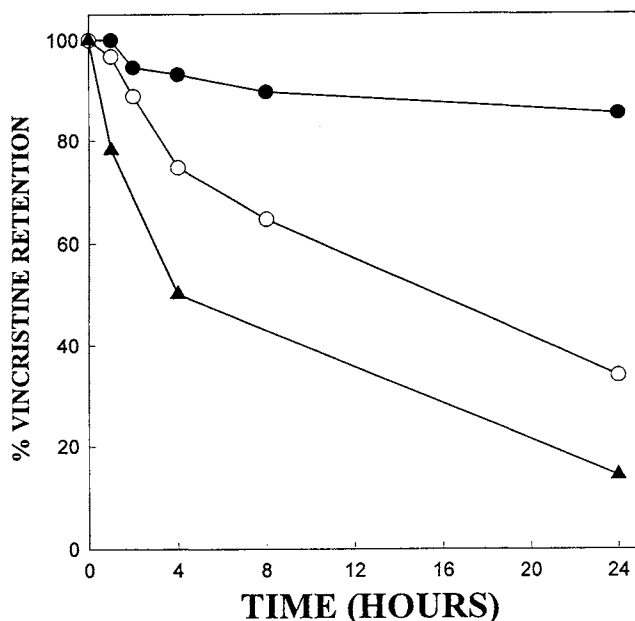


Fig. 1. Release of vincristine from 100 nm DSPC/cholesterol (55:45, mol: mol) liposomes encapsulated via pH gradient-dependent loading at a drug to lipid ratio of 0.05:1 (wt: wt). Liposomes were dialyzed in the presence of HEPES buffered saline, pH 7.5 (●) or bovine serum (○) at 37°C. Liposomal vincristine was injected i.v. into BDF1 mice at a drug dose of 2 mg/kg and the plasma drug to lipid ratio was determined and used to calculate the percent vincristine leakage from the liposomes in the circulation (▲).

to physiological fluids. While most liposomes can withstand a significant transmembrane osmotic gradient in the absence of extraneous proteins, exposure of liposomes exhibiting large osmotic gradients to plasma or purified lipoprotein fractions results in a burst of leakage from the liposomes while osmotic balance is re-established.²⁰ This effect is more pronounced with less ordered membranes where, for example, DSPC/Chol liposomes can withstand osmotic gradients of far greater magnitude than EPC/Chol liposomes in the presence of proteins.²⁰ This may, in part, explain the differences observed between DSPC/Chol and EPC/Chol liposomal doxorubicin formulations *in vivo* where the circulating drug-to-lipid ratio (used to assess drug leakage) observed for EPC/Chol liposomes drops approximately 50% within 1 h of injection and subsequently decreases to a release rate comparable to that observed for DSPC/Chol (Figure 2).

III.1.2. Liposome-protein interactions and circulation longevity

A significant amount of attention has focused on identifying the protein components in the circulation that, upon binding to liposomes, mark them for clearance from the circulation (for review see Ref. 21). Studies have demonstrated that increased protein binding to liposomes after i.v. administration is associated with

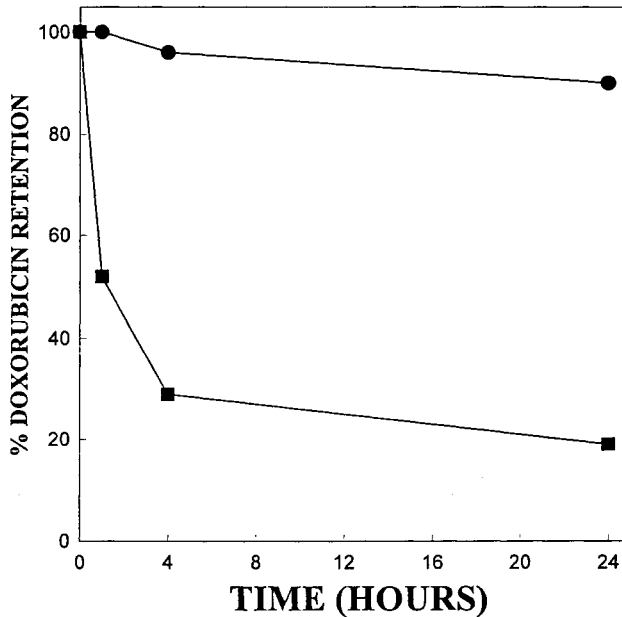


Fig. 2. Release of doxorubicin from DSPC/cholesterol (●) and EPC/cholesterol (■) liposomes in plasma after i.v. injection to mice. The 100 nm liposomes were prepared at a phospholipid to cholesterol molar ratio of 55:45. Doxorubicin encapsulation was completed using the pH gradient entrapment technique at a drug to lipid weight ratio of 0.2:1 (wt:wt). Lipid levels in plasma were determined using tritiated cholesterylhexadecyl ether and doxorubicin was quantified by fluorescence detection of extracted samples.

increased elimination from the blood.^{19,21,22} Increased protein binding and clearance is observed for liposomes prepared with phosphatidylserine, cardiolipin and phosphatidic acid. Certain proteins, most notably complement proteins, serum albumin and beta 2 glycoprotein 1 have been associated with increased recognition or “opsonization” of these liposomes.^{18,22–24} In contrast, liposomes prepared with other anionic lipids such as phosphatidylglycerol and phosphatidylinositol exhibit extended circulation lifetimes following i.v. administration despite having reasonable levels of adsorbed serum proteins. Variations in the fluidity (acyl chain composition) of neutral liposomes containing $\geq 30\%$ cholesterol do not result in substantial differences in the types of proteins adsorbed and correspondingly these liposomes are cleared from the circulation at similar rates.^{18,22}

III.1.3. Inhibition of liposome-protein interactions

Clearly, rapid release of a liposomal encapsulated agent following i.v. administration negates the value of using liposomes as drug carriers. In addition, unless the target disease is localized in organs such as the liver and spleen,^{25,26} rapid removal of liposomes from the central blood compartment seriously compromises their ability to provide pharmacological improvements as drug delivery systems. In this regard, one significant breakthrough in liposome technology over the past decade

has been the identification of lipids, in particular polyethylene glycol (PEG)-derivatized PE, that can be incorporated into conventional liposomes which reduce protein interactions with liposomes.^{19, 27–30} It is believed that these lipids act by providing a steric barrier that limits the exposure of the liposome surface to macromolecules in bulk solution.^{31,32} Liposomes which are prepared using these lipids exhibit extended circulation times relative to conventional liposomes of similar bulk lipid composition. The application of this technology is discussed in greater detail in Chapter 4.3 by Goren and Gabizon. Of interest are recent observations where reduced protein binding and increased circulation longevity of neutral liposomes can be achieved without incorporating PEG by utilizing sphingomyelin rather than phosphatidylcholine as the main bilayer forming lipid.³³ Given that both lipids contain the same phosphorylcholine head group, we can suggest that the decreased protein binding and clearance result from altered lipid packing properties for sphingomyelin which may limit adsorption and insertion of protein domains into the hydrophobic region of the bilayer. It should be noted that work completed by Parr et al.³³ and Holland et al.³⁴ suggest that PEG-modified lipids exchange out of the liposomal membrane at a rate that is dependent on the acyl chain composition. PEG-modified lipids prepared using unsaturated or short (<14 carbons) acyl chains are lost rapidly following i.v. administration.

III.1.4. Is there a therapeutic advantage to increased circulation lifetimes?

Although it is generally believed that liposomes for systemic drug delivery should contain either PEG-lipids or other “stabilizing” lipids (e.g., sphingomyelin), such generalizations can be misleading and at times inappropriate depending on the disease site and drug being delivered. This is perhaps best exemplified by reviewing the biological properties of liposomal formulations developed for the anticancer drugs doxorubicin, mitoxantrone and vincristine.

III.1.4.1. Doxorubicin Doxorubicin is an anthracycline antineoplastic agent that is actively taken up by cells and is retained with high avidity by many tissues, most notably those associated with drug toxicity (heart and epithelial cells of the gut) and tumors. Liposome encapsulation can significantly reduce the toxicity of doxorubicin by decreasing drug accumulation in drug sensitive normal tissue, presumably by decreasing peak levels of free doxorubicin that are experienced after administration in conventional (unencapsulated) form.^{11,12,35–37} The degree of toxicity buffering is directly related to the ability of the liposomes to retain their entrapped doxorubicin where increased phospholipid acyl chain saturation results in decreased toxicity.^{37,38}

The antitumor activity of liposomal doxorubicin, however, is much less sensitive to drug leakage or circulation longevity. Liposomal formulations with widely varying doxorubicin retention properties have been shown in some preclinical models to exhibit comparable antitumor activities when compared on an equal dose basis.³⁸ In this case, increased efficacy for the less permeable liposomes is achieved by the ability to administer elevated drug doses due to their reduced toxicity. Further, while the inclusion of PEG-PE increases the circulation longevity

of liposomal doxorubicin,^{39,40} the magnitude of increased liposome levels in the blood (compared to conventional liposomes) is far less than that observed for empty (drug-free) liposomes.^{40,41} This is related to an RES blockade effect that is observed for doxorubicin loaded conventional liposomes (see Section III.2.1 for further discussion). Controversy still exists as to the overall therapeutic benefit of incorporating steric stabilizing lipids like PEG-PE into conventional liposomes since examples of significant as well as negligible improvements in efficacy have been documented.³⁹⁻⁴² (For comparison, see Chapter 4.3 by Goren and Gabizon.)

III.1.4.2. Vincristine In contrast to the observations made with doxorubicin, altering the physical properties of liposomal vincristine formulations results in dramatic changes in antitumor activity while only minimally affecting drug toxicity characteristics. Increasing the retention of vincristine inside 100 nm liposomes by changing the phosphorylcholine-containing lipid component from EPC to DSPC to sphingomyelin (while maintaining cholesterol content at 45 mol%) leads to dramatic increases in antitumor activity, particularly when compared to the efficacy obtained with free vincristine.⁴³⁻⁴⁵ This is consistent with the steep dependence of vincristine antitumor potency on the duration of drug exposure^{45,46} as well as the fact that retention of vincristine in most tissues, including tumors, is rather poor.⁴⁷ In this case it appears that the ability to prolong the exposure of vincristine *in vivo* is more important than peak drug concentrations. Furthermore, although inclusion of PEG-PE in the liposomes increases the circulating liposomal lipid levels at extended time periods, this steric stabilizing lipid does not improve the vincristine pharmacokinetic or therapeutic properties over conventional DSPC/Chol or sphingomyelin/Chol systems.⁴⁵ This is due to the fact that PEG-PE increases the permeability of the lipid bilayer to vincristine, thus offsetting the potential benefits provided by increased longevity of the liposomal carrier. The reasons for this increased drug leakage are not well understood. It may be related to the fact that PEG-modified phosphatidylethanolamine is negatively charged and this may alter drug partitioning properties at the inner monolayer membrane surface. In addition, it is not yet clear whether this phenomenon is specific for vincristine encapsulated via pH gradient techniques employing citrate buffers, compared to ammonium sulfate entrapment systems.⁴⁸

III.1.4.3. Mitoxantrone The final example, derived from recent reports describing liposomal formulations of mitoxantrone, illustrates how a balance between efficient liposome delivery to the disease site and controlled drug release can work synergistically to achieve optimum therapeutic results.^{25,26} Mitoxantrone is less cardiotoxic than doxorubicin and is not capable of generating free radical mediated toxicities on non-dividing cell populations.⁴⁹ The liposome mediated increases in mitoxantrone MTD observed for formulations (phosphorylcholine and cholesterol based systems) described by Chang et al.²⁵ and Lim et al.²⁶ are comparable to those reported for liposomal mitoxantrone formulations prepared using an anionic lipid-drug complex.^{50,51} In contrast to the results of Schwendener et al., liposomal mitoxantrone formulations prepared using DSPC or DMPC and cholesterol

(45 mol%) exhibit significantly better drug retention characteristics. This is reflected in higher blood levels and improved circulation lifetimes for mitoxantrone encapsulated in the PC/Chol based liposomal carriers. These differences may be due to protein binding and rapid clearance of anionic liposome formulations. Alternatively, differences in drug release characteristics may, as suggested above for vincristine, be a consequence of the use of anionic lipids, which have been shown to enhance release of the anthracycline doxorubicin even in the absence of serum.⁵²

Studies evaluating the therapeutic activity of DSPC/Chol and DMPC/Chol liposomal mitoxantrone focused on treatment of an i.v. L1210 and/or P388 tumor model, where cells seeded primarily in the liver and spleen following i.v. administration.²⁶ These studies illustrated how controlled drug release effected significant improvements in therapeutic activity of the anticancer drug mitoxantrone. It is well established that the liver is a primary site of liposome accumulation, and that the rate of accumulation for DSPC/Chol liposomes in liver is comparable to DMPC/Chol liposomes. Based on this information, a relatively simple question was asked: Is a liposome (DSPC/Chol) which retains drug following i.v. administration therapeutically more active than a liposome (DMPC/Chol) that releases drug when tested against a tumor that progresses in the liver? Despite being less effective in terms of delivering drug to the site of tumor progression, the DMPC/Chol liposomes, which release drug steadily following administration, were strikingly more efficacious than the DSPC/Chol formulations. A natural extension of the previous question was: What effect would incorporation of PEG-modified lipids have on the therapeutic activity of either of these formulations when used to treat disease in the liver? For both formulations, addition of PEG-PE resulted in significant reductions in antitumor activity²⁵ (Lim et al., unpublished observation).

It can be concluded from such data that it is not necessarily sufficient to develop drug carriers that accumulate at the disease site to high levels; one must also engineer appropriate drug release rates. Controlled drug release must, however, be balanced with liposome mediated drug delivery to the site of tumor growth. Regardless, it is apparent that whether or not additional components (i.e., PEG-PE) should be incorporated into conventional liposomes must be re-evaluated for each therapeutic agent and one must consider the site of disease progression.

III.2. Barriers to extravasation

While in the circulation, liposomes are continually exposed to cells lining the vasculature. The inner lining, or intima, of blood vessels is composed primarily of endothelial cells which form a contiguous layer on the interior surface of all blood vessels. Underlying this layer is the basement membrane and in larger (non-capillary) vessels the vasculature is supported by smooth muscle cells.⁵³ The endothelial cells in most normal vasculature exhibit intact intercellular junctions and only small molecules are able to readily permeate across capillaries of this type. However, this structure is significantly altered in certain normal tissues, most notably the liver and spleen, as well as in disease sites such as infection and tumor

growth. The latter are characterized by the presence of capillaries that exhibit fenestrae or larger intercellular openings and can be devoid of the basement membrane layer. The gaps in these endothelial layers can range in size from 30 nm for fenestrated capillaries to greater than 500 nm in liver, tumor and inflammation site vascular beds.⁵⁴⁻⁵⁶ In the liver, these openings provide access to sinusoids wherein the phagocytic Kupffer cells lie. In disease sites, the fenestrated/discontinuous capillary beds and post-capillary venules allow direct exposure of the underlying epithelial cells to the circulation. It is the unique nature of vascular structures that exist in liver/spleen and disease tissues which significantly impacts the pharmacological behavior of liposomal drug delivery systems.

III.2.1. The reticuloendothelial system (RES) and liposome clearance

The RES has long been recognized as the major site of liposome accumulation after systemic administration. The primary organs associated with the RES are the liver, spleen and lung. The liver exhibits the largest capacity for liposome uptake while the spleen can accumulate liposomes such that the tissue concentration (liposomal lipid/gm tissue) is 10-fold higher than that which can be achieved in other organs. Assuming that liposomes are designed to minimize protein binding and cell interactions, the extent of liposome accumulation in the lung is typically below 1% of the injected dose. Early studies demonstrated that large as well as charged liposomes (particularly those containing negatively charged lipids like PS, PA or cardiolipin) were removed very rapidly by the liver and spleen with clearance half-lives of less than 1 hour.^{57,58} The rate of clearance from the circulation could be reduced to some extent by increasing the administered lipid dose, however, only when small (approx. 100 nm), neutral liposomes containing $\geq 30\%$ cholesterol were utilized at doses of at least 10 mg/kg or more could circulation lifetimes in the range of several hours be achieved.⁵⁹ The removal of liposomes from the blood is attributed to phagocytic cells that reside in the RES and appears to be mediated through direct interactions between the phagocytic cell and the liposomes. In vitro studies have shown that liposome uptake into macrophages can occur in the absence of serum proteins, however recognition mediated by protein elements that associate with liposome surfaces is likely playing a dominating effect on interactions with the RES (see Section III.1.2).

The identification of certain naturally occurring lipids (e.g., ganglioside GM₁ and PI) that increase the circulation lifetime of liposomes in which they are incorporated spawned what is often referred to as the "second generation" of liposome technology. Analogous to the development of polymer surfaces that exhibit reduced protein binding characteristics, it is believed that these carbohydrate containing lipids act by limiting the interaction of liposome surfaces with proteins and this, in turn, inhibits the rate of uptake by phagocytic cells.^{29,30,60} As indicated in Section III.1.3, a variety of synthetic lipids have been developed to prevent protein binding. The most notable are based on hydrophilic polymers, such as PEG, which are attached to phospholipids such as PE. Perhaps the most widely utilized steric stabilizing lipid is one composed of 2,000 MW linear PEG attached to DSPE and it is incorporated at levels of 2 to 10 mol% in the bilayer

of conventional liposomes. Inclusion of PEG-PE into conventional empty neutral (PC/cholesterol) liposomes can result in 3- to 20-fold increases in plasma liposome content 24 h after i.v. injection.^{30,32,40} This is accompanied by significant decreases in liposome uptake by the liver and spleen at early times post-injection. It is important to note that the difference in cumulative uptake of liposomes by the RES organs between conventional and sterically stabilized liposomes become less significant over time, indicating that the effect of PEG-PE is to reduce the rate of liposome removal by cells of the RES. It has not been determined whether eventual removal of these liposomes by the RES is due to time dependent increases in protein association or the loss of PEG from the surface of the liposomes.³³

Although liposome elimination rates differ greatly between conventional and sterically stabilized liposomes in the absence of encapsulated agents, this difference can be significantly reduced for liposomes containing entrapped drugs, particularly drugs that impair the ability of cells to accumulate or process liposomes.⁶¹⁻⁶³ This is perhaps best exemplified in the case of the anticancer drug doxorubicin. As shown in Figure 3, when empty 100 nm DSPC/Chol liposomes are injected i.v. at a lipid dose of 100 mg/kg into C3H mice, inclusion of PEG-DSPE results in circulating liposomal lipid levels 24 h post injection that are approximately 20-fold higher than that observed in the absence of the PEG-lipid. However, when the

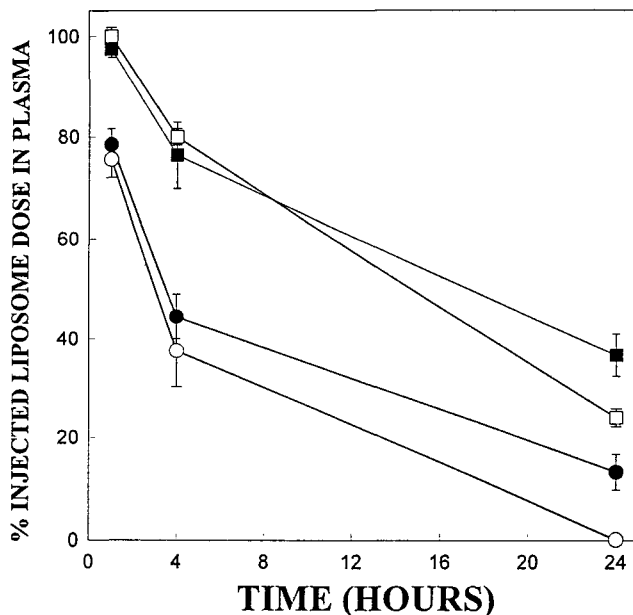


Fig. 3. Circulating levels of 100 nm empty (open symbols) or doxorubicin loaded (closed symbols) DSPC/cholesterol liposomes after i.v. injection to C3H mice. Liposomes were prepared in the absence (●, ○) or presence (■, □) of 5 mol% PEG₂₀₀₀-DSPE. Doxorubicin was encapsulated using the pH gradient-dependent entrapment technique and quantification of plasma levels of liposomes and doxorubicin were accomplished as described in the legend to Figure 2.

DSPC/Chol liposomes are loaded with doxorubicin, the 24 h plasma liposome concentrations are significantly increased and are only 2.8-fold less than those observed for 5 mol% PEG-DSPC containing DSPC/Chol liposomal doxorubicin systems.⁴¹

Significant increases in circulating levels of empty liposomes can also be achieved by pre-dosing animals with a low dose (10 mg lipid/kg) of liposomal doxorubicin.^{61,62} This effect, referred to as RES "blockade", has raised concerns over potential deleterious side effects resulting from altered RES phagocytic capacity. In vitro studies have demonstrated that liposomal doxorubicin uptake by cultured macrophages can result in cell death,⁶⁴ and exposure of macrophages in culture to concentrations of doxorubicin that are not cytotoxic significantly impairs the ability of these cells to accumulate particles (M. Bally, unpublished observation). Although a substantial amount of doxorubicin can accumulate in liver tissue,⁶⁵ indications of significant liver toxicity arising from this uptake have only been observed pre-clinically under conditions of extremely high doses (80 mg doxorubicin/kg) and in clinical situations where pre-existing liver impairment was a factor.⁶⁶

Investigators have been able to demonstrate macrophage and Kupffer cell depletion following administration of high doses of large and/or negatively charged liposomes containing doxorubicin or other agents such as clodronate.⁶³ RES blockade induced by low doses (<10 mg/kg lipid and 2 mg/kg drug) of small, uncharged liposomal doxorubicin formulations, however, does not appear to result in reduced numbers of Kupffer cells.⁶⁵ This was determined by histological evaluations of thin sections of liver stained with hematoxylin and eosin as well as on the basis of carbon particle uptake in livers of mice that have been previously (4 days) treated with liposomal doxorubicin. This information suggests that our understanding of the mechanisms whereby liposomes (particularly small liposomes) are recognized, cleared from the blood and processed may be somewhat simplistic.

In light of the observations cited above, steric stabilizing lipids are likely to provide the greatest RES avoidance benefits at low liposome doses and for liposome formulations containing drugs that do not lead to reduced liposome clearance. Regarding the latter, it has been shown that encapsulation of vincristine, doxorubicin or cisplatin results in a reduction in liposome elimination. In contrast, liposomal mitoxantrone formulations exhibit circulation characteristics identical to liposomes without entrapped drug. It should also be stressed that the theoretical "benefits" arising from decreased liposome elimination by the RES is typically assumed to be related to the increased circulating concentrations of liposomes obtained. However, we suggest that it is not the plasma concentration of liposomes that dictates therapy, but rather the amount of liposomal drug that penetrates the vascular barrier and gains access to diseased tissue. In the following section we will focus on this extravasation event.

III.2.2. Liposome extravasation through vascular endothelium

If liposomes are designed in an appropriate manner, with respect to size, lipid composition, and/or use of PEG-modified lipids, they can remain in the blood

compartment for a period of several days. The fact that under such circumstances the vast majority of liposomes administered can be accounted for in the blood, liver and spleen demonstrates that liposomes are inefficient at crossing the endothelial cell barrier present in most tissues. The property of long circulating liposomes that is exploited for therapeutic purposes relies on changes in the endothelial cell barrier, prevalent in many disease states, that allow liposomes to traverse out of the blood compartment and into the tissue.

Major diseases, such as bacterial infection, inflammation and tumors, have the common feature of altered vasculature permeability at the site of disease progression. The mediators that lead to increased permeability of the vascular barrier are quite distinct for different disease states. For example, chemotactic factors and adhesion molecules overexpressed at sites of inflammation attract infiltrating lymphocytes and granulocytes that subsequently release factors which can directly damage endothelial cells and/or cause defects in intercellular junctions.⁶⁷ In hypoxic environments, such as those that arise during rapid cell proliferation or through vascular injury, cells can release vascular endothelial growth factor (VEGF).^{68,69} VEGF is an endothelial cell specific mitogen and its release can lead to the development of neovasculature. Interestingly, VEGF has proven to be identical to vascular permeability factor,^{70,71} a protein first identified as a factor capable of inducing defects in the permeability barrier of blood vessels. Regardless of the mediator, the end result for all of these conditions is the presence of blood vessels that are permeable to large molecules. This may be a consequence of fenestrae or larger “gaps” occurring between adjacent endothelial cells through which macromolecules can pass⁷² or, alternatively, may involve increases in endothelial cell mediated transcytosis.⁷³

Increases in vascular permeability give rise to the accumulation of small liposomes in sites of infection, inflammation and tumor growth. However, this is not a selective process and there is also a general increase in extravascular fluids in these regions. The hydrostatic pressure within these sites is elevated relative to the vascular pressure, resulting in a pressure gradient that impedes movement of molecules from the blood into the tissue interstitium.^{74,75} We must therefore assume that additional features lead to selective accumulation of macromolecules in the diseased extravascular space. Studies, for example, have demonstrated that the lack of a developed lymphatic system in conjunction with the large openings in the vascular endothelial cell lining may lead to an extravascular “trapping” phenomenon.⁷⁵ In the absence of lymphatic drainage, interstitial diffusion of molecules leads to egress from the disease site and this diffusion rate is dependent on molecule size, small molecules exiting more rapidly than large molecules.

Liposome extravasation and accumulation in solid tumors has been well studied and there is a great deal of phenomenological evidence demonstrating that liposomes can enter an extravascular site in regions of tumor growth following i.v. administration. Although evidence for endothelial cell uptake of liposomes and transcytosis across endothelial cells have been documented, videomicroscopy investigations in solid tumor models indicate that the majority of liposome extravasation occurs directly through the openings present in tumor neovasculature.^{76,77}

Table 2

Tumor accumulation efficiency (T_e) for conventional and steric stabilized (PEG-containing) liposomal anticancer drug formulations

Tumor model	Preparation ^a	Plasma AUC ^b	Tumor AUC	T_e ^c
Lewis lung (murine solid tumor)	DSPC/Chol ^d	2,118 $\mu\text{gh/ml}$	819 $\mu\text{gh/g}$	0.39
	DSPC/PEG-PE/Chol ^d	7,910 $\mu\text{gh/ml}$	1,432 $\mu\text{gh/g}$	0.18
Fsa-N fibrosarcoma (murine solid tumor)	DSPC/Chol ^e	10,560 $\mu\text{gh/ml}$	2,981 $\mu\text{gh/g}$	0.28
	DSPC/PEG-PE/Chol ^e	18,500 $\mu\text{gh/m}$	2,892 $\mu\text{gh/g}$	0.16
P388 (murine ascitic tumor)	DSPC/Chol ^e	16,530 $\mu\text{gh/ml}$	1,720 $\mu\text{gh/peritoneum}$	0.10
	DSPC/PEG-PE/Chol ^e	37,600 $\mu\text{gh/ml}$	2,037 $\mu\text{gh/peritoneum}$	0.05
	SM/Chol ^f	5,116 $\mu\text{gh/ml}$	206 $\mu\text{gh/peritoneum}$	0.041
	SM/PEG-PE/Chol ^f	6,762 $\mu\text{gh/ml}$	184 $\mu\text{gh/peritoneum}$	0.027

^aArea under the curve (AUC) values were calculated as trapezoidal AUC over the time period 0–24 h.

^bAll liposomes were 100 nm in size and contained 45 mol% cholesterol. PEG-DSPE was incorporated at 5 mol% when utilized.

^cTumor accumulation efficiency was calculated as the 0–24 h liposome AUC in the tumor divided by the 0–24 h liposome AUC in plasma.

^dEmpty liposomes injected at a dose of 100 mg/kg.

^eLiposomal doxorubicin preparations constituted by pH gradient encapsulation at a drug to lipid weight ratio of 0.2:1.

^fLiposomal vincristine preparations constituted by pH gradient encapsulation at a drug to lipid ratio of 0.1:1.

This extravasation process appears to be quite heterogeneous within the tumor and does not appear to be associated with any specific histological characteristics in the tumor mass. The net result of this phenomenon is that peak drug concentrations achieved are greater and drug exposure as measured by concentration vs time AUCs is increased when the drug is administered in a liposome, compared to free form.

The design of liposomes that will exhibit maximal extravasation in disease sites associated with leaky vasculature has received considerable attention and is an area of some controversy. As summarized in Section III.1.3, the inclusion of PEG-modified lipids in conventional liposomes can significantly increase the circulating liposome levels over extended times by decreasing the rate of clearance by the RES. It has generally been assumed that increases in the concentration of liposomes in plasma over time will lead to increased accumulation of liposomes in extravascular disease sites and experimental evidence supporting this has been reported.⁴² Videomicroscopy has also suggested that the permeability coefficient of tumor vasculature is greater for PEG-PE containing liposomes compared to conventional liposomes.⁷⁷ In contrast, studies conducted in our laboratories as well as others have demonstrated that although plasma levels of PEG containing liposomes are several fold higher than for comparable conventional liposomes, this often does not result in increased extravasation and accumulation in solid tumor tissue.^{40,41,78}

As shown in Table 2, we have examined the tumor uptake properties for

conventional and steric stabilized liposomal formulations of doxorubicin and vincristine in a variety of tumor models. Three important observations can be made on the basis of the comparative biological properties of conventional and sterically stabilized liposomes. First, sterically stabilized liposomes uniformly display increased circulation longevity compared to conventional liposomes, regardless of the presence of encapsulated drug. Second, the rate and extent of liposome accumulation in tumor tissue are often comparable for both conventional and sterically stabilized liposomes. Third, the tumor targeting efficiency or T_E (defined as the AUC in the tumor divided by the AUC in plasma) is higher for conventional liposomes compared to sterically stabilized systems. It is important to note that the relationship between tumor liposome uptake and plasma liposome AUC is linear for conventional and sterically stabilized liposomes, respectively (M. Bally, unpublished observation). This suggests that mass action does appear to drive the accumulation of specific types of non-targeted small liposomes into tumors. However, inclusion of lipids such as PEG-DSPE appears to decrease the efficiency of liposome extravasation from the blood into tumor tissue as indicated by the decreased T_E values observed for sterically stabilized liposomes in several solid tumor models (Table 2).

The basis for discrepancies in tumor extravasation comparisons between conventional and sterically stabilized liposomes may be related to one of several potential explanations. The tumor models utilized may exhibit different vascular structures⁷⁹ and it is reasonable to assume that increases or decreases in conventional liposome extravasation in comparison to sterically stabilized liposomes may be tumor specific. However, many different tumor types have been evaluated and it would appear that preferential accumulation efficiency of conventional liposomes is prevalent in most tumor types, regardless of differences in vascular structure. Another factor that may contribute to the discrepancies concerns the techniques utilized to monitor liposome extravasation. In the study by Dewhirst and co-workers, a fluorescent lipid label was employed to follow liposome distribution using fluorescent videomicroscopy in a breast carcinoma skin flap window chamber model.⁷⁶ Vascular permeability measurements were based on extravasation events that occurred over 90 minutes post injection. In such studies it is important to demonstrate that the fluorescent lipid label is equally representative as a marker for conventional and sterically stabilized liposomes, particularly when considering the potential for such lipids to exchange.⁸⁰

Our comparisons are typically based on extended AUC measurements of total tumor liposome uptake (following a non-exchangeable, non-metabolizable lipid label and correcting for blood volume contributions) and we place great emphasis on measuring both liposomal lipid and drug over the specified time course. Simultaneous measurements of drug and liposomal lipid can be used to assess drug retention, which is a determining factor in terms of accumulation of entrapped contents in tumors. It should be noted that the lipid compositions utilized in our studies for conventional and sterically stabilized liposomes contain 45 mol% cholesterol whereas studies by others often utilize liposomes with 33 mol% cholesterol. Reduced cholesterol content will result in increases in drug permeability

(see Section III.1). Several comparative studies demonstrating improved tumor accumulation for sterically stabilized liposomes have relied on the use of entrapped aqueous markers such as Ga^{67} which are rapidly cleared when released from the liposomes. Consequently, it is difficult to determine if differences in tumor accumulation are due to altered elimination and/or extravasation properties affected by lipid composition or a result of lipid composition effects on drug retention. It is important to resolve these issues and this will require a concerted effort to standardize the tumor models, liposome compositions and methods/parameters for evaluating liposome extravasation into tumors.

It should not be unexpected that conventional and sterically stabilized liposomes exhibit different efficiencies in extravasation. Videomicroscopy studies with steric stabilized liposomal doxorubicin systems have identified that some endothelial cells can take up liposomes.^{76,77} Endothelial cell interactions may contribute to the extravasation process either directly via transcytosis or indirectly by facilitating an increase in the local liposome concentration at the endothelial cell surface, thereby increasing access to openings in the vasculature. Given the effects of PEG on inhibiting liposome-cell interactions, this polymer may reduce endothelial cell interactions and this, in turn, would reduce the rate of extravasation. In contrast, conventional liposome extravasation could be facilitated through increased interactions with the endothelial cell lining of the neovasculature in tumors. This is, of course, highly speculative but is consistent with the surface properties of conventional liposomes compared to steric stabilized liposomes. A logical extension of this argument, however, is that improved extravasation may be possible by designing liposomes which interact more extensively with vascular endothelium in tumors.

III.3. The behavior of liposomes in interstitial tissue compartments

Once liposomes have moved through the vascular endothelial barrier, their fate in the interstitial spaces is tissue specific. Generally, negligible levels of liposomes extravasate into tissues such as muscle and kidney.⁴⁵ Presumably the liposomes that have distributed into these sites migrate slowly through the intercellular matrix until they are removed via the lymphatics. Interestingly, liposomes administered i.v. do appear to accumulate to high levels in lymph nodes (on a per weight basis), where combined filtration and presence of phagocytic cells act to concentrate liposomes.⁸¹ In liver and spleen, fixed macrophages actively take up liposomes and these cells process the carrier via the intracellular phagolysosomal system. However, for the purpose of this discussion we will focus on the behavior of liposomes that have extravasated into disease sites, and in particular, into solid tumors.

As cited in Section III.2, the distribution of liposomes which have extravasated into the tumor interstitium is heterogeneous. This is not unexpected given the irregular and often redundant organization of tumor vasculature. Tumor vascular structure often engenders highly variable blood flow properties and evaluation of histological sections from tumors reflect this heterogeneity. This would be more apparent for liposomes compared to unencapsulated small molecules due to the

decreased diffusion through the interstitial space for large macromolecules. This slow diffusion after extravasation has been documented by fluorescence video microscopy where fluorescently labeled liposomes could be seen to accumulate in the perivascular spaces primarily associated with the roots of capillary sprouts.⁷⁷ Diffusion away from these sites was observed to be very slow and significant perivascular clustering was observed for several days. This is consistent with the data from several tumor models which demonstrate that tumor accumulation levels of liposomes reached a maximum approximately 24 h after injection and these levels are maintained for extended time periods. Importantly, evaluations of drug accumulation properties can suggest remarkably different behavior, where drug release from the liposomes in the extravascular site results in greater drug penetration into the tissue and more rapid loss of the drug from the site when compared with the loss of liposomal lipid.

The preferential extravasation and accumulation of liposome encapsulated anticancer drugs in solid tumors results in tumor drug levels that can be as much as 15-fold higher than achieved with free (non-liposomal) drug.^{38,40,45} An example of this increased tumor drug delivery is shown in Figure 4. These fluorescent micrographs illustrate the dramatic increase in tumor doxorubicin levels obtained at 24 h after injection when the drug is encapsulated inside 100 nm DSPC/Chol liposomes compared to free doxorubicin. In addition, the prolonged residence of liposomes in tumors also significantly increases the duration of tumor drug exposure and AUC relative to free agents.⁴⁰ In some tumor models, such properties have been shown to correlate with increased antitumor activity for liposomal formulations of drugs such as doxorubicin and daunorubicin. It is not clear from these studies, however, what the relative increase in therapeutic potency is in the context of tumor drug delivery improvements. Specifically, studies have typically compared the efficacy and tumor drug accumulation following administration of equal doses of free and liposomal drug. A comparison of efficacy under conditions where tumor drug accumulation is comparable for free and liposomal drug has not been completed, but would likely demonstrate that the liposomal drug is less potent. Other studies have demonstrated comparable antitumor efficacy for free and liposomal doxorubicin under conditions where tumor drug levels were as much as 5-fold higher for liposomal systems.⁴⁰ Such observations have raised obvious questions about the bioavailability of anticancer drugs carried inside liposomes that have extravasated into solid tumors as well as the mechanisms that lead to drug release in the interstitial compartment.

The consensus emerging from studies in several laboratories on the mechanism of action of liposomal anticancer drug formulations is that liposomes exert their effect on therapeutic activity by providing an *in situ* drug infusion reservoir within the tumor. Once released, the anticancer drug can diffuse through the tumor and has direct access to tumor cells where it can act in a manner that presumably is similar to drug in the absence of a liposomal carrier. *In vitro* studies have demonstrated that macrophages can engulf doxorubicin loaded liposomes, process them and re-release doxorubicin extracellularly in free form.⁸² In view of the high macrophage content residing in some tumors,⁸³ such phenomena led to the pro-

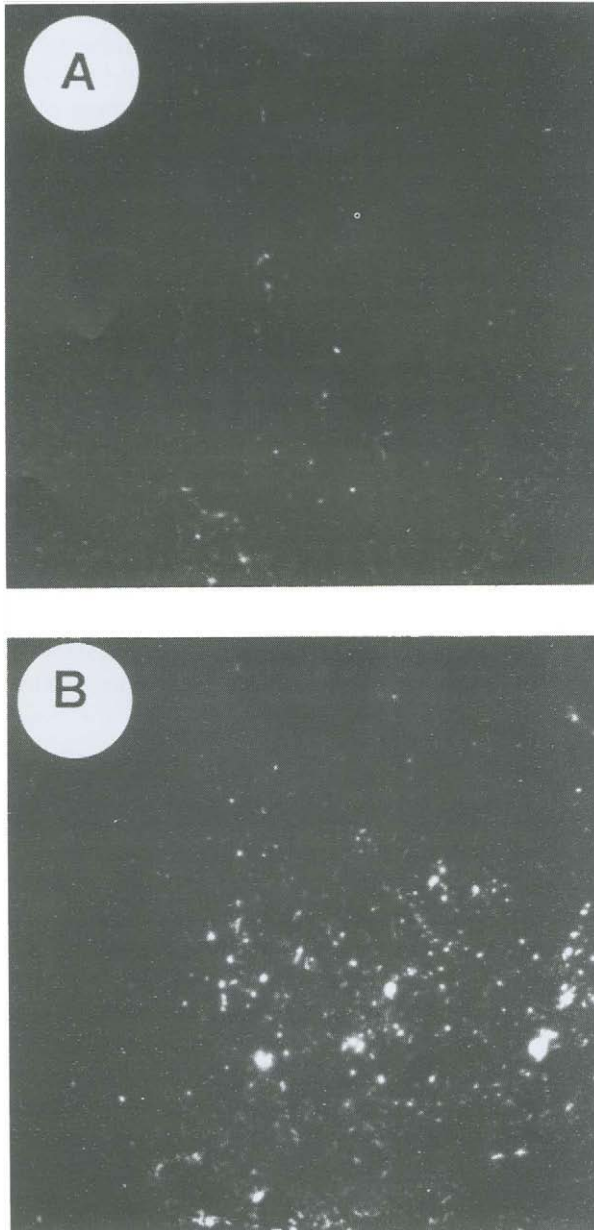


Fig. 4. Fluorescent micrographs of Fsa-N fibrosarcoma frozen thin sections 24 hours after i.v. injection of free (A) and 100 nm DSPC/cholesterol liposome encapsulated (B) doxorubicin at a drug dose of 20 mg/kg. Images were viewed employing a 10 \times objective lens.

posal that liposomal anticancer drug release may involve macrophage processing after extravasation. However, recent studies have shown that in solid tumors there are limited interactions between tumor associated macrophages and extravasated liposomes.⁴¹ Although macrophage enriched tumors do accumulate higher levels of liposomal doxorubicin, this effect appears more related to increased vascular permeability rather than direct uptake and processing of the liposomes by the macrophages. This was further supported by the fact that both conventional and sterically stabilized liposomes displayed comparable distribution properties (as determined by fluorescence microscopy of tumor thin sections) after extravasation into the tumor.

III.4. Intracellular delivery and processing of liposomes and their contents

As mentioned in Section III.3, for most applications, investigators exploit the ability of liposomes to provide a disease site localized depot of drug, which is slowly released and taken up by target cells. However, current efforts to improve the therapeutic properties of liposomes are focusing on designing systems that will not only localize selectively in a disease site, but will also specifically deliver their encapsulated contents into a defined target cell population. Strategies for targeting liposomal anticancer drugs are reviewed in the chapter by Theresa Allen and these will not be considered in any detail here. However, it is important to think about the general approaches that are being taken to accomplish cell specific delivery.

Targeting concerns the use of liposomes with surface associated targeting ligands that can bind molecules over-expressed on the surface of disease cells in an extravascular site. It is important that such targeting information does not inherently alter the extravasation events required for the liposomes to reach their cellular target. Consequently, the pharmacodistribution benefits provided by targeted liposomes should arise from a decreased rate of egress from the disease site (rather than increased influx) and cell specific binding. Given that liposomes migrate slowly through interstitial spaces in disease sites such as solid tumors, one must question whether liposomes within the interstitial compartment will be able to interact with a target cell. In addition, the avidity of liposome binding to target cells may actually inhibit liposome migration and subsequent drug exposure in areas more distant from blood vessels. This is anticipated on the basis of the binding barrier effects which have been described by Saga et al.⁸⁴ Targeting approaches may be most appropriate for small foci of disease where extensive interstitial diffusion is not required to expose all of the diseased cells to the therapeutic agent. This has been demonstrated with immunoliposomes targeted to lung cancer metastases growing in mice where small tumors could be treated much more effectively with targeted liposomes compared to conventional liposomes or free drug.⁸⁵ (See also Chapter 4.7 for contrasting results.)

When liposomes are being designed with targeting ligands there has been an emphasis on targeting cell surface molecules known to be internalized via the endocytic pathway.^{86,87} When preparing these targeted carriers additional lipid components can be included to make the liposomal carrier pH sensitive⁸⁸ or

fusogenic.⁸⁹ Release of entrapped contents or fusion with the organelle membrane occurs when the liposomes are exposed to the low pH of the late endosome/lysosome. This has been shown to dramatically increase the potency of liposomal anticancer drugs *in vitro*.⁹⁰ A second approach for intracellular delivery is based on the use of fusogenic lipids. In this case, introduction of liposome encapsulated agents into the cytoplasm of targeted cells is via membrane fusion of the liposome bilayer with the disease cell's plasma membrane. This first requires binding to the cell surface. Recent reports suggest that highly fusogenic lipid mixtures can be stabilized by incorporation of small amounts of exchangeable or cleavable PEG lipids.^{91,92} Loss of the PEG moiety leads to destabilization of the liposome membrane which, in turn, will have the potential to fuse with nearby cell membranes. While these novel approaches for intracellular delivery of liposomal contents are providing exciting data in cell culture systems, their utility *in vivo* will depend on maintaining or increasing access and delivery of the liposomal carrier and encapsulated drug to the disease tissue.

IV. The dilemma faced when designing optimized liposomal anticancer drugs

Investigators designing liposomal anticancer drug carrier technology, rationalized on the basis of improved tumor drug delivery, must contend with a dilemma of opposing goals in the different biological compartments that the formulations experience. Since uptake of liposomes in tumors appears to be passive, extended circulation times (irrespective of conventional vs. steric stabilized comparisons) appear necessary to facilitate liposome accumulation. It follows that drug leakage from the liposomes must be minimized in order to avoid toxicities associated with free drug as well as to optimize drug delivery to the tumor. However, the characteristics that are well suited for these aims in the circulation (biologically inert, non-leaky liposomes) seriously limit the bioavailability of the encapsulated agent. This is due to the fact that tumor cells do not actively take up liposomes and, in the absence of any targeting or internalization information on the liposome surface, encapsulated drugs must be released in order to exert their antitumor activity. It should also be emphasized that the design of liposomes with optimal drug release kinetics, in either the blood compartment or disease site interstitial compartment, will also be highly dependent on the specific drug encapsulated. This could explain, in part, why the antitumor potency of vincristine, a drug whose activity is extremely sensitive to the duration of tumor cell exposure, is dramatically improved by reducing its leakage from liposomes whereas the antitumor potency of liposomal doxorubicin is much less dependent on drug release rates.

The inability to differentially control drug release rates in the plasma compartment and disease site is perhaps the most significant limitation of presently available liposomes. Ideally, one would be able to completely eliminate drug leakage in the circulation and then increase the release rate at the disease site to a level that would provide the optimal concentration vs. time profile for the specific drug being utilized. While this may seem to be a very onerous task, initial indications

suggest that such approaches may be very fruitful. Early attempts to selectively increase drug leakage at tumor sites centered on the fact that liposomes can be constructed to become leaky in the acidic interstitial pH of some solid tumors,⁹³ which can drop to values of 6.5. More direct evidence of the importance of site-specific drug release has been obtained using localized hyperthermia.^{94,95} Liposomal doxorubicin preparations, for example, can be prepared such that there is an increase in drug release at 42°C, compared to 37°C. These liposomes are administered i.v. to tumor bearing mice and the tumor site is then heated using a topical microwave heating device placed on the subcutaneous tumor. Application of a transient heating pulse after the liposomal doxorubicin had accumulated into the solid tumor resulted in a significant increase of therapeutic activity compared to free drug with hyperthermia and liposomal doxorubicin in the absence of heating. Although hyperthermia may not be applicable to many multifocal or deep seated tumors, this technique provides encouraging indications that liposomes exhibiting controlled or triggered release of their contents will significantly augment the pharmacological improvements provided by liposomes.

V. Closing comments

As our understanding of the processes that dictate the fate of liposomes after i.v. injection has increased, we have been better able to design formulations that will optimize the selectivity of action for encapsulated agents. Inclusion of additional components into conventional liposomes can now be done on the basis of extensive data describing the in vivo behavior of various liposome types. Although some questions still remain in areas such as the uptake and processing of liposomes in extravascular sites, we can now more reliably predict how such specific manipulations of liposomes should affect therapeutic activity. This increased understanding has also helped to identify new directions that may improve the therapeutic activity of liposomal drug formulations. Greater control of drug leakage rates within disease sites and the use of targeted and/or fusogenic liposomes for intracellular delivery offer opportunities to dramatically increase the efficiency and specificity of liposome encapsulated agents. The challenge for the future will be to develop systems that are actually therapeutically superior and not just technologically sophisticated.

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