CHAPTER 3

Liposomal Vincristine: The Central Role of Drug Retention in Defining Therapeutically Optimized Anticancer Formulations

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Introduction

Vincristine is an alkaloid derived from *Vinca rosea* that is effective against a wide variety of human carcinomas.[1,2] When used in conjunction with corticosteroids, vincristine is the treatment of choice to induce remissions in childhood leukemia. Vincristine is also part of a complex protocol used in the treatment of adult patients with Hodgkin's disease or non-Hodgkin's lymphomas. In addition, vincristine has some effectiveness against Wilms' tumor, neuroblastoma, brain tumors, rhabdomyosarcoma, and carcinomas of the breast and the bladder, as well as the male and female reproductive systems.

Vincristine is a cell-cycle specific antiproliferative agent, arresting cell growth exclusively during metaphase by attaching to the growing end of microtubules and terminating further assembly.[3,4] As a consequence of this cell-cycle specificity, methods which extend exposure of vincristine to neoplastic cells should increase therapeutic activity. This prediction is supported by preclinical experiments by Jackson and Bender[5] and later by our research group.[6] We demonstrated that the concentration of vincristine required to achieve a 50% inhibition in cell proliferation (IC_{50}) in vitro decreased 10^5-fold when the drug exposure time was increased from 1-72 h. In contrast, the anthracycline doxorubicin, another anticancer drug, shows only a 40-fold decrease in the IC_{50} for the same increase in exposure time (see Table 3.1).

Systemic chemotherapy treatment is required to treat systemic disease and a central premise of cancer chemotherapy is to achieve maximum dose intensity under conditions where drug related toxicities are manageable. In the absence of a defined drug delivery technology, attempts to optimize both plasma concentration and the time of exposure to the diseased tissue to chemotherapy agents have relied on the manipulation of variables such as drug dose and dosing schedules. However, such strategies are of limited benefit when the drugs used are rapidly eliminated from the

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Table 3.1. Effect of exposure time on the cyfotoxicity of doxorubicin and vincristine on L1210 cells in vitro.

Exposure Time (hours)	IC_{50} (nM) Doxorubicin	Vincristine
1	370	12,000
6	55	2,400
24	18	7.3
72	9.2	0.12

Data obtained from Mayer et al[6] and Masin and Nayer (unpublished).

plasma compartment, actively metabolized into inactive species, rapidly released from cells exhibiting low drug binding affinities and/or are toxic due to poor disease tissue specificity. Since most drugs used to treat cancer are afIlicted with one or more of these problems, methods to maximize drug exposure following systemic administration must fundamentally improve both drug specificity and the bioavailability of the active drug.

For vincristine, a drug that exhibits a dose limiting neurotoxicity, two separate approaches have been pursued clinically to enhance drug exposure:

1) Use of intravenous infusions.
2) Applications of drug carrier technology.

Long-term infusions attempt to achieve a balance between toxicity and maintenance of therapeutic drug levels in the plasma compartment over extended infusion times (typically in excess of 96 h). Drug carriers have the potential to increase plasma concentration and AUC while also facilitating increased specificity of drug delivery to the disease site. The latter attribute distinguishes drug carrier technology from intravenous drug infusions, since drug delivery to the disease site is a consequence of the characteristics of the drug carrier as well as the vasculature within the disease tissue.

We strongly believe that increases in drug exposure in regions of disease can best be achieved using long circulating liposomal carriers, a systemic delivery system that can be designed to give exquisite control over drug levels in the plasma, the site of disease progression and, most importantly, drug bioavailability within the diseased tissue. In this context, we wish to emphasize that systems described as long circulating include those with minimal interactions with serum proteins and RES cells which also must have good drug retention properties. They may refer to liposomes with PEG surface coatings but also include other forms such as with highly cohesive bilayers, specific glycolipids, or drugs that effect RES uptake (see chapter 1). In this chapter we review our studies leading to a liposomal vincristine formulation that is now undergoing clinical testing. In addition, we will use vincristine as a model drug to illustrate how regulated blood circulation and drug release can be achieved by careful control of liposomal lipid composition and trapping characteristics. In turn, optimized release characteristics are correlated to significant improvements in therapeutic activity. It is important to recognize that the membrane permeability characteristics of vinaistine are such that it has been a challenge to develop

a carrier system that effectively retains the drug for extended time periods. This research has been developed on the basis of two assumptions:

1) Rapid release of the drug from the carrier will be of limited benefit, resulting in formulations that are therapeutically no better than free vincristine.
2) Complete retention of the drug will result in a therapeutically inactive liposomal formulation.

Therefore the goal of this research is to identify long circulating liposomal formulations that release vincristine at an optimal rate for maximum therapeutic activity. The focus of the following sections is:

1) Attributes that effect drug release.
2) Liposome circulation and disease exposure.
3) Clinical utility of the optimized formulation.

Vincristine Encapsulation and In Vitro Drug Release

Vincristine can be loaded into liposomes by several methods. So-called passive techniques rely on entrapping the drug during liposome formation, followed by removal of drug that was not sequestered in the liposome. Alternatively, vincristine can be actively encapsulated in liposomes that exhibit ion gradients.74 As noted by Mayer et al,[7] ion gradient based loading procedures significantly improve encapsulation efficiency and decrease the rate of drug release.

We have focused our research on the use of transmembrane pH gradients to actively load vincristine into liposomes. It is well established that many drugs which are weak bases can be encapsulated within liposomes in response to a transmembrane pH gradient.[8] Vincristine is one of these compounds that can be rapidly loaded into liposomes and the physicochemical basis for encapsulation of weak bases such as vincristine using a transmembrane pH gradient is well characterized.[10,11] Encapsulation efficiencies approaching 100% in response to a transmembrane pH gradient have been achieved for formulations with vincristine/lipid (wt/wt) ratios in the *range* between 0.05 and 0.20. Typically, we prepare liposomes in a citric acid buffer and increase the **external** pH of the liposomes by titration with sodium phosphate. Alternatively, the liposomes can be eluted through a desalting column to exchange unencapsulated citrate with a defined buffer adjusted to a pH that is higher than that of the encapsulated citrate solution.

Release of vincristine from liposomes has been measured by the change of the vincristineilipid ratio during dialysis. This formulation property is influenced by a number of factors dictated by the loading procedure, including the initial internal pH, the magnitude of the pH gradient, the internal buffering capacity and the residual pH gradient after drug loading. In general, the rate of vincristine release is a consequence of the ability of the formulations to maintain the transmembrane pH gradient during loading and under conditions used to evaluate drug release. As shown in Figure 3.1, increasing the magnitude of the transmembrane pH gradient by decreasing the internal pH, significantly decreased vincristine release from DSPC/Chol liposomes." This observation is consistent with a proposed mechanism of leakage where the rate constant for vincristine movement across a lipid bilayer is proportional to the inverse square of the proton concentration. This mechanism predicts a loo-fold reduction in leakage rate-for an increase in the transmembrane PH gradient by one unit."

The most important factor controlling vincristine release in vitro and in vivo, however, is the lipid composition of the vesicles. For in vitro studies, liposomal lipid

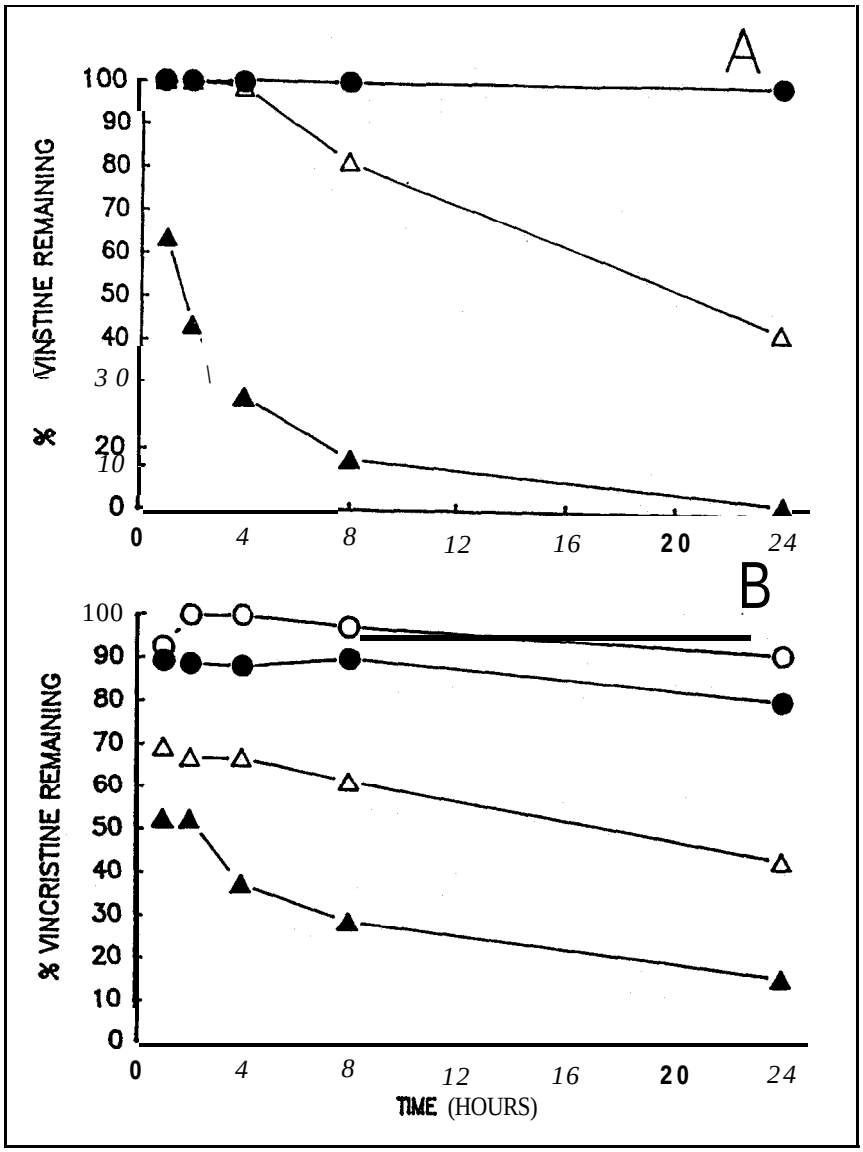


Fig. 3.1. Vincristine release from 100 nm DSPC/Chol vesicles incubated in buffer (A) and mouse serum (B) at 37°C for internal pH of 2.0 (○), 3.0 (●), 4.0 (△), and 5.0 (▲). Internal buffering capacity was 300 mM citrate for all systems. Initial drug/lipid ratios were 0.1/1 (wt/wt). Graph from Boman et al[12] with permission.

composition influences drug permeability rates as well as stability of the pH gradient which together determine drug accumulation. The intrinsic permeability of the drug through the liposomal membrane controls both drug loading rate and its subsequent release rate while the pH gradient stability affects both the liposome loading capacity as well as the rate constant for drug release. In vitro, the slower release

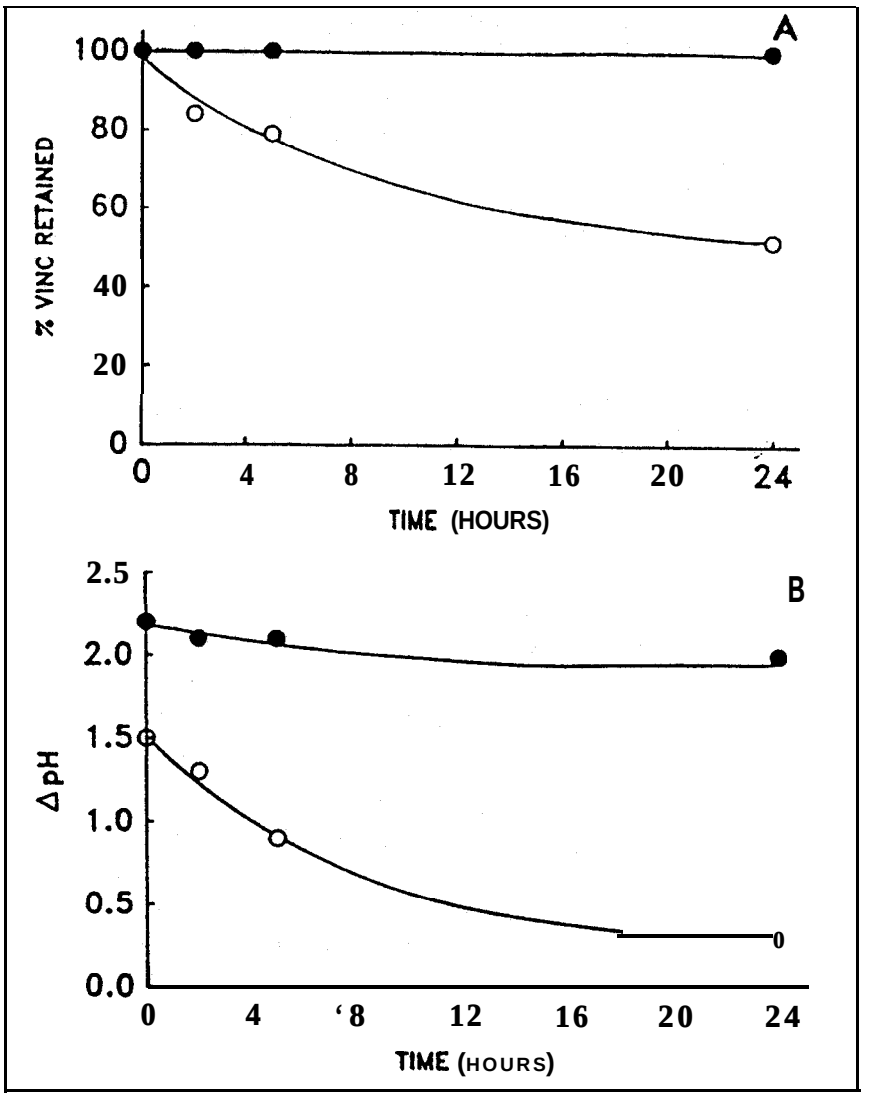


Fig.3.2.A;vincristine release from **DSPC/cholesterol (●)** and **EPC/cholesterol (O)** vesicles subsequent to ApH-dependent drug accumulation.Vmcristine was encapsulated at a drug/lipid ratio (w/w) of 0.19 (± 0.01). Liposomes were incubated at **21°C** and at the indicated times liposome-associated,drug was determined. B; transmembrane pH gradient dissipation at **21°C** in DSPC/cholesterol(0) and **EPC/cholesterol (O)** vesicles subsequent to vincristine encapsulation at a drug/lipid ratio (w/w) of 0.19 (± 0.01). Incubation conditions were identical to those described in A. Graph from Mayer et **al**[13] with permission.

of vincristine from DSPC/Chol liposomes compared to that from EPC/Chol liposomes (Fig. **3.2A)**[13] is due, in part, to enhanced stability of the transmembrane pH gradient (Fig. 3.2B) with the saturated acyl chain. Generally, in vitro release of vincristine is decreased in liposomes containing longer saturated acyl chain phosphatidylcholines, such as DAPC/Chol and DBPC/Chol, in comparison to liposomes

containing short chain phosphatidylcholine, such as DMPCKhol." These in vitro studies have been extremely useful in defining mechanisms which govern transmembrane transport of vincristine in response to pH gradients, and thus provide methods to control its loading and release.

As described in the following section, the factors which decrease the rate of vincristine release in vitro have proven to have similar effects on the release of vincristine from liposomes in vivo. However, drug leakage from liposomes in vivo is usually much more rapid than observed in vitro. Altogether, these studies, and the subsequent findings in vivo, have defined the parameters which influence vincristine release from long circulating liposome formulations permitting optimization of the therapeutic value of these liposomal formulations.

Drug and Liposome Plasma Levels and Disease Exposure

It is well established that encapsulation of drugs in long circulating liposomes is an effective *means* of increasing the circulation lifetime of a drug, the concentration of drug in the blood compartment, and the amount of therapeutic agents delivered to tumors (see chapter 1). Increased blood levels and circulation longevity of liposomal drugs are controlled primarily by the characteristics of the carrier which, when administered iv, persist in the blood compartment for extended time periods. Elimination of a liposome encapsulated drug from the circulation is dependent on both accumulation in the RES through recognition of the liposomes as foreign and on extravasation of the liposomes at sites where the blood vessels exhibit pores or gaps in the vascular endothelium large enough to allow the liposome to cross.

It is important, however, to distinguish plasma elimination of the drug carrier from release of drug from the carrier and its elimination. In this section we illustrate the importance of drug release rates, showing that increased liposome circulation longevity is of little value when the entrapped drug is released too quickly. It is our opinion that the permeability characteristics of encapsulated compounds from liposomes residing within the blood compartment and within sites of disease are the most important attributes defining an efficacious liposomal anticancer agent. As summarized below, we have demonstrated this for vincristine, a drug for which it has been difficult to achieve adequate in vivo retention within liposomes. Importantly, progressive increases in antitumor efficacy are observed as the rate of vincristine release from the liposomal formulation decreases.

Liposome Elimination and Vincristine Blood Levels

When using long circulating liposomes as drug carriers, as outlined in the Introduction, increased drug concentrations within the blood compartment are affected by both the physical and chemical attributes of the carrier. In practice, decreased liposome elimination rates are obtained when using liposomes that are small (~100 nm diameter), neutral (presence of selected anionic or cationic lipids significantly enhances liposome elimination), and contain polyethylene glycol (PEG)-containing lipids.[14-16] Decreases in the rate of vincristine release have been achieved by careful selection of the encapsulation parameters (as summarized above) and the choice of lipids used in the preparation of the liposomes. The influence of liposome encapsulation on the blood levels of vincristine achieved following a single intravenous dose of 2 mg/kg is shown in Figure 3.3. These results illustrate two important points. First, free drug is rapidly eliminated from the plasma compartment in comparison to the liposomal drug formulations, with the exception of the EPC/Chol liposomal lipid

composition. Second, retention of vincristine in the blood is strongly dependent on the choice of liposomal lipid composition. Lower drug levels are observed when the drug is encapsulated in EPC/Chol liposomes in comparison to DSPC/Chol liposomes. Furthermore, lower levels are observed at 24 h with DSPC/CChol compared to DSPC/Chol/PEG-PE or SM/Chol. At least some of these results were predicted on the basis of the data shown in Figure 3.2A. Thus, differences in plasma drug levels are due in a large part to differences in drug release rates. This is supported by parallel measurement of liposome levels, permitting calculation of the vincristine/lipid ratios within the plasma compartment, shown in Figure 3.4. These results indicate that vincristine is released **rapidly** from the EPC/Chol liposomes, slowly from DSPC/Chol liposomes, and slowest from SM/Chol liposomes (Fig. 3.4). Therefore the observed differences in plasma clearance of vincristine (Fig. 3.3) are due primarily to differences in leakage during liposome circulation rather than differences in liposome circulation.

Since the liposome elimination rates are comparable for the various long circulating liposomal vincristine formulations studied, our emphasis here is on how changes in the vincristine/lipid ratio and vincristine release influence both drug delivery to sites of disease progression and antitumor efficacy. It is important to note, however, that all the liposomes used in these studies exhibit extended circulation lifetimes in comparison to large liposomes (>200 nm) or liposomes that contain anionic lipids such as phosphatidylserine, phosphatidylglycerol and cardiolipin. Further, the pres-

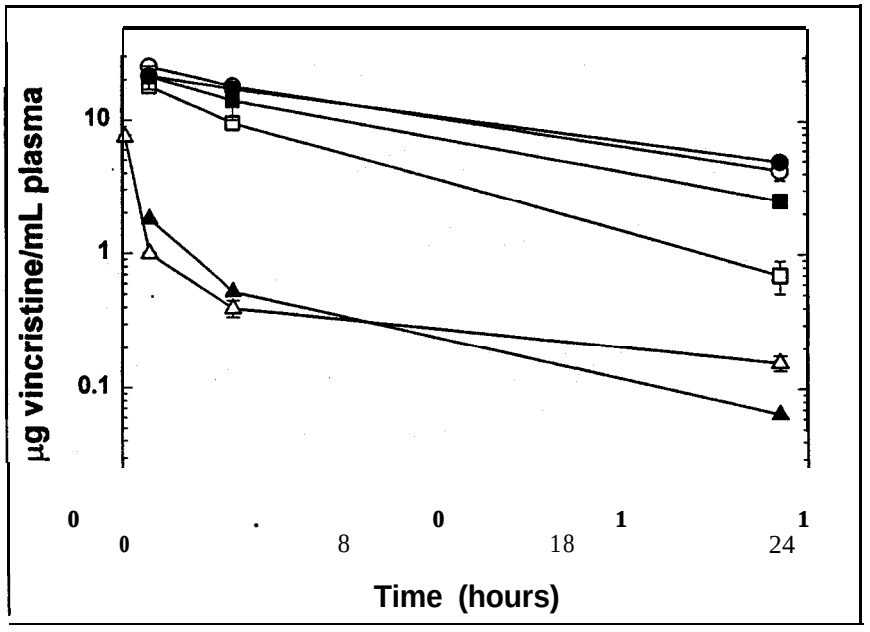


Fig. 3.3. In vivo plasma concentrations of vincristine after the intravenous administration of free vincristine (△) or vincristine encapsulated in hposomes composed of EPC/Chol (A), DSPC/Chol (❒), DSPC/Chol/PEG$_{2000}$-DSPE (■), SM/Chol (○) or SM/Chol/PEG,,-DSPE (●). Vincristine dose was 2 mg/kg in BDF1 (G, ❒, ■, ○, ●) or DBA/2J mice (A). Data summarized from Webb et al[24] (G, ❒, ○), Webb, Bally and Mayer (unpublished data) (■, ●) and from Mayer et al[6](▲).

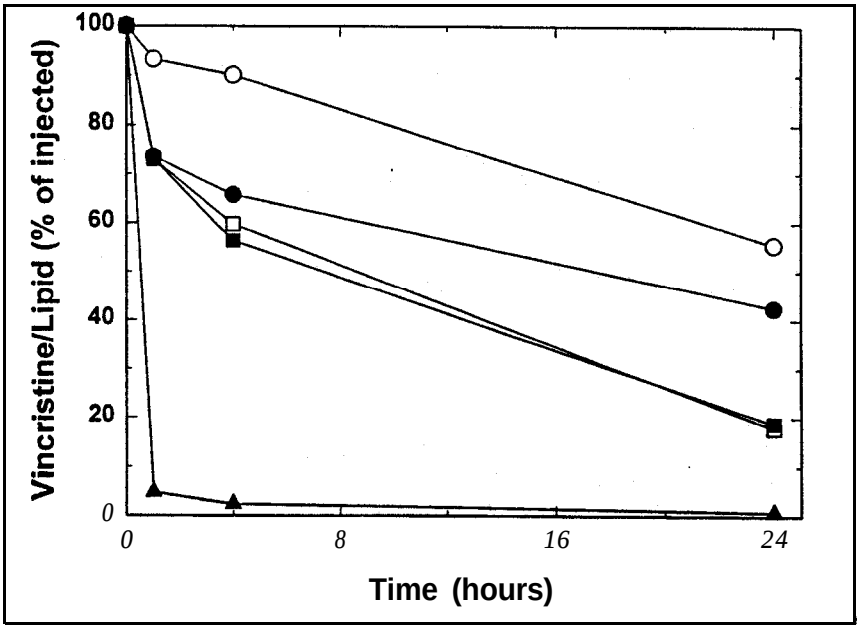


Fig. 3.4. Retention of vincristine in liposomal carriers after intravenous administration in mice. Vincristine retention is expressed as vincristine/lipid ratio in the plasma at various times after administration as a percentage of the administered vincristine/lipid ratio. Vincristine was encapsulated in liposomes composed of EPC/Chol (A), DSPC/Chol (0), DSPC/Chol/PEG_{2000}-DSPE (■), SM/Chol (0) or SM/Chol/PEG_{2000}-DSPE (0). Vincristine dose was 2 mg/kg in BDF1 (□, ■, ○, ●) or DBA/2J mice (A). Data summarized from Webb et al[24] (□, ○), Webb, Bally and Mayer (unpublished data) (■, ●) and from Mayer et al[6] (A).

ence of entrapped vincristine has the effect of decreasing carrier elimination rate. This effect has been attributed to the now well-established RES blockade phenomenon that occurs with selected drugs encapsulated in liposomes.[17,18] RES blockade is observed for liposomal carriers of doxorubicin and vincristine with and without surface associated PEG. Longer times are, however, required following iv administration to observe the maximum level of RES blockade when drug is administered in PEG liposomes.

Factors Influencing Drug-to-Lipid Ratio Following ***iv Administration***

The factors which increase vincristine retention in vitro also improve the retention of vincristine in vivo. For example, release of vincristine from DSPC/Chol liposomes after iv administration in DBA/2J mice was significantly slower than that from EPC/Chol liposomes (see Figs. 3.2 and 3.3). Similarly,vincristine release from DSPC/Chol liposomes after iv administration in mice was significantly slower when the transmembrane pH gradient was increased by two orders of magnitude. A comparison of the data in Figure 3.1 with that shown in Figure 3.5 indicates that decreasing the internal pH (pH_i) to 2.0 from 4.0 leads to reductions in drug release in vitro (Fig. 3.1) and in vivo (Fig. 3.5).

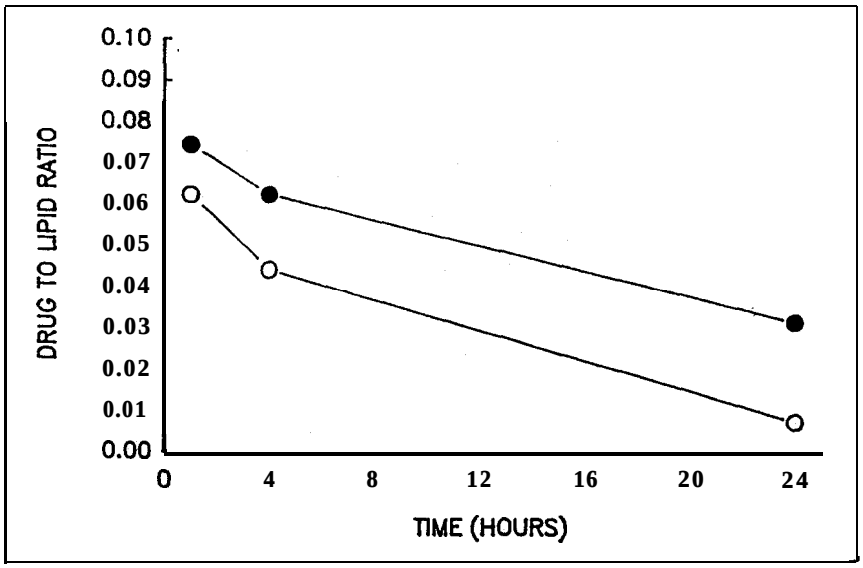


Fig. 3.5. Vincristine/lipid ratios for DSPC/Chol vesicles in vivo with internal pH of 2.0 (●) and 4.0 (0). Both systems were loaded at an initial drug/lipid ratio of 0.1/l (wt/wt). Each point represents the average value obtained from four BDF1 mice. Graph from Boman et al[12] with permission.

Importantly, lipid composition and loading conditions can act together to achieve significant improvements in drug retention and a slower drug elimination rate. This important point is supported by recent data obtained with liposomal vincristine formulations prepared using G_{M1} containing and SM/Chol liposomes.[19-24] **The** SM/Chol formulation was initially developed for pharmaceutical reasons. Specifically, phospholipids such as DSPC are susceptible to acyl hydrolysis when stored at low pH. We therefore chose SM, which is known to be less sensitive to extended storage at pH 4, for reasons summarized elsewhere.* Fortuitously, however, the SM/Chol formulation also exhibited a drug release rate that was significantly slower than that observed for a DSPC/Chol formulation (Figs. 3.3,3.4,3.6). That is, the vincristine concentration in the plasma was significantly greater after administration of the drug in SM/Chol liposomes (Figs. 3.3,3.6).

The balance between liposome circulation and drug permeability is well illustrated by studies attempting to further increase the vincristine concentration in plasma by addition of sterically-stabilized lipids (PEG--DSPE) to the SM/Chol formulation. The results, shown in Figure 3.7, demonstrate that incorporation of PEG_-DSPE into SM/Chol liposomes engendered a significant decrease in the liposome elimination rate (Fig. 3.7A) but facilitated increased drug release from the liposomes (Fig. **3.7B). These** two opposing effects resulted in no net change in plasmavincristine concentrations (Fig. 3.7C and we concluded from these data that there would be no pharmacokinetic benefit achieved through incorporation of PEG-modified lipids in this formulation.[24]

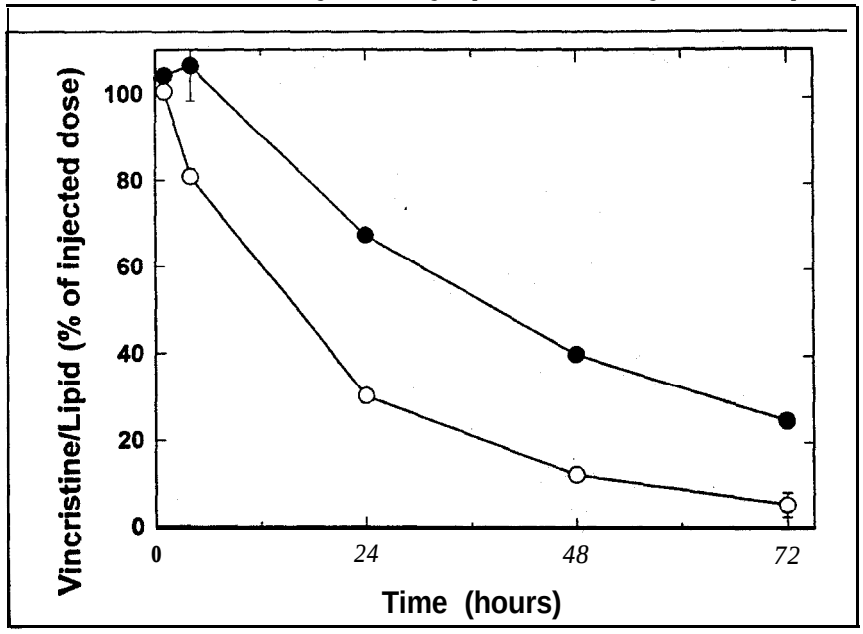


Fig. 3.6. Vincristine/lipid ratio, expressed as a percentage of the injected ratio, in the plasma of BDFl mice at various times after the injection of large unilamellar liposomes of **DSPC/Chol (O)** or SM/Chol **(●).** Mice were injected with liposomes at a vincristine/lipid ratio of approximately 0.1/l, corresponding to a lipid dose of 20 mg/kg and a vincristine dose of 2.0 mg/kg. Total amounts injected were approximately 430 **µg** of lipid and 43 **µg** of vincristine. Data represent means **(±** s.e.) of three mice; where standard error bars are not visible, they are smaller than the size of the symbol. Graph from Webb et al[24] with permission.

Influence of Drug **Retention on the** *Therapeutic* **Activity of Vincristine**

An initial antitumor study comparing EPC/Chol to DSPC/Chol liposomal vincristine demonstrated the importance of increased drug plasma levels in achieving **increased *anticancer*** activity. This study clearly indicated that the DSPC/Chol formulation was significantly more active than either free vincristine or vincristine encapsulated in EPC/ChoI liposomes when tested against the murine L1210 lymphocytic leukemia model (Fig. 3.8).[6] The EPC/Chol formulation exhibited no significant activity, consistent with data showing that drug encapsulation in this liposome formulation effected negligible increases in the level of vincristine in the plasma (Fig. 3.3). We interpreted these results as indicating the inabiity of the vincristine-permeable EPC/Chol formulation (Fig. 3.4) to maintain vincristine levels in the plasma above a therapeutic threshold (Fig. 3.8). Of greater interest, however, was the *re*markable improvement in therapeutic activity observed for the DSPC/Chol liposomal formulation of vincristine. We have subsequently demonstrated that the DSPC/Chol liposomal formulation is significantly more active than free drug in treating a variety of murine and human xenograft tumor modles.[6,23-25]

In the preceding example, the antitumor efficacy of vincristine in DSPC/Chol liposomes was compared to that of a vincristine-permeable formulation, EPC/Chol. Two studies comparing the therapeutic activity of DSPC/Chol liposomal vincristine to formulations with even slower drug release characteristics are, however, impor-

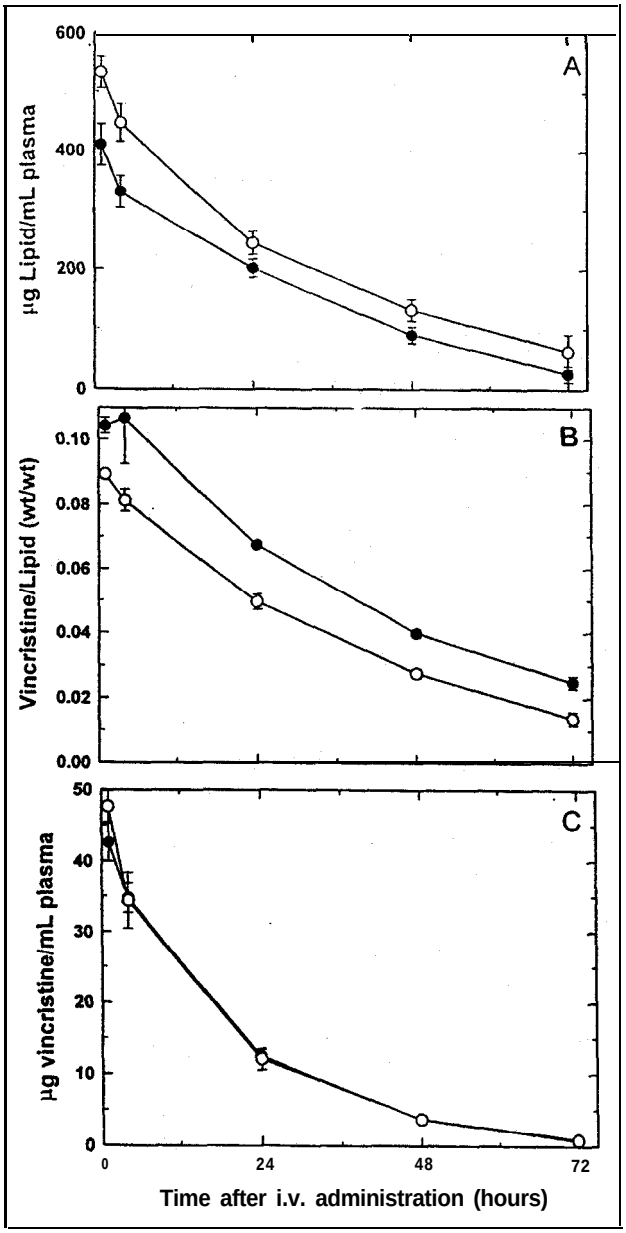


Fig. 3.7. Lipid clearance (A), vincristine/lipid ratio (B) and vincristine clearance (C) in the circulation of mice after the iv administration of vincristine encapsulated at a drug/lipid ratio of 0.1/l (wt/wt) in liposomes composed of either SM/Chol (●) or SM/Chol/PEG,,,,-DSPE (0). Vmcristine was injected at a dose of 2.0 mg/kg, representing a lipid dose of 20 mg/kg. Data represent means (± s.e.) of three mice; where standard error bars are not visible, they are smaller than the size of the symbol. Data from Webb et al[24] and Webb, Masin, Bally and Mayer (unpublished).

tant to note. The incorporation of the ganglioside G_{M1} and use of an initial PH_i of 2.0 to enhance circulation and decrease the rate of vincristine release from DSPC/Chol liposomes also significantly increased the antitumor activity of vincristine when tested against the murine P388 leukemia model (Fig. *3.9).* These studies indicated that 100% of tumor-bearing animals could be effectively cured of the disease when using the formulation which exhibited the slowest rate of drug release. The DSPC/

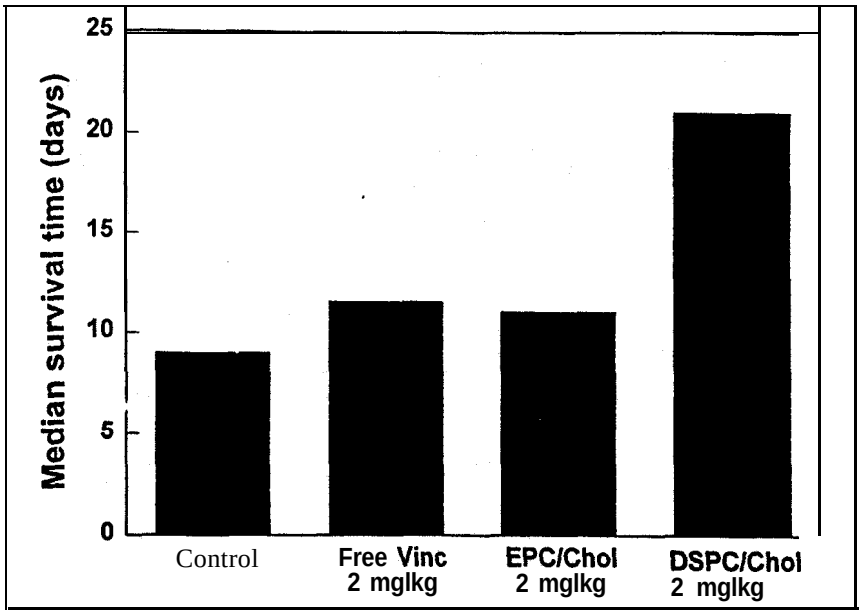


Fig. 3.8. Autitumor efficacy of free and liposomal vincristine in DBA/2J mice bearing the L1210 ip leukemia and treated iv with saline, free vincristine or vincristine encapsulated in either EPC/Chol or DSPC/Chol liposomes. Data summarized from Mayer et al.6

Chol/G_{M1} (pH_i 2.0) formulations administered at vincristine doses of 2,3, and 4 mg/kg all produced long-term survivors with median survival times of > 70 d. It must be stressed that this increase in antitumor activity was due to decreased drug release from the liposomes and was not simply a consequence of increased liposome circulation longevity. This conclusion was also supported by studies evaluating the more pharmaceutically viable SM/Chol liposomal vincristine formulation; another formulation with drug release that is significantly slower than that observed for DSPC/Chol liposomes (Figs. 3.4,3.6). This result predicted that the therapeutic activity of SM/Chol liposomal vincristine would be improved in comparison to DSPC/Chol. Studies using the murine P388 tumor model (data not shown) confirmed this prediction.* In addition, we demonstrated that the SM/Chol liposomal formulation exhibited significant therapeutic activity when tested against the human A431 xenograft tumor model **(Fig.** 3.l0).Treating these animals with free vincristine resulted in a 2-3 d delay in initiation of tumor growth while treatment with DSPC/Chol liposomal vincristine effected a 15 d delay. The SM/Chol liposomal vincristine treated animals, however, exhibited no tumor growth for at least 40 d after drug administration (Fig. *3.10).*

In an attempt to directly correlate vincristine release fromliposomes and plasma levels of vincristine with antitumor activity we have summarized data obtained from P388 tumor bearing mice treated with the nine different liposome vincristine formulations tested to date. The results are presented in Figure 3.11. The efficacy of free or liposomal vincristine against the P388 tumor, as determined from the median days of survival after a single administration at 2 mg vincristine/kg, was very well

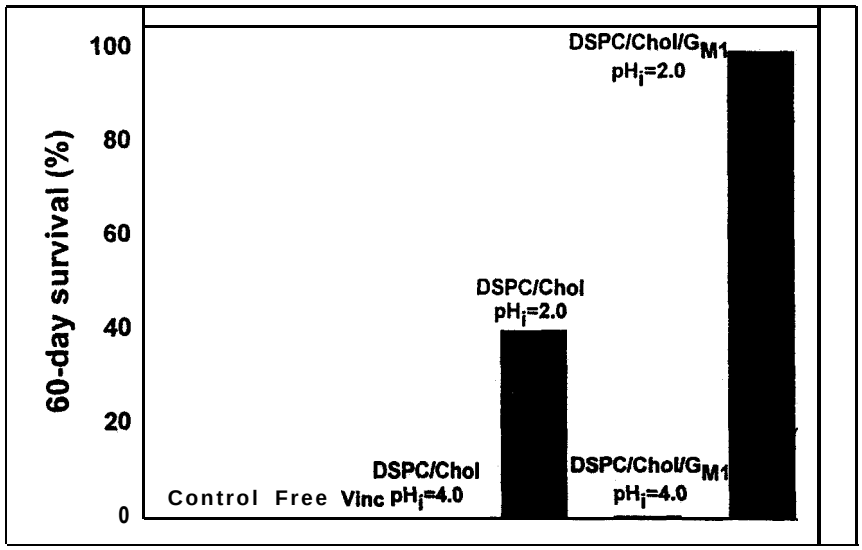


Fig. 3.9. Antitumor efficacy of free and liposomal vincristine in BDF1 mice bearing the P388 ip leukemia and treated iv with saline, free vincristine or vincristine encapsulated in either DSPC/Chol or DSPC/Chol/G_{M1} liposomes with pH_i values of either 2.0 or 4.0. Vincristine dose was 3 mg/kg and the vincristine/lipid ratio was 0.1/1 for the liposomal formulations. For saline, free vincristlne, DSPC/Chol pH_i = 4.0 and DSPC/Chol/GM_{M1} pH_i = 4.0 treatments, the 60-day survival was 0%; small bars are plotted solely for visibility. Data summarized from Boman et al.[23]

correlated (r^2 = 0.95) with increases in the circulation half-life of the drug (Fig. 3.11A). It is worth noting in this analysis the similarity of SM/Chol and SM/Chol/PEG_{2000}-DSPE liposomal vincristine (points 6 and 9) in both vincristine half-life and efficacy, despite the differences between these formulations in both lipid circulation lifetime and vincristine retention (Fig. 3.7A,B). In our experience, the primary factor influencing the vincristine half-life in the circulation is the retention of vincristine by the liposomal carrier. Consequently, for a variety of different liposomal formulations of vincristine, the antitumor efficacy is also strongly correlated with the half-life for the release of vincristine from the liposomes in the circulation (r^2 = 0.84) (Fig. 3.13B). In contrast, antitumor efficacy was poorly correlated with the circulation lifetime of the lipid carriers (r^2 = 0.49) (Fig. 3.11C). Taken in sum, these data clearly indicate that the primary pharmacokinetic parameter associated with increased antitumor efficacy is the circulation half-life of vincristine

DrugExposure at the Disease Site

Any analysis of antitumor efficacy and circulation half-life of vincristine must also take into consideration the propensity for liposomes to move from the plasma compartment into extravascular sites in regions of disease progression. This phenomena has been well documented for liposomal vincristine formulations[6,23-25] and is best illustrated by results obtained using the SM/Chol liposomal vincristine formulation. As indicated in the previous section, the therapeutic activity of vincristiue in SM/Chol liposomes is better than that observed for a DSPC/Chol formulation. This has been demonstrated for ascitic tumors (Fig. 3.11A) as well as solid tumors

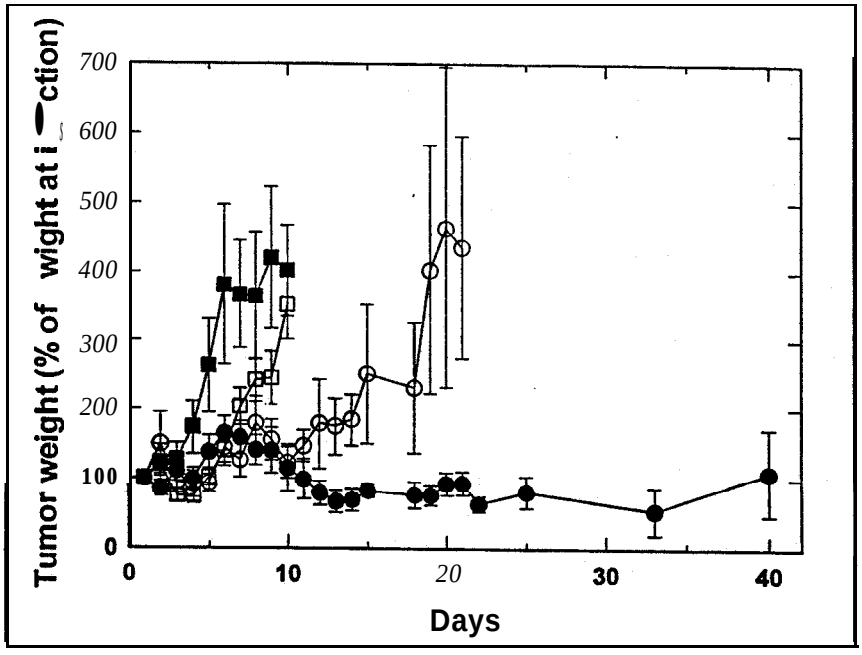
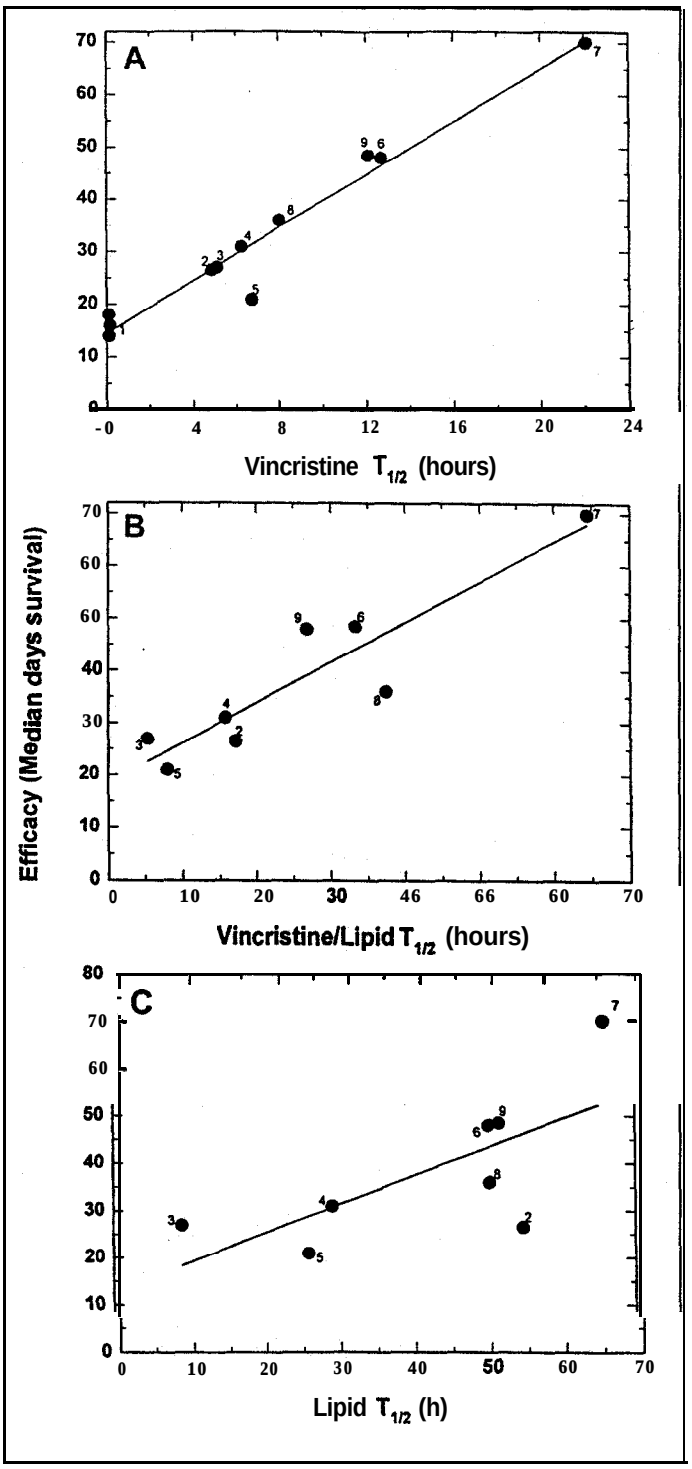
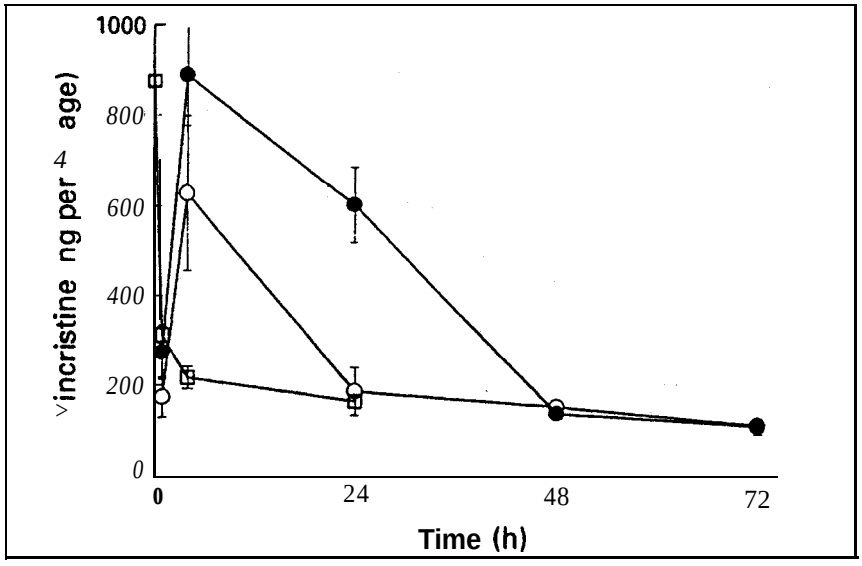


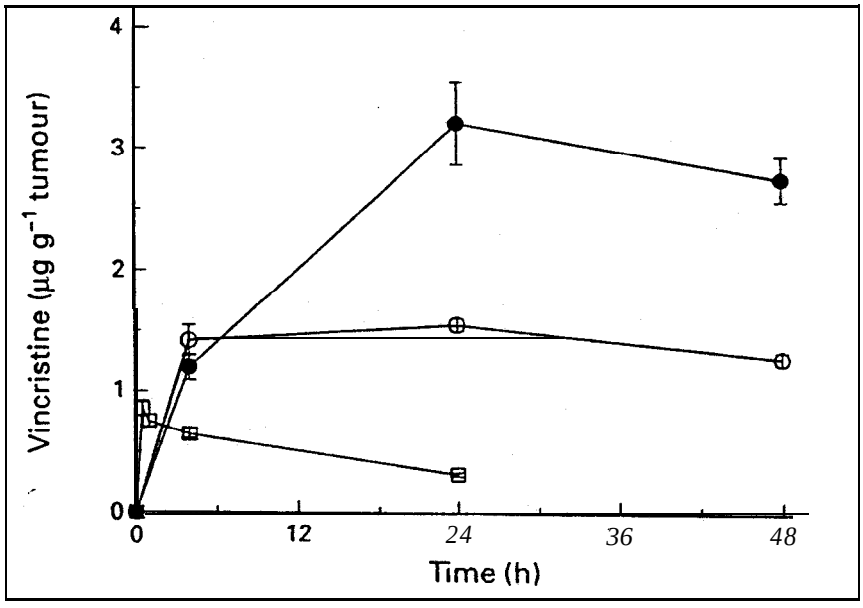
Fig. 3.10.Antitumor efficacy of free and liposomal vincristine in SCID mice bearing A431 tumors. SCID mice bearing two A431 tumors received no treatment (■) or were injected iv with free vincristine (❑) or with large unilamellar liposomes of **DSPC/Chol (O)** or SM/**Chol (●)** containing vincristine at a drug/lipid ratio of 0.1/1 (wt/wt). Vincristine was injected at a dose of 2.0 mg/kg, representing a lipid dose of 20 mg/kg. Data represent the weight of A431 tumors (expressed as a percentage of the tumor weight immediately before treatment) and are the means (± s.e.) of 8-10 tumors in 4-5 mice. Graph from Webb et al[24] with permission.

Fig. 3.11. (Opposite page) Summary of the pharmacokinetic parameters of free and liposomal formulations of vincristine that are associated with alterations in antitumor efficacy. Pharmacokinetic parameters are the half-life ($T_{1/2}$) for vincristine in the circulation (A); half-life ($T_{1/2}$) for the retention of vincristine during liposome circulation (B), and; half-life ($T_{1/2}$) for the circulation of the liposomal carriers in the circulation (C). Antitumor efficacy is reported for activity against an ip P388 tumor in BDF1 mice and is expressed as median days of survival after treatment. All animals were treated with an iv administration of either free or liposomal vincristine at a vincristine dose of 2.0 mg/kg, all liposomes were 0.1 pm. Data are presented for 1) free vincristine from Boman et al,[23] Mayer et al,[13] and Webb et al;[24] 2) DSPC/Chol (pH_i = 4.0) from Webb et al;[24] 3) DSPC/Chol (pH_i = 4.0) from Boman et al;[23] 4) DSPC/Chol (pH_i = 2.0) from Boman et al;[23] 5) DSPC/Chol/G_{M1} (pH_i = 4.0) from Boman et al;[23] 6) SM/Chol (pH_i = 4.0) from Webb et al;[24] 7) DSPC/Chol/G_{M1} (pH_i = 2.0) from Boman et al;[23] 8) DSPC/Chol/sphingosine (pH_i = 2.0) from Boman (unpublished); and 9) SM/Chol/PEG_{2000}-DSPE (pH_i = 4.0) from Webb,Masin, Sally and Mayer (unpublished).


A
Vincristine $T_{1/2}$ (hours)
B
Efficacy (Median days survival)
Vincristine/Lipid $T_{1/2}$ (hours)
C
Lipid $T_{1/2}$ (h)



Pig. 3.12. **Accumulation** of vincristine in the peritoneal cavity of **BDF1** mice bearing peritoneal P388 tumor cells after iv administration of free **vincristine (❑)** or of large unilamellar liposomes of **DSPC/Chol (O)** or **SM/Chol (●) containing** vincristine at a drug/lipid ratio of **0.1/1** and a vincristine dosage of **2.0 mg/kg.** Data represent means **(± s.e.)** of four mice; where standard error bars are not visible, they are smaller than the size of the symbol. Graph from Webb et **al**[24] with permission.



(Fig. 3.10). Biodistribution studies that have measured vincristine levels in the site of ascitic tumor development (Fig. 3.12) and within the solid tumor (Fig. 3.13) have clearly demonstrated that increased drug levels in the plasma are associated with increased drug accumulation at the disease site.

It was anticipated that liposomal vincristine formulations that deliver more vincristine to the tumor for an extended time would also be associated with increased antitumor efficacy. However, it must be emphasized that increased vincristine accumulation in sites of tumor growth occurs as a consequence of liposome accumulation.[26] Further, an analysis of the vincristine/lipid ratio in the tumors of treated animals indicated that liposomes which have extravasated to the tumor site release drug at a rate comparable to that observed within the plasma compartment.":

We conclude from these data that successful optimization of the therapeutic activity of liposomal anticancer drugs, through changes in drug release rates, must balance two conflicting attributes. Since the process of extravasation of the lipo somes out of the blood compartment to the tumor site is slow, drug release rates must also be sufficiently slow to ensure that maximal quantities of drug are contained within the extravasated liposomes. In contrast, once the liposomes have extravasated the drug must be released at rates sufficient to achieve therapeutic activity.

Alternative Liposomal Formulations of Vincristine

The pharmacokinetics and therapeutic activity of a liposomal vincristine formulation prepared using sterically stabilized PEG-containing liposomes has recently been described.[27] Encapsulation of vincristine into these sterically stabilized liposomes increased the vincristine half-life in the circulation from the initial half-life of 20 min seen for a conventional liposome formulation to approximately 10.5 h (Fig. 3.14).[27] The cause of the extremely rapid clearance of vincristine in the conventional liposome formulation used in this report is unclear and not representative of the clearance seen for the PC/Chol and SM/Chol formulations described by our group.[6,13,24,25] We, however, would suggest that such differences are due to either faster liposome elimination rates or increases in the rate of drug release. Allen's studies, for example, administered liposomes prepared with 5 mol% EPG to rats. It has been shown that PG liposomes bid the complement protein C3bi and induce platelet aggregation mediated through the presence of the C3bi receptor on rat platelets.[28,29] Alternatively, we demonstrated that the presence of anionic lipids facilitates drug release from liposomes that have been prepared using the pH gradient drug loading procedure.7 It would be of interest to determine whether the differences in plasma drug levels reported by Allen et al were due to liposome clearance or faster drug release rates. Although this study did not compare the efficacy of sterically-stabilized liposomes to conventional liposomes, it did show that the efficacy of vincristine

Fig. 3.13. (Opposite page, bottom) Tumor levels of vincristine after administration of free and liposomal vincristine in SCID mice bearing A431 tumors. SCID mice bearing two A431 tumors were injected iv with free vincristine (❑) or with large unilamellar liposomes of DSPC/Chol (○) or SM/Chol (●) containing vincristine at a drug/lipid ratio of 0.1/1 (w/w). Vincristine was injected at a dose of 2.0 mg/kg, representing a lipid dose of 20 mg/kg. Data represent means (± s.e.) of three mice (six tumors); where standard error bars are not visible, they are smaller than the size of the symbol. Graph from Webb et al[24] with permission.

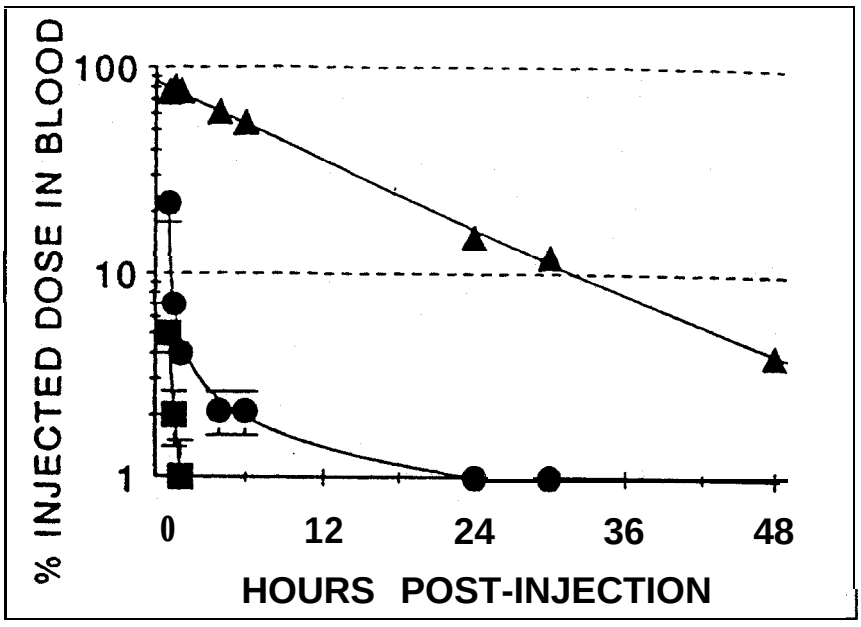


Fig. *3.14.* Pharmacokinetics of long circulating liposomal vincristine (A), conventional liposomal vincristine **(●),** and free vincristine **(■);** 0.25 mg vincristine/kg body weight was injected iv into Sprague-Dawley rats. Graph from Allen et al[27] with permission.

encapsulated in PEG-containing liposomes against ip and SC tumors was greater than that of free vincristine.~ The median survival times for the sterically stabilized liposomes[27] were comparable to the median survival times seen previously with the DSPC/Chol pH_i 4.0 formulation against P388 leukemia and shorter than for the nonsterically stabilized SM/Chol formulation,* The PEG-containing formulation showed lower efficacy, in terms of median survival times against L1210 leukemia, compared to that of a DSPC/Chol formulation.[6] The PEG-containing formulation was also shown to decrease growth of the C26 colon carcinoma solid tumor, compared to free drug, and increase the survival time of the mice.[27] Similar results have also been reported by Vaage et al,[9] for sc mouse mammary carcinomas (MC2) treated with free vincristine or vincristine encapsulated in sterically stabilized liposomes.

Another modification which has been shown to produce long circulation times for liposomes is the incorporation of the uronic acid derivative palmitoyl-D-glucuronide (PGlcUA).[30,31] Liposomes composed of DPPC/Chol/PGlcUA (4:4:1 mol%) and containing vincristine were observed to inhiiit the growth of the Meth A sarcoma in Balb/c mice to a greater extent than observed for vincristine encapsulated in "control" liposomes? However, in this study PG-containing liposomes were used as a control. As mentioned above, negatively charged liposomes containing PG activate complement in the circulation, rendering them less stable in terms of both drug retention and circulation longevity." In order to clearly determine the factors responsible for tumor efficacy, we suggest that it is necessary to determine the serum clearance rates for the liposomes and the drug/lipid ratios. Our summary *results,* shown in Figure 3.11, would suggest that regardless of the liposome clearance rate, the plasma concentration must be elevated for extended time periods in order for

improved therapeutic activity to be observed. We would argue that this can be achieved using relatively simple liposomal formulations that provide adequate plasma circulation and stability of encapsulation.

Clinical Utility of Liposomal Vincristine

The preclinical toxicity and efficacy data obtained by our research group were sufficient to warrant clinical development of a liposomal vincristine formulation. Comprehensive summaries of these preclinical studies of liposomal vincristine have been published elsewhere.[11,26] It is important to recognize that the beneficial effects observed preclinically which occur as a consequence of vincristine encapsulation within liposomes are not due to a decrease in toxicity but rather to improved antitumor activity. As emphasized in this chapter, the latter is associated with increased vincristine circulation longevity and accumulation of the liposomal drug at the tumor site. The rationale advancing this research effort continues to be based on the fact that vincristine is a cell-cycle specific agent whose activity can be enhanced by prolonged cell exposure. This rationale, supported by the exceptional pharmacokinetic, toxicologic and antitumor properties of liposomal vincristine, led to development of a Phase I Clinical Trial at the British Columbia Cancer Agency. The results from the Phase I toxicity study, briefly summarized elsewhere,* were encouraging and prompted initiation of a Phase II efficacy study that is presently ongoing.

Summary

The antitumor activity of vincristine in a variety of preclinical murine tumor models is strongly correlated with increased vincristine circulation longevity and increased vincristine tumor accumulation. Dramatic improvements in vincristine circulation lifetime and vincristine accumulation at tumor sites have been effected by vincristine encapsulation in liposomal carriers. However, vincristine is distinct from antineoplastic agents such as doxorubicin in that it leaks much more readily from liposomal carriers. Consequently, further increases in vincristine circulation longevity, drug accumulation at tumors and antitumor efficacy have been achieved primarily through improvements in vincristine retention within the liposomal carrier. The relationship between circulation lifetime and antitumor efficacy is the direct result of extravasation of the intact liposome, with the encapsulated vincristine, from the circulation to the tumor site. Liposomal carriers with enhanced drug retention characteristics will carry a greater therapeutic payload per extravasated liposome than those with poor drug retention characteristics. We have observed no additional pharmacokinetic or therapeutic benefit in liposomal vincristine formulations that have been rendered "long circulating" by the presence of PEG_{2000}-DSPE. Bather, these preclinical studies with well-characterized "conventional" but still relatively long circulating formulations which exhibit long drug circulation lifetimes (i.e. not necessarily equivalent to long circulation lifetimes) are the foundation supporting an encouraging Phase I/II Clinical Trial of liposomal vincristine.

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