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THE ROLE OF DRUG-LIPID INTERACTIONS IN BIODISTRIBUTION AND THERAPEUTIC EFFECTS FOR DRUGS INCORPORATED INTO LIPOSOMES

By

Claudette R. Bethune

A thesis submitted in partial fulfillment of the
requirements for the degree of

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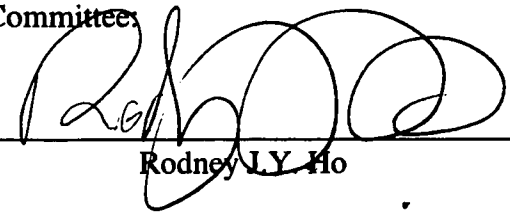

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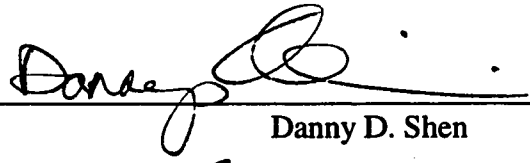

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Abstract

The Role of Drug-Lipid Interactions in Biodistribution and Therapeutic Effects for Drugs Incorporated into Liposomes

by Claudette Renee Bethune

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While a number of drugs formulated in liposomes or lipid vesicles are approved for human use, many are formulated empirically, without a full or complete understanding the role of drug-lipid interactions. Hence, the primary goal of this thesis is to study the role of drug-lipid interactions in modulating biodistribution and therapeutic effects when drugs are formulated in liposomes.

We first investigated drug-lipid interactions by studying the interactions of a series of opioids with varying hydrophobicity to associate to and release from liposomes. While highly lipophilic sufentanil associated completely with liposomes, the hydrophilic agent morphine sulfate had low association (30%). The dissociation rate of morphine from liposomes was significantly lower (37-fold) than for sufentanil in the presence of CSF. Studies with a catheterized pig model equipped with microdialysis probes confirmed that in vitro results parallel the in vivo drug release profiles of respective liposome-associated drugs. Our results also suggest that drug incorporation into the aqueous compartment of liposomes and the degree of insertion to lipid membranes, all may modulate a drugs association and dissociation in vitro and in vivo.

With this knowledge of drug-lipid interactions, we chose to determine whether the antitumor agent 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) incorporated liposomes may increase the stability of this highly volatile agent. Our results showed that lipid association increased CCNU's stability and efficacy against tumor cell lines. In rats,

liposome-formulated CCNU enhanced total drug exposure in plasma with significant decreases in behavioral neurotoxicities. Mechanistic studies in rats suggested that intact drug-liposome complexes, rather than free-drug molecules dissociated from liposomes in systemic circulation, accumulated in tumors. Liposome-associated CCNU reduced plasma free fractions of CCNU by one-half as compared to free drug. It is probable that a combination of increased drug accumulation in tumors and reduced plasma free fractions contributed to the enhanced tumor-growth suppression of lipid-associated CCNU.

In conclusion, a systematic study to understand drug-lipid interactions improves our ability to design and optimize drug formulations that use liposomes to improve the therapeutic index of highly potent drugs.

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DEDICATION

The author wishes to dedicate this dissertation to her brother Sean and his wife Sandy, for their unyielding encouragement, love and support during this work.

CHAPTER 1: BACKGROUND

Liposomes ("lipo" meaning lipid, "soma" meaning body) are microscopic vesicles composed of amphipathic lipids arranged in concentric bilayers (lamellae) that resemble myelin figures which form when dried phospholipids are exposed to aqueous solutions (sterile water, normal saline, etc.) [Stockenius, 1959]. It was not until 1965, when the existence of the cell bilayer was confirmed, when studies showing that liposomes could trap a range of ions and release them at various rates, that the potential for drug delivery of liposomes was realized [Bangham et al., 1965].

While it has long been recognized that liposomes form spontaneously when appropriate lipids are exposed in sufficient concentration to an aqueous solution, in practice, however, there are a large number of variables to consider in producing a liposomal preparation of a particular drug. Critical variables of drug-lipid interactions include the efficiency of drug entrapment, lability of the drug, liposome size, drug-to-lipid ratios, liposome permeability, and stability of the final preparation. While the role of these drug-lipid interactions have been recognized and taken into account in the development of lipid-based drugs, they have not been studied systematically.

For example, the marketed drug "ABELCET® " of The Liposome Co. was originally thought to be a formulation of liposome-encapsulated amphotericin B [Lopez-Berestein et al., 1985], yet as the drug reached Phase II clinical trials, the lack of liposomes in the formulation was realized [Anaissie et al., 1995]. The ultimate ABELCET® formulation is now characterized as an amphotericin B lipid complex composed of lipid sheets, rather than vesicles (table 1). Its therapeutic efficacy is comparable to free amphotericin B, but it has substantially reduced nephrotoxicity [Clark et al., 1991]. The true liposomal amphotericin B product is marketed by another manufacturer as "AmBisome®" (table 1).

During the course of developmental and efficacy studies of liposomal amphotericin B, numerous investigations on the role of drug-lipid interactions were made using novel formulations, varying in lipid composition and vesicle size. Among the

salient observations made were that saturated lecithins, as compared to unsaturated ones, were better in reducing the toxicity of amphotericin B [New, 1981]. Chen and Bittman [1977] reported a decrease in the rate of uptake of the drug into liposomes with an increase in the saturation of the phospholipids. In a separate study on the effect of lipid composition on the ion fluxes in red cells, it was shown that when amphotericin B was associated with liposomes composed of unsaturated phosphatidylcholine, the drug was much more potent than when incorporated into saturated liposomes [Juliano et al., 1987]. Inclusion of cholesterol in neutral egg phosphatidylcholine liposomes was found to drastically reduce the toxicity of the drug [Ahmad et al., 1990]. On the other hand, cholesterol had no effect on the toxicity in the case of positively and negatively charged liposomes. As explained in more detail later in this chapter, the addition of cholesterol into the phospholipid membrane of liposomes can reduce drug leakage, an attribute which may be offset by the charge of the vesicles, with charged vesicles tending to be more leaky than neutral liposomes in general. In addition, small unilamellar vesicles (SUVs) show higher levels of circulating amphotericin B in plasma as compared with larger or multilamellar vesicles (MLVs) [Juliano and Stamp, 1975; Payne et al., 1987; Szoka et al., 1987]. The difference in efficacy for SUV and MLV preparations is explained by the fact that amphotericin B does not cross the bilayers of liposomes rapidly and hence the drug is unable to easily transfer from internal lamellae in MLVs as compared to SUVs [van Hoogevest and de Kruijff, 1978].

The use of liposomes to improve the therapeutic index of agents employed in cancer chemotherapy has found increasing interest. With some understanding of drug-lipid interactions in physiological conditions, the successful marketing of two liposomal drugs, which use a highly effective transmembrane pH gradient for encapsulating the anticancer agents daunorubicin and doxorubicin, has been achieved (table 1). The marketed liposomal daunorubicin product, DaunoXome®, is a SUV formulation composed of distearoylphosphatidylcholine and cholesterol with a total lipid concentration of approximately 27 mM and 1 mg/ml drug concentration [Lasic, 1998]. This formulation was found to be stable in the circulation because the liposomes are small (45 nm) and their membrane is electrically neutral and mechanically strong [Lasic

et al., 1995]. The liposomal doxorubicin formulation, Doxil®, was the first liposomal drug approved by the FDA and was created by encapsulating the drug into preformed liposomes by an ammonium-sulfate-gradient technique, which additionally precipitates the entrapped drug inside the liposomes [Lasic, 1998]. Doxil® is composed of SUVs with a surface polyethylene glycol (PEG) coating the distearoylphospatidylethanolamine hydrogenated-soya-bean phosphatidylcholine-cholesterol vesicles [Lasic, 1998]. This liposomal formulation has been found to circulate in patients for several days, owing to the polymer coating and mechanically stable lipid bilayers [Kuhl et al., 1994; Lasic et al., 1995].

As reviewed by Swenson and Bolcsak [1999], the importance of controlling critical physico-chemical properties for liposomal drugs has been recognized by the U.S. Food and Drug Administration (FDA) [FDA, 1994]. The structure of the lipid-drug complex or liposome, charge distribution, lamellarity, volume of entrapment, particle size and distribution profile, in vitro drug release rate, phase transition temperature, and the ratio of drug substance to lipid(s) were specifically mentioned as properties that were important for the adequate characterization of a liposomal drug. Further discussion of these variables at the American Association of Pharmaceutical Scientists meeting in the Fall of 1996 resulted in the suggestion that the list reflect those characteristics that may be useful but which may not be absolutely required for adequate characterization of the liposomal drug [Kling, 1996]. In any event, it appears that a clear understanding and systematic characterization of drug-lipid interactions is required to produce a viable liposomal drug product.

Hence, the goal of this thesis is to systematically determine and examine the role of drug lipophilicity in drug-lipid interactions and the resulting altered pharmacokinetics and pharmacodynamics of highly potent therapeutic agents when associated with liposomes. In this chapter, I will first discuss the role of drug-lipid interactions by examining liposome composition, size, surface characteristics, and disposition of liposomal drugs in vivo. Also, I have included sections which describe the drugs considered most applicable for liposomal drug delivery, the underlying mechanisms behind the improved efficacy found compared to free drug therapy, and discuss current

targeting strategies used for drugs incorporated into liposomes. The objectives of the dissertation conclude this chapter.

1.1. CLASSIFICATION OF LIPOSOMES

1.1.1. COMPOSITION

Liposomes can be made from a large variety of natural and synthetic lipids. Major liposome constituents can range from:

1) saturated fatty acids such as myristic, lauric, stearic and palmitic acid;
2) unsaturated fatty acids such as oleic, linoleic, and arachidonic acid;
3) membrane forming phospholipids such as phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS);
4) neutral lipids such as cholesterol and tocopherols.

Among the phospholipids, dimyristoyl phosphatidylcholine (DMPC) and dipalmitoyl phosphatidylcholine (DPPC) are the archetypal and most intensively studied of the biological lipids [Yager, 1996].

Lipid composition can directly affect the bilayer permeability of liposomes due to the fact that phospholipid membranes can exist in different phases at different temperatures. Phase transitions in liposome bilayers range from a gel phase at low temperatures to a liquid-crystal phase at higher temperatures. The gel phase is characterized by a thicker bilayer and low permeability while the liquid crystal phase is observed as thinner bilayer with high permeability [Laggner and Kriechbaum, 1991; Koynova and Caffrey, 1998]. Gel-phase liposomes are rigid and retain their shape. Maximum bilayer permeability occurs at the phase-transition temperature (T_m) [Kim, 1993]. This has important practical implications, since liposomes may pass back and forth through the T_m if temperature is not strictly controlled during manufacture and

storage, leading to leakage of encapsulated drug at the phase transition temperature. With the understanding that temperature effects carrier permeability, the T_m can be engineered to promote the release of encapsulated drug at inflamed or locally heated tissue sites [Khoobehi et al., 1988; Gaber, 1995].

The incorporation of cholesterol into the lipid bilayers of phoshatidylcholine vesicles is known to counteract phase transitions and increase the rigidity of the bilayer (with a consequent reduction in permeability), thereby increasing the retention of hydrophilic solutes and stability of liposomes in vivo [Papahadjopoulos, 1973]. The incorporation of cholesterol, as well as other types of lipids, into phospholipid vesicles has allowed unique pharmaceutical formulations to be developed for specific therapeutic applications.

1.1.2. LIPOSPOME PREPARATION AND VESICLE SIZE

A liposome is a structure consisting of one or more lipid bilayers completely enclosing one or more aqueous compartments. Many types of lipid vesicles and their formation techniques have been described which influence liposome size. The definitions for liposomes vary, but the primary ones are based size and number of lamellae. Small unilamellar vesicles (SUVs) are those vesicles that are 25 to 100 nm in diameter while multilamellar vesicles (MLVs) have diameters ranging from 100 nm to 200 μm [Yager, 1996]. Note that vesicles in MLV preparations can exhibit a nearly 2,000-fold range in diameter and 6×10^6- fold range in vesicle volume!

Liposome morphology depends on the method of preparation used. As reviewed by Szoka and Papahadjopoulos [1980], the major liposome formation technique for MLVs include suspending lipids in buffer and vortex mixing. This is the simplest method for forming liposomes, where the size of the MLVs produced decreases with the vigor of the agitation. The MLVs are very heterogeneous in size and lamellarity. Forming SUVs is the same as MLVs, but the method is followed by sonication (exposure to ultrasound). This method produces the most homogeneous preparation with respect to

vesicle size. Other methods of vesicle formation include making MLVs then following with extrusion or other high shear processes to create small vesicles. To increase the incorporation of drugs into liposomes, any of the above methods may be followed by freeze-thaw cycling to create FATMLVs. Additionally, as described by Mayer and co-workers [1993], the use of transmembrane pH gradients to encapsulate certain drugs with titratable groups has advanced drug trapping efficiencies to nearly 100% [Mayer et al., 1986].

1.1.3. Surface Characteristics and Cellular uptake

While most liposomal constituents have no net charge, negatively charged lipids such as PG, PS, and cholesterol sulfate, or positively charged lipids such as stearylamine, are frequently used to reduce the tendency of uncharged liposomes to aggregate in aqueous suspension [Fielding, 1991; Gabizon, 1988]. However, charged liposomes tend to be more leaky than those that are uncharged, and they may interact electrostatically with oppositely charged surfaces. The cationic liposomes were developed for delivery of nucleic acids into cells. The anionic nucleic acids initially bind to the surface of the cationic vesicles, forming multi-lamellar lipid-DNA complexes [Radler et al., 1997]. In addition to electostatic attraction, hydrophobic interactions are believed to aid in complex formation [Wong et al., 1996].

The inclusion of certain glycoproteins (such as monosialoganglioside GM1) also confers a negative charge to liposomes and reduces leakage of liposome contents in the presence of plasma [Allen, 1985]. Including glycoproteins or lipids which are conjugated to ethylene glycol renders a steric barrier outside the vesicle membrane, and these formulations have been termed "sterically stabilized" liposomes [Naper, 1983 and Gabizon, 1992]. Other membrane coatings such as virus coat proteins, synthetic amphiphilic peptides, and immunoglobulins can also be incorporated into liposomes to enhance drug delivery [Dass, 1997].

Negatively charged liposomes have been shown to interact with cells, in delivery of polio RNA [Wilson et al., 1979], carboxyfluorescein [Straubinger et al., 1983], SV40 DNA [Fraley et al., 1980] or proteins [McIntosh and Heath, 1982] more effectively than neutral liposomes. Heath and co-workers [1985] noted that for methotrexate-γ-aspartate (a dihydrofolate reductase inhibitor), incorporation into phosphatidylserine (negatively charged) liposomes yielded much more efficient delivery to cells than that observed with neutral liposomes. These authors also suggested that the optimal size for cellular uptake of these negatively charged vesicles is 0.05 to 0.1 μm, a size range which would allow endocytic uptake mediated by cellular surface pits that have a diameter of 0.15 μm [Pastan and Willingham, 1981]. Wasan and co-workers [1993] have observed that when amphotericin B is incorporated into negatively charged liposomes, there is an increase in the level of association of the drug with human serum high density lipoproteins (HDLs) as compared to free drug, which may provide a degree of targeting to those cells bearing HDL receptors.

It seems clear from several reports that the most likely mechanism of cellular drug delivery involves the endocytosis of the liposomes [Straubinger et al., 1983; Leserman et al., 1981; Heath et al., 1983; Huang et al., 1983; Matthay et al., 1984; Machy and Leserman, 1983]. The primary mode by which liposomes are incorporated into certain types of cells is known to be endocytosis via the coated pit pathway [Straubinger et al., 1983]. The involvement of clatherin-coated pits in liposome endocytosis has been demonstrated by microinjection of anti-clatherin antibody into cells [Chin et al., 1989]. In a recent in vitro study, Wasan and Lopez-Berestein [1994] showed that the reduction of toxicity of liposomal amphotericin B formulations to pig kidney cells was related to predominant distribution of the formulation to low affinity serum HDL receptors. However, the nature of the membrane component responsible for liposome binding and internalization is unknown at present. Most approaches for investigating the mechanism of liposome uptake is to make specific changes in the lipid composition of liposomes and document the conditions which promote or reduce their uptake by cells.

In a report by Lee and co-workers [1992], the investigators examined how various negatively charged liposomes are recognized by endocytic cells in culture. Their results

obtained by a quantitative analysis of binding and endocytosis with two cell lines (monkey kidney and murine macrophages) demonstrated that these cells have the capacity to distinguish small differences in the lipid headgroup structure on the bilayer of the liposome and suggested that such specificity describes a receptor-mediated uptake. These authors hypothesized that these cells have different liposome-binding sites, which was supported by a competition experiment with various poly-anions. Additionally, these researchers also considered that the liposome binding sites were the same in the two cell lines and that the cell surface density of the liposome binding sites may be higher in one cell line. In this case, a multi-ligand effect could explain the difference in the uptake of charged liposomes. The binding of a liposome may require several negatively charged lipids and clustering of one or several types of membrane binding components. Also, previous studies with the monkey kidney cells (J774) indicated that a significant percentage of liposomes are taken up through smooth pits [Lee et al., 1992] contrary to the case with the murine macrophages (CV1) which take up liposomes exclusively through coated pits [Straubinger et al., 1983], and suggest a different route of liposome uptake exists in each of these cells.

Apparently, the cellular uptake of liposomes is more complicated than a pure electrostatic interaction between the liposome surface and the cell membrane surface. Lee and co-workers [1992] concluded that the rate of liposome uptake is specifically controlled by the headgroup of the lipid rather than simply the net negative charge on the liposome surface. Furthermore, a higher surface charge density also promotes uptake, but the concentration of negatively charged lipids needed for high levels of uptake is cell type dependent.

1.2. DISPOSITION OF LIPOSOMES

In general, in vivo studies are usually initiated only when a liposomal formulation exhibits the necessary chemical and physical stability properties to be considered pharmaceutically viable. In vivo analysis must also consider that liposomal drugs will

interact with a number of distinct physiological processes and associated barriers between tissues. For the following discussion, I will focus on systemic administration and, specifically, the fate of liposomes after intravenous (i.v.) injection.

This section describes the current understanding of liposome behavior in vivo which is characterized by their: 1) interactions with plasma constituents; 2) clearance by the reticuloendothelial system (RES); 3) microvascular permeability and extravastion capabilities; 4) plasma kinetics; and 5) systemic toxicities.

1.2.1. INTERACTIONS WITH PLASMA CONSTITUENTS

When liposomes are injected intravenously they are immediately exposed to a wide variety of circulating cells, lipoproteins, and soluble factors which include proteins, carbohydrates and small ions. The fate of liposomes in this compartment is dictated by the interactions between the liposome surface and serum protein components. The primary effect of proteins adsorbing onto liposomes is increased membrane permeability (which compromises drug retention) and recognition with subsequent clearance of liposomes by the RES [Mayer et al., 1998].

Early experimental work has shown that when liposomes are incubated with cells in either serum-free [van Renswoude and Hoekstra., 1981; Szoka et al., 1979] or serum-containing [Allen and Cleland, 1980] media, the vesicles become more permeable, with subsequent leakage of their entrapped contents. Decreasing the fluidity of the liposomes (by the addition of cholesterol) decreases the cell-induced liposome leakage [Allen and Cleland, 1980]. In addition to increasing the permeability of liposome bilayers, protein adsorption can also lead to increased susceptibility to transmembrane stresses caused by ion gradients or high concentrations of encapsulated drugs [Mayer et al., 1998]. The high concentrations of buffer and/or drug entrapped in liposomes often results in substantial osmotic gradients across the liposome membrane when exposed to physiological fluids. While most small unilamellar liposomes (SUVs) can withstand a significant osmotic gradient in the absence of extraneous proteins, exposure of liposomes with a large

osmotic gradient to plasma or purified lipoproteins results in a burst of leakage from the vesicles until osmotic balance is re-established [Mui et al., 1994].

Liposomes are destabilized by plasma lipoproteins through the insertion of the apoprotein into the liposomal phospholipid bilayer, resulting in an exchange of lipids between lipoprotein and liposome [Senior, 1987; Williams and Tall, 1988]. While interactions have been observed with all the types of plasma lipoproteins (VLDL, LDL, and HDL), the lipoprotein mainly responsible for liposome destabilization is HDL [Williams and Tall, 1988; Scherphof et al., 1978].

Other components found in plasma which destroy liposomes are phospholipases and complement proteins. Phospholipase A_2, commonly obtained from snake venom, has been extensively studied. Normal serum and plasma from man and several animal species contain phospholipase A_2 (PLA_2) activity [Kaplan-Harris et al., 1980]. The enzymatic products of phospholipids with PLA_2 are fatty acids and 2-hydroxy lysophospholipid [Jain and Berg, 1989]. Both fatty acids and lysophospholipids are considered fusogens and their addition to liposome bilayers (above a critical mole ratio) results in aggregation, fusion, and lysis of the original liposomes.

Complement proteins have been associated with liposome destruction by coating the vesicles such that they are identified by the immune system (opsonization) and quickly removed from the circulation [Semple et al., 1996]. Increased opsonization has been noted for liposomes prepared from negatively charged lipids such as phoshatidylserine, cardiolipin and phosphatidic acid [Oja et al., 1996]. In contrast, liposomes prepared with other anionic lipids such a phosphatidylglycerol and phosphatidylinositol exhibit extended circulation times following i.v. administration despite having reasonable levels of adsorbed serum proteins [Chonn et al., 1992]. The mechanisms behind such different observations are most likely due to the different liposome compositions, where anionic lipid headgroups as well as a net negative charge are known to influence cellular uptake mechanisms [Lee et al., 1992]. Also, variations in bilayer fluidity of neutral liposomes containing more than 30% cholesterol (to make the vesicles more rigid) does not result in significant differences in the types of proteins

adsorbed, and these liposomes are cleared from the circulation at similar rates to those liposomes without cholesterol [Semple et al., 1996].

1.2.2. Clearance by cells of the Reticuloendothelial System

The importance of this clearance mechanism cannot be overstated since the removal of liposomes, as well as other exogenous particles, is primarily through this cellular system. The rate of uptake by the reticuloendothelial system (RES) depends on liposome size, fluidity, composition, and dosage. It has been demonstrated previously that SUVs are taken up much more slowly than are MLVs [Allen, 1988; Gregoriadis, 1988]. When liposomes are prepared with certain naturally occurring lipids (ganglioside GM1 and phosphatidylinositol), an increase in liposome circulation times were observed [Mayer, 1998]. Similar to the development of polymer surfaces that exhibit reduced protein binding, it is believed that these carbohydrate containing lipids act by limiting the interaction of liposome surfaces with plasma proteins and this inhibits the rate of uptake by cells of the RES. As the liposome dose increases, the mechanisms of liposome uptake by the RES become progressively saturated and liposome clearance decreases [Senior, 1987; Gregoriadis, 1988].

The bulk of cells considered to be responsible for removing liposomes from the circulation are the phagocytic cells which comprise the RES. The concept of grouping a population of cells together as a RES was first proposed by Aschoff in 1924, and is based primarily on the histological similarity between various cell populations and secondly on the fact that the cells take up intravital dyes from the bloodstream [Senior, 1987]. The main features of the cells (all termed macrophages) of the RES are that they are highly phagocytic, mononuclear, and are present in many different organs of the body [Bradfield, 1984], with some of these cells being found settled in tissue components while others are found migrating through the circulation. Also, it is thought that macrophages within tissues are replenished by replacement from circulating blood monocytes derived from bone marrow [Carr and Wright, 1978].

For the wide range of tissues containing phagocytic cells of the RES, by far the most studied and perhaps the most responsible for particle uptake are the Kupffer cells in the sinusoids of the liver [Senior, 1987]. In addition to the liver, connective tissue histiocytes, lung alveolar macrophages, spleen free and fixed macrophages and sinusoidal lining cells, lymph node free and fixed macrophages, peritoneal macrophages, bone tissue osteoclasts, and nervous system microglia, all posses highly phagocytic macrophages as well [Bradfield, 1984]. When considering the i.v. route for liposome administration, only those macrophages which line the blood circulatory vessels contribute to blood clearance. Liver, spleen, and bone marrow are the major sites for liposome clearance [Bradfield, 1984], with each of these sites acting independently so that the clearance of liposomes administered i.v. is determined by the organ's relative blood flow, extraction ratio, and capacity for uptake.

The ability to internalize particles by phagocytosis or by endocytosis is a common feature of RES macrophages. In studies on the ingestion of liposomes by these cells, the terms endocytosis and phagocytosis have often been used interchangeably. This looseness of nomenclature seems to be the result of poor differentiation between the processes of pinocytosis, macropinocytosis, endocytosis, and phagocytosis. These different processes can be distinguished by the size of the ingested particle and by the presence or absence of receptor mechanisms. Many investigators [Mangala and Alving, 1998] consider phagocytosis to be restricted to relatively large particles (those greater than 200 nm) while pinocytosis and endocytosis are restricted to soluble proteins and small particles (those less than 200 nm). Also, phagocytosis of liposomes may be associated with the presence of specific receptors on phagocytic cells, such as the complement C3b receptor or the Fc receptor [Allen and Aderem, 1996; Alving and Wassef, 1992; Aragnol and Leserman, 1986]. In contrast, macropinocytosis, a process that can cause the nonphagocytic ingestion of soluble particles greater than 200 nm, is not associated with any receptor activity [Swanson and Watts, 1995].

Internalization of phospholipid liposomes is also found in non-phagocytic cells such as fibroblasts, kidney cells, lymphocytes, and hepatocytes [Margolis, 1988; Poste, 1980]. This non-receptor-mediated phagocytosis appears to be strictly dependent on the

size of liposomes, the optimal size being 50-100 nm [Machy and Leserman, 1983; Heath et al., 1985]. Once inside the cell, the final destination of liposomes is generally thought to be in the low pH compartments of endosomes and lysosomes [Straubinger et al., 1983].

1.2.3. MICROVASCULAR PERMEABILITY AND EXTRAVASATION

While in the blood circulation, liposomes are exposed to cells lining the vasculature. The inner lining (intima) of blood vessels is composed primarily of endothelial cells which form a continuous layer on the interior surface of all blood vessels. Under this layer is the basement membrane and in larger (non-capillary) vessels the vasculature is supported by smooth muscle cells [Jain, 1987]. The microvascular endothelium and the basement membrane are the two major barriers for liposome extravasation [Yuan et al., 1994].

The endothelial cells in most normal vasculature exhibit intact intercellular junctions and only small molecules are able to pass through capillaries of this type. However, this structure is significantly altered in certain normal tissues, most notably the liver and spleen, as well as in disease sites of infection and tumor growth. Areas of tumor growth are characterized by the presence of capillaries that exhibit fenestrae or larger intercellular openings and can be missing the basement membrane layer entirely [Mayer, 1998]. The gaps in these endothelial layers can range in size from 30 nm for fenestrated capillaries to greater than 500 nm in liver, tumor and inflammation site vasculature [Dvorak et al., 1988; Yuan et al., 1995]. In the liver, these openings provide access to sinusoids where the phagocytic Kupffer cells lie. In disease sites, the fenestrated/discontinuous capillary beds allow direct exposure of the underlying epithelial cells to the circulation.

Major diseases, such as bacterial infection, inflammation and tumors, have the common feature of altered vasculature permeability at the site of disease progression. The factors that lead to increased permeability of the vascular barriers are distinct for

different disease states. Chemotactic factors and adhesion molecules overexpressed at sites of inflammation attract infiltrating lymphocytes and granulocytes that subsequently release factors which can directly damage endothelial cells and/or cause defects in intercellular junctions [Chen et al., 1996]. In hypoxic environments, such as those found during rapid cell proliferation or through vascular injury, cells can release vascular endothelial growth factor (VEGF) [Hanahan et al., 1996; Rak et al., 1996]. VEGF is an endothelial cell specific mitogen and its release can lead to the development of neovasculature. Also, VEGF has proven to be identical to the vascular permeability factor [Dellian et al., 1996; Dvorak et al., 1995], a protein first identified as a factor capable of inducing defects in the permeability barrier of blood vessels. Regardless of the factor, the end result for all of these conditions is the presence of blood vessels that are permeable to large molecules. This may be due to the presence of fenestrae or larger gaps occurring between adjacent endothelial cells through which macromolecules can pass or, alternatively, may also involve increases in endothelial cell mediated transcytosis [Huang et al., 1993].

To summarize, it is the unique nature of vascular structures and chemical mediators which exist in liver/spleen and diseased tissues that can significantly impact the pharmacological behavior of liposomal drug delivery systems.

1.2.4. Plasma Kinetics

A biphasic plasma decay curve is often observed after an intravenous injection of phospholipid liposomes. To investigate whether such a plasma concentration-time profile is the result of two simultaneous processes, reversible distribution to the extravascular space (causing the initial rapid decay) and elimination, Hwang et al. [1982] injected intravenouslyc SUVs (25 nm) into mice. These liposomes are suitable for detecting a possible reversible distribution, since they are small enough to pass out of discontinuous capillaries and remain intact in body fluids for some time. A biphasic decay was observed with these vesicles and the exrapolated apparent volume of

distribution (V_d) was found to be 1.3 times larger than the volume occupied by erythrocytes. It was speculated from these results that small liposomes may equilibrate in a volume larger than the vascular space. However, considering that the extrapolation method overestimates the V_d [Gibaldi and Perrier, 1982], it may be concluded that even with the smallest of liposomes the extravascular space that equilibrates with the plasma compartment is very limited. For practical purposes, the one-compartment model is successfully used for agents such as liposomes where the V_d approximates the volume of the vascular compartment [Wagner, 1983].

From the above considerations, it is evident that factors other than reversible distribution must be responsible for the biphasic concentration-time profile for liposomes injected intravenously. Juliano and Stamp [1975] were the first to demonstrate that a biphasic plasma decay may result from heterogeneity in the size of the injected liposomes. Accordingly, they observed a biexponential clearance pattern with heterogeneous vesicle populations and a monoexponential decay with liposomes samples homogeneous in size. Gregoriadis and Neerunjun [1974] observed that the biphasic decay of i.v. injected MLVs converted to a linear one when the liposome dose was increased. These authors suggested that the biphasic decline may be due to the presence of two elimination pathways: a faster one, due to a saturable uptake by the RES, and a slower one, due to uptake by the liver parenchymal cells. When the retention capacity of the RES becomes saturated, then only the slower elimination pathway remains operating and the clearance rate decreases. Also, when the storing capacity of the RES is rapidly exhausted by the administration of very large doses, then only the slower decay phase is detected. It was later shown that MLVs cannot pass out of the endothelial barrier, and it is unlikely that the slow uptake observed by Gregoriadis and Neerunjun [1974] was due to liposome uptake by the liver parenchymal cells. More likely, the biphasic decay pattern was the result of the heterogenous size distributions of the injected MLVs.

As noted earlier, it is not necessary to postulate the existence of two distinct elimination pathways to explain the different elimination rates of large and small liposomes, since they are taken up at different rates by the RES. After i.v. administration, smaller liposomes have slower clearance rates than larger liposomes [Allen et al., 1995].

However, a time-dependent saturation of the RES cannot be ruled out. Conversion of a biexponential decline into a linear one with an increase in the liposome dose has been confirmed by other authors using more homogeneous MLV preparations [Kao and Juliano, 1981; Souhami et al., 1981]. While the presence of two parallel elimination pathways is an unlikely possibility for MLVs, it is theoretically possible for SUVs that can be extravasated. Beaumier et al. [1983] observed a downwardly curved plasma decay after intravenous administration of SUVs (mean diameter 25 nm). This decay profile is typical of substances eliminated either exclusively through a capacity-limited (Michaelis-Menten) pathway or through parallel first-order and capacity-limited pathways [Kume et al., 1991]. On the basis of these considerations, Beaumier et al. [1983] proposed that the elimination kinetics of SUVs are best described by a model involving two parallel pathways: a capacity-limited one due to uptake by the Kupffer cells, and a linear one due to pinocytosis by the liver parenchymal cells.

To summarize, the observations described so far make it apparent that until more general concepts of liposome clearance mechanisms evolve, a pharmacokinetic model encompassing all the aspects of liposome disposition is likely to be very complex. Although a one-compartment model may be satisfactory from a practical point of view, various assumptions of non-linear kinetics must be built into the model. These assumptions are: 1) Time- and dose-dependent decreases in MLV clearance are due to the saturation of the retention capacity of the RES and 2) Dose-dependent decrease in SUV clearance is due to the saturation of the uptake mechanism of the RES. In the absence of specific information on the behavior in vivo of a given liposomal preparation, a model independent analysis appears safest. In addition, model-independent parameters should be cautiously evaluated with the specified amount of lipid administered and mean vesicle diameter in the liposome formulation.

1.2.5. TOXICITY

It has generally been assumed that lipids, because they are natural components of all cells, are likely to be relatively safe excipients. Based on the current understanding of the fate and behavior of liposomes in vivo, Storm and co-workers [1991 and 1993] list a number of areas that are of concern to those studying the safety of liposomal formulations. These included effects on the RES, the potential for interaction with plasma proteins and lipoproteins, the clotting system and cellular components of the blood, the potential for capillary plugging, the possibility that liposomes may be immunogenic, the potential pharmacologic activity of novel lipids and the problems that may be associated with chemical or physical instability of the formulation. Additional areas of concern include the possibility of new toxicities due to an altered distribution of the encapsulated drug, nutritional effects of high lipid doses, and interactions with other drugs. Many of these areas have been investigated with research liposomal preparations, but systematic studies with well characterized systems have been few and generalizations are difficult to make.

Natural phospholipids, such as egg phosphatidylcholine, have been administered i.v. to man for years in the form of i.v. fat emulsions. These emulsions are generally believed to be well tolerated and biodegradable. However, many effects have been reported with total parenteral nutrition with fat emulsions including alterations in serum lipoproteins, lung dysfunction, changes in liver and RES function and increased susceptibility to infection [Hardin, 1994]. Some of these effects may be due to the triglyceride component of the fat emulsion, but hepatic and RES effects are generally thought to be due to the phospholipid component [Bach et al., 1996]. Fat emulsions are typically given in doses of 1 to 3 g/kg/day [Intralipid® insert, 1991]. All contain 1.2% egg phospholipids, thus, the total egg phospholipid dose is generally 12 to 36 mg/kg/day. Studies have shown that these emulsions contain chylomicron-like emulsion particles with diameters of 300-400 nm and liposomes (phospholipids not associated with the triacylglycerols in chylomicron-type particles) with diameters of 70-100 nm. A typical daily dose of 10% Intralipid® would result in delivering 22 mg/kg egg phospholipid

liposomes, usually over 4 to 6 hours. The amount of lipid administered to humans with several i.v. liposomal drugs that are marketed (Table 1) or have entered clinical trials is estimated to be in the same range as the liposomal phospholipid dose administered with Intralipid [Swenson and Bolcsak, 1999].

It is clear that rapid administration of large quantities of parenteral lipid emulsions [Hardin, 1994] or liposomes [Storm, 1993] can affect RES function. Unfortunately, it is not possible to give an exact lipid dose that results in "RES blockade" or impaired macrophage function in a mouse (or any other species for that matter). This is because the effects on the RES are also related to infusion rate, particle size, actual number of particles, as well as the lipid composition and nature of the particle. Accumulation of lipid in the macrophages of the liver and spleen may be seen with i.v. administration of liposomes as well as Intralipid. This may be described as a "pigment" within the cells [vanHaelst and Sengers, 1976] and may be accompanied by increases in liver and spleen weight. These changes are considered "adaptive" since they are not accompanied by an inflammatory or other response and are generally reversible in animal studies. However, the detection of altered RES function (unless it is accompanied by alterations in liver enzymes, hepatomegaly or splenomegaly, or profound immunosuppresion leading to increased infections or tumors) may be difficult in standard, repeated dose toxicology studies [Swenson and Bolcsak, 1999].

Lastly, while such a toxicity has not been yet been encountered, the potential for large liposomes or intravascular aggregates of small liposomes to cause emboli has long been a concern among those working with these delivery systems. Sites that might be expected to be adversely effected by embolism include the lung, kidneys, eyes, and the brain and these sites should be carefully examined, grossly and microscopically, in toxicological studies of liposomal drugs [Swenson and Bolcsak, 1999].

1.2. CHARACTERISTICS OF DRUGS INCORPORATED INTO LIPOSOMES

1.3.1. DRUGS SUITABLE FOR LIPOSOMAL FORMULATION

Many drugs, either in clinical use or in development, have properties which are far from ideal. They may have poor solubility, rapid metabolism, and instability under physiological conditions or unfavorable biodistribution leading to toxicities. One attempt at achieving a solution to these problems has been to associate the drugs with a variety of drug carriers. Liposomes are the most advanced of the drug carriers with several products approved for clinical use and in advanced clinical trials (Table 1). Liposomes can be used as a drug depot for controlled release [Mayhew et al., 1976] and for reducing the non-specific toxicity of the drug [Rahman et al., 1980; Lopez-Berestein et al., 1983; Szoka et al., 1987].

Drugs which benefit most from association with liposomal drug carriers are those drugs which have unfavorable pharmacokinetic, biodistribution or toxicity profiles. Anticancer drugs have proven particularly suitable for liposomal delivery [Ranson et al., 1996; Woodle and Storm, 1998]. Liposomal formulations of several antibacterials, anti-inflammatory drugs, analgesic, and antiviral drugs are also in clinical development [Wasan and Lopez-Berenstein, 1995; Abraham et al., 1996; Deol and Khuller, 1997; Dipali et al., 1997; Senior, 1998]. Members of other drug classes whose efficacy is characterized by a low therapeutic index may also benefit from liposomal formulations. In addition, liposomal formulations will generally be more suitable for drugs with higher, rather than lower, potencies since the formulation will limit the amount of particulate material which much be given in order to achieve a therapeutic concentration of the drug in vivo.

1.3.2. HYDROPHOBIC VS. HYDROPHILIC AGENTS

Liposomes can function as carriers for both water-soluble and lipid-soluble drugs [Defrise-Quertain et al., 1984]. Hydrophilic drugs, such as aminoglycosides and morphine sulfate, are readily trapped in the liposome interior and are only released over several hours to several days in cerebral spinal fluid (CSF) at 37° [Bethune et al., In preparation]. Hydrophobic drugs, such as the alkylating agent lomustine (CCNU) and the analgesic opioid sufentanil, associate rapidly with the hydrophobic fatty acyl chain region of the liposome bilayer with release rates of minutes to hours in plasma and CSF at 37° [Bethune et al., 1999; Bethune et al., In preparation]. Nuclear magnetic resonance (NMR) studies also suggest that the uncharged form of a drug can, because of its greater hydrophobicity and its chemical/physical structure, penetrate deeply into the lipid bilayer of liposomes [Kelusky and Smith, 1984; Boulanger et al., 1981].

The antifungal agent amphotericin B, due to its aqueous insolubility, is typically formulated into detergent micelles for systemic applications [Lasic, 1998]. This micellular formulation is unstable in the circulation and severe neuro- and nephrotoxicity limit the doses which can be administered [Lasic, 1998]. With the rationale that if the drug is formulated in liposomes, the drug would be delivered more efficiently to the infection site and the dose-limiting toxicities could be reduced, three lipid based amphotericin B are now commercially available. One successful amphotericin B formulation, AmBisome (Table 1), contains the drug formulated into SUVs consisting of hydrogenated soybean phosphatidylcholine (a saturated phospholipid) and cholesterol along with distearoyl phosphatidylglycerol which is added to increase the extent of encapsulation and also to achieve an appropriate bilayer fluidity of the membrane structure [Gulati et al., 1998; Adler-Moore, 1994].

Drugs with intermediate solubility, such as doxorubicin, can be more difficult to formulate into liposomes. These drugs rapidly equilibrate between the liposomes and other in vivo membranes unless special formulation techniques are used [Allen, 1998]. As described previously for the liposomal doxorubicin (Doxil®), these techniques involve manipulation of the pH in the liposome interior, or the formation of insoluble molecular

complexes with the drug. These techniques are also applicable to drugs which are weak bases or weak acids, with the resulting formulations showing excellent drug retention [Haran et al., 1993; Cullis et al., 1997; Gabizon et al., 1994].

Drugs associated with liposomes receive substantial protection from interaction with degradative enzymes, physiological processes or unfavorable pHs which would lead to rapid breakdown of the non-associated drug. For example, in physiological conditions, CCNU decomposes in a span of minutes to form inactive products [Reed et al., 1975]. We found that incorporating CCNU into negatively charged liposomes protected the drug against decomposition in serum by nearly 2-fold, when compared to free drug [Bethune et al., 1999].

1.3.3. MECHANISMS OF DRUG RELEASE

Liposomes can be engineered to release their associated drugs over short or long periods of time, depending on the liposome composition, the presence of pH gradients or molecular complexes in the liposome interior, the in vivo environment of the liposomes (in plasma, spinal fluid, or localized in a solid tumor, etc.) and the in vivo stability of the liposomes. The ability to control drug release rates, combined with the ability to protect associated drugs from degradation, allows liposomal formulations to act as sustained release systems, continually releasing their store of drugs over an extended period of time [Allen et al., 1992; Horowitz et al., 1992; Lim et al., 1997]. The rate of this drug release is often non-linear, in contrast to free drug infusions [Allen et al., 1992]. Manipulation of liposome composition can also result in formulations in which drug release can be triggered at the intended site of action when liposomes are exposed to membrane destabilizing factors such as fusogenic peptides, transient hyperthermia, local pH changes or ultrasound [Ho et al., 1986; Chu and Szoka, 1991; Srinath and Jain, 1994].

The most commonly understood form of drug release from liposomal formulations is through pH dependent leakage [Weinstein et al., 1986]. For liposomes containing neutral phospholipids such as PC, the liposomal bilayer destabilizes at acidic

pH (below pH 6.3) thereby releasing its contents. This has been observed intracellularly with the acidic vacuoles of endosomes where liposomal contents were released for entry into the cytoplasm [Legendre and Szoka, 1992]. With the premise that the pH in the vicinity of certain tumors or in pockets of a tumor might be lower than in normal tissues [Murray and Carmichael, 1995], liposomes can be formulated to release their contents at lower pH (i.e. pH < 5.5), preferentially releasing their contents in the proximity of the target site.

Some of the best characterizations for mechanisms of liposomal drug release can be found with the liposomal doxorubicin formulations. Encapsulated doxorubicin is released from the liposomes after extravasation in tumors [Dewhirst and Needam, 1995]. Several possible factors may contribute to liposome breakdown and drug release in tumors: (1) conditions in the interstitial fluid surrounding tumors may cause breakdown of the liposomes, such as low pH [Stubbs et al., 1992], and lipases released from dead or dying tumor cells [Sakayama et al., 1994]; (2) inflammatory cells (which are often found in tumors) may release factors that lead to liposome destabilization such as enzymes or superoxide and other oxidizing agents [Dvorak et al., 1981], or (3) phagocytic cells residing in tumors which are known to engulf liposomes [Hunag et al., 1995] may digest the lipid matrix intracellularly and release doxorubicin (or its active metabolites) back into the interstitial fluid. A combination of these possibilities is likely to be responsible for the observed release of doxorubicin after extravasation of liposomal doxorubicin in tumors. Recent investigations with multivesicular liposomes (DepoFoam™) indicate that this formulation releases drug by gradual breakdown or reorganization of the lipid membranes [Yaksh et al., 1999; Kohn et al., 1998].

1.3.4. ALTERED PHARMACOKINETICS AND PHARMACODYNAMICS

Perhaps the most compelling property of liposomes is their ability to significantly alter the pharmacokinetics and the biodistribution of many of their associated drugs [Hwang, 1987; Allen et al., 1995; Scherphof et al., 1997]. Drugs, when associated with

liposomes, are sequestered away from interaction with their normal site of action in the body until they are released from the liposomes. Upon release from the liposome, the released drug becomes free drug and has the pharmacokinetic and pharmacodynamic parameters similar to free drug administered at a similar location and at a similar concentration to that of released drug. For a drug which is rapidly released from liposomes in the vasculature, the pharmacokinetic parameters will be very similar to that of free drug administered intravenously.

For drugs which are released slowly from liposomes, their pharmacokinetics will be similar to that of the liposomes themselves. These drug formulations are characterized by a low volume of distribution (approximating the volume of the compartment they are delivered into), a slow rate of clearance and a low tendency for distribution into normal tissues with the exception of the RES. For drugs with intermediate rates of release, the kinetics will be a combination of the pharmacokinetics of the free drug and that of the carrier. For the purpose of illustrating the substantial changes which can accompany liposome encapsulation of drugs, a comparison of the pharmacokinetics of free doxorubicin and liposomal doxorubicin is shown in table 2.

Because liposomes and their associated drug(s) are usually confined to the central compartment into which they are administered, uptake of the drug into normal tissues is decreased, leading to decreased toxicities in some sensitive normal tissues [Boman et al., 1996]. For example, when entrapped in liposomes, the anthracycline class of anticancer drugs with high avidity to cardiac tissue, doxorubicin and daunorubicin, have much lower cardiac toxicity than the respective free drugs, allowing for higher cumulative doses of the drug to be administered [Uziely et al., 1995; Northfelt et al., 1996; Forssen et al., 1997]. It was thought that altered distribution of liposomal drugs towards the RES, relative to free drugs, might lead to increased RES impairment [Parr et al., 1993]. However, to date, this has not been observed clinically. Bone marrow toxicity appears to be similar or less for the liposomal anthracyclines as for the free drugs and liver and spleen toxicities have not been evident for the liposomal formulations [Allen, 1998].

One of the well established goals of encapsulating or complexing drugs with lipids is to reduce acute toxicity. This has been demonstrated for several classes of

highly toxic drugs where lethality after a single dose can be substantially reduced when the drugs are incorporated into liposomes (Table 3). Studies from our laboratory also indicate that, compared to free drug formulations, liposomal CCNU grants more systemic exposure with reduced side effects by abrogating acute neurotoxicity after intra-arterial administration in addition to decreasing delayed hematological toxicity after intraperitoneal injection in rats [Bethune et al., 1999].

The highly potent antifungal agent amphotericin B acts by binding to membrane sterols, causing the formation of trans-membrane channels through which the cytoplasmic contents (vital ions, small metabolites) leak out, resulting ultimately in metabolic disruption and cell death [Holz, 1979]. The main cause of cellular toxicity of free amphotericin B is due to increased ionic flux to the outside of the cell. When liposomal amphotericin B is used, there is substantial reduction in ionic flux which decreases the toxicity. This was proposed in studies by Mehta and co-workers [1984] and Juliano and co-workers [1987]. They showed that prior to pore formation, a threshold level with amphotericin B transfer between donor and target membranes is required. With free amphotericin B, the threshold membrane transfer (likely through diffusion) was easily achieved. However, with liposomal amphotericin B, the process is hindered and the flux and cytolysis is minimal. The authors found that the selective transfer process was regulated by the physical characteristics of both the donor and target membranes.

Szoka and co-workers [1987] suggested that the liposomes reduce the toxicity of amphotericin B by slowing its rate of transfer to a sensitive cellular target. This conclusion was drawn by the authors after experimentation on three possibilities: (1) change in gross organ distribution which they thought could divert the drug from its lethal targets, (2) alteration in the fundamental nature of the interaction between the drug and receptor, and (3) alteration in the rate at which the drug reaches its lethal target. While the kinetics of lethality were found to be different for amphotericin B incorporated into liposomes, there was no change in the disposition in mice after administration of either free or liposomal amphotericin B. Also, the intrinsic interaction of amphotericin B

with the putative cell receptor in mammalian cells remained the same under both conditions.

Another proposed mechanism for the decreased toxicity observed with liposomal amphotericin B formulations is that liposomal encapsulation prevents the direct interaction of drug with cell membranes. This was suggested by Mehta and co-workers [1985], who observed a protection of macrophages and lymphocytes from the toxic effects of amphotericin B following its encapsulation into liposomes. According to these authors, incorporation of amphotericin B into liposomes bypasses the direct interaction of the drug with the macrophage plasma membrane, and through phagocytosis, the drug is directed to the endosome compartment, where it has less potential for toxicity. Wasan and co-workers [1993] have observed that liposomal amphotericin B predominantly associates with high density lipoproteins (HDLs) in human serum and that the amount of amphotericin B associated with HDLs increases when the drug is incorporated into negatively charged liposomes. The association with HDL may serve as a secondary means of delivering drugs incorporated into negatively charged liposomes to cells bearing HDL receptors and away from cells that lack these receptors.

Apparently, all of the above described mechanisms for amphotericin B are based around a hindered release of the drug from liposomes. While the slower and controlled release of amphotericin B from liposomes has been credited mainly to mechanical entrapment of the drug in the vesicle, the same effect may also be due to other factors which include lipid-induced change in the aggregation state of the drug [Dufoure et al., 1984; Janoff et al., 1988] and stabilization of the bilayer [Proffitt et al., 1991]. The restricted release of amphotericin B from liposomal vesicles is thought to be mostly due to the complexation of the drug with lipid material [Gulati et al., 1998]. This also suggests that a closed structure of the liposome is not required for an increased selectivity in the action of amphotericin B.

However, toxicities which have been described for sustained, high-dose therapy with free drugs may also be observed with liposomal drugs [Allen, 1998]. It has been observed that long-circulating liposomal doxorubicin (Caelyx®) given in a high dose regimen can cause desqualmation of the skin in areas of stress (hands and feet), a toxicity

which has also been described for high dose free doxorubicin [Uziely et al., 1995; Goebel et al., 1996]. With high dosing regimens for liposomal CCNU in the rat, we observed similar hematological toxicity and weight loss to that of free drug with the same dosing regimen [Bethune et al., in preparation].

For many applications, liposomal delivery systems are employed to improve the therapeutic index of encapsulated drugs by selectively accumulating in extravascular disease sites. Further, there is increasing evidence which indicates that drug released from liposomes in the circulation does not contribute significantly to the therapeutic activity of liposomal anticancer agents [Mayer et al., 1998]. However, sustained drug exposure alone may not justify the development of liposomal formulations of drugs. This is due to advances being made in the area of portability drug infusion technology. Compact and cost effective infusion pumps are now widely used and these can provide well controlled systemic drug exposure over several days. It seems clear that the most significant advantage for the use of liposome drug carriers in systemic applications arises as a consequence of disease specific changes in vascular permeability that favor accumulation of the intact liposome and associated drug into the site of disease progression. These properties can be differentiated from the benefits of drug infusion technology, which are primarily concerned with the maintenance of circulating blood levels of free drug.

1.4. TARGETING OF LIPOSOMAL DRUGS

It has long been a goal of many scientists to deliver therapeutic agents as close as possible to, or within a target cell. Such targeting would achieve two major aims of drug delivery, the maximum dose of drug to the target cell and avoidance of uptake by non-target tissues to reduce side effects. In this section we will examine the targeting strategies currently in use for liposomal drug delivery.

1.4.1. SPATIAL

As described in a previous section herein, liposomes delivered to the blood circulation are efficiently taken up by macrophages of the RES. This localization pattern agrees with the well-documented role of the RES in the clearance of particulate materials from the circulation. In some instances, this RES trapping is beneficial, such as in the delivery of antibiotics to hepatic and splenic macrophages that are infected with intracellular organisms in some disease states. Specifically, liposomal drug delivery has proved beneficial in the experimental treatment of listerosis [Bakker-Woudenberg, et al., 1988] and in the use of immune stimulants to activate macrophages to a tumoricidal state [Killion and Fidler, 1998]. For busulfan, a drug used for hematologic malignancy and conditioning prior to hematopoietic stem-cell transplantation, a liposomal formulation has shown significantly higher concentrations of the drug in target organs (3-fold in bone marrow and 2-fold in spleen) compared with free busulfan delivery after i.v. administration in the rat [Hassan, et al., 1998].

Another way to spatially target liposomes is to directly inject them to the site of interest, where, because of their size, they would tend to remain localized. This strategy has been used successfully by Kim and co-workers who have used large multivesicular liposomes (MVLs), known commercially as DepoFoam™, as drug depots for sustained delivery of drugs such as the anti-cancer agent cytarabine, the aminoglycoside antibiotic amikacin, and the opiate analgesic morphine [Kim et al., 1987, 1994; Grayson et al., 1995; Perkins, 1999]. The routes for liposomal drug administration include intratumoral, intrathecal, epidural, intraperitoneal, and subcutaneous injection [Perkins, 1999].

If the blood-brain barrier (BBB) or blood-cord barrier remain intact, intrathecal or intracranial delivery of liposomal anti-cancer agents would be more effective than conventional chemotherapy at maintaining therapeutic drug concentrations in the central nervous system (CNS) for an extended period of time. A number of studies in laboratory animals have shown that liposomes do not cross the normal BBB [Tokes et al., 1980; Micklus et al., 1992]. McKeran and co-workers [1985] examined the intracerebral delivery (by Ommaya reservoir) of bleomycin and vincristine [Oliver et al., 1985]

entrapped in negatively charged liposomes to treat human cerebral gliomas. After liposomal drug delivery, these investigators found the accumulation of bleomycin and vincristine in blood and urine in patients with malignant gliomas to be decreased when compared with levels when free drug was injected. Studies investigating the intrathecal delivery of Carmustine (BCNU), in hybrid liposomes (phospholipid:Tween 20, 10:1, m/m) to dogs, indicated that lipid-vesicle delivery of BCNU increased the cerebro spinal fluid (CSF) half-life of BCNU by approximately 2.8-fold as compared to drug solubulized with 5% dextrose/water [Kitamura et al., 1996]. Currently, the FDA has approved a DepoFoam™-encapsulated sustained-release cytarabine (DepoCyt™) for the treatment of neoplastic meiningitis [Senior, 1998]. The Phase I studies in humans found CSF exposure ($AUC_{0-\infty}$) with DepoCyt after lumbar and ventricular injection to be nearly 10-fold higher in patients when compared to free cytarabine injection [Chamberlain et al., 1995].

Another application of liposomal drugs in the CNS is for sustained analgesia and reduced toxicity with opioid analgesic agents. DepoFoam™ encapsulated morphine sulfate has been tested in preclinical studies in both rat and dog models and a Phase I clinical trial was initiated in early 1997 [Senior, 1998; Kim et al., 1996; Yaksh et al., 1999]. The data described by Kim and co-workers [1996] show that single epidural doses of DepoFoam™ encapsulated morphine (C0401) results in equivalent time to peak analgesia compared with free morphine sulfate at doses ranging from 10 to 250 μg in the rat. However, the duration of analgesia was significantly prolonged and the area under the analgesia effect vs. time curve was increased 3 to 19-fold compared with morphine sulfate. In contrast to free morphine sulfate, hemoglobin oxygen saturation was decreased minimally after administration of DepoFoam™ encapsulated morphine and the incidences of catalepsy and loss of corneal reflex were minimal even at large doses. In the dog, epidural administration of C0401 resulted in restrained and persistent release of morphine from the epidural space. This extended release corresponded with an extended duration of analgesia with an attendant increase in the incidence of side effects.

Similar beneficial effects of prolonged analgesia, without concomitant increases in supraspinal side effects, have been observed with intrathecal delivery of liposomal

formulations of the synthetic opioid alfentanil in the rat [Bernards et al., 1992; Wallace et al., 1994]. As described in chapter 2, my investigations with sufentanil and morphine sulfate incorporated into MLVs indicate that drug hydrophobicity plays an important role in drug release from liposomes. In a catheterized pig model, we found, compared to free drug, significantly lower peak drug concentrations and slower elimination-rate profiles after morphine or sufentanil was administered in a MLV preparation to the epidural space.

1.4.2. PASSIVE

The length of time that liposomes are present in the vasculature can affect their biodistribution, and increases in circulation time for small liposomal drugs appears to increase their localization into certain diseased tissues [Papahadjopoulos et al., 1991; Vaage et al., 1997]. This observation has led to clinical approval of several liposomal anticancer agents, including liposomal daunorubicin (DaunoXome®) and doxorubicin (Doxil®) for the treatment of refractory Kaposi's sarcoma and liposomal doxorubicin and vincristine for the treatment of advanced breast and prostate cancer, respectively [Gill et al., 1995 and 1996; Lasic, 1998; Cowens et al., 1993]. Regions of solid tumor growth, infection, and inflammation, have capillaries with increased permeability because of the disease process. Therefore, drug-containing liposomes with long circulation times are able to localize in greater quantities in these regions than in normal tissues, which have intact capillaries that are essentially impermeable to liposomes [Proffitt et al., 1983; Forssen, 1997]. The concentration of drugs can thus be increased several-fold in tissues with increased capillary permeability when delivered in a liposome as compared with free-drug administration [Meyhew et al., 1992; Vaage et al., 1992]. This method of increasing the localization of drugs to diseased sites has been called "passive targeting" [Allen, 1997].

The passive accumulation of small liposomes in diseased tissue is not a selective process as there is also a general increase in the extravascular fluids of these regions. The hydrostatic pressure within these extravascular sites is elevated relative to the

vascular pressure, resulting in a pressure gradient that impedes movement of molecules from the blood into the tissue interstitium [Jain, 1993; Buocher and Jain, 1992]. It must be assumed that additional features lead to selective accumulation of liposomes in the diseased extravascular space. Studies have demonstrated that the lack of a developed lymphatic system in addition to large openings in the vascular endothelial lining may lead to an extravascular "trapping" phenomenon [Boucher and Jain, 1992]. In the absence of lymphatic drainage, interstitial diffusion of molecules leads to egress from the disease site and this diffusion rate is dependent on molecule size, with small molecules exiting more rapidly than large molecules.

Liposome extravasation and accumulation in solid tumors has been well studied and there is a great deal of phenomenological and quantitative evidence demonstrating that liposomes can enter an extravascular site in regions of tumor growth following i.v. administration [Gabizon and Papahadjopoulos, 1988; Papahadjopoulos et al., 1991; Huang et al., 1992]. Although evidence for endothelial cell uptake of liposomes and transcytosis across endothelial cells have been documented, videomicroscopy investigations in solid tumor models indicate that the majority of liposome extravasation occurs directly through the openings present in tumor neovasculature [Wu et al., 1993; Yuan et al., 1994]. This extravasation process appears to be heterogeneous within the tumor and does not appear to be associated with any specific histological characteristics in the tumor mass. The net result of this phenomenon is that, for solid tumors, the peak drug concentrations achieved in tumorous tissues are greater and drug exposure as measured by concentration vs time AUCs is increased when the drug is administered i.v. in a liposomal form as compared to free form.

The treatment of Kaposi's sarcoma (an angiogenic tumor) with long-circulating polyethylene glycol expressed (pegylated) liposomal doxorubicin is an excellent example of passive targeting. Kaposi's sarcoma lesions are characterized by greatly increased vascular permeability in the region of the tumor. Long-circulating pegylated liposomes were able to deliver to patients between 5 to 11 times more doxorubicin to the Kaposi's sarcoma lesions than to normal skin [Northfelt, et al., 1996], leading to overall response rates (complete plus partial responses) of as high as 80% [Bogner et al., 1994].

Encapsulation of drug in liposomes decreased the uptake of doxorubicin by sensitive tissues such as the heart [Uziely et al., 1995]. Localization of the free doxorubicin to cardiac tissue is responsible for a major dose-limiting toxicity of the free drug. In patients treated with liposomal doxorubicin, skin lesions flattened during 2 to 3 weeks of treatment, and rapid reduction of visceral lesions was also observed in some patients [Northfelt et al., 1996; Bogner et al., 1994]. Thus, the improved therapeutic effect depends on both increased delivery of doxorubicin to the Kaposi's sarcoma lesions and decreased delivery of the drug to the heart.

1.4.3. ACTIVE

As we have seen a considerable clinical benefit is possible with passively targeted liposomes, an important question then arises: is it possible to further improve on the results achieved with non-targeted or passively targeted liposomes through the strategy of attaching targeting molecules such as antibodies or other ligands to the liposome surface? Liposomal delivery can be enhanced by incorporation of synthetic amphiphilic peptides into the vesicle surface. For example, lactosylceramide can be used for increasing delivery to hepatocytes with natural galactose receptors [Scherphof et al., 1989]. Results from our laboratory with lactosylceramide liposomes containing the aminoglycoside antibiotic G418 sulfate indicated that this delivery vehicle could accumulate the drug to a greater extent in liver than free drug after i.v. administration in mice [see chapter 5]. Additional work from our laboratory also shows another example of ligand targeting through the use the non-toxic pentameric B subunit of cholera toxin (CTB) [Lian and Ho, 1997]. These colleagues found that CTB could mediate specific binding to liposomes incorporating the ganglioside GM1 and also found this complex provided specific targeting to mucosal cells and tissues expressing GM1 in the mouse.

Possibly the greatest potential for an actively targeted drug delivery vehicle is in the field of cancer chemotherapy. Malignant cells often overexpress receptors for growth factors or other molecules which will help maintain the aggressive growth pattern

characteristic of cancer cells. Ligands for growth factor receptors, the folate receptor, the transferrin receptor, and the low density lipoprotein (LDL) receptor have all been coupled to liposomes for targeting cancer cells in vitro [Allen et al., 1998]. For example, folate targeted liposomes were shown to be internalized into cultured tumor cells by receptor-mediated endocytosis after effective ligand mediated delivery [Lee and Low, 1994]. Also, an increased in vitro cytotoxicity was shown for doxorubicin-loaded folate targeted liposomes over non-targeted liposomal doxorubicin and free drug [Lee and Low, 1995].

The most widely used molecules for targeting liposomes in cancer chemotherapy have been the monoclonal antibodies. Monoclonal antibodies, selected for their ability to bind cancer-associated epitopes on the surface of cancer cells, have been coupled to liposomes to produce targeted drug carriers, often referred to as immunoliposomes [Connor et al., 1985]. However, the use of whole anitbodies, or antibody fragments as targeting ligands is not without problems. The production of monoclonal antibodies is time consuming and expensive. Antibodies are large proteins, which may be denatured or otherwise inactivated by some of the procedures used in the formation and sizing of liposomes, or in the procedures used in the formation of immunoliposomes [Ho et al., 1986]. Also, cancer cells are notorious for their ability to down-regulate, shed or alter their surface epitopes. It will be hard to effectively target tissues when the targeted epitopes have been down-regulated, but a clear understanding of the factors leading to up- or down-regulation of the epitopes may allow a more effective targeting strategy to be developed.

1.5. OBJECTIVES OF THE DISSERTATION

The objectives of this dissertation are to examine the role of drug lipophilicity in drug-lipid interactions and to elucidate the mechanisms behind the enhanced drug efficacy observed with liposomal analgesic, anticancer, and cytotoxic agents compared with free drug delivery through pharmacokinetic and pharmacodynamic studies. The

central hypothesis is that when these drugs are optimally associated with liposomes, the biodistribution and pharmacodynamic profile can be positively modified. The following chapters of this thesis will address: 1) The role of drug-lipid interactions on disposition of liposome-formulated analgesic agents with different hydrophobicity in a pig model, 2) Lipid association increasing the in vitro potency of CCNU against glioma cells and systemic exposure in rats, 3) Lipid association improves the therapeutic index of CCNU and increases 36B-10 tumor uptake in rats, and lastly, a chapter devoted to summary and conclusions.

Table 1. Lipid-based human pharmaceuticals approved for Marketing in the U.S.

Description	Product Name	Size (nm)	Composition	Manufacturer
Amphotericin B Lipid complex	ABELCET® ABLC™	3000	Amphotericin B DMPC: DMPG	The Liposome Co.[a]
Amphotericin B cholesterol sulfate complex	Amphotec™ Amphotericin B Colloidal Dispersion	93	Amphotericin B Na-chol sulfate	Sequus Pharmaceuticals, Inc.[b]
Amphotericin B liposome	AmBisome® VS104	<100	Amphotericin B HSPC Cholesterol DSPG	Nexstar Pharmaceuticals, Inc.[c]
Doxorubicin HCl liposome	Doxil®; Caelyx®	100	Doxorubicin HCl; MPEG-DSPE; HSPC; Cholesterol	Sequus Pharmaceuticals, Inc.[d]
Daunorubicin citrate liposome	DaunoXome® VS103	45	Daunorubicin DSPC Cholesterol	Nexstar Pharmaceuticals, Inc.[e]
Cytarabine liposome injection	DepoCyt™	2000	Cytarabine DOPC; DMPG; Triolein; Cholesterol	DepoTech (SkyePharma Inc.)

DMPC= dimyristoylphosphatidylcholine; DMPG=dimyristoylphosphatidylglycerol; HSPC= hydrogenated soy phosphatidylcholine; DSPG= distearylphosphatidylglycerol; MPEG-DSPE= methoxypolyethylene-glycol distearoylphosphatidylethanolamine; DSPC= distearoylphosphatidylcholine; DOPC=dioleoylphosphatidlylcholine.

[a] October, 1996; [b]October, 1996; [c]August, 1997; [d]November, 1995; [e]April, 1996

Table 2. A comparison of the pharmacokinetics of free and liposomal doxorubicin in humans at a dose of 25 mg/m^2*.

Formulation	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	AUC (mg•h/L)	C_{max} (mg/L)	CL (ml/min)	V_d (L)
Free Drug	0.07	8.7	1.0	3.3	755	254
Liposomal Drug	3.2	45.2	609	12.6	1.33	4.1

*Adapted from Gabizon et al. [1994].

AUC = area under the plasma concentaion-time curve from 0 to ∞; CL = total body clearance; C_{max} = peak plasma concentration; $t_{1/2\alpha}$, $t_{1/2\beta}$ = initial and terminal half-lives; V_d = volume of distribution

Table 3. Comparisons of free and liposomal drugs in single dose, acute lethality studies in mice.

		Free drug		Liposomal drug		
Drug type	Drug class	Name	LD_{50} (mg/kg)	Name	LD_{50} (mg/kg)	Reference
Antibacterial	Aminoglycoside	Gentamicin	40	TLC G-65	>150	Swenson et al., 1991
		Amikacin	~150	VS 107	>240	Petersen et al., 1996
Antifungal	Polyene	Amphotericin B	3	ABELCET®	>40	Clark et al., 1991
		Amphotericin B	2.3	Amphotec™	37	Guo et al., 1991
		Amphotericin B	2.3	AmBisome®	>175	Proffitt et al., 1991
		Nystatin	<4.4	Nyotran	>16	Mehta et al., 1987
Antineoplastic	Anthracycline	Doxorubicin	26	TLC D-99	40	Kanter et al., 1993
		Doxorubicin	10-15	Doxil®	38	Working et al., 1996
		Annamycin	8.8	L-Ann	15.7	Zou et al., 1995

CHAPTER 2: THE ROLE OF DRUG-LIPID INTERACTIONS ON DISPOSITION OF LIPOSOME-FORMULATED ANALGESIC AGENTS WITH DIFFERENT HYDROPHOBICITY IN A PIG MODEL

2.1. INTRODUCTION

Opioid analgesic agents are frequently given epidurally to provide selective spinal analgesia in a variety of clinical situations, including postoperative pain, postpartum pain, and cancer pain [Yaksh, 1987; Cousins and Mather, 1984]. Morphine sulfate, a hydrophilic opioid (octanol:buffer distribution coefficient = 1), is typically given epidurally because it exhibits a relatively long duration of action [Mather, 1983; Bernards et al, 1992]. However, a disadvantage of morphine analgesia is that, to control postoperative or postpartum pain which usually lasts several days, repeated bolus injections are administered that can result in respiratory depression due to either vascular redistribution or rostral cerebrospinal fluid (CSF) movement of the drug [Bernards et al, 1992]. While the more hydrophobic analgesic sufentanil (octanol:buffer distribution coefficient = 1787) partitions into spinal cord tissue more readily, it is cleared more rapidly from the CSF than morphine sulfate; as a result, significant supraspinal side effects are observed due to rapid efflux of this agent to the vasculature and redistribution into the brain [Mather, 1983; Payne, 1987; Cousins et al., 1988; Cohen et al., 1988; Cohen et al., 1993].

Analgesics administered into the epidural space must distribute from this site of administration to reach their sites of action in the spinal cord. A pharmacokinetic model for epidural opioids has been described previously [Cousins and Mather, 1984]. This model includes opioid administration into the epidural space and deposition in epidural fat. From the epidural space, the opioid can cross the dural membrane into the CSF and migrate directly or through bulk flow to gain access to the opioid receptors in the spinal cord. Followed by vascular uptake in the cord tissue, uptake of drug into the vascular system has been demonstrated as the major route of distribution of epidural opioids

[Glynn et al., 1981; Sjostrom et al., 1987; Sjostrom et al., 1988]. Vascular uptake of the opioid occurs due to the concentration gradient that exists with the presence of the numerous thin-walled venous plexi (Batson's plexus) in the epidural space [Batson, 1957]. The relative contributions of these potential routes of elimination of the opioid from the epidural space have been suggested to depend upon the physical and chemical properties of the particular opioid agent.

Lipid solubility has been suggested as an important chemical property for determining a drug's permeability through the spinal meninges [Cousins et al., 1988; Cousins and Mather, 1984; Bromage, 1989]. Previous studies have demonstrated a biphasic relationship between a drug's octanol:buffer distribution coefficient and its meningeal permeability coefficient [Bernards and Hill, 1992; Schoenwald and Ward, 1978; Kishida and Otori, 1980; Yano et al., 1986]. This profile showed that permeability coefficients are found to be low when octanol:buffer coefficients of the opioids are both relatively low or high and the highest permeability coefficient was found for opioids with an intermediate octanol:buffer coefficient such as with alfentanil (octanol:buffer distribution coefficient = 129). Bernards and Hill [1992] explain the biphasic relationship between hydrophobicity and a drug's permeability coefficient is the result of the dual nature of the meningeal permeability barrier. The meningeal cellular barrier can be considered to consist of a hydrophilic fluid domain and hydrophobic cell membrane domain such that passage across the barrier is by a two-step partition process. Therefore, if the drug molecule is highly hydrophobic or hydrophilic, it may be excluded by either the aqueous or lipid barriers, respectively. Drugs of intermediate hydrophobicity move more readily between the lipid and aqueous phases, and their partition coefficients across the meninges are correspondingly greater [Bernards and Hill, 1992].

To improve the therapeutic efficacy of epidurally administered opioids, researchers have investigated liposomes as drug-delivery vehicles. Previous animal studies have suggested that multilamellar liposomes (MLVs) remain as a depot in the epidural space after epidural administration, allowing the sustained-release of analgesics into the CSF [Bernards et al., 1992; Boogaerts et al., 1995; Legros et al., 1990; Umbrain et al., 1995; Umbrain et al., 1997; Kim et al., 1996; Yaksh et al., 1999]. The selection of opioids for

incorporation into liposomes has typically been made empirically rather than through systematic determination of optimum release rate and biodistribution profiles.

Previous studies by Wallace et al. [1994] indicated that when alfentanil was incorporated into synthetic dipalmitoyl phosphatidylcholine (D-DPPC) multilamellar liposomes, this liposomal formulation showed improved efficacy with inhibited rostral neuroaxial spread of the opioid as well as diminished supraspinal redistribution in rats. The use of D-DPPC liposomes eliminated the touch-evoked agitation produced with the use of certain natural L-isomeric phospholipids. Initial studies in our laboratory examined the degree of drug association to D-DPPC multilamellar liposomes and in vitro liposome-release rates of four opiates (morphine, alfentanil, fentanyl, and sufentanil) which exhibited a wide range of hydrophobicity (Table 1). We found liposome-associated morphine and sufentanil to exhibit the lowest and highest degree of drug association and rate of release from the liposomes, respectively.

Based on these findings, the relative epidural, CSF, and plasma exposure to the hydrophilic agent morphine and the highly lipophilic agent sufentanil were studied in a microdialysis pig model. The placement of microdialysis probes in the epidural and intrathecal spaces of pigs allows the direct quantitation of drug release from the liposomal formulation as it passes through the meninges after epidural administration. Given the extreme differences in hydrophobicity between morphine and sufentanil and their distinct interactions with lipid membranes, we hypothesized that incorporating these agents into liposomes would create two distinct liposomal formulations with altered biodistribution compared to that observed with free drug formulations.

For liposome-associated morphine, we predicted the drug would be retained in the epidural space and distribute drug to the intrathecal space and plasma with a sustained-release profile. This is in contrast to free morphine, which would be expected to be quickly released to CSF and plasma. For liposome-associated sufentanil, we anticipated a degree of sustained release to be seen in the epidural space when compared to free sufentanil. Compared to liposomal morphine, we expected liposome-associated sufentanil to show a somewhat lower degree of drug retention in the epidural compartment.

Since it is possible that vascular absorption of highly hydrophobic opioids may be affected by the presence of fat in the epidural space, we further hypothesized that a change in drug:lipid ratio for liposomal morphine and sufentanil would further alter the disposition of liposomal drug. Since sufentanil is considerably more lipophilic than morphine, we anticipated sufentanil to be affected to a greater extent by epidural fats than morphine. Therefore, an increase in lipid content of liposomal sufentanil dosage forms, compared to liposomal morphine, may increase the ability of the liposomes to form a sustained-release depot in the epidural space.

Our results show that opioid hydrophobicity and drug interactions with lipid membranes play a significant role in drug association and dissociation to D-DPPC liposomes both in vitro and in vivo.

2.2. METHODS

2.2.1. MATERIALS

The opioids, morphine, alfentanil, fentanyl, and sufentanil were supplied by the National Institute on Drug Abuse through the Research Triangle Institute (Research Triangle Park, NC). D-α-dipalmitoyl phosphatidylcholine (D-DPPC) was purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were of analytical grade or better.

2.2.2. PREPARATION OF LIPOSOMAL DRUGS

Two distinct types of lipid formulations were used in the two animal experiments described herein. For the in vitro studies and first set of animal experiments, formulations of opioids with multilamellar vesicles (MLVs) were prepared by dissolving

200 mg of D-α-dipalmitoyl phosphatidylcholine (D-DPPC) with 8 mg ^{3}H-sufentanil (0.014 mmol, specific activity = 9 Ci/mmol), 36 mg ^{14}C-morphine (0.054 mmol, specific activity = 671 μCi/mmol), 25 mg ^{3}H-alfentanil (0.055 mmol, specific activity = 12.5 Ci/mmol), or 20 mg ^{3}H-fentanyl (0.054 mmol, specific activity = 11 Ci/mmol) in 1 ml chloroform. The solutions were then evaporated to dryness in a rotary evaporator at room temperature under a low vacuum. The dried lipid and drug mixture was then resuspended in 1 ml sterile phosphate buffered solution (PBS, pH 7.4, composed of 8 g/l NaCl, 0.2 g/l KCl and KH_2PO_4, and 0.16 g/l Na_2HPO_4), vortexed, and incubated for 30 minutes in a 55°C water bath to create MLVs. The resultant suspensions were rapidly frozen in an acetone dry-ice bath and then thawed to room temperature. To efficiently load the drugs into the lipid vesicles, the freeze-thaw cycle was repeated six times. The suspensions were then heated to 60°C in a water bath for 30 min to anneal the membrane, and subsequently cooled to room temperature. The resulting suspension was then diluted with 10 ml PBS and centrifuged at 22,000 x g for 10 min to remove unbound/unencapsulated drugs, this was repeated three times. Removal of unencapsulated drug was verified by measuring drug levels found in the supernatant using radiotracer techniques.

With this procedure, the percent trapping of drug encapsulation from total drug added averaged 30 to 35% for liposomal morphine, with a final drug:lipid ratio (m/m) of 1:17 and 100% for lipid-associated sufentanil with a final drug:lipid ratio of 1:61. The percent trapping for liposome-encapsulated alfentanil was found to be 25% with the final drug:lipid ratio of 1:20, and 30% for liposome-encapsulated fentanyl with a final drug:lipid ratio of 1:17. All procedures were carried out under sterile conditions. The radiochemical purity of morphine, alfentanil, fentanyl, and sufentanil was found to be greater than 98%. The effect of storage (3 months) on drug leakage was minimal, with percent trapping remaining unchanged for each formulation.

For the second set of animal studies, liposomal formulations of morphine and sufentanil with an increased final drug:lipid ratio were examined. Briefly, to 200 mg of D-DPPC lipid, 63 mg ^{3}H-sufentanil and 72 mg ^{14}C-morphine was added, suspended in 1 ml of chloroform, and processed as described above to formulate liposome-encapsulated

drug. The percent trapping of this formulation remained unchanged from the initial set of liposome preparations, with 30 to 35% found for liposomal morphine and 100% for liposomal sufentanil. For this set of liposome preparations, the final drug:lipid ratio achieved was 1:37 and 1:329 for liposome-encapsulated morphine and sufentanil, respectively.

2.2.3. In vitro release rates of liposomal drugs in CSF

To determine the first-order rate of drug release from the D-DPPC liposomes in CSF, 10 µl of prepared liposomes containing morphine (drug:lipid ratio 1:17), alfentanil (1:20), fentanyl (1:17), and sufentanil (1:61) were added to 10 µl of porcine CSF and incubated in a water bath at 37°C. Quadruplicate samples were then removed at adequate intervals over 5 release-rate half-lives for the opioids examined. The samples were then diluted with 1 ml PBS and centrifuged at 22,000 x g for 10 min to separate free (supernatant) from lipid-associated drug in the sample suspension. The entire supernatant was removed from the samples and counted for ^{14}C or ^{3}H radioactivity (TRI-CARB 2200 CA liquid scintillation analyzer, Packard Instrument Company, Downers Grove, IL).

The first-order release-rate constant (K) for each formulation was estimated from the slope of linear regression of the natural logarithm of the free drug concentration versus time plots. The release rate half-life values were derived from the relationship: half-life = 0.693/K.

2.2.4. Animal Model

The cannulated pig model has been described previously [Bernards and Sorkin, 1994]. Briefly, pigs (n=15) weighing 4-7 kg were anesthetized with halothane (1-2%) and nitrous oxide (70%) in oxygen. The animals were orotracheally intubated and their lungs mechanically ventilated. The right femoral and right brachial arteries were

cannulated for blood pressure measurement and blood sampling. Microdialysis probes were prepared from cellulose microdialysis fibers (Spectrum Medical Industries, Houston, TX) with a 215 μm inside diameter, a 235 μm outside diameter, and a molecular weight cut-off of 6,000 daltons. A catheter was placed in the lumbar epidural space, for drug administration, and microdialysis probes were placed in the thoracic epidural and intrathecal space for sample collection. At t = 0, drugs were injected into the epidural space with epidural and intrathecal samples collected every 5 min to 1 hour, and every 10 min to 4 hours to determine drug concentrations. Plasma samples were collected at 0, 2, 5, 10, 20, 40, 60, 90, 120, 150, 180, and 240 min.

To determine drug concentrations in epidural and intrathecal dialysate samples, 5 ml of hydrofluor (National Diagnostics, Mannville, NJ) scintillation fluid was added to the samples. The samples were counted in a Packard liquid scintillation counter (Tri-Carb 2000) for 15 min or until the standard deviation of the depurations per minute was less than or equal to 2%.

2.2.5. Analysis of morphine in plasma

Morphine concentration in plasma was analyzed by a selected-ion monitoring (SIM) GC/MS method originally described by Grinstead [1991]. Samples (0.5 ml) were placed in screw-cap glass culture tubes (13 x 100 mm), to which 20 ng of internal standard (nalorphine, Alltech Associates, Inc., Deerfield, Il.), 1 ml pH 8.9 borate buffer (105 mM boric acid and 28.8 mM sodium borate), and 4 ml of chloroform with isopropyl alcohol (95:5 v/v) were added. The tubes were capped, shaken on a reciprocating shaker at 150 RPM for 15 min and centrifuged for 10 min at 1000 x g. The aqueous layer was removed by suction and the organic layer was transferred to a fresh tube and evaporated to dryness under a stream of nitrogen at 65°C. After cooling to room temperature, 50 μl of pentafluoropropionic anhydride (PFPA, Fluka Chemical Corp. Ronkonkoma, NY) was added to derivatize the samples. The tubes were immediately capped and heated at 65°C for 45 min. Subsequently, the excess PFPA was evaporated under a stream of nitrogen at

room temperature. The residue was reconstituted in 100 µl ethyl acetate, and 2 µl was injected on the GC/MS.

An HP-5MS column (30 m x 0.25 mm x 0.25 µm, Hewlett-Packard Co., Wilmington, DE.) was used with an initial head pressure of 25 psi at 150°C. The injector operated a in splitless constant-flow mode with vacuum compensation. The injector (model 7673B autosampler, Hewlett-Packard Co., Wilmington, DE.) and transfer line temperatures were maintained at 280°C. The GC oven temperature (model 5890II, Hewlett-Packard Co., Wilmington, DE.) was programmed for 150°C for 1 min then 15°C /min to 270°C until nalorphine eluted. The mass spectrometer (model HP5989A, Hewlett-Packard Co., Wilmington, DE.) was operated in SIM mode and monitored at 414.2 m/z for morphine and 440.2 m/z for nalorphine with a dwell time of 200 msec per ion. Concentrations of morphine in the sample were calculated by comparison of peak area or peak height ratios from the unknown samples to standard curves (ratio vs. concentration) prepared by the addition of known amounts of morphine to blank plasma. With this method, the recovery of morphine from plasma after extraction was found to be 95%, with a detection limit of 1.0 ng/ml and a between run coefficient of variation of 8% from 35 to 150 ng/ml.

2.2.6. ANALYSIS OF SUFENTANIL IN PLASMA

Quantitation of sufentanil in plasma was performed using GC/MS in the SIM mode according to the procedure reported by Woestenborghs and co-workers [1981]. First, 1 ml of sample was placed in screw-cap glass culture tubes (16 x 125 mm). Then, 20 ng of internal standard for sufentanil (Janssen Life Science Products, Geel, Belgium), 1.5 ml of 0.1 N NaOH, and 4 ml of heptane:isoamyl alcohol (98.5:1.5 v/v) were added. The tubes were then capped, shaken on a reciprocating shaker at 150 RPM for 15 min, and centrifuged for 10 min at 1000 x g. The aqueous layer was frozen in crushed dry ice and the organic layer was decanted into a fresh tube. The extraction was repeated on the remaining aqueous fraction, and the organic layers were combined. To the organic

fraction, 2 ml of 0.05 M sulfuric acid was added and the tubes were capped and shaken for 30 min. The tubes were centrifuged for 10 min at 1000 x g, and placed in crushed dry ice. The organic layer was discarded and 200 µl of 30% ammonium hydroxide and 2 ml of the heptane with isoamyl alcohol was added to the thawed aqueous layer. The tubes were capped, shaken for 15 min, centrifuged for 10 min at 1000 x g, and placed in crushed dry ice. The organic layer was then poured into a fresh culture tube (12 x 75 mm), evaporated to dryness under a stream of nitrogen at 65°C. The residue was reconstituted in 40 µl methanol, and 3 µl was injected onto the GC/MS.

A DB-1701 column was used (20 m x 0.18 mm x 0.4 µm, J&W Scientific, Folsom, CA.) with a head pressure of 25 psi. The injector (model 7673A autosampler, Hewlett-Packard Co., Wilmington, DE.) operated in a splitless mode with transfer line temperatures maintained at 290°C. The GC oven (model 5890, Hewlett-Packard Co., Wilmington, DE.) was programmed for 150°C for 1 min then 30°/min to 300°C until the IS eluted. The mass spectrometer (model HP5970B, Hewlett-Packard Co., Wilmington, DE.) was monitored at 289.2 m/z for sufentanil and 282.2 m/z for the internal standard and at a dwell time of 200 msec per ion. Concentrations of sufentanil in plasma were calculated by comparison of peak area and peak height ratios from the unknown samples to standard curve (ratio vs. concentration) prepared from the addition of known amounts of sufentanil to blank plasma. With this method, the recovery of sufentanil from plasma after extraction was found to be 85%, with a detection limit of 0.03 ng/ml and a between run coefficient of variation of 10% at 0.5 ng/ml.

2.2.7. PHARMACOKINETIC ANALYSIS

For animal studies, all dialysate concentration determinations were expected to reflect extracellular drug as the result of collecting samples by microdialysis. Plasma, considered a sink compartment towards drug elimination from the spinal sites, was monitored for total drug (plasma protein and/or liposome associated) concentration.

The area under the concentration-time curves ($AUC_{0\text{-}240\ min}$) were calculated from the time of epidural drug administration to the last measured concentration (240 min) in the epidural, intrathecal, and plasma samples by employing the linear trapezoidal rule [Gibaldi and Perrier, 1982]. To normalize for the small differences in liposomal drug doses, free and liposome-encapsulated morphine AUCs were normalized to a 1 mg dose, while free and liposome-encapsulated sufentanil AUCs were normalized to a 52.8 μg dose, levels which reflect the lowest dose administered. The terminal half-life ($t_{1/2\beta}$) of free and liposome formulations were derived from the apparent first-order terminal phase of the concentration-time profiles. At least 8 points in the log-linear terminal phase of each profile was used to estimate half-lives.

2.2.8. Statistical analysis

Data are expressed as mean values ± SD. All statistical comparisons between free and liposomal drugs were made with paired Student's t-tests (two-tail) assuming uniform variance and ANOVA (two-factor without replication) for means was performed to compare experiments with one drug:lipid ratio to another. Differences were considered statistically significant when p values were less than 0.05.

2.3. RESULTS

2.3.1. Opioid association and release rates with liposomes

To determine whether the lipophilicity of analgesics may influence the drug's interaction with the multiple lipid membranes of MLVs, we first prepared liposomes for four analgesic agents with significantly different lipid:buffer partitioning. We then characterized the degree of drug incorporation into the MLVs and drug release from the

lipid carrier. As shown in table 1, the lipophilicity of the analgesic influenced the ability of the MLVs to incorporate the drug as well as the rate of drug release from liposomes in CSF. Morphine sulfate (octanol:buffer distribution coefficient = 1) shows a low degree of drug entrapment in liposomes (approximately 30%) and the slowest rate of drug release, while sufentanil (the most lipophilic analgesic tested with an octanol:buffer distribution coefficient of 1737) shows complete incorporation with the MLVs and the greatest rate of drug release. The opioids alfentanil (octanol:buffer distribution coefficient = 129) and fentanyl (octanol:buffer distribution coefficient = 955) had trapping efficiencies in the liposomes similar to that of morphine sulfate, with approximately 25 and 30% drug incorporation for alfentanil and fentanyl, respectively. Liposome incorporated or lipid-associated sufentanil had a release half-life ($t_{1/2}$) 37-fold shorter than that observed for morphine sulfate. These data suggest that sufentanil may bind peripherally to the surface of the MLVs such that lipid-bound drug may be readily dissociated in the presence of CSF proteins. In contrast, the more hydrophilic morphine sulfate may be incorporated into the aqueous solution encapsulated within the liposomes, allowing drug to be retained within the liposomal bilayers. With drug incorporation similar to morphine sulfate and average drug release rates similar to sufentanil, the opioids alfentanil and fentanyl may be associated to the liposomes in a fashion intermediate to that morphine sulfate and sufentanil. Based on these findings, we chose to examine morphine and sufentanil as model drugs with a large difference in hydrophobicity to determine the role of drug lipophilicity and drug:lipid interactions in modifying drug release and drug disposition after epidural delivery of drug formulated with MLVs.

2.3.2. Pharmacokinetics of free and liposome-formulated morphine (drug:lipid ratio 1:17) and sufentanil (drug:lipid ratio 1:61)

In this pig model, microdialysis probes were placed in epidural and intrathecal sites and the femoral artery was catheterized to allow continuous sampling from these

sites. Hence, this model allows for a detailed examination of diffusible drug concentrations in the respective sampling sites after epidural administration of free and liposomal opioids. Figure 1 shows the mean concentration-time course profiles in epidural, intrathecal, and plasma compartments after epidural administration of free and liposomal formulations of morphine and sufentanil. For this experiment, the drug:lipid ratio was 1:17 and 1:61 for liposome-formulated morphine and sufentanil, respectively. When compared to free drug, mean drug concentrations after administration of either liposomal morphine or sufentanil showed a pronounced sustained-release effect in the epidural space (Fig. 1 A and B). For the liposomal opioids, there is an initial slow accumulation period before maximum concentrations (C_{max}) are reached, followed by a slow elimination phase. In contrast, both free opioids show an immediate rapid decline (distribution plus elimination) followed by a somewhat slower elimination phase from the sampled compartments.

In epidural and intrathecal spaces, liposomal morphine (Fig. 1 A and C, Table 2) shows average C_{max} values to be approximately 10-fold lower than after free drug administration, and liposomal sufentanil (Fig. 1 B and D, and Table 2) shows approximately 5-fold lower C_{max} values as compared to free drug. In plasma, C_{max} values for liposomal morphine and sufentanil (Table 2) were about 4- and 2-fold lower than those of free morphine and sufentanil, respectively. Figure 1 also shows the time of peak concentrations in all sampling sites was similar for morphine and sufentanil in free or liposome groups. These results may indicate a rapid distribution of diffusible opioid occurred from the epidural site of drug administration to the intrathecal and plasma compartments. In contrast to the biphasic decline of drug concentrations observed after free opioid administration, drug levels after liposomal opioid administration declined in a monophasic fashion after C_{max} levels were reached.

To examine the relative exposure and possible mechanisms underlying the altered pharmacokinetics observed with liposome-formulated opioids, the AUC for respective opioids were determined over 240 min in epidural, intrathecal, and plasma samples. This sampling period represents the limit to which free opioids could be confidently detected in all samples. For morphine, a significant difference ($p < 0.05$) was found in the $AUC_{(0-}$

$_{240\ min)}$ values between free and liposomal drug delivery while for sufentanil, the mean partial AUC values were nearly equal after free and liposomal drug administration in epidural, intrathecal, and plasma compartments (Table 2). These results are consistent with the in vitro liposome release-rate profiles observed for liposomal morphine and sufentanil (Table 1).

Collectively, we observed significant reductions in C_{max} values in all three sampling sites when morphine and sufentanil were administered in the MLV formulation. Concentration-time profiles presented here indicate that epidural administration of liposome-formulated opioids results in sustained intermediate concentration levels of free drug in epidural, intrathecal and plasma spaces. The apparent slow elimination rate of drug observed after liposomal drug administration is likely due to the contribution of liposomal release rates offsetting free-drug elimination rates.

2.3.3. PHARMACOKINETICS OF FREE AND LIPOSOME-FORMULATED MORPHINE (DRUG:LIPID RATIO 1:37) AND SUFENTANIL (DRUG:LIPID RATIO 1:329)

To investigate the hypothesis that increased lipid-to-drug ratios may modulate drug elimination profiles after epidural injection in this animal model, the lipid content in lipid-encapsulated morphine formulation was raised 2-fold, to achieve a final drug:lipid ratio of 1:37 and the lipid content in the liposomal sufentanil formulation was raised 5-fold, to achieve a final drug:lipid ratio of 1:329. These ratios represent the maximum concentrations of lipid that could be used without altering the percent of drug incorporation observed with the initial drug:lipid ratio. As shown in Table 3, an increase in the lipid:drug ratio yielded an even greater difference in mean drug exposure levels between free and liposomal drugs for both morphine sulfate and sufentanil. Compared to lower drug:lipid ratios (Table 2), the most significant changes are found with liposomal morphine in the epidural space. Here, liposomal morphine sulfate shows approximately 3-fold lower mean exposure ($AUC_{(0\text{-}240\ min)}$) and $t_{1/2\beta}$ as compared to the liposome formulation with a 2-fold lower drug:lipid ratio. Table 4 illustrates the effect of changing

the drug:lipid ratio on liposomal drug exposure in epidural, intrathecal, and plasma compartments after normalizing for drug exposure when free drug is administered. Statistical analysis between the two drug:lipid ratios examined for both liposome-formulated morphine sulfate and sufentanil shows significant differences ($p < 0.05$) in the epidural space for liposomal morphine sulfate only, while the intrathecal space shows significant differences for both liposome formulations of morphine sulfate and sufentanil with approximately 2-fold lower exposure levels observed for the higher drug:lipid ratio. There were no significant differences in exposure found in plasma for both liposomal morphine sulfate and sufentanil when the drug:lipid ratio was altered.

2.4. DISCUSSION

The studies presented here illustrate that liposomes, when used as drug carriers, can create a sustained-release dosage form for opioid analgesics. As with alfentanil [Wallace et al., 1994], it is believed that the incorporation of morphine sulfate and sufentanil into multilamellar lipid vesicles may enhance analgesic effects and reduce systemic toxicity by slowing the rate of drug clearance from the CNS. Specifically, the opioids morphine sulfate and sufentanil have been used in this report since they show vastly different physical properties. Morphine sulfate, with an octanol/buffer distribution coefficient of 1, is on the low end of lipophilicity, while sufentanil with a lipid-partitioning coefficient of 1737 is extremely lipophilic [Mather, 1983].

We have shown here that the physical differences in hydrophobicity between morphine sulfate and sufentanil have, in part, created distinct association and dissociation profiles to MLVs. Previous pharmacokinetic and pharmacologic studies by Bernards and coworkers in rats[1992], using alfentanil associated with the same liposome vehicle and intrathecal administration, led to the conclusion that drug release from these MLVs in an environment such as CSF may occur by simple diffusion down a concentration gradient from the liposome to CSF and/or the progressive dissolution of the liposome carrier. Also, these investigators speculated that the early analgesia observed in this model was

the result of rapid drug release from the outer layers of the liposomes, and that persistent analgesic effects in the liposome group were the result of continued slow release of drug from the inner liposomal membranes. For a proprietary multivesicular liposome (MVL) preparation (DepoFoam™ drug-delivery system, SkyePharma Inc., San Diego), drug release in the epidural and intrathecal space is thought to be due to a gradual breakdown or reorganization of the lipid membranes [Yaksh et al., 1999; Kohn et al., 1998]. While morphologic studies utilizing techniques such as nuclear magnetic resonance (NMR) should be performed with the liposomal formulations described in this study, morphine sulfate, due to its low hydrophobicity, would be expected to be found encapsulated within the aqueous content of the lipid vesicles, while sufentanil is likely to be incorporated primarily with the acyl chains of the liposome bilayers.

The encapsulation efficiency of opioids into liposomes is thought to be a function of the liposome preparation method, lipid composition, and opioid hydrophobicity [Reig et al., 1989]. The interaction of opiate molecules (buprenophine, codeine, dextromethorphan, diprenophine, etorophine, meperidine, methadone, morphine, and naloxone) with lipids (phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and cholesterol) by using liposomes and monomolecular layers as membrane models has been described previously [Reig et al., 1992]. In these studies, the ability of opiates to induce carboxyfluorescin leakage from liposomes was highly dependent on the hydrophobicity of the opiate molecules, with increased membrane permeability found with opioids of increased lipophilicity (buprenorphine and etorphine). In our studies, liposome-encapsulated morphine showed a relatively low degree of drug entrapment (30%) and a slow rate of drug release from liposomes when the formulation was placed in CSF at 37°C (Table 1). In contrast, sufentanil showed complete incorporation with the liposomes and a fast rate of drug release. Liposomal sufentanil had a drug release half-life nearly 37-fold faster than that observed for liposome-encapsulated morphine sulfate (Table 1). These results are consistent with the hypothesis that morphine sulfate, being more hydrophilic, is predominately incorporated within the aqueous interiors of the MLVs and, therefore, the time to exit the vesicles should be greater than that of sufentanil. While sufentanil is more hydrophobic than morphine, it may be structurally

incompatible for binding deeply into the acyl chains of the lipid bilayer. As a result, sufentanil may be primarily associated peripherally with the liposome bilayers. This type of association may result in a more rapid rate of drug release from liposomes when liposomal membranes containing sufentanil are exposed to dilution in CSF. These results are also consistent with transmembrane (TM) protein models, where peripherally membrane-bound proteins are found to be unstable in their association to lipid bilayers [Killian, 1998].

Epidural and intrathecal administration of liposomal drugs has been used successfully for therapeutic applications. In man, liposome-associated bupivacaine administered epidurally induced a prolonged postsurgical analgesia without concurrent motor blockade and side effects [Boogaerts et al., 1994]. Liposome-encapsulated alfentanil administered intrathecally in rats prolonged analgesia and improved the therapeutic ratio of this drug [Bernards et al, 1992], but the liposome composition in this formulation (L-DPPC) also induced supraspinal side effects on behavior, such as touch-evoked agitation [Wallace et al., 1994]. After a single epidural dose, a DepoFoam™ formulation of morphine sulfate, numbered C0401, showed increased CSF exposure and prolonged duration of analgesia with minimal supraspinal toxic effects when compared with formulations of free morphine sulfate in rats [Kim et al., 1996] and dogs [Yaksh et al., 1999]. All of these studies have used multilamellar vesicles (MLVs).

It has been shown that MLVs as well as small unilamellar vesicles (SUVs) do not cross the intact blood-brain barrier nor the subarachnoid membrane when administered intravenously or in the epidural space [Tokes et al., 1980; Muller and Munz, 1988]. Depending on their size, liposomes may be cleared from an extravascular space by vascular and/or lymphatic routes. The epidural space is very vascular with venous plexi which drains through the azygos vein into the vena cava [Batson, 1957; Durant and Yaksh, 1986]. The epidural space also contains a lymphatic network [Rouviere, 1974] which can effectively remove liposomes [Umbrain et al., 1995]. Umbrain and co-workers [1995] found ^{3}H-cholesterol-labelled SUVs with sizes averaging 50 nm drained unmodified into the systemic circulation through epidural lymphatics in rats. These

results have led to the selection of MLVs for clinical trials using liposome-associated local anaesthetics such as bupivacaine.

After intrathecal drug administration, the major routes of liposome clearance appear to be through arachnoid granulations (villi) in the cervical spine which project into the intracranial venous sinsuses [Ferrante, 1993]. The arachnoid membrane is metabolically active and can form giant vacuoles which communicate with the subdural or the epidural space and provide a system for rapid drainage of the CSF and clearance of cells, debris and drugs [Waggener and Beggs, 1967; Horwath, 1952; Tripathi and Tripathi, 1974]. After intrathecal administration, MLVs are cleared more slowly from the spinal space than SUVs in rats [Umbrain et al., 1997]. These differences were explained in terms of vesicle sizes and volumes. Umbrain and co-workers [1997] found SUVs to be rapidly absorbed into the blood through the arachnoid granulations. In contrast to SUVs, these investigators found MLVs could accumulate in the central nervous system (CNS) with a slow elimination rate. Additionally, previous studies indicated that when alfentanil was incorporated into MLVs composed of L-DPPC, this liposomal formulation could inhibit the rostral neuroaxial spread of the drug as well as diminish the agents supraspinal redistribution after intrathecal administration in rats [Wallace et al., 1994].

After epidural administration in this microdialysis pig model, concentration-time profiles (Figure 1) revealed peak drug concentrations (C_{max}) to be approximately 10-fold lower in epidural and intrathecal spaces and about 5-fold in plasma for liposome-formulated morphine when compared to free drug. For liposomal sufentanil, approximately 5-fold lower C_{max} values were observed in epidural and intrathecal spaces and about 2-fold in plasma when compared to free opioid administration (Table 2). For alfentanil in a similar liposome vehicle, peak plasma concentrations were approximately 2-fold lower than that after intrathecal administration of free drug [Bernards et al., 1992]. Similar decreases in C_{max} have also been observed in rats [Kim et al., 1996] and dogs [Yaksh et al., 1999] after epidural administration of morphine in DepoFoam™. In the rat, C0401 administration resulted in C_{max} levels approximately 3- and 16-fold lower than that observed after free morphine sulfate administration in CSF and serum, respectively. For

the dog, C_{max} levels after C0401 administration were decreased approximately 3-fold in lumbar CSF and serum when compared to free morphine administration.

The observed similar time of peak drug concentrations in epidural, intrathecal and plasma sampling sites after administration of free or liposomal formulations of morphine sulfate and sufentanil (Fig. 1), indicates that efficient transfer exists for free drug between spinal compartments and plasma. These results are consistent with previously described studies for the analgesic agents morphine, alfentanil, meperidine, pethidine, and sufentanil [Wallace et al., 1994; Herz and Tesch, 1971; Gustafsson et al., 1985; Durant and Yaksh, 1986; Nordberg et al., 1984; Sjostrom et al., 1987; Hansdottir et al., 1991]. When delivering liposomal morphine sulfate and sufentanil to the epidural space, there appears to be a lag time for morphine sulfate to reach peak concentrations as compared to liposomal sufentanil in all sampled compartments (Figure 1). These discrepancies in times to reach C_{max} levels reflect a rate limitation set by the distinct liposome release rates (Table 1). Additionally, morphine sulfate's lower meningeal permeability (0.62 ± 0.09 versus 0.75 ± 0.06 cm/min x 10^{-3}) and transmeningeal flux (1.71 ± 0.2 versus 4.84 ± 0.7 $\rho M \cdot min^{-1} \cdot cm^{-2}$) compared to sufentanil may contribute to the observed concentration-time profiles [Bernards and Hill, 1992; Bernards and Kern, 1996].

Examination of opioid exposure and persistence in the CNS indicated a sustained-release formulation was achieved with liposome-formulated morphine and sufentanil when compared with free opioid administration (Table 2). By monitoring microdialysis dialysate in the epidural and intrathecal compartments, we observed the concentration-time course of free drug (free from proteins and liposomes) in these sites. Table 2 shows the relative bioavailability of morphine to the CNS and plasma after liposomal drug delivery to be approximately half-that of free morphine administration. This table also shows the apparent elimination half-life ($t_{1/2\beta}$) values after liposomal morphine to be approximately double that of free morphine delivery. These results indicate that the epidural space retains the MLV preparation of morphine sulfate and slowly releases free drug pools into this compartment. The clinical implication of these findings is that, when liposomal opioid formulations are administered, less frequent dosing may be required to maintain therapeutic drug concentrations in the intrathecal space.

After epidural drug administration, it is likely that liposome-released drug in free form distributes to the intrathecal and plasma spaces in a similar pattern to that observed after free morphine sulfate administration. As a result, elimination half-lives are practically the same between CNS and plasma for free drug and liposome formulations with varied drug:lipid ratios. The relative bioavailability and half-life ($t_{1/2\beta}$) values in epidural, intrathecal, and plasma spaces after free and liposomal sufentanil delivery were approximately equal (Table 2). These results are consistent with the liposome drug-release rates observed in vitro (Table 1), which indicated that liposomal sufentanil may provide a greater degree of CSF exposure than liposomal morphine when partial exposure levels (0 to 4 hours) are measured.

By altering the drug:lipid ratio in the liposomal opioid formulations, by increasing the lipid composition, we anticipated an alteration in drug disposition. We hypothesized that the competition of drug association between liposomal lipid and epidural fats would be shifted such that the drugs would remain associated with the liposomes for a longer period of time. For liposome-formulated morphine, a 2-fold increase in the lipid content appeared to significantly sequester drug in the epidural space. Table 4 reveals exposure to freely diffusible morphine was significantly reduced in epidural and intrathecal sites, with levels approximately 50% lower than those found with the original drug:lipid ratio. In addition to reducing drug exposure, the mean apparent $t_{1/2\beta}$ for morphine was reduced by 2-fold in the liposome group so that values closely resembled those observed after free morphine administration (Tables 2 and 3). These results possibly reflect a decrease in the liposome release rate to such a degree that the contribution of this rate on the free drug elimination profile is not apparent during the experimental period. In this study, the monitored concentrations of morphine are likely to be those which are released from the liposomal formulation during drug administration or perhaps were the result of free drug present in the liposomal dosage. For liposomal sufentanil, even a 5-fold increase in lipid content only slightly altered this opioid's in vivo exposure and apparent elimination half-life compared to the original drug:lipid ratio (Tables 3 and 4). These results may further indicate that sufentanil unstably associates with the lipid bilayers of the liposomes and

that diffusion through these membranes is not impeded by lipid content or the number of bilayers present.

In conclusion, we found that the hydrophobicity of opioids influenced drug association and drug-release rates from D-DPPC liposomes. In addition to hydrophobicity, the degree of drug insertion and orientation in liposome membranes may influence drug release from liposomal formulations. For morphine, using liposome formulations with varied lipid content may represent an important method for systematically evaluating opioid loads that can be delivered into the epidural space without increasing the amount of freely diffusible drug. For the more lipophilic opioid sufentanil, the significant reduction in peak plasma concentrations observed after the administration of liposome formulations may significantly reduce the respiratory depression associated with initial high-doses of this drug. For both morphine and sufentanil, liposomal formulations provided an epidural depot which allowed progressive exposure to local tissues to relatively steady-state concentrations of drug over an extended period. Regardless, incorporation of both opioids in liposomes and administrating them into the epidural space may provide a safe and effective method to prolong the otherwise short duration of action of these agents.

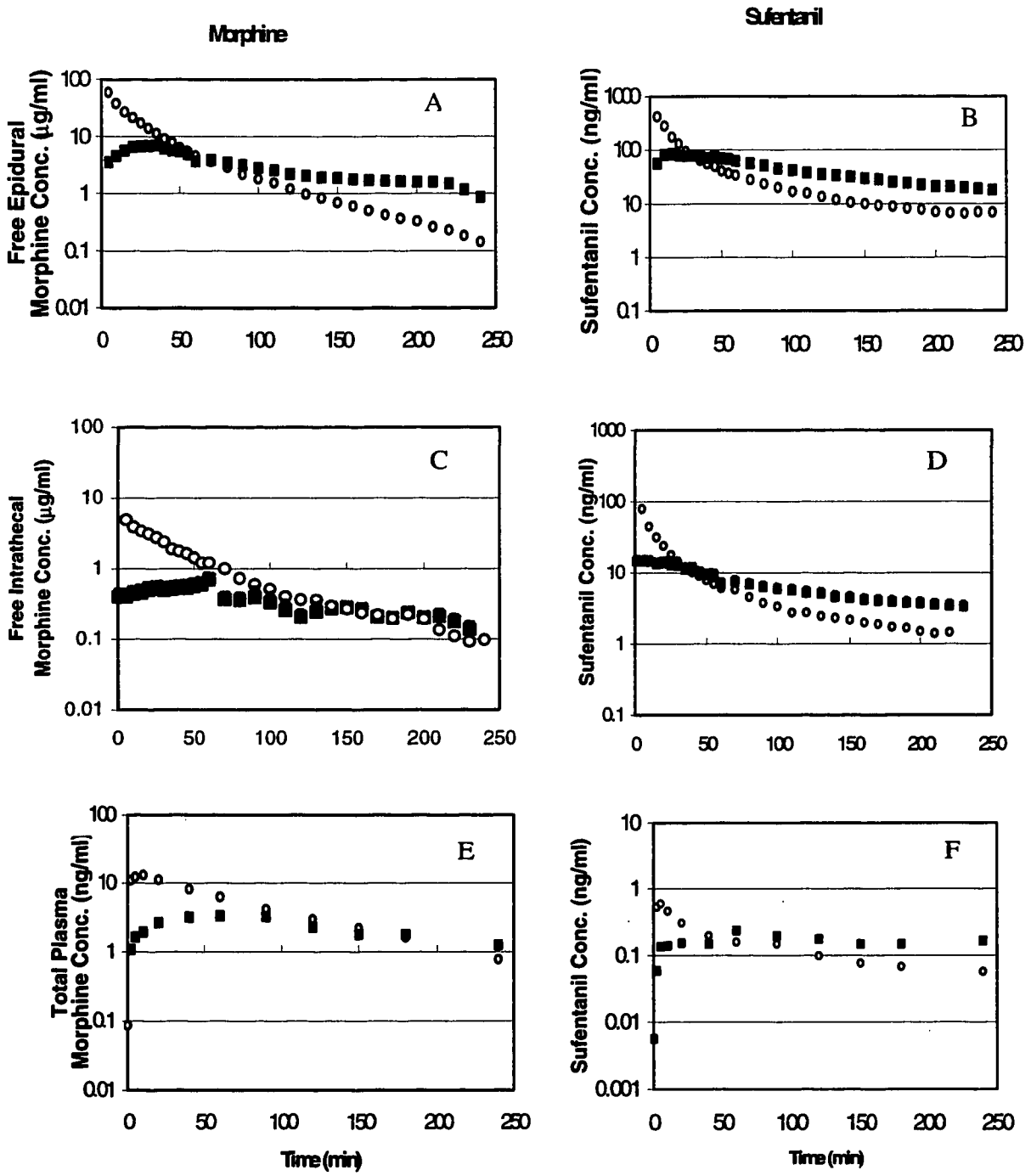

Figure 1. Concentration-time profiles after a single epidural dose of free (○) and liposomal (■) morphine sulfate and sufentanil. Data shown for epidural and intrathecal compartments are derived from microdialysis dialysate and indicate unbound (from protein and liposomes) opioid concentrations. Data points represent the mean from 10 individual pig studies.

Table 1. Liposome encapsulation and average release-rate of opioids in CSF at 37°C.

Opioid[a]	Octanol:Buffer Distribution Coefficient[b]	Drug Incorporation (%)	Release Rate Constant ($hour^{-1}$)	Release Half-life (hours)
Morphine	1	30 ± 5	0.04 ± 0.02	17.30 ± 6.80
Alfentanil	129	25 ± 6	0.90 ± 0.09	0.77 ± 0.07
Fentanyl	955	30 ± 4	0.70 ± 0.08	0.90 ± 0.02
Sufentanil	1737	100 ± 5	1.50 ± 0.10	0.46 ± 0.03

[a]Drug:lipid ratios: 1:17 for morphine, 1:20 for alfentanil, 1:17 for fentanyl, 1:61 for sufentanil.
[b]Values derived from Mather [1983].

Table 2. Relative CNS and plasma exposure to morphine and sufentanil after epidural drug administration of liposomal and free drug formulations.

Compartment		Morphine Formulation[a]		Sufentanil Formulation[a]	
		Free	D-DPPC Liposomes	Free	D-DPPC Liposomes
Epidural					
C_{max}	(μg/ml)	59.0 ± 34.0	6.8 ± 5.6*	0.4 ± 0.3	0.1 ± 0.1*
$AUC_{(0\text{-}240\ min)}$	(μg·min/ml)	1333 ± 609	742 ± 652*	11.1 ± 6.8	10.6 ± 12.0
$t_{1/2\beta}$	(min)	41 ± 11	119 ± 47*	144 ± 100	144 ± 35
Intrathecal					
C_{max}	(μg/ml)	4.9 ± 11.8	0.7 ± 1.0*	0.1 ± 0.1	0.2 ± 0.0*
$AUC_{(0\text{-}240\ min)}$	(μg·min/ml)	190 ± 121	93 ± 90*	2.0 ± 3.9	1.9 ± 2.8
$t_{1/2\beta}$	(min)	48 ± 21	95 ± 50*	96 ± 55	160 ± 102
Plasma					
C_{max}	(ng/ml)	13.1 ± 3.3	3.3 ± 1.9*	0.6 ± 0.3	0.3 ± 0.2*
$AUC_{(0\text{-}240\ min)}$	(μg·min/ml)	1.0 ± 0.3	0.5 ± 0.3*	0.03 ± 0.01	0.05 ± 0.03
$t_{1/2\beta}$	(min)	64 ± 10	135 ± 81*	119 ± 39	287 ± 136
Intrathecal/Plasma					
$AUC_{(0\text{-}240\ min)}$		213 ± 162	180 ± 95	70 ± 87	44 ± 60

[a]For morphine, drug:lipid ratio 1:17 and for sufentanil, drug:lipid ratio 1:61.
* $p < 0.05$ between free and D-DPPC liposome formulations; n = 10 each formulation.

Table 3. Relative CNS and plasma exposure to morphine and sufentanil after epidural drug administration of liposomal and free drug formulations. For morphine, drug:lipid ratio = 1:37 and for sufentanil, drug:lipid ratio = 1:329.

		Morphine Formulation		Sufentanil Formulation	
Compartment		Free	D-DPPC Liposomes	Free	D-DPPC Liposomes
Epidural					
C_{max}	(μg/ml)	177 ±103	10.3 ± 5.9*	0.5 ± 0.3	0.1 ± 0.01*
$AUC_{(0\text{-}240\ min)}$	(μg·min/ml)	1536 ± 682	255 ± 128*	14.0 ± 7.9	8.4 ± 3.6*
$t_{1/2\beta}$	(min)	20 ± 14	46 ± 45*	147 ± 52	178 ± 55
Intrathecal					
C_{max}	(μg /ml)	17.8 ± 11.3	2.8 ± 1.3*	0.1 ± 0.1	0.01 ± 0.02*
$AUC_{(0\text{-}240\ min)}$	(μg·min/ml)	455 ± 124	115 ± 55*	3.3 ± 1.6	1.7 ± 1.5*
$t_{1/2\beta}$	(min)	32 ± 28	46 ± 38	77 ± 7	152 ± 35*
Plasma					
C_{max}	(ng/ml)	12.6 ± 3.4	2.4 ± 1.6*	0.4 ± 0.3	0.1 ± 0.02*
$AUC_{(0\text{-}240\ min)}$	(μg·min/ml)	0.9 ± 0.2	0.4 ± 0.1*	0.03 ± 0.01	0.02 ± 0.01*
$t_{1/2\beta}$	(min)	61 ± 20	83 ± 24*	136 ± 90	300 ± 129*
Intrathecal/Plasma					
$AUC_{(0\text{-}240\ min)}$		480 ± 265	319 ± 185	110 ± 68	84 ± 62

* $p < 0.05$ between free and D-DPPC liposome formulations; n = 5 each formulation.

Table 4. The effect of drug:lipid ratio on the exposure of liposomal opioids in CNS and plasma compartments.

Compartment	Drug:Lipid Ratio	Liposomal AUC[a] / Free AUC	
		Morphine	Sufentanil
Epidural			
	Low	0.6 ± 0.6	0.8 ± 0.5
	High	0.2 ± 0.1*	0.6 ± 0.4
Intrathecal			
	Low	0.6 ± 0.5	1.3 ± 1.1
	High	0.3 ± 0.1*	0.5 ± 0.4*
Plasma			
	Low	0.5 ± 0.2	1.0 ± 0.3
	High	0.4 ± 0.2	0.8 ± 0.1

[a]Liposomal $AUC_{(0 \text{ to } 240 \text{ min})}$ normalized with Free drug $AUC_{(0 \text{ to } 240 \text{ min})}$ values.
[b]Low and high drug:lipid ratios are 1:17 and 1:37 for morphine, 1:61 and 1:329 for sufentanil, respectively.
* $p < 0.05$ between drug:lipid ratios.

CHAPTER 3: LIPID ASSOCIATION INCREASES THE IN VITRO POTENCY OF CCNU AGAINST GLIOMA CELLS AND SYSTEMIC EXPOSURE IN RATS

3.1. INTRODUCTION

There are approximately 17,500 primary brain tumor cases with about 14,000 deaths reported annually, placing the brain cancer mortality rate (80%) second only to lung cancer (85%) in all cancer-related deaths [Poledak and Flannery, 1995]. Malignant brain tumors are the second most common form of solid tumors during childhood and the leading cancer-related cause of death and illness in children [Packer, 1996]. Medulloblastoma, a malignant neuroepithelial tumor of the central nervous system (CNS), affects 14-25% of children and 4-7% of adults with brain tumors. In addition, medulloblastoma has a high degree of intraspinal dissemination, spinal metastasis, and recurrence, which results in a 70-75% mortality rate [Tomlinson et al., 1992]. The propensity of medulloblastoma to undergo cell exfoliation and spread into ventricular cerebrospinal fluid (CSF) is well documented [Provias and Becker, 1996]. The exfoliated tumor cells travel along the CSF to the lumbar sac and adhere to surface invaginations of arachnoidal matter, allowing the cancer to spread throughout the CNS.

Medulloblastoma continues to represent a formidable therapeutic challenge despite increasing neurological surgery and radiotherapeutic techniques for intervention. In general, standard treatment involves surgical removal (which is generally subtotal) of the primary tumor followed by intense craniospinal irradiation and adjunct chemotherapy. Unfortunately, radiation therapy can result in significant neurological and neuroendocrine side effects that are especially detrimental to young children. Therefore, chemotherapy is often substituted for radiotherapy in pediatric cases. However, current chemotherapy is not effective against medulloblastoma since the optimal dose necessary for control of tumor growth is limited by the systemic toxicity of these drugs [Cohen and Packer, 1996]. Dose limitations due to central organ toxicity is a significant barrier to

successful chemotherapy, particularly with alkylating agents of nitrosourea derivatives [Dunkel and Finlay, 1996]. If the toxicity of these alkylating agents can be reduced without compromising their potency, the dose-limiting effects of these chemotherapeutic agents can be overcome.

Most of the alkylating agents used clinically for treating CNS tumors, including medulloblastoma, are of intermediate hydrophobicity [octanol/saline partition coefficients for CCNU = 3; Thiotepa = 2.4; 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) = 1.5] which facilitates penetration across the blood-brain barrier. Once in the biological milieu, the chloroethylnitrosoureas are thought to undergo general base catalysis to generate the reactive alkylating chloroethyl-carbonium and isocyanate-ion intermediates [Chatterji, et al., 1978]. In aqueous buffer at physiological temperature and pH, CCNU decomposes rapidly into the inactive products 2-chloroethanol (18-25%), acetaldehyde (5-10%), and cyclohexylamine (32%) [Reed et al., 1975]. Also, it has been shown that when steady-state conditions are achieved through intravenous infusions of both CCNU and BCNU in humans, the CSF:plasma drug ratios produced are near unity [Lind and Ardiet, 1993]. Under these conditions, tumor as well as normal tissue and cells are indiscriminately exposed to these potent drugs.

The use of liposomes as a drug carrier has been extensivley studied in cancer chemotherapy, and a number of advantages and disadvantages have been discussed. One of the major advantages of using liposomes as a carrier is that they change the pharmacokinetic and pharmacodynamic behavior of a drug, resulting in not only prolongation of plasma half-life but also an improved tissue distribution, potency, and reduction in toxicity of the drug [chapter 1]. The design of liposomes for targeting cytotoxic drugs to tumors after systemic asministration requires inhibition of their fast and avid clearance by the reticuloendothelial system (RES) and avoidance of any premature leakage of their contents in the blood stream [Papahadjopoulos and Gabizon, 1983; Hwang, 1987]. It has been demonstrated previously that small unilamellar vesicles (SUVs, 50-150 nm) are taken up by the RES and removed from the circulation much more slowly than are multilamellar vesicles (MLVs, 200-10,000 nm) [Allen, 1988; Gregoriadis, 1988]. Increased circulation times for liposome-associated drugs can allow

for increased uptake of liposomes (and associated drugs) through extravasation into tissues with enhanced capillary permeability such as solid tumors [Proffitt et al., 1983; Forssen, 1997; Papahadjopoulos et al., 1991; Gabizon et al., 1990; Huang et al., 1992]. It is clear that the most likely mechanism of liposome drug delivery involves the endocytosis of the liposomes [Straubinger et al., 1983; Leserman et al., 1981; Heath et al., 1983; Huang et al., 1983; Matthay et al., 1984; Machy and Leserman, 1983]. The primary mode by which liposomes are incorporated into certain types of cells is known to be endocytosis via the coated pit pathway [Straubinger et al., 1983]. Machy and Leserman [1983] concluded that smaller liposomes are more effective for drug delivery because they are more readily endocytosed by cells. In addition to size, the potency of a liposome-associated drug is affected by liposome charge, and potency varies among different cell lines. For a number of cancer cells tested in vitro, negatively-charged liposomes have been found to associate more effectively and deliver their contents more effectively than neutral liposomes, with optimal delivery observed with negatively charged liposomes of 50-100 nm diameter [Ritter and Rutman, 1980; Heath et al., 1985]. This range of liposome size would allow endocytic uptake mediated by cellular surface pits that have a typical diameter of 150 nm [Pastan and Willingham, 1981].

If lipophilic alkylating agents, such as CCNU, can be incorporated into negatively-charged SUVs, we hypothesized that the drug may be preferentially localized (via liposome endocytosis and/or extravasation at vascularized tissues) in tumor cells, and the rapid decomposition rate of these agents in physiological environments may be decreased. This strategy of drug delivery may reduce the systemic toxicity of these alkylating agents while enhancing their overall therapeutic potency, thus permitting a successful high-dose therapy for CNS tumors.

For this chapter, the majority of which was published in the journal Pharmaceutical Research [Bethune et al., 1999], we have characterized the interactions of CCNU with negatively charged SUVs, determined the ability of this lipid-drug complex to inhibit glioma or normal cell growth in culture, and examined the changes in plasma drug exposure in rats. The next chapter examines the therapeutic effect of liposome-formulated CCNU in rats bearing subcutaneous rat tumors and investigates the possible

mechanisms behind the increased efficacy observed over free drug administration.

3.2. METHODS

3.2.1. REAGENTS AND CHEMICALS

The CCNU was kindly provided by the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Division of Cancer Treatment from the National Cancer Institute. The phospholipids, DMPC and DMPG were purchased from Sygena, Inc. (Cambridge, MA). All other reagents were of analytical grade.

3.2.2. PREPARATIONS OF LIPOSOMAL CCNU

To prepare formulations of CCNU encapsulated in lipid vesicles, we first mixed 100 mg DMPC and DMPG (1:1, mol/mol) with adequate amounts of CCNU to achieve drug:lipid molar ratios of 1:5, 1:10, or as indicated. The dry materials were mixed in 1 ml chloroform in a test tube and the solvent was subsequently evaporated off with a stream of N_2 gas to create a dry film. The dry mixture was then vacuum desiccated for at least 30 min. To prepare desired lipid concentrations, a 1 ml volume of sterile phosphate buffered saline (PBS), pH 7.4, composed of 8 g/l NaCl, 0.2 g/l KCl and KH_2PO_4, and 0.16 g/l Na_2HPO_4 was then added to create a 100 mg/ml lipid suspension. The mixture was then sonicated at room temperature in a bath type sonicator (Laboratory Supplies, Inc., Hicksville, NY) until a uniform, translucent suspension of small unilamellar vesicles (SUVs) was obtained. Typically, total sonication time ranged from 20 to 30 min. To prepare formulations of CCNU admixed to lipid vesicles, appropriate amounts of CCNU dissolved in absolute ethanol were mixed with empty sonicated lipid vesicles to achieve desired drug:lipid ratios (final ethanol concentration <10%).

3.2.3. Determination of CCNU Concentrations in Buffers, Media, and Plasma

To determine CCNU concentrations in samples derived from size-exclusion chromatography, discontinuous sucrose gradients, and degradation studies, we used a colorimetric assay based on the Bratton-Marshall method originally for sulfonamides [Loo and Dion, 1965; Bratton and Marshall, 1939]. Briefly, 25 µl samples were diluted with 175 µl PBS in microwell plates (VWR Scientific Products, Brisbane, CA). Then 25 µl of sulfanilamide reagent (1.5 g sulfaniliamide in 100 ml of 2 N HCl) was added and the samples were incubated in a water bath at 50°C for 45 minutes. Once cooled to room temperature, 16 µl of Bratton-Marshall reagent [30 mg N-(1-Napthyl) ethylene diamine in 10 ml distilled water] was added to each sample and absorbance at λ = 540 nm was measured with a microplate reader (Series 750, Cambridge Technology, Inc., Watertown, MA).

To determine CCNU concentrations in rat plasma samples and cell growth media, ethyl acetate was first added to the samples (5:1, v/v) to precipitate proteins. After centrifuging at 2,000 x g for 5 min to remove precipitated materials, CCNU concentrations in the sample supernatants were determined with a reverse-phase high-performance liquid chromatography (HPLC) system fitted with an octadecylsilica column (Spherisorb 5 µm, 4.6 mm inside diameter and 250 mm length, Phenomenex, Torrance, CA). The isocratic mobile phase consisted of acetonitrile and 0.05 M ammonium acetate (60:40, v/v, adjusted to pH 4.0 with glacial acetic acid). With a flow rate of 1.0 ml/min (pump model 510, Waters Assoc., Millford, MA) at 22°C, CCNU was detected at λ = 232 nm (Waters 486 tunable absorbance detector, Waters Assoc., Milford, MA) and eluted with a retention time of 5.3 ± 0.1 min. A 25 µl injection volume was delivered with an autosampler (Wisp 712, Waters Assoc., Milford, MA). With this method, the typical recovery of CCNU after extraction from plasma or media was determined to be 94 - 106%. Standard curves were generated with reference to the peak heights found with 0-170 µM CCNU. The detection limit for CCNU, as determined by the signal to noise ratio of 3 or greater, was found to be 8 ng for a 25 µl injection.

3.2.4. CHARACTERIZATION OF DRUG-LIPID INTERACTIONS.

To detect the ability of CCNU to induce lipid vesicle aggregation, we first incubated (at room temperature) 200 µl samples consisting of 20 mM empty vesicles with varing concentrations of CCNU in ethanol (total ethanol concentration <10%) to achieve drug:lipid ratios ranging from 1:1 to 1:15. After 30 min, the samples were diluted up to 2 ml, transferred to a quartz cuvette (1 cm x 1 cm) and 90° light-scattering was measured with a fluorescence spectrophotometer (Perkin-Elmer MPF-37, Hitachi, Ltd., Tokyo, Japan), where the excitation and emission wavelengths were both set at 660 nm (excitation slit width = 5 nm, emission slit width = 2 nm). Changes in vesicle diameter due to CCNU incorporation were estimated by photon-correlation spectroscopy. Empty vesicles, vesicles admixed or encapsulated with CCNU were diluted in PBS to a final lipid concentration of 1 mM and mean diameter was measured at room temperature with a Coulter N_4 sub-micron particle analyzer (Coulter Electronics, Inc., Hialeah, FL). Estimated diameters are mean values from 4 runs (each run consisted of 3 measurements).

To measure the incorporation of CCNU into the lipid vesicles, we utilized size-exclusion gel chromatography (Method A) using biogel A-0.5 M with a 10 cm x 1 cm column (Bio-Rad Laboratories, Hercules, CA). A 50 µl aliquot, with 1 mM of free drug in ethanol (10%) or lipid-associated CCNU, was placed onto the column and 250 µl fractions of the running buffer (PBS) were collected and assayed for CCNU with the colorimetric assay as described above. The percentage of CCNU associated with the lipid vesicles was determined by taking the ratio of the amount excluded (vesicle fractions) to the total amount loaded. The recovery of free and lipid-associated CCNU from the column, in four runs of quadruplicate samples, was 87 - 105%.

Further characterization of CCNU's incorporation into lipid vesicles was carried out by a discontinuous sucrose-gradient (Method B). The discontinuous sucrose gradient consisted of 100 µl 65% sucrose and 1 ml of 10% sucrose. 50 µl of 1 mM free or lipid-associated CCNU was placed on top of the gradient and centrifuged for 45 min at 4°C and 20,000 x g in an Eppendorf 5810 R Centrifuge (Brinkman Instruments, Inc.,

Westbury, NY). Under these conditions, free CCNU was found in the bottom fraction while lipid-associated CCNU remained in the top fraction of the tubes. The percentage of lipid-associated CCNU was determined by colorimetric assay. The recovery of CCNU from the gradient, in four runs of quadruplicate samples, was 82 - 99%.

The effect of lipid association on CCNU degradation was investigated in aqueous buffer (PBS), growth media (Minimum-Essential-Media containing 5% antibiotic/antimycotic, 2 mM glutamine, and 10% fetal bovine serum) and rat serum (Sigma Chemical Company, St. Louis, MO) at 37°C. Briefly, 2 ml samples, containing an initial concentration of 86 μM of CCNU from formulations of free (in PBS with 10% ethanol), free with 2% Tween 80 in 0.9% NaCl, and lipid vesicles (admixed or encapsulated with drug:lipid ratios 1:5 and 1:10), were placed in a 37°C water bath. At indicated time points (0, 10, 20, 30, 40, 50, 60, 120, 150, 180, 210, and 240 min), individual samples were removed and immediately flash-frozen to -80°C. Subsequently, the CCNU concentration in each sample was analyzed in aggregate using the colorimetric method as described above. The first-order degradation rate-constant (K) for each formulation was estimated from the disappearance of CCNU by linear regression of the natural logarithm of the CCNU concentration versus time plots. All values were the result of averaging K from each CCNU formulation run in quadruplicate. The $t_{1/2}$ values were derived from the relationship $t_{1/2} = 0.693/K$.

3.2.5. Inhibition of Cell Growth with CCNU

The studies here utilized three types of cancer cells as well as two types of non-cancerous cells. The tumor cells included the medulloblastoma cell line D 283 [He et al., 1989] from the American Type Culture Collection (Rockville, MD), the diploid primary human medulloblastoma cells UW 228-3 which have been characterized previously [Keles et al., 1995], and the rat glioma 36B-10, a malignant astrocytoma also described in detail elsewhere [Spence and Coates, 1978]. Both UW 228-3 and 36B-10 cells were

generated in the Department of Neurological Surgery, University of Washington (Seattle, WA). The primary human diploid fibroblast cells, HDF, were derived from non-cancerous embryonic tonsillar tissue and were kindly provided by the Virology lab of Children's Medical Center (Seattle, WA). The other non-cancerous human cells utilized, FB_3, were derived from a primary culture of fetal brain which resembled astrocytes of normal brain tissue (isolated by J. R. Silber). All cells were cultured in T175 tissue culture flasks (Becton Dickinson Labware, Franklin Lake, NJ) with Minimum-Essential-Media containing 5% antibiotic/antimycotic, 2 mM glutamine, and 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C.

To determine the ability of free and lipid-associated CCNU formulations to inhibit cell growth, 1 x 10^4 cells in 100 µl culture medium were first seeded into flat-bottom microwell tissue culture plates and incubated overnight. Then, 0 to 100 µM of CCNU in preparations of free (in ethanol, ≤ 10%) or lipid vesicles (admixed or encapsulated with drug:lipid ratios 1:5 and 1:10), were added to the cells in 50 µl media and incubated for 3 days. After drug incubation, 1 µCi of [^{3}H]thymidine (^{3}H-dT) with a specific activity of 87.6 Ci/mmol (NEN Products, Boston, MA) in 50 µl media was added to each well and the total ^{3}H-dT incorporated into cellular DNA was determined 18 hours later by harvesting the ^{3}H-DNA onto glass fibers with a cell harvester (PHD Cell Harvester, Cambridge Technologies, Inc., Watertown, MA). The ^{3}H-DNA incorporated into the glass fibers was determined by counting ^{3}H radioactivity (TRI-CARB 2200 CA liquid scintillation analyzer, Packard Instrument Company, Downers Grove, IL). The cellular ^{3}H-dT incorporation was compared with untreated cells to determine the percent of growth inhibition. The drug concentration at which 50% growth inhibition occurred (IC_{50}) was estimated using a maximum effect model described as:

$$I = \frac{I_{max} \bullet C}{IC_{50} + C}$$

where I is equal to the percent inhibition of cell growth, I_{max} the maximum effect (100% inhibition), C the concentration of CCNU, and IC_{50} the concentration at which 50% of the maximal effect was detected. IC_{50} values for free and lipid-associated

CCNU were estimated by a non-linear regression method according to the above model; data were expressed as means ± SD from 8 replicate curves of each CCNU formulation.

As originally described by Wheeler et al. [1975], we sought to access the cytotoxicity of free and liposomal CCNU relative to the total exposure of drug. Doses of free and liposomal CCNU were normalized by the degradation rate of the dosage form in growth media with the equation:

$$\text{Exposure} = C°/K$$

Where C° is equal to the dose of free and liposome-formulated CCNU administered (0-100 μM) to the cells at time = 0, and K is equal to the first-order degradation rate in growth media at 37°C. The K values were 0.0126, 0.0062, and 0.0073 min^{-1} for CCNU in free, liposome admixed, and liposome-encapsulated formulations, respectively.

3.2.6. Effect of lipid association on in vivo exposure and toxicity of CCNU

Normal male Sprague-Dawley rats, specified pathogen free and weighing approximately 200 grams, were used for the in vivo experiments (Charles River Laboratories, Wilmington, MA). The rats were maintained in a pathogen-free environment and fed sterile laboratory pellets and water *ad libitum*. The animals were anesthetized by i.m. injection of 44 mg/kg ketamine and 5 mg/kg xylazine prior to right jugular vein and left carotid artery cannulation. The method described by Bakar and Niazi [1983] was followed for catheter implantation procedures.

Lipid vesicles admixed with CCNU (drug:lipid ratio 1:5) or free-drug suspension (containing 10% ethanol and 2% Tween 80 in sterile 0.9% NaCl), were prepared in dosages of 5 or 10 mg/kg of body weight for intra-arterial dosing. The free-CCNU carrier of ethanol and Tween 80 has been shown to not induce any detectable neurologic side effects [Russo et al., 1984]. Blood was sampled from the right jugular vein just before drug administration and at 3, 8, 15, 20, 26, 34, 43, 50, 63, 75, 90, and 120 min intervals after dosing animals with free-drug suspension (n=12) or admixed vesicles

(n=12). These samples were processed immediately to collect plasma and quickly frozen to -80°C. Plasma samples were analyzed for total CCNU concentration (CCNU that is protein and/or liposome bound) by HPLC analysis within 20 days of collection.

The total systemic exposure to CCNU was estimated by plasma AUC values. The AUC values were calculated using the trapezoidal rule from 0 to 120 min (the detection limit) from CCNU plasma concentrations normalized to a 5 mg/kg dose. The terminal half-life ($t_{1/2\beta}$) was estimated from the slope of the log-linear portion of the CCNU concentration versus time profiles. The level of acute neurotoxicity after drug administration was made from immediate observations of neurological deficit, which progressed from marked disorientation, to zigzag gait, full hindlimb tonic extension, to whole body seizure. Marked neurotoxicity was noted when two or more of the symptoms described above were observed.

To compare the hematological toxicity of lipid-associated CCNU to free drug therapy in rats, a dose-escalation study was conducted to determine the day and extent of white blood cell (WBC) and platelet count suppression (nadir effect). Single i.p. doses of 0, 20, 35, and 50 mg/kg CCNU encapsulated in lipid vesicles (drug:lipid ratio 1:10) or free dosage form (10% ethanol and 2% Tween 80 in 0.9% NaCl) were administered to rats and blood was collected (Unopette microcollection system, Becton-Dickinson, Rutherford, NJ.) from tail veins at -1, 2, 4, 9, and 14 days after drug administration for WBC and platelet count determination. Animals treated with lipid-associated CCNU or free drug were compared to non-treated controls (n=4 each group).

3.2.7. Statistical Analysis

Data were expressed as mean values ± SD. Student's *t* test (two-tailed) for means was performed comparing each experimental group in the particle size determinations, cell growth inhibition experiments, and degradation studies. Differences were considered statistically significant when p values were less than 0.05.

3.3. RESULTS

3.3.1. CHARACTERIZATION OF LIPID-ASSOCIATED CCNU

To determine whether CCNU binds to phospholipid vesicles, we first incubated lipid vesicles composed of DMPC and DMPG with increasing amounts of CCNU in an admixed suspension. This combination of lipids (1:1, m/m) confers a negative net charge at physiological pH through the glycerol group of DMPG. We used DMPG in the DMPC vesicles to increase membrane fluidity and confer the negative charge to these vesicles. To determine the binding interaction between CCNU admixed to DMPC:DMPG lipid vesicles, we monitored the particle size of the lipid vesicles by 90° light-scattering measurement. As shown in Fig. 1, an increase in light-scattering was detected when 20 mM lipid vesicles were exposed to increasing concentrations of CCNU. Near maximal degrees of light-scatter were detected at 4 mM CCNU. With 20 mM lipids in the suspension, the molar ratio of CCNU:lipid which exhibited the maximum light-scatter was estimated to be 1:5. Increasing the concentration of CCNU beyond 4 mM did not produce significant additional increases in light-scatter.

The particle size of CCNU admixed to lipid vesicles was further analyzed with photon-correlation spectroscopy and the data are summarized in Table 1. Incubation of CCNU with lipid vesicles increased the mean particle size while solvent controls (10% ethanol) did not. Similarly, when CCNU was added to the phospholipids during vesicle preparation (encapsulated formulation), we also found an increase in vesicle size due to CCNU incorporation. Even after an extended sonication time of 40 min, vesicles with encapsulated CCNU were significantly larger than vesicles without drug. In fact, the mean lipid-vesicle size was similar for both the admixed and encapsulation method of incorporating CCNU into vesicles (Table 1). These data suggest that CCNU binds to the lipid bilayer of the phospholipid vesicles, leading to an increase in particle size regardless of whether CCNU was added prior to or after lipid vesicles are formed.

To evaluate the extent of CCNU association with the lipid vesicles, we used size-exclusion chromatography (Method A) and discontinuous sucrose-gradient fractionation

(Method B) to separate free CCNU in the suspension from vesicle-associated form. These techniques were validated with a control of CCNU in PBS containing 10% ethanol, for which no CCNU was found in gel-excluded (lipid-associated) fractions. As shown in Table 1, for formulations of CCNU either admixed or encapsulated with lipid vesicles, practically all of the CCNU in suspension was associated with lipid fractions when vesicles were made with drug:lipid mole ratios between 1:5 and 1:10. Taken together, these data indicate that CCNU binds to lipid vesicles with sufficient avidity to be co-purified in the lipid vesicle fractions under these chromatographic conditions.

To examine whether the incorporation of CCNU into lipid bilayers may increase drug stability in suspension, we monitored the rate of drug disappearance in PBS and serum at 37°C. In addition to free drug in ethanol (10%) and lipid-associated CCNU, we also studied the degradation of the in vivo dosage form of free CCNU in suspension (containing 10% ethanol and 2% Tween 80 in 0.9% NaCl). For CCNU admixed or encapsulated with lipid vesicles, we found significant drug stability when compared to free drug in both PBS and serum (Table 2). The formulation of CCNU in suspension with 2% Tween 80 exhibited similar stability to free drug in 10% ethanol in both PBS and serum.

Collectively, these results indicate CCNU was readily incorporated into the lipid vesicles, resulting in increased vesicle size and stabilization of CCNU.

Because the degree of drug incorporation into vesicles and increased drug stability were similar for both the 1:5 and 1:10 drug:lipid ratios, we primarily used 1:5 as the drug:lipid ratio for all the subsequent experiments in this chapter.

3.3.2. Effect of lipid association on CCNU cytotoxicity

To evaluate the effect of the lipid-vesicle carrier on CCNU's ability to inhibit cell growth, we examined brain tumor cells derived from primary cultures (UW 228-3 and 36B-10) and a commercially available cell line (D 283). In addition to tumor cells, primary cultures derived from non-cancerous tonsillar (HDF) and brain (FB_3) tissue were

included in these studies. For these experiments, we fixed the drug:lipid mole ratio for lipid-associated CCNU at 1:5 and 1:10 and varied the concentration of drug incubated with the cells. With a drug:lipid ratio of 1:5, a typical dose-titration curve for medulloblastoma cells (UW 228-3) is shown in Figure 2. We observed that lipid-associated CCNU was significantly more effective in inhibiting medulloblastoma cell growth than free CCNU. In addition, the dose-titration curve was practically the same for the two lipid-associated CCNU preparations (admixed and encapsulated), with dose IC_{50} values estimated to be 18.0 ± 4.9 and 14.0 ± 2.2 μM for UW 228-3 cells and 23.0 ± 3.2 and 25.0 ± 3.6 μM for D 283 cells, respectively (Table 3). We also found that at CCNU doses greater than 35 μM, up to 90% inhibition of cell growth was observed for lipid-associated CCNU (Fig. 2). This compared to approximately 80% cell growth inhibition achieved with concentrations of 100 μM free CCNU.

Due to the substantial differences in drug stability between free and liposome-associated CCNU, we have additionally examined the relative exposure of CCNU present in these growth inhibition studies. Figure 3 shows the effect of CCNU exposure found after incubating UW 228-3 cells with free CCNU and liposome admixed and encapsulated CCNU. This figure indicates that liposome-associated CCNU was still considerably more potent than free CCNU in these cells when drug stability was accounted for. As summarized in Table 4, we observed an approximately 2- to 3-fold decrease in IC_{50} values for liposome-associated CCNU as compared to free CCNU in medulloblastoma cell lines UW 228-3 and D 283. In contrast to human medulloblastoma cells, rat glioma cells (36B-10) did not show an increased sensitivity to lipid-associated CCNU when compared to exposure with free drug (Table 4). In addition to cancer cells, the potency of lipid-associated CCNU was tested against cells derived from non-tumorous human tissues. As shown in Table 4, both lipid-associated CCNU formulations exhibited a nearly 2-fold decreased potency against fibroblasts (HDF) and brain cells (FB_3) when compared to free drug exposure. The exposure IC_{50} values for free drug, CCNU admixed to lipid vesicles, and vesicle-encapsulated CCNU in HDF cells were found to be 825 ± 35, 1629 ± 33, and 1370 ± 21 (nmoles·min)/ml, respectively. The FB_3 cells showed increased sensitivity over the fibroblasts, with exposure IC_{50} values

recorded at 294 ± 32, 581 ± 38, and 438 ± 16 (nmoles·min)/ml for free, admix, and encapsulated CCNU, respectively. When evaluating lipid-associated CCNU formulations with a drug:lipid ratio of 1:10 (data not shown), we found essentially identical results to the drug:lipid ratio of 1:5 (Tables 3 and 4) in all cells.

Thus, these data indicate that lipid-associated CCNU was biologically active and exhibited an equal or higher potency than free CCNU against medulloblastoma cells. In non-tumorous cells, a significant decrease in CCNU lethality due to lipid-association was found.

3.3.3. Effect of lipid association on CCNU exposure in vivo

To determine whether lipid-association can modify the disposition of CCNU in rats, we first studied CCNU admixed to phospholipid vesicles with a drug:lipid mole ratio of 1:5. The plasma AUC, terminal half-life ($t_{1/2\beta}$), and toxic side effects were examined after systemic administration of 5 and 10 mg/kg free or lipid-associated drug. A typical time course of plasma concentrations after i.v. administration of 5 mg/kg free or lipid-associated CCNU is shown in Fig. 4. The results from these experiments are summarized in Table 4. We found that two animals, administered 10 mg/kg free CCNU, exhibited marked and acute neurotoxicity in the form of pronounced disorientation and seizure activity. Therefore, we reduced the free CCNU dosage to 5 mg/kg. At this dose, 7 of 10 animals treated with free CCNU produced similar behavioral neurotoxicity, but without seizure, while the fraction of animals treated with 10 mg/kg dose of lipid-associated CCNU did not exhibit such toxicity (Table 5).

The systemic drug exposure, presented here as AUC, was significantly higher for lipid-associated CCNU (38.53 ± 1.93 μg· min/ml) compared to free drug (20.46 ± 2.15 μg· min/ml) at 5 mg/kg (Table 5). Also, we found the AUC values observed with a 5 mg/kg dose to increase proportionally when the dose was raised to 10 mg/kg for both free and lipid-associated CCNU. In addition, there was a nearly 9-fold increase (17 ± 9 to 147 ± 48 min) in the $t_{1/2\beta}$ when CCNU was given as a lipid-associated form (Table 5).

Taken together, these data indicate that lipid-association increased the total systemic drug exposure and $t_{1/2\beta}$ of CCNU without increasing acute neurotoxicity.

3.4. DISCUSSION

While surgery and radiotherapy are effective for treating patients with large localized brain metastases, both strategies are ineffective for tumors which have spread throughout the CNS. Adjunct chemotherapy with high doses of systemically administered alkylating agents allows effective drug concentrations to reach the CNS [Bobo et al., 1992; Chabner et al., 1973]. However, the high degree of peripheral toxicity associated with high-dose chemotherapy often limits their usefulness. In addition, intrathecal delivery of alkylating agents to reduce peripheral toxicity have, thus far, failed to provide sufficient therapeutic outcomes [Kochi et al., 1993; Edwards et al., 1981; Fulton et al., 1982; Gutin et al., 1976; Gutin et al., 1977].

To overcome these limitations, we have constructed a CCNU-lipid vesicle complex. CCNU associated with negatively charged SUVs increased the drug's stability and enhanced cytotoxicity against medulloblastoma cells (Tables 2 and 3). Administration of lipid-associated CCNU increased systemic drug exposure (AUC) and significantly reduced acute neurotoxicity in rats (Table 5). The exact mechanisms leading to enhanced systemic exposure of CCNU when the drug is associated with lipid vesicles is not clear. In addition to increased drug stability, it is possible that liposome-formulated CCNU has significantly altered distribution and clearance than free drug. This change in drug disposition could result from the large particle size of the lipid-associated CCNU complex (Table 1), as well as through a reduction of nonspecific binding of CCNU to blood components and endothelial cells. Lipid-associated CCNU may reduce the decomposition of the drug in blood, leading to the apparent increase in residence times observed (Table 5). The decomposition rate of CCNU in blood has been shown to be reduced significantly when increased concentrations of lipids are found in plasma [Weinkam, et al., 1980]. In our experiments, we found that lipid-associated

CCNU degraded at a 2-fold lower rate than that of free CCNU in serum (Table 2). While it is likely that both altered distribution and increased drug stability contribute to the observed increase in systemic exposure seen in rats (Table 5), the relative contribution of each mechanism remains to be directly determined.

The intracarotid administration of chemotherapeutic agents has been advocated for the treatment of malignant gliomas, but intracarotid nitrosourea (BCNU) administration has a definite dose limitation because of its toxicity to the central nervous system [Tyler et al., 1986]. Patchy necrosis in the white matter, hemorrhagic necrotizing encephalitis, and hemorrhagic necrosis in the brain have been observed as the end results of toxic doses in animal studies and in clinical trials [Nagahiro et al., 1991]. It is noteworthy that the observed 2-fold increase in systemic CCNU exposure, detected with lipid-associated CCNU formulations of equal dose to free drug, did not lead to an increase in acute neurotoxicity after intercarotid administration in rats (Table 5). Even at a two-fold higher dosage (10 mg/kg versus 5 mg/kg) than free CCNU, none of the animals treated with lipid-associated CCNU exhibited acute neurotoxicity while 7 of 10 animals treated with 5 mg/kg free drug did (Table 5). In an effort to explain the reduced toxicity found with liposome-formulated amphotericin B, Wasan and co-workers [1993] have observed that liposomal amphotericin B predominantly associates with high density lipoproteins (HDLs) in human serum and that the amount of amphotericin B associated with HDLs increases when the drug is incorporated into negatively charged liposomes. The association with HDLs may serve as a secondary means of delivering drugs incorporated into negatively charged liposomes to cells bearing HDL receptors and away from cells that lack these receptors.

While lipid-associated CCNU reduced the observed neurotoxicity in rats, CCNU-lipid vesicles did not reduce the ability of the drug to inhibit tumor cell growth. In our in vitro assay, we found the CCNU complexed to lipid vesicles enhanced drug potency when compared to free CCNU (Fig. 2), even after accounting for the increased stability of CCNU in the media afforded by liposome association (Fig. 3). It is likely that increased uptake of drug, possibly through phagocytosis of the lipid-CCNU vesicles, played a role in reducing the dose and exposure IC_{50} values 2- to 3-fold when compared

to free drug for medulloblastoma cells (Tables 3 and 4). Previous in vitro cytotoxicity studies in mammalian cell lines have demonstrated that liposome uptake into cells was dependent both on the surface properties of the liposomes and on the cell line [Lee et al., 1992]. Lee and coworkers [1992] found negatively charged phospolipids incorporated into egg phosphatidylcholine liposomes were recognized by cell lines to different extents depending on the lipid headgroup and its charge density in the liposome bilayer. These investigators offered that a simple explanation for cell specificity is that exposed cells have different binding sites (or receptors) or, alternatively, similar binding sites but with different surface density for negatively charged liposomes. Apparently, liposome uptake is more complicated than a pure electrostatic interaction between the liposome surface and the cell membrane surface. Whether CCNU dissociates from the lipid vesicles at the cell surface, or inside endosomes or lysosomes, to become cytotoxic is not clear, and the exact mechanism remains to be determined.

In addition, when accounting for CCNU's rapid degradation in aqueous media, we found lipid-associated CCNU increased the exposure IC_{50} values against normal cells (HDF and FB_3) compared to free CCNU (Table 4). These results indicate liposome-formulated CCNU was considerably less toxic to normal cells than free CCNU and that these cells lacked the liposome recognition sites that may be present on the medulloblastoma cells. Whether normal cells from other tissues will exhibit similar or higher sensitivity to liposomal CCNU remains elusive. However, even cancer cells derived from different tissue origins exhibit varying sensitivity to free CCNU [NCI, 1997]. In particular, for medulloblastomas, the greatest difference in nitrosourea sensitivity is seen among genetically similar sublines [Keles et al., 1995].

While the exact mechanisms of altered disposition and enhanced cytoxicity for lipid-associated CCNU remain undetermined, it is clear that CCNU binds to lipid vesicles with high avidity. We found that CCNU remained associated to the lipid vesicles even under conditions of gel-permeation chromatography, where fluid-flow and gel-matrix drug interactions counteract the CCNU-lipid association. Under these conditions, we found that almost all of the CCNU remained in the lipid-associated fractions, provided the drug:lipid ratio was maintained at 1:5 or higher. The detailed molecular interactions,

including depth of CCNU insertion into lipid membranes, remain to be determined. We have concluded that if CCNU is inserted into the lipid membrane at sufficient depth, thereby shielding the drug from the surrounding water, its decomposition in aqueous suspension can be reduced (Table 2). These mechanisms warrant further investigation.

Regardless of the mechanism of enhanced cytotoxicity, reduced neurotoxicity, and increased systemic exposure, these positive results due to lipid association of CCNU may permit dose escalation in patients requiring high-dose chemotherapy. Using lipid-associated CCNU may reduce the acute toxicity of nitrosoureas while enhancing efficacy against tumor cell growth. In a recent study, intrathecally administered BCNU in lipid/micellular vesicles was shown to significantly increase survival for rats implanted with meningeal glioma [Kitamura et al., 1996]. It is possible that a high degree of lipid association for BCNU provided a significant therapeutic advantage. Subsequent experiments in our laboratory [chapter 4] will investigate the mechanisms of the increased therapeutic advantages of liposome-formulated CCNU by examining drug biodistribution and plasma free fractions of drug.

In summary, we have shown that CCNU incorporated readily with phospholipid vesicles to form a tightly bound complex that significantly increased the stability and potency of the parent drug, and enhanced systemic exposure while reducing acute toxicity in rats. This strategy may be used to improve delivery of other short-acting lipophilic chemotherapeutic agents.

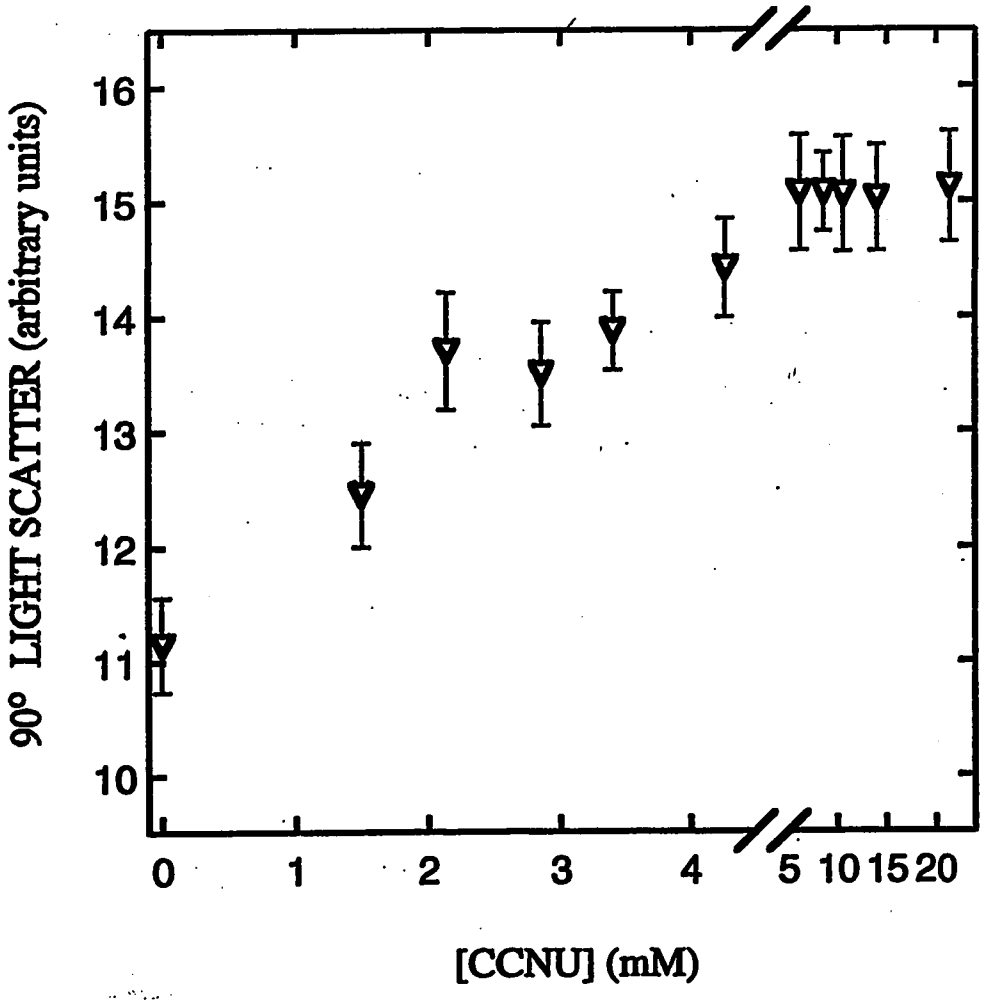

Fig. 1. Effect of CCNU on turbidity of lipid vesicles in suspension. An increasing concentration of CCNU (0-20 mM) was added to 20 mM small unilamellar lipid vesicles composed of DMPC:DMPG (1:1, m/m). The drug-induced lipid aggregation was detected at λ_{ex} and λ_{em} = 660 nm using a spectroflurometer as described in Methods section. Data were expressed as means ± SD of quadruplicate samples.

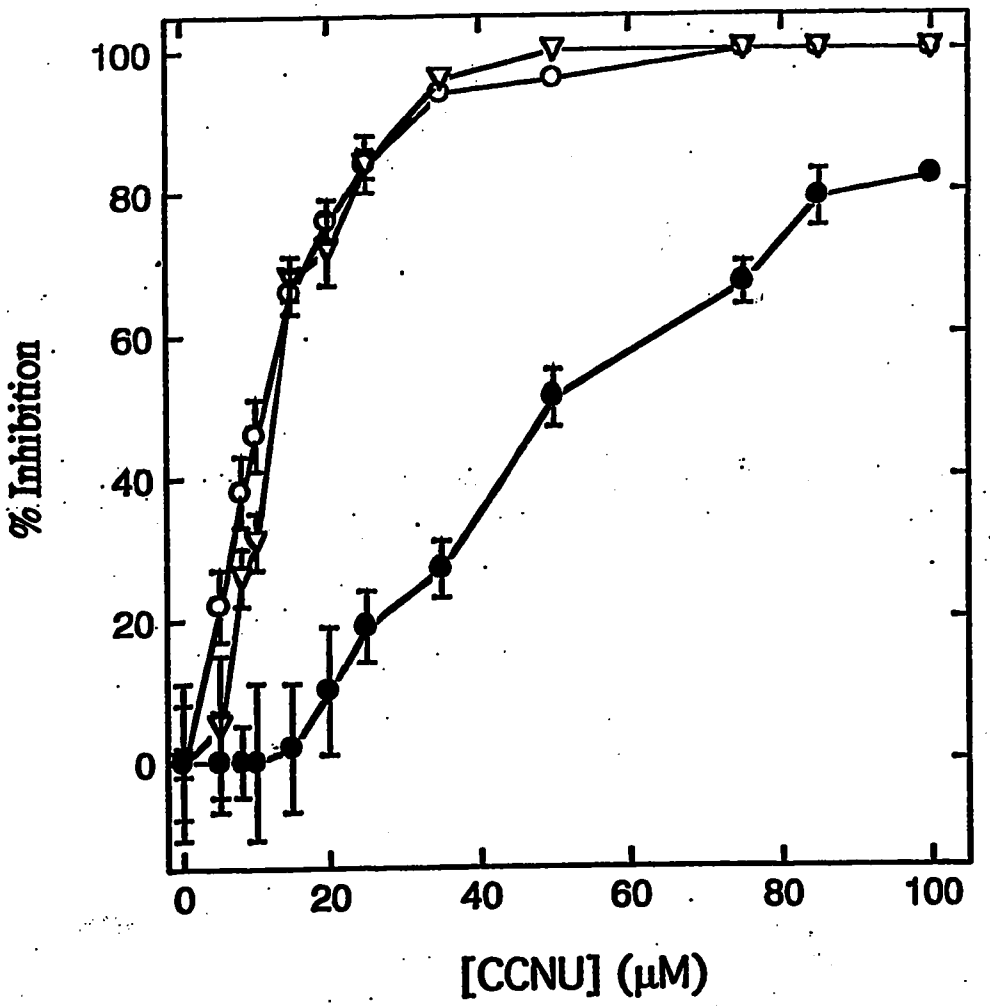

Fig. 2. Effect of lipid association on CCNU's ability to inhibit medulloblastoma cell growth. Medulloblastoma cells (UW 228-3) were incubated with increasing concentrations of CCNU in free (●), encapsulated (○), or admixed vesicle (▽) formulation (drug:lipid ratio 1:5). Cell growth inhibition was determined by ^{3}H-dT incorporation assay. Data were expressed as means ± SD of a typical experiment of four repeated curves.

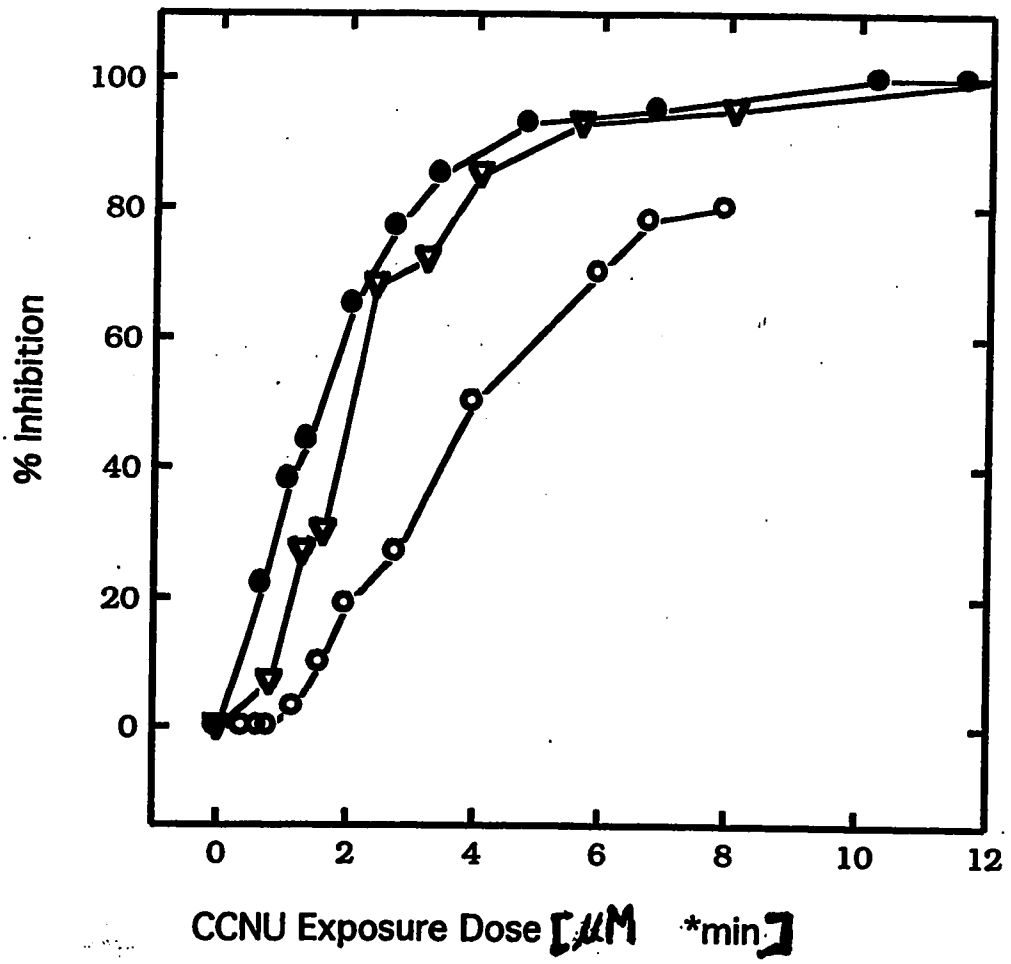

Fig.3. Effect of liposomal CCNU exposure to inhibit medulloblastoma cell growth. Medulloblastoma cells (UW 228-3) were incubated with increasing concentrations of CCNU in free (○), encapsulated (●), or admixed vesicle (▽) formulation (drug:lipid ratio 1:5). Cell growth inhibition was determined by ^{3}H-dT incorporation assay. Data were expressed as means ± SD (error bars included in symbols) of a typical experiment of four repeated curves.

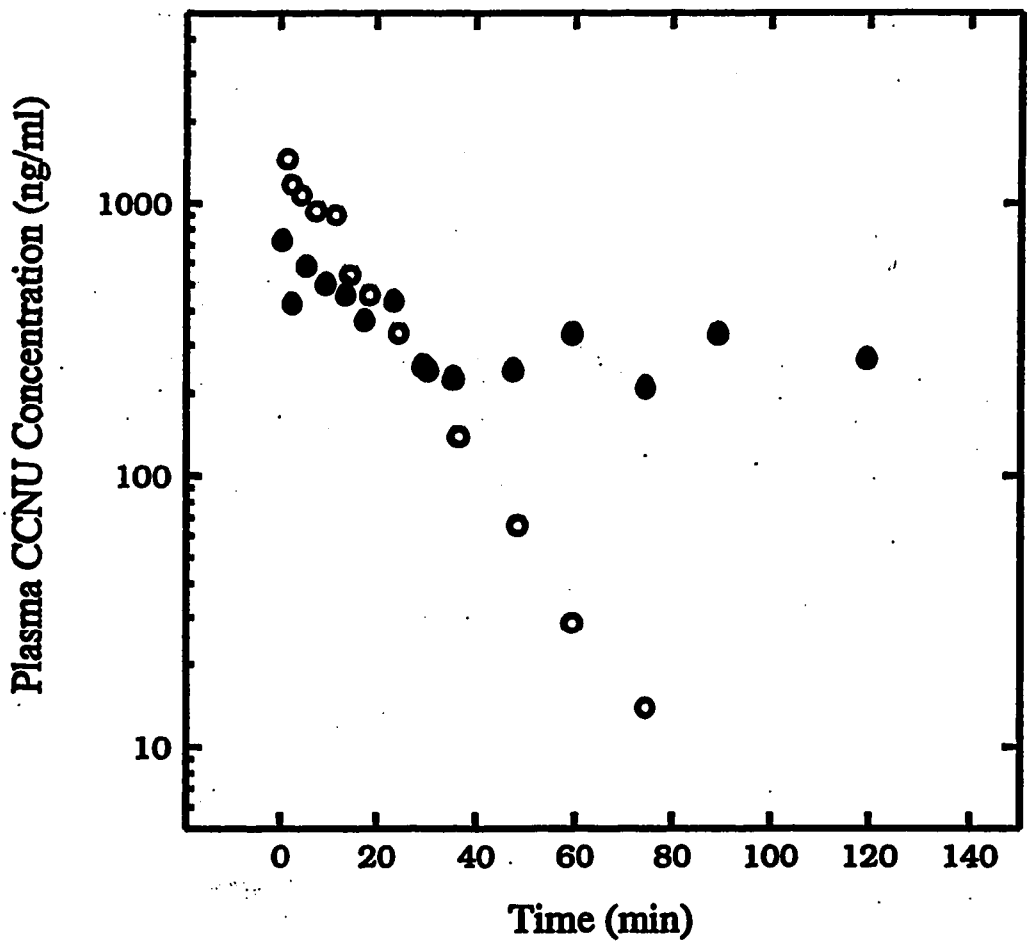

Fig. 4. A representative time course of total drug concentrations in plasma after intravenous administration of 5 mg/kg CCNU in free form (O) or (●) lipid-admixed vesicles (as described Methods section).

Table 1. Effect of drug:lipid ratios on CCNU association to lipid vesicles and vesicle size.

Formulation	Drug:Lipid Ratio	% CCNU Associated with Lipid Vesicles		Mean Diameter (nm)
		Method A[a]	Method B[b]	
Empty vesicles	---	---	---	75 ± 17
Empty vesicles in 10% ethanol	---	---	---	87 ± 7
CCNU Encapsulated in vesicles	< 1:4	12 ± 4	ND[c]	ND
	1:5 - 1:10	95 ± 5	92 ± 4	105 ± 15[d]
CCNU Admixed to vesicles	<1:4	< 20	ND	ND
	1:5	90 ± 9	86 ± 4	123 ± 8[d,e]
	1:6 - 1:10	96 ± 7	90 ± 3	103 ± 15[d]

[a] Determined by size-exclusion chromatography as described in Methods section.
[b] Determined by discontinuous sucrose-gradient centrifugation as described in Methods.
[c] ND = not determined.
[d] $p < 0.05$ when compared to empty vesicles.
[e] $p < 0.05$ when compared with drug:lipid ratios > 1:5.

Table 2. Effect of lipid association on CCNU degradation in suspensions.

CCNU Formulation	PBS		Rat Serum	
	K ($\times 10^{-3}$ min^{-1})	$t_{1/2}$ (min)	K ($\times 10^{-3}$ min^{-1})	$t_{1/2}$ (min)
Free drug in 10% ethanol	11.2 ± 0.6	62 ± 3	15.4 ± 0.6	45 ± 3
Free drug dosage form[a]	11.4 ± 2.0	61 ± 10	14.1 ± 1.0	49 ± 5
Admixed to vesicles				
1:5[b]	5.7 ± 0.4[c]	121 ± 18[c]	7.0 ± 0.4[c]	99 ± 6[c]
1:10	2.5 ± 0.4[c]	278 ± 38[c]	8.1 ± 0.4[c]	86 ± 5[c]
Encapsulated in vesicles				
1:5	6.5 ± 0.5[c]	107 ± 9[c]	8.2 ± 0.2[c]	85 ± 3[c]
1:10	3.5 ± 0.2[c]	201 ± 11[c]	6.2 ± 0.7[c]	112 ± 12[c]

[a]Free-drug suspension containing 10% ethanol and 2% Tween 80 in 0.9% NaCl, used for rat administration.
[b]Drug:lipid ratio.
[c]$p < 0.05$ when compared to free drug in 10% ethanol.

Table 3. Effect of lipid-associated CCNU formulation dose on cell growth inhibition.

		Dose IC_{50}[a] (μM)		
		CCNU Formulation		
Cells	Origin	Free[b]	Admixed	Encapsulated
UW 228-3	Medulloblastoma[c]	83.0 ± 11.0	18.0 ± 4.9[h]	14.0 ± 2.2[h]
D 283	Medulloblastoma[d]	92.5 ± 4.0	23.0 ± 3.2[h]	25.0 ± 3.6[h]
36B-10	Astrocytoma[e]	2.98 ± 1.7	2.2 ± 0.4	2.0 ± 0.3
HDF	Fibroblast[f]	10.4 ± 0.7	10.1 ± 0.5	10.0 ± 0.2
FB_3	Fetal Brain[g]	3.7 ± 0.5	3.6 ± 0.3	3.2 ± 0.2

[a]IC_{50} represents the drug concentration at which 50% growth inhibition was observed after a 3 day cytotoxicity assay. Admixed and encapsulated-CCNU vesicle formulations composed with a drug:lipid ratio of 1:5.
[b]Free-drug formulation containing 10% ethanol in media.
[c]Human cells derived from a primary culture.
[d]Human cell line derived from metastatic medulloblastoma.
[e]Rat glioma.
[f]Human cells derived as primary culture from fetal tonsillar tissue.
[g]Human cells derived from a primary culture of brain tissue.
[h]$p < 0.05$ when compared to free CCNU formulation.

Table 4. Effect of CCNU exposure on cell growth inhibition.

		Exposure IC_{50}[a] (μM·min)		
		CCNU Formulation		
Cells	Origin	Free[b]	Admixed	Encapsulated
UW 228-3	Medulloblastoma[c]	6587 ± 104	2903 ± 41*	1918 ± 34*
D 283	Medulloblastoma[d]	7341 ± 86	3709 ± 40*	3425 ± 42*
36B-10	Astrocytoma[e]	237 ± 28	355 ± 24	274 ± 19
HDF	Fibroblast[f]	825 ± 35	1629 ± 33*	1370 ± 21*
FB_3	Fetal Brain[g]	294 ± 32	581 ± 38*	438 ± 16*

[a]IC_{50} represents the drug exposure at which 50% growth inhibition was observed after a 3 day cytotoxicity assay. Admixed and encapsulated-CCNU vesicle formulations composed with a drug:lipid ratio of 1:5.
[b]Free-drug formulation containing 10% ethanol in media.
[c]Human cells derived from a primary culture.
[d]Human cell line derived from metastatic medulloblastoma.
[e]Rat glioma.
[f]Human cells derived as primary culture from fetal tonsillar tissue.
[g]Human cells derived from a primary culture of brain tissue.
*$p < 0.05$ when compared to free CCNU formulation.

Table 5. Effect of lipid association on exposure and neurotoxicity of CCNU in rats.

CCNU Formulation	Dose (mg/kg)	Neurotoxicity[a]	$AUC_{0\text{-}120min}$[b] (μg·min/ml)	$t_{1/2ß}$ (min)
Free[c]	5	7/10	20.46 ± 2.15	17 ± 9
	10	2/2	ND[d]	ND
Admixed[e]	5	0/4	39.59 ± 1.87*	152 ± 33*
	10	0/8	38.53 ± 1.93	147 ± 48

[a]Fraction of animals showing marked behavioral neurotoxicity (as described in Methods).
[b]AUC (area under the drug concentration versus time curve) values were normalized to a 5 mg/kg dose.
[c]Free-drug dosage form containing 10% ethanol and 2% Tween 80 in 0.9% NaCl.
[d]ND = Not Determined.
[e]CCNU admixed to lipid vesicles with a drug:lipid ratio of 1:5.
*$p < 0.05$ between free and liposome-associated CCNU

CHAPTER 4: LIPID ASSOCIATION IMPROVES THE THERAPEUTIC INDEX OF CCNU AND INCREASES 36B-10 TUMOR UPTAKE IN RATS

4.1. INTRODUCTION

Liposome encapsulation of anticancer drugs has been studied extensively both in the laboratory and in the clinic, with reports of increased plasma drug concentrations, improved tumor delivery, decreased toxicity, and increased efficacy for a variety of cytotoxic agents [Bethune et al., 1999; Mayer et al., 1998; Gabizon, 1994]. The benefits associated with liposome applications in the area of anticancer drug delivery have been related primarily to reduced toxicity associated with the encapsulated form of the drug. Improvements in antitumor potency have been modest, perhaps because most efforts to date have focused on anthracyclines, which exhibit only a modest degree of cytotoxic potency relative to the duration of tumor drug exposure [Boman et al., 1995].

The properties of liposome formulations to increase circulation times and tumor drug accumulation in conjunction with decreased toxicity would be particularly well suited for delivery of highly active alkylating agents such as CCNU. CCNU is an extremely potent nitrosourea, a class of drugs which are some of the most widely used chemotherapeutic compounds in the treatment of CNS tumors [Lesser and Grossman, 1994]. The intermediate lipophilicity of these agents allows high levels to partition into the CNS after systemic administration. However, the clinical use of these drugs is restricted by dose-related toxicities, which produce neurotoxicity and delayed and cumulative myelosuppression [Nagahiro et al., 1991; Carter et al., 1972]. Moreover, the nitrosoureas do not produce durable long-term responses and, even in combination with radiation therapy, are not favorable to the survival of most patients [Walker et al., 1980]. The development of novel agents with increased antitumor activity and decreased toxicity is imperative to improving the treatment of CNS tumors.

Toward this end, we have developed and optimized liposome-formulated CCNU that could enhance the drug's antitumor activity without exacerbating toxic side effects. Incorporating CCNU into negatively charged, small unilamellar vesicles (SUVs) resulted in profound increases in antitumor potency against malignant medulloblastoma cells while exhibiting decreased toxicity to fetal brain cells when compared to free drug in vitro. Additionally, systemic administration of liposomal CCNU in normal rats led to dramatic increases in plasma exposure without concomitant increases in neurotoxicity [Chapter 3].

In light of the improvements in in vitro antitumor potency and plasma exposure for CCNU accompanied by a decrease in toxicity in normal rats [Chapter 3], here we further characterize the efficacy of liposome-encapsulated CCNU in controlling the growth of tumors in a rat model. In addition, we have sought to elucidate the possible mechanisms underlying the improved therapeutic effects of liposomal CCNU in this model by examining tumor-to-plasma accumulation and plasma free fraction levels.

4.2. METHODS

4.2.1. FREE AND LIPOSOMAL CCNU FORMULATIONS

CCNU was kindly provided by the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Division of Cancer Treatment from NCI. The phospholipids, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) were purchased from Sygena, Inc. (Cambridge, MA). Lipid-encapsulated CCNU (drug:lipid molar ratio 1:10) was prepared by first mixing 100 mg DMPC and DMPG (1:1, mol/mol) with CCNU in 1 ml chloroform in a test tube and evaporating off the solvent with a stream of N_2 gas to form a dry film. The dry film was then vacuum desiccated for at least 30 minutes. To prepare desired lipid concentrations, a 1 ml volume of sterile phosphate buffered saline (PBS),

pH 7.4, composed of 8 g/liter NaCl, 0.2 g/liter KCl and KH_2PO_4, and 0.16 g/liter Na_2HPO_4 was then added to create a 100 mg/ml suspension. The mixture was then sonicated at room temperature in a bath type sonicator (Laboratory Supplies, Inc., Hicksville, NY) until a uniform translucent suspension of small unilamellar vesicles approximately 80 nm in size was obtained. Typically, total sonication time ranged from 20 to 30 min. Free CCNU dosages were prepared just prior to drug administration and consisted of dissolving CCNU in a carrier solution of sterile 0.9 % NaCl with 10% ethanol and 2% Tween 80.

4.2.2. ANIMAL MODEL AND TUMOR CELLS

The ethylnitrosourea-induced Fisher F-344 rat malignant astrocytoma, 36B-10 cells were generated in the Department of Neurological Surgery, University of Washington (Seattle, WA) and has been described in detail elsewhere (Spence and Coates, 1978). The glioma cells were cultured in tissue culture flasks (Becton Dickinson Labware, Franklin Lake, NJ) in Waymouth's medium containing 5% antibiotic/antimycotic, 2 mM glutamine, and 10% fetal-bovine serum in a humidified atmosphere of 5% CO_2 at 37°C. In vitro growth inhibition due to CCNU exposure was performed with a ^{3}H-Thymidine uptake assay and is described in detail in the preceding chapter.

Fisher F-344 female rats, weighing approximately 180 g and obtained from Charles River Laboratories (Wilmington, MA), were used in these experiments. The rats were maintained in a specific pathogen-free environment and fed sterile laboratory pellets and water *ad libitum*. Cultured 36B-10 cells were harvested, centrifuged at 900 rpm for 5 min, the resulting supernatant was removed and the cells resuspended with plain Waymouth's media and kept on ice until the suspension was drawn into 1 ml syringes for subcutaneous (s.c.) injection. For subcutaneous tumor inoculation, 0.2 ml of a cell suspension containing 5×10^5 cells was inoculated s.c. to each flank (4 sites) of rats using 22-gauge needles. Animals were monitored daily for weight and tumor development.

There was 100% tumor take in all animals 14 days after inoculation. Tumor measurements were performed with vernier calipers and volume was determined with the following equation:

$$\text{Tumor Volume } (mm^3) = (a^2 \times b)/2$$

where a = shorter length and b = longer length.

4.2.3. CHEMOTHERAPY

Rats bearing s.c. gliomas received free and lipid-encapsulated CCNU, as well as control carrier solutions intraperitoneally (i.p.) when tumors were detected, typically day 14 post tumor innoculation. Controls consisted of animals inoculated with tumors which received free drug carrier solution and blank liposomes. Single and multiple dosing schedules were examined for the effect on tumor volume and hematological toxicity. Single dose-response studies were done with treatments of 0, 20, 35, and 50 mg/kg free and lipid-encapsulated CCNU (n=4 animals each treatment). Multiple dosing schedules consisted of (A), 40 mg/kg on day 14 post tumor inoculation followed by 20 mg/kg 7 and 14 days later and (B), 20 mg/kg 14 days after tumor inoculation followed by 40 mg/kg 7 and 14 days later (n=8 animals for each treatment schedule). Tumor volume data reported 21 days after treatment was initiated was normalized by dividing these final values with pre-treatment tumor volumes.

4.2.4. TOXICITY

Before and during the course of chemotherapy, blood was collected from tail veins using the Unopette Microcollection System (Becton-Dickinson, Rutherford, NJ) and white blood cells (wbc) and platelets were manually counted. In addition, animal weight was monitored daily throughout each experiment.

4.2.5. PHARMACOKINETIC STUDIES

For these studies, 50 mg/kg of free or liposome-encapsulated CCNU was administered i.p. to tumor bearing rats and blood (by cardiac puncture) and tumorous tissues were collected 5, 10, 15, 20, 30, 120, 240, and 360 min after dosing. Plasma was quickly separated from the blood samples after collection and placed with the tumors in dry ice to freeze. Plasma and tumor tissue concentrations were determined by first diluting and homogenizing the samples with chilled homogenizing buffer (50 mM Tris-HCl; 0.1 mM EDTA; pH 2 with HCl). Sample homogenates were extracted (3 times) with 5 volumes of ethyl acetate and centrifuged at 2000 rpm to separate the organic phase from the aqueous layer. The organic supernatants were combined and evaporated to dryness under a stream of nitrogen. As described previously, dried extracts were reconstituted with HPLC mobile phase (acetonitrile:acetate, 60:40, v/v, pH 4) and analyzed by isocratic reverse-phase chromatography [Chapter 3].

Separation of free from liposomal and protein-bound drug was performed using Centrifree (1.0 ml capacity) ultrafiltration devices (Amicon, Inc., a division of Millipore, Bedford, MA) with a molecular weight cutoff of 30,000 daltons. As previously characterized for liposome-associated doxorubicin and vincristine, these devices separate free from bound drug under equilibrium conditions. Centrifree devices hold filtration membranes which are anisotropic, hydrophilic, exhibit low adsorption properties, and have a sample hold-up volume of 10 µl or less [Mayer and St.-Onge, 1995]. After i.p. administration of free CCNU, plasma samples (n=2) were collected at 10, 15, 20, and 30 min. After dosing with liposomal CCNU, plasma samples were collected at 30, 60, 120, 240, and 360 min. Centrifree devices containing rat plasma were spun at 2000*g* for 20 min in a refrigerated (4°C) Beckman J21-C centrifuge equipped with a fixed-angle rotor (JA-20). The ultrafiltrate and bound fractions were then frozen on dry ice and stored at -80°C until drug concentrations were analyzed by HPLC analysis. The ultrafiltrate and bound concentrations of CCNU were compared to CCNU concentrations in total plasma to determine % free and % bound fractions of drug.

4.2.6. STATISICAL ANALYSIS

Data were expressed as mean values ± SD or SE (standard error). Student's t-test (two-tailed) for means was performed comparing each experimental group. Differences were considered significant when p values were less than 0.05.

4.3. RESULTS

To investigate the relative sensitivity of 36B-10 rat glioma cells to free and liposome-encapsulated CCNU, we first verified the sensitivity of 36B-10 cells to both free and liposomal CCNU. Figure 1 shows the effect of free and liposome-encapsulated CCNU exposure on 36B-10 cell growth. The growth inhibition curves for both formulations of CCNU, after normalizing for the slower degradation rate of liposome-formulated CCNU, appear to be identical. However, in Figure 2 and results in the previous chapter (Fig. 4) indicate that, for the same dose, plasma exposure to CCNU in normal rats was increased at least 2-fold after liposomal as compared to free drug administration. Figure 2 shows a comparison of plasma CCNU concentrations at indicated time points in tumor-bearing rats receiving the same dose of free or liposome-encapsulated CCNU. We observed approximately 5-fold higher CCNU plasma concentrations after liposomal drug delivery as compared to free drug injection from 10 to 30 minutes after intraperitoneal dosing (Fig. 2). CCNU concentrations (μg/ml) at 10 min were 10.2 ± 1.7 vs 47.0 ± 9.3, at 30 min 2.2 ± 0.3 vs 14.4 ± 0.6 after free and liposomal drug delivery, respectively. At extended times, 2 and 4 hours after drug administration, CCNU is detected only in plasma of animals treated with liposome-formulated CCNU.

With the hypothesis that increased plasma exposure of liposomal CCNU may also lead to increased tumor exposure, and therefore increased efficacy over free drug, we compared the effects of free and liposome-encapsulated CCNU on tumor progression in rats implanted with 36B-10 tumors. Figure 3 shows the time course of tumor progression after a single bolus dose of 0, 20, 35, or 50 mg/kg CCNU administered in free (panel A) or liposome formulation (panel B). Tumor progression was substantially delayed when CCNU was delivered in liposomes as compared to free formulation. Figure 4 compares the dose-response of free and liposomal CCNU on tumor volumes measured 21 days after treatment. There is a clear dose-response effect up to 35 mg/kg for both free and liposomal CCNU. Increasing the dose to 50 mg/kg did not provide further tumor growth reduction. which is not significantly increased with an increase in dose. Table 1 summarizes the antitumor effects and hematological toxicity of single-dose administration of free and liposomal CCNU. For each dose examined, tumor volumes observed after liposomal CCNU treatment were found to be approximately 2-fold lower than that observed for free CCNU therapy.

In addition to evaluating tumor response, systemic toxicity between free and liposome-encapsulated CCNU was evaluated by monitoring peripheral white blood cells (wbc) and platelets as well as body weight. Table 1 shows the systemic toxicity observed 4 days after single-dose treatments of free and liposomal CCNU. In these studies, the lowest wbc and platelet counts were found at 4 days post drug administration, however, even at the highest dose of 50 mg/kg, no significant differences in toxicity were found between free and liposomal CCNU. Also, single-dose treatments with both free and liposomal CCNU did not inhibit tumor progression beyond 12 days after drug administration.

To optimize tumor response and systemic toxicity with a more aggressive dosing regimen, two multiple-dosing regimens with free and liposomal CCNU were performed. The first regimen consisted of single weekly doses of 40, 20, and 20 mg/kg (80 mg/kg total dose) and was designed with the intention to suppress early tumor progression with an initial dose of 40 mg/kg, followed weekly by 20 mg/kg maintenance doses. The second regimen, with weekly doses of 20, 40, and 40 mg/kg (100 mg/kg total) was

chosen with the intention of controlling the exponential tumor growth found at the later stage of therapy. Table 2 shows the results from a weekly dosing regimen with cumulative doses of 80 and 100 mg/kg free and liposome-encapsulated CCNU. As with the single-dose treatments (Table 1), endpoint tumor volumes from multiple-doses of liposome-encapsulated CCNU treatments were approximately 2-fold lower than those found after free drug treatments (Table 2) and the time course of tumor progression was significantly altered (data not shown) from single-dose studies. As Table 2 shows, hematological toxicity 7 days after a cumulative dose of 80 mg/kg liposome-encapsulated was found to be significantly lower than that after the same dosing regimen of free drug. However, with a cumulative dosage of 100 mg/kg, both free and liposomal CCNU showed equivalent decreases in wbcs, platelets, and body weight.

To elucidate the mechanisms of increased antitumor activity provided by liposome formulated CCNU, we monitored tumor concentrations of CCNU after free and liposome-encapsulated drug administration. In contrast to free CCNU treatment, we hypothesized that liposomal CCNU may accumulate in tumors as a liposome-associated complex. If CCNU levels in tumors exceed those in plasma, after delivery of the liposome formulation, it would suggest retention of the drug with liposomes in plasma and subsequent passive uptake of liposomal drug by the tumor. If drug levels in the tumors are the same as those found in plasma, it may indicate rapid dissociation of CCNU from the liposomes occurs in plasma and the tumor-to-plasma ratio would be similar to that found after free CCNU administration. Therefore, we examined drug concentrations in tumors relative to plasma after both free and liposome-encapsulated CCNU administration. Figure 5 shows the ratio between tumor and plasma concentrations after free and liposomal CCNU delivery. Initial time points indicate that, after administration of free CCNU, there is rapid drug accumulation in the tumor, such that the ratio between tumor and plasma concentrations has reached unity by 30 min. By 2 hours after free drug administration, there were no detectable concentrations of CCNU in plasma or tumors. In contrast, tumor accumulation from plasma takes considerably longer after liposome-encapsulated drug delivery and tumor concentrations exceed plasma levels by nearly 10-fold from 2 to 6 hours after liposomal CCNU administration.

Next, we explored some of the possible mechanisms behind the lower hematotoxicity (observed in this report) and neurotoxicity [Chapter 3] for animals treated with liposomal CCNU. We examined the free and protein bound fractions of CCNU found in rat plasma. The data presented in Table 3 are mean ± SD of the % free and % bound fractions. After a 50 mg/kg dose, we found plasma free fractions of 5.0 ± 1.5 % and 13.2 ± 1.4 % after free and liposome-encapsulated CCNU administration, respectively. These results indicate that, compared to free drug formulations, plasma free fractions were halved with liposome-formulated CCNU.

4.4. DISCUSSION

We have shown here that the antitumor activity of liposome-encapsulated CCNU is significantly greater than that of free drug in the s.c. inoculated 36B-10 rat glioma model. Previous studies with this rat-tumor model revealed that increased survival times could not be achieved with escalating doses of BCNU, a more hydrophillic analog of CCNU [Spence and Geraci, 1981]. These investigators suggested that the effectiveness of the nitrosourea is in part limited by drug delivery problems. We have substantiated these speculations through our results between the potency of liposomal CCNU with 36B-10 cells in culture and that found in tumor-bearing rats. While we found liposomal CCNU to inhibit 36B-10 cell growth to the same extent as free CCNU in vitro (Fig. 1), treatments to tumor-bearing rats indicate a significant 2-fold decrease in tumor progression was found with liposomal CCNU therapy as compared to free drug (Tables 1 and 2).

One of the mechanisms for this increased therapeutic effect is the increased circulation time found with lipid-associated CCNU, which allows increased tumor exposure. When CCNU is formulated in SUVs of approximately 80 nm diameter, we found substantially greater and prolonged plasma concentrations of CCNU compared to free drug (Fig. 2). Many studies have shown that the size of liposomes is one of the most important determinants of their longevity in the bloodstream [Huang et al., 1992; Proffitt

et al., 1983]. With decreased recognition by phagocytic cells of the RES, small unilamellar liposomes (SUVs) can remain significantly longer in the circulation than larger types of liposomes [Allen and Everst, 1983; Juliano and Stamp, 1979; Huang, 1992]. The observation that long-circulating liposomes of small size (<100 nm) accumulate in the interstitial fluid of transplanted tumors at levels comparable to those in RES-rich organs, such as liver, is the basis for a renewed momentum in the search of liposomal drug formulations with potential applications in cancer therapy [Proffit et al., 1983; Ogihara-Umeda and Kojima, 1988; Gabizon et al., 1990; Huang et al., 1992]. The enhanced drug accumulation in tumors is apparently related to prolonged circulation times of liposomes in vivo. When we compared 36B-10 tumor concentrations of CCNU to those in plasma, after free and liposome-encapsulated drug administration, we found greater than 10-fold higher levels of CCNU reaching the tumor from plasma when the drug was administered in the liposomal formulation (Fig. 5). The results of tumor drug concentrations reaching, but not exceeding, the concentrations in plasma after free CCNU delivery, is consistent with the knowledge that all clinically available nitrosoureas enter tumor cells via passive diffusion [Begleiter et al., 1977].

For liposome-encapsulated CCNU, there are two likely mechanisms of drug delivery to tumors; (1) release of drug from circulating liposomes followed by free distribution into all body compartments and/or (2) extravasation of liposomes into the tumor interstitial fluid followed by in situ release of drug. Pharmacologically, the latter mechanism is the most interesting since it represents first order targeting. Many types of tumors are well vascularized, increasing the opportunity for liposome delivery. In general, vessels within tumors have wide endothelial junctions, a large number of fenestrae, and discontinuous or absent basement membranes [Jain, 1987]. Also, tumor angiogenesis includes the formation of capillary sprouts which are highly permeable due to gaps between adjacent endothelial cells and openings at the vessel termini [Folkman and Haudenschild, 1981]. The 36B-10 glioma, when implanted s.c., is well vascularized and grows rapidly (Fig. 3). The ability of liposome-encapsulated CCNU to extravasate into s.c. 36B-10 tumors is supported in this study through the slow increases in tumor drug concentrations which exceed that in plasma at extended time points (Fig. 5),

consistent with a liposome extravasation process, and similar to what has been described in other preclinical models [Gabizon, 1992; Huang et al., 1992]. We found that tumor-to-plasma ratios were much higher than unity after liposomal CCNU administration (Fig. 5). This can only be achieved if a significant fraction of the liposome-CCNU complex accumulates intact in the tumor. However, we cannot rule out concomitant drug release from a small fraction of liposomes in circulation. Additional studies are needed to examine whether CCNU is released exclusively in the plasma compartment, and/or after transport to tumor sites.

While liposome-formulated CCNU was found to increase therapeutic effects by reducing tumor volumes, we did not observe a concomitant increase in side effects. We found the hematoxicity observed after liposomal CCNU administration to be equal or less than that of free CCNU (Tables 1 and 2). Similar results of increased efficacy and decreased toxicity have also been observed with liposomal formulations of doxorubicin [Gabizon et al., 1982; Gabizon et al., 1989; Mayer et al., 1990]. The relationship between plasma exposure to free drug and hematological toxicity has been demonstrated for a number of commonly used anticancer agents, including CCNU [Ratain et al., 1990]. For liposomal drug delivery, bone marrow is considered a major site of interest for liposome localization due to possible drug toxicity and attendant hematotoxicity. In a detailed study of liposome biodistribution, Huang and coworkers [1992] observed SUVs to reside exclusively in the resident macrophages, and not the hematopoietic cells, in the bone marrow of mice after intravenous injection. This selective uptake in bone marrow may also be present with liposome-formulated CCNU, and may explain the equal or decreased hematological toxicity when compared with free CCNU administration. Additional studies are needed to elucidate these mechanisms.

Moreover, to investigate potential mechanisms for the equal or decreased toxicity observed with liposomal CCNU, we quantitated the % free and % protein bound drug after intraperitoneal dosing. In this study, we found plasma free fractions of CCNU to be approximately half that after liposomal drug delivery as compared to free CCNU delivery (Table 3). These findings may provide a possible mechanism to explain the hematoxicity observed with liposomal CCNU in this tumor model (Tables 1 and 2). It is likely that

both altered cellular distribution and decreased plasma free fractions of CCNU may be involved in the reduction side effects observed here.

The plasma free-fraction results found here also have relevance on clinical applications of systemic delivery of liposome-formulated CCNU to treat brain tumors. Whether a decrease in plasma free-fraction found after liposome delivery of CCNU may decrease the availability of CCNU to the brain remains to be determined. However, a number of recent studies with indicate that plasma-protein bound agents, such as tryptophan, oxicams, and methylprednisolone, are as available to cross the BBB and enter the CNS as free pools of drug [Pardridge, 1988; Jolliet et al., 1997; Chen et al., 1996]. We found that the decrease in plasma free fractions, observed after liposomal CCNU treatment, did not result in a decreased tumoricidal effect in s.c. brain tumors (Tables 1 and 2). Therefore, the degree of transfer of CCNU to the brain, after systemic delivery of liposomal CCNU, is likely to be strongly influenced by the relative affinity of CCNU for the liposomes compared to the affinity for the cellular membranes of brain capillary endothelium. To elucidate if lipid association alters CNS availability of CCNU, additional studies investigating the brain uptake of CCNU in normal and tumor-bearing animals after systemic administration of liposome-formulated CCNU should be performed. Additionally, direct administration of liposomal CCNU to tumors, possibly through Ommaya reservoirs, should be examined to evaluate if this formulation can provide localized and sustained release of CCNU to brain tumors.

In conclusion, liposome-encapsulated CCNU provided increased plasma exposure and tumor selectivity in tumor-bearing rats without exacerbating the systemic toxicity seen with free drug treatment. The evidence presented here provides possible mechanisms for the increased therapeutic efficacy of CCNU found in a rat tumor model. The superior margin of safety and activity of liposome-associated CCNU to reduce tumor volumes, compared with free drug delivery, suggests that liposomal CCNU may be effective in the treatment of solid tumors in the clinical setting.

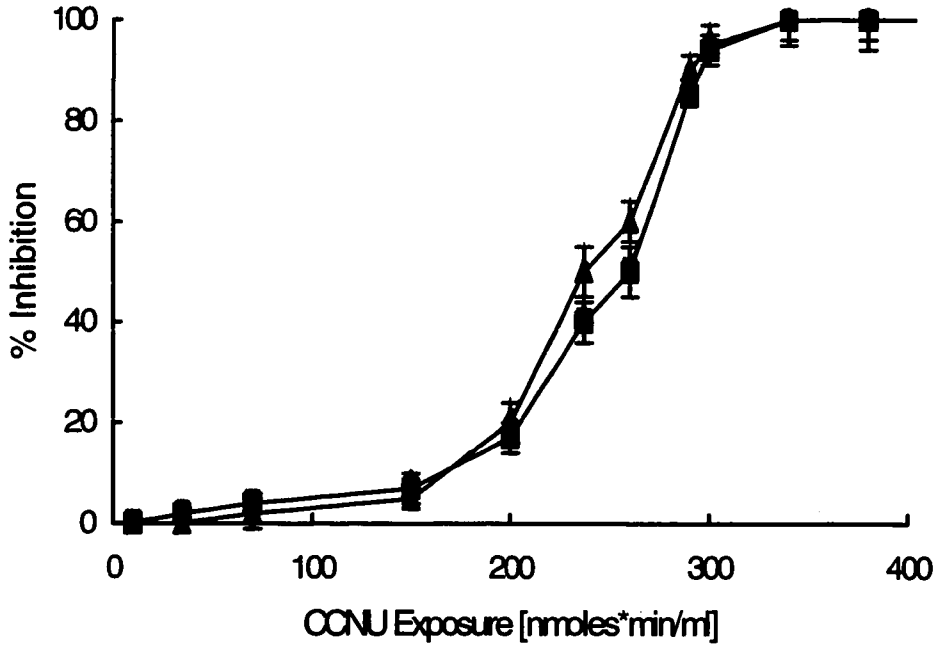

Figure 1. Effect of free (▲) and liposome-encapsulated CCNU (■) exposure to inhibit rat glioma cell growth in vitro. Cell growth inhibition was determined by ^{3}H-dT incorporation assay. Data expressed as means ± SD of a typical experiment of four repeated curves.

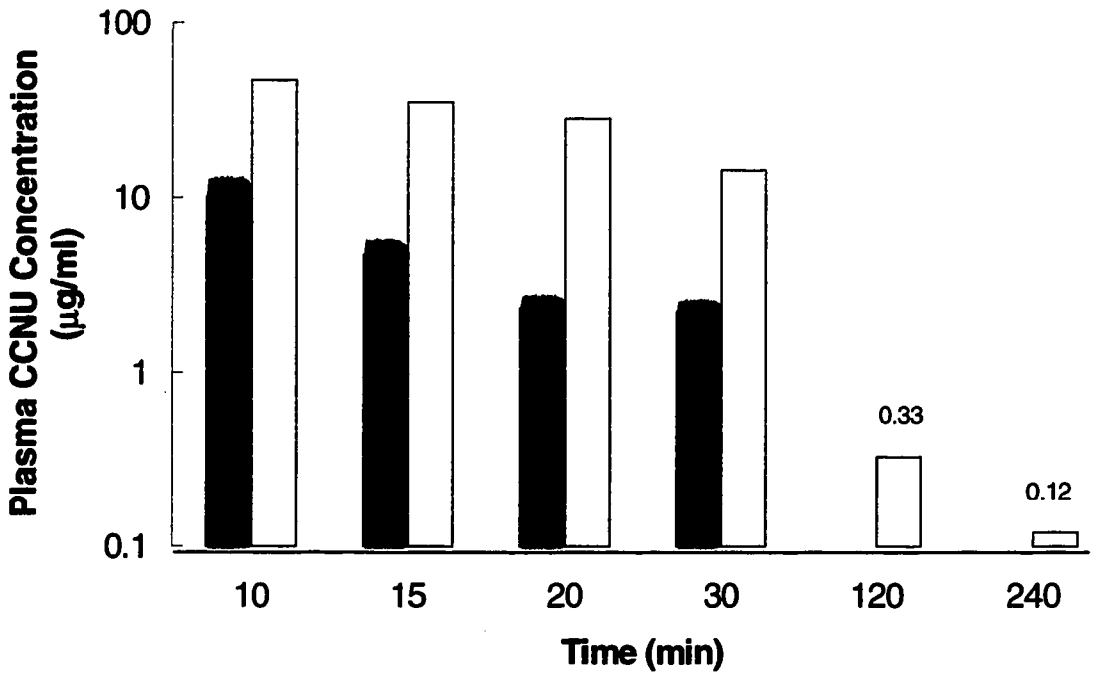

Figure 2. Comparision of CCNU plasma concentrations in rats administered free (solid bars) and liposome-encapsulated (open bars) CCNU at indicated time points. Tumor-bearing rats were given 50 mg/kg by intraperitoneal route. Data represents mean values from n = 3 animals each time point. Range of values included within symbols.

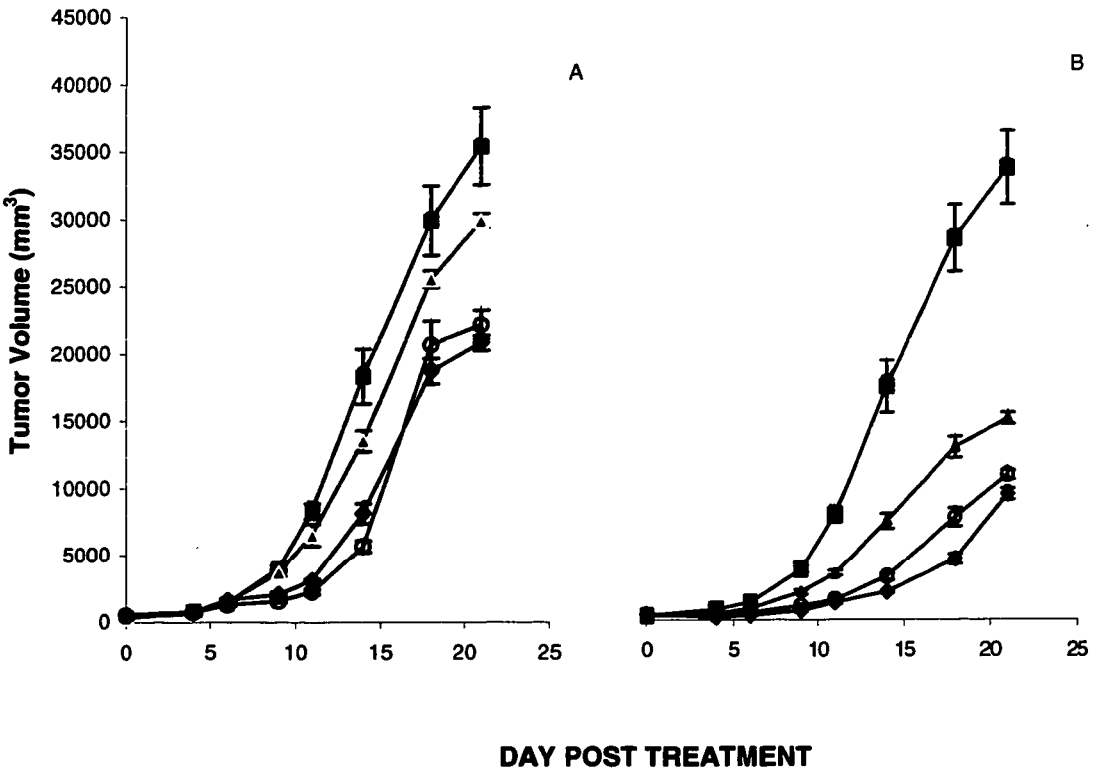

Figure 3. The time course of tumor progression after a single-dose treatment of 0 (■), 20 (▲), 35 (○), and 50 (◆) mg/kg free (A) and liposome-encapsulated CCNU (B). Data points represent mean values ± SE for each treatment (n = 4 animals each treatment).

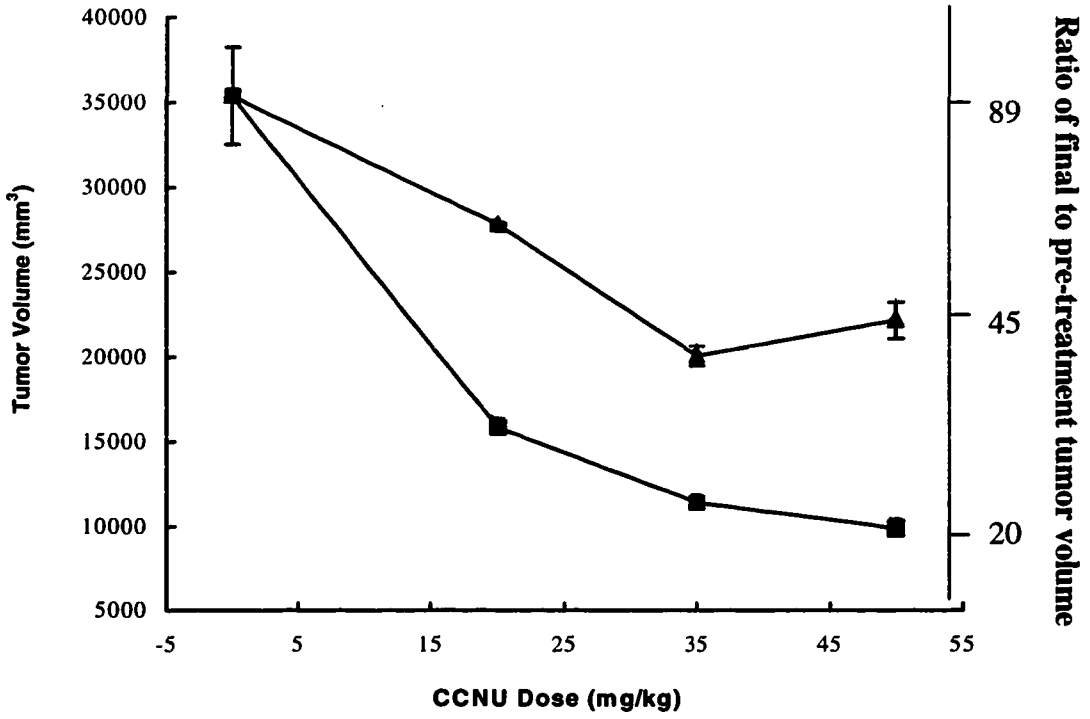

Figure 4. The effect of dose escalation on tumor progression after a single dose of free (▲) or liposome-encapsulated CCNU (■). Data represent mean ± SE for tumor volumes measured 21 days after treatment, n=4 each treatment.

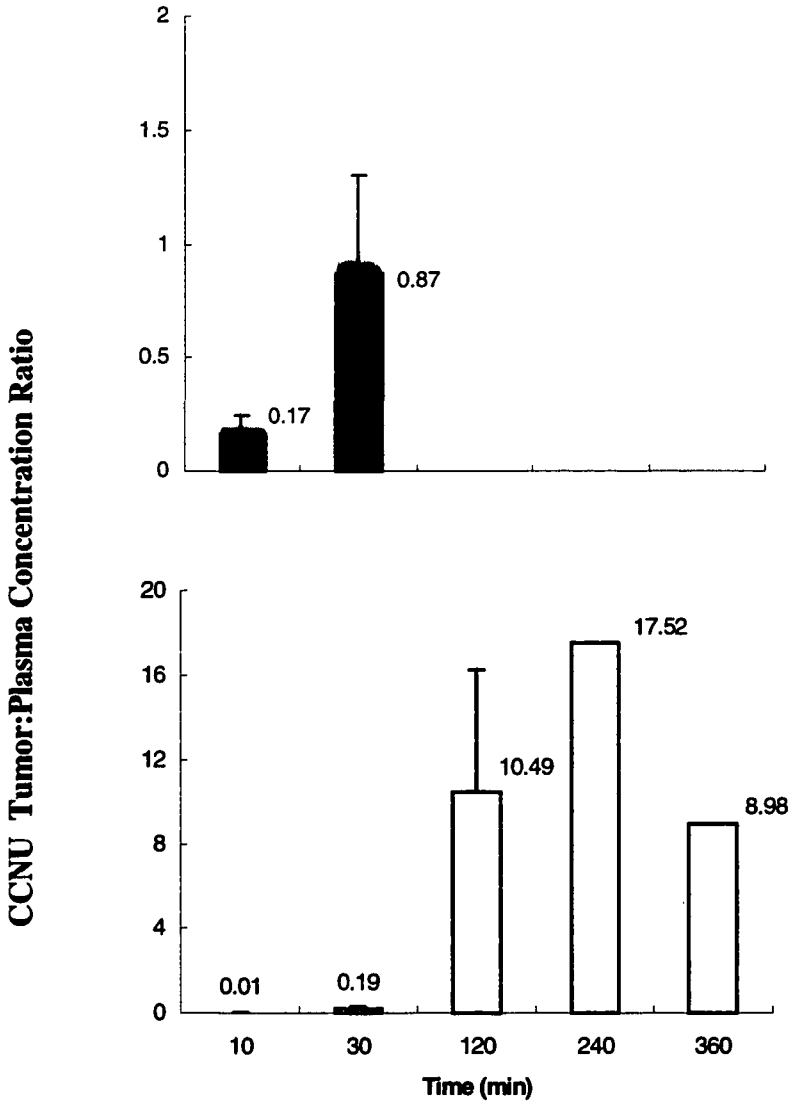

Figure 5. The relative tumor-to-plasma concentration ratio at indicated time points for free (solid bars) and liposome-encapsulated (open bars) CCNU in tumor bearing rats receiving 50 mg/kg CCNU i.p.. Data represent means ± SD.

Table 1. Effect of single-dose treatment with free and liposome-encapsulated CCNU on tumor response and systemic toxicity.

Treatment [a]	Dose (mg/kg)	Tumor Volume [b] [Fold Increase]	WBC [c] (% Decrease)	Platelet [c]	Body Weight [d] (%)
Control	0	89 ± 20	0	0	+ 11 ± 1
Free CCNU	20	54 ± 2	17 ± 7	23 ± 5	+ 13 ± 3
	35	39 ± 8	29 ± 6	19 ± 4	+ 7 ± 5
	50	45 ± 6	28 ± 8	14 ± 6	+ 14 ± 3
Liposome- encapsulated CCNU					
	20	30 ± 8 *	31 ± 5	16 ± 4	+ 4 ± 7
	35	23 ± 4 *	20 ± 4	18 ± 4	0 ± 2
	50	20 ± 3 *	35 ± 4	7 ± 3	+ 2 ± 1

[a] Each treatment n = 4 animals
[b] Tumor volume is expressed as the fold increase over the 21 day study period. Data expressed as mean ± SD.
[c] Mean % ± SD change from untreated control group 4 days after treatment.
[d] Mean % ± SD change from pre-treatment value to 21 days post treatment.
[e] $p < 0.05$ compared to free CCNU treatment.

Table 2. Effect of multiple-dose treatment with free and liposome-encapsulated CCNU on tumor response and systemic toxicity.

Treatment	Dose[a] (mg/kg)	Tumor Volume[b] [Fold Increase]	WBC[c] (% Decrease)	Platelet[c] (% Decrease)	Body Weight[d] (% Decrease)
Control	0	82 ± 13	0	0	0
Free CCNU					
A:	80 (40,20,20)	20 ± 9	73 ± 12	49 ± 10	15 ± 2
B:	100 (20,40,40)	18 ± 5	60 ± 8	63 ± 7	36 ± 2
Liposome-Encapsulated CCNU					
A:	80 (40,20,20)	11 ± 2 *	49 ± 3*	18 ± 5*	12 ± 3
B:	100 (20,40,40)	6 ± 4 *	87 ± 6	87 ± 5	39 ± 4

[a] Total dose administered , n = 8 animals for each treatment.
[b] Tumor volume is expressed as the fold increase over the 21 day study period. Data expressed as mean ± SD.
[c] Mean % ± SD change from control group 7 days after last dose.
[d] Mean % ± SD change from pre-treatment value to 22 days post treatment.
* $p < 0.05$ compared to free CCNU treatment.

Table 3. Effect of liposome association on free and protein bound fractions of CCNU in rat plasma.

CCNU Formulation	Free Fraction (%)	Bound Fraction (%)
Free	13.2 ± 1.4[a]	86.8 ± 1.4
Liposome-encapsulated	5.0 ± 1.5[b,*]	95.0 ± 1.5*

[a]Mean ± SD from animals (n=2) measured at 10, 15, 20, and 30 min after dosing.
[b]Mean ± SD from animals (n=2) measured at 30, 60, 120, and 360 min after dosing.
*$p<0.05$ between free and liposome-encapsulated CCNU, n=8 for each formulation.

CHAPTER 5: SUMMARY AND CONCLUSIONS

The overall goal of this dissertation was to determine the role of drug-lipid interactions as the mechanism behind the improved therapeutic potential found with liposomal formulations of the opioid analgesics morphine sulfate and sufentanil and the anticancer agent CCNU. The central hypothesis was that when these drugs are optimally associated or incorporated with liposomes, the biodistribution and pharmacodynamic profile can be positively modified. We found that drug-lipid interactions in liposomal-drug formulations play a major role in drug efficacy.

Since the first century, lipophilicity has been recognized as a meaningful parameter in structure-activity relationship studies and has become the single most informative and successful physiochemical property in medicinal chemistry [Helmer et al., 1968; Hansch and Leo, 1979; Rekker, 1977]. Hence, lipophilicity analysis and measurement has become a major experimental and theoretical tool in drug design.

The first objective of this dissertation was to investigate how, under physiological conditions, drug lipophilicity modulates a drug's association and dissociation to liposomes optimized for epidural drug administration. Given the extreme differences in hydrophobicity between morphine and sufentanil and their distinct interactions with lipid membranes, we reasoned that incorporating these agents into liposomes would create two distinct liposomal formulations with altered biodistribution compared to that observed with free drug formulations.

Our results show that opioid hydrophobicity plays a significant role in drug association and dissociation to D-DPPC multilamellar liposomes. We found in vitro opioid release rates for liposomal sufentanil (sufentanil octanol:buffer distribution coefficient = 1787) in CSF to be 37-fold faster than that for liposomal morphine (morphine octanol:buffer distribution coefficient = 1). After epidural administration of liposome-formulated morphine and sufentanil in a pig catheterized with microdialysis probes at epidural and intrathecal sites, significant sustained-release profiles with substantial decreases in peak concentrations were observed in epidural, intrathecal and

plasma spaces for both drugs. As predicted from in vitro release rates, liposomal sufentanil showed a more modest sustained-release profile in vivo when compared to liposome-formulated morphine. Unexpectedly, an increase in the lipid content of the liposomal dosage forms revealed an increase in epidural depot for morphine to a higher degree than sufentanil. Future studies which can determine the placement of sufentanil within lipid bilayer should be performed to elucidate the nature of sufentanil's association to liposomes. For morphine, using liposome formulations with varied lipid content may represent an important method for systematically evaluating opioid loads that can be delivered into the epidural space without increasing the amount of freely diffusible drug.

In regard to drug-lipid interactions, while hydrophobicity of drugs may strongly influence drug association and drug-release rates from liposomes, structural complimentation of drugs in liposome membranes may play a significant role also. For CCNU (octanol:water coefficient = 3), the stuctural complimentation (tighter/stable binding) to lipid bilayers may be significantly greater than that of the more hydrophobic agent sufentanil. For example, we found sufentanil to be efficiently incorported into DPPC liposomes, but rapid release from the vesicles was also apparent. Figure 1 shows the structure of DPPC and sufentanil. We hypothesize that sufentanil does not associate in a complimentary fashion to DPPC bilayers, due to steric and structural hindrances. In a report by Forrest and Mattai [1985], the effects of the general anesthetics alpha-chloralose and chloral hydrate on the order of phosphatidylcholine and phosphatidylcholine-cholesterol liposomes was examined by 2H nuclear magnetic resonance. Chloral hydrate was found to interact primarily with the hydrophilic head-group region of the lipid bilayer, causing a change in the torsion angle of the C alpha-C beta bond. The membrane interior was also found to be disordered by the presence of this agent. alpha-Chloralose, on the other hand, disorders only the central position of the membrane. Taken together, systematic studies which investigate the structure and orientation of drug classes in liposomal membranes should be performed.

With regard to therapeutic efficacy, the significant reduction in peak concentrations observed after the administration of the liposome formulations may significantly reduce the respiratory depression associated with initial high-doses of these

drugs. For both morphine and sufentanil, liposomal formulations provided an epidural depot which allowed progressive exposure to local tissues to relatively steady-state concentrations of drug over an extended period. These findings suggest that liposomal drug administration to the epidural space may provide a safe and effective method to prolong the otherwise short duration of action of these agents.

Using the information gathered with opioids, we searched for a hydrophobic chemotherapeutic agent with known antitumor activity to incorporate into a liposomal carrier. CCNU was chosen for its lipid association, potency, and rapid decomposition potential. We hypothesized that CCNU, due to its small size and conformation (Fig. 1), would be readily and stably associated to the acyl chains of the lipid bilayers. While fat emulsions have been previously used with CCNU [Litterst et al., 1974; Davigon et al., 1973], none have produced significant improvements in pharmacokinetic and pharmacodynamic profiles compared to free-drug treatment. The dissertation presented here represents the first work to design a liposomal CCNU formulation that could be stably incorporated into small unilamellar vesicles (SUVs), and that CCNU is preferentially localized (via liposome endocytosis and/or extravasation at vascularized tissues) in tumor cells, and the rapid decomposition rate of these agents in physiological environments may be decreased. As a result, this strategy of drug delivery reduces the systemic toxicity of this alkylating agent while enhancing its overall therapeutic potency, thus may permit a successful high-dose therapy for CNS tumors.

For CCNU, in vitro studies indicated that incorporating the drug into SUVs could decrease this agents rapid degradation in serum by approximately 2-fold. Increased drug stability was also seen in vivo, where plasma exposure to CCNU was found to be 2-fold greater after liposomal, as compared to free, drug administration. Additional studies found that the relative potency of liposomal CCNU against cultured brain tumor and normal cells to be significantly different, with IC_{50} values 2 to 3-fold lower against certain glioma cells and less toxic (IC_{50} values 2-fold higher) towards normal cells as compared to free CCNU. We conclude from these results that distinct cellular handling may exist for liposome-associated CCNU which is significantly different from that of free drug.

Additionally, we hypothesized that we would observe increased safety and efficacy with liposome-associated CCNU when compared to free drug in an animal tumor model. While hematological toxicity and weight loss for both liposomal and free CCNU formulations were similar for the 20, 35, and 50 mg/kg dosages examined, in the same dose range lipid-associated CCNU resulted in about twice the extent of tumor suppression achievable with free drug. Dose optimization studies showed that a cumulative dose of 80 mg/kg, given as weekly doses of 40, 20, and 20 mg/kg, produced approximately half the hematological toxicity as seen with the free drug. At a cumulative dose of 100 mg/kg (20, 40, 40 mg/kg weekly dose), pronounced hematological suppression and weight loss comparable to free CCNU treatment was observed. For both dosing schedules, lipid-associated drug may enhance antitumor activity by 2-fold.

Mechanistic studies of liposomal CCNU to enhance antitumor activity reveals that lipid-associated drug accumulates in tumor tissue. CCNU concentrations in tumor tissue, relative to plasma was approximately 10- to 17-fold higher when the drug was delivered in liposomes as compared to free-drug treatment. This can only be achieved if lipid-associated drug accumulated in the tumors as a complex. If lipid-associated drug was released in blood or at the injection site, the tumor-to-plasma CCNU concentration ratios would be less than or equal to 1, the values found after free-drug administration. These results indicate that drug distribution mechanisms beyond passive diffusion, possibly passive targeting, that may lead to enhanced accumulation of liposomal CCNU in tumors. Regardless, future studies should investigate, with labeled liposomes, the distribution pattern and cellular uptake of liposomal CCNU in tumor, bone marrow, and brain tissues.

We sought to determine if the reduced neurotoxicity, observed after intra-arterial administration in normal rats, and reduced nadir (delayed hematological) effect, observed in tumor-bearing animals after intraperitoneal administration, may be the result of a lower free fraction of CCNU present in plasma when the drug is administered as a liposomal formulation. We found free fractions of CCNU in plasma after liposomal CCNU administration to be about one-half that after free CCNU treatment. We conclude that the reduced tumor progression and toxicity, observed after liposomal CCNU administration, is due to increased concentrations of CCNU accumulating in tumors and possibly lower

free fraction of drug in plasma, respectively. It is also possible that lipid-associated CCNU would minimally distribute into bone marrow and /or reduce cellular uptake by hematopoietic cells. Regardless of the mechanism, clinical use of lipid-associated CCNU may produce a higher margin of safety and probably produce better therapeutic effects by suppressing tumor growth than free drug treatment.

The plasma free-fraction results found here also have relevance on clinical applications of systemic delivery of liposome-formulated CCNU to treat brain tumors. Whether a decrease in plasma free-fraction found after liposome delivery of CCNU may decrease the availability of CCNU to the brain remains to be determined. However, a number of recent studies indicate that plasma-protein bound agents, such as tryptophan, oxicams, and methylprednisolone, are available to cross the BBB and enter the CNS [Pardridge, 1988; Jolliet et al., 1997; Chen et al., 1996]. We found that the decrease in plasma free fractions, observed after liposomal CCNU treatment, did not result in a decreased tumoricidal effect in s.c. brain tumors (Tables 1 and 2). Therefore, the degree of transfer of CCNU to the brain, after systemic delivery of liposomal CCNU, is likely to be strongly influenced by the relative affinity of CCNU for the liposomes compared to the affinity for brain capillary endothelium. To elucidate if lipid association alters CNS availability of CCNU, additional studies investigating the brain uptake of CCNU in normal and tumor-bearing animals after systemic administration of liposome-formulated CCNU should be performed. Additionally, direct administration of liposomal CCNU to tumors, possibly through Ommaya reservoirs, should be examined to evaluate if this formulation can provide localized and sustained release of CCNU to brain tumors. For direct intratumoral or intrathecal administration, future studies should investigate formulating CCNU with MLVs, to possibly increase drug residence time in the tumors and CNS.

In conclusion, this systematic study of drug-lipid interactions has yielded increased information that can be readily utilized in designing and optimizing liposome-formulated drugs.

FUTURE DIRECTIONS

Based on current understanding of drug-lipid interactions and liposome disposition in vivo, one can design liposomes to increase circulation times and reduce clearance by the RES. Increased circulation of SUVs may permit one to consider targeting liposomes via ligand-receptor interactions. Hence, we designed lactoceramide expressing liposomes to deliver an aminoglycoside, G418, to liver cells expressing galactose receptors. Since no assay was available to detect G418 in tissue, we developed a sensitive HPLC method which is now published and attached as Appendix 1. The goal of these targeting experiments is to permit selection of foreign DNA or vectors designed to treat genetic disorders. Recombinant virus or plasmid DNA construct used for gene delivery often requires the use of antibiotic-resistance genes to afford selection pressure to induce the expression of a target gene product. Targeted delivery of G418 antibiotic to liver cells may enhance the expression of foreign genes (in hepatocytes) which are essential for treating respective genetic disorders. Preliminary results, as shown in Figure 2, indicate that SUVs loaded with G418 and expressing lactoceramide ligands could target liver hepatocytes. Additional experiments are needed to further characterize and optimize this liposomal formulation.

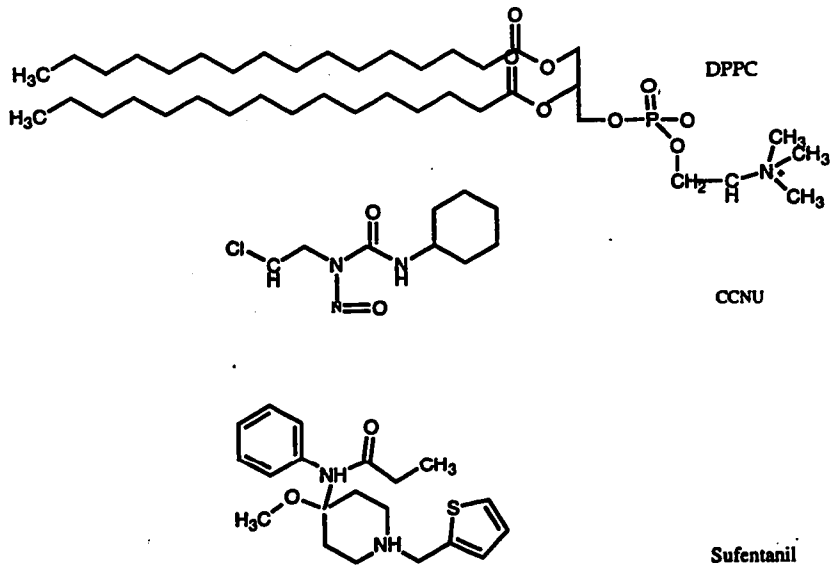

Figure 1. Chemical structures of the lipid DPPC, opioid sufentanil, and antitumor agent CCNU.

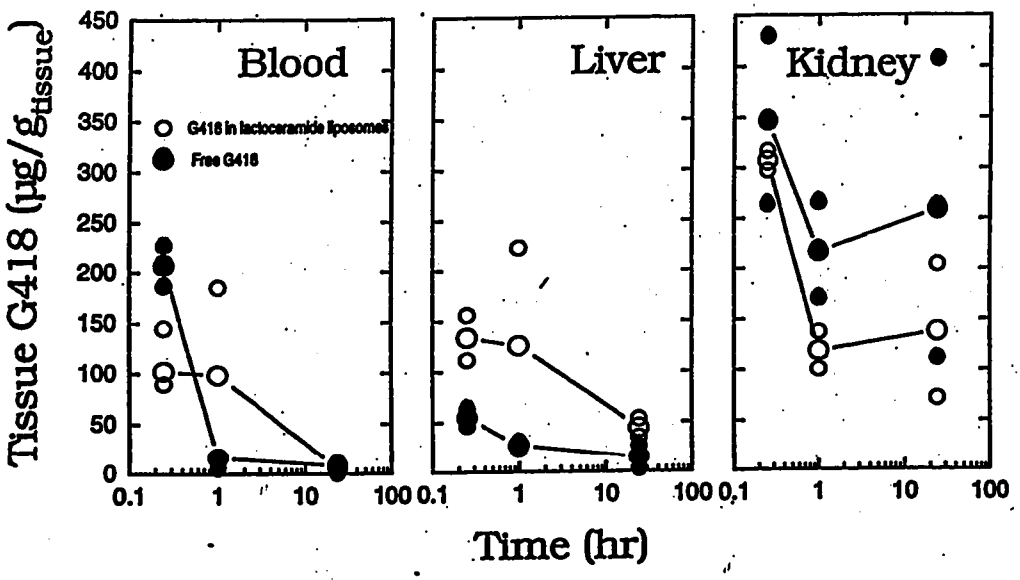

Figure 2. Relative concentrations of G418 in mouse blood, liver, and kidney tissue after a 3 mg intravenous dose of G418 sulfate in free form or encapsulated in lactosyl ceramide liposomes. Data represent individual animals (n=2 per time point) and average values (larger circles).

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APPENDIX 1: DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR G418 SULFATE (GENETICIN)

A.1 INTRODUCTION

Geneticin (G418 sulfate), a 2-deoxystreptamine antibiotic produced by *Microspora rhodorangea,* is structurally related to the aminoglycoside gentamicin but has inhibitory activity against a greater variety of pro- and eukaryotic organisms [Jimenez and Davies, 1980; Loebenberg et al., 1975]. This antibiotic has been tested as an antiparasitic agent; specifically, it has shown potent antiamoebic activity. Its clinical use as an antiparasitic has been limited, however, by the significant toxicities found in host animals and parasitic cells. With the increased use of bacterial plasmids which contain neomycin resistance determinants to introduce foreign genes in human gene therapy, the use of G418 as a drug marker for selecting DNA transfected cells has become a key factor in the successful development of human gene therapies. Expression of aminoglycoside phosphotransferase 3' (I or II), the neomycin resistance gene product, in eukaryotic cells enables transfected cells to grow in media containing G418 sulfate [Jimenez and Davies, 1980]. The ability of G418 to eliminate nontransformed eukaryotic cells, compared with neomycin, which mainly affects prokaryotic cells, has made this antibiotic indispensable for selection of eukaryotic cell transformants [Hadfield et al., 1990].

With the advent of human trials in human gene therapy, new approaches designed to improve human gene transfer or expression *in vivo* has become increasingly important. Thus, the need for a selectable marker that can be used *in vivo* as well as *in vitro* is evident. Combination use of G418 with neomycin resistant plasmid determinants is currently one of the most widely used strategies to isolate transfected eukaryotic cells *in vitro* [Kotani et al., 1994]. Also, the recent success for stable expression of foreign gene products of therapeutic interest has been due in part to the transfected cell population

containing the resistance genes being increased or maintained by applying selection pressure through the administration of drugs such as G418 sulfate [Fang et al., 1995; Kay and Woo, 1994]. Recent reports of four adenosine deaminase (ADA) deficient children treated with gene therapy indicated that the ADA gene can be successfully transduced in peripheral blood lymphocytes [Blaese et al., 1995; Bordigon et al., 1995]. However, a major limitation with ADA and other gene therapies is the relatively low number of genetically-modified cells which are available to alleviate genetic defects in animals or humans [Kay and Woo, 1994]. Strategies for selecting genetically-modified cells are being developed for rapidly dividing cell populations with the selectable human multidrug resistance gene MDR1 [Aran et al., 1994; Metz et al., 1996; Sokolic et al., 1996]. For non-dividing cells, other means of selection are needed. Towards these goals we have considered using antibiotic selection with G418 sulfate as a model.

In order to determine if therapeutic and safe concentrations of G418 sulfate are achieved *in vivo*, it is important to monitor the tissue and blood levels of this potent drug. Although microbiological assays permit an estimation of G418 potency, currently there is no quantitative chemical assay available for characterizing the chemical purity and concentration of G418 within a biological matrix. One of the major limitations in developing a chemical assay for G418 is its property of poor UV absorption. While G418 can be separated with high-performance liquid chromatography (HPLC), the poor UV absorption limits its utility. Following the work of Tsuji and co-workers [1979] for neomycin, we hypothesized that since G418 also contains several primary amines, that derivatization with a chromophore such as Sanger's reagent, 1-fluoro-2,4-dinitrofluorobenzene (DNFB) would dinitrophenylate these amines and make them visible in the UV range [Sanger, 1945]. With this reversed-phase HPLC assay, we found that DNFB derivatized G418 can be sensitively and selectively detected in biological samples.

A.2 MATERIALS AND METHODS

A.2.1 REAGENTS AND LIPOSOMAL G418 SULFATE

G418 sulfate was purchased from GIBCO BRL, Gaithersburg, MD; the Sanger's reagent, DNFB, was obtained from Aldrich Chemical Company Inc., Milwaukee, WI; HPLC grade methanol and acetonitrile were purchased from Baker Analyzed, Phillipsburg, NJ. Multilamellar vesicles were composed with egg phosphatidylcholine, cholesterol (Avanti Polar Lipids, Inc., Birmingham, Ala.), and lactosyl ceramide (Sigma Chemical Co., St. Louis, MO), mole:mole ratio of 7:3:3, and prepared as described previously [Utsumi et al., 1984].

A.2.2 G418 SULFATE QUANTITATION AND CHROMATOGRAPHIC CONDITIONS

The standard curves for G418 sulfate in tissue were prepared by the addition of 100 µl of G418 sulfate in borate buffer to 100 µl unclarified homogenized mouse tissue or serum. To detect G418 in the livers of mice administered this compound, the tissue samples were first homogenized in the presence of borate buffer (1 g tissue per 5 ml buffer) with a motor driven Potter-Elvehjem style tissue homogenizer (VWR, Seattle, WA) operating at 300 rpm for 1 minute.

To detect G418 in tissue and plasma, 100 µl of G418 containing samples were deproteinized with either absolute methanol (1:2, v/v, tissue homogenate: methanol) or with 6% (w/v) trichloroacetic acid. Protein precipitate was separated from the supernatant by centrifugation in a model 15 Biofuge (Baxter Scientific Products, McGraw Park, IL) at 15, 000 x *g* for 10 minutes. The G418 in the supernatant was lyophilized and reconstituted in 50 µl borate buffer. The dried samples were then used for precolumn derivatization.

The samples containing G418, either from the standards in buffer or deproteinated tissue homogenates, in 50 µl borate buffer were mixed with 150 µl of 0.15 M DNFB and incubated at 100° C for 45 minutes. At the end of the incubation, the samples were completely evaporated. The samples, cooled to room temperature, were dissolved in 500 µl of acetonitrile:water (1:1, v/v). Then 200 µl of these samples were transferred to 300 µl glass vial inserts (Alltech [part # 98118] Deerfield, IL), and 50 µl was injected into the HPLC system fitted with an auto injector (Waters Assoc., Milford, MA). DNFB-derivatized G418 samples were stored in the dark, where they remained stable at room temperature for 1 week

The DNFB-derivatized G418 sulfate was separated on a reverse-phase HPLC system (Waters Assoc.). The samples were introduced with a WISP model 712 autosampler. Separation of the G418-DNFB conjugate was performed with a reverse-phase C_{18} column (8 mm x 100 mm with 5 mm i.d., cat. no. 85721, Waters Assoc.) at room temperature. Elution was with a linear gradient using acetonitrile:water as the mobile phase.

Mobile phases consisted of solvent A, acetonitrile and water (50:50), and solvent B, acetonitrile. Both solvents were prefiltered with a 0.45 µm nylon membrane (Alltech Assoc., Inc.) to reduce interference. With the Maxima 820 computer controlled work station (version 3.3, Millipore Corp., Milford, MA), the solvent flow rate was set at 1 ml/min. The linear gradient between the two mobile phases was set as follows: (1) from 0-to-2 min, 100% solvent A; (2) from 2-to-6 min, a linear increase of 0-to-100% solvent B; (3) from 8-to-12 min, 100% solvent B; (4) from 12-to-12.5 min, return to 100% solvent A; and finally, (5) from 12.5-25 min, the column was re-equilibrated with 100% solvent A. Using a UV detector (model 440, Waters Assoc.) with the wavelength set at 340 nm, DNFB-derivatized G418 sulfate was eluted with a retention time of 13.60 ± 0.09 minutes.

The chromatograms were recorded and analyzed with the Maxima 820 computer workstation. For initial experiments, the linearity of the assay was determined with both peak height and peak area response. We found that both methods produced similar results and hence, only peak height data are presented in this report. Slopes of the standard curves were fitted by least-squares linear regression. Statistical comparisons of

the standard curves were performed with a two-sided Students t-test with a significance level set at 0.05.

A.3 RESULTS AND DISCUSSION

We first determined whether derivatization of G148 sulfate with DNFB would provide a chromophore sufficient for UV detection of G418 using a reversed-phase HPLC system. Based on the methods developed for amikacin by Barends et al. [1983], DNFB-derivitized G418 was eluted isocratically with an acetonitrile:water mobile phase. We found, however, that an isocratic mobile phase was not sufficient to separate G418 from other co-eluting DNFB-derivitized components found in mouse tissue and plasma samples.

Therefore, we developed a linear gradient system with increasing acetonitrile concentration from 50% to 100% to adequately separate the DNFB-derivatized G418 from contaminants. Under these conditions, G418 eluted with a retention time of 13.6 ± 0.09 minutes. Typical chromatograms are presented in Figure 1. The free, hydrolyzed DNFB eluted near the injection peak; therefore, it did not interfere with G418 sulfate detection. Also, since the concentration of DNFB is always in excess, the derivitization reaction proceeded to completion.

With the G418 sulfate standards prepared in borate buffer, the linearity and detection limit of the assay were determined. Figure 2 shows a mean standard curve constructed from four replicates of varying concentrations of G418 sulfate in borate buffer. Within the 0.078 to 10 μg/ml concentration range, this HPLC assay produced low variability and excellent linearity with a correlation ($r^2 = 0.999$) based on resultant peak height analysis of chromatograms. The linearity of the method was observed at concentrations up to 100 μg/ml (data not shown). The detection limit, which was greater than two standard deviations above the baseline, was estimated to be 78 ng/ml.

To determine the reproducibility of the assay, we have determined intraday and interday variation of samples containing 0.25, 1.5, and 3.0 μg/ml G418 in borate buffer. These concentrations were chosen based on estimated levels that would be achieved with

our animal studies. Intraday variation was tested on 1 day in quadruplicate while interday reproducibility was examined over 5 separate days. As shown in Table 1, both interday and intraday variation was low with the resulting coefficient of variation (CV) being below 6% for both. These results indicate that reproducibility of this assay was excellent.

For the detection of G418 in tissue homogenate or plasma samples, a deproteinization technique is essential to isolate this highly water soluble compound. Of the various deproteinizing agents used (including methanol, acidified methanol, and acetonitrile) to remove proteins, methanol (1:2 (v/v); sample:methanol) or 6% trichloroacetic acid (TCA) was found to be the most useful. The efficiency of G418 detection using methanol and TCA deproteination methods is presented for mouse liver homogenate containing (0 - 20 μg/ml) G418 sulfate in Table 2. The percent of G418 recovered after protein precipitation was determined from the ratio of the slope from G418 standard curves prepared in the tissue homogenate compared with that prepared in borate buffer (without tissue). As can be seen in Table 2, removal of protein with methanol produced a higher (81.8 vs. 65.6, $p < 0.016$) percent recovery of G418 with less variation (6.3% vs. 12.5% CV) when compared with the TCA method. Therefore, the methanol deproteination method was used subsequently.

Using the optimized deproteinization technique, we next determined whether G418 in various tissues can be separated under the chromatographic conditions described above. A typical chromatogram of G418 recovered from a liver sample is shown in Figure 1A. Under these assay conditions, the liver homogenate produced minimal background at the region where DNFB derivatized G418 peak was detected (Figure 1C).

Therefore, we used this method to determine whether this assay can be used to detect G418 in other tissues, including kidney and plasma. As shown in Figure 3A, the deproteination procedure produces practically the same standard curve as that of neat samples. Furthermore, the same method can be used to detect G418 in plasma and kidney samples with similar efficiency, which is evident from similar concentration-dependent peak height responses (slopes equal to 378 - 392 mV/μg) detected irregardless of the tissue tested.

Of the various methods used for detecting antibiotics in biological samples, liquid chromatography has been increasingly utilized to determine aminoglycoside levels. In the development of HPLC methods for antibiotics, comparisons and correlations of bulk products are usually made with antimicrobial potencies estimated from biological assays. While these may be more sensitive than HPLC assays, the chemical identity cannot be directly discerned without elaborate and time consuming analysis. With this HPLC assay, we can sensitively and rapidly detect G418 in a quantitative manner. Also, Barends et al. [1983] has shown with amikacin that concentrations estimated by HPLC and bioassay are in excellent agreement ($r^2 = 0.993$).

Currently, no microbiological assay for the direct quantitation of G418 exists, so a comparison of this HPLC assay with a bioassay could not be evaluated. It is likely that a high correlation would also exist between this HPLC method for G418 and a bioassay because the derivitization reagent and detection methods are similar to those used for amikacin. Also, the linearity, sensitivity, and recovery of G418 in this assay matches or exceeds those for HPLC methods developed for amikacin [Barends et al., 1983] and gentamicin [Barends et al., 1980].

In general, HPLC assays for other aminoglycosides utilize pre or postcolumn derivitization with o-phthalaldehyde to form a fluorogenic compound. Theoretically, G418 can be derivitized with flourescence reagents such as o-phthalaldehyde by a postcolumn derivitization technique to further reduce the detection limit. However, postcolumn derivitization with o-phthalaldehyde may increase baseline noise as well as the cost of detection because: the flow cell may be contaminated by reaction products; consumption of reagent is high; and reaction time can only be prolonged by increased dead volume, resulting in additional peak broadening [Essers, 1984].

In summary, it may be possible to use G418 sulfate as a means to selectively expand genetically modified cells in vivo to increase transgene expression. We have developed and characterized a HPLC assay to detect G418 sulfate with specificity and high sensitivity suitable for its detection in biological samples.

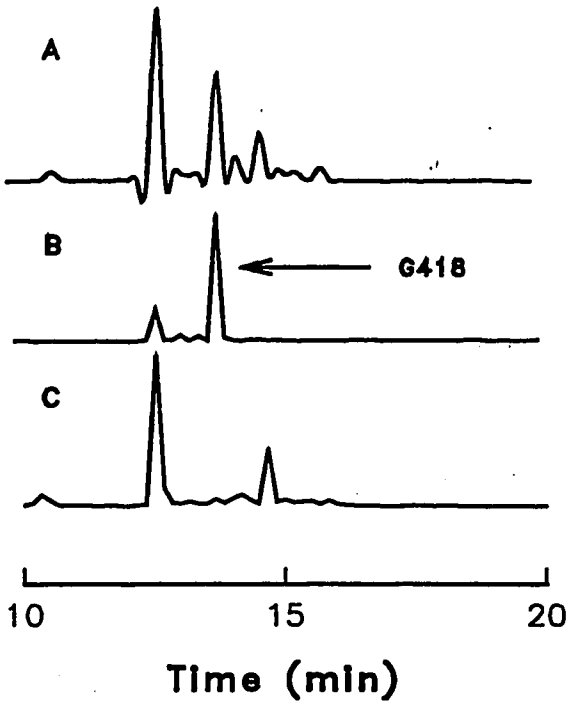

Figure 1. Representative chromatograms for G418 derivatized with DNFB. G418 in either liver homogenate (A) or borate buffer (B) or a liver homogenate control (without G418) (C) were deproteinated, derivatized with DNFB, and chromatographed as described in Materials and Methods section. The concentration of G418 sulfate was 2.5 μg/ml for panels A and B.

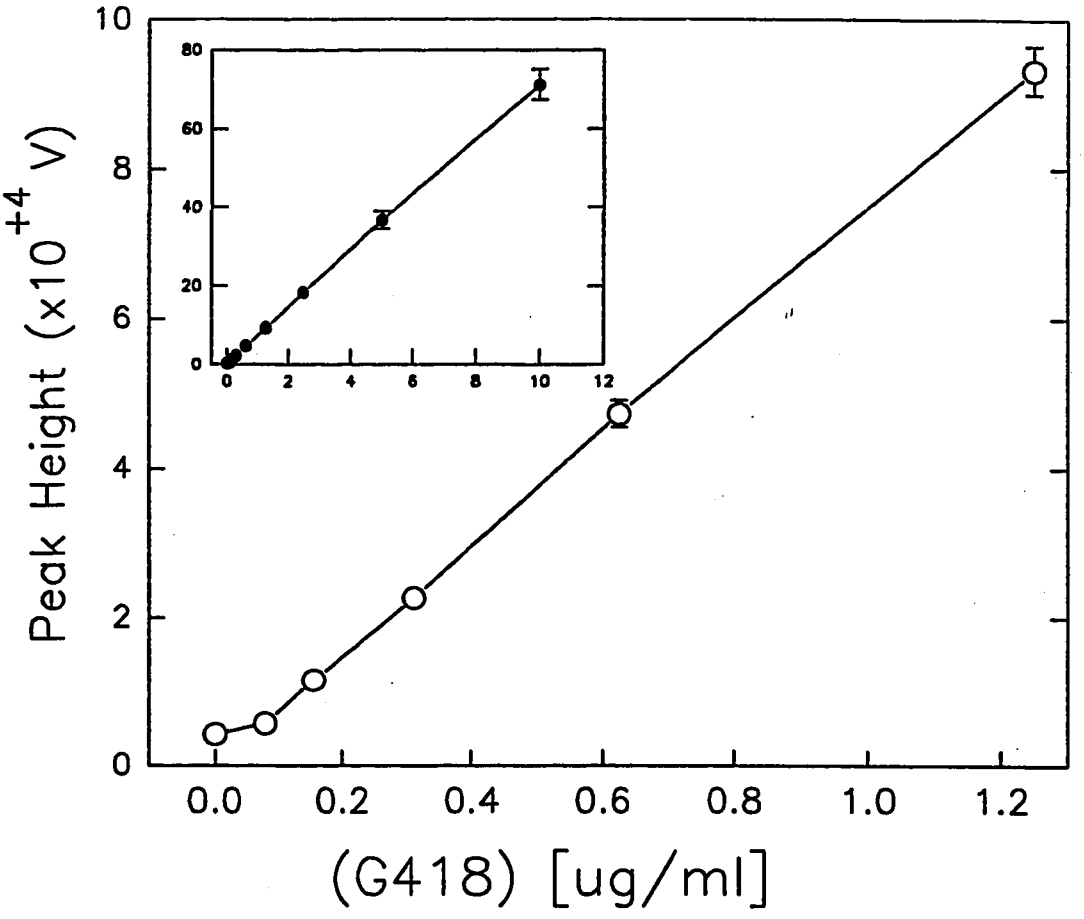

Figure 2. Linearity and detection limit for the HPLC assay detecting G418 derivatized with DNFB. The indicated concentrations of G418 sulfate in borate buffer were chromatographed as described in the Material and Methods and analyzed based on the peak height detected for the DNFB-derivatized G418. Each data point represents the mean ± SD from quadruplicate samples. The figure insert shows a wider linear concentration range (0 to 12 μg/ml) of G418 standards.

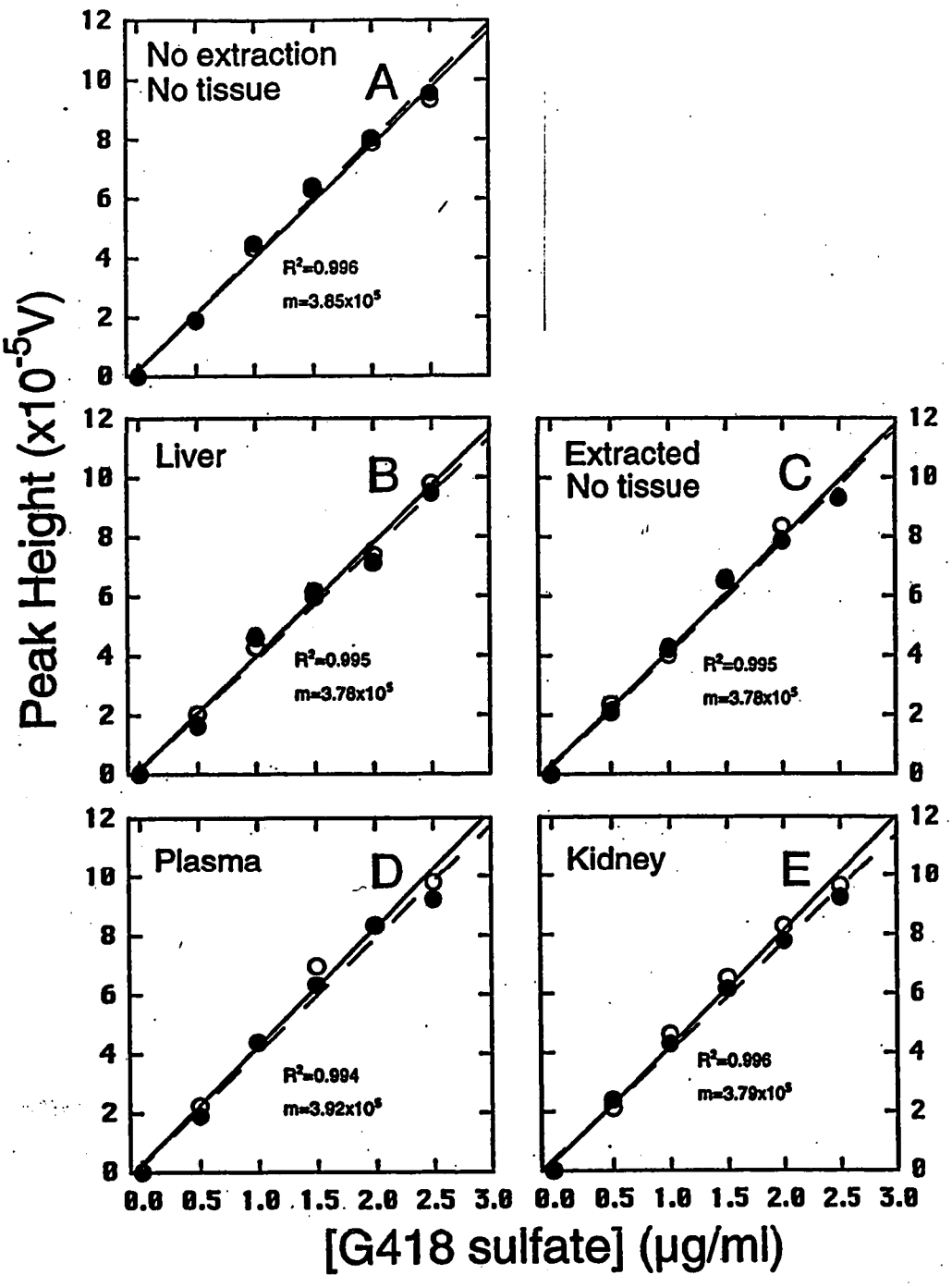

Figure 3. Detection of G418 in mouse tissues. The various concentrations of G418 sulfate in liver homogenate (B), borate buffer (C), plasma (D), or kidney (E) were deproteinated with methanol, derivatized, and assayed for G418 by the HPLC as described in the text. In parallel, as a control to determine the effect of the deproteination procedure, G418 sulfate in borate buffer was derivatized without methanol deproteination (A). Open and closed circles represent mean data from run 1 and run 2, respectively.

TABLE 1. Precision of the HPLC assay for G418 sulfate[a].

Type of variation	Retention time	Slope (mV/μg)
Run to Run[b]	13.76 ± 0.02	438.8 ± 1.9
Day to Day[c]	13.70 ± 0.09	424.9 ± 14.9

[a]The intraday and interday variation for the assay was determined from analysis of 0 - 3 μg/ml standard curves of G418 sulfate spiked into borate buffer. The resulting retention times and slope of peak heights (mV/μg) were used to evaluate the variation of the assay.

[b]Values represent the average ± standard deviation from four runs of standard curves containing 0 - 3 μg/ml G418 sulfate spiked into borate buffer.

[c]Values represent the mean ± standard deviation from five runs of standard curves prepared as above but analyzed on separate days.

TABLE 2. Recovery of G418 between two protein precipitation procedures[a].

Method	Recovery	% CV
TCA	65.6 ± 8.2	12.5
Methanol[b]	81.8 ± 5.1	6.3

[a]The values here represent % recovery for four runs of TCA and methanol methods in comparison with nonprecipitated standard curves for 0.5, 1.0, 2.0 μg.

[b]t-test of $p < 0.016$ between the two methods.

Vita

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