OPTIMIZATION OF LIPOSOMAL RETENTION OF VINCristine

by

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Department of Biochemistry

We accept this thesis as conforming
to the required standard

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ABSTRACT

Vincristine is a widely used antineoplastic agent. It is a cell cycle specific drug arresting cell growth during metaphase. Since tumors contain dividing cells distributed throughout the cell cycle, it is important to achieve as long an exposure time as possible. Encapsulation of drugs within liposomes can prolong circulation time \textit{in vivo} as well as reduce toxic side effects. This thesis examines the effect of liposomal encapsulation on reducing toxicity as well as improving antineoplastic activity of vincristine by altering drug pharmacokinetic and biodistribution behaviour.

The first focus of investigation demonstrates that encapsulation of vincristine within liposomes greatly reduces soft-tissue toxicity of the drug. Subcutaneous injections of liposomal vincristine are shown to demonstrate minimal toxic effects whereas similar injections of free vincristine result in gross necrosis and ulceration. Free drug is rapidly cleared from the area of injection. Liposomal drug remains at the area of injection much longer, but remains trapped within the liposomes. Slow release rates presumably prevent it from exerting cytotoxic effects.

The next topic concerns improving the retention of vincristine within liposomes. The influence of lipid composition, internal pH and internal buffering capacity on the retention properties of vincristine loaded into LUVs in response to transmembrane pH gradients has been assessed. It is shown that increasing the (saturated) acyl chain length of the phosphatidylcholine molecule, increasing the internal buffering capacity, and decreasing the internal pH all result in increased drug retention. Further, a study of the pH dependence on
the rates of accumulation indicate that uptake proceeds via the neutral form of the vincristine molecule. This uptake is associated with an activation energy of 37 kcal/mol for DSPC/Chol LUVs. It is shown that the major improvement in drug retention in vitro is achieved by employing low initial internal pH values, where 90% retention is obtained over 24 h for an initial internal pH of 2. Improved retention over the same system with an internal pH of 4 in vivo was also observed where a drug-to-lipid ratio approximately 4-fold greater at 24 h was maintained.

The third area of investigation concerns the incorporation of cationic lipids to further improve vincristine retention within liposomes. The influence of both the incorporation of 10 mol% cationic lipid (AL-1, stearylamine, or sphingosine) into DSPC/Chol (55:45; mol:mol) vesicles and lowering the internal pH to pH 2.0 on the circulation life-time and antitumor activity of liposomal vincristine systems (drug-to-lipid ratio of 0.1:1) has been examined. With an internal pH of 2.0, the incorporation of 10 mol% cationic lipid is shown to significantly increase drug retention within the liposomes without affecting lipid clearance times. The resulting increase in plasma drug concentration seen by the incorporation of 10 mol% sphingosine results in a significant increase in therapeutic activity against the P388 lymphocytic leukemia cell line in vivo.

The final area of investigation examines two different methods for increasing the circulation longevity of vincristine encapsulated in liposomes. The first involves incorporation of the ganglioside G\textsubscript{M1}, which acts to increase the circulation longevity of liposomal carriers, while the second approach relies on modification of the vincristine encapsulation procedure to enhance drug retention. It is shown that these approaches are synergistic and increase the circulation half-life of vincristine from approximately one hour
to greater than 12 hours. This results in a dramatic improvement in the therapeutic activity of liposomal vincristine as measured using a murine P388 lymphocytic leukemia model. At doses above 2 mg/kg the optimized liposomal vincristine formulation cures greater than 50% of mice bearing the P388 tumor, whereas free vincristine results in no cures. The optimized formulation is also shown to significantly increase solid tumor uptake of vincristine within the Lewis Lung tumor model and result in improved therapeutic activity.
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<thead>
<tr>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL-1</td>
<td>rac 1,2-dioleoyl-3-N,N-dimethylaminopropane</td>
</tr>
<tr>
<td>ApoA-1</td>
<td>apolipoprotein A-1</td>
</tr>
<tr>
<td>Chol</td>
<td>cholesterol</td>
</tr>
<tr>
<td>DAPC</td>
<td>diarachidoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DBPC</td>
<td>dibehenoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DMPC</td>
<td>dimyristoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DOPA</td>
<td>dioleoyl phosphatidic acid</td>
</tr>
<tr>
<td>DPPC</td>
<td>dipalmitoyl phosphatidylcholine</td>
</tr>
<tr>
<td>DSPC</td>
<td>distearoyl phosphatidylcholine</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>egg-PC/EPC</td>
<td>egg phosphatidylcholine</td>
</tr>
<tr>
<td>G\text{M1}</td>
<td>monosialoganglioside G\text{M1}</td>
</tr>
<tr>
<td>HBS</td>
<td>hepes buffered saline</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>[4-(2-hydroxyethyl)]-piperazine ethanesulfonic acid</td>
</tr>
<tr>
<td>i.m.</td>
<td>intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravascular</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
</tr>
<tr>
<td>MLV</td>
<td>multilamellar vesicle</td>
</tr>
<tr>
<td>NBD-PC</td>
<td>N-(4-nitrobenzo-2-oxa-1,3-diazoly)- phosphatidylcholine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEG-PE</td>
<td>phosphatidylethanolamine-polyethyleneglycol</td>
</tr>
<tr>
<td>pH&lt;sub&gt;i&lt;/sub&gt;</td>
<td>internal pH</td>
</tr>
<tr>
<td>ΔpH</td>
<td>transmembrane pH gradient</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
<td>SA</td>
<td>stearylamine</td>
</tr>
</tbody>
</table>
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TO MY OLDEST FRIENDS, MY MOM AND DAD;

MY BEST FRIEND, MY HUSBAND BILL;

AND MY NEWEST FRIEND, MY DAUGHTER KYLIE.
CHAPTER 1
INTRODUCTION

1.1 Liposomes as Drug Delivery Vehicles

Liposomes have played an important role in the understanding of the structural and physical properties of lipids in biological membranes. The aqueous interior of lipid vesicles is able to contain ions and small, water-soluble molecules, sequestering them from the external environment, and thus provide simple "models" of biological membranes. It has also been demonstrated that liposomes can encapsulate biologically active molecules such as proteins, DNA, or conventional drugs (Gregoriadis, 1984; Kabanov et al., 1990; Frezard and Garnier-Suillerot, 1991; Madden et al., 1990). As lipids are biologically compatible and liposomes can potentially be targeted to disease sites, the possibility of utilizing liposomes as \textit{in vivo} drug carrier systems holds much appeal.

The appeal of liposomes as drug delivery systems is particularly strong for anticancer applications. At the present time, anticancer chemotherapeutic drug therapy is often limited by the devastating toxic side effects observed in organs or tissues not associated with the site of disease. However, encapsulation of various antineoplastic agents within lipid vesicles has been shown to decrease toxic side effects while maintaining or increasing therapeutic activity (Herman et al., 1983; Forssen and Tokes, 1981; Gregoriadis and Neerunjun, 1975; Woo et al., 1983; Mayhew and Rustum, 1985; Gabizon et al., 1982a; Kobayashi et al., 1977; Hunt et al., 1979; Rahman et al., 1982; Gregoriadis, 1988; Fichtner et al., 1981; Gabizon et al., 1982b). Most of this work has been focused on doxorubicin, however similar observations
have been made for vincristine (Mayer et al., 1990c). Vincristine is an antineoplastic agent derived from the periwinkle plant (see Figure 1.1). It is an important anticancer drug in that it displays effectiveness against a wide variety of neoplasms including both the Hodgkin's and non-Hodgkin's lymphomas, acute lymphoblastic leukemia, embryonal rhabdomyosarcoma, neuroblastoma, breast carcinoma, and Wilm's tumor (Carter and Livingston, 1976; Sieber et al., 1976). Vincristine is a cell-cycle specific drug which arrests cell growth exclusively during metaphase by attaching to the growing end of microtubules and terminating their assembly (Owellen et al., 1976; 1972). For this reason, it is presumably advantageous to expose neoplastic cells to the drug for prolonged periods of time. This effect has been demonstrated in vitro by Jackson and Bender (1976), and has been confirmed in our laboratory using the L1210 leukemic cell line (see Table 1.1, Mayer et al., 1993). Table 1.1 demonstrates that by increasing the exposure time for the drug from 1 to 72 h, there is a 10^5-fold decrease in the concentration of vincristine necessary to yield 50% cytotoxicity. Further, the potential importance of this relationship in the treatment of human malignancies is supported by clinical trials where patients refractory to bolus vincristine therapy exhibited increased response rates when the drug was administered as a 5-day infusion (Jackson et al., 1986a; 1986b). These data indicate that therapy with vincristine can be improved with the same dosage by prolonging tumor cell exposure time.

This must be balanced against the threshold level required to maintain antimitotic ability, as illustrated in Figure 1.2.

Previous work has shown that liposomal formulations of vincristine can exhibit reduced toxicity and enhanced efficacy compared to free drug (Mayer et al., 1990c). This has been related to the enhanced residence times of the drug in the circulation which is
Figure 1.1
Structure of the vincristine molecule.
Table 1.1

Effect of exposure time on the in vitro cytotoxicity of vincristine against L1210 cells.

The drug concentration required to yield 50% cytotoxicity (IC$_{50}$) decreases from 12 $\mu$M to 0.12 nM as the duration of drug exposure is increased from 1 to 72 h. (Taken from Mayer et al., 1993).

<table>
<thead>
<tr>
<th>Exposure time (h)$^a$</th>
<th>IC$_{50}$ (nM)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.000</td>
</tr>
<tr>
<td>4</td>
<td>2.400</td>
</tr>
<tr>
<td>6</td>
<td>2.400</td>
</tr>
<tr>
<td>24</td>
<td>7.3</td>
</tr>
<tr>
<td>72</td>
<td>0.12</td>
</tr>
</tbody>
</table>

$^a$ Duration of drug exposure starting at $t = 0$ over a total incubation period of 72 h

$^b$ Vincristine concentration required to achieve 50% cytotoxicity
Figure 1.2
Model for vincristine sensitivity of tumors.

This figure indicates that for therapy, cellular levels of drug must be maintained above the concentration required to inhibit mitotic spindle formation (M phase of the cell cycle). Further, since tumors contain dividing cells distributed throughout the cell cycle it is important to achieve as long an exposure time as possible.
achieved when using liposomal carriers, which in turn may be expected to lead to longer exposure of the drug to the tumor. An added advantage is that there tends to be increased accumulation within tumors for liposomes which exhibit longer circulation residence times (Vaage et al., 1993; Olson et al., 1979; Bally et al., 1990; Parr et al., 1993; Mayer et al., 1990b; Allen et al., 1991a). However, a major problem associated with liposomal vincristine preparations concerns drug leakage from the liposomal carrier. For example, the best available retention achieved in previous work still results in approximately 85% release of entrapped vincristine from DSPC/Chol liposomes in the blood within 24 h of i.v. administration (Mayer et al., 1990c). Despite this rapid release of vincristine from its liposomal carrier, significant improvements in therapeutic activity are observed over free drug. It could therefore be assumed that improvements in drug retention would result in increased therapeutic activity.

The main objective of this thesis is to examine the effect of various parameters on improving vincristine retention within liposomes with the aim of reducing toxicity and improving antitumor potency. Chapter 2 examines the ability of liposomal encapsulation of vincristine to reduce drug toxicity after extravasation. Chapter 3 focuses on the use of parameters such as lipid composition, internal buffering capacity, and internal pH to improve retention while Chapters 4 and 5 focus on the incorporation of specialized lipids to improve drug retention.

This introductory chapter will first describe liposome structure and the effects of varying lipid composition on the physical properties of these systems. The second part reviews the in vivo behaviour of liposomal drug delivery systems.
1.2 Liposomes

The term "liposome" refers to one or more lipid bilayers forming an enclosed vesicle which is able to encapsulate a distinct aqueous volume. Liposomes can be multilamellar or unilamellar (see Figure 1.3) and can be large (>1 μm diameter) or small (<50 nm diameter) (see Hope et al., 1986; Szoka and Papahadjopoulos, 1980). The stability of liposomes (both circulation longevity and the ability to retain molecules) is strongly influenced by the lamellarity and size of the liposomes.

1.2.1 Multilamellar Vesicles (MLVs)

MLVs spontaneously form by mixing bilayer forming membrane lipids in water (Bangham et al., 1965; Bangham, 1983). MLVs are typically heterogeneous in size, usually in the range of 1-10 μm diameter, and can exhibit unequal solute distributions across the bilayers where the concentration of trapped solute is lower than that in the external medium (Mayer et al., 1985). Trapped volumes of MLVs can be increased by successive cycles of freezing and thawing (called FATMLVs, Mayer et al., 1985). MLVs are at a disadvantage for experimental and practical usage due to their large and heterogeneous size as well as their variable lamellarity. This precludes quantitative studies on the permeability properties of bilayers to various solutes, for example, and limits their use as drug delivery vehicles as large systems are rapidly cleared from the circulation. Because of these disadvantages, procedures have been developed to generate unilamellar liposomes of defined size.

1.2.2 Small Unilamellar Vesicles (SUVs)

SUVs range in size from 25-50 nm diameter and can be formed by the sonication of
Figure 1.3
Multilamellar and unilamellar liposomes.

MULTILAMELLAR VESICLES

Diameter: 0.2 - 10 \( \mu \text{m} \)

UNILAMELLAR VESICLES

Diameter: 0.02 - 0.2 \( \mu \text{m} \)
MLVs (Huang, 1969). They can also be prepared using a "French press" process which involves forcing the MLV suspension through a narrow orifice, employing very high pressures (Barenholz et al., 1979; Szoka and Papahadjopoulos, 1980). SUVs are relatively easy to prepare and are homogeneous in size. However, due to their high degree of membrane curvature they are often unstable and tend to fuse to form larger systems. Another disadvantage is that they have very small trapped volumes (<0.2 μL/μmol phospholipid). These disadvantages make SUVs inappropriate for many applications as model membrane systems as well as most forms of drug delivery. Therefore, several procedures have been developed to produce unilamellar vesicles of larger sizes.

1.2.3 Large Unilamellar Vesicles (LUVs)

There are several techniques which have been developed to produce LUVs. The simplest method involves the sequential extrusion of MLVs through polycarbonate filters of various sizes (Olson et al., 1979; Hope et al., 1985). With this procedure, homogeneously sized liposomes can rapidly be produced with vesicle diameters in the range from 50-200 nm. The resulting vesicles are called LUVETs (Large Unilamellar Vesicles by Extrusion Techniques). The major advantages of this technique include its speed, the avoidance of solvents or detergents, the high trapping efficiencies obtained, the homogeneous nature of the resulting vesicle populations, and the ability to select liposome size through filter pore size.

Other methods for producing LUVs include ethanol injection (Kremer et al., 1977), ether infusion (Deamer and Bangham, 1976), reverse phase evaporation (Szoka and Papahadjopoulos, 1978), and detergent dialysis (Mimms et al., 1981). In these methods,
lipid is solubilized in organic solvent or detergent, followed by injection of the mixture into buffer. The organic solvent or detergent can then be removed by one of several different techniques such as distillation, dilution, or dialysis. LUVs prepared by these methods have average diameters of 50-200 nm and trapped volumes of between 1-3 L per mol of lipid (Szoka and Papahadjopoulos, 1980). All of these techniques are time consuming, result in liposome populations of heterogeneous size and may contain residual detergent or organic solvents (Parente and Lentz, 1984). Due to these disadvantages, extrusion techniques (using 100 nm pore size filters) were used to produce the vesicles used for all experiments presented in this thesis. Electron micrographs of EPC LUVs can be seen in Figure 1.4.

1.3 Lipid Structures

1.3.1 Phosphatidylcholine

This section will deal only with the primary lipids used for producing liposomes employed in this thesis. These are phosphatidylcholine (PC) and cholesterol. Structures of the most common phospholipids can be seen in Figure 1.5. Phosphatidylcholine is a zwitterion composed of a glycerol-phosphate ester with a choline headgroup and two acyl chains esterified to the sn-1 and sn-2 positions (Small, 1986). Phosphatidylcholine is the most common phospholipid in eukaryotic plasma membranes. Usually, the fatty acid at the sn-1 position tends to be saturated, while the sn-2 acyl chain tends to be unsaturated (Small, 1986). In this thesis, PCs with saturated fatty acids at both the sn-1 and sn-2 positions were the primary lipid species employed. The variety of saturated fatty acids attached to these two positions is illustrated in Table 1.2. Variations in the acyl chain length and unsaturation can produce marked effects on the physical properties of the lipid bilayers comprising these
Figure 1.4
Freeze-Fracture Electron Micrographs of LUVs
Produced by Extrusion

Vesicles were prepared by extruding frozen and thawed egg-PC MLVs, at a concentration of 100 mM lipid, 10 times through polycarbonate filters of various pore sizes. (A) 400 nm, (B) 200 nm, (C) 100 nm, and (D) 50 nm.
Figure 1.5
Structures of common phospholipids.

phosphatidyl choline

phosphatidyl ethanolamine

phosphatidyl serine

phosphatidyl glycerol

phosphatidyl inositol

sphingomyelin
Table 1.2  
Names and Structures of Saturated Fatty Acids.

<table>
<thead>
<tr>
<th>No. of Carbon Atoms</th>
<th>Structural Formula</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>CH₃(CH₂)₁₂CO₂H</td>
<td>myristic acid</td>
</tr>
<tr>
<td>16</td>
<td>CH₃(CH₂)₁₄CO₂H</td>
<td>palmitic acid</td>
</tr>
<tr>
<td>18</td>
<td>CH₃(CH₂)₁₆CO₂H</td>
<td>stearic acid</td>
</tr>
<tr>
<td>20</td>
<td>CH₃(CH₂)₁₈CO₂H</td>
<td>arachidic acid</td>
</tr>
<tr>
<td>22</td>
<td>CH₃(CH₂)₂₀CO₂H</td>
<td>behenic acid</td>
</tr>
</tbody>
</table>
phospholipids, as indicated below.

1.3.2 Phase Transitions

Lipid molecules in a bilayer can exist in a gel or frozen state which is ordered and rigid or the liquid-crystalline state which is disordered and fluid (see Figure 1.6). In the gel state all the carbon-carbon bonds are in the extended, all-trans conformation, while in the liquid-crystalline state some are in the gauche conformation. Lateral diffusion occurs rapidly in the liquid-crystalline state but much more slowly in the gel state. For example, dipalmitoyl PC in the liquid-crystalline state exhibits a lateral diffusion rate of $D_t = 2 \times 10^{-8} \text{ cm}^2/\text{sec}$, whereas in the gel state, $D_t < 10^{-9} \text{ cm}^2/\text{sec}$ (Cullis, 1976). Employing the relation $x^2 = 4D_t t$, where $x$ is the distance diffused in time $t$, it is straightforward to show that in 1 sec a liquid-crystalline DPPC molecule will diffuse approximately $2.8 \times 10^{-6} \text{ m}$, whereas in the gel state the distance diffused is less than $0.3 \times 10^{-6} \text{ m}$. In the more fluid liquid-crystalline state, the lipid acyl chains undergo fluctuations about individual carbon-carbon segments. There is a specific temperature at which a lipid transforms between these two states. Increasing the length of the fatty acyl chains and increasing their degree of saturation raises this transition temperature (see Table 1.3).

The transition between the ordered gel state and the fluid liquid-crystalline state can be detected by calorimetric techniques, which measure the heat required to raise the temperature of a sample containing the lipid dispersion. As illustrated in Figure 1.4, for dipalmitoyl PC, this transition occurs at approximately 41°C.
Figure 1.6

Phase transition of bilayer lipids.

The phase transition of a lipid bilayer from the gel state \( (L_\beta) \) to the liquid crystalline state \( (L_\alpha) \) upon reaching the transition temperature \( (T_c) \) of the lipid.

Gel State
\[
\begin{align*}
L_\beta \\
T<T_c
\end{align*}
\]

endothermic transition

Liquid Crystalline State
\[
\begin{align*}
L_\alpha \\
T>T_c
\end{align*}
\]
Table 1.3
Effect of Fatty Acyl Chain Length and Degree of Saturation on Phase Transition Temperature

<table>
<thead>
<tr>
<th>Lipid Species</th>
<th>Transition Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dimyristoyl PC (14:0, 14:0)</td>
<td>24</td>
</tr>
<tr>
<td>dipalmitoyl PC (16:0, 16:0)</td>
<td>41</td>
</tr>
<tr>
<td>distearoyl PC (18:0, 18:0)</td>
<td>55</td>
</tr>
<tr>
<td>palmitoyl, oleoyl PC (16:0, 18:1)</td>
<td>-1</td>
</tr>
<tr>
<td>dioleoyl PC (18:1, 18:1)</td>
<td>-19</td>
</tr>
<tr>
<td>stearoyl, oleoyl PC (18:0, 18:1)</td>
<td>6</td>
</tr>
<tr>
<td>stearoyl, linoleoyl PC (18:0, 18:2)</td>
<td>-16</td>
</tr>
<tr>
<td>stearoyl, linolenoyl PC (18:0, 18:3)</td>
<td>-13</td>
</tr>
<tr>
<td>stearoyl, arachidonyl PC (18:0, 20:4)</td>
<td>-13</td>
</tr>
</tbody>
</table>
1.3.3 Cholesterol

In all liposomal preparations employed in this thesis, cholesterol is a major component (45 mol%). Cholesterol is the major neutral lipid component of eukaryotic plasma membranes. Part of the molecule has a rigid steroid structure, with a polar 3-β-hydroxyl group on one end of the molecule (see Figure 1.7).

In liposomal systems, the addition of cholesterol to saturated phosphatidylcholine progressively decreases the enthalpy of the gel-liquid crystalline phase transition. At about 30 mol% cholesterol or higher, the transition can no longer be detected (Chapman, 1975). An example of this progression for dipalmitoyl PC can be seen in Fig. 1.8. Cholesterol increases the "order" in the acyl chains of PCs which are in the liquid-crystalline state and decreases the order for PCs which are in the gel state (Demel and de Kruyff, 1976). Also, cholesterol can have a condensing effect with PC, where the volume of a mixture of liquid-crystalline PC and cholesterol is less than the volume of the two components separately (Hyslop et al., 1990).

It has previously been shown that cholesterol stabilizes liposomal membranes as evidenced by an increase in liposomal solute retention (Papahadjopoulos et al., 1973; Inoue, 1974). In vivo uptake of liposomes by RES cells in the liver and spleen is also reduced by the addition of cholesterol (Patel et al., 1983). Further, there is decreased uptake and intracellular degradation of cholesterol-containing liposomes by cultured Kupffer cells (Roerdink et al., 1989).

Several studies have established that liposome interactions with HDL result in destabilization of the liposomes and the transfer of liposomal phospholipid to HDL (Tall and Green, 1981; Chobanian et al., 1979; Scherphof et al., 1978; Krupp et al., 1976). Cholesterol
Figure 1.7
Structure of cholesterol.
Figure 1.8
Calorimetric tracings of DPPC dispersions (fully hydrated) with and without cholesterol.

ENDOTHERMIC

Temperature (K)
appears to reduce the net transfer of phospholipid to high density lipoproteins in the presence of serum (Kirby et al., 1980a; Kirby et al., 1980b; Gregoriadis and Davis, 1979). Kirby and coworkers monitored the fate of $^3$H-phosphatidylcholine and $^{14}$C-cholesteryl oleate in the liposomal membrane and of 6-carboxyfluorescein trapped in the aqueous interior of liposomes. They found that by incorporation of increasing amounts of cholesterol into liposomes, there was a greatly diminished release of 6-carboxyfluorescein in the presence of serum and a decrease in the amount of phospholipid associated with HDL.

### 1.4 Transmembrane pH Gradients

Many biological compounds or pharmaceutical agents (such as vincristine) have proton accepting (basic) groups. The dissociation constant ($K_a$) for the protonated base can be written as follows:

$$K_a = [H^+] [B] / [BH^+]$$  \hspace{1cm} (1)

where $[H^+]$ is the hydrogen ion concentration, $[B]$ is the concentration of the neutral form of the weak base and $[BH^+]$ is the concentration of the protonated base. Assuming that $K_a$ is the same on both sides of the liposomal membrane, it follows that:

$$K_a = [H^+]_i [B]_i / [BH^+]_i = [H^+]_o [B]_o / [BH^+]_o$$  \hspace{1cm} (2)

where the subscripts $i$ and $o$ refer to the inside and outside of the liposome. Since it is known that the neutral form of ionizable molecules are usually orders of magnitude more membrane permeable than the charged species, (Addanki et al., 1968; Rottenberg, 1979), at equilibrium, the concentration of the neutral species will be the same on both sides of the membrane. Therefore,

$$[BH^+]_i / [BH^+]_o = [H^+]_i / [H^+]_o$$  \hspace{1cm} (3)
This result indicates that the presence of a pH gradient across a liposome membrane (inside acidic) will drive the net uptake of weak bases. For example, for a three-unit pH gradient (ΔpH=3) (inside acidic), an equilibrium concentration of weak base inside the vesicle 1000 times higher than outside should be achieved (see Figure 1.9).

1.5 Drug Trapping in Liposomes

1.5.1 Passive Entrapment

This thesis focuses on the potential of liposomes as drug delivery vehicles for vincristine. Passive entrapment of drugs can be accomplished by simply preparing liposomes in a solution of the desired molecule for entrapment (Taylor et al., 1990). This procedure results in extremely low drug-to-lipid ratios requiring a high dose of lipid administration into patients. Previous work with doxorubicin, for example, has show as low as 4% liposomal trapping efficiencies and drug-to-lipid ratios of 0.004:1 (wt:wt) (Shinozawa et al., 1981). If drug-to-lipid ratios of only 0.01:1 or less are achieveable, to inject a therapeutic dose of vincristine in mice of 2 mg/kg, a lipid dose of 200 mg/kg would need to be administered. Drugs which have been passively entrapped within liposomes tend to leak out quite rapidly. Between 20-50% of passively loaded doxorubicin is released from EPC/Chol (55:45; mol:mol) liposomes by 1 h at 37°C (Gabizon et al., 1982a; Mayer et al., 1985).

1.5.2 Active Entrapment

Drugs which are weak bases can be actively entrapped within liposomes in response to a pH gradient (inside acidic). This occurs essentially as outlined for weak bases in Section
Figure 1.9
Redistribution of weak bases in response to transmembrane pH gradients;
where D represents the drug/weak base of interest.

At equilibrium, if:

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

Then:

$$\frac{[DH^+]_i}{[DH^+]_o} = \frac{[H^+]_i}{[H^+]_o}$$
A survey of drug accumulation within liposomes exhibiting a pH-gradient has been performed (Madden et al., 1990). These studies reveal that a large number of drugs which are weakly basic in character could accumulate within unilamellar vesicles in the presence of a pH-gradient (interior acidic). A similar procedure had earlier been utilized to entrap catecholamines in liposomes (Nichols and Deamer, 1976).

Active entrapment is advantageous in that it allows for much higher drug-to-lipid ratios and there is much slower drug leakage (Mayer et al., 1986b). For example, under appropriate conditions doxorubicin can be liposomally encapsulated in response to a pH gradient yielding transmembrane drug concentration gradients in excess of $10^3$ and trapping efficiencies approaching 100% (Mayer et al., 1986b; Mayer et al., 1990d). In addition to inducing efficient encapsulation of doxorubicin, the transmembrane pH gradient also enhances drug retention within the liposomes. Less than 5% of doxorubicin is released from EPC/Chol (55:45; mol:mol) liposomes by 24 h at 37°C (Mayer et al., 1985). Doxorubicin release from liposomes increases significantly upon dissipation of the pH gradient caused either by decreasing the external pH or by addition of proton gradient uncouplers (Mayer et al., 1990d).

1.6 Lipid Asymmetry

It is generally agreed that many biological membranes exhibit asymmetric transmembrane distributions of lipids (Houslay and Stanley, 1982; Op den Kamp, 1979). An example of this can be seen in the erythrocyte membrane where phosphatidylethanolamine (PE) and phosphatidylserine (PS) are localized to the inner
membrane (Houslay and Stanley, 1982; Op den Kamp, 1979). The asymmetric transbilayer distributions of lipids commonly observed in biological membranes may be expected to play a role in membrane fusion in vivo. For example, erythrocytes which have lost lipid asymmetry fuse more readily than asymmetric erythrocytes with the fusogenic PE and PS molecules localized to the inner monolayer (Tullius et al., 1989).

The ability of transmembrane pH gradients to generate lipid asymmetry in model membrane systems has been described for both basic and acidic lipids (Hope and Cullis, 1987; Eastman et al., 1989; Hope et al., 1989; Eastman et al., 1991). Specifically, the complete migration of both stearylamine and sphingosine to the inner monolayer has been observed for LUVs with acid interiors (Hope and Cullis, 1987). Since both stearylamine and sphingosine are amino-containing lipids which behave as weak bases, they permeate the vesicle membranes in their neutral form to reside on the inner monolayer in their protonated state.

Chapter 4 examines the use of cationic lipids to enhance vincristine retention within liposomes. These lipids are utilized to create a positive charge at the inner membrane surface thus decreasing the concentration of drug at that site. The charge repulsion between the vincristine and the inner surface is expected to reduce the ability of vincristine to partition into the inner monolayer of the liposomes, decreasing the rate of release from the liposome. It is important to describe how these lipids behave under the experimental conditions employed in these studies.
1.7 Interaction of Liposomes In Vivo

It has previously been demonstrated that MLVs are cleared from the circulatory system in a biphasic pattern, with an initial rapid rate followed by a slower rate of elimination (Gregoriadis and Ryman, 1972). The same biphasic clearance pattern was later seen for LUVs and SUVs (for reviews see Gregoriadis, 1988; Hwang and Beaumier, 1988). Although the exact reasons for this clearance pattern are unclear, it is generally believed that liposome clearance is a result of two major factors: (1) interactions with plasma proteins causing liposomal membrane destabilization, and (2) the ingestion of liposomes by cells of the RES, primarily in the liver and spleen (Gregoriadis, 1988; Hwang and Beaumier, 1988).

In order for liposomes to be effective drug delivery vehicles, they require extended circulation half-lives, in order to be able to accumulate within target areas. Because of this, the effect of varying physical characteristics on liposome clearance times will be addressed in this section.

1.7.1 Plasma Protein-Liposome Interactions

It has been shown that certain liposome preparations become unstable in the presence of plasma (Senior, 1987; Gregoriadis, 1973; Gregoriadis and Senior, 1980). Thus, plasma must contain factors responsible for inducing this instability in liposomes. This section will deal with the types of proteins which interact with liposomes. These include lipoproteins, complement factors, and other serum proteins.

In vitro studies on the role of plasma lipoproteins in inducing liposome instability have been extensively performed (Allen and Cleland, 1980; Mui et al., 1994). There is a net transfer of liposomal lipids to lipoproteins (HDL) resulting in an increased leakage of
entrapped solutes (reviewed by Senior, 1987) (see Section 1.3.3). The major apolipoprotein of HDL, ApoA-1 has been implicated in this destabilization effect (Klausner et al., 1985).

Liposome instability can also be induced by complement factors as first suggested by Finkelstein and Weissmann (1979). When the complement cascade is activated, the formation of membrane attack complexes occurs which leads to membrane lysis (reviewed by Muller-Eberhard, 1986). The mechanism whereby the membrane becomes damaged has been extensively studied (reviewed by Alving and Richards, 1983; Muller-Eberhard, 1986). Studies indicate that release of solute from LUVs occurs through stable pores of approximately 10 nm diameter (Malinski and Nelsestuen, 1989). Complement damage can also result in the release of phospholipids from liposomal membranes (Shin et al., 1978; Kinoshita et al., 1977; Shin et al., 1977).

Many other plasma proteins have been shown to bind to liposomes (reviewed by Bonte and Juliano, 1986; Chonn et al., 1992a). An example is the binding of vitamin K dependent serum proteins via Ca$^{2+}$ ions to anionic phospholipids (reviewed by Jackson, 1980). They enhance the conversion of the zymogen forms of the clotting proteins to active proteases. This may enhance the removal of negatively charged liposomes from the circulation. As another example, C-reactive protein binding to lipid membranes causes agglutination of liposomes and may affect their structural integrity (Richards et al., 1979).

The most prominent protein that is associated with rapidly cleared liposomes is a 50 kDa protein (Chonn et al., 1992a). Purified apolipoprotein H exhibits very similar electrophoretic mobility to this 50 kDa protein (Chonn et al., unpublished). Apolipoprotein H has been shown to act as a cofactor for antiphospholipid antibody binding (McNeil et al., 1990; Galli et al., 1990).
1.7.2 Interaction of Liposomes With Cells of the RES

Following i.v. injection of liposomes, there is rapid uptake of liposomes by the cells of the RES (Poste et al., 1982, 1983). The majority of these liposomes accumulate in the fixed RES cells of the liver and spleen as well as in the lung, lymph nodes, and bone marrow (Poste, 1983). Evidence of this can be seen from the biodistribution of DSPC/Chol liposomes (see Figures 5.2 and 5.3). The pattern of liposomal uptake can be seen in Figure 1.10. This affinity of liposomes for RES cells can be utilized to enhance the therapeutic potential of liposomally entrapped drugs. It has also been shown that liposome encapsulated molecules can accumulate at sites of inflammation (Williams et al., 1986), infection (Morgan et al., 1981), and in solid tumors (Ogihara et al., 1986). For example, Morgan and coworkers determined that approximately 13% of total i.v. injected anionic liposomes accumulated in a site of thigh abscess within 30 min following administration. This is in comparison to 4% accumulation in an uninfected thigh. Likewise, experiments in J6456 lymphoma tumor-bearing mice demonstrated a 25-fold increase of the liposome concentration in the tumor when formulations with long and short blood residence times were compared (Gabizon and Papahadjopoulos, 1988). This passive targeting effect is clearly a potential therapeutic advantage as a higher concentration of drug accumulates at the site of disease.

1.8 Factors Affecting Liposome Circulation Lifetimes

It is important for liposomes in therapeutic applications to have extended circulation times for several reasons. Firstly, they can provide a long-term release of encapsulated drug. Also, passive targeting of liposomes may be enhanced by extending liposome circulation
Figure 1.10
Biodistribution of liposomes within the organs of the RES (spleen, liver, bone marrow, lungs, and lymph nodes).
time. Usually, 100 nm diameter vesicles composed of long chain saturated lipids such as DSPC combined with cholesterol are used as basic drug delivery vehicles (Bally et al., 1990; Gabizon and Papahadjopoulos, 1988). The circulation half-lives of these systems can be extended by incorporation of the ganglioside $G_{M1}$ (Allen and Chonn, 1987) or polyethyleneglycol derivatives (Blume and Cevc, 1990; Klibanov et al., 1990; Allen and Hansen, 1991; Lasic et al., 1991; Allen et al., 1989). These "extended lifetime" formulations yield dose-independent clearance kinetics, with 5 to 30% of the injected dose remaining in the (mouse) circulation at 24 h post injection. There are six major factors which play a significant role in promoting liposome circulation lifetime. These include, as emphasized previously, the presence of cholesterol in liposome membranes (Gregoriadis and Davis, 1979) and using phospholipids with high phase transition temperatures (Blok et al., 1975; Senior et al., 1982). Liposomes are also rendered more long-lived by decreasing their size (Senior, 1987). For example, for DSPC/Chol systems, small unilamellar vesicles and vesicles passed through 400 nm diameter pores exhibited circulation half-lives of 7.5 and 0.2 h, respectively. They can also be rendered more long-lived by injecting higher lipid doses to saturate cells of the RES (Abra et al., 1980). A saturation of liver uptake with increasing lipid dose has been demonstrated for liposomes of various sizes, together with a corresponding increase in blood levels. An alternative theory suggests that saturation of the RES, per se, does not occur due to inhibition of uptake by RE cells. Rather, the decreased clearance rates observed for liposomes injected at higher doses may be due to a decrease in protein binding to these liposomes (Chonn et al., 1992a; Oja et al., unpublished). Also, the presence of encapsulated cytotoxic drug can enhance liposome circulation time (Bally et al., 1990; Parr et al., 1993). Lastly, as previously mentioned, recent studies have shown that
increasing the hydrophilic nature of the liposome surface can prolong liposome circulation
time, as summarized in the next section.

1.8.1 Increasing Liposome Surface Hydrophilicity

Liposome surface hydrophilicity can be increased by the incorporation of molecules
such as monosialoganglioside G\textsubscript{M1} (see Figure 1.11) (Allen and Chonn, 1987; Gabizon and
Papahadjopoulos, 1988) and phosphatidylethanolamine-polyethyleneglycol (PEG-PE)
derivatives (Blume and Cevc, 1990; Klibanov et al., 1990; Allen and Hansen, 1991; Lasic
et al., 1991; Allen et al., 1989). These molecules are postulated to provide a steric barrier
that inhibits the liposome association with plasma proteins (Chonn et al., 1992b; Allen et al.,
1989; Klibanov et al., 1991; Senior et al., 1991a). Allen and coworkers have demonstrated
that addition of 10 mol% G\textsubscript{M1} into DSPC/Chol (2:1; mol:mol) vesicles of 100 nm diameter
increases the recovery of liposomes in the circulation from 0% to 28% 24 h after i.v.
injection. This is for an injected lipid dose of 25 mg/kg. Klibanov and coworkers have
demonstrated similar results with the incorporation of 10 mol% PEG-PE.

1.9 Summary

Earlier work with liposomal vincristine demonstrated that encapsulation within
DSPC/Chol liposomes decreased the toxicity seen with free drug (Mayer et al., 1990). The
50% lethal dose of 1.9 mg/kg in CD-1 mice observed for free vincristine increased to 4.8
mg/kg upon encapsulation of the drug within these liposomes. Liposomal encapsulation of
vincristine also enhanced the antitumor activity against murine P388 and L1210 lymphocytic
leukemia models. This resulted from increased efficacy for liposomal vincristine at doses
Figure 1.11
Structure of Monosialoganglioside $G_{M1}$. 

![Structure of Monosialoganglioside $G_{M1}$]
equal to free drug as well as the ability to administer increased doses of liposomal vincristine.

These improvements were observed for systems which released approximately 85% of the drug within 24 h after injection. It may be expected that further therapeutic improvements should be achievable for vincristine preparations with improved retention properties. Prior to this work, however, a liposomal system has not been developed which is capable of retaining vincristine for longer periods of time. The purpose of this study is to determine the factors necessary for prolonging vincristine retention within liposomes and using these factors to improve vincristine retention, and to correlate this with the antitumor effects of such improved systems.

The effect of liposomal encapsulation on decreasing drug toxicity is discussed in Chapter 2. Physical factors such as lipid composition, internal buffering capacity, internal pH, and temperature and their role in improving drug retention are discussed in Chapter 3. In Chapters 4 and 5, the incorporation of novel lipids such as sphingosine and monosialoganglioside G_{M1} and their effects on vincristine retention in liposomes is examined.
CHAPTER 2
DECREASING VINCRIStINE TOXICITY By ENCAPSULATION WITHIN LIPOSOMES

2.1 Introduction

As mentioned in Section 1.1, vincristine is a widely used antineoplastic agent that displays effectiveness against a wide variety of neoplasms (Carter and Livingston, 1976; Sieber et al., 1976). A dose limiting toxicity associated with vincristine use is its neurotoxic effects which are manifest mainly as peripheral neuropathy. Vincristine is also known for its ability to produce soft tissue necrosis and ulceration if accidently extravasated during i.v. administration or injected i.m. (Bellone, 1981; Choy, 1979).

Liposomal encapsulation of several antineoplastic drugs has been shown to decrease toxic side effects while maintaining their therapeutic potential (Herman et al., 1983; Forssen and Tokes, 1981; Gregoriadis and Neerunjun, 1975; Woo et al., 1983; Mayhew and Rustum, 1985; Gabizon et al., 1982a; Kobayashi et al., 1977; Hunt et al., 1979; Rahman et al., 1982; Gregoriadis, 1988; Fichtner et al., 1981; Gabizon et al., 1982b). This includes the vinca alkaloid vincristine (Mayer et al., 1990c). It has previously been demonstrated that liposomal encapsulation of doxorubicin decreases the vesicant properties of the drug (Balazsovits et al., 1989; Forssen and Tokes, 1983). However, minimal attention has been given to the ability of liposomal encapsulation to diminish the vesicant properties of vincristine.

Several studies have been devoted to analyzing the effectiveness of antidotes in the
treatment of accidental extravasation of vincristine (Loth and Eversman, 1986; Barr and Sertic, 1981; Dorr and Alberts, 1985). However, in severe cases of extravasation, no antidote injections have been shown to significantly alter the outcome of the accident (Dorr and Fritz, 1982; Knoben and Anderson, 1983). In this chapter we assess the effect of liposomal encapsulation on reducing the vesicant properties of vincristine, thereby alleviating the need for antidotes if inadvertant extravasation of the drug should occur.

2.2 Materials and Methods

2.2.1 Lipids and Chemicals

Distearoyl phosphatidylcholine (DSPC) was purchased from Avanti Polar Lipids and was >99% pure. Cholesterol, HEPES, and citric acid were obtained from Sigma (St. Louis, MO, USA). Vincristine sulfate was purchased from Lymphomed (Markham, ON). [14C]cholesteryl hexadecyl ether was produced by special order from New England Nuclear (Ontario, Canada) and was >95% pure. It was chosen as a lipid marker due to its stability in vivo (Derksen et al., 1987). [3H]vincristine was obtained from Amersham (Oakville, Ontario, Canada). Female BALB/c mice (retired breeders) were purchased from Charles River Laboratories.

2.2.2 Liposomes

DSPC/Chol (55:45; mol:mol) liposomes were prepared by first dissolving the lipid mixture in 95% ethanol at 60°C for 30 min. (100 mg lipid/mL). Multilamellar vesicles (MLVs) were formed by adding 300 mM citric acid pH 4.0 and vortex mixing vigorously (25 mg lipid/mL final mixture). The resulting MLVs were then incubated at 60°C for an
additional 30 min to ensure equilibration of citrate buffer across the lipid bilayers. Following incubation, large unilamellar vesicles (LUVs) were produced by extruding the MLVs through an extruder containing 2 polycarbonate filters with 100 nm pore size. The extrusion device was obtained from Lipex Biomembranes (Vancouver, British Columbia, Canada) and was equilibrated at 60°C. Following extrusion, the liposomes were dialyzed against two changes of 100 volumes of citric acid buffer (pH 4.0) over a 24-h period. Spectra/Por 2 dialysis tubing was used (cutoff 12-14 kDa).

2.2.3 Drug Entrapment Procedure

Vincristine was loaded into the liposomes as follows. The vesicles were passed down a G-25 Sephadex column equilibrated with HBS pH 7.4 to exchange the external buffer. Vincristine sulfate was then added to the liposomes to achieve a drug-to-lipid ratio of 0.1:1. The resulting drug/lipid mixture was then incubated at 60°C for 10 min. This procedure results in greater than 95% trapping efficiency of the drug.

2.2.4 Gross and Histological Studies

The procedure used for skin toxicity experiments has been previously described (Dorr et al., 1980). Briefly, an approximately 3-cm² area of hair above the hindleg of the adult BALB/c mouse is removed by vigorous rubbing with Neet topical depilatory lotion (Whitehall Laboratories, New York, NY). This procedure causes no adverse skin effects in itself (Dorr et al., 1980). Twenty-four hours following hair removal, 10 μg of either free or liposomal vincristine (diluted to 50 μL in normal saline) was injected subcutaneously using a 25-gauge needle (bevel up). Mice were visually monitored twice daily for any evidence
of skin damage or gross ulceration. Mice appearing to be in any distress were immediately sacrificed by cervical dislocation following anaesthetic.

For histological examination, at varying time points, mice were anaesthetized with an i.p. mixture of ketamine 160 mg/kg and xylazine 20 mg/kg. Following anaesthetic, skin around the site of injection was removed and immediately placed in 10% formalin as a fixative. Histological sectioning and analysis were subsequently performed. Haematoxylin and eosin were used for staining.

2.2.5 Skin Retention Studies

The presence of liposomal vincristine in the skin was monitored at varying time points by radiolabelling. Skin was removed from the mice as in section 2.2.4, removing a circular section with a diameter as close as possible to 2 cm. The skin was then homogenized in saline using a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ont.). Skin homogenates (total volume) were then digested with 500 μL of "Solvable" (DuPont Canada, Inc., Mississauga, Ont.) for 3 h at 50°C. Subsequently, the samples were cooled to room temperature before decolorizing with 200 μL of 30% hydrogen peroxide. Samples were then analyzed using dual label liquid scintillation counting.

2.3 Results

2.3.1 Gross Studies

Figures 2.1 and 2.2 are photographs of mice given subcutaneous injections of free and liposomal vincristine, respectively, on day 9. Neither treatment group displayed any evidence of erythema or edema at the site of injection over the first 7 days following
Figure 2.1
Female BALB/c mouse given s.c. free vincristine.

The mouse was given a single s.c. injection (50 μL) of free vincristine (10 μg). The necrosis and ulceration was prominent on day 9 following injection.
Figure 2.2
Female BALB/c mouse given s.c. liposomal vincristine

The mouse was given a single s.c. injection (50 µL) of liposomal vincristine (10 µg drug, 100 µg lipid). There was no evidence of inflammation or ulceration at any time following the injection. This picture was taken on day 9 following injection.
injection. On day 7, 5 of the 10 mice given free vincristine showed ulcerations with diameters of 2-3 mm. By day 10, 9 mice showed evidence of ulceration, 4 of which had ulcerations of 5-6 mm diameter. By day 11, all mice had ulcerated, 6 mice having ulcerations of 5-8 mm diameter. The progressive severity of the lesions at this point necessitated sacrificing the animals. In contrast, mice injected with liposomal vincristine showed no evidence of skin necrosis or ulceration throughout the time course of the experiment. Control animals given saline or empty liposomes showed no evidence of inflammatory response.

2.3.2 Histological Studies

Skin sections were examined at 1, 3, 5, and 7 days following subcutaneous injection of both free and liposomal vincristine. Injection of free drug resulted in a rapid infiltration of inflammatory cells in the subcutaneous tissues (day 1) followed by a rapid resolution by day 3 (Figure 2.3). In contrast, when injected with liposomal vincristine, the initial inflammatory response in the subcutaneous area is less intense but is more prolonged, lasting throughout the 7 days studied (Figure 2.4). The numbers of inflammatory cells in the dermal area are relatively constant for both vincristine preparations and are 2-3 fold higher for the liposomal preparation. The estimated number of inflammatory cells is shown in Table 2.1.

2.3.3 Skin Retention Studies

Skin samples were analyzed at 1, 3, 5, and 7 days following subcutaneous injection of free or liposomal vincristine. Free drug was cleared from the site of injection very rapidly with only 0.4% of the drug remaining (0.04 μg) on day 1 after injection (Figure 2.5).
Figure 2.3
Histological skin sections from BALB/c mice given free vincristine.

Each mouse was given a single s.c. injection (50 μL) of free vincristine (10 μg). All sections are shown at 25X magnification and stained with haematoxylin and eosin. Photographs represent sections at 1 (A), 3 (B), 5 (C), and 7 (D) days following injection.
Figure 2.4
Histological skin sections from BALB/c mice given liposomal vincristine.

Each mouse was given a single s.c. injection (50 µL) of liposomal vincristine (10 µg). All sections are shown at 25X magnification and stained with haematoxylin and eosin. Photographs represent sections at 1 (A), 3 (B), 5 (C), and 7 (D) days following injection.
Table 2.1
Inflammatory Response Following Subcutaneous Injection of Free or Liposomal Vincristine

<table>
<thead>
<tr>
<th>Sample Injected</th>
<th>Dermal inflammatory cells (cells/mm²)</th>
<th>Subcutaneous inflammatory cells (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>8.2 x 10⁴</td>
<td>9.8 x 10³</td>
</tr>
<tr>
<td>Free vincristine (10µg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td>1.9 x 10⁵</td>
<td>1.3 x 10⁶</td>
</tr>
<tr>
<td>day 3</td>
<td>1.8 x 10⁵</td>
<td>6.8 x 10⁴</td>
</tr>
<tr>
<td>day 5</td>
<td>1.5 x 10⁵</td>
<td>9.0 x 10⁴</td>
</tr>
<tr>
<td>day 7</td>
<td>1.1 x 10⁵</td>
<td>9.0 x 10⁴</td>
</tr>
<tr>
<td>Liposomal vinc (10µg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td>4.3 x 10⁵</td>
<td>6.9 x 10⁵</td>
</tr>
<tr>
<td>day 3</td>
<td>2.0 x 10⁵</td>
<td>5.4 x 10⁵</td>
</tr>
<tr>
<td>day 5</td>
<td>4.6 x 10⁵</td>
<td>6.6 x 10⁵</td>
</tr>
<tr>
<td>day 7</td>
<td>7.2 x 10⁵</td>
<td>9.0 x 10⁵</td>
</tr>
</tbody>
</table>
Cutaneous retention of vincristine (A) and lipid (B) following s.c. injection of 10 μg of free (■) or liposomal (●) vincristine. The drug-to-lipid ratio of liposomal vincristine was 0.1:1 (wt:wt). Error bars represent standard deviations of four mice.
residual amount remained constant over the 7 day time course. By comparison, there was approximately 6% of the drug remaining at the site of injection for the liposomal preparation on day 1 (0.6 μg). This remaining drug level gradually declined over the time course of the experiment until approximately 0.3% remained on day 7. Approximately 45% of the liposomes remained at the subcutaneous site on day 1 and decreased to approximately 30% by day 7. This corresponds to a drug-to-lipid ratio of approximately 0.01 on day 1 following injection or 90% leakage of the drug. This value at 24 h corresponds to the same drug-to-lipid ratio seen in the circulation for this liposomal preparation 24 h after i.v. administration (Mayer et al., 1990c).

2.4 Discussion

This chapter investigates the effect of liposomal encapsulation on the vesicant properties of vincristine. It has previously been shown that encapsulation of the drug reduces vincristine toxicity as evidenced by a decrease in weight loss over time in mice (Mayer et al., 1990c, Boman et al., 1994). The soft tissue toxicity of liposomal vincristine, however, has received little attention until now. In this chapter we focus on the vesicant properties of the drug since extravasation can be devastating and seriously affect the patient's quality of life, particularly since vincristine is widely used in paediatric patients for the treatment of acute lymphoblastic leukemia. Extravasation injuries have been shown to be highest in the paediatric and geriatric age groups (Upton et al., 1979). Extravasation is also a relatively common occurrence, occurring in as many as 1-2% of chemotherapy infusions (Spiegel, 1981).

In patients, following antineoplastic drug extravasation, blistering and skin loss
become apparent in a few days followed by a progressive tissue necrosis which can continue for as long as 3 months (Reilly et al., 1977). Full thickness skin necrosis can eventually ensue, exposing underlying tendons and neurovascular structures (Spiegel, 1981). Due to the seriousness and frequency of antineoplastic extravasation, it is felt that minimizing these effects by liposomal encapsulation is an important factor to be studied.

The mechanism by which soft-tissue necrosis occurs is widely assumed to be due to a directly cytotoxic effect of the drug. Histologic analysis has been reported on two patients following inadvertent extravasation of cytotoxic drugs (Rudolph et al., 1976; Chait and Dinner, 1975). These studies revealed a nonspecific chronic inflammation with a patent microvasculature. Regardless of the mechanism and severity of vincristine-induced necrosis, various antidote therapies have questionable efficacy (Loth and Eversman, 1986; Dorr and Alberts, 1985).

Liposomal encapsulation of doxorubicin has been shown to dramatically reduce the vesicant properties of the drug (Balazsovits et al., 1989; Forssen and Tokes, 1983). Using the same approach to decrease the vesicant properties of vincristine, it was found that liposomal encapsulation also dramatically reduced soft-tissue damage by the drug. There was virtually no evidence of inflammatory response seen grossly when liposomal vincristine was administered subcutaneously. In contrast, free drug produced gross ulceration by day 9 following injection. On histological analysis, liposomal vincristine produced a chronic and mild inflammatory response whereas free drug produced an acute and more intense inflammatory response. This is likely due to the fact that the drug was shown to remain in the area of injection for a much longer time period when liposomally encapsulated. The drug leaks slowly from the liposome interior, resulting in a long-term, low dose of free drug
being exposed to the tissue. In comparison, when free drug is administered, there is a brief exposure of the full drug dose to the tissue before being absorbed by the circulatory system.

In summary, it is clear by this study that the liposomal encapsulation of vincristine greatly reduces its potential for producing tissue necrosis upon accidental extravasation. As previously mentioned, the drug's therapeutic potential remains the same. This could dramatically improve the quality of life of the cancer patient and allow the drug to be administered more safely.
CHAPTER 3
OPTIMIZING VINCRISTEINE RETENTION IN LIPOSOMES

3.1 Introduction

Since the vinca alkaloids are cell-cycle-specific cytotoxic drugs, it is likely that an ability to maintain high plasma levels of vincristine for extended periods would be advantageous. A liposome system which provides extended drug retention in the circulation is therefore desirable. Previous work with doxorubicin has shown that increased drug retention within liposomes leads to an increase in drug circulation time in vivo (Bally et al., 1990) and increased anti-tumor activity (Mayer et al., 1990b). However, currently available retention properties for vincristine are not optimal, as the best available formulation of liposomal vincristine releases drug in vivo with a half-life of less than 8 h, leading to more than 90% release by 24 h.

In this chapter we explore three different parameters for improving the liposomal retention of vincristine. These include the use of phospholipids with increased acyl chain length, reduction of the interior pH, and increased interior buffering capacity. The latter two parameters are important factors in the loading of lipophilic amines, such as vincristine, into vesicles exhibiting a transmembrane pH gradient (ΔpH; inside acidic) (Mayer et al., 1990a; 1990c; 1990d). It is shown that the major factor resulting in improved retention is the interior pH, where initial interior pH values of 2.0 result in nearly 50% retention at 24 h in vivo.
3.2 Materials and Methods

3.2.1 Lipids and Chemicals

Dimyristoylphosphatidylcholine (DMPC), dipalmitoyl PC (DPPC), distearoyl PC (DSPC), diarachidoyl PC (DAPC), and dibehenoyl PC (DBPC) were purchased from Avanti Polar Lipids, while cholesterol and all salts were obtained from Sigma (St. Louis, MO, USA). Vincristine sulfate was obtained from the British Columbia Cancer Agency (Vancouver, British Columbia, Canada).

\(^{[14}C]\)cholesteryl hexadecylether was chosen as a lipid marker due to its stability in vivo (Derksen et al., 1987). It was purchased from New England Nuclear (Ontario, Canada) and was >95% pure. Tritiated vincristine was obtained from Amersham (Oakville, Ontario, Canada).

Normal mouse serum was purchased from Cedar Lane Laboratories and female BDF1 mice (6-8 weeks old) were purchased from Charles River Laboratories.

3.2.2 Liposomes

PC/cholesterol (55:45; mol:mol) liposomes were prepared as outlined in Section 2.2.2. Each mixture was incubated at or above the transition temperature for each particular PC derivative (65°C for DMPC, DPPC and DSPC; 85°C for DAPC; and 100°C for DBPC). These MLVs were then extruded ten times through two stacked 0.1 μm polycarbonate filters. The extrusion device was heated to the appropriate temperature for each sample.
3.2.3 Drug Uptake and Release Experiments

For the vincristine uptake experiments, drug was added to the various liposome preparations to achieve a drug-to-lipid ratio of 0.1:1 (wt:wt). The external pH of the vesicles was then raised to pH 7.0-7.2 with 0.5 M Na₂HPO₄ and incubated at 37°C over a 4 h period. Aliquots were removed at various time points for determination of vincristine uptake. External untrapped vincristine was removed by running the samples over G-50 Sephadex columns prior to dual label scintillation counting of the liposomal fractions contained in the void volume.

For the drug release studies, vincristine was initially loaded into the liposomes using the same procedure as for the drug uptake experiments, except that the samples were immediately heated to their lipid transition temperatures for 10 min. This ensured >95% trapping efficiencies at a drug-to-lipid ratio of 0.1:1 (wt:wt) for all lipid compositions studied (Mayer et al., 1990c). The liposomal vincristine was then diluted 10-fold in either HBS (pH 7.4) or normal mouse serum. These samples were dialyzed using Spectra/Por 2 dialysis tubing against 200 vols. of HBS (pH 7.4) at 37°C. Aliquots were removed at various time points, run down G-50 Sephadex columns, and retained vincristine analyzed by dual label scintillation counting.

3.2.4 In Vivo Pharmacokinetics Studies

Liposomal vincristine was injected into BDF1 mice via a lateral tail vein (2 mg/kg vincristine, 20 mg/kg lipid). At varying time points, mice were anaesthetized with i.p. ketamine (160 mg/kg) and xylazine (20 mg/kg). Blood was removed via cardiac puncture
and placed into EDTA-coated microtainer tubes (Becton Dickenson). Samples were then centrifuged and plasma was analyzed for lipid and vincristine content by dual label liquid scintillation counting.

3.2.5 Kinetic Analysis of Vincristine Uptake and Release

An initial-rates treatment of the uptake of lipophilic amino containing drugs into vesicles with an acidic interior has been previously developed for doxorubicin (Harrigan et al., 1993). Assuming that only the neutral form of the drug can cross the lipid bilayer, we consider first weak bases with a single ionizable group with a dissociation constant ($K_d$) where $K_d << [H^+]_o$ where $[H^+]_o$ is the external proton concentration. Assuming that $V_o >> V_m$, where $V_o$ is the aqueous volume and $V_m$ is the volume of the membrane, it can be shown that

$$[D(t)]_i = [D(eq)]_i (1-e^{-kt})$$

(1)

where $[D(t)]_i$ is the interior concentration of the drug at time t, $[D(eq)]_i$ is the equilibrium interior concentration at $t = \infty$ and k is the rate constant associated with the uptake process. The rate constant k can be written as (Harrigan et al., 1993)

$$k = \frac{PA_mK}{V_o[H^+]_o} K_d$$

(2)

where P is the membrane permeability coefficient for the neutral form of the drug, $A_m$ is the
area of the membrane, and $K$ is the lipid-water partition coefficient of the drug. A similar analysis for a drug such as vincristine, which contains two basic functions can be performed under the assumption that $[H^+]_o \gg K_{d1}, K_{d2}$ (where $K_{d1}, K_{d2}$ are the dissociation constants associated with the two basic groups). It can be shown that

$$k = \frac{P_{Am}K}{V_o[H^+]_o^2} K_{d1}K_{d2}$$  \hspace{1cm} (3)

### 3.3 Results

#### 3.3.1 Influence of Acyl Chain Length on Vincristine Retention

It has previously been shown that both doxorubicin (Mayer et al., 1990a; 1990d) and vincristine (Mayer et al., 1990c) display enhanced retention within liposomes composed of DSPC-cholesterol as compared to EPC-cholesterol after loading in response to a pH-gradient (inside acidic). One could extend this observation to predict that the presence of longer saturated acyl chain PCs will further improve the retention properties of vincristine. The uptake and release properties of LUVs composed of DAPC and DBPC, in combination with cholesterol, were therefore investigated.

LUVs for drug loading were prepared from mixtures of cholesterol with DMPC ($C_{14}$), DPPC ($C_{16}$), DSPC ($C_{18}$), DAPC ($C_{20}$), and DBPC ($C_{22}$). The uptake of vincristine into these LUVs at 37°C is shown in Fig. 3.1. As expected, the rate of uptake was fastest for the DMPC-cholesterol system, and decreased progressively for DPPC-cholesterol and DSPC-cholesterol systems. Surprisingly, this progression was reversed for DAPC- and DBPC-
Vincristine uptake across a pH gradient at 37°C for DMPC/Chol (○), DPPC/Chol (●), DSPC/Chol (△), DAPC/Chol (▲), and DBPC/Chol (□). Vincristine was added to vesicle preparations at a potential drug-to-lipid ratio of 0.1:1. Internal pH was 4.0 and external pH was 7.5. All vesicles were sized through 100 nm filters. Error bars representing standard deviations of three trials are too small to be visible.
cholesterol systems, which exhibited rates of vincristine uptake which increased as the acyl chain length increased.

Vincristine release from the same liposomal formulations was also investigated (Fig. 3.2). Liposomal vincristine was incubated at 37°C for 24 h in the presence of buffer and mouse serum. The DMPC-cholesterol system exhibits the most rapid leakage, as expected, whereas the DAPC-cholesterol and DBPC-cholesterol exhibit the best drug retention, with approximately 40% of the drug remaining at 24 h in the presence of mouse serum.

3.3.2 Influence of Internal Buffering Capacity on Vincristine Retention

It has been previously documented that the accumulation of weakly basic drugs in response to a pH-gradient is extremely dependent on the internal buffering capacity (Harrigan et al., 1993). This can easily be explained by the fact that the drugs permeate across the liposome bilayer in the neutral form and are protonated on reaching the interior, thus consuming a proton and raising the interior pH. This, consequently, will limit the equilibrium uptake of drug. In the case of a drug which contains two basic functions, such as vincristine, it can be shown that, in the absence of membrane partitioning effects, and assuming that $[H^+]_o, [H^+]_i \gg K_{d1}, K_{d2}$ that

$$\frac{[\text{Drug}]}{[\text{Drug}]} \leq \frac{[H^+]_i^2}{[H^+]_o^2}$$

and thus the amount of drug entrapped will decrease as the square of the internal proton concentration as the internal pH rises.
Figure 3.2
Vincristine Release as a Function of Acyl Chain Length.

Vincristine release over time at 37°C from liposomes incubated in HBS (A) and mouse serum (B) for DMPC/Chol (○), DPPC/Chol (▲), DSPC/Chol (■), DAPC/Chol (▼), and DBPC/Chol (♦). Initial drug-to-lipid ratio was 0.1:1 (wt:wt). Error bars representing standard deviations of three trials are too small to be visible.
The drug retention properties of DBPC-cholesterol LUVs with varying internal citrate concentrations was investigated (Fig. 3.3). Samples were incubated in both buffer and serum at 37°C. As expected, higher initial internal citrate levels resulted in improved retention, however, these improvements were not significant for internal citrate concentrations in excess of 400 mM. At internal citrate concentrations of both 400 and 500 mM, >50% drug retention was achieved at 24 h when incubated in mouse serum.

3.3.3 Influence of Internal pH on Drug Retention

From the model outlined in Section 3.2.5, the rate constant for transbilayer movement of vincristine should be proportional to the inverse square of the proton concentration. We would therefore predict that the efflux of entrapped vincristine would be significantly slower if the internal pH is lowered. The dependence of the rate constant k on the pH was initially examined. As shown in Section 3.2.5, when \([H^+]_0 \gg K_{d1}, K_{d2}\) it is expected that \(k \propto [H^+]_0^{-2}\) and, thus, \(\log k \propto 2pH_0\). Thus, a plot of \(\log k\) vs. \(pH_0\) should result in a straight line with a slope of 2. Uptake of vincristine into DSPC-cholesterol vesicles over the external pH range 4-5 was observed. The uptake rates were seen to vary significantly (Fig. 3.4A). The rate constants \(k\) can be calculated from the slopes of the semilogarithmic plots shown in Fig. 3.4B, leading to a plot of \(\log k\) vs. \(pH_0\) (Fig. 3.4C) which exhibits a slope of 1.6. Since the first pK of vincristine is so low (pK1=5.0), the condition \([H^+]_0 \gg K_1\) is not well observed. Therefore, there is deviation from the predicted slope of 2.

These results predict that vincristine retention within liposomes is extremely sensitive to the internal pH. Lower internal pH values should dramatically improve drug retention. This prediction was therefore tested by preparing DSPC-cholesterol (55:45) LUVs with
Figure 3.3
Vincristine Release as a Function of Internal Buffer Salt Concentration.

Effect of varying the internal buffer salt concentration on vincristine release from DBPC/Chol vesicles incubated at 37°C in HBS (A) and mouse serum (B). Internal citrate concentrations were 500 mM (●), 400 mM (■), 300 mM (▲), and 200 mM (▼). Internal pH was 4.0 for all preparations. Initial drug-to-lipid ratios were 0.1:1 (wt:wt). Error bars representing standard deviations of three trials are too small to be visible.
Figure 3.4
The Effects of pH on Vincristine Uptake.

(A) Time-course for vincristine uptake into 100 nm DSPC/Chol vesicles for different external pH values. The internal pH for all systems was 3.0. The external pH values were 4.00 (〇), 4.25 (△), 4.50 (□), 4.75 (▽), and 5.00 (●). All samples were loaded at 60°C with a potential drug-to-lipid ratio of 0.1:1.

(B) Plot of \( \ln\left\{ \frac{[A(\text{eq})]}{[A(t)]} \right\} \) vs. time, where \([A(t)]\) is the internal concentration of the accumulated drug at time \(t\) and \([A(\text{eq})]\) is the internal concentration at equilibrium. The slopes of these lines give the rate constant \((k)\) for the transport of vincristine across the liposome membrane.

(C) Plot of \( \log k \) vs. external pH. The slope of this line is 1.60.
initial interior pH values of 2.0, 3.0, 4.0, and 5.0, using 300 mM citrate buffer. As evidenced in Fig. 3.5, the internal pH has a profound effect on vincristine retention. With (initial) pH values of 3 or less, there is essentially complete retention of contents for 24-h incubations in the presence of both buffer and serum. Lowering the internal pH appears to exhibit its greatest effect on improving drug retention initially. The slopes of the curves in Fig. 3.5 are not as markedly different after the 1 h time point.

3.3.4 Influence of Temperature on Vincristine Uptake

A final variable which would be expected to influence vincristine uptake (and, by extension, release) is temperature. It has been shown elsewhere that weak bases such as doxorubicin (unpublished data), as well as amino acid and peptide derivatives (Chakrabarti et al., 1992) can exhibit high activation energies for uptake rates in the range of 30 kcal/mol. An activation energy of 30 kcal/mol corresponds to an uptake rate which increases by approximately a factor of 5 for every 10°C increase in temperature. Vincristine uptake into DSPC-cholesterol LUVs was therefore monitored over the temperature range 30-60°C which resulted in remarkable differences in uptake rates as shown in Fig. 3.6A. An Arrhenius plot (Fig. 3.6C) of the rate constants derived from these data resulted in an activation energy of 37 kcal/mol.

3.3.5 Vincristine Retention in DSPC/Cholesterol LUVs in vivo

A basic aim of these studies was to identify parameters which would lead to a formulation of liposomal vincristine which is able to better retain the drug in vivo to allow for extended circulation lifetime and payout characteristics. It is clear from the studies
Figure 3.5
Vincristine Release as a Function of Internal pH.

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 buffer salt concentration was 300 mM citrate for all systems. Initial drug-to-lipid ratios were 0.1:1. Error bars representing standard deviations of three trials are too small to be visible.
Figure 3.6
Vincristine Uptake as a Function of Temperature.

(A) Time-course of vincristine uptake into 100 nm DSPC/Chol vesicles exhibiting a ΔpH (pH$_i$ = 3.0; pH$_o$ = 5.0). Uptake was conducted at 30 (○), 40 (●), 45 (△), and 60°C (▲). (B) Plot of ln{([A(eq)]$_i$ - [A(t)]$_i$) / [A(eq)]$_i$} vs. t, where [A(t)]$_i$ and [A(eq)]$_i$ are the same as for Fig. 3.4. (C) Arrhenius plot of the rate constants (k) for vincristine uptake. The activation energy calculated from the slope of this plot is 37 kcal/mol.
presented above that the internal pH is the most important variable for retention, and that DSPC/cholesterol LUVs prepared with an (initial) pH$_i$ of 3 or less exhibit retention of 90% or more over 24 h in the presence of buffer or serum. However, it is also known that liposome leakage *in vivo* is usually more extensive than *in vitro* (Mayer et al., 1989). The release properties *in vivo*, of DSPC/cholesterol LUVs with (initial) interior pH values of 2 and 4 and loaded with vincristine were assessed by monitoring the drug-to-lipid ratio in plasma (Fig. 3.7). It may be observed that whereas the 90% retention over 24 h obtained *in vitro* was not achieved for the pH$_i$ = 2 formulation, a value of 40% was achievable. This is approximately a factor of 5 higher than obtained with the pH$_i$ = 4.0 formulation. It is once again apparent, as *in vitro*, that the most marked difference in vincristine release rates is seen before 1 h post-injection. It is of interest that DBPC/cholesterol (55:45) preparations with 0.5 M internal citrate at pH$_i$ = 2.0 did not result in improved retention *in vivo* over the DSPC/cholesterol systems (results not shown). It should be noted that >99% of injected free vincristine (no liposomal carrier) is cleared from the circulation within 5 min post injection (Mayer et al., unpublished data).

### 3.4 Discussion

This chapter presents a detailed study of factors leading to improved retention of vincristine in liposomal systems. These results may also be expected to extend to other members of the large class of drugs which are lipophilic weak bases. Here we discuss the influence of the experimental parameters investigated on vincristine uptake and release and the implications for design of liposomal formulations of lipophilic amino containing drugs.

Increases in acyl chain length are shown to exhibit the type of retention
Figure 3.7
In Vivo Drug Retention.

Drug-to-lipid ratios for DSPC/Chol vesicles in vivo with internal pH of 2.0 (●) and 4.0 (○). Both systems were loaded with an initial drug-to-lipid ratio of 0.1:1. Each point represents the average value obtained from four BDF1 mice.
improvements expected. Thus, the half-times for vincristine release at 37°C in buffer increase from approximately 1 h for DMPC/Chol LUVs to approximately 12 h for DAPC- and DBPC-containing systems. The uptake of vincristine into these systems exhibits anomalous behavior in that the rates of uptake first decrease, as expected, as acyl chain length is increased to 18 carbons (DSPC), and then increase markedly for the DAPC and DBPC systems. It is possible that this reflects an increased lipid-water partition coefficient for vincristine for the outer monolayer of the DAPC and DBPC systems. An increased hydrophobicity of this interface would be consistent with packing effects expected for longer chain lipids in small vesicular systems, and this is reflected by the increased tendency of the longer chain DAPC and DBPC LUV systems to aggregate after extrusion. Conversely, for the inner monolayer, it would be expected that these effects would result in tighter packing in the headgroup region, and correspondingly reduced leakage, as shown experimentally.

With regard to interior buffer capacity, the results presented here show that increasing the interior citrate concentration above 400 mM does not result in significant improvements in vincristine retention. This is consistent with the osmotic properties of extruded LUV systems. As shown for 100 nm egg PC/cholesterol systems (Harrigan et al., 1993), extruded LUVs exhibit tubular 'sausage' shapes and respond to osmotic gradients (high interior osmolality) first by 'rounding up' to increase the interior volume and subsequently undergo osmotically induced lysis. For EPC/Chol LUVs the 'effective' osmotic difference that can be sustained is approximately 650 mosmol/kg. The osmolality of 400 mM citrate, pH 4.0, is 700 mosmol/kg, resulting in an effective osmotic imbalance of 400 mosmol/kg when the liposomes are in normal saline solution (osmolality 300 mosmol/kg). This initial effective osmotic imbalance will increase on drug loading. Vincristine, after crossing the liposomal
bilayer in its neutral form, becomes protonated in the vesicle interior due to the low internal pH, consequently raising the internal osmolality.

Lower internal pH values are clearly critical for improving vincristine retention in liposomal systems. This is due to the dependence of the rate constant for vincristine movement on the inverse square of the proton concentration (Eqn. 3). This predicts a 100-fold reduction in leakage rates for every unit the interior pH is lowered.

In conclusion, the internal pH is the most important parameter in enhancing liposomal vincristine retention both in vitro and in vivo. These effects can likely be extended to enhance retention of other basic drugs in liposomes in vitro and in vivo.
CHAPTER 4
THE USE OF CATIONIC LIPIDS TO IMPROVE LIPOSOMAL VINCristINE RETENTION

4.1 Introduction

Several mechanisms for improved vincristine retention within liposomes were examined in Chapter 3. Of these, lowering the internal pH to 2.0 had substantial effects on improving drug retention both in vitro and in vivo. This improved retention may be partially due to the fact that at pH 2.0, the phospholipid phosphate groups are nearing their pKₐ value. The phosphatidylcholine molecules will thus exhibit some positive charge, creating a charge repulsion with the positively charged vincristine molecules on the vesicle interior.

In order to further develop the hypothesis that lowering the internal pH increases drug retention by a charge repulsion effect, it was decided to incorporate cationic lipids into the liposomal membrane to determine their effect on vincristine retention. A potential problem with this approach is that liposomes containing positively charged lipids are recognized more readily by the RES and are cleared from the circulation more rapidly than neutral liposomes (Senior et al., 1991). It was necessary, therefore, to utilize positively charged lipids that could be induced to reside only in the inner monolayer of the liposomes, thereby preventing their recognition by the RES. It has previously been shown that weakly basic simple lipids such as stearylamine and sphingosine can rapidly and completely migrate to the inner monolayer in the presence of a transbilayer pH-gradient (inside acidic) (Hope and Cullis, 1987; Hope et al., 1989; Eastman et al., 1989; Eastman et al., 1991). Since these are the same conditions necessary for loading the weakly basic drug into the vesicles (Mayer et al., 1990c), one would expect to see the migration of these lipids to the inner monolayer.
as the drug is loaded into the vesicles.

In this Chapter, it is demonstrated that the incorporation of positively charged lipids can indeed prolong vincristine retention within liposomes and that this incorporation of weakly basic lipids has no effect on liposome circulation time in vivo.

4.2 Materials and Methods

4.2.1 Lipids and Chemicals

DSPC was obtained from Avanti Polar Lipids. Cholesterol, stearylamine, and sphingosine were purchased from Sigma Chemical Company (St. Louis, MO). rac 1,2-dioleoyl-3-N,N-dimethylaminopropane (AL-1) was synthesized in our laboratory using standard procedures (Bailey and Cullis, 1994). Vincristine sulfate was obtained from Adria Laboratories of Canada. [14C]-cholesteryl hexadecylether was specially synthesized for us by Amersham (Oakville, Ontario). It was chosen as a lipid marker since it is not exchanged or metabolized in vivo (Derksen et al., 1987). [3H]-vincristine was also purchased from Amersham. Female BDF1 mice (18-22 g) were obtained from Charles River Laboratories.

4.2.2 Liposomes

DSPC/Chol (55:45; mol:mol), DSPC/Chol/SA (45:45:10; mol:mol:mol), DSPC/Chol/AL-1 (45:45:10; mol:mol:mol), or DSPC/Chol/sphingosine (45:45:10; mol:mol:mol) solutions were prepared by the same procedure used in Section 2.2.2.

4.2.3 Drug Entrapment Procedure

Vincristine was entrapped in the liposomes using the ΔpH loading procedure.
described elsewhere (Mayer et al., 1990c). Liposome preparations were run over G-25
Sephadex columns equilibrated with HBS (pH 7.4) to achieve a pH-gradient across the
vesicle membranes. The vesicles were then added to vincristine (vincristine sulfate solution,
1 mg vincristine/mL) to achieve a drug-to-lipid ratio of 0.1:1. The resulting exterior pH of
the liposome/vincristine mixture was raised to pH 7.4 with 0.5 M Na2HPO4 and immediately
heated to 60°C for 10 min. This procedure ensured >95% trapping efficiencies in all cases.

4.2.4 Pharmacokinetic Studies

Pharmacokinetic studies were performed by injecting liposomal vincristine into
BDF1 mice via a lateral tail vein (2 mg/kg vincristine, 20 mg/kg lipid). It has previously
been shown that this dose of liposomal vincristine exhibits measurable levels of antitumor
activity in the P388 ascites tumor model (Mayer et al., 1990c). At varying time points, mice
were anaesthetized with i.p. ketamine (160 mg/kg) and xylazine (20 mg/kg). Blood was
removed via cardiac puncture and collected in EDTA-coated microtainer tubes (Becton
Dickenson). The samples were then centrifuged (500 x g for 10 min.) to pellet the blood
cells and obtain plasma samples. Liposomal lipid and/or vincristine were then assayed using
dual label scintillation counting.

4.2.5 Biodistribution Studies

Biodistribution studies were performed on the same mice used for pharmacokinetic
studies. Following heart puncture, mice were terminated by cervical dislocation, and the
liver and spleen were removed from each animal and weighed. 10% (w/v) homogenates in
saline were achieved using a Polytron homogenizer (Brinkmann Instruments, Rexdale,
500 µL of each homogenate was digested with 500 µL of "Solvable" (DuPont Canada, Inc., Mississauga, Ontario) for 1 h at 50°C. After cooling to room temperature, the samples were decolorized with 200 µL of 30% hydrogen peroxide and maintained at 4°C overnight to prevent excessive foaming. Samples were then counted using Ultima Gold (Packard) scintillation cocktail. The statistical significance of both the plasma clearance and biodistribution results were determined using the student's t-test.

4.2.6 Antitumor Studies

The antitumor effects of liposomal vincristine were monitored using the P388 lymphocytic leukemia model. BDF1 mice (5 per group) were injected i.p. with $1 \times 10^5$ P388 cells. The indicated doses of saline or liposomal vincristine were administered (i.v.) 24 h after tumor inoculation. Animal weights and mortality were monitored daily. Median survival times as well as the statistical significance of the results were determined using the Mann-Whitney-Wilcoxon procedure.

4.3 Results

4.3.1 Plasma Clearance and Biodistribution Studies

Previous work has shown that pH-gradients across liposomal membranes (inside acidic) induce the complete migration of cationic lipids such as sphingosine and stearylamine to the inner monolayer (Hope and Cullis, 1987). It has also been shown that the presence of cationic lipids on the liposome surface decreases blood circulation times (Senior et al., 1991). For these reasons, the plasma clearance of empty DSPC/Chol/sphingosine (45:45:10; mol:mol:mol) vesicles in the presence or absence of a pH-gradient was investigated (Figure
4.1). Vesicles with an interior and exterior pH of 7.5 had more rapid clearance times than vesicles with an interior pH of 4.0 and exterior pH of 7.5 ($\Delta$pH = 3.5). This was particularly evident at the 1 hr time point indicating that the presence of cationic lipid on the liposome surface increases initial clearance rates. The presence of the pH-gradient drives sphingosine migration to the inner monolayer, creating an asymmetric liposomal membrane which exhibits essentially identical liposome clearance times to vesicles composed solely of DSPC/Chol (55:45; mol:mol).

The previous chapter demonstrated that lowering the internal pH to pH, 2.0 of DSPC/Chol (55:45; mol:mol) liposomes significantly improves vincristine retention both in vitro and in vivo. This is, in part, due to the fact that a larger proportion of vincristine molecules remain protonated (and hence, positively charged) on the interior of the liposomes. It was thought that if a small percentage of cationic lipids were incorporated into the liposomal membrane, this would repel the positively charged vincristine molecules, preventing their partitioning into the inner monolayer and, therefore, leakage from the vesicles.

The data presented here strongly indicate that the incorporation of cationic lipids increase vincristine retention within DSPC/Chol (55:45; mol:mol) liposomes in vivo. Liposomes containing 10% stearylamine result in a substantial increase in vincristine circulation times with an internal pH of 2.0 (Fig 4.2A) without changing liposome clearance times (Fig 4.2B). This is due to a significant increase in liposomal retention of drug as evidenced by an increase in drug-to-lipid ratios over a 24 h period (Fig 4.2C). At 24 h, there is approximately a 6-fold higher drug-to-lipid ratio for the stearylamine-containing liposomes at pH 2.0 than for the DSPC/Chol (55:45; mol:mol) liposomes at pH 4.0. There
Figure 4.1
Plasma Clearance of Sphingosine-Containing Vesicles

Plasma clearance of DSPC/Chol/sphingosine empty liposomes with internal pH of 7.5 (●) and internal pH of 4.0 (■), and DSPC/Chol pHi 7.5 (▲). Error bars represent standard deviations of four mice.
Figure 4.2
Influence of Stearylamine and Internal pH on Lipid and Drug Clearance In Vivo.

Vincristine clearance (A), liposome clearance (B), and drug-to-lipid ratios (C) were determined following i.v. administration in BDF1 mice of DSPC/Chol pH\(_i\) 4.0 (○), DSPC/Chol pH\(_i\) 2.0 (●), DSPC/Chol/SA pH\(_i\) 4.0 (△), and DSPC/Chol/SA pH\(_i\) 2.0 (▲). Vincristine was encapsulated at a drug-to-lipid ratio of 0.1:1 (wt:wt). Error bars represent standard deviations of four mice.
is also a 2-fold increased drug retention over the DSPC/Chol pH 2.0 vesicles at 24 h.

Essentially identical results are seen with the incorporation of 10 mol% AL-1 or sphingosine (Fig 4.3). The presence of cationic lipid substantially improves drug retention at pH 2.0 but not at pH 4.0. Liposome clearance times are not affected by the addition of these lipids (results not shown).

Since sphingosine is a naturally occurring lipid found in biological membranes (Sabbadini et al., 1993; Tao et al., 1973; Wang and Schick, 1981), it was decided to perform further in vivo experiments solely with this lipid. Biodistribution studies were performed to witness any differences in liposomal drug accumulation in the liver and spleen, two major organs of the RES.

Liposome accumulation in the liver was found to be independent of internal pH and independent of liposome composition (results not shown). However, due to the increased drug retention seen with the sphingosine-containing pH 2.0 preparation, there are substantially higher drug levels in the liver at the 24 h time point (Fig 4.4A). Interestingly, despite seeing the highest drug accumulation in the spleen at 24 h for the sphingosine-containing pH 2.0 preparation (Fig. 4.4B), we see similar liposome uptake for all vesicle compositions. Any differences at the 1 and 4 h time points are insignificant.

4.3.2 Antitumor Activity of Liposomal Vincristine

It was decided to compare the antitumor activity of the DSPC/Chol/sphingosine pH 2.0 formulation to that of the DSPC/Chol pH 4.0 preparation. Consistent with previous results (Boman et al., 1994; Mayer et al., 1990c), both liposomal formulations of vincristine were significantly more efficacious in the P388 tumor model when compared to free drug
Figure 4.3
Influence of AL-1 or Sphingosine on Vincristine Retention In Vivo.

Drug-to-lipid ratios were determined with the incorporation of 10 mol% AL-1 (A) or 10 mol% sphingosine (B). Vincristine retention was determined following i.v. administration in BDF1 mice for both systems at pH 4.0 (○) and at pH 2.0 (●). Vincristine was encapsulated at a drug-to-lipid ratio of 0.1:1 (wt:wt).
Drug accumulation in the liver (A) and spleen (B) were determined 24 hours after i.v. injection in BDF1 mice. Error bars represent standard deviations of four mice.
Most interestingly, the combined incorporation of sphingosine into liposomes and the lowering of the internal pH to 2.0 significantly increased the antitumor activity of vincristine against P388 leukemia when compared to DSPC/Chol liposomal vincristine (pH 4.0) at drug dosages of 2, 3, and 4 mg/kg (p<0.01 at all drug dosages). There was also a decrease in drug toxicity, as judged by drug-induced weight loss, for the DSPC/Chol/sphingosine (pH 2.0) vesicles compared to DSPC/Chol vesicles (pH 4.0). The DSPC/Chol/sphingosine (pH 2.0) formulations at drug dosages of 3 and 4 mg/kg produced long-term survivors with median survival times of >60 d. When these surviving mice were again challenged with i.p. inoculation of 1 x 10^5 P388 cells on day 62, they exhibited median survival times similar to the control group (data not shown).

4.4 Discussion

The results presented in this Chapter demonstrate that in the presence of a pH-gradient (inside acidic), cationic lipids such as sphingosine are completely transferred to the inner monolayer of DSPC/Chol vesicles, creating a liposomal clearance pattern essentially similar to vesicles containing no cationic lipid. It has also been demonstrated that vincristine retention within liposomes is greatly increased by the simultaneous incorporation of sphingosine in DSPC/Chol vesicles as well as lowering the internal pH to 2.0. Further, this increased drug retention results in a substantial increase in vincristine circulation time. Finally, the large increase in drug circulation time seen with the sphingosine-containing pH 2.0 preparation results in a significant increase in antitumor activity over liposomal vincristine without sphingosine (p<0.01 at all drug dosages). Each of these observations shall be discussed below.
TABLE 4.1

P388 Antitumor Activity of Free and Liposomal Vincristine in BDF1 Mice

<table>
<thead>
<tr>
<th>Sample</th>
<th>Drug Dose (mg/kg)</th>
<th>Lipid Dose (mg/kg)</th>
<th>% wt change on day 7</th>
<th>60-day Survival</th>
<th>Median Survival (days)</th>
<th>%ILS*</th>
<th>L/Fb</th>
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<td>Saline control</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Free vinc</td>
<td>2.0</td>
<td></td>
<td>+ 6.0</td>
<td>0/10</td>
<td>14.0</td>
<td>40</td>
<td></td>
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<tr>
<td></td>
<td>3.0</td>
<td></td>
<td>- 3.6</td>
<td>0/10</td>
<td>12.0</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td></td>
<td>- 29.8</td>
<td>0/5</td>
<td>8.5</td>
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<td></td>
</tr>
<tr>
<td>DSPC/Chol pH 4.0</td>
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<td>- 2.1</td>
<td>0/10</td>
<td>27.0</td>
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<tr>
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<td>- 12.0</td>
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<td>31.0</td>
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<tr>
<td></td>
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<td>0/10</td>
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<td>DSPC/Chol/ sphingos pH 2.0</td>
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<td>1/5</td>
<td>36.0</td>
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<td>- 18.5</td>
<td>3/5</td>
<td>&gt;60.0</td>
<td>ND</td>
<td>ND</td>
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*Percentage of ILS values were determined from median survival time comparing treated and saline control groups. If greater than 50% of the animals survived for greater than 70 days, median survival times and %ILS were not calculated.

*L/F (liposomal/free) values were calculated by dividing the median survival time for the liposomal vincristine group by the median survival time for the equivalent dosage of free drug.

*N.D. - not determined.
As shown in Chapter 3, when the internal pH of liposomes is lowered to pH 2.0, there is a dramatic increase in vincristine retention both *in vitro* and *in vivo*. At this pH, the headgroups of the phosphatidylcholine molecule is beginning to become positively charged, perhaps creating a charge separation between the inner monolayer of the liposome and the positive charge on the vincristine molecule at low pH. In order to examine this hypothesis, the incorporation of cationic lipids into liposomal membranes was investigated. A potential problem with this approach is that cationic lipids have been shown to increase liposome clearance from the circulation (Senior et al., 1991). Therefore, it was necessary to utilize cationic lipids which could be induced to migrate to the liposome inner monolayer, producing an asymmetric vesicle membrane.

Previous work has demonstrated that positively charged lipids such as sphingosine and stearylamine can be induced to migrate to the inner monolayer in the presence of a pH-gradient (inside acidic) (Hope and Cullis, 1987; Hope et al., 1989; Eastman et al., 1989; Eastman et al., 1991). We examined this effect on the *in vivo* clearance times of sphingosine-containing vesicles in the presence and absence of a pH-gradient across the liposome bilayer. It is clearly evident that in the presence of a pH-gradient, the vesicles display an essentially identical clearance pattern to DSPC/Chol vesicles containing no cationic lipid, indicating that the sphingosine is completely migrating to the inner monolayer of the vesicle.

When the internal pH of cationic lipid-containing vesicles is decreased from pH 2.0, there is a substantial increase in drug retention *in vivo*. This is likely due to the fact that the strong positive charge on the sphingosine molecules (and stearylamine or AL-1) at the inner
monolayer at this pH ($pK_a = 5.30$ for sphingosine) repels the positive charge on the vincristine molecules on the liposome interior. This charge separation prevents the drug from partitioning into the inner monolayer of the liposome, the first step necessary for diffusion across the lipid bilayer to the exterior of the vesicle.

It is of interest to note that with an internal pH of 4.0 there is no increase in drug retention with the incorporation of stearylamine, AL-1, or sphingosine. With the simultaneous movement of cationic lipids to the inner monolayer and the loading of vincristine across a pH-gradient, there is a greater drop in the internal proton concentration than for the loading of drug alone. This increase in the internal pH allows for not only a higher percentage of the vincristine molecules to be in their neutral form, but also for a higher percentage of the sphingosine (or other cationic lipid) molecules to be in the neutral form. This results in a lower charge density for the liposome inner monolayer, being less able to repel the positive charges on the vincristine molecules. This allows the vincristine to more easily partition into the inner monolayer and diffuse across the membrane into the external compartment.

All liposome compositions studied displayed essentially identical lipid clearance times in vivo. Since the liver and spleen are major organs of the RES, one would expect all liposomal systems studied to have similar patterns of uptake in these organs. This is indeed the case. Neither the lowering of the internal pH to 2.0 nor the incorporation of sphingosine into DSPC/Chol liposomes appears to increase liposome recognition by the cells of the RES.

The pattern of drug uptake by the liver and spleen is quite different. The vincristine uptake by these organs is determined by two parameters: the liposome uptake by these organs and the drug retention within the liposomes. Since it's shown that liposome uptake
is essentially identical for all four preparations studied, any differences should be attributed to vincristine retention within the liposomes. Therefore, we would expect to see the highest drug accumulation in both organs for the sphingosine-containing pH 2.0 preparation. This is indeed the case as we see a 2 to 3-fold increase in drug accumulation in both the liver and spleen at 24 h for the sphingosine-containing pH 2.0 preparation.

By analogy with previous studies with liposomal anticancer agents (Mayer et al., 1990b; 1990c; Bally et al., 1990; Mayer et al., 1993; Vaage et al., 1993), increased drug concentration in the plasma would be expected to result in an increase in antitumor activity. Such an effect has been seen previously for P388 and L1210 leukemia when the vincristine circulation time was increased by encapsulation within DSPC/Chol vesicles (Mayer et al., 1990c). The encapsulated drug displayed increased antitumor activity over free drug as well as drug encapsulated in leaky liposomes (eggPC/Chol). The increased plasma drug concentrations observed here by incorporating sphingosine into vesicle membranes and decreasing the internal pH results in a substantial increase in antitumor activity in the P388 leukemia model. This is particularly evident at drug dosages above 3 mg/kg. At these dosages, we see long term survivors with median survival times in excess of 60 days. Thus, the benefits from extended circulation times are clearly evident.

The DSPC/Chol/sphingosine pH 2.0 system also displays a considerable decrease in toxicity as evidenced by decreased weight loss. This is possibly due to the fact that the drug is more tightly held within the liposome, preventing toxic side effects.

In summary, this Chapter has demonstrated that, in the presence of a pH-gradient (inside acidic), sphingosine, stearylamine, or AL-1 can be induced to migrate to the inner monolayer of liposomes, where, at low enough pH, the increase in the charge density of the
vesicle inner monolayer repels the positively charged vincristine molecules, reducing drug leakage. This results in substantially increased drug circulation times. The system containing sphingosine at pH 2.0 displays dramatic improvement in therapeutic activity against the P388 leukemia cell in *in vivo* resulting in long term survival.
CHAPTER 5
THE USE OF MONOSIALOGLANGLIOSIDE GM1 TO INCREASE LIPOSOme
CIRCULATION LONGEVIbTY AND IMPROVE LIPOSOmAL VINCRISTINE
RETENTION

5.1 Introduction

As previously mentioned, liposomal formulations of vincristine can exhibit reduced
toxicity and enhanced efficacy compared to free drug (Mayer et al., 1990c). This has been
related to the enhanced residence times of the drug in the circulation which is achieved when
using liposomal carriers. The incorporation of monosialogangliosides (GM1) and
polyethyleneglycol (PEG) derivatives can further enhance liposome circulation time
(Gabizon et al., 1988; Klibanov et al., 1991; Allen et al., 1989; Senior et al., 1991a; Liu et
al., 1990; Klibanov et al., 1990; Allen et al., 1987; Allen et al., 1991b). Incorporation of
these lipids reduces protein/liposome association (Chonn et al., 1992), and results in a
decreased uptake by the reticuloendothelial system (RES) (Allen et al., 1987; Allen et al.,
1991b). They also have been shown to decrease macrophage uptake when coated on
colloidal particles (Illum et al., 1986). An added advantage is that there tends to be increased
accumulation within tumors for liposomes which exhibit longer circulation residence times
(Gabizon et al., 1988; Gabizon et al., 1983; Fichtner et al., 1981; Richardson et al., 1978;
Proffitt et al., 1983; Gabizon et al., 1990). However, until recently, such extended
circulation lifetimes have been of questionable value for liposomal vincristine preparations
since approximately 85% of the entrapped vincristine is released from DSPC/Chol pH 4.0
liposomes in the blood within 24 h of i.v. administration.

In Chapter 3, we identified conditions which may circumvent the drug leakage
problem cited above (Boman et al., 1993). Specifically, decreasing the pH of the entrapped
citrate buffer to 2.0 dramatically reduces vincristine leakage in the presence of serum in vitro. In view of this development, we have investigated the pharmacokinetic, biodistribution, and efficacy properties of vincristine entrapped in DSPC/Chol liposomes containing pH 4.0 and pH 2.0 citrate buffers. Further, we have examined the effects of including the ganglioside G_{M1} in the liposomes on the in vivo properties of these systems. These studies reveal that combining the effects of decreased entrapped pH and including G_{M1} synergistically enhance the pharmacological and therapeutic activity of liposomal vincristine.

5.2 Materials and Methods

5.2.1 Lipids and Chemicals

"Oncovin" (vincristine sulfate) was obtained from the B.C. Cancer Agency (Vancouver, British Columbia, Canada). DSPC was purchased from Avanti Polar Lipids, and was greater than 99% pure. Monosialoganglioside G_{M1}, cholesterol, and all salts were obtained from Sigma Chemical Company (St. Louis, MO). Cholesteryl hexadecylether (^{14}C) was specially synthesized for us by Amersham (Oakville, Ontario). Female BDF1 mice (6-8 weeks old) were purchased from Charles River Laboratories.

5.2.2 Liposomes

DSPC/Chol (55:45; mol:mol) or DSPC/Chol/G_{M1} (45:45:10; mol:mol:mol) were prepared by the same procedure outlines in Section 2.2.2.

5.2.3 Drug Entrapment Procedure

Vincristine was entrapped in the liposomes using the ΔpH loading procedure
described in Section 4.2.3.

5.2.4 Pharmacokinetic Studies

Plasma clearance studies were performed as outlined in Section 4.2.4.

5.2.5 Biodistribution Studies

Biodistribution studies were performed as outlined in Section 4.2.5 except that the lung and kidney were also removed from the animals.

5.2.6 Antitumor Studies

The antitumor effects of liposomal vincristine were monitored using the P388 lymphocytic leukemia model. BDF1 mice (5 per group) were injected i.p. with $1 \times 10^5$ P388 cells. The indicated doses of saline or liposomal vincristine were administered (i.v.) 24 h after tumor inoculation. Animal weights and mortality were monitored daily. Mean and median survival times as well as the statistical significance of the results were determined using the Mann-Whitney-Wilcoxon procedure.

5.2.7 Solid Tumor Loading and Efficacy Studies

Tumor loading of liposomal vincristine was determined in BDF1 mice using the Lewis Lung tumor model. $3 \times 10^5$ tumor cells were injected subcutaneously above the hind leg. Free vincristine, DSPC/Chol pH 4.0 liposomal vincristine, and DSPC/Chol/GM1 pH 2.0 liposomal vincristine were injected i.v. 7 to 9 days after inoculation of Lewis Lung tumor cells. At varying time points, tumors were excised, digested, and lipid and drug contents
were determined by liquid scintillation counting. Efficacy studies were performed by daily monitoring of tumor size following drug injection.

5.3 Results

5.3.1 Plasma Clearance and In Vivo Drug Release Studies

It was shown in Chapter 3 that the retention of vincristine entrapped inside DSPC/Chol liposomes in response to a transmembrane pH gradient (inside acidic) could be improved by decreasing the pH of the intravesicular citrate buffer below that previously employed for such systems (300 mM citrate, pH 4.0) (Boman et al., 1993). These observations were expanded on here to determine whether such in vitro results translated to increased circulation lifetimes for vincristine in vivo. Also, G_{M1}-containing liposomes were employed to determine whether increasing the circulation lifetime of the liposomal carrier system could provide additional improvements in the longevity of vincristine in the blood compartment after i.v. administration.

Figure 5.1A demonstrates that decreasing the interior pH of DSPC/Chol 100 nm liposomes from 4.0 to 2.0 has a modest effect on circulating vincristine levels over 24 h post i.v. injection (2 mg/kg drug, 20 mg/kg lipid) to BDF1 mice. This effect appears to be related primarily to the enhanced ability of the pH 2.0 liposomes to retain entrapped vincristine. This is evidenced by the fact that circulating liposomal lipid levels for these two systems are comparable over the time course studied (Figure 5.1B) whereas the circulating drug-to-lipid ratio is increased for the pH 2.0 system 1.2, 1.3, and 9.1-fold over the pH 4.0 system at 1 h, 4 h, and 24 h respectively (Figure 5.1C).

Inclusion of 10 mol% G_{M1} in the DSPC/Chol liposomes with an entrapped pH 4.0
Figure 5.1
Influence of $G_{M1}$ and Internal pH on Lipid and Drug Clearance In Vivo.

Vincristine clearance (A), liposome clearance (B), and drug-to-lipid ratios (C) were determined following i.v. administration in BDF1 mice of DSPC/Chol pH$_i$ 4.0 (●), DSPC/Chol pH$_i$ 2.0 (▼), DSPC/Chol/$G_{M1}$ pH$_i$ 4.0 (■), and DSPC/Chol/$G_{M1}$ pH$_i$ 2.0 (▲). Vincristine was encapsulated at a drug-to-lipid ratio of 0.1:1 (wt:wt). Error bars represent standard deviations of four mice.
citrate buffer results in circulating vincristine levels that are increased compared to the carrier system in the absence of GM\(_{\text{M1}}\) and equivalent to those observed employing DSPC/Chol liposomes with the pH\(_{\text{i}}\) 2.0 entrapped buffer (Figure 5.1A). In this case the increased plasma vincristine concentrations arise from the extended circulation lifetime of the DSPC/Chol/G\(_{\text{M1}}\) liposomes. Plasma liposomal lipid levels are increased approximately 2.5-fold 24 h after i.v. administration when G\(_{\text{M1}}\) is incorporated into DSPC/Chol liposomes prepared at pH\(_{\text{i}}\) 4.0 (Figures 5.1B). Corresponding circulating drug-to-lipid ratios for these two systems are very similar over the time course studied (Figure 5.1C).

Incorporation of G\(_{\text{M1}}\) into the vesicle membrane in combination with the use of the pH\(_{\text{i}}\) 2.0 entrapped citrate buffer resulted in unexpected pharmacokinetic effects for both the lipid and drug components. Plasma vincristine levels for this system were approximately 1.5-fold higher than the DSPC/Chol pH\(_{\text{i}}\) 2.0 and DSPC/Chol/G\(_{\text{M1}}\) pH\(_{\text{i}}\) 4.0 preparations at 1 and 4 h after i.v. injection and approximately 6-fold higher at 24 h (Figure 5.1A). As may be expected, the G\(_{\text{M1}}\) pH\(_{\text{i}}\) 2.0 liposomes display enhanced circulation longevity compared to systems devoid of G\(_{\text{M1}}\) (Figure 5.1B), however, significantly lower liposomal lipid concentrations than the G\(_{\text{M1}}\) pH\(_{\text{i}}\) 4.0 preparation are seen over 24 h (p<0.01 at all time points). Consequently, the increased plasma vincristine levels arise from the ability of the G\(_{\text{M1}}\) pH\(_{\text{i}}\) 2.0 liposomes to retain vincristine in the circulation. This is shown in Figure 5.1C where, in contrast to the pH\(_{\text{i}}\) 4.0 systems, pH\(_{\text{i}}\) 2.0 G\(_{\text{M1}}\)-containing liposomes display circulating drug-to-lipid ratios that are 1.3-, 1.4-, and 2.2-fold higher than observed for the DSPC/Chol pH\(_{\text{i}}\) 2.0 preparation at 1 h, 4 h, and 24 h after injection, respectively. This ratio is increased to 7.2-fold at 24 h when compared to the pH\(_{\text{i}}\) 4.0 liposomal systems.

The increase in circulation longevity of the liposomes is reflected by a decreased
uptake in the liver and spleen, two major organs of the RES. This is illustrated in Figures 5.2B and 5.3B where it is shown that $G_{M1}$-containing liposomes are accumulated in these organs much less than the DSPC/Chol liposomes. The data indicate significant differences (p<0.05) at 24 h in the liver and for all time points in the spleen (p<0.01). Lung and kidney uptake were identical for all liposomal compositions (data not shown). Lowering the internal pH of the DSPC/Chol/$G_{M1}$ vesicles to pH 2.0 increases both liver and splenic uptake (p<0.005) as would be expected by the decreased circulation time of these liposomes. Lowering the internal pH of the DSPC/Chol vesicles, however, does not appear to affect their biodistribution.

For liposomal drug preparations at pH 4.0, there was increased vincristine uptake by both the liver and spleen in the absence of $G_{M1}$ (p<0.005 for all time points) (Figures 5.2A and 5.3A). The opposite effect, however, was seen for the pH 2.0 preparations. For the DSPC/Chol/$G_{M1}$ systems, lowering the internal pH to 2.0 results in increased liver and splenic drug accumulation. This is likely due to the high drug-to-lipid ratios seen for this formulation. Again, no significant differences were seen in uptake by the lung or kidney (data not shown).

5.3.2 Antitumor Activity of Liposomal Vincristine

Tumor efficacy studies were conducted to determine whether the pharmacokinetic effects described above influenced the antitumor activity of liposomal vincristine. The antitumor activity of the DSPC/Chol/$G_{M1}$ pH 2.0 formulation was compared with that of the DSPC/Chol pH 4.0 preparation. Consistent with previous results (Mayer et al., 1990c), both liposomal formulations of vincristine were significantly more efficacious in the P388 tumor.
Figure 5.2
Liver Accumulation of Liposomal Vincristine.

Drug accumulation (A) and lipid accumulation (B) were determined following i.v. administration in BDF1 mice of DSPC/Chol pH 4.0 (O), DSPC/Chol pH 2.0 (□), DSPC/Chol/GMI pH 4.0 (●), and DSPC/Chol/GMI pH 2.0 (■). Vincristine was encapsulated at a drug-to-lipid ratio of 0.1:1 (wt:wt). Error bars represent standard deviations of four mice.
Figure 5.3
Spleen Accumulation of Liposomal Vincristine.

Drug accumulation (A) and lipid accumulation (B) were determined following i.v. administration in BDF1 mice of DSPC/Chol pH 4.0 (○), DSPC/Chol pH 2.0 (□), DSPC/Chol/GMI pH 4.0 (●), and DSPC/Chol/GMI pH 2.0 (■). Vincristine was encapsulated at a drug-to-lipid ratio of 0.1:1 (wt:wt). Error bars represent standard deviations of four mice.
model when compared to free drug (Table 5.1) (p<0.01 for all drug dosages). Most interestingly, the combined incorporation of GM1 into liposomes and the lowering of the internal pH to 2.0 significantly increased the antitumor activity of vincristine against P388 leukemia when compared to DSPC/Chol liposomal vincristine (pH 4.0) at drug dosages of 2, 3, and 4 mg/kg (p<0.01). There was no improvement in therapeutic activity, however, at the 1 mg/kg drug dose. There was also a decrease in drug toxicity, as judged by drug-induced weight loss, for the DSPC/Chol/GM1 (pH 2.0) vesicles compared to DSPC/Chol vesicles (pH 4.0). The DSPC/Chol/GM1 (pH 2.0) formulations at drug dosages of 2, 3, and 4 mg/kg all produced long-term survivors with median survival times of >70 d. When these surviving mice were again challenged with i.p. inoculation of 1 x 10^5 P388 cells on day 72, they exhibited median survival times similar to the control group (data not shown).

5.3.3 Solid Tumor Loading and Efficacy Studies

Vincristine loading in the Lewis Lung tumor as well as tumor efficacy studies were performed to determine any correlations which may be present. Lewis lung tumor loading of free vincristine, DSPC/Chol pH 4.0 liposomal vincristine, and DSPC/Chol/GM1 pH 2.0 liposomal vincristine was determined. Both preparations of liposomal drug displayed significantly higher levels of tumor drug uptake than when free drug was administered (Figure 5.4A). Free vincristine initially accumulated in the solid tumor before decreasing to a level near zero 24 h following i.v. administration. In contrast, when vincristine was encapsulated within liposomes composed of DSPC/Chol pH 4.0, much higher tumor levels of drug were achieved initially as well as a much slower release of drug from the site of tumor growth resulting in drug levels nearing zero at approximately 72 h following i.v.
TABLE 5.1

P388 Antitumor Activity of Free and Liposomal Vincristine in BDF1 Mice

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<thead>
<tr>
<th>Sample</th>
<th>Drug Dose (mg/kg)</th>
<th>Lipid Dose (mg/kg)</th>
<th>% wt change on day 7</th>
<th>60-day survival</th>
<th>Median survival (days)</th>
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*Percentage ILS values were determined from median survival times of treated and saline control groups. If more than 50% of the animals survived for more than 70 days, median survival times and % ILS are indicated as greater than 70 days.

*L/F* (liposomal/free) values were calculated by dividing the median survival time for the liposomal vincristine group by the median survival time for the group receiving the equivalent dosage of free drug.

*N.D. - not determined.
Figure 5.4
Lewis Lung Tumor Accumulation of Liposomal Vincristine

Drug accumulation (A) and lipid accumulation (B) were determined following i.v. administration in BDF1 mice of free vincristine (○), DSPC/Chol pH 4.0 liposomal vincristine (■), and DSPC/Chol/GMI pH 2.0 (▲). Vincristine was encapsulated at a drug-to-lipid ratio of 0.1:1 (wt:wt). Error bars represent standard deviations of eight tumors.
injection. Vesicles composed of DSPC/Chol/GM1 pH 2.0 resulted in even better drug retention within the site of tumor growth. For this liposomal drug preparation, despite similar levels of tumor drug accumulation to DSPC/Chol pHi 4.0 liposomal vincristine at 24 h, the release of drug from the tumor site is much slower, resulting in drug levels of approximately 2 μg/g tumor at 72 h following injection.

Initial tumor drug loading for the two liposomal preparations show quite different patterns of uptake. For the DSPC/Chol pHi 4.0 preparation, initial tumor drug uptake is very rapid reaching close to its maximum value within the first hour following i.v. administration. The DSPC/Chol/GM1 pH 2.0 formulation, however, displays a much slower drug accumulation within the site of tumor growth. At 1 h following i.v. injection, even lower levels of drug accumulation are seen than for the administration of free drug (Figure 5.4A). These vesicles result in peak tumor drug concentrations at approximately 24 h following i.v. injection. The reasons for these differences may be due to the ability of the different liposomes to gain access to the tumor. This is reflected in Figure 5.4B. At 1 h following administration, the concentration of DSPC/Chol pHi 4.0 liposomes is approximately 5-fold higher than the concentration of DSPC/Chol/GM1 pH 2.0 liposomes within the tumor. The concentration of DSPC/Chol pHi 4.0 liposomes increases to reach a maximum at 24 h before steadily declining. In contrast, the accumulation of of DSPC/Chol/GM1 pH 2.0 liposomes within the tumor progressively increases over the 72 h experiment.

The accumulation of liposomal vincristine within sites of tumor growth is determined not only by the ability of the liposomes to accumulate within the tumor, but also on the ability of the liposome to retain the drug within its aqueous interior. In order to better understand the factors affecting tumor uptake of drug, it is of interest to examine the drug-to-
lipid ratios of both liposomal vincristine preparations within the site of tumor growth (Figure 5.5). DSPC/Chol/G_{M1} pH 2.0 liposomes retain vincristine much better than DSPC/Chol pH_{4.0} liposomes within the Lewis Lung tumor. It appears that this difference is mainly dependent on the initial drug retention since vincristine release rates for both systems following 1 h post injection are essentially similar.

Lewis Lung tumor efficacy studies are shown to reflect the differences seen in tumor drug accumulation (Figure 5.6). Control mice demonstrate a continual increase in tumor weight until all mice are dead by day 9 following i.v. injection of saline. When 2 mg/kg of free vincristine is administered, a minimal decrease in tumor growth is observed but all mice are dead by day 9 following drug administration. The administration of DSPC/Chol pH_{4.0} liposomal vincristine results in a substantial decrease in tumor growth as compared to controls and an increase in survival time of approximately 2-fold. The best results, however, are seen with the DSPC/Chol/G_{M1} pH_{2.0} preparation where tumor growth is essentially halted for the first 10 days following i.v. administration of the drug. Tumor growth then begins to progress resulting in mouse death by day 21 following drug administration.

5.4 Discussion

A large amount of attention in liposomal drug delivery has been focussed recently on the use of "stealth" lipids such as G_{M1} and PEG-derivatized phospholipids to increase the circulation lifetime and therapeutic activity of certain liposomal drugs. This approach has been utilized successfully for liposomal formulations of doxorubicin and cytosine arabinoside (Gabizon and Papahadjopoulos, 1988; Allen et al., 1992). However, an implicit feature of the "stealth" strategy is that the entrapped drug must be efficiently retained inside
Drug-to-lipid ratios within the Lewis Lung tumor over time for DSPC/Chol pH\textsubscript{1} 4.0 liposomal vincristine (■) and DSPC/Chol/G\textsubscript{M1} pH\textsubscript{1} 2.0 liposomal vincristine (▲). Vincristine was encapsulated at an initial drug-to-lipid ratio of 0.1:1 (wt:wt).
Figure 5.6
Lewis Lung Tumor Growth Following Vincristine Administration

Tumor weight (as calculated from size) was measured daily following i.v. administration of saline (O), free vincristine (●), DSPC/Chol pH 4.0 liposomal vincristine (■), and DSPC/Chol/GM1 pH 2.0 liposomal vincristine (▲). Vincristine was administered at a dose of 2 mg/kg. Error bars represent standard errors of four mice.
the liposome in order to take advantage of the long circulation lifetime of such systems. Previously, there has been minimal interest in utilizing \( G_{\text{M1}} \) in liposomal vincristine preparations due to the relatively rapid release of the drug from liposomes in the circulation (Mayer et al., 1993). Recent developments in the use of pH-gradient liposome systems to enhance vincristine entrapment, however, has now made the use of stealth lipids for \textit{in vivo} vincristine delivery of interest.

Decreasing the pH of the entrapped citrate buffer for DSPC/Chol liposomes leads to increased vincristine retention in the circulation as evidenced by the 3.1-fold increase in the circulating drug-to-lipid ratio compared to DSPC/Chol pH 4.0 preparations 24 h after i.v. administration. These results, together with the fact that including \( G_{\text{M1}} \) into DSPC/Chol liposomes at pH 4.0 results in a 2.4-fold increase in circulating liposomal lipid levels at 24 h, suggest that plasma vincristine concentrations for \( G_{\text{M1}} \) pH 2.0 liposomes should be approximately 7.5-fold greater than observed for the DSPC/Chol pH 4.0 systems studied extensively in previous investigations (Mayer et al., 1990c; 1993). The data presented here indicate, however, that lowering the pH of the entrapped buffer to 2.0 and including \( G_{\text{M1}} \) in the membrane synergistically combine to dramatically increase the vincristine concentration in the plasma. Specifically, 24 h after i.v. administration, circulating drug levels are increased 18.3-fold when \( G_{\text{M1}} \)-containing pH 2.0 DSPC/Chol liposomes are utilized compared to DSPC/Chol pH 4.0 systems.

It is interesting to note that the enhanced pharmacokinetic properties of \( G_{\text{M1}} \) pH 2.0 liposomal vincristine systems are obtained even though liposomes prepared at pH 2.0 display somewhat decreased circulation lifetimes (Figure 5.1B) and corresponding increased RES uptake (Figure 5.2). The reasons for this difference are not yet understood, however it is
clear that being able to eliminate this effect could potentially result in further increases in circulating vincristine levels.

The studies described in this chapter demonstrate that the enhanced pharmacokinetic characteristics provided by the $G_{M1}$ pH 2.0 liposomal vincristine preparations translate to substantially improved antitumor activity. We have previously shown that increasing the circulation lifetime of vincristine entrapped in PC/Chol systems improved antitumor efficacy (Mayer et al., 1990c; 1993; Boman et al., 1994). The results obtained with the long-lived, non-leaky $G_{M1}$ pH 2.0 liposomes extend from this initial observation, thereby transforming a drug with minimal activity against the murine P388 tumor model into one where high cure rates are achieved.

The mechanisms whereby the use of $G_{M1}$ and pH 2.0 entrapped buffer synergistically stabilize liposomal vincristine preparations is not well understood. One possibility is that $G_{M1}$-containing vesicles are able to withstand an osmotic gradient approximately 10% greater than that withstood by DSPC/Chol liposomes when incubated in mouse serum (results not shown). On exposure to large osmotic gradients, liposomal membranes form transient pores allowing entrapped solute to escape (Mui et al., 1993). By the $G_{M1}$-containing vesicles being able to withstand a greater osmotic gradient, they are less likely to release entrapped drug.

It is of interest to note that with an internal pH of 4.0 there is no increase in drug retention with the incorporation of $G_{M1}$. However, at pH 2.0, there is a substantial increase in drug retention when $G_{M1}$ is added. This is likely due to the fact that lowering the internal pH and adding $G_{M1}$ act via different mechanisms to increase drug retention. It has been shown previously that vincristine is released from liposomes as the pH gradient across the liposome membrane decreases (Boman et al., 1993). By increasing the initial pH gradient
across the membrane, the drug can be retained by the liposome more efficiently. In contrast, the incorporation of $G_{M_{1}}$ decreases the amount of plasma protein binding to the liposome (Chonn et al., 1992). This enables the membrane to remain more stable thereby preventing drug leakage. At pH 4.0, drug leakage is so rapid that the stabilizing effects of $G_{M_{1}}$ are unable to further enhance the efficiency of drug retention in vivo.

By analogy with previous studies with liposomal anticancer agents (Mayer et al., 1990b; Mayer et al., 1989), increased drug concentration in the plasma would be expected to result in an increase in antitumor activity. Such an effect has been seen previously for P388 and L1210 leukemia when the vincristine circulation time was increased by encapsulation within DSPC/Chol vesicles (Mayer et al., 1990c). The encapsulated drug displayed increased antitumor activity over free drug as well as drug encapsulated in leaky liposomes (eggPC/Chol). The increased plasma drug concentrations observed here by incorporating $G_{M_{1}}$ into vesicle membranes and decreasing the internal pH results in a substantial increase in antitumor activity in the P388 leukemia model. This is particularly evident at drug dosages above 2 mg/kg. At these dosages, we see long term survivors with median survival times in excess of 70 days. Thus, the benefits from extended circulation times are clearly evident.

The extended drug circulation times seen with the incorporation of $G_{M_{1}}$ with an internal pH of 2.0 also prove to be beneficial in the treatment of a murine solid tumor model. Not only are increased tumor drug levels observed but also a substantial decrease in tumor growth is noted in vivo. This improvement in tumor drug accumulation appears to be due not only to the ability of the liposomes to retain the drug, but also to the ability of the liposomes to remain within the site of tumor growth for extended periods of time. This is
likely due to the extended plasma circulation times seen for this preparation.

It is entirely possible that similar liposomal systems can be utilized to increase drug retention of other remote loading drugs. By increasing their retention within liposomes, there would likely be an increase in therapeutic activity.

The DSPC/Chol/GM1 pH 2.0 system also displays a considerable decrease in toxicity as evidenced by decreased weight loss. This could be due to the fact that the drug is more tightly held within the liposome, thus preventing toxic side effects.

It is important to emphasize that the improved therapeutic activity observed here is obtained following a single i.v. dose of encapsulated vincristine. It could be argued that similar results would be achieved with a systemic infusion of free drug. Results from this laboratory (Mayer et al., 1990b) and others (Gabizon and Papahadjopoulos, 1988), however, suggest that increased therapeutic activity achieved with liposomal anticancer drugs is due primarily to accumulation of drug loaded liposomes in the region of tumor cell growth. Further, recent pharmacological studies (Mayer, L.D., unpublished observations) indicate that systemic exposure to free vincristine is lower for drug administered in liposomal form compared to unentrapped vincristine. Therefore, improved drug retention characteristics should result in improved specificity of drug delivery to the tumor and hence, increased therapeutic activity. Comparable drug delivery to a defined region of disease growth would not be expected using a continuous i.v. infusion of free vincristine.

In summary, this chapter demonstrates that lowering the internal pH and incorporation of GM1 into liposomal membranes results in a synergistic increase in drug retention in vivo and increased drug circulation times. This system shows substantial improvement in therapeutic activity against the P388 leukemia cell line in vivo resulting in
long term survival. It also displays a considerable decrease in solid tumor growth rate in mice inoculated with Lewis Lung tumor cells. Although the synergistic effects of $G_{M1}$-incorporation and lowering the internal pH to 2.0 are not well understood, many of these areas warrant further investigation.
CHAPTER 6
SUMMARY

Until recently (Mayer et al., 1990c), liposomal encapsulation of vincristine has been of marginal benefit due to the inability to retain the drug within the vesicles for extended periods of time. This thesis has first shown that liposomal encapsulation employing the technique of Mayer et al. (1990) ameliorates the toxic side effects usually observed on extravasation, and has then examined factors which further improve the ability of liposomes to retain the drug in vivo.

Firstly, it was shown in Chapter 2 that the use of liposomal encapsulation, employing the procedures of Mayer et al., 1990, dramatically decreases the soft-tissue necrosis and ulceration seen for the free drug when extravasated. This observation is important due to the devastating necrotic effects that can occur on accidental extravasation of vincristine (Bellone, 1981; Choy, 1979). Although the liposomally encapsulated drug remains in the tissue for extended periods of time after extravasation, the trapped drug is unable to exert cytotoxic effects. The tissue is exposed to a low, long-term dose of free drug as the vincristine slowly leaks from the liposomes.

Next, Chapter 3 investigated physical parameters such as lipid composition, internal buffering capacity, internal pH, and temperature on vincristine uptake and release from vesicles. Through these studies, it was determined that reduction of the internal pH had the most profound effect on improving vincristine retention within liposomes in vivo.

Chapters 4 and 5 examined the incorporation of factors into liposomes to improve vincristine circulation time. In Chapter 4 the hypothesis that cationic lipids would improve
drug retention was studied, the thought being that the presence of positively charged lipids on the inner monolayer of vesicles would repel the positive charge on the vincristine molecule at low pH, thus inhibiting release. Effects consistent with this interpretation were observed. Vincristine retention was dramatically improved by the incorporation of 10 mol% cationic lipid at low pH. The resultant increase in vincristine circulation time translated to a substantial improvement in therapeutic activity.

Chapter 5 examined a different approach for improving vincristine circulation time which involved the use of monosialoganglioside G\textsubscript{M1}. Surprisingly, it was found that the incorporation of G\textsubscript{M1} combined with the lowering of the internal pH to 2.0 showed a synergistic effect in improving vincristine retention within the liposomes. This effect combined with the effect of G\textsubscript{M1} on increasing liposome circulation time, greatly improved the circulation time of the drug, resulting in high cure rates when employed against P388 lymphocytic leukemia in mice.

There are several directions for future research. The first is to characterize how monosialoganglioside G\textsubscript{M1} contributes to improved drug retention. It would be of interest to examine the use of other lipids which improve liposome circulation time such as polyethyleneglycols and phosphatidylinositol (Gabizon and Papahadjopoulos, 1988). It has previously been demonstrated that G\textsubscript{M1} increases liposome circulation time by reducing the total amount of blood protein bound to the liposome surface (Chonn et al., 1992). It would be interesting to determine if any correlation exists between plasma protein binding and vincristine leakage from liposomes. A wide variety of liposome compositions could be studied with varying abilities to bind plasma proteins.

To this point, we have demonstrated that the slower the leakage of vincristine from
liposomes and the more drug associated with the vesicles, the lower the toxicity and the higher the therapeutic potential. Previous work by Layton and Trouet (1980) demonstrated no advantage with liposomal encapsulation of vincristine either in terms of reduced toxicity or increased therapeutic index. This was likely due to the fact that preparations were utilized with low drug trapping efficiencies and poor drug retention abilities. Since vincristine must gain access to tumor cells in order to exert its cytotoxic effects, there is likely an optimal level of drug retention within liposomes, above which therapeutic activity will again decrease. It would be of considerable interest to develop means of further improving vincristine retention within liposomes to determine the optimum level of drug retention for therapeutic activity.

The next area which warrants further investigation is the ability of various liposomes to access sites of disease, such as tumors. Although a liposome may be better able to retain a drug, if that drug is less able to access the target cells, it may be of little or no benefit. Since the majority of this thesis has focused on the antineoplastic effects against P388 lymphocytic leukemia injected intraperitoneally, access to the peritoneal cavity for the various liposomal compositions studied should be determined. Factors such as liposome size, liposome composition, and the presence or absence of internal drug should be investigated as to their effects on peritoneal accumulation. The accumulation should also be investigated in both the presence and absence of tumor growing in the mouse. Another interesting avenue for further investigation would include the use of drugs which promote vascular permeability, thereby allowing the liposomes to pass through the vasculature and achieve better access to the tumor.

Along the same lines, various factors should be investigated for their abilities to
promote or inhibit liposome accumulation within solid tumors. In Chapter 5 it was seen that although G\textsubscript{m1} resulted in higher vincristine accumulation within the Lewis Lung tumor, the lipid accumulation was drastically decreased.

Very high drug retention levels could be important for targeting these liposomal systems directly to tumor cells. At the present time liposomes have successfully been targeted to some tissues (Gabizon et al., 1990; Ahmad and Allen, 1992; Lundberg et al., 1993; Ahmad et al., 1993). If targeting to tumor cells could be accomplished with very high drug retention levels, the drug could then be released to expose the target area to a very high concentration of drug, leaving non-target tissues with lower potential drug toxicity. Means by which the drug could be released at its target site by dissipating the pH-gradient across the liposomal membrane. This could be accomplished with the use of proton gradient uncouplers (Mayer et al., 1990d). More effectively, the targeted liposomes could be induced to fuse with the membranes of target cells, thereby releasing the drug directly into the cytosol of tumor cells. Liposome fusion has already been accomplished with the use of fusogenic lipids (Allen et al., 1990; Wilschut et al., 1992; Eastman et al., 1992) (see Figure 6.1).

In conclusion, it has been shown that improved retention of vincristine in liposomes leads to decreased drug toxicity and improved antineoplastic activity. These studies may be used as a model for the liposomal encapsulation of other drugs of weakly basic nature which can be loaded into liposomes across a pH gradient. The potential for reduced toxicity and improved therapeutic activity is of obvious benefit.
Figure 6.1
Targeted fusion of liposomes.

The targeted carrier binds to the target cell and fuses with it. The entrapped material is then released directly into the cell interior.
REFERENCES


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