ROLE OF LIPOSOME MEDIATED DRUG DELIVERY AND DRUG RELEASE IN

DETERMINING THE THERAPEUTIC ACTIVITY OF LIPOSOMAL FORMULATIONS OF

MITOXANTRONE

by

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B.Sc., University of British Columbia. 1994

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENT FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

DEPARTMENT OF PATHOLOGY AND LABORATORY MEDICINE

THE UNIVERSITY OF BRITISH COLUMBIA

September. 1999

0-612-46372-9

Canada

ABSTRACT

Although liposomal accumulation at the target site is an important issue, the critical parameter defining the activity of a liposomal formulation is drug release, a factor that includes where, when, and how fast the therapeutic agent dissociates from the liposomal carrier. This point was investigated using two liposomal formulations of the anti-cancer drug mitoxantrone. Mitoxantrone was encapsulated via a pH gradient method in liposomes prepared of 1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC)/cholesterol (Chol) (55:45 mol ratio) or 1,2 dimyristoyl-sn-glycero-3-phosphocholine (DMPC)/Chol (55:45 mol ratio), the latter exhibiting a greater rate of drug release *in vivo*. Using a model of liver localized cancer consisting of BDF1 mice inoculated with either P388 or L1210 cells intravenously (*i.v.*), it was demonstrated that a single dose of DMPC/Chol mitoxantrone (10 mg/kg) administered *i.v.* resulted in 100% 60 day survival. In contrast, no long-term survivors were obtained in animals treated with free or DSPC/Chol mitoxantrone. Drug levels in the liver were determined and demonstrate that greatest drug delivery was achieved with the DSPC/Chol liposomal formulation. In an effort to address whether liposome mediated delivery or drug release is the dominant factor determining therapeutic activity, additional experiments examined the role of drug release at tumour sites where liposome accumulation is slow. As demonstrated in subcutaneous LS180 and A431 tumours grown on the backs of SCID/RAG-2 mice, the DMPC/Chol formulation demonstrated greater activity in the LS180 tumour model and was as efficacious as the DSPC/Chol formulation when treating A431 tumours. These data emphasize the importance of designing liposomal formulations that optimize drug biological availability rather than drug delivery.

In an effort to understand factors that are important in governing the activity of DMPC/Chol liposomal mitoxantrone used to treat liver localized disease, studies modulating liposomal accumulation in the liver were completed. Two methods were used to effect reductions in

liposome delivery to the liver: the use of PEG modified lipids and hepatic mononuclear phagocyte system (MPS) blockade. Both methods reduced liposomal drug accumulation in the liver by a factor of 2 to 3 fold. A significant reduction in therapeutic activity was observed when PEG-modified lipids were incorporated into the DMPC/Chol mitoxantrone formulation; however, MPS blockade did not affect anti-tumour activity. Long term survival (>60 days) was still observed in animals where hepatic MPS blockade effected elimination of liver Kupffer cells. It is concluded that reductions in therapy observed for the PEG-modified DMPC/Chol mitoxantrone are likely due to inhibition of cell binding and processing. Conversely it is suggested that the activity of the DMPC/Chol mitoxantrone is dependent on cell processing, but the Kupffer cells do not play a significant role in this processing event.

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ABBREVIATIONS

% ILS	percentage increase in lifespan.
^{3}[H]-CDE	[^{3}H] Cholesteryl hexadecyl ether
AUC	area under the curve
BSA	bovine serum albumin
Chol	cholesterol
CRP	C reactive protein
DiI	1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMPC	1,2-Dimyristoyl-sn-glycero-3-phosphocholine
DOX	doxorubicin
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
DSPE	distearoyl phosphatidylethanolamine
EDTA	ethylenediaminetetra-acetic acid
EPC	egg phosphatidylcholine
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	centrifugal force
HBS	HEPES buffered saline
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid
IC_{50}	Concentration necessary for 50% inhibition of growth
I.L.S.	Increase in life span
$i.p.$	intraperitoneal
$i.v.$	intravenous
LUV	large unilamellar vesicles
MAC	membrance attack complex
Mitox	mitoxantrone
MLV	multilamellar vesicles
mol	mole
MPS	mononuclear phagocytic system
MTT	3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide
MTD	maximum tolerated dose
PBS	phosphate buffered saline
PE	phosphatidylethanolamine
PEG	poly(ethylene glycol)
PEG-PE	poly(ethylene glycol)-modified phosphatidylethanolamine
QELS	quasielastic light scattering
s.c.	subcutaneous
S.D.	standard deviation
S.E.M.	standard error of the mean
SUV	small unilamellar vesicles
T_c	gel-liquid crystalline phase transition temperature
Vinc	vincristine

ACKNOWLEDGEMENTS

Is Life worth living? That depends on the liver.
- Anonymous.

Essentially it will be difficult to thank everyone who helped make it to this point since there have been so many people who supported me throughout this journey and I would like to say a heartfelt - Thank You!

A very special thanks to Marcel for trusting me 1) with your house 2) with your horse 3) as a grad student! I've had a great time and learned more than I could possibly imagine. Thank you for 1) your tremendous support 2) convincing me to do a Ph.D. 3) teaching me the big picture to science and research and 2) showing me how to teach as well. (I hope I haven't corrupted too many kids on Bowen....). And of course, thanks to Jlonka. I'm sure my thesis took up some of Marcel's time, which could have been better spent with you.

Thanks to Lawrence for great laughs and for sharing your philosophy on science.

Thanks also to Kirsten Skov, Tom Madden, and Paul Sorenson – What a great committee!!! Thanks for referring me to Marcel (Tom) and for the encouragement from my whole committee.

Thanks to Gwyn Bebb for sharing his wisdom and kind words over some sort of dessert.

Of course I can't forget all those past and present who have lived down in the deep pit known as the Lower main floor of the B.C. Cancer Research Centre. Thanks to Troy, Murray, Shane, and Sandy who I must have annoyed endlessly with questions those first few months. Many thanks to Dana, Natashia, Nancy, and Rebecca for their help with the animals and cell cultures. And of course Kelly, Frances, Hafiza, Raj, Peirrot, Lynn, Spencer, Guoyang, Y.P., Zaihui, Gary, Dody, Ellen, Dawn, the NHL, every restaurant on the Broadway strip, Safeway, the QC group for their reagents and glassware, Don Phillips and Ernie Hildman of Analytical Cytology, Maryesse St. Louis for her work on the confocal microscope at U.B.C. and of course my poor summer students (who kept me honest): Julie, Daniel, and Anne.

Many thanks to the Division of Medical Oncology for giving me opportunity to learn the clinical aspects of cancer as well as for allowing me access to the seminar room for all those committee meetings.

Of course, I will also remember those who helped me survive the non-science parts of my degree: Lillian, Julie, and Penny.

Special thanks to two ladies who made my life so much easier by washing my glassware: Maria and Shelley.

Thanks to Mr. and Mrs. Tan who also provided a home away from home and their words of encouragement. And of course all my friends (new and old) who put up with me all this time.

Thanks to my Dad and to Auntie Maggie for all their support.

P.S. Marcel - If you ever come up with liposomal ADH, I might be able to drink some beer.

A special Thank You:

I want to thank the teachers and professors who have made this whole journey exciting and most of all fostered my desire to learn. I would not be here if it were not for the effort of these people:

Thank you very much.

David Llyod George Elementary School
Ms. Mitchell - Grade 2

Gilpin Elementary School
Ms. Nelson - Grade 4
Ms. Horne - Grade 5 and 6
Mr. Bell - Grade 7

Magee Secondary School:
Ms. McColl - Drama and Acting
Mr. Nakashima - Chemistry
Mr. Baptie and Mr. Rollins - Biology
Mr. Jung, Mr Cheeseman, and Mr. Miller – Mathematics and Calculus
Mr. Keenlyside - Social Studies

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Dr. Mayer
Dr. Bally

DEDICATION

To my mom,

Thank you for raising me to be the person I am today. Thank you for teaching me the value of learning both inside and outside the academic world. Your support, patience and love, has been truly remarkable.

And

To Lisa,

Thank you for being there for those late night calls and panic attacks. You are truly a great friend.
Who knew I would need all those highlighters.

Enjoy!

CHAPTER 1

INTRODUCTION

1.1 Foreword

In their present form, liposomal carriers primarily impact drug biological availability and this, in turn, results in a number of important therapeutic benefits. This includes the well-established reduction in toxicity for liposomal formulations of drugs such as the anti-cancer agents doxorubicin (Gabizon *et al.*, 1982; Olson *et al.*, 1982; Mayer *et al.*, 1994;) and vincristine (Mayer *et al.*, 1993; Boman *et al.*, 1994; Kanter et al, 1994) and the anti-fungal agent amphotericin B (Graybill *et al.*, 1982; Krause and Juliano, 1988). This reduced toxicity does not occur at the expense of therapeutic activity and, as a result, the therapeutic index of these drugs is improved through liposomal encapsulation.

The reasons that liposomal drug carriers improve the therapeutic properties of an associated drug are not well understood. Pre-clinical studies suggest that free drug (drug released from liposomes) remains the biologically active agent and that therapeutic improvements arise from liposome mediated changes in drug circulation lifetime and tissue distribution (Hwang, 1987; Mayer *et al.*, 1994; Gabizon and Martin, 1997). Reduced toxicity may be related to reduced availability of drug to sensitive tissues. For example, in the case of doxorubicin where cardiotoxicity represents a significant treatment limiting toxicity, reduced levels of drug in cardiac tissue are observed when the drug is administered in liposomal form (Gabizon *et al.*, 1982; Olson *et al.*, 1982). Furthermore, it is believed that therapy results from enhanced drug accumulation at the disease site and this is mediated by extravasation and localization of the drug loaded liposomal carrier ("passive targeting"). This appears to be a relatively general phenomenon in that liposomes preferentially accumulate in sites of inflammation (O'Sullivan *et*

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al., 1988), infection (Bakker-Woudenberg *et al.*, 1992); and tumour growth (Richardson *et al.*, 1979; Proffitt *et al.*, 1983; Gabizon and Papahadjopoulos, 1988).

Perhaps the most significant liposome characteristic to consider, in addition to the carrier's effect on drug delivery, is the carrier's drug retention attributes. This thesis is principally concerned with the importance of controlled drug release. Drug release attributes must, however, be considered in the context of when, where and how rapidly drug release occurs. The illustration shown in Figure 1.1 is useful in orienting the reader to the fundamental premise guiding the research described. It is believed that for an intravenously administered liposomal anti-cancer drug to be optimal it must possess different attributes depending on where the liposome is localized. While in the blood compartment the liposome should retain drug. This will serve two purposes: 1) to minimize systemic exposure of free drug and 2) to maximize delivery of the liposomal drug to sites outside the blood compartment. The latter is typically a slow process and if the drug release rates are too rapid, liposomes which have left the blood compartment may contain little drug. Once localized in the site of disease development, the liposomes ideally must undergo a transformation process resulting in drug release from the liposome. This chapter reviews how this model of liposome delivery was developed and the results presented in subsequent chapters support the contention that drug release combined with liposome-mediated changes in drug distribution work together to enhance the therapeutic activity of an associated anti-cancer drug.

It is important to recognize that the research described in this thesis was developed using simple liposome formulations. In fact it is argued here that the primary advantage of using liposomal carriers, as opposed to other carrier technologies, is due to the fact that it is not complicated. Procedures for making physically and chemically well-defined liposomes as well as procedures for encapsulating certain drugs in liposomes such that extremely high trapping efficiencies and

Figure 1.1

Liposome target site accumulation

To allow for passive accumulation to the target site appropriately designed liposomal carriers must be retained in the blood compartment for extended time periods (A). While in the blood compartment liposomes interact with the cells lining the endothelium (B) or with specific target cells (C). Passive targeting is dependent on the presence of altered vascular endothelium, alterations that permit extravasation of circulating macromolecules (D). Following extravasation liposomes can release drug while residing in the interstitial space (E) or can be taken up by tumour associated macrophages (F). The potential to achieve specific interactions with target cells (active targeting) through use of targeting ligands is also feasible (G). (Figure reproduced from Harasym, 1997)

Endothelial cell
Tumor cell
Macrophage
Erythrocyte
Liposome
Drug release

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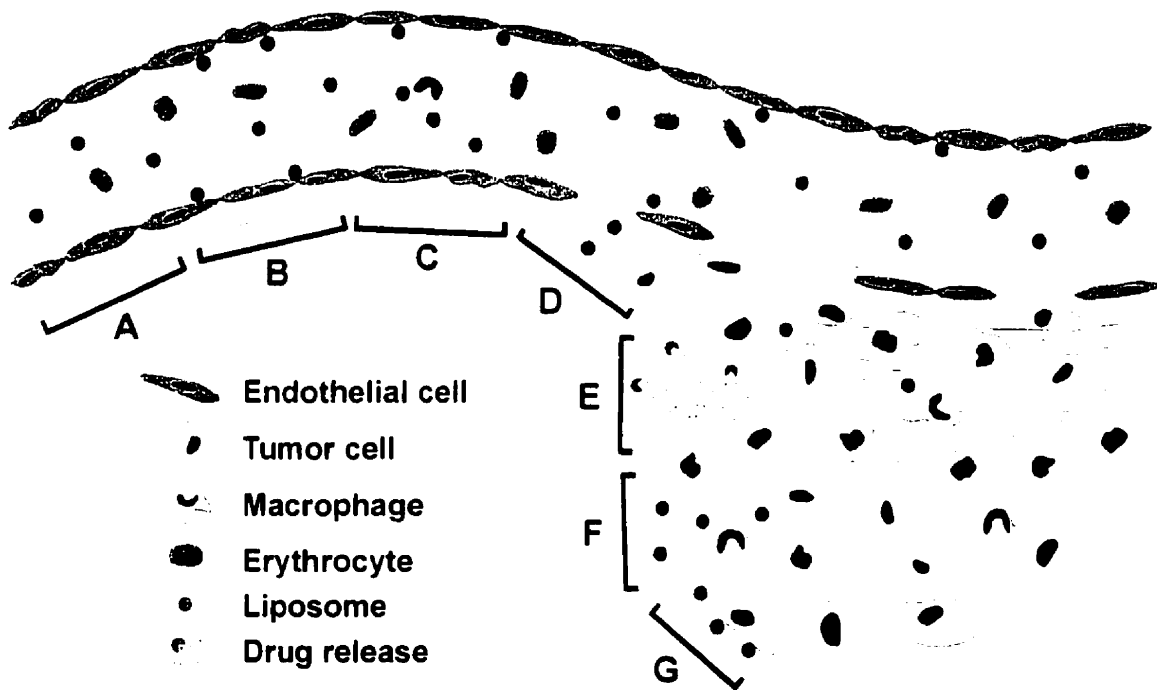
high drug-to-liposome ratios have been developed and these procedures are reviewed in this introduction. Although many investigators are working towards improved technology, developing liposome carriers that exhibit modified surface features (Allen et al., 1989; 1991; Gabizon, 1992), targeting ligands (Leserman et al., 1981; Ahmad et al., 1993) and/or membrane fusing attributes (Holland et al., 1996b; Kirpoitin et al., 1996), it is important to recognize that our understanding of the mechanisms governing the activity of simple liposome formulations is relatively poor. The research described in this thesis leads to a better understanding of how liposomes function as anti-cancer drug carriers and this information is essential for those interested in developing improved technology.

1.2 Liposomes

Lipids can be extracted from natural sources (e.g. cellular membranes) and upon hydration orient into a spheres of bilayers, resulting in the formation of liposomes. First observed by Bangham et al. (1965), these multilamellar vesicles (MLV) consisted of concentric lipid bilayers separated by aqueous channels. The bilayer configuration arises due to the amphipathic nature of the lipid: the hydrophilic head group and hydrophobic tail of the lipid molecule orient the lipid molecules such that the head groups face the aqueous environment and the fatty acyl chains are oriented toward one another.

Liposomes were first used as model membrane systems because they form closed spheres which have a defined interior aqueous space separated by lipid bilayers. making them a valuable tool for study of the structural and functional role of lipids in the biological membrane. This included investigations of membrane fusion (Dunham et al., 1977; van Meer et al., 1985; Bailey and Cullis, 1994), membrane-protein interactions (Rogers and Strittmatter, 1975; Sogor and Zull,

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1975; Bortoleto *et al.*, 1998; Yamaji *et al.*, 1998), complement activation (Devine *et al.*, 1994), and multi-drug resistance (Shapiro and Ling, 1995). Liposomes are also used to study ion gradients and membrane permeability (Deamer and Nichols, 1983; Viero and Cullis, 1990). This section will focus on the two components, phospholipid and cholesterol, which typically are used in the preparation of liposomal drug delivery systems.

1.2.1 Phospholipids

Phospholipids (or glycerophospholipids) consist of a glycerol backbone with a phosphate group esterified at the C(3) position and fatty acids esterified at the C(1) and C(2) positions as shown in Figure 1.2. Changes in the headgroup and/or in the fatty acids dictate the properties exhibited in the lipid bilayer. For example, liposomes containing the lipids phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid will have a negative surface charge at physiological pH. Liposomes with phosphatidylserine and phosphatidic acid are rapidly eliminated from the circulation following intravenous (*i.v.*) administration, in part because of serum protein binding effects attributed to the negative charge (Moghimi and Patel, 1989). Since the ability of liposomes to move from the blood compartment to an extravascular site is dependent on maintaining a sufficient plasma concentration of liposomes for extended time periods, anionic phospholipids are not typically used when developing liposomal drug carriers. Instead there has been a focus on using zwitterionic phospholipids, in particular phosphatidylcholine.

In addition to the importance of phospholipid head group charge, the acyl chain composition of the phospholipid can dramatically effect the characteristics of liposomes and their use as drug carriers. A key property of phospholipids is the temperature of the gel to liquid-crystalline phase

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Figure 1.2

Structure of a phospholipid

(Figure reproduced from Parr, 1995)
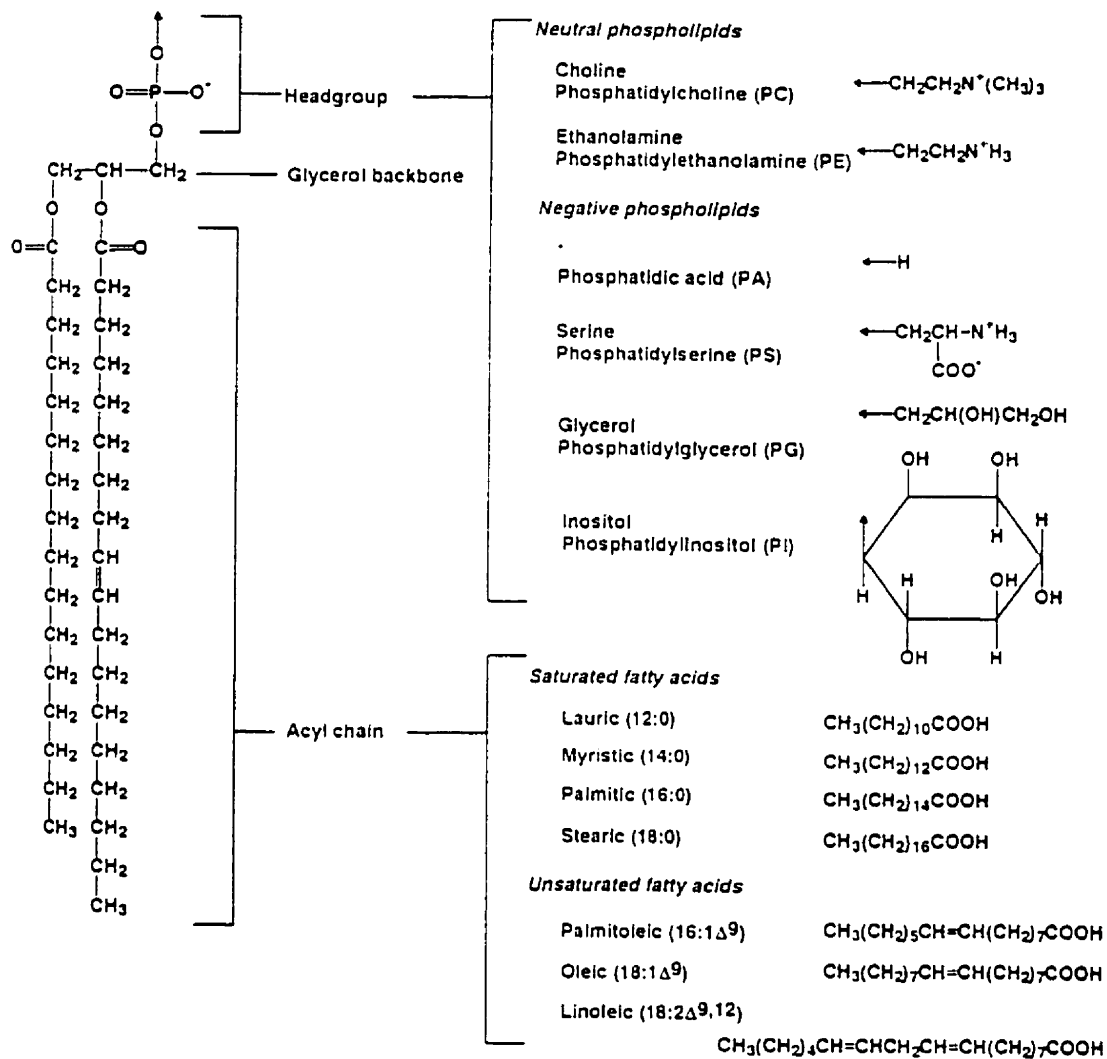
transition (T_c) and this property is determined by both head group chemistry (Kruyff *et al.* 1973; Chowdry and Dalziel, 1985) and acyl chain composition (McElhaney, 1982; Wang *et al.*, 1997; Huang *et al.*, 1993). The temperature where phospholipids undergo the transition from the gel to liquid-crystalline phase is referred to as the T_c and the length and saturation of acyl chains is a determining factor where the T_c is observed. Typically, longer, more saturated acyl chains give rise to higher phase transition temperatures. The acyl chains are characterized by an order parameter "s" where s = 1 for no motion, and s = 0 for rapid isotropic motion. Below the T_c, the acyl chains have a high "order" (s ~ 1), meaning that the chains are packed together in a frozen or "gel" phase where motion of the acyl chains is restricted. At temperatures above the T_c, the acyl chains are more fluid and less ordered in a "liquid-crystalline" phase. Longer acyl chains have increased order whereas unsaturated acyl chains disrupt packing and reduce the acyl chain order of the membrane. The phospholipid head group can also affect the T_c, as seen in Table 1.1. In general, membranes are more permeable to a variety of solvents and solutes at or above the T_c than below (Bittman and Blau, 1972) and increased unsaturation or shorter acyl chains have been correlated with increased membrane permeability (Papahadjopoulos *et al.*, 1973).

A simple example of how the T_c can be used in designing effective liposomal carriers concerns development of what have been termed temperature sensitive liposomes (Weinstein *et al.* 1980; Magin *et al.*, 1986). These liposomes are composed primarily of dipalmitolylphosphatidylcholine, which has a T_c of 41°C. These liposomes can be induced to release entrapped contents by inducing local hyperthermia at regions where these liposomes accumulate following *i.v.* administration and result in increased drug availability. The studies described in this thesis also take advantage of differences in acyl chain composition to promote drug release. In particular, dimyristoyl- (T_c = 24 °C) and distearoyl- (T_c = 55 °C) phosphatidylcholine are used as the primary phospholipid components of the liposomes

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Table 1.1

Transition temperature (T_c) of various combinations of acyl chain length, degree of saturation, and headgroup moiety

Lipid Species	Transition Temperature T_c (°C)
Dilauroyl PC (12:0, 12:0)	-1
Dimyristoyl PC (14:0, 14,0)	24
Dipalmitoyl PC (16:0, 16:0)	41
Distearoyl PC (18:0, 18:0)	55
Stearoyl, oleoyl PC (18:0, 18:1)	6
Stearoyl, linoleoyl PC (18:0, 18:2)	-13
Dipalmitoyl PA (16:0, 16:0)	67
Dipalmitoyl PE (16:0, 16:0)	63
Dipalmitoyl PS (16:0, 16:0)	55
Dipalmitoyl PG (16:0, 16:0)	41

characterized in this thesis. Differences observed in drug release from these liposomes are attributable, at least in part, to difference in permeability ascribed to acyl chain composition.

1.2.2 Cholesterol

Cholesterol is the major neutral lipid component of eukaryotic biological membranes and is composed of a rigid steroid ring and a polar 3-β-hydroxyl group. It orients itself with the hydroxyl group toward the lipid/water interface and the rigid steroid ring associated with the acyl chains. The flexible aliphatic tail extends into the membrane. Incorporation of cholesterol into the bilayer results in a decrease in the membrane order for phospholipids in the gel phase and increases the order of the membrane for lipids in the liquid-crystalline phase (De Kruyff et al., 1973; Demel and de Kruyff, 1976). At amounts above 7 mol %, the enthalpy of the gel to liquid crystalline phase transition is reduced until at 33 mol % and greater, the phase transition can no longer be detected (Hubbell and McConnel, 1971). Addition of cholesterol to unsaturated and saturated phosphatidylcholine (PC) membranes above their phase transition temperatures

decreases membrane permeability, while increasing the membrane permeability for membranes composed of saturated PC below the T_c (Bittman and Blau, 1972).

Cholesterol is an essential component of liposomes if they are to be used as drug carriers. The presence of cholesterol at levels in excess of 30 mol% reduces serum protein binding (Patel et al., 1983; Semple et al., 1996). This, in turn, increases the circulation lifetime of the carrier (Kirby et al., 1980, Patel et al., 1983) and decreases release of entrapped contents (Fielding and Abra, 1992). The stabilizing role of cholesterol has been best illustrated by studies completed in Scherphof's laboratory (Scherphof et al., 1978; 1979). These investigators demonstrated that liposomes prepared of dimyristoylphosphatidylcholine were completely "dissolved" when incubated with serum at the T_c ($24^{\circ}C$), an effect attributed to interactions with lipoproteins. Addition of cholesterol eliminated the serum-mediated destruction of these liposomes.

1.2.3 Preparation of liposomes

Upon hydration of lipids, multilamellar vesicles (MLVs) are formed. These liposomes are heterogeneous and range in diameter from 1-10 microns. MLVs have proven to be of limited value for pharmaceutical applications, particularly those involving i.v. administration. These liposomes are rapidly eliminated from the plasma following injection due to their large size (Rahman et al., 1982). In addition, these liposomes tend to have a low trapped volume due to the tight packing of the bilayers (Perkins et al., 1988). This trapped volume can increase with the incorporation of charged lipids that promote swelling of the liposomes due to the electrostatic replusion between the bilayers (Hope et al., 1986). In addition, methods that promote more efficient hydration of the lipids can also increase the trapped volume of MLVs. These methods would include reverse phase procedures (Szoka and Papahadjopoulos, 1978; 1980), dehydration-

rehydration methods (Shew and Daemer, 1985), and those that use repeated freeze/thaw cycles (Mayer *et al.*, 1985a; Ohsawa *et al.*, 1985). Although the large size and heterogeneous nature of MLVs make them unsuitable for systemic applications, the steps used in the preparation of the MLVs define some of the attributes and the ease of manufacturing of the unilamellar liposomes that are commonly used for drug carrier applications. MLV precursors used in this thesis typically were subject to the freeze-thaw procedure to ensure equilibrium solute distribution and optimal trapped solute concentration. The latter term refers to circumstances where the trapped solute concentration is equivalent to the solute concentration used when hydrating the dried lipids (Mayer *et al.*, 1985a).

1.2.3.1 Unilamellar vesicles (LUV and SUV)

Small unilamellar vesicles (SUVs) range in size from 25-50 nm in diameter and are produced by sonicating MLVs or by forcing MLVs under high pressure through small openings. The latter refers to a method that originally used a French press (Barenholz *et al.*, 1979) or as more recently developed for large-scale manufacturing of SUVs, an automatic high-pressure system called a Microfluidizer (Cheng *et al.*, 1987). Although relatively easy to prepare and scalable to large (>10 L) batch size, vesicles produced by these techniques tend to be unstable due to the curvature of their membranes and an associated propensity to fuse and form larger membrane structures. In addition, these systems tend to have small trapped volumes (<0.2 μl/μmol) making them less suitable as drug carriers. Finally, following *i.v.* administration SUVs are small enough to penetrate the fenestrations that exist in the blood vessels of the liver (Hwang and Beaumier, 1986) and are accumulated in this organ at a faster rate then unilamellar liposomes that exhibit a mean diameter just 2- to 3- times larger. Combined, these properties make SUVs less useful as drug carriers.
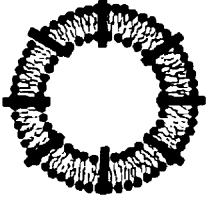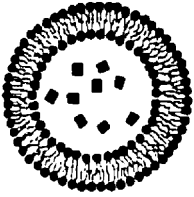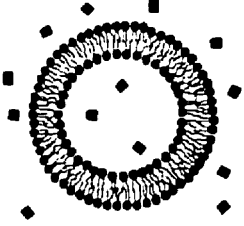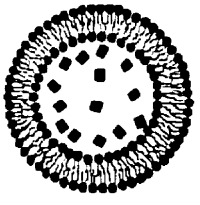
Large unilamellar vesicles (LUV's) exhibit a mean diameter of between 50 and 400 nm and the majority of the vesicles consist of one bilayer enclosing an aqueous space. Many procedures have been described for the preparation of LUVs. but the most versatile and frequently utilized technique involves extruding MLV's through polycarbonate filters using high pressures of an inert gas (Olson *et al.*, 1979; Hope *et al.*, 1985; Mayer *et al.*, 1986). This procedure forms a homogeneous population of unilamellar liposomes of well defined sizes depending on the pore size of the filter used (50 nm –200 nm). LUVs are most suitable for drug delivery applications because of their higher trapped volumes (1.5 to 10 μl/μmole lipid), stability and pharmacokinetic/ biodistribution characteristics. This size has been found to be optimal for stability in the circulation as well as extravasation through vasculature. Unless otherwise indicated the remaining sections of this introduction refer to the preparation and *in vitro/in vivo* characterization of LUVs designed for intravenous applications as drug carriers.

1.2.4 Drug encapsulation

There are essentially two techniques available for drug encapsulation: passive trapping and active trapping. These are illustrated in Figure 1.3. Passive trapping involves the addition of drug during the hydration of lipid. The efficiency of this encapsulation procedure depends on the nature of the compound, where the level of hydrophobic compound association is governed by the capacity of the bilayer to incorporate the agent and the level of hydrophilic compound encapsulation is dependent on the aqueous trapped volume of the liposome used. Active trapping refers to techniques that involve addition of the therapeutic agent to pre-formed liposomes.

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Figure 1.3

Illustration of passive and active entrapment

Type	Method	Distribution
Passive encapsulation of hydrophobic drug	Drug added to solvents used to solubilize lipids or added during hydration of lipids	
Passive encapsulation of hydrophilic drug	Drug added during hydration of lipids	
Active encapsulation of drugs exhibiting a protonizable amine function	Generation of an ion gradient (e.g. pH gradient) followed by addition of selected drug	 - pH gradient + pH gradient

Compounds that are hydrophobic will partition into the lipid bilayer. Alternatively techniques have been developed that rely on the chemical attributes of the drug and use of transmembrane ion gradients.

1.2.4.1 Passive entrapment (Figure 1.3)

Passive entrapment of drugs is accomplished by the preparation of liposomes in the solution of the agent that is to be entrapped (Taylor *et al.*, 1990). Use of this method generally results in poor drug entrapment and low drug-to-lipid ratios. For example, passive encapsulation of the anti-cancer drug doxorubicin results in a 4% trapping efficiency and a drug-to-lipid ratio of 0.004:1 (wt:wt) (Shinozawa *et al.*, 1981). Trapping efficiency and drug-to-lipid ratio attributes are, of course, dependent on the aqueous trapped volume of the liposome as well as the lipid concentration when preparing the liposome. Mayer *et al.* (1986), for example, demonstrated 80% trapping efficiency using liposomes extruded through 100 nm pore size polycarbonate filters. Considering the trapped volume of these liposomes is typically between 1.5 and 2.5 µl/µmole lipid. 80% trapping efficiency can only be obtained by preparing the liposomes at high lipid concentration (up to 400 µmol/ml).

Hydrophobic drugs, such as cyclosporin A, are also entrapped in this manner (Ouyang *et al.*, 1995). In this case, drug incorporation is dependent on the packing constraints of the drug in the membrane and the lipid characteristics. This procedure can result in high drug entrapment efficiency, but low drug-to-lipid ratios. Drugs of this class often exchange into other membranes rapidly and thus, *in vivo* the drug leaves the carrier quickly (Choice *et al.*, 1995).

Liposomal anti-cancer drug formulations described in this thesis use transmembrane ion gradient based trapping methods (see next section), but it is important to recognize that these gradient techniques rely on passive trapping procedures to prepare the liposomes for drug loading. As indicated above, the method should promote equilibrium solute distribution and the trapped volume of the liposome should be sufficient to insure an adequate trapping capacity (Boman *et al.*, 1993). For these reasons, liposomes prepared for use in active drug loading procedures are typically generated by extrusion of frozen and thawed MLVs through 100 nm pore size polycarbonate filters. The lipid concentration used when preparing these liposomes is not as critical as that required for passive encapsulation of hydrophilic drugs.

1.2.4.2 Active entrapment (Figure 1.3, 1.4)

The active trapping procedure is identified with any technique where drugs are loaded into preformed liposomes. For this reason any procedure using hydrophobic drugs that partition into the membranes of pre-formed liposomes can be defined as an active loading technique. It is more common, however, to associate active loading procedures with drugs that exhibit protonizable amine functions which can accumulate inside preformed liposomes exhibiting a transmembrane pH gradient (Mayer *et al.*, 1985b; Madden *et al.*, 1990; Mayer *et al.*, 1993). The mechanism for accumulation (see Figure 1.4) is in response to a proton gradient where the interior of the liposomes have acidic pH. In the external environment, the neutral form of the weak base is membrane permeable and crosses the lipid bilayer. Once it enters the internal acidic environment, the weak base becomes protonated. The protonated form is then unable to permeate back across the lipid bilayer and is effectively "trapped" within the interior of the liposome. Assuming the pK_a is the same on both sides of the membrane, the intravesicular and

external drug concentration can be derived from the Henderson-Hasselbach equation as:

$[HA^-]_{in}/[HA^-]_{out} = [H^+]_{in}/[H^+]_{out}$

Therefore, a difference of 3 pH units between the exterior and interior of the liposome will permit drug accumulation up to a maximum drug gradient of 10^3 fold higher inside versus outside.

There are many advantages to the use of this procedure. First, this technique allows for trapping efficiencies approaching 100%. In addition, the rate of drug efflux is decreased by approximately 30-fold (Mayer *et al.*, 1986) when compared to the same drug (doxorubicin) passively encapsulated in liposomes. Finally, provided the buffering capacity of the internal buffer has not been depleted, this trapping method works independently of the starting drug to lipid ratio and can be used with almost any liposome formulation which is capable of maintaining an ion gradient. As noted in Chapter 2, anti-cancer drug loaded liposomes where prepared using the pH gradient based loading procedure, where 300 mM citrate buffer (pH 4.0) was trapped inside. Many variations of the ion gradient based loading procedures have been developed (Mayer *et al.*, 1985b; Lasic *et al.*, 1992; Haran *et al.*, 1993; Cheung *et al.*, 1998; Fenske *et al.*, 1998), and as indicated in the following section these active loading procedures played a fundamental role in the development of clinically viable anti-cancer drug formulations.

1.3 Liposomes as drug carriers

Research on liposomes as model membrane systems and as drug carriers facilitated the design of pharmaceutically viable lipid-based drugs. In fact much of the research and technology required to prepare liposomal carriers for testing in clinical trials was well established by 1987 (Cullis *et*

Figure 1.4
Active drug entrapment in liposomes

Redistribution of a lipophilic amine (weak base) in response to a pH gradient (ΔpH) across the liposome membrane. Only the neutral form of the molecule is capable of crossing the lipid bilayer. (Figure reproduced from Parr, 1995)

$$K_a = \frac{[B]_o[H^+]_o}{[BH^+]_o} \qquad\qquad K_a = \frac{[B]_i[H^+]_i}{[BH^+]_i}$$

At equilibrium, if: $\qquad [B]_o = [B]_i$

Then: $\qquad\qquad\quad \dfrac{[BH^+]_i}{[BH^+]_o} = \dfrac{[H^+]_i}{[H^+]_o}$
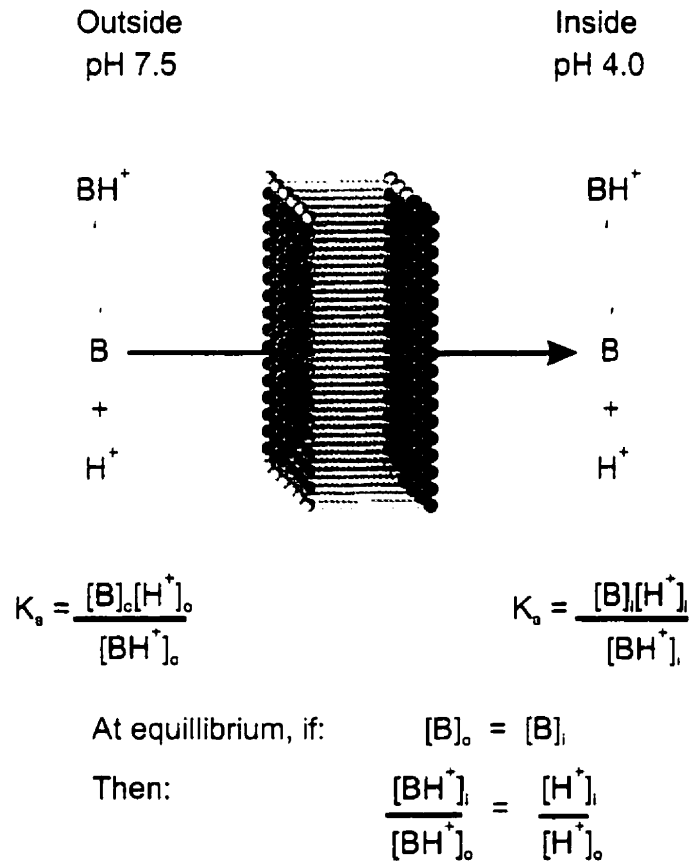
al., 1987; Ostro and Cullis, 1989; Perez-Soler, 1989). By that time, four pivotal hurdles were overcome. First, the importance of carefully assessing structure activity relationships through analysis of physiochemical characteristics was proven to be essential in product development. This is exemplified by studies contributing to the characterization of the amphotericin-B lipid complex (Janoff *et al.*, 1988; Grant *et al.*, 1989). Second, biological barriers previously believed to limit the distribution properties of systemically administered macromolecular drug carriers, such as liposomes, proved to be penetrable. In 1983, John Baldeschwieler and co-workers recognized that liposomal drugs could effectively deliver contents to tumours (Proffitt *et al.*, 1983), a phenomena that continues to be a fundamental rationale for development of systemically administered liposomal anti-cancer drugs (Gabizon and Martin, 1997). Third, manufacturing issues for preparing pharmaceutically acceptable formulations were resolved (Lichtenberg and Barenholz, 1988; Swenson *et al.*, 1988; Vuillemard, 1991). This included identification of sources for inexpensive raw materials, the elucidation of procedures for storing lipid-based carriers for extended time periods (Madden et al, 1985) and the development of methods for reproducibly preparing large batches of liposomes with attributes that could be characterized according to the rigorous guidelines of health boards such as the FDA. Fourth, procedures for loading liposomes with pharmaceutically active agents that relied on the chemical attributes of the lipids prior to liposome formation (e.g. doxorubicin/cardiolipin complex) and/or involved loading of pre-formed liposomes were developed (Wizke and Bittman, 1984; Gootmaghtigh *et al.*, 1987 Mayer *et al.*, 1990; Schwendener *et al.*, 1991; Haran *et al.*, 1993). The latter involves the use of ion gradients to effect drug loading (see section 1.2.4.2), a procedure that has proven to be particularly useful and versatile.

At the end of the 1980's investigators confidently suggested that liposomes could be designed to achieve specific therapeutic benefits for a broad range of disease targets. It is perhaps

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disappointing, therefore, that improvements in the therapeutic properties of liposomal drugs have been relatively incremental since 1990. The most significant revisions of lipid-based carrier technology that have guided research efforts during the 1990's involved three breakthroughs made in the late 1980's: 1) the observation that surface associated polymers (i.e. polyethylene glycol or the ganglioside GM1) cause changes in the liposome surface properties that contribute to increased circulation lifetimes (Allen and Chonn, 1987; Papahadjopoulos et al., 1991); 2) the discovery that positively charged liposomes can be used to transfer polynucleotides into cells (Brigham et al., 1989; Felgner and Ringold, 1989); and 3) the identification of certain lipids that can act as therapeutic molecules (Berdel et al., 1986).

1.3.1 Liposomal anti-cancer drugs

There are two general reasons for developing a liposomal anti-cancer drug. First, the drug may be hydrophobic and difficult to dissolve in aqueous solutions, and thus a hydrophobic environment is required in order for the drug to remain in solution/suspension. Second, the liposome can serve as a carrier that will improve drug specificity by increasing delivery to the site of disease and/or decrease delivery to a site where toxicity is manifested. The former is an important, perhaps underdeveloped, role for lipid-based carriers. However, the methods and characterization studies required for development of lipid-based formulations optimal for drug solubilization are distinct from those used in the development of liposome drug carrier technology. Differences in the two approaches can be defined primarily through *in vivo* studies that determine plasma elimination behavior of both drug and liposomal lipid. If the drug dissociates from the liposome immediately following administration then the lipid-based carrier is acting as an excipient for drug solubilization. When drug elimination parameters are dictated by the elimination behavior of the liposomes, then the systems are acting as true delivery vehicles.

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This thesis focuses on use of liposomes developed as drug carriers. The primary consequence of anti-cancer drug encapsulation is liposome-mediated changes in drug elimination and biodistribution. It is important to recognize that therapeutic responses obtained following administration of anti-cancer drugs, in free form or associated with a drug carrier, are dependent on tumour physiology and tumour cell heterogeneity. Ideally, an effective drug must access the target cell populations at levels sufficient to cause cytotoxic effects and should be effective in all microenvironments present within tumours. In humans, strategies designed to maximize the anti-tumour activity of chemotherapeutic agents must, therefore, contend with a heterogeneous population of proliferating cells. Tumour cells are proliferating at different rates, are governed by differences in cell cycle control and are capable of adapting rapidly to the chemotherapeutic stresses exerted on them. In practical terms this means that chemotherapy typically involves the use of multiple drugs that exert anti-tumour activity via different mechanisms (De Vita, 1997). Vincristine is a cell cycle specific agent that acts by destabilizing microtubules and is almost always used in combination with two or three other anti-cancer drugs. The therapeutic action of vincristine is complemented by drugs such as doxorubicin (an anthracycline that acts as a topoisomerase II inhibitor) as well as cyclophosphamide (a nitrogen mustard pro-drug and strong alkylating agent). The mechanisms of therapeutic action of these drugs are quite different; this complementary nature and the side effects of each drug are sufficiently different such that they can be used in combination, thereby increasing the reduction in tumour burden and decreasing the risk of drug resistance.

In addition to the necessity of using multiple agents to achieve optimal therapy, another general principle of cancer chemotherapy concerns maximizing dose intensity (Livingston, 1994). Tumour cells must be exposed to the highest levels of drug for the longest time periods if maximum therapeutic effects are to be achieved (Mulder and de Wit, 1995). The advantage of anti-cancer drug carrier technology is based on carrier characteristics that give rise to increased

drug exposure in sites of tumour growth. An example of how liposome drug carrier technology can improve the pharmacodynamic behavior of an anti-cancer agent is evident when evaluating studies with doxorubicin. Efforts to maximize the dose intensity of this chemotherapeutic agent (in free form) have been limited due to non-specific toxic side effects. Therapeutic doses must, therefore, be limited to schedules and amounts that do not compromise regeneration of blood cells or cells of the immune system. In addition. doxorubicin exhibits a dose limiting cardiotoxicity (Minow et al., 1975) restricting the total dose to approximately 450 mg/m². Myelosuppression can be counteracted using the hemopoietic growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) (Elias et al., 1993). Administering the drug in a liposomally encapsulated form, on the other hand, can reduce cardiotoxicity (Gabizon et al., 1982; Herman et al., 1983; Balazsovits et al., 1989). It has also been shown that the therapeutic activity of the liposomal drug is greater than or equal to free doxorubicin in a variety of pre-clinical and clinical studies (Mayhew et al., 1987; Mayer et al., 1990; Elias et al., 1993; Northfelt et al., 1997; Vail et al., 1997).

Table 1.2 summarizes information on some of the major anti-cancer drugs that have been evaluated in a liposomal formulation. The formulations that have advanced the furthest along the clinical development pathway includes those used for doxorubicin (approved for clinical use in AIDS related Kaposi's sarcoma), daunorubicin (approved for clinical use in AIDS related Kaposi's sarcoma); cisplatin (Phase I clinical trials; Perez-Soler et al., 1990), and mitoxantrone (Phase I/II clinical trials. Pestalozzi et al., 1995). The studies developed in this thesis have focused on the anti-cancer drug mitoxantrone. Some of the rationale for selecting this drug have been summarized below. In addition, data summarized in Chapters 3-5, add to these rationale and suggest that mitoxantrone is a excellent drug to consider for development as a liposomal formulation.

Table 1.2

Major antineoplastic agents evaluated in a liposomal drug carrier system

Class/Drug	# of Different Liposomal Formulations	Pre-clinical Evaluations	Clinical Testing
Plant Alkaliods-			
Vincristine	<10	extensive	Phase II
Vinblastine	<5	very limited	---
Anthracyclines-			
Doxorubicin	>10	extensive	Approved
Daunorubicin	<5	extensive	Approved
Mitoxantrone	<5	extensive	Phase II
Antimetabolites-			
Methotrexate	<5	limited	---
5-Fluorouracil	<5	limited	---
Cytosine arabinoiside	<5	limited	---
Other			
cis-diamminedichloroplatinum	<5	limited	---

1.3.2 Mitoxantrone

Mitoxantrone is a dihydroxyanthracenedione (as shown in Figure 1.5) that was developed in an effort to produce new agents with similar modes of action to doxorubicin without the cardiotoxic side effects. It has demonstrated activity in a wide range of experimental tumours such as P388 and L1210 leukemias. ADJ PC6 plasmacytoma. B16 melanoma. colon and mammary adenocarcinomas. transitional cell bladder carcinoma. and M5076 carcinoma (Johnson et al.. 1979; Wallace et al.. 1979; Corbett et al.. 1982; Fujimoto and Ogawa. 1982; Schabel et al.. 1983 a,b; Ballou and Tseng, 1986). It has been investigated in the treatment of advanced breast cancer (Brambilla et al.. 1989; Harris et al.. 1990). non-Hodgkin's lymphoma (Bajett et al.. 1988. Ho et al., 1990), acute leukemia (Bezwoda et al., 1990; Amadori et al., 1991; Archimbaud et al., 1991; Hiddemann et al., 1991; Wahlin et al., 1991), and hepatocellular carcinoma (Dunk et al., 1985;

Yoshida *et al.*, 1988; Lai *et al.*, 1989; Colleoni *et al.*, 1992). Mitoxantrone has proved useful as palliative therapy in patients with hepatocellular carcinoma (Civalleri *et al.*, 1996), advanced breast cancer (Roberston *et al.*, 1989), or prostate cancer (Tannock *et al.*, 1996).

Figure 1.5

Structure of mitoxantrone hydrochloride

OH   O   H N   H
                       N   OH

. 2 HCL

OH   O   N   OH
          H   N
                        H
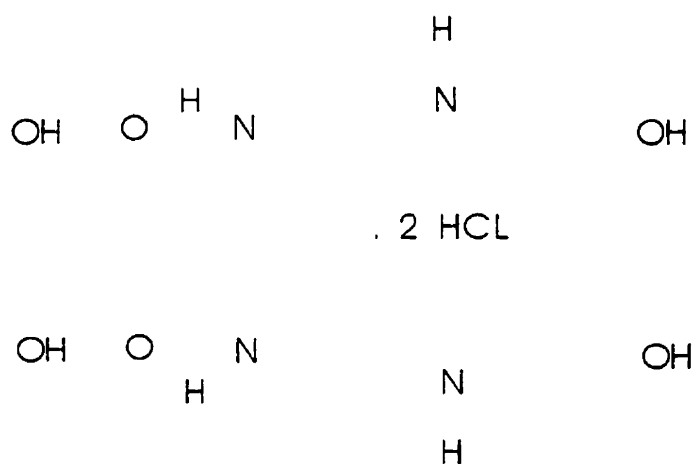
There are several mechanisms of action that have been identified: 1) DNA intercalation, 2) stabilization of the topoisomerase-DNA complex, 3) DNA condensation *via* electrostatic cross-linking, and 4) non-protein-associated DNA strand breaks induced by free radical generation *via* oxidative activation. Structurally mitoxantrone is similar to doxorubicin in that it contains a planar polycyclic aromatic ring structure which allows it to intercalate within the DNA and inhibit DNA and RNA synthesis (Safa *et al.*, 1983; Durr, 1984). In addition, mitoxantrone induces protein associated strand breaks *via* stabilization of the topoisomerase II complex. Topoisomerase enzymes are responsible for the catalysis of the breaking and rejoining of DNA *via* an enzyme-DNA intermediate, topoisomerase I for single strands and topoisomerase II for

double strand breaks. Mitoxantrone appears to inhibit topoisomerase II by binding to the enzyme-DNA complex, thereby preventing rejoining of the DNA. Unlike doxorubicin, which is reduced to a semiquinone free radical via NADPH cytochrome P-450 reductase, mitoxantrone does not produce any free radicals by this pathway and acts as a potent antioxidant (Fisher et al., 1989; Vile and Winterbourn, 1989). However, mitoxantrone can undergo peroxidative conversion to an unstable diimino compound which then generates a radical cation. This oxidative activation results in DNA damage as demonstrated by Fisher and Patterson (1989).

Several liposomal formulations of mitoxantrone have been developed for the treatment of cancer. Two groups have focused on passive entrapment of mitoxantrone in liposomes (Schwendener et al., 1991; Law et al., 1996). Phase II clinical trials have been conducted but demonstrated disappointing activity in the treatment of breast cancer. The formulation tested, however, exhibits rapid release characteristics and /or liposome elimination with the majority of the liposomes eliminated from the plasma compartment within the first 10 minutes (Schwendener et al., 1994). Thus, the benefits of using a liposomal carrier were not observed. In addition, the use of charged liposomes increased the accumulation of the liposomes to the liver and spleen. Other formulations developed have utilized the pH gradient encapsulation of mitoxantrone (Madden et al., 1990; Schwendener et al., 1994; Chang et al., 1997). These formulations demonstrate significant increases in drug circulation lifetime and levels of drug within the plasma compartment when compared to the free drug (Schwendener et al., 1994; Chang et al., 1997;), leading to improvements in efficacy over the free drug (Chang et al., 1997; Lim et al., 1997).

1.4 Biological fate of liposomes following intravenous administration

In vivo studies are usually initiated only after the development of a liposomal formulation that exhibits the necessary chemical and physical stability properties to be considered pharmaceutically viable. As suggested in section 1.3, technological advances as well as an increased understanding of lipid chemistry have, to large extent, overcome many pharmaceutical hurdles. This section will focus on systemic administration and, in particular, on the fate of lipid-based delivery systems injected intravenously (*i.v.*).

1.4.1 Barriers and compartments

In vivo analysis must consider the fact that a liposomal drug will interact with a number of distinct physiological "compartments" and associated barriers between compartments. After injection, liposomes are exposed to a variety of circulating protein and cellular components that reside within the central blood compartment, many of which can destabilize the liposomes through interactions with the lipid bilayer or initiate biological processes that lead to increased liposome leakage and/or clearance *via* the mononuclear phagocyte systems (Allen and Cleland, 1980; Bronte *et al.*, 1986; Liu *et al.*, 1997). To gain access to a disease site in an extravascular compartment, liposomes must cross the vascular endothelium, the blood vessel lining which is composed primarily of endothelial cells and, in most cases, an underlying basement membrane and associated smooth muscle cells (See Figure 1.6). This vascular barrier represents the greatest obstacle for liposomal drug delivery to extravascular disease sites, however, at the same time it offers properties that can be utilized to differentiate between normal and diseased tissue. Should liposomes traverse this barrier, a second compartment is encountered consisting of the interstitial space and associated fluids and cells. This compartment can vary significantly not only between normal and disease tissues but also among normal tissues in different organs of the

Figure 1.6

Structure of a capillary: continuous endothelium type

Electron micrograph of a capillary composed of continuous endothelium. The capillary is supported by a basement membrane (BM) and collagen fibrils (C). A pericyte (P) embraces the capillary and is supported by its own basement membrane (BMp). The endothelial cells are seen encircling the capillary lumen with cytoplasmic flaps called marginal folds (M) extending across the intercellular junctions. (Reproduced from Burkitt et al., 1993)
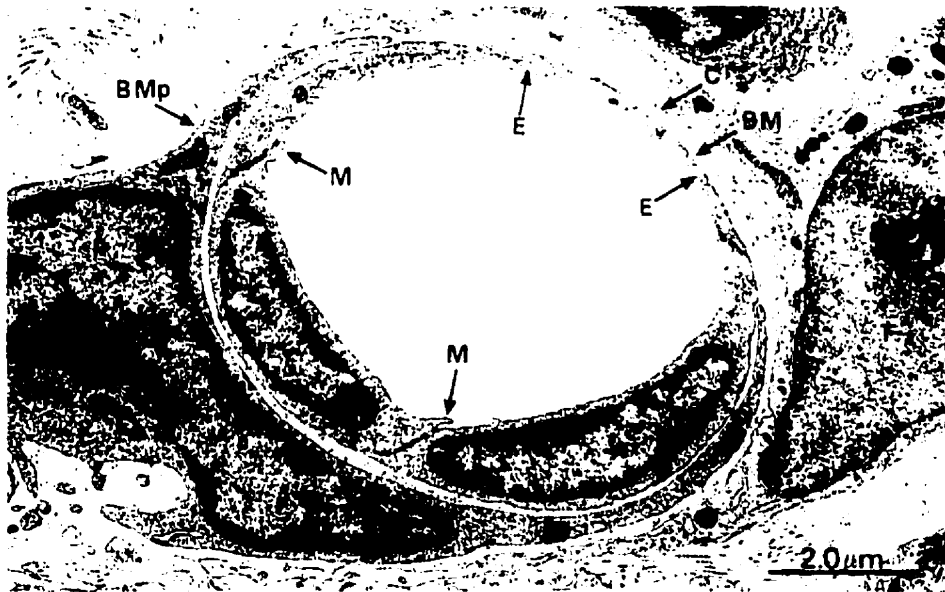
body. Within this compartment, the barriers to liposome movement and distribution are varied and include factors such as interstitial volume, interstitial pressure, and the presence (or absence) of a lymphatic system. The final physiological compartment(s) is the cells into which liposomes and/or their associated agents are taken up. This includes intracellular organelles that may be involved in processing of the administered agent or that contain the molecular target through which the drug exerts its therapeutic activity. The critical barrier that must be crossed in order to access this final compartment is the cell membrane. Similar to the vascular endothelium, crossing this barrier is a significant obstacle to the development of therapeutically optimized liposomal anti-cancer drugs.

In the following sections, the fate of liposomes will be discussed as they enter these physiological compartments and pass through the various barriers. The focus will be on specific interactions between liposomes and the biological milieu in the various compartments that directly impact on the delivery of encapsulated agents to their therapeutic target. Further, sections will highlight strategies that have been employed to augment conventional liposomes (defined as un-derivatized membrane bilayers composed of naturally occurring lipids) with components that alter these interactions.

1.4.2 Liposome serum protein interactions

Within the blood vessels, liposomes are exposed to circulating cells, lipoproteins, other serum proteins as well as other small molecules such as carbohydrates and divalent cations. As indicated in section 1.2.2, liposomes designed for intravenous application typically contain 30 to 50 mol % cholesterol, a required component to minimize the protein-liposome interactions (Patel *et al.*, 1983; Semple *et al.*, 1996). It is important to recognize that cholesterol-containing liposomes bind other serum proteins (Bonte and Juliano, 1986; Chonn *et al.*, 1992) and the

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biological fate of the liposome is determined. in part, by these associated proteins. Serum protein binding can increase membrane permeability as well as play a role in defining the liposome elimination rate and biodistribution characteristics. These two effects are discussed in the following sections.

1.4.2.1 Serum-induced increases in membrane permeability

Serum protein binding can increase liposome permeability non-specifically and specifically. The latter is best illustrated by studies assessing liposome-complement protein interactions (Silversmith and Nelsestuen. 1986a. b. Malinski and Nelsestuen. 1989. Shiver *et al.*, 1991). It is known. for example. that anionic liposomes (those containing PS or cardiolipin) can activate the alternative complement pathway that is associated with C3b binding and formation of the membrane attack complex (MAC). The MAC is a complex of the complement proteins C5b-6. C7, C8 and C9 and its formation has been associated with increased membrane permeability (Malinski and Nelsestuen. 1989) attributed to ion channel formation and/or pore formation as well as transbilayer flip-flop of lipids (Van der Meer *et al.* 1989). In addition. complement binding has been shown to influence the binding of C-reactive protein (CRP) (Li *et al.*, 1994) and vise-versa (Richards *et al.*, 1977. Richards *et al.* 1979). CRP is known to localize in sites of inflammation or other regions displaying membrane damage and it plays a role in recruitment of inflammatory cells including those phagocytic cells involved in removal of damaged cells. Binding of CRP will, therefore, play a role in immune recognition of certain liposome formulations. This is typically not an important factor when the liposomal formulations being developed are composed of neutral lipids. such as PC and cholesterol.

In the context of this thesis. non-specific effects of serum protein binding on liposome permeability are those that can not be attributed to a defined protein or complex of proteins.

Serum-induced increased release of encapsulated drugs (e.g. vincristine) and markers (e.g. calcein) (Allen and Cleland, 1980; Boman *et al.* 1993) can be determined *in vitro* and *in vivo*. Interestingly, release rates measured *in vitro* in the presence of serum are often much slower than those measured following *i.v.* administration. The *in vivo* data are determined by monitoring changes in the drug to lipid ratio of liposomes within the plasma compartment (Figure 1.7). As shown in Chapter 3, drug leakage rates can be significantly greater *in vivo* than *in vitro*. This is consistent with other reports that stress that *in vivo* drug retention properties and drug release kinetics for different liposomal formulations can not be predicted on the basis of *in vitro* data (Bally *et al.*, 1993). Another serum protein mediated effect on membrane permeability is particularly unique to the active loading procedure, such as that used in this thesis. The high concentrations of buffer components and/or entrapped drug in liposomes can result in a significant osmotic gradient across the liposome membrane when exposed to physiological fluids. It has been shown that liposomes can withstand a transmembrane osmotic gradient of greater than 100 mOsm/kg in the absence of serum proteins; however, these liposomes release a portion of their contents when diluted into serum containing buffers (Mui *et al.*, 1994). This is typically seen as a burst of entrapped-content release that occurs while an osmotic balance across the membrane is re-established.

1.4.2.2 Serum protein binding and liposome elimination

In a general context, there appears to be a direct correlation of increased protein binding to liposomes and increased liposome elimination rates (Chonn *et al.*, 1992). Increased protein binding and clearance are, in particular, identified with liposomes composed of anionic lipids (e.g. phosphatidylserine, cardiolipin and PA) (Spanjer *et al.*, 1986; Chonn *et al.*, 1992) and cationic lipids (e.g. stearylamine) (Mold *et al.*, 1981; Oku *et al.*, 1996). Certain proteins such as complement proteins, serum albumin and beta 2 glycoprotein 1 have been associated with

Figure 1.7

Liposomal drug release

Drug release from liposomes *in vivo* can be estimated by measuring drug-to-lipid ratio of liposomes in the blood compartment. It is important to recognize that two events are being monitored as a function of time after i.v. administration. Liposomes are being eliminated from the plasma compartment and, in addition, drug is being released from the liposomes.
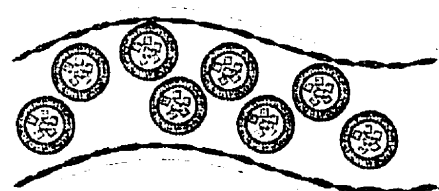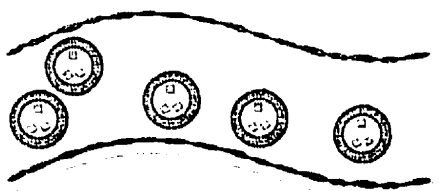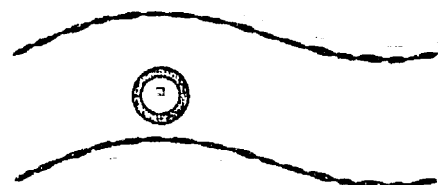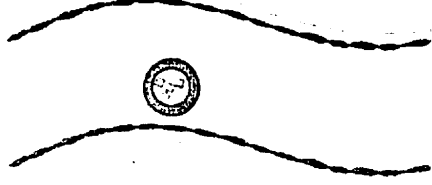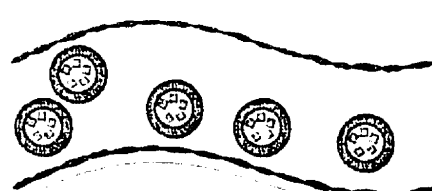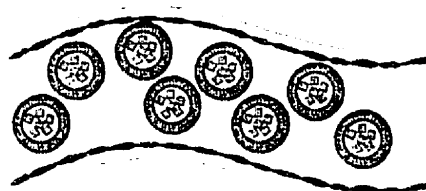
increased elimination rates and these have, in turn, been attributed to their role as opsonizing proteins that are instrumental in "labeling" foreign macromolecules in the blood compartment (Chonn *et al.*, 1995). This is an essential component of the immune system that facilitates recognition of bacteria and damaged/dead cells by cells of the mononuclear phagocytic system (MPS) (see section 1.4.4). It is notable that not all anionic lipids cause an increase in liposome clearance. Phosphatidylglycerol and phosphatidylinositol containing liposomes exhibit plasma elimination rates that are comparable to or slower than neutral liposomes and this is in spite of having increased levels of absorbed serum proteins (Chonn *et al.*, 1992). It is also worth noting that protein binding can have a direct effect or an indirect effect on liposome elimination from the plasma compartment. The indirect effect is one related to "opsonization" of liposomes and subsequent recognition by the MPS. PS containing liposomes, for example, are eliminated rapidly following *i.v.* administration due to this opsonization effect (Spanjer *et al.*, 1986). In contrast, PG containing liposomes (when administered to rats) bind the complement protein C3b which is subsequently converted to C3bi. The presence of bound C3bi facilitates liposome binding to platelets that express the C3bi receptor, and an associated platelet aggregation reaction occurs (Reinish *et al.*, 1988; Doerschuk *et al.*, 1989). This aggregation reaction leads to removal of the aggregates in certain vascular beds such as those in the lung and spleen. This elimination mechanism is not affected by the MPS.

It has also been postulated that saturation of serum protein binding can occur, i.e. that there is a limited amount of blood protein that is available to bind to liposomes (Oja et al., 1996). It has been demonstrated that at liposome doses ranging from 10 to 100 mg lipid/kg animal weight, circulation lifetime increases. In addition, the amount of protein bound to the liposome decreases as the dose increases. However, it is believed that at higher doses saturation of the MPS system occurs, resulting in the increased circulation lifetime (Abra and Hunt, 1981; Bosworth and Hunt, 1982). To date the exact mechanism of liposome elimination has yet to be

elucidated but two factors affecting liposome elimination kinetics are: 1) the role of the MPS system and 2) the degree of protein binding.

1.4.3 Extravasation through blood vessels

A microvascular structure is a capillary network composed of endothelial cells, a basement membrane, connective tissue elements, associated marginated leukocytes and the presence of certain serum proteins which function collectively as a selective barrier to circulating cells and macromolecules. In addition, the microvascular structure serves to selectively determine what size macromolecules can penetrate the blood vessel and this in turn is dependent on the tissue type and/or the presence of disease (Dvorak *et al.*, 1988).

With regards to liposomal drug carriers systems there is compelling evidence (both theoretical and experimental) that these circulating macromolecules will have limited access to extravascular sites (Jain and Baxter, 1988; Yuan *et al.*, 1995). Liposomes with a mean diameter in excess of 50 nm will only leave the blood compartment in tissues where large pores or fenestrations exist in the associated blood vessels. Blood vessels of the liver and spleen provide examples of such tissues. However, there is also substantial evidence, albeit phenomenological, that liposomes can access extravascular sites within tumours following intravenous administration (Gabizon A.A., 1988; 1992; Yuan *et al.*, 1994).

1.4.3.1 Tumour vasculature

It is established that tumours can exhibit unique microvascular structures that are often incapable of maintaining a complete permeability barrier between the vascular compartment and the growing tumour mass (Heuser and Miller, 1986; Dvorak *et al.*, 1988). Thus there are potential

sites where large drug carriers can escape from the circulation. Blood vessels of particular interest include: 1) sinusoidal vessels which are extremely porous, exhibiting large gaps between endothelial cells that are not closed by any membrane structure providing a discontinuous endothelium; 2) capillaries which exhibit fenestrated endothelium characterized by pores between endothelial cells, which allow macromolecules in the range of 20 to 100 nm to pass; 3) blood channels which lack an endothelial cell lining, allowing blood to percolate around and between tumour cells; and 4) postcapillary venules in tumours which are characterized by vessel walls composed of endothelial cells, devoid of basement membrane, supported by some fibrous tissue. The presence of these blood vessels in tumours will promote leakage of circulating liposomes.

As indicated above, the organization endothelial cells adopt in different blood vessels influences the permeability characteristics of blood vessels. Endothelial cells also participate more directly in normal physiological processes regulating microvascular permeability (Simionescu, 1983; Simionescu et al., 1987; Pearson, 1991; Crone, 1986). These cells, for example, are known to have a direct role in the transport of serum components to extravascular compartments. Proteins and other circulating macromolecules can be taken up by endothelial cells via receptor mediated and fluid phase endocytosis (Simionescu, 1983; Simionescu et al., 1987). Subsequently the internalized material can be either degraded by transfer to lysosomal compartments (Ryan, 1988) or alternatively the endosome contents can be moved through the cell and released into the interstitial space on the opposite side of the cell (Kohn et al., 1992; Dvorak et al., 1996). This latter process is referred to as transcytosis. Further, it is known that endothelial cells are capable of phagocytosis and can actively accumulate particles in excess of 5 μm in diameter (Ryan, 1988). Given these characteristics it is reasonable to postulate that endothelial cells play an important role in governing the fate of liposomal drug carriers.

In addition, factors secreted by the tumour associated cells also affect the permeability of the vasculature. The most dominant factor is vascular endothelial growth factor (VEGF). This protein has been associated with several characteristics of tumour blood vessels such as increased vascular permeability (Dvorak *et al.*, 1991), increased transcytotic activity and angiogenesis (Folkman and Shing, 1992). The endothelial cells express a high-affinity receptor for VEGF (De Vries *et al.*, 1992; Takagi *et al.*, 1996) which is a member of the platelet-derived growth factor receptor family, *flt*. Expression of these high-affinity receptors can be induced in tumour vascular endothelial cells (Senger *et al.*, 1993) and in endothelial cells maintained under hypoxic conditions (Stein *et al.*, 1995).

1.4.4 Role of the mononuclear phagocytic system (MPS)

The MPS [previously referred to as the reticuloendothelial system (RES)] has long been recognized as the major site of liposome accumulation after systemic administration. The primary organs associated with the MPS are the liver, spleen and lung. The liver exhibits the largest capacity for liposome uptake while the spleen can accumulate liposomes such that the tissue concentration (liposomal lipid/g tissue) is as much as 10-fold higher than that which can be achieved in other tissues. Assuming that liposomes are designed to minimize protein binding (see section 1.4.2.2) and cell interactions, the extent of liposome accumulation in the lung is typically below 1% of the injected dose. Early studies demonstrated that large, as well as charged liposomes (particularly those containing negatively charged lipids like PS, PA or cardiolipin), were removed very rapidly by the liver and spleen with more then 50% of the injected liposomes being eliminated from the plasma compartment in less than 1 hour (Chonn *et al.*, 1992). However, when small (approx. 100 nm), neutral liposomes containing $\geq$ 30% cholesterol are injected at doses of at least 10 mg/kg or more the plasma elimination rate is

substantially reduced (Patel *et al.*, 1983; Semple *et al.*, 1996;). The removal of liposomes from the blood is attributed to phagocytic cells that comprise the MPS and uptake of liposomes by cells of the MPS is mediated through direct interactions between the phagocytic cell and the liposomes and is stimulated by the binding of certain serum proteins (Chonn *et al.*, 1992). When the dose of the liposomes is increased to levels of 100 mg/kg, there is a further increase in the circulation longevity of the liposome carrier. This is due to two effects: saturation of the MPS (Abra et al., 1981; Bosworth et al., 1982) and depletion of circulating opsonins which mark the liposomes for elimination (Oja et al., 1996). If liposomes are designed in an appropriate manner, whether with respect to size or lipid composition, liposomes can remain in the blood compartment for a period of several days (Parr *et al.*, 1997). The fact that under such circumstances the vast majority of liposomes administered can be accounted for in the blood, liver and spleen demonstrates that liposomes are relatively inefficient at crossing the endothelial cell barrier present in most other normal tissues.

1.4.4.1 Liposome accumulation in the liver

The liver represents a major obstacle for liposomal formulations that are being designed for extravascular sites such as tumours residing in sites located away from the liver. Liposomes rapidly accumulate in this organ due to 1) the blood supply and vessel structure and 2) the presence of Kupffer cells. The liver is unique in that it has a dual blood supply from the hepatic artery and the portal vein. Therefore, any intravenous injection will pass through the liver. Further, the liver functions as a filter, removing unwanted debris (e.g. senescent erythrocytes, bacteria, and toxins), as well as a detoxification organ. The cells responsible for this filtration process are the Kupffer cells which phagocytose and remove any foreign elements. Thus, following *i.v.* injection of a liposomal formulation, the liposomes will naturally accumulate in the liver due to the blood supply and be processed by the Kupffer cells.

There have been many studies investigating liposomal interactions with the cells of the liver including hepatocytes. endothelial cells and Kupffer cells (Hu and Liu. 1996: Spanjer *et al.*. 1986; Kamps *et al.*. 1997;). The architecture of the liver is such that the blood percolates through sinusoids lined by endothelial cells and Kupffer cells. The endothelial layer which lines the sinusoids is discontinuous allowing macromolecules, ranging in size from 70 nm to 120 nm, to access to the hepatocytes. These molecules then enter the space of Disse between the endothelium and hepatocytes allowing for interaction with liver cells outside the blood compartment (see Figure 1.8). Hepatocytes are organized into one or two-cell-thick plates that are separated by sinusoids. They are responsible for the major functions of the liver such as detoxification, protein synthesis. metabolism. and storage.

Numerous studies have been performed to understand the role of liver in liposome clearance and many pathways have been postulated for liposome uptake in the cells of the liver (Hu and Liu. 1996: Scherphof and Kamps. 1998). Many of these pathways involve receptor mediated endocytosis (Scherphof and Kamps, 1998), or serum protein binding (Hu and Liu, 1996). As indicated in section 1.4.2.2. complement proteins and ApoE have been implicated in the removal of liposomes from the circulation (Chonn *et al.*. 1995: Devine and Bradley, 1998: Scherphof and Kamps, 1998) and liposomes that exhibit a negative charge, such as phosphatidylserine containing liposomes, are rapidly taken up by the Kupffer cells due to the increased amount of protein bound to these liposomes. In general. neutral liposomes composed of PC with a mean diameter of 100 to 200 nm will also localize in the Kupffer cells: however. the rate at which these liposomes accumulate in this cell population is much slower. Any population of liposomes that exhibit a diameter of less than 50 nm do. however. have the potential to interact with the hepatocyte population in the liver (Scherphof *et al.*, 1987). The interaction of liposomes with

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Figure 1.8

Diagram of a classic liver lobule

Branches of the hepatic artery (HA) and hepatic portal vein (PV) empty blood into hepatic sinusoids (S), through which it flows toward the central vein. The endothelial lining of the sinusoids is discontinuous and is separated from the radial plates of hepatocytes by the space of Disse. Bile canaliculi receive bile from the hepatocytes that border them and convey it toward the bile ducts in the portal triads. The arrows show that blood (dark arrows) and bile (open arrows) flow in opposite directions. (Figure reproduced from Paulsen, 1996)
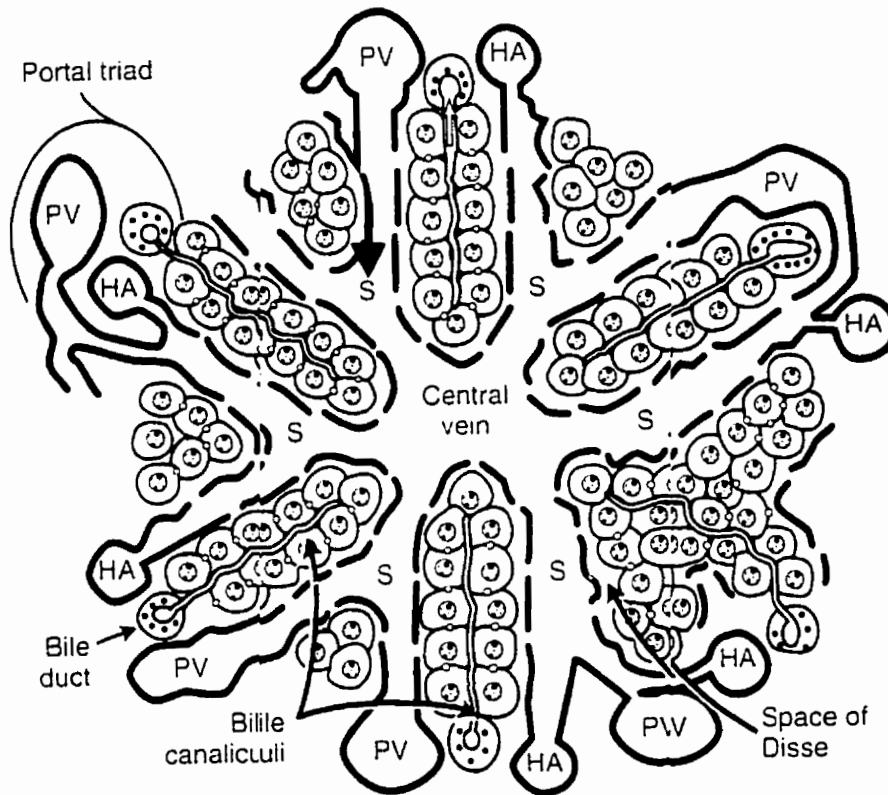
Kupffer cells and hepatocytes and the relationship with therapeutic activity is still not well understood as demonstrated by the data presented in Chapter 5.

1.4.4.2 Decreasing liposome interactions with the MPS

The identification of certain naturally occurring lipids (e.g. ganglioside GM_1 and PI) (Allen and Chonn, 1987) and synthetic lipids with selected polymers linked to the head group (Allen *et al.*, 1991; Yuda *et al.*, 1996;) that decrease the plasma elimination rate of liposomes has provided a fundamental advance in liposome technology. It is believed that these lipids act by limiting the interaction of liposome surfaces with proteins and this, in turn, inhibited the rate of uptake by phagocytic cells (Chonn *et al.*, 1991, 1992). The best characterized example of these lipids is based on the hydrophilic polymers PEG which can be chemically linked to the reactive amine function of the PE. The steric stabilizing lipid that is used most frequently is composed of 2.000 mean molecular weight linear PEG moiety attached to DSPE. This lipid is incorporated into the liposomes while being prepared. typically at levels less than 10 mol %. Inclusion of PEG-PE into neutral (PC/cholesterol) liposomes can result in 3 to 20-fold increases in plasma liposome content 24 hour after *i.v.* injection (Allen *et al.*, 1991; Parr *et al.*, 1997). This is accompanied by significant decreases in liposome uptake by the liver and spleen at early times post-injection. It is important to note that the difference in cumulative uptake by the liver and spleen of liposomes with and without PEG-PE are reduced as a function of time. indicating that the effect of PEG-PE is to reduce the rate of liposome removal by cells of the MPS. It has been demonstrated recently that PEG-modified lipids can be lost from the outer monolayer of the liposomal membrane due to lipid transfer or cleavage of the PEG-linker and it is not clear whether eventual removal of PEG liposomes by the MPS is due. in part. to the loss of PEG moiety (Parr *et al.*, 1994).

Significant increases in circulating levels of liposomes can also be achieved by strategies that eliminate phagocytic cells of the MPS. This effect, referred to as MPS (RES) "blockade", can be achieved by pre-dosing animals with a low dose (10 mg lipid/kg) of liposomal doxorubicin (Bally *et al.*, 1990; Daemen *et al.*, 1995,) or alternatively through use of the encapsulated bisphosphonate clodronate (Van Rooijen and Claassen, 1989; Van Rooijen *et al.*, 1990) (see Chapter 5). Investigators have been able to demonstrate macrophage and Kupffer cell depletion following administration of high doses of large and/or negatively charged liposomes containing doxorubicin or other agents such as clodronate (Van Rooijen *et al.*, 1990; Daemen *et al.*, 1995). MPS blockade induced by low doses (<10 mg/kg lipid and 2 mg/kg drug) of small, uncharged liposomal doxorubicin formulations, however, does not result in complete elimination of Kupffer cells (see Chapter 5). The MPS blockade effect observed for liposomal anti-cancer drugs has raised concerns over potential harmful side effects resulting from altered phagocytic cell activity. Although a substantial amount of doxorubicin can accumulate in liver tissue, indications of significant liver toxicity arising from this uptake have only been observed pre-clinically with high drug doses (80 mg doxorubicin/kg) and in clinical situations where pre-existing liver impairment was a factor. It should also be stressed that the theoretical "benefits" arising from decreased liposome elimination by the MPS is typically assumed to be related to the increased circulating concentrations of liposomes obtained. For example it has been suggested that maintenance of the plasma concentration of liposomes for extended time periods is essential to maximize the amount of liposomal drug that penetrates the vascular barrier and gains access to diseased tissue. In this thesis MPS blockade is used to address the importance of liver phagocytic cells in mediating the therapeutic activity of a liposomal formulation of mitoxantrone (see Chapter 5).

1.4.5 Liposome extravasation

As discussed in section 1.4.3, diseases such as bacterial infection, inflammation and cancer share a common feature in that the diseases induce regional increases in vasculature permeability. The mediators that lead to increased permeability of the vascular barrier are quite distinct and can be attributed to transendothelium migration of inflammatory cells (Thureson-Klein *et al.*, 1986; Kling *et al.*, 1987) or to the release of vascular endothelial growth factor (VEGF) (Senger *et al.*, 1993). Regardless of the mediator, the end result for all of these conditions is the presence of blood vessels that are permeable to large molecules. This may be a consequence of fenestrations or "gaps" occurring between adjacent endothelial cells through which macromolecules can pass (Jain, 1987). Alternatively, liposome extravasation may involve increases in endothelial cell mediated transcytosis (Kohn *et al.*, 1992; Dvorak *et al.*, 1996).

Increases in vascular permeability give rise to the selective accumulation of small liposomes at sites of infection, inflammation and tumour growth. However, this is not a selective process. There is also a general increase in extravascular fluids in these regions. The hydrostatic pressure within these sites is elevated relative to the vascular pressure, resulting in a pressure gradient that impedes movement of molecules from the blood into the tissue interstitium (Baxter and Jain, 1989). It must therefore be assumed that additional features lead to selective accumulation of macromolecules in the diseased extravascular space. Studies, for example, have demonstrated that the lack of a developed lymphatic system in conjunction with the large openings in the vascular endothelial cell lining may lead to an extravascular "trapping" phenomenon (Baxter and Jain, 1990). In the absence of lymphatic drainage, interstitial diffusion of molecules leads to egress from the disease site and this diffusion rate is dependent on molecule size, small molecules exiting more rapidly than large molecules.

Designing liposomes that will exhibit maximal extravasation in disease sites associated with leaky vasculature is of considerable interest and is an area of some controversy. The inclusion of PEG-modified lipids in conventional liposomes can significantly increase the circulating liposome levels over extended times by decreasing the rate of clearance by the MPS. It has generally been assumed that increases in the concentration of liposomes in plasma over time will lead to increased accumulation of liposomes in the extravascular disease sites, and experimental evidence supporting this has been reported (Gabizon. 1992). However. there are studies contrasting these reports. It has been demonstrated that although plasma levels of PEG containing liposomes are several fold higher than for comparable conventional liposomes, this often does not result in increased extravasation and accumulation in solid tumour tissue (Parr *et al.*, 1997).

It should not be unexpected that conventional and sterically stabilized liposomes exhibit different efficiencies in extravasation. Endothelial cell interactions may contribute to the extravasation process either directly *via* transcytosis or indirectly by facilitating an increase in the local liposome concentration at the endothelial cell surface. Given the effects of PEG on inhibiting liposome-cell interactions (Du *et al.*, 1997), this polymer may reduce endothelial cell interactions and this, in turn, would reduce the rate of extravasation.

1.5 Dissociation of the active agent from the carrier: the critical parameter

The distribution of liposomes that have extravasated into the tumour interstitium is heterogeneous and these large carriers diffuse slowly within the perivascular spaces (Yuan *et al.*, 1994). Slow diffusion within the site of extravasation has also been associated with very slow loss of the liposomes from the site. Data from several tumour models, including results shown in

Chapter 4, demonstrate that the level of liposomes achieved following extravastion can be maintained for extended time periods (Parr et al., 1997). Importantly, drug accumulation properties in solid tumours or within other disease sites can exhibit remarkably different behavior in comparison to the liposomal carrier. Drug release from the liposomes in the extravascular site can result in greater drug penetration into the tissue and more rapid loss of the drug from the site when compared with the loss of liposomal lipid (see Chapter 4 for example).

It is not clear from studies correlating anti-cancer activity and increased liposome mediated drug delivery, what is the critical parameter to consider when optimizing a liposomal anti-cancer drug. This thesis has the primary goal of addressing this problem. Studies demonstrating improvement in liposomal anti-cancer drug activity in comparison to free drug have typically compared the efficacy and drug accumulation following administration of drug doses that are equivalent on a weight basis (equal mg/kg dose) or toxicity basis (at the maximum tolerated dose). Under these conditions, there can be 3- to 100-fold increases in drug exposure achieved for the liposomal formulations. It is anticipated, however, that efficacy measured under conditions where tumour drug accumulation levels are comparable for free and liposomal drug that the liposomal drug would be less active. This assumption is made on the basis of studies that demonstrate significant (100-fold) increases in drug exposure, but only marginal (20%) increases in therapeutic activity (Parr et al., 1997). Such observations have raised obvious questions about the biological availability of anti-cancer drugs carried inside liposomes that have extravasated into solid tumours as well as the mechanisms that lead to drug release in the interstitial compartment.

It can be suggested that liposomes exert their effect on the therapeutic activity of an associated anti-cancer drug by providing a drug infusion reservoir within the tumour. Once released, the anti-cancer drug can diffuse through the tumour, directly accessing tumour cells in a manner that

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is comparable to drug in the absence of a liposomal carrier. There are questions regarding how and where drug release occurs and, as suggested in Chapters 4 and 5, a model consisting of drug release from liposomes in the plasma compartment or from a site distant from the disease can not be discounted. *In vitro* studies, for example, have demonstrated that macrophages can engulf doxorubicin-loaded liposomes, process them and re-release doxorubicin extracellularly in free form (Storm *et al.,* 1988). Since the macrophage content within tumours can be significant, it can be suggested that liposomal anti-cancer drug release may involve macrophage processing after extravasation. Interestingly, however, recent studies have shown that interactions between tumour-associated macrophages and extravasated liposomes are minimal (Mayer *et al.,* 1997).

1.5.1 Drug release - importance of drug type

As indicated in section 1.4.2.1, it is not possible to predict drug release rates *in vivo* on the basis of *in vitro* studies even when the *in vitro* release studies are completed in the presence of serum. It is also not suitable to determine release rates using a trapped "marker" (e.g. radiolabeled inulin) to predict the release characteristics for an encapsulated therapeutic agent (Bally *et al.,* 1993). Drug release rates are dependent on the chemical properties of the entrapped drug. This is perhaps best illustrated using liposomal formulations of vincristine and doxorubicin as described below.

Reducing the drug release rate is advantageous for encapsulated formulations of vincristine but is of questionable benefit for doxorubicin. Liposome encapsulation can significantly reduce the toxicity of doxorubicin by decreasing drug accumulation in drug sensitive normal tissue, presumably by decreasing peak levels of free doxorubicin that are experienced after administration in the conventional (unencapsulated) form (Mayer *et al.,* 1994). The degree of toxicity buffering is directly related to the ability of the liposomes to retain their entrapped

doxorubicin where increased phospholipid acyl chain saturation results in decreased toxicity (Mayer *et al.*. 1994). The anti-tumour activity of liposomal doxorubicin, however, is much less sensitive to drug leakage or circulation longevity. Liposomal formulations with widely varying doxorubicin retention properties have been shown in some preclinical models to exhibit comparable anti-tumour activities when compared on an equal dose basis (Mayer *et al.*. 1994). In this case, increased efficacy for the less permeable liposomes is achieved by administering elevated drug doses due to their reduced toxicity. Further, while the inclusion of PEG-PE increases the circulation longevity of liposomal doxorubicin. the magnitude of increased liposome levels in the blood (compared to conventional liposomes) is far less than that observed for empty (drug-free) liposomes (Parr *et al.*, 1997). This is related to the MPS blockade effect described in section 1.4.4.2.

In contrast to the observations made with doxorubicin. altering the physical properties of liposomal vincristine formulations results in dramatic changes in anti-tumour activity while only minimally affecting drug toxicity characteristics. Increasing the retention of vincristine inside 100 nm liposomes by changing the phosphatidylcholine-containing lipid component from EPC to DSPC to sphingomyelin (while maintaining cholesterol content at 45 mol%) leads to dramatic increases in anti-tumour activity. particularly when compared to the efficacy obtained with free vincristine (Webb *et al.*, 1995). This is consistent with the steep dependence of vincristine anti-tumour potency on the duration of drug exposure as well as the fact that retention of vincristine in most tissues, including tumours. is rather poor. It appears that the ability to prolong the exposure of vincristine *in vivo* is more important than peak drug concentrations. Furthermore. although inclusion of PEG-PE in the liposomes increases the circulating liposomal lipid levels at extended time periods. this steric stabilizing lipid does not improve the vincristine pharmacokinetic or therapeutic properties over conventional DSPC/Chol or sphingomyelin/Chol systems (Webb *et al.*, 1998). This is due to the fact that PEG-PE increases the permeability of the lipid bilayer to vincristine. thus offsetting the potential benefits provided by increased

longevity of the liposomal carrier. It should be noted that perhaps the best example of how a balance between efficient liposome delivery to the disease site and controlled drug release can work synergistically to achieve optimum therapeutic results is provided by the liposomal mitoxantrone data presented in this thesis.

1.5.2 Future considerations for the next generation of liposomes

It can be suggested that the drug retention properties required to minimize systemic exposure of drugs encapsulated inside long circulating liposomes significantly limits biological availability of the agent once it has reached the disease site. This conclusion arises from results in several model systems that show that significant increases in disease site drug delivery often translate into only incremental increases in drug potency. It has been demonstrated in pharmacodynamic studies with liposomal anti-cancer agents that the circulating drug pool itself has little direct impact on therapeutic activity (Mayer et al., 1994). Instead, it appears that once extravasated, the lipid carrier provides a localized source of drug infusion within the disease site. While the liposomal drug formulations used to date have given rise to significant improvements in therapeutic activity, many results suggest that drug within the tumour is not freely biologically available. *In vitro* studies measuring the doxorubicin concentrations necessary for 50% inhibition of growth (IC_{50}) of tumour cells in culture indicate a range in doxorubicin IC_{50}'s of 100 nM in MCF-7 breast tumour cell line (Formari et al., 1994) to 190 nM and 24 µM in parental and DOX- resistant P388 cells, respectively (De Jong et al., 1995). Parr *et al.* (1997) has demonstrated that drug concentrations of 250 nmoles per gram tumour can be achieved using doxorubicin loaded drug liposomes and it can be suggested that drug concentrations within the tumour are in excess of that required to achieve maximum cytotoxic effects, even for drug resistant tumours. However, calculated rates of drug release from liposomes in tumour (0.60 to

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0.65 nmol drug/µmol lipid/h for doxorubicin encapsulated in DSPC/Chol liposomes) may not be sufficient for inhibition or elimination of the tumour cells (Parr *et al.*, 1997).

The inability to differentially control drug release rates in the plasma compartment and disease site is perhaps the most significant limitation of presently available liposomes. As suggested in section 1.1, it would be ideal if one could design liposomes that have little or no drug leakage in the circulation and increased release rate at the disease site. Early attempts to selectively increase drug leakage at tumour sites centered on the fact that liposomes can be constructed to become leaky in the acidic interstitial pH of some solid tumours (Connor *et al.*, 1984; Aicher *et al.*, 1994), which can drop to values of 6.5. More direct evidence of the importance of site-specific drug release has been obtained using localized hyperthermia (Chelvi et al, 1995; Gaber *et al.*, 1996; Kakinuma *et al.*, 1996). Liposomal doxorubicin preparations, for example, can be prepared such that there is an increase in drug release at 42°C, compared to 37°C. These liposomes are administered *i.v.* to tumour bearing mice and the tumour site is then heated using a topical microwave heating device placed on the subcutaneous tumour. Application of a transient heating pulse after the liposomal doxorubicin had accumulated into the solid tumour resulted in a significant increase of therapeutic activity compared to free drug with hyperthermia and liposomal doxorubicin in the absence of heating. Although hyperthermia may not be applicable to many multifocal or deep-seated tumours, this technique provides encouraging indications that liposomes exhibiting controlled or triggered release of their contents will significantly augment the pharmacological improvements provided by liposomes.

1.5.3 Other methodology considerations

For many applications, liposomal delivery systems are employed to improve the therapeutic index of encapsulated agents by selectively accumulating in extravascular disease sites. As

suggested above, there is also evidence indicating that drug released from liposomes in the circulation does not contribute significantly to therapeutic activity of liposomal anti-cancer agents. There is no question that liposomes can provide sustained exposure of therapeutic agents in the blood compartment through controlled release kinetics of encapsulated drugs; however, it is difficult to justify development of liposomal drugs using a rationale that involves sustained systemic exposure. This is largely due to significant advances made in the area of drug infusion technology. Compact and cost effective infusion pumps are now widely used and these can provide well-controlled systemic drug exposure over several days. It is argued that the most significant advantage for the use of liposome drug carriers arises as a consequence of disease specific changes in vascular permeability that favor accumulation of the intact liposome and associated drug into the site of disease progression. This property is differentiated from the benefits of drug infusion technology, which are primarily concerned with maintenance of circulating blood levels of free drug.

1.6 Thesis objectives and hypotheses

The aims of this thesis were to 1) characterize DSPC/Chol and DMPC/Chol formulations of mitoxantrone. 2) evaluate the compensating roles of drug delivery and drug release following i.v. administration of liposomal mitoxantrone. and 3) define the role of Kupffer cells and liposome mediated drug delivery to the liver in governing the efficacy of liposomal mitoxantrone used to treat liver localized cancer. Three connected hypotheses are addressed in this thesis which is focused on the development and characterization of liposomal mitoxantrone. The work emphasizes use of this formulation in the treatment of cancer that is progressing in the liver. Many groups have tried to take advantage of the natural tendency of liposomes to accumulate in the liver for the treatment of liver localized disease (Gabizon et al., 1983; Asao et al., 1992) but have met with limited success. Hepatocellular carcinoma has the highest rate of incidence

among all cancers worldwide. Current therapies, such as resectional therapy, radiation therapy, chemoembolization, cyrotherapy, are ineffective with remaining options being palliative for the patient. The only current course of action is focused on prevention through the use of vaccination of the hepatitis B virus, as the incidence of hepatocellular carcinoma has been causally linked to the viral infection (Lee and Ko, 1997). In addition to hepatocellular carcinoma, the liver is also a major site of metastasis. The majority of the cases are due to metastasis from colorectal carcinoma because of the gastrointestinal venous drainage to the liver. There is clearly a need to develop effective agents to treat liver cancer.

It is argued that liposomal formulations should be more effective in treatment of liver disease because these carriers accumulate in liver tissue to high levels. However, the results presented in this thesis suggest that drug delivery alone is not sufficient to treat liver localized disease. The first research chapter (Chapter 3) addresses the hypothesis that in a site where liposome accumulation is rapid, drug biological availability is more critical in defining therapeutic activity than drug delivery. Using liposome lipid composition as the primary regulator of drug release, it is demonstrated that DMPC/Chol mitoxantrone is much more active in the treatment of liver disease in comparison to DSPC/Chol mitoxantrone. It is concluded that mitoxantrone release is the dominating factor controlling biological activity of the liposomal drug in tissues where the rate of liposome accumulation is rapid. In Chapter 4 the question of whether drug release or liposome-mediated drug delivery becomes the dominant factor controlling therapeutic activity under conditions where the rate of liposome accumulation is slow and tumour development is within a site outside the liver is addressed. DSPC/Chol mitoxantrone and DMPC/Chol mitoxantrone delivery in tumours established following s.c. injection of human LS180 and A431 cell lines is measured and then compared to the anti-tumour activity of the drug. The results suggest that liposomal mitoxantrone induced delays in tumour growth are achieved using a

liposomal formulation that is selected on the basis of drug release attributes, even when the liposome accumulation rate in the site of tumour growth is slow. The research focus returns to liver localized disease in the final research chapter (Chapter 5) which documents the fact that liposomal mitoxantrone is particularly well suited for treatment of cancer that is progressing primarily in the liver. It also addresses two simple hypotheses: 1) strategies which result in reduced delivery of mitoxantrone to liver will result in decreased therapeutic activity and 2) Kupffer cells play a significant role in defining the therapeutic activity of liposomal mitoxantrone. Surprisingly the second hypothesis was not supported by data that used MPS blockade to effect decreases in liver delivery of liposomal mitoxantrone. The results clearly indicate that Kupffer cells are not responsible for mediating the therapeutic activity of liposomal mitoxantrone. In addition, the results with formulations prepared with PEG-modified lipids where the anti-tumour activity of the entrapped mitoxantrone is significantly reduced in comparison to the formulations which do not contain the lipid, imply that cell processing may be necessary for the formulation to be therapeutically active.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Novantrone® (mitoxantrone hydrochloride), Adriamycin® (doxorubicin hydrochloride), and Oncovin® (vincristine sulphate) were obtained from the British Columbia Cancer Agency and are products of Wyeth Ayerst, (Montreal, PQ), Adria Laboratories (Mississauga, ON), and Faulding (Vaudreuil, PQ) respectively. Clodronate (dichloromethylene-bisphosphonate) was generously donated by Boehringer Manneheim (Mannheim, Germany). 1,2 Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2 dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2 distearoyl-*sn*-glycero-3-phosphatidylethanolamine–polyethylene glycol 2000 (PEG), and egg phosphatidycholine (EPC) were purchased from Avanti Polar Lipids (Alabaster, AL) and Northern Lipids (Vancouver, BC). 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Molecular Probes (Eugene, OR). Citric acid, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid (HEPES), hydrogen peroxide (H_2O_2), Sephadex G50 (medium), nigericin and cholesterol were purchased from Sigma Chemical Company (St. Louis, MO). Dibasic sodium phosphate, sodium chloride, sodium citrate, and 10N hydrochloric acid were obtained from Fisher Scientific (Fair Lawn, NJ). Hank's buffer (with and without calcium and magnesium) was purchased from Stem Cell Technologies (Vancouver, BC). Rat – anti mouse F4/80 antibodies and FITC conjugated goat-anti rat antibodies were purchased from Serotec (Mississauga, ON). O.C.T. was purchased from Tissue-Tek. (Miles Inc., USA). Solvable™ was obtained from NEN (New England Nuclear) Research Products (Dupont Canada, Mississauga,

ON). [^{14}C]-Mitoxantrone, used as a tracer, was generously donated by Wyeth Ayerst (Montreal, PQ) . [^{3}H]-Cholesteryl hexadecyl ether (CHE), a lipid marker that is not exchanged or metabolized *in vivo* (Stein *et al.*, 1980), and [^{3}H] thymidine were purchased from Amersham (Oakville, ON). Aquacide II was purchased from Terochem Laboratories Ltd. (Edmonton, AB). A431 (a human squamous carcinoma cell line) and LS180 cells (a human colon carcinoma cell line) were purchased from the ATCC (Manassas, VA) and maintained in culture. The L1210 and P388 tumour cell lines were originally purchased from the NCI tumour repository (Bethesda, MD) and cells were obtained from ascites fluid generated weekly by passage in BDF1 mice. Cells were used for experiments after the third passage and before the twentieth. Once the cells reach the twentieth passage, these care discarded and the new cell lines revert back to the original NCI tumour stock. Female CD1, DBA2 and BDF1 mice (8-10 weeks old) were purchased from Charles River Laboratories (St. Constant, PC). Female SCID/RAG-2 were bred at the British Columbia Cancer Agency Animal Breeding Facility.

2.2 Preparation of liposomes

DSPC/Chol (55:45; mol:mol), DMPC/Chol (55:45; mol:mol), and DMPC/Chol/PEG (50:45:5;mol:mol:mol) liposomes were prepared using well established extrusion technology (Hope *et al.*, 1985). When DiI was used as a fluorescent lipid marker, it was added at a ratio of 0.4 mg to 100 mg total lipid. The indicated phospholipid and cholesterol mole ratios were dissolved in chloroform and dried down to a homogenous lipid film under a stream of nitrogen gas. This lipid film further was dried under vacuum for 3 hours to remove any residual chloroform. Subsequently, the lipid film was hydrated in a 300 mM citric acid buffer (pH 4.0) to a final lipid concentration of 100 mg/ml. The resulting multilamellar vesicle mixture was frozen in liquid nitrogen and thawed five times (Mayer *et al.*, 1986) and extruded through three 100 nm

(pore size) stacked polycarbonate filters (Nuclepore, Pleasanton, CA; Poretics Corp., Mississauga, ON) using an extrusion device (Lipex Biomembranes Inc., Vancouver, BC). The resulting large unilamellar vesicles (LUVs) were sized by quasielastic light scattering using a Nicomp 270 submicron particle sizer (Pacific Scientific, Santa Barbara, CA) operating at 632.8 nm. The mean diameter of these liposomes was 100-120 nm.

2.3 Transmembrane pH gradient loading of mitoxantrone

Mitoxantrone was encapsulated using a transmembrane pH gradient driven loading procedure (Madden *et al.*, 1990; Mayer *et al.*, 1985). The procedure used is analogous to that employed for vincristine (Boman *et al.*, 1993) and consisted of incubating liposomes at 65°C for 10 minutes prior to addition of sufficient mitoxantrone to achieve a final drug to lipid weight ratio of 0.1. The pH of this mixture was then increased from pH 4.0 to 7.2 by the addition of 350 μl of 0.5 M Na_2HPO_4 buffer to 1.0 ml of the drug liposome mixture. The resulting mixture was incubated at 65 °C for an additional 15 minutes. Encapsulation efficiency for mitoxantrone was determined at 3 different temperatures: 37 °C, 50 °C, and 65 °C using size exclusion chromatography on mini-spin columns made of Sephadex G-50 (Madden *et al.*, 1990). Aliquots of the sample (100 μl) were taken at intervals over a 2 hour time period and assayed for drug encapsulation. Drug and lipid concentrations in the samples collected in the void volume of these columns were determined by measuring [^{3}H]-CHE and [^{14}C]-mitoxantrone. Radioactivity was assessed by mixing the sample with 5 ml Pico-Fluor 40 (Packard, Meriden, CT) scintillation cocktail and counted with a Packard 1900 scintillation counter (Packard, Meriden, CT).

2.4 Transmembrane pH gradient loading of vincristine

Vincristine was encapsulated using a transmembrane pH gradient driven loading procedure as described by Boman *et al.* (1993). The procedure consisted of incubating liposomes at 65 °C for 10 minutes prior to addition of sufficient vincristine to achieve a final drug to lipid weight ratio of 0.1. The pH of this mixture was then increased from pH 4.0 to 7.2 by the addition of 350 μl of 0.5 M Na_2HPO_4 buffer to 1.0 ml of the drug liposome mixture. The resulting mixture was incubated at 65 °C for an additional 15 minutes. Encapsulation efficiency was approximately >95% for vincristine.

2.5 Transmembrane pH gradient loading of doxorubicin

Doxorubicin (DOX) was encapsulated in the liposomes using the transmembrane pH gradient loading procedure (interior acidic) employing sodium carbonate as the alkalinizing agent and a drug to lipid weight ratio of 0.2:1 (Mayer *et al.*, 1994). Empty preformed liposomes with interior pH of 4.00 (300 mM citrate buffer) were titrated with 0.5 M sodium carbonate to a pH of 7.8 - 8.0. Doxorubicin, solubilized in HBS, and the titrated vesicle solution were heated at 65°C for 2 min prior to addition of doxorubicin to the liposome solution. The mixture was vortexed for 2-3 min at 65°C and then maintained at this temperature for an additional 10 min to facilitate complete drug loading. Encapsulation efficiency was > 95%. Liposomal DOX preparations were diluted with saline as necessary prior to *in vivo* administration.

2.6 Preparation of EPC/Chol clodronate liposomes

Clodronate liposomes were prepared as outlined by Van Rooijen and Sanders (1994) with minor modifications. An EPC/Chol (86:8 wt:wt) lipid film was prepared by weighing out the required amounts of EPC and cholesterol. Chloroform was then added to the lipids and the solution was dried down under a stream of nitrogen. The resulting film was then kept under vacuum for 3 hours. The EPC/Chol film was hydrated in a 5 ml solution of clodronate (2 mg/ml) and subsequently subjected to 5 freeze-thaw cycles in order to increase encapsulation efficiency (Mayer et $al.$, 1986). The resulting suspension was centrifuged at 6,000 x g for 20 minutes to separate the unencapsulated clodronate from the clodronate MLVs. The MLVs form a milky band on top of the suspension. The lower suspension was removed and the liposomes were resuspended in PBS. This liposome were washed in PBS and centrifuged at 20,000 x g for 30 minutes three times. The resulting pellet of clodronate MLVs were then resuspended in 4 mls of phosphate buffered saline (PBS).

2.7 Microculture tetrazolium assay

The modified microculture tetrazolium (MTT) assay was used to determine the IC_{50} values for mitoxantrone, doxorubicin, and vincristine on L1210, LS180, and A431 cells (Alley et $al.$, 1988). Briefly, L1210 cells were obtained through in $vivo$ cultivation in the mouse peritoneum. Typically, 10^6 cells were inoculated intraperitoneal ($i.p.$) and the tumour progressed for 7 days prior to isolation of cells to be used for cytotoxicity assays. Cells were isolated from the mice by peritoneal lavage and the collection of ascitic fluid into EDTA containing tubes. L1210 cells were then separated from lymphocytes and RBCs by Ficoll-Hypaque density gradient

centrifugation, where cells at the interface were collected and placed into RPMI 1640 medium containing 10% FBS. The cells were washed three times prior to transferring the cells into a T75 culture flask. The resulting cell suspension was incubated at $37^{\circ}C$ in a humidified incubator with 5% CO_2 for 4 hours. All non-adherent cells were transferred into T75 flasks and diluted to a concentration of approximately 10^5 cells/ml. The cells were incubated for 24 hours prior to use in a cytotoxicity study.

A431 and LS180 cells were harvested from exponential phase cultures and counted by Trypan blue exclusion (cell preparations demonstrating viability >90% were used) prior to dispensing the cells into 96-well flat-bottomed CostarR (Cambridge, MA) culture plates (2000 cells/100 μl/well for a 3-day incubation). The cells were exposed to defined concentrations of the anti-cancer drug (diluted with RPMI 1640 supplemented with 10% heat-inactivated FBS) over a 3-day incubation at $37^{\circ}C$, 5% CO_2 and 100% relative humidity.

The MTT assay consisted of adding 50 μl MTT (5 mg/ml PBS, filtered through 0.45 μm filter units, and stored at $4^{\circ}C$ for not longer than 1 month) to each well and the plates were further incubated for 4 hours at $37^{\circ}C$. Subsequently, plates were centrifuged and the supernatant aspirated slowly through a blunt 18-gauge needle. The reaction product retained in the viable cells was thoroughly solubilized by the addition of 150 μl DMSO. The plates were read spectrophotometrically at 570 nm in a Dynex Technologies MRX multiplate reader (Dynex Technologies, Chantily, VA). Cytotoxicity was expressed in terms of percentage of control absorbance (mean ± s.d.) following subtraction of background absorbance. The IC_{50} was determined from a plot of percentage absorbance vs. log drug concentration of the data obtained in triplicate.

2.8 *In vitro* characteristics of liposomal mitoxantrone

For release studies, liposomal mitoxantrone formulations were prepared as outlined in section 3. The resulting drug loaded liposomes were transferred into 25 mm diameter Spectrapor dialysis tubing (10,000-12,000 molecular weight cut off, Spectrum Medical Industries, Los Angeles,CA) and the samples (3 ml) were dialyzed against 1 liter of HBS at 37 °C. At the indicated time points, 100 μl samples were taken from the dialysis bag and assayed for drug and lipid using the mini-spin columns as described above. The experiment was then repeated in the presence of nigericin, an ionophore that collapses the pH gradient by promoting exchange of a monovalent cation (eg. K^+, Na^+) with H^+. The ionophore was added to the sample and external buffer to a concentration of 120 nM. Further release experiments were carried out in the presence of serum. Liposomal mitoxantrone was prepared as above. Liposomal mitoxantrone (200 μl) was incubated with 800 μl of 100% fetal bovine seurm at 37°C for 24 hours. After incubation, 500 μl of the mixture was applied to a Biogel A-15 column in order to separate released drug from liposomal drug.

2.9 Plasma elimination and distribution studies

Female CD1 mice (20-25 g, 4 per group) were injected with a 10 mg/kg drug dose *via* the lateral tail vein. At 1, 4, 24, and 48 hours animals were terminated by CO_2 asphyxiation and whole blood was collected *via* cardiac puncture and placed into EDTA coated tubes (Microtainers, Becton Dickinson). Plasma was isolated following centrifugation of whole blood at 500 x *g* for 10 minutes. Aliquoted plasma samples (100 μl) were mixed with 5 ml Pico-Fluor 40 and counted for [^3H] and [^{14}C].

Tissue weights were determined by placing (isolated and saline washed) tissues into pre-weighed glass tubes before reweighing and freezing at -70 °C. Appropriate volumes of distilled water were added to the tissues and homogenized with a Polytron tissue homogenizer (Kinematica, Switzerland) to achieve a 10% homogenate (w/v). Aliquots of the homogenate (200 µl) were mixed with 500 µl of Solvable™ and incubated at 50 °C for 3 hours. After the resulting mixture was cooled to room temperature, 50 µl of 200 mM EDTA, 200 µl of 30% H_2O_2 and 25 µl of 10 N HCl were added. Five ml of Pico-Fluor 40 was added to the samples and radioactivity ([³H]-CDE and [¹⁴C]-mitoxantrone tracer) was determined using a Packard 1900 scintillation counter.

2.10 A431 and LS180 tumour accumulation and plasma elimination studies of liposomal mitoxantrone

Female SCID/RAG-2 mice (18-20 g, 4 per group) were inoculated bilaterally with 2 x 10⁶ A431 cells or 1 x 10⁶ LS180 cells subcutaneously on the hind regions of the back. Once the tumours reached a measurable size (tumour volume > 0.05 cm³), as measured using calipers, mice were injected with a 10 mg/kg drug dose of free mitoxantrone, DSPC/Chol mitoxantrone, or DMPC/Chol mitoxantrone via the lateral tail vein. At 4, 24, 48, and 96 hours animals were terminated by CO_2 asphyxiation and whole blood was collected via cardiac puncture and placed into EDTA coated tubes (Microtainers, Becton Dickinson). Plasma was isolated following centrifugation of whole blood at 500 x g for 10 min. Aliquoted plasma samples (100 µl) were mixed with 5 ml Pico Fluor 40 (Packard, Meriden, CT) and [³H] and [¹⁴C] were measured using a Canberra Packard 1900 scintillation counter. Isolated tissues were processed as outlined in Section 2.9.

2.11 Plasma elimination and biodistribution studies in MPS blockaded mice

Female CD1 mice (20-25 g, 4 mice per group) were injected with a 10 mg/kg drug dose of DMPC/Chol mitoxantrone or DMPC/Chol/PEG mitoxantrone *via* the lateral tail vein. To achieve hepatic MPS blockade to alter the plasma elimination and biodistribution of DMPC/Chol liposomal mitoxantrone, animals were injected *i.v.* with a 2 mg/kg drug dose of DSPC/Chol doxorubicin (10 mg/kg lipid dose) 24 hours prior to injection of the DMPC/Chol mitoxantrone. At 1 and 4 hours, 25 μl of blood was collected in EDTA coated microcapillary tubes from the tail vein which had previously been given a small cut with a scalpel. Blood was mixed with 250 μl of 5% EDTA and spun for 15 minutes at 500 x g. The supernatant was collected and the resultant pellet was resuspended in Hanks buffered saline solution (250 μl) and spun again at 500 x g. The supernatant was collected and pooled with the first supernatant prior to addition of 5 ml of scintillation fluid. Radioactivity in the sample was assessed by scintillation counting. At 24 hours, mice were terminated by CO_2 asphyxiation, and whole blood was collected *via* cardiac puncture and processed as outlined in section 2.9. Livers were harvested and processed as outlined in section 2.9.

2.12 Liposome mediated drug delivery to region of tumour cell inoculation

In order to measure mitoxantrone and liposomal lipid accumulations under conditions where the tumour was not established, the following protocol was used. Prior to inoculation, LS180 cells were incubated with [³H]-thymidine for 48 hours. The adherent cells were rinsed with RPMI media and cell suspensions were prepared by adding trypsin-EDTA followed by a brief (< 1 minute) incubation. The radiolabeled cells were then resuspended in RPMI media to a concentration of 20 x 10⁶ LS180 cells/ml. Viability was assessed using Trypan blue and cells

were counted using a hematocytometer. The injection sites on female SCID/RAG-2 mice were shaved and marked. LS180 cells (1×10^6) in 50 µl of RPMI media were injected bilaterally subcutaneously into the inferior dorsal region of the mice. An equivalent amount of the cell suspension was taken for scintillation counting. Forty-eight hours after tumour cell inoculation, 10 mg/kg drug dose of DSPC/Chol mitoxantrone, DMPC/Chol mitoxantrone, or free mitoxantrone was injected i.v. [^{14}C]-mitoxantrone was used as a tracer. Twenty-four hours later, mice were terminated using CO_2 asphyxiation and blood was collected via cardiac puncture and processed as outlined in the plasma elimination studies. A 1.5 cm $\times$ 1.5 cm section of skin and underlying muscle area surrounding the inoculation site was removed and processed as outlined in section 2.9. This study was repeated using cells which were not labeled with [^{3}H]-thymidine so that liposomal lipid ([^{3}H]-CHE) and drug ([^{14}C]-mitoxantrone) delivery to the region of cell inoculation could be measured simultaneously. Values obtained using this technique were reported as total delivery to the site of cell inoculation.

2.13 Establishing the maximum tolerated drug dose and L1210 and P388 efficacy studies

The maximum tolerated drug dose (MTD) was determined in limited dose ranging studies where female BDF1 mice in groups of two were given drug by a single i.v. injection. Weight loss and signs of stress/toxicity were monitored for 30 days. If individual animals lost greater than 20% of the original body weight, they were terminated. If animals appeared severely stressed as judged by appearance and/or behavior, as assessment made by qualified animal care technicians, they were terminated. The MTD was estimated as the dose where tumour-free animals survived for a period of 30 days after drug administration. At the end of the 30 day period, animals were terminated by CO_2 asphyxiation and necropsies were completed to identify any additional toxicities. The exact LD_{10} dose of the different mitoxantrone formulations was not determined as

such toxicity studies are not approved by the Canadian Council on Animal Care or the institutional Animal Care Committee.

For L1210 and P388 efficacy studies, female BDF1 mice (19-21 g, typically 2 sets of 5 mice per group were used providing an n value of at least 10) were injected with 10^4 L1210 cells or 10^5 P388 cells i.v. 24 hours before a single treatment of the indicated drug dose and formulation. When these cells are injected i.v., they seed primarily in the liver and spleen (See Chapter 3). For animals injected with L1210 cells, tumour progression is characterized by increased liver and spleen weight and histological studies indicate the presence of massive, diffuse infiltration of the liver. For animals injected with P388 cells, liver and spleen mass increase and the histopathology reveals discrete foci of tumour cells that progressively become larger over time. Mice were given the specified drug dose in a volume of 200 µl and, where required, drug loaded liposomes were concentrated (using Aquacide II) prior to administration. The animals were monitored daily for any signs of stress and were terminated when body weight loss exceeded 20% or when the animals exhibited signs of lethargy, scruffy coat, dehydration or labored breathing. When animals were terminated, the survival time was recorded as the following day. Survival times were monitored for sixty days and drug induced increases in life span (% ILS) were calculated by dividing the median survival time of the treated by the median survival time of the control mice (saline treated).

2.14 Liposomal mitoxantrone anti-tumour efficacy using the human A431 and LS180 solid tumour models

SCID/RAG-2 mice were inoculated bilaterally with 2×10^6 A431 cells or 2×10^6 LS180 cells 14 days prior to initiation of drug treatment. Tumour bearing animals (tumour size > 0.05 cm^3) were

given a single *i.v.* injection of free mitoxantrone. DSPC/Chol liposomal mitoxantrone, or DMPC/Chol liposomal mitoxantrone. Control mice were injected with saline. Previous results obtained with immunocompetent BDF1 mice indicated that free mitoxantrone was tolerated at 10 mg/kg and liposomal formulations were tolerated at 20 mg/kg. In contrast, both liposomal drug formulations proved to be toxic (non-tumour related deaths were observed in 100% of the animals within 15 days after administration) in tumour bearing SCID/RAG-2 mice when administered at 20 mg/kg and free drug was toxic at the 10 mg/kg dose. It should be noted that SCID/RAG-2 mice were selected because they tolerated DNA damaging agents much better than other SCID mice (e.g. Toronto SCID and NOD/SCID mice). Based on drug dose titrations from 5 to 20 mg/kg, the maximum therapeutic dose of drug when given as a single *i.v.* injection was defined as 5 and 10 mg/kg for the free and liposomal drugs, respectively. Animal weights and tumour volumes were measured daily until the tumour mass exceeded 10% of the animals original body weight or until the tumours showed any sign of ulceration. Tumour volume was determined by measuring tumour dimensions and calculating volume with the equation (Tomayko and Reynolds. 1989):

$$(\pi / 6) \times \text{length} \times \text{width}^2$$

2.15 Treatment of non-established LS180 and A431 tumours

In an effort to establish optimal conditions for treating SCID/RAG-2 mice inoculated with LS180 and A431 cells, studies evaluating treatment of animals two days after tumour cell inoculation were completed. Treatment was based on single (5 mg/kg free drug and 10 mg/kg liposomal drug) and multiple (1.5 mg/kg free drug and 3.5 mg/kg liposomal drug) doses. The latter consisted of intravenous injections on days 2. 3 and 4. Other dose schedules were evaluated (e.g.

days 2, 4 and 6; days 2, 6 and 10) but under the conditions employed, optimal therapy was obtained using days 2, 3 and 4 schedule. Control mice were injected with saline.

2.16 Efficacy of liposomal mitoxantrone in the *i.v.* L1210 tumour model with and without MPS blockade

Female BDF1 or DBA2 mice were inoculated with 1 x 10⁴ L1210 tumour cells *i.v.* and 24 hours after tumour cell inoculation mice were treated with a 10 mg/kg drug dose of DMPC/Chol mitoxantrone or DMPC/Chol/PEG mitoxantrone. Mice were given the specified drug dose in a volume of 200 µl. In order to assess the impact of hepatic MPS blockade therapeutic activity, mice were injected *i.v.* with either DSPC/Chol doxorubicin (2mg/kg drug), DSPC/Chol vincristine (1mg/kg drug), or EPC/Chol clodronate 2 hours after tumour cell inoculation. Controls indicated that the agents used to blockade the hepatic MPS blockade had no therapeutic activity at the doses administered. The animals were monitored and terminated as described in Section 2.13.

2.17 F4/80 staining of macrophages in the liver

CD1 mice were injected with either 2 mg/kg drug dose of DSPC/Chol doxorubicin or EPC/Chol clodronate. Control livers were left untreated. Twenty-four hours after treatment, mice were terminated *via* CO_2 asphyxiation and livers were harvested. The livers were rinsed in ice cold PBS buffer and placed in O.C.T. embedding solution for 30 minutes before frozen at −70 °C. Cryostat sections (5 µm)were prepared using a Frigocut 2800E microtome from Leica. The slides were then washed in PBS and incubated with the rat-anti mouse F4/80 primary antibody and then a FITC goat anti-rat secondary antibody. A Leitz Dialux fluorescence microscope (at

40 × magnification) was used to evaluate FITC fluorescence of the sections (430-490 nm cut off filter) with fluorescent photomicrographs obtained using a Orthomat microscope camera. All images were recorded on Fuji color ASA400 negative film.

2.18 Hepatocyte isolation

Hepatocytes were isolated from female CD1 mice as described by (Klaunig et al., 1981) with slight modification. Mice were terminated via CO_2 asphyxiation. Livers were harvested and kept in ice cold Hank's buffer (without calcium and magnesium). Using two scalpel blades, the livers were minced to a fine mixture and this was transferred to a 15 ml culture tube. Hank's buffer (without calcium and magnesium) was added to final volume of 5 ml. Three hundred μl of collagenase (4 mg of collagenase/ml of Hank's with calcium and magnesium) was then added to the solution and incubated on a rotating tube rack at 37°C for 30 minutes. The resulting solution was then strained through a 40 μm nylon filter and 40 ml of Hank's buffer added. This was spun for 1 minute at 50 x g. The supernatant was extracted and the resulting pellet was reconstituted in another 40 ml of Hank's buffer (without calcium or magnesium). This solution was spun for 1 minute at 50 x g. This step was repeated twice. The final pellet was reconstituted in 5 ml of Hank's buffer (without calcium and magnesium). Viability was assessed using Trypan Blue and was found to be greater than 90%. Hepatocytes were counted using a Coulter cell counter ZM 901 (Coulter, Burlington, ON).

2.19 Confocal microscopy

DiI [a fluorescent lipid label that is not exchanged or metabolized (Claassen, 1992; Honig and Hume, 1986)] was added to liposomes as described in Section 2.2. DMPC/ Chol.

DMPC/Chol/PEG liposomes were loaded with mitoxantrone and injected at a drug dose of 10

mg/kg (100 mg/kg lipid dose). Twenty-four hours after injection, mice were terminated and the

livers were gentlely harvested and rinsed in PBS. Subsequently, the livers were placed in O.C.T.

for 30 minutes before freezing at $-70^{\circ}C$. Sections (5 µm in thickness) were made using a

cryostat and imaged using confocal micoscopy.

Confocal images were collected on a Optiphot 2 research microscope (Nikon Japan) attached to a

confocal laser scanning microscope (MRC-600, BioRad Laboratories, Hercules CA) using

COMOS software (BioRad Laboratories). The laser line on the krypton/argon laser was 488 nm.

Filterblock BHS (568 nm) was used to detect DiI (549 nm excitation, 565 nm emission). The

numerical aperture was 0.75 on the 10x air objective and 1.2 on the 60x oil objective. The

images were captured such that the xyz dimensions were 0.4 mm cubed (20x) and 0.2 mm pixel

(60x). NIH Image version 1.61 was used for image analysis, and all images were based on

maximum intensity projection. Projections made in NIH image were saved in TIFF format and

then imported to Adobe Photoshop version 4.0 where final modifications were performed.

2.20 Statistical analysis

ANOVA (analysis of variance) was performed on the results obtained after administration of the

two liposomal formulations and free mitoxantrone. Common time points were compared using

the post hoc comparison of means, Scheffé test. Differences were considered significant at $p <$

0.05. Area under the curve analysis was performed using trapezoidal integration from the time

points indicated. The zero point is a theorical point and was calculated as the injected dose over

the plasma compartment of the mouse and then corrected for 100 µl.

CHAPTER 3

INFLUENCE OF DRUG RELEASE CHARACTERISTICS ON THE THERAPEUTIC ACTIVITY OF LIPOSOMAL MITOXANTRONE

3.1 Introduction:

It is well established that the therapeutic activity of anti-cancer agents can be improved through application of liposomal drug carrier technology (Fielding, 1991; Sugarman and Perez-Soler, 1992; Kim, 1993). In general, liposomes engender pharmacokinetic and biodistribution characteristics which lead to increases in therapeutic activity and/or reductions in drug related toxicities (Fielding, 1991; Mayer et al., 1994). Although the mechanism of therapeutic activity for liposomal anti-cancer drugs is not well understood, studies have suggested that increased drug exposure at the site of tumour growth is important (Gabizon and Papahadjopoulos, 1988; Wu et al., 1993; Bally et al., 1994; Mayer et al., 1994). These increases in tumour drug levels result from preferential accumulation of the liposome carrier within tumours (Gabizon, 1992; Wu et al., 1993; Bally et al., 1994; Ogihara-Umeda et al., 1994; Uchiyama et al., 1995). It is important to note, however, that there is no evidence suggesting that the encapsulated form of the drug is therapeutically active. It is postulated, therefore, that anti-tumour activity is mediated by drug released from regionally localized liposomes (Mayer et al., 1994).

The emphasis of investigators developing liposomal anti-cancer agents has been, for the reasons cited above, on the use of liposomal lipid compositions that are less permeable to the encapsulated agent and exhibit increased circulation lifetimes. Liposomes that are retained in the plasma compartment for extended periods of time exhibit a greater tendency to accumulate in regions of tumour growth (Gabizon and Papahadjopoulous, 1988; Gabizon, 1992; Wu et al.,

1993). However, the kinetics of this extravasation process, where liposomes leave the blood compartment and enter an extravascular site, are slow (Nagy *et al.*, 1989; Bally *et al.*, 1994). Efficient drug delivery can, therefore, only be achieved with liposomes that effectively retain the drug following systemic administration. The problem that arises through applications of liposomal carriers that are optimized for enhanced drug retention concerns evidence from studies with liposomal doxorubicin that demonstrate reduced therapeutic activity, despite efficient delivery of drug to tumours (Parr *et al.*, 1997). A balance between doxorubicin retention (to maximize drug accumulation in a site of tumour growth) and release (to effect therapy) has not been established.

Attempts to improve the therapeutic properties of liposomal doxorubicin formulations through changes in drug release characteristics have been unsuccessful due to specific adverse effects of free doxorubicin, including cardiotoxicity (Minow *et al.*, 1975) and drug mediated free radical damage (Rajagopalan *et al.*, 1988). More specifically, effective modulation of doxorubicin release rates has been achieved with relatively simple changes in liposomal lipid composition (Mayer *et al.*, 1989; Bally, *et al.*, 1990); however, liposomal formulations of doxorubicin that release drug following *i.v.* administration, exhibit enhanced toxicity and increased doxorubicin accumulation in cardiac tissue. This effect is most dramatic for doxorubicin formulations prepared using DMPC/Chol liposomes, which release greater than 90% of the encapsulated contents in the blood compartment within 24 hours after *i.v.* administration and are 3 times more toxic than free drug (Mayer *et al.*, 1994).

The studies in this chapter examine the influence of liposome drug release properties on the biological activity of mitoxantrone. The rationale for selecting mitoxantrone is based on the fact that this drug is less cardiotoxic than doxorubicin (Weiss, 1989) and is not capable of generating

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free radical damage in non-proliferating cells (Durr, 1984). It is demonstrated that the *in vivo* rate of mitoxantrone release from DMPC/Chol liposomes is at least 68-fold greater than that obtained from DSPC/Chol liposomes. The pharmacodynamic characteristics of these formulations have been characterized using murine tumour models where the primary site of tumour progression is in the liver. The data illustrate how a balance between drug release characteristics and liposome mediated drug delivery to sites of tumour progression is required for optimal therapeutic activity.

3.2 Results

3.2.1 *In vitro* mitoxantrone uptake and release characteristics

Studies evaluating *in vitro* drug accumulation in liposomes prepared from DMPC (C_{14}) /Chol and DSPC (C_{18}) /Chol at 37 °C, 50 °C and 65 °C are shown in Figure 3.1. At 37 °C, less than 15% of the drug was encapsulated in either liposome formulation over the 2 hour time course. In contrast, >98% of the drug was efficiently entrapped when the incubation temperature was increased to above 50 °C. The time required to achieve maximum uptake was 45 minutes and less than 5 minutes when the incubation temperature was 50 °C and 65 °C, respectively. Uptake rate was enhanced slightly at 50 °C for the DMPC/Chol when compared to the DSPC/Chol systems. The results suggest that the phase transition temperature (T_c) of the phospholipid species does not markedly affect mitoxantrone loading characteristics. This result is consistent with the *in vitro* drug release studies (Figure 3.2) that demonstrate no difference in drug release from either liposomal formulation. The *in vitro* release assay used is based on dialysis against a large volume (1 L) of HBS buffer. Under these conditions, free mitoxantrone equilibrates across the dialysis membrane in less than 8 hours. In contrast, less than 2% drug release was observed

Figure 3.1

Effect of temperature on pH gradient loading of mitoxantrone into DSPC/Chol (A) and DMPC/Chol (B) liposomes

Loading was evaluated at three temperatures: 37 °C (●); 50 °C (▲); 65 °C (▼). At time zero, mitoxantrone and the liposomes were mixed together at a drug to lipid ratio of 0.1 (wt:wt). Encapsulated drug was determined by the mini spin column procedure described in Chapter 2, section 8. Duplicate samples were taken and [^{3}H]-CDE and [^{14}C]-mitoxantrone were measured. Data represents the average values ± S.D. of four measurements.
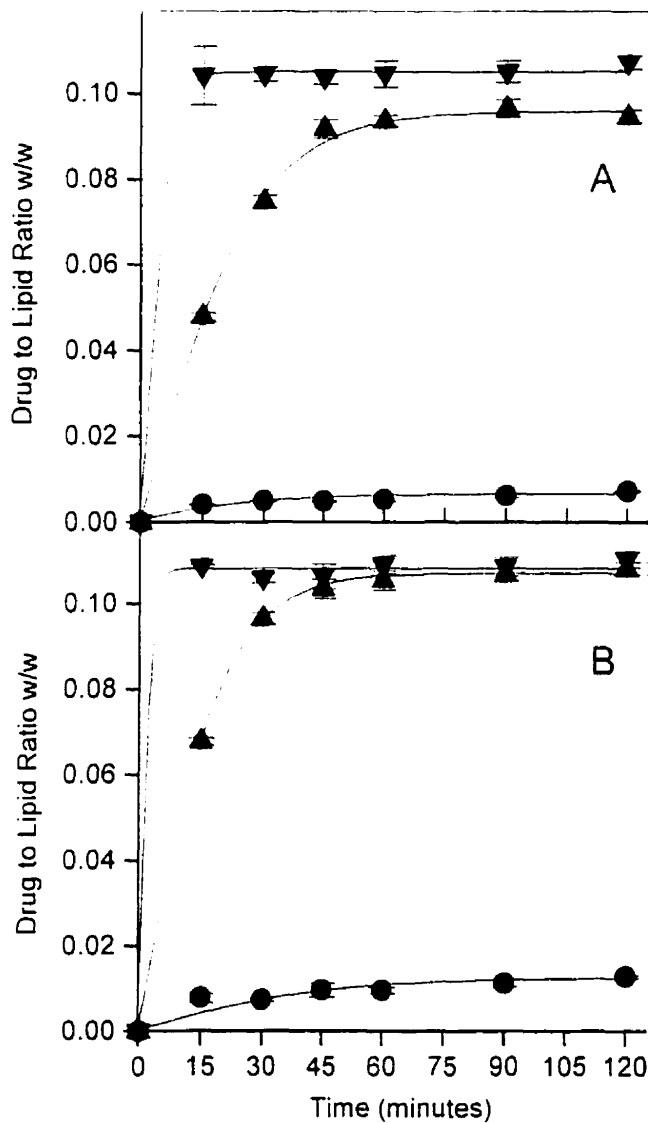
from the liposomal formulations over a 72 hour incubation period at 37 °C. Figure 3.2 also

incorporates data obtained for mitoxantrone loaded liposomes incubated with nigericin, a

H⁻/monovalent cation exchanger (dashed lines). Although drug release rates were increased in

the presence of nigericin, there were minor differences in release rates observed for the two

liposomal systems studied. After the 48 hour incubation period the DMPC/Chol liposomes

released less than 30% of the encapsulated drug in comparison to 20% drug release observed for

the DSPC/Chol system. Figure 3.3 demonstrates release of mitoxantrone from DMPC/Chol

liposomes after incubation with fetal bovine serum for twenty-four hours. The results in figure

3.3B demonstrate that the presence of serum proteins also did not enhance mitoxantrone release

from DMPC/Chol liposomes.

3.2.2 *In vivo* plasma elimination of liposomal lipid and mitoxantrone

Results in Figure 3.4 show that the plasma elimination of liposomal lipid, following *i.v.*

administration of mitoxantrone loaded DMPC/Chol and DSPC/Chol liposomes, is similar (Figure

3.4A). An estimation of the amount of mitoxantrone retained in the liposomes remaining in the

circulation can be made by determining the ratio of mitoxantrone to lipid at the indicated time

points; an estimation that assumes the level of free drug in the plasma of animals given liposomal

mitoxantrone is negligible. The results shown in Figure 3.4B demonstrated greater release of

mitoxantrone from DMPC/Chol liposomes than DSPC/Chol liposomes (p < 0.05 for 24 and 48

hour time points). For DMPC/Chol liposomes, 73% of the mitoxantrone originally associated

with the carrier was released within 48 hours. In contrast, less than 5% of the drug was released

from DSPC/Chol liposomes. Between the 4 and 48 hour time points, the rate of mitoxantrone

release was estimated to be 1.7 and < 0.025 μg lipid/100μl plasma/hour for DMPC/Chol and

DSPC/Chol liposomes, respectively. These results are consistent with those obtained using

Figure 3.2

Release of mitoxantrone from DSPC/Chol (●)and DMPC/Chol (■) liposomes in HEPES buffered saline at 37 °C

Solid lines indicate the absence of Nigericin. Dashed lines indicate the addition of Nigericin at time zero. Samples (100 μl) were taken from the dialysis bags and applied to Sephadex G-50 mini spin columns in duplicate and spun at 500 xg for 2 minutes. Duplicate samples were taken from the resulting mixture and [^{3}H] and [^{14}C] were measured as described in Chapter 2, section 8. Data represents the average values ± SD of at least four measurements for studies in the presence of Nigericin.
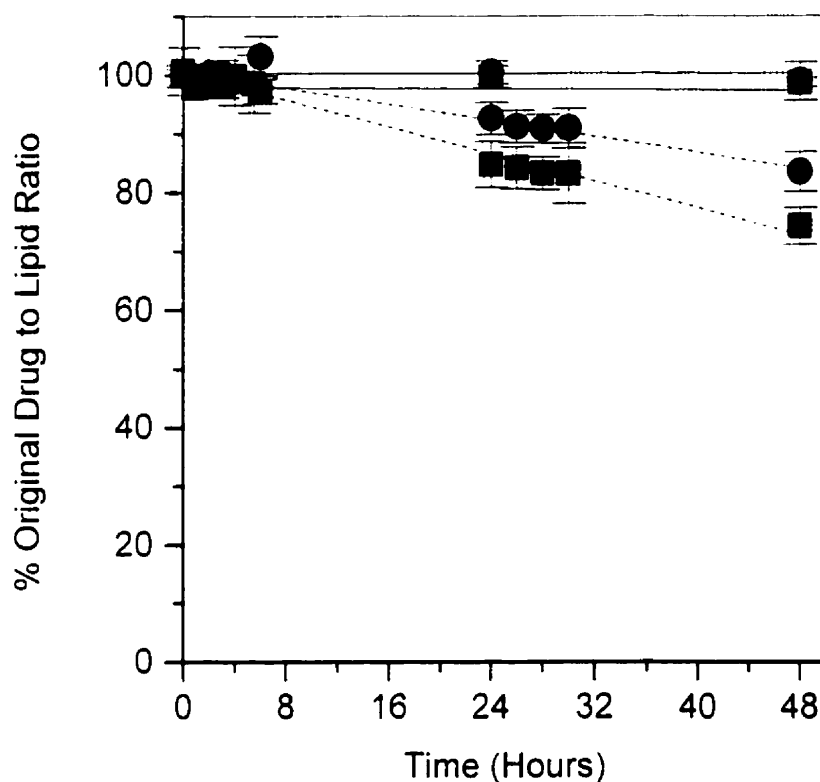
Figure 3.3

Release of mitoxantrone from DMPC/Chol liposomes incubated with fetal bovine serum at 37°C for 24 hours

DMPC/Chol liposomes (200 µl) were incubated with 800 µl of fetal bovine serum for 24 hours at 37°C. The solution (500µl) was applied to a BioGel A-15 column and fractions were collected. Panel A represents the fractions collected when empty DMPC/Chol liposomes (☐) and free mitoxantrone [mitox(Δ)] were passed down the column. Panel B represents the lipid (■) and mitoxantrone (▲) fractions collected with loaded liposomes passed down the column.
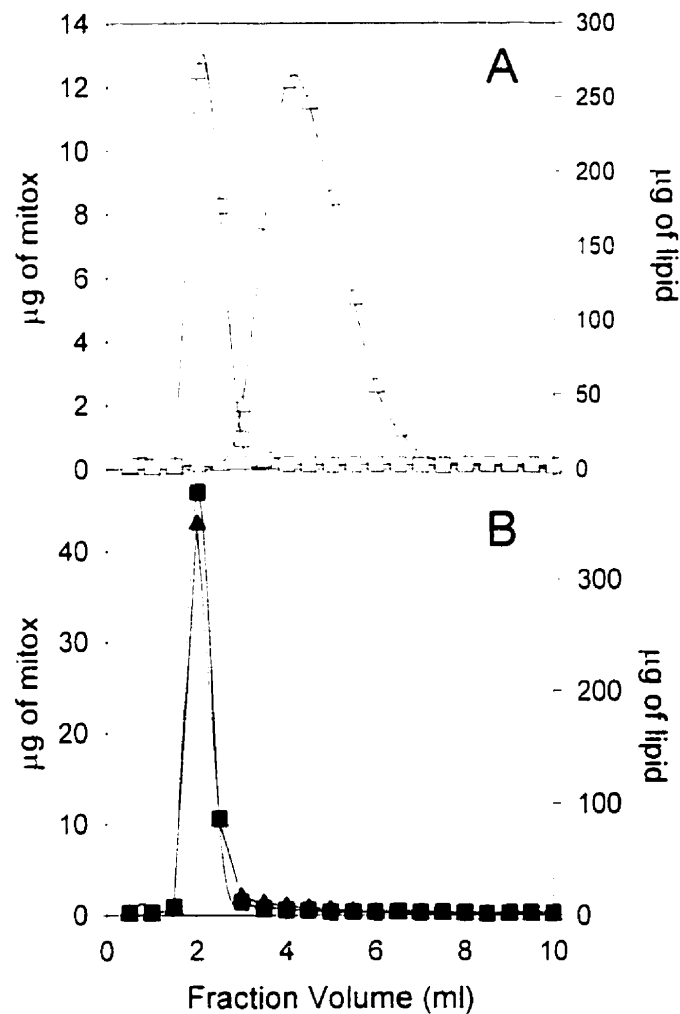
entrapped doxorubicin (Mayer *et al.*, 1994) and clearly demonstrate that control of *in vivo* mitoxantrone release rates can be achieved through simple changes in liposomal lipid composition. It should be noted that plasma drug levels obtained following administration of free drug are significantly less than those obtained with the liposomal formulations. This is indicated in Figure 3.4C, a plot of plasma drug levels measured following *i.v.* administration of the indicated formulation. Trapezoidal area-under-the-curve (AUC) analysis of these plasma drug levels, from 1 to 48 hours, indicate plasma AUCs of 0.01, 167.86 and 229.86 µg mitox/100µl plasma/hour following administration of free mitoxantrone, DMPC/Chol mitoxantrone and DSPC/Chol mitoxantrone, respectively.

3.2.3 Acute toxicity of free and liposomal mitoxantrone

Formal LD_{10} and LD_{50} studies are not sanctioned by the Canadian Council of Animal Care; therefore, toxic dose range finding studies in tumour free female BDF1 mice were conducted using only 2 mice per dose. These limited dose escalation studies suggested that the MTD of free drug was approximately 10 mg/kg. When drug was encapsulated in DSPC/Chol or DMPC/Chol, the MTD increased to approximately 30 mg/kg. At this dose, 100% of the animals treated survived for greater than 30 days. Necropsies suggested no gross abnormalities in any of the tissues examined. An evaluation of drug induced weight loss, however, suggested that the DMPC/Chol liposomal formulation was more toxic than the DSPC/Chol system. This result was confirmed in efficacy experiments, where changes in weight were measured over 14 days following initiation of treatment. For animals given 10^4 L1210 cells *i.v.* and treated 24 hours later with mitoxantrone, the maximum therapeutic dose of free and liposomal mitoxantrone was 10 and 20 mg/kg, respectively. The nadir in weight loss following treatment of tumour bearing animals occurred between day 12 and 13 and at this time point animals treated with free drug (10

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In vivo **release of mitoxantrone from DSPC/Chol (●), DMPC/Chol (■) liposomes, and free mitoxantrone (▲)**

Liposomes were loaded with mitoxantrone at a drug to lipid weight ratio of 0.1 (wt:wt). Female CD1 mice were injected at a 10 mg/kg drug dose *i.v. via* lateral tail vein. Panel A shows elimination of lipid from the plasma compartment over 48 hours. Panel B shows the change in the drug to lipid ratio over the 48 hour time period. Panel C shows the elimination of the free drug from the plasma compartment over 48 hours. Data represents the mean and S.E.M. obtained from at least 4 animals. (*) indicates $p < 0.05$.
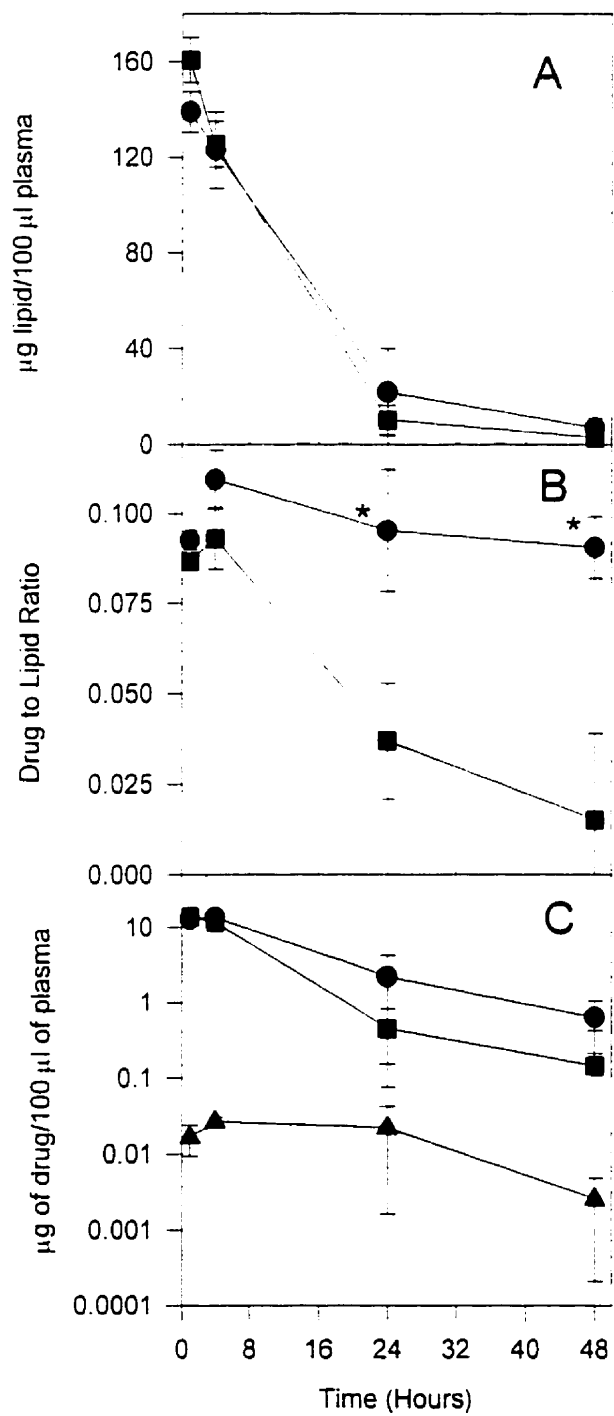
mg/kg) lost almost 30% of their original body weight and had to be killed. In contrast, animals treated with DSPC/Chol and DMPC/Chol mitoxantrone (20 mg/kg) exhibited a body weight loss of 8% and 25%, respectively.

3.2.4 L1210 and P388 anti-tumour activity of free and liposomal mitoxantrone

The murine tumour models used for evaluating the anti-tumour activity of liposomal mitoxantrone were based on *i.v.* injection of L1210 or P388 cells. Although these cells are typically used to initiate ascitic tumours following *i.p.* inoculation, the cells can also be given by alternate routes of administration. When given *i.v.*, primary sites of cell seeding include the liver and spleen. Evidence to support this is provided in Figures 3.5 and 3.6. Seven days following *i.v.* inoculation of 10^4 L1210 cells, the liver and spleen of the recipient animals showed greater than a 2- and 3-fold increase in liver and spleen weight, respectively as shown in Figure 3.5. Untreated animals must be terminated as a result of significant tumour related disease within 10 days. Histological studies indicated the presence of massive, diffuse cell infiltration throughout the liver. There were no other gross abnormalities in any other organs or tissues derived from these animals. For mice injected with P388 cells, liver and spleen weight increases were also observed. The histopathology, however, revealed discrete foci of tumour cells that progressively became larger over a 7 day time course (Figure 3.6). These *i.v.* tumour models were typically not responsive to chemotherapy with doxorubicin or vincristine [free or liposomally encapsulated drug (Refer to Chapter 5, Table 5.1)], hence these models were employed as a stringent measure of mitoxantrone anti-tumour activity.

Figure 3.5

Liver and spleen weights of untreated BDF1 mice (open bars) and BDF mice previously (7 days) injected *i.v.* with 10⁴ L1210 cells (hatched bars)

On day 7, livers and spleens were taken from BDF1 mice and weighed. The results were obtained from 4 animals and error bars indicate the S.E.M.
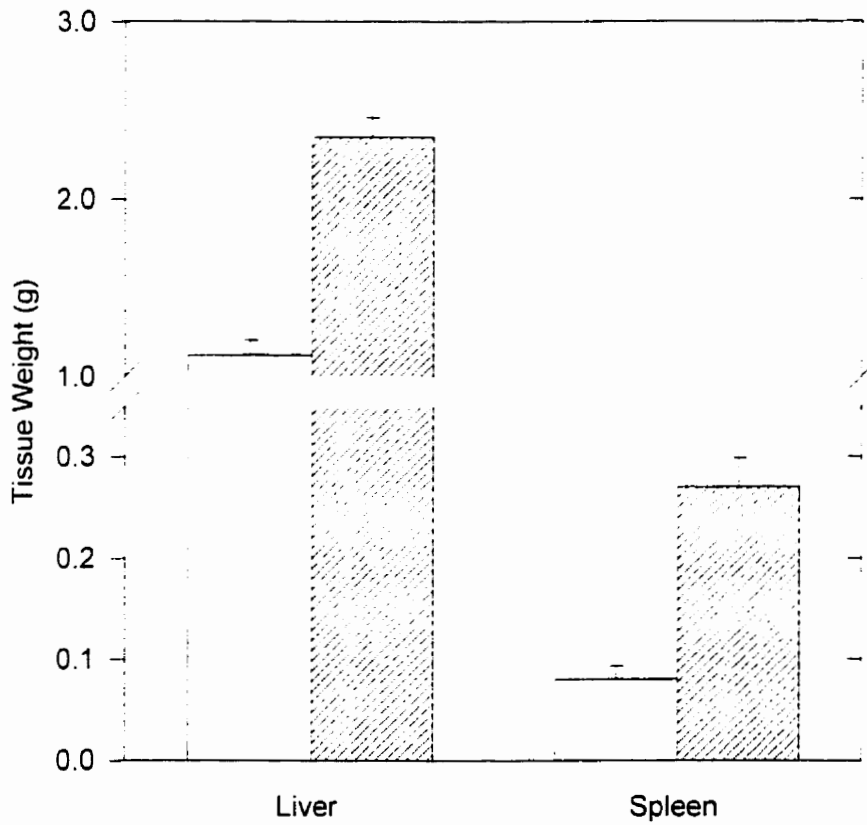
Figure 3.6

Progression of the P388 *i.v.* tumour model in the liver

Hematoxylin and eosin staining of paraffin embedded livers of untreated BDF1 mice (Panel A), and BDF1 mice previously injected *i.v.* 1 day (Panel B), 3 day (Panel C), and 7 days (Panel D) with 10^5 P388 cells. Structural features are pointed out as: H - Hepatocytes, S- Sinusoid, V - Blood vessel, rbc - red blood cell, K - Kupffer cell. Arrowheads indicate inflammatory infiltrate and arrows show the disorganization and lack of hepatocytes during tumour cell invasion. The bar in Panel C represents 30 μm.
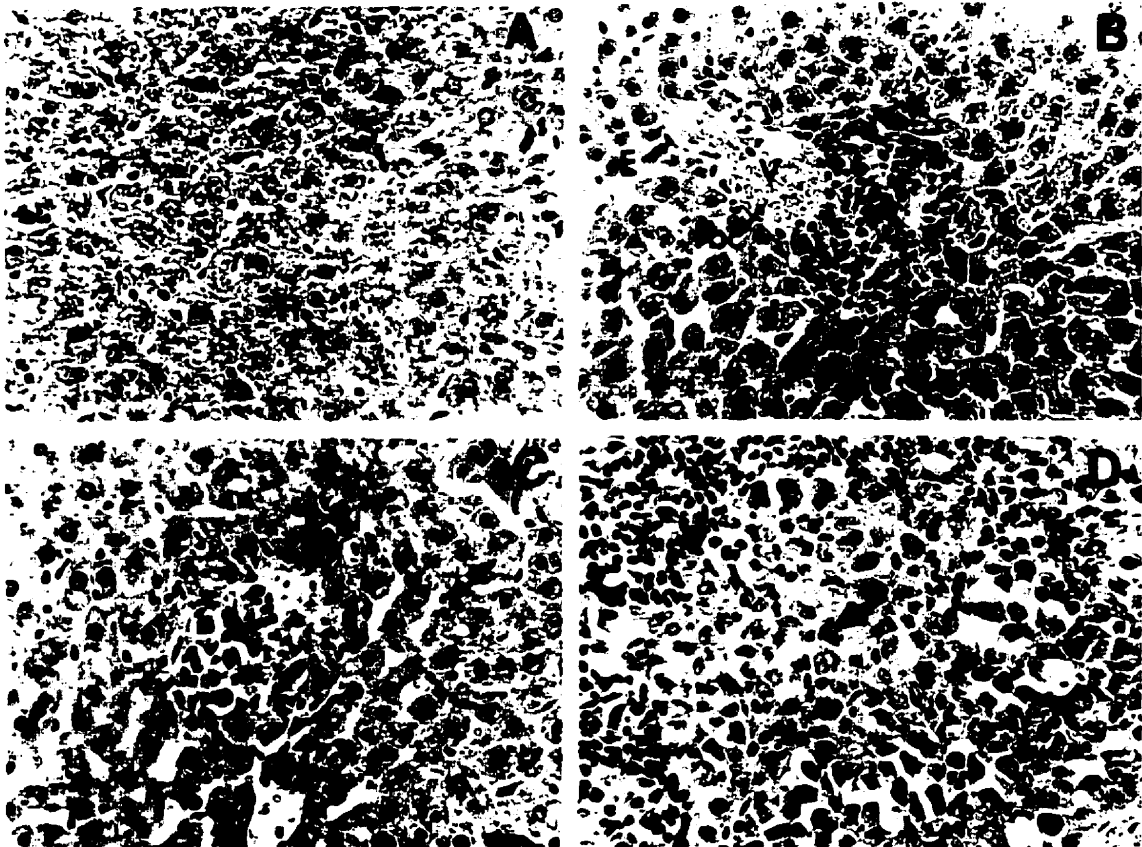
The L1210 anti-tumour studies summarized in Table 3.1 and Figure 3.7A clearly demonstrate that the DMPC/Chol liposomal formulation was therapeutically more active than free drug and drug encapsulated in DSPC/Chol liposomes. As shown in Table 3.1, the maximum % ILS achieved with free drug was 98%. Enhanced therapy was observed for drug encapsulated in DSPC/Chol liposomes, where a maximum % ILS value of 189 was obtained at a dose of 20 mg/kg. Improved therapy achieved with DSPC/Chol liposomal drug was primarily a consequence of liposome mediated reductions in drug toxicity. At 10 mg/kg, for example, the L1210 anti-tumour activity of this liposomal formulation was significantly lower than that obtained with free drug. Remarkably, treatment with DMPC/Chol liposomal mitoxantrone resulted in 100% long term (>60 day) survival at drug doses of 10 and 20 mg/kg. The survival curves obtained for animals treated at a dose of 10 mg/kg (Figure 3.7A) clearly show that the therapeutic activity of mitoxantrone was significantly enhanced when encapsulated in DMPC/Chol liposomes. These results were confirmed using a similar tumour model derived following i.v. injection of P388 cells. These results, shown in Figure 3.7B, demonstrate that animals treated with the DMPC/Chol liposomal mitoxantrone formulation were effectively cured when the drug was administered at a dose of 10 mg/kg.

3.2.4 Drug and liposomal lipid uptake in liver

The results presented to this point demonstrate that 1) the rate of mitoxantrone release from DMPC/Chol liposomes following i.v. administration was significantly greater than that measured for DSPC/Chol liposomes and 2) DMPC/Chol liposomal mitoxantrone was significantly more efficacious than free drug or DSPC/Chol liposomal mitoxantrone when tested against a tumour model where the primary site of disease progression is in the liver and spleen. It has been proposed that differences in the therapeutic activity of encapsulated anti-cancer drugs will be a

Table 3.1: L1210 anti-tumour activity of free and liposomal mitoxantrone in BDF1 mice

Sample	Drug Dose (mg/kg)	Lipid Dose (mg/kg)	60 Day Survival	Mean Survival (days)	%ILS[a]	L/F[c]
Control	-	-	0/25	8.7	N/A	N/A
Free Mitoxantrone	5	-	0/10	13.6	56	N/A
	10	-	0/10	17.2	98	N/A
	20	-	0/5	12.6	45	N/A
DSPC/Chol	10	100	0/9	14.7	69	0.85
	20	200	0/10	25.1	189	1.99
DMPC/Chol	5	50	0/5	17.2	98	1.26
	10	100	10/10	>60	ND[b]	ND
	20	200	10/10	>60	ND	ND

[a] Percentage ILS (Increase in Life Span) Values were determined from mean survival times of treated and untreated control groups. If the animals survived more than 60 days the % ILS was not determined

[b] ND can not be determined based on a 100% survival rate for 60 days

[c] L/F (Liposomal/Free) values were calculated by dividing the mean survival time of the liposomal formulation by the mean survival time of the free drug at the equivalent dose.

consequence of liposomal characteristics that regulate the drug exposure within sites of disease progression. Therefore, in addition to assessing drug release from liposomes in the plasma compartment, it is also important to correlate anti-tumour activity with drug levels at the site of tumour progression. For this reason, drug delivery to the liver, a primary site of disease progression for the *i.v.* tumour models employed, was evaluated. Results, shown in Figure 3.7, were obtained in tumour free CD1 mice. It should be noted that drug/liposome plasma elimination and biodistribution data were similar in tumour free CD1 and tumour bearing BDF1 mice. As shown in Figure 3.8A, liposomal lipid accumulation in the liver was similar for both DSPC/Chol and DMPC/Chol liposomal mitoxantrone formulations over 48 hours. Unlike doxorubicin (Bally *et al.*, 1990), the presence of entrapped mitoxantrone did not cause significant

reductions in liposomal lipid accumulation in the liver. Empty DMPC/Chol liposomal lipid uptake in the liver, for example, was not significantly different from mitoxantrone loaded DMPC/Chol liposomes. Figure 3.8B demonstrates that the level of mitoxantrone achieved in the liver following i.v. administration of DMPC/Chol liposomal mitoxantrone is less than that observed for DSPC/Chol mitoxantrone (p < 0.01 for the 48 hour time point). AUC analysis of liver drug levels, from 1 to 48 hours, indicates liver AUCs of 2564, 1810, and 1070 µg drug/g liver/hr following i.v. administration of DSPC/Chol mitoxantrone, DMPC/Chol mitoxantrone, and free mitoxantrone, respectively. Notably the liposomal formulation that engenders the greatest level of drug exposure in the liver (DSPC/Chol) did not provide the greatest therapeutic benefit.

3.3 Discussion

The therapeutic index of most anti-cancer drugs is narrow, with severe toxic side effects occurring within the same dose range required to mediate effective therapy. Although a variety of experimental strategies have been developed to improve the therapeutic index of anti-cancer drugs, these strategies have a common aim: to improve drug specificity. The principle benefit postulated for the use of liposomes as carriers of anti-cancer drugs is liposome mediated increases in drug delivery to the disease site and decreases in drug delivery to healthy tissues and organs (Sugarman and Perez-Soler, 1992; Mayer et al., 1994). Using this as a rationale, emphasis is placed on the importance of designing liposomes that have a greater propensity to accumulate within disease sites (Gabizon and Papahadjopoulos, 1988; Gabizon, 1992; Ogihara-Umeda et al., 1994; Uchiyama et al., 1995). In this regard, liposome carriers have been optimized with respect to maximizing the amount of drug contained per liposome (Mayer et al.,

Figure 3.7

Survival times of BDF1 mice injected with 10^4 L1210 cells (Panel A) or 10^5 P388 cells (Panel B) _i.v. via_ the lateral tail vein and treated with mitoxantrone

Twenty four hours after tumour cell inoculation, the mice were treated with 10 mg/kg dose of free mitoxantrone (▲), DSPC/Chol (●), and DMPC/Chol (■) liposomal formulations. Untreated (saline) animals served as controls (▼).
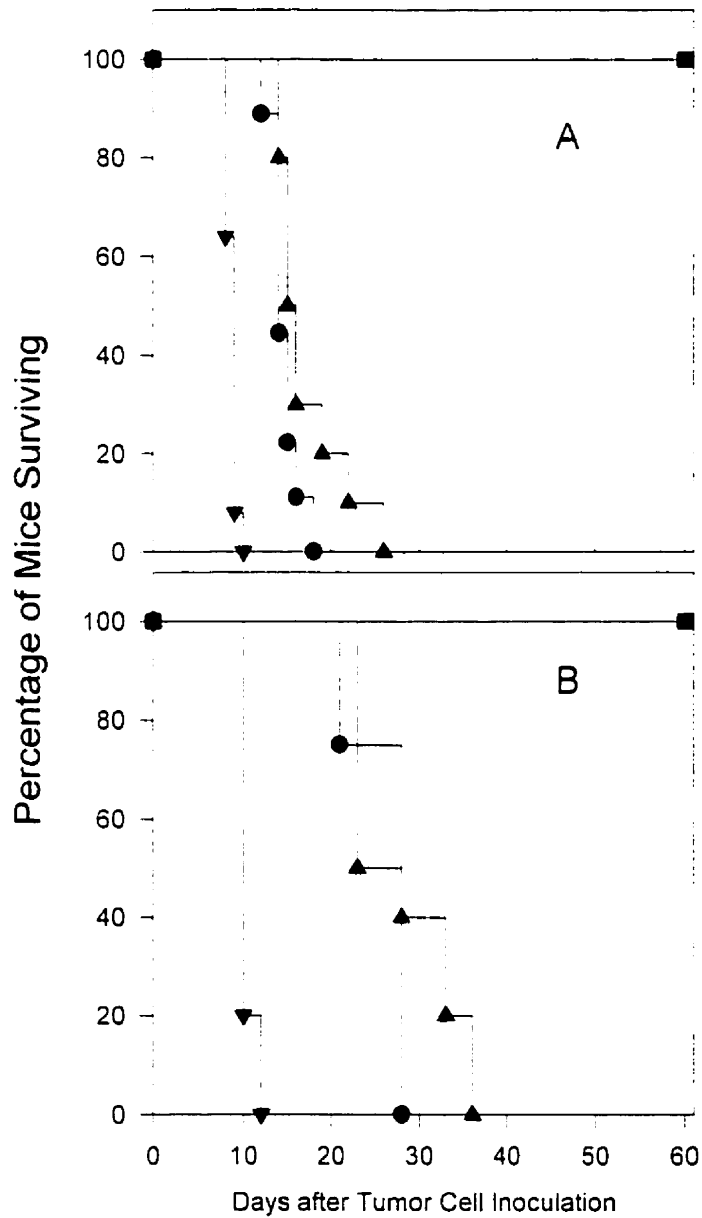
Figure 3.8

Lipid and drug levels in the liver of mice after injection of DSPC/Chol mitoxantrone (●), DMPC/Chol mitoxantrone(■), empty DMPC/Chol liposomes (□), and free mitoxantrone (▲)

The liposomal lipid dose was 100 mg/kg and the drug dose was 10 mg/kg. Panel A shows the amount of lipid per gram of liver and panel B shows the amount of mitoxantrone per gram of liver measured over 48 hours. Drug and lipid levels were determined as described in Chapter 2, section 9. The data represents the mean ± S.E.M. from at least 3 animals. (**) indicate $p < 0.01$ when compared to free drug.
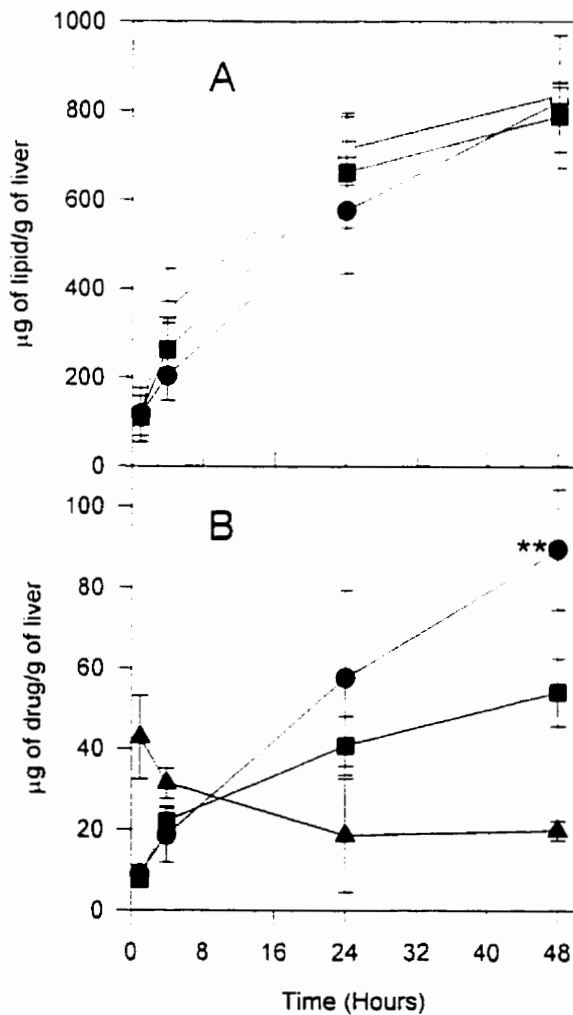
1989; Mayer *et al.*, 1994), increasing drug retention characteristics (Mayer *et al.*, 1989; Boman *et al.*, 1994) and augmenting the circulation lifetime of the drug loaded carrier (Gabizon, 1992; Wu *et al.*, 1993). However, it can be suggested that the therapeutically active component of a liposomal anti-cancer drug formulation is the free drug. It is believed that the primary source of free drug arises from regionally localized liposomes (Mayer *et al.*, 1994). Therefore, this research has attempted to establish a balance between efficient liposome delivery to the disease site and controlled drug release. The latter can be achieved for certain drugs by changing the liposomal lipid composition (Boman *et al.*, 1994; Mayer *et al.*, 1994;). This study illustrates how controlled drug release can engender significant improvements in therapeutic activity of the anti-cancer drug mitoxantrone.

It was surprising that differences in drug accumulation and leakage rates for DSPC/Chol and DMPC/Chol liposomes were not substantial when evaluated *in vitro*, even when the liposomes were incubated in the presence of nigericin. The phase transition temperatures (T_c) for DSPC and DMPC are 55.3 °C and 23.9 °C, respectively (Lewis *et al.*, 1987) and it was anticipated that differences in the gel to liquid crystalline phase transition of these phospholipids would be reflected by changes in permeability characteristics. This was evident for liposomal formulations of vincristine, where a good correlation between phospholipid T_c and drug leakage, *in vitro*, was observed (Boman *et al.*, 1993). Collapse of the transmembrane pH gradient did increase drug release from the liposomal formulations; however, no substantial differences in the rate of drug release from the DSPC/Chol and DMPC/Chol liposomes were noted. Following pH gradient mediated uptake, it is believed that drugs such as mitoxantrone can form insoluble precipitates within the liposome (Madden *et al.*, 1990). If this is the case, permeability characteristics of the drug in a precipitated form may be less dependent on membrane characteristics or the presence of

a residual transmembrane pH gradient. It is not understood. however, why differences in drug permeability become apparent *in vivo*.

Mitoxantrone was selected as a model drug for these studies for two reasons. First. the drug loading and release characteristics of mitoxantrone are comparable to doxorubicin (Madden *et al.*, 1990). Second. mitoxantrone is less cardiotoxic than doxorubicin (Dukart *et al.*, 1985: Neidhart *et al.*, 1986: Bennett *et al.*, 1988: Weiss *et al.*, 1989). Liposome mediated increases in mitoxantrone MTD observed in this report are comparable to those reported for a liposomal mitoxantrone formulation prepared using an anionic lipid-drug complex (Schwendener *et al.*, 1991: Schwendener *et al.*, 1994). The liposomal formulations evaluated here. however. exhibit significantly better drug retention characteristics than those formulations described by Schwendener *et al.*. This is reflected in higher blood levels and improved circulation lifetimes for mitoxantrone encapsulated in the PC/Chol based liposomal carriers. Differences in drug release characteristics may be a consequence of the use of anionic lipids. Anionic lipids will increase liposome elimination rates (Hwang. 1987) and have been shown to enhance release of the anthracycline doxorubicin even when encapsulated using the transmembrane pH gradient loading procedure (Mayer *et al.*, 1989). Clearly. when the rate of drug dissociation from the liposomal carrier is very rapid. carrier mediated changes in drug pharmacokinetics and biodistribution will not be significant and changes in biological activity (relative to drug administered in free form) will be minimal.

Studies evaluating the therapeutic activity of DSPC/Chol and DMPC/Chol liposomal mitoxantrone (Figure 3.7 and Table 3.1) establish that both drug delivery and drug release are important attributes of an optimal liposomal anti-cancer drug formulation. The *i.v.* L1210 tumour model was selected for these studies. in part. because L1210 cells seed primarily in the

liver and spleen following *i.v.* administration. It is well established that these tissues are primary

sites of liposome accumulation (Hwang, 1987; Sugarman and Perez-Soler, 1992). Further, other

investigators have shown using experimental models of liver cancer that the therapeutic activity

of liposomal formulations of a novel platinum compound and doxorubicin analogue is enhanced

compared to free drug (Perez-Soler, 1989; Gabizon, 1992). It is perplexing, therefore, that

models of liver cancer have not been used more frequently to characterize the pharmacodynamic

behavior of liposomal anti-cancer drugs. These studies have shown that mitoxantrone delivery to

the liver is enhanced when using DSPC/Chol liposomes in comparison to DMPC/Chol liposomes

(see Figure 3.8B). Increased liposomal drug exposure in this tissue, however, does not result in

improved therapeutic activity. In fact, the DMPC/Chol liposomal formulation, which exhibits

controlled release characteristics and a reduced capacity to deliver drug to the liver, was

significantly more effective. Thus, it is not sufficient to develop drug carriers that accumulate at

the disease site in high levels, one must also engineer appropriate drug release rates.

Studies completed and summarized in Chapter 5 have demonstrated using the *i.v.* L1210 tumour

model that EPC/Chol liposomal doxorubicin, DSPC/Chol liposomal doxorubicin, and

DSPC/Chol liposomal vincristine are relatively ineffective in treating this model, typically

producing increases in lifespan of less than 50% at the maximum therapeutic doses (see Table

5.1). A possible explanation for the effectiveness of liposomal mitoxantrone may be related to

the fact that this encapsulated drug does not appear to affect the liver Kupffer cells. These

studies have shown, for example, that empty and mitoxantrone loaded liposomes exhibit

comparable plasma elimination profiles and comparable levels of uptake in liver (see Figure 3.8).

This is contrary to effects observed with vincristine (Boman *et al.*, 1994) or doxorubicin (Bally *et*

al., 1990) loaded liposomes, where encapsulated drug significantly increases the circulation

lifetime of the liposomal carrier. This effect is due, in part, to drug mediated blockade of

phagocytic cells in the liver. It can be suggested that the blockade effect may adversely affect the therapeutic activity of liposomal anti-cancer drugs in treating tumours that are progressing in the liver and that phagocytic cells in the liver may have a significant role in defining the anti-tumour activity of liposomal mitoxantrone.

In conclusion, a liposomal mitoxantrone formulation has been developed which has significant therapeutic activity. The plasma elimination curves and biodistribution data demonstrate that effective control of both drug release characteristics and target site delivery can work synergistically to achieve optimal therapy. The research described in the following two chapters will continue to study liposomal formulations of mitoxantrone with the aims of: 1) further improving the therapeutic index of the drug; 2) targeting the liposomal drug for use in treatment of specific cancers, such as hepatocellular carcinomas and/or 3) developing novel formulations that effect delivery of the drug loaded carrier to tumour cells, thereby, bypassing normal cellular drug uptake mechanisms. The DMPC/Chol formulation described here meets the first objective.

CHAPTER 4

FACTORS AFFECTING THE THERAPEUTIC ACTIVITY OF LIPOSOMAL MITOXANTRONE FOLLOWING INTRAVENOUS ADMINISTRATION IN SCID/RAG2 MICE BEARING ESTABLISHED HUMAN A431 AND LS180 SOLID TUMOURS: DRUG RELEASE VERSUS LIPOSOME MEDIATED DRUG DELIVERY

4.1 Introduction

Liposome formulations developed in an effort to enhance the therapeutic properties of anti-cancer drugs have traditionally focused on lipid compositions that allow for retention of the liposomes in the circulation for extended periods of time and exhibit slow drug release rates (Mayer et al., 1989; Mayer et al., 1993; Boman et al., 1994; Gabizon et al., 1996). This strategy has been pursued based on a putative biological mechanism relying on the inherent ability of liposomes to be preferentially taken up in disease sites such as tumours (Proffitt et al., 1983; Mayer et al., 1990; Allen et al., 1991; Bally et al., 1994). This uptake is interrelated with increases in tumour blood vessel permeability that occur as a consequence of angiogenesis and associated expression of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) in tumours (Folkman, 1985; Dvorak et al., 1988; Dvorak et al., 1991).

Given the emphasis placed on maximizing liposome-mediated drug delivery to tumours, this chapter assesses the role of liposome delivery compared to drug release from liposomes in enhancing the therapeutic activity of associated anti-cancer drugs. This research has focused on the anti-cancer drug mitoxantrone for several reasons including data that suggests that mitoxantrone is: 1) less cardiotoxic compared to doxorubicin (Dukart et al., 1985; Neidhart et al., 1986; Bennett et al., 1988; Weiss et al., 1989) and 2) effective in the treatment of breast cancer, leukemia, and lymphoma (Smith et al., 1983; Durr, R.B., 1984). Mitoxantrone has proved to be a suitable substitute for doxorubicin in clinical settings where alopecia and/or

cardiotoxicity are concerns (Dukart *et al.*, 1985; Bennett *et al.*, 1988; Neidhart *et al.*, 1986; Weiss *et al.*, 1989).

Another property of mitoxantrone which makes it an ideal choice for the pharmacodynamic studies developed in this thesis is that the encapsulated drug does not influence the plasma elimination and biodistribution characteristics of the liposomal carrier (See Chapter 3). This is in contrast to other anti-cancer drugs such as vincristine and doxorubicin, which, when encapsulated in liposomes, engender reductions in elimination rate of the associated carrier following intravenous administration. This effect has been attributed to a direct toxicity of the encapsulated drug on phagocytic cells that play an important role in effecting liposome elimination from the plasma (Bally *et al.*, 1990; Boman *et al.*, 1994; Daemen *et al.*, 1995). For this reason, mitoxantrone biodistribution and elimination parameters are dictated solely by attributes of the liposomal carrier rather than by combined effects induced by encapsulated-drug dependent changes in liposome pharmacokinetic behavior.

Using liposomal formulations of mitoxantrone differing in their *in vivo* drug retention characteristics, it was demonstrated in Chapter 3 that drug release is required for optimal therapeutic activity when the tumour model grows in the liver. The previous chapter addressed a hypothesis suggesting that drug release is the dominating factor controlling biological activity of liposomal drugs in tissues where the rate of liposome accumulation is rapid. The studies in this chapter addresses the question of whether drug release or liposome-mediated drug delivery becomes the dominant factor controlling therapeutic activity under conditions where the rate of liposome accumulation is slow and tumour development is within a site outside the liver. DSPC/Chol mitoxantrone and DMPC/Chol mitoxantrone delivery were evaluated in tumours established following s.c. injection of human LS180 and A431 cell lines. These cell lines were

selected on the basis of empirical observations that indicated more rapid liposome uptake in LS180 tumours compared to A431 tumours. The results suggest that delays in tumour growth induced by liposomal mitoxantrone are achieved using a liposomal formulation that is selected on the basis of drug release attributes, even when the liposome accumulation rate in the site of tumour growth is slow.

4.2 Results

4.2.1 Lipid and drug accumulation in solid LS180 and A431 tumours.

Lipid and drug levels were measured in established (≥ 0.05 cm^3) A431 and LS180 solid tumours over a 96 hour time period following a single *i.v.* injection of free mitoxantrone (10 mg/kg), DSPC/Chol liposomal mitoxantrone (10 mg drug/kg, 100 mg total lipid/kg) and DMPC/Chol liposomal mitoxantrone (10 mg drug/kg, 100 mg total lipid/kg) and the results are summarized in Figure 4.1. The level (μg lipid/g tumour) of liposomal lipid in the LS180 and A431 tumours is shown in panels A and B, respectively, and the tissue concentration (μg drug/g tumour) of mitoxantrone in the LS180 and A431 tumours is shown in panels C and D, respectively. There are two important conclusions that can be made from the data shown in Figure 4.1, panels A and B. First, the accumulation rates of DMPC/Chol and DSPC/Chol liposomes are comparable in the LS180 tumours and they are comparable in the A431 tumours. Second, the rate of liposomal lipid accumulation in the LS180 tumour is significantly faster than that observed in the A431 tumour. In the LS180 tumour (Panel A) the maximum concentration (C_{max}) of liposomal lipid observed is approximately 100-μg lipid/g tumour and at 4 hours following *i.v.* administration. In contrast, the C_{max} of liposomal lipid observed in the A431 tumour (Panel B) is approximately 70-μg lipid/g tumour and at 48 hours after drug administration. Two important conclusions can be

inferred from the data shown in panels C and D. First, mitoxantrone accumulation in the solid tumours is increased when the drug is given encapsulated in liposomes in comparison to free drug. Following administration of free drug, the C_{max} observed is at the 4 hour time point, a level of drug that is equivalent to that obtained following administration of the liposomal formulations of mitoxantrone. Subsequently the level of mitoxantrone observed in tumours decreases in animals given free mitoxantrone while the drug level increases or is maintained in tumours from animals given the liposomal formulations. Second, following administration of the liposomal formulations of mitoxantrone, the total concentration of drug achieved in the tumour is greater when drug is entrapped in DSPC/Chol liposomes compared to DMPC/Chol liposomes. This result is consistent with results from Chapter 3 demonstrating that the DMPC/Chol liposomal formulation releases mitoxantrone more rapidly than DSPC/Chol liposomes.

Differences in the drug release attributes of these two liposomal formulations are emphasized in Figure 4.2 where the percentage of initial drug-to-lipid ratio is determined at the 48 hour time point. Panel A shows the percentage of initial drug-to-lipid ratio in the plasma compartment while panel B shows the percentage of initial drug-to-lipid ratio measured in isolated tumours. The plasma results are consistent with the results in Chapter 3, indicating that DSPC/Chol liposomes retain 97% and 85% of the initial drug to lipid ratio in the plasma of mice bearing A431 or LS180 tumours, respectively. In contrast, the DMPC/Chol formulations exhibit 22% and 16% of the initial drug to lipid ratio in the plasma from mice bearing A431 or LS180 tumours, respectively. These values are comparable to those obtained in non-tumour bearing animals (see Chapter 3), suggesting that the presence of established tumours does not affect the release properties of the liposomes. Changes in drug to lipid ratios are less evident when the data are obtained from isolated tumours; however, these results (Panel B) are consistent with the plasma data and demonstrate a greater reduction in drug-to-lipid ratio for the DMPC/Chol

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liposomal mitoxantrone formulation. Data from A431 and LS180 tumours obtained from animals injected with the DSPC/Chol formulation suggest that 90% and 78% of the entrapped mitoxantrone is still associated with the liposome, respectively. Tumours from animals injected with the DMPC/Chol formulation have 53% (A431) and 62% (LS180) of the drug associated with the liposome. It should be noted that the estimates of drug-to-lipid ratio in tumours are equivocal considering free mitoxantrone (or mitoxantrone that has been released from liposomes) will localize in these regions of tumour growth (Figure 4.1, panel C and D).

The extent of drug exposure in the two tumours is best summarized by the data in Table 4.1, which provides the area under the liposomal-lipid (AUC_L) and mitoxantrone (AUC_D) concentration-time curve values obtained in tumours from 0 to 96 hours following $i.v.$ administration of free and liposomal drug (10 mg/kg drug dose). In the LS180 tumours, AUC_L values of 10167 and 9926 µg of lipid/g of tumour/hour were measured following administration of mitoxantrone encapsulated in DSPC/Chol and DMPC/Chol liposomes, respectively. In the A431 tumour model, the DSPC/Chol and DMPC/Chol have AUC_L values of 5728 and 5150 µg of lipid/g of tumour/hour, respectively. A comparison of the tumour AUC_D values obtained after administration of the two liposomal formulations demonstrates that more drug is delivered using the DSPC/Chol formulation (504 and 1000 µg drug/g of tumour/hour for the A431 and LS180 tumours, respectively) as compared to the DMPC/Chol formulation (304 and 749 µg drug/g of tumour/hour for the A431 and LS180 tumours, respectively). It should be noted that the tumour AUC_D values obtained after administration of free mitoxantrone are only 3 to 5 times lower than that measured for the liposomal formulations. This is in contrast to the area under the mitoxantrone concentration-time curves obtained in plasma from 0 to 96 hours, where the plasma AUC_D is 20- to 30-fold lower following $i.v.$ administration of free in comparison to that measured following injection of the liposomal formulations.

Figure 4.1

Lipid and mitoxantrone accumulation in A431 and LS180 tumours in SCID/RAG-2 mice over a 96 hour time period

SCID/RAG-2 mice were injected bilaterally with 2×10^6 A431 cells and 1×10^6 LS180 cells subcutaneously. Once the tumours reached a size of approximately 0.05-0.2 cm^3, mice were injected with a 10 mg/kg drug dose of free mitoxantrone ($\triangle$). DSPC/Chol mitoxantrone ($\bullet$), or DMPC/Chol mitoxantrone ($\blacksquare$) via the lateral tail vein. Mice were terminated using CO_2 asphyxiation and tumours were removed and processed as described in Chapter 2, section 10. Panels A and B demonstrate lipid accumulation in both the LS180 and A431 tumours respectively and Panels C and D demonstrate drug accumulation in the LS180 and A431 tumours respectively. Data points represent the average and standard error of the mean of at least 4 animals.
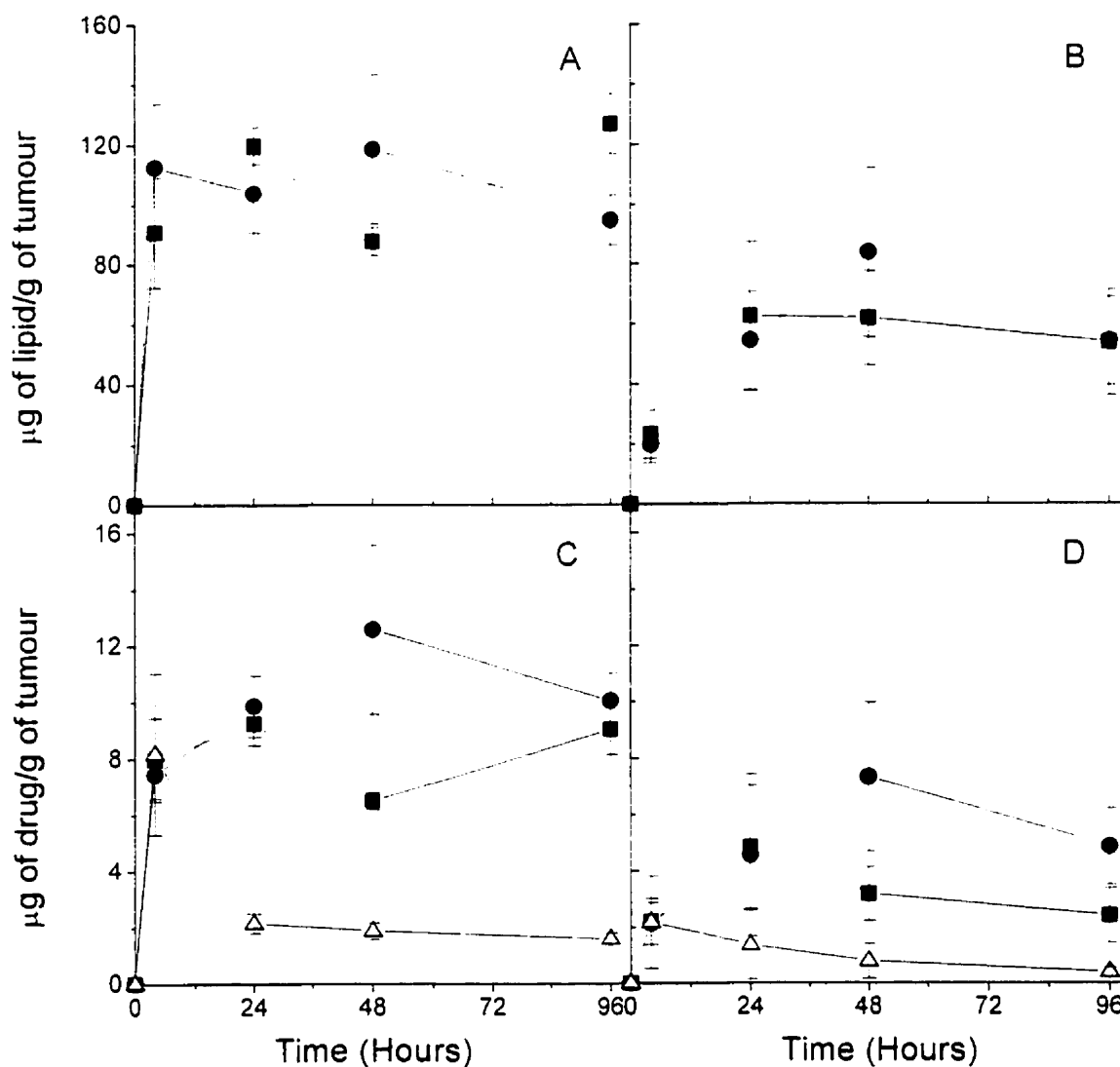
Figure 4.2

Percentage of initial drug to lipid ratio of DSPC/Chol mitoxantrone and DMPC/Chol mitoxantrone after 48 hours in plasma.

SCID/RAG-2 mice were injected bilaterally with 2 x 10^6 A431 cells or 1 x 10^6 LS180 cells subcutaneously. Once the tumours reached a size of approximately 0.05 cm^3, mice were injected with a 10 mg/kg drug dose of DSPC/Chol mitoxantrone (shaded bars) or DMPC/Chol mitoxantrone (open bars) via the lateral tail vein. Plasma and tumours were collected and processed as outlined in Chapter 2. section 10. Panel A shows the drug-to-lipid ratio in plasma and Panel B shows the drug-to-lipid ratio in the tumour. Data points represent the average and standard error of the mean of the data collected from at least 4 animals.

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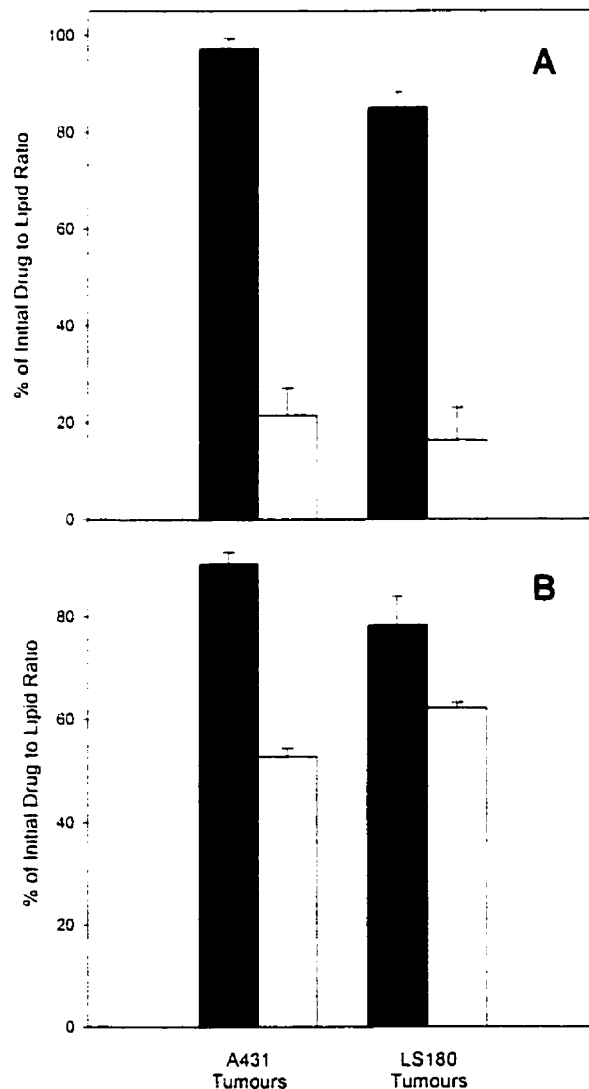
The distribution of drug from the plasma compartment to the tumour site can be described employing a drug targeting efficiency parameter, T_e, relating the AUC in the circulation to the tumour AUC ($T_e = AUC_T/AUC_P$). Using this parameter (see Table 4.1) it can be suggested that drug accumulation is more efficient in the LS180 tumours, an observation that is consistent with this tumour's extensive vascularization. The T_e value obtained for the LS180 tumour is 2.3- to 2.8-fold greater than that observed for the A431 tumour. The T_e values for the DSPC/Chol and DMPC/Chol liposomal mitoxantrone formulations are comparable for each tumour type and the greatest T_e values obtained are for the free drug, and these values are at least 8-fold higher than those obtained for either liposomal formulation. This higher T_e value for free drug is a reflection of drug distribution characteristics associated with small molecules (free drug) in comparison to large molecules (liposomal drug).

4.2.2 Efficacy of single dose administration of liposomal and free mitoxantrone in established A431 and LS180 human solid tumours

In Chapter 3, the studies demonstrated that treatment of mice bearing L1210 and P388 liver tumours with DMPC/Chol liposomal mitoxantrone resulted in 100% long term survivors. Although the DSPC/Chol liposomal mitoxantrone formulation delivered more mitoxantrone than the DMPC/Chol formulation to the tumour site, treatment with this formulation proved to be less effective due to drug release characteristics. It is important to determine whether these carrier-associated differences in mitoxantrone efficacy extend to solid tumours. As indicated in the previous section, the A431 and LS180 tumours provided suitably different liposome uptake characteristics so that comparisons between the liposomal formulations could be made. It is important, however, to recognize that the selected tumour cells exhibit different growth characteristics and drug sensitivity (Table 4.2). Particularly, the LS180 tumours exhibit a growth

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Table 4.1

Area under the liposomal-lipid and mitoxantrone concentration-time curves obtained in tumours and plasma from 0 to 96 hours following *i.v.* administration of free and liposomal drug (10 mg/kg dose) in SCID/RAG-2 mice bearing established A431 and LS180 tumours.

Tumour	Lipid μg lipid/g of tumour/hour		Drug μg drug/g of tumour/hour		
	DSPC/Chol	DMPC/Chol	DSPC/Chol	DMPC/Chol	Free
A431	5728	5149	505	304	93
LS180	10167	9925	1000	749	251

Plasma	Drug μg drug/ml of plasma/hour			Targeting efficiency (T$_e$)[a]		
	DSPC/Chol	DMPC/Chol	Free	DSPC/Chol	DMPC/Chol	Free
A431	373	226	11.6	1.35	1.35	8.01
LS180	254	264	11.8	3.94	2.84	21.3

[a] Targeting efficiency is a term that has been developed to characterize the distribution of drug between the plasma compartment and the tumour site. It is calculated by relating the AUC in the plasma compartment to the tumour AUC (T$_e$ = AUC$_T$/AUC$_P$).

rate that is approximately 2-times faster than that measured for the A431 tumours. In contrast to the A431 tumours, LS180 tumours are highly vascularized and the LS180 cells are about 5-times more sensitive to free mitoxantrone in comparison to A431 cells. Gross observations indicated that the LS180 tumours are less cohesive than the A431 tumour and the LS180 tumours ulcerated more rapidly than A431 tumours.

Results obtained following treatment of mice with established LS180 and A431 tumours are summarized in Figure 4.3. As shown in Panel A, free mitoxantrone and the DSPC/Chol

mitoxantrone formulation demonstrate minimal effects on the LS180 tumours. Tumour growth in animals treated with these formulations could not be distinguished from untreated controls. other than perhaps a reduction in the rate of tumour ulceration observed when animals were treated with free mitoxantrone. Animals that developed ulcerated tumours were killed as required by the Canadian Council for Animal Care guidelines and DSPC/Chol mitoxantrone and saline treated animals typically exhibited tumour ulcerations when the volume exceeded 0.5 cm^3. Reductions in tumour growth were observed when LS180 tumour bearing animals were treated with the DMPC/Chol mitoxantrone formulation. It should be noted that treatment with this formulation did not result in a reduction in tumour size and the tumour growth rate measured after day 17 was equivalent to that observed for control mice. Although the LS180 cells are more sensitive to mitoxantrone than A431 cells *in vitro* (see Table 4.2) and LS180 tumours exhibited increased drug exposure (see Table 4.1) in comparison to the A431 tumours, the A431 tumours were more responsive to treatment with free mitoxantrone (Figure 4.3. Panel B). Control mice exhibited 0.5 cm^3 tumours 12 days after initiation of treatment. whereas mice treated with free mitoxantrone exhibited similar tumour sizes after 16 days. The therapeutic activity of the liposomal formulations was better than free drug: however. there were slight differences in the therapeutic activity measured between liposomal formulations in the A431 tumours. Mice treated with DSPC/Chol liposomal mitoxantrone exhibited 0.5 cm^3 tumours 18 days after initiation of treatment versus 21 days with the DMPC/Chol liposomal mitoxantrone.

4.2.3 Efficacy of multiple dose administration of liposomal and free mitoxantrone in non-
established A431 and LS180 human solid tumours

The studies summarized in Figure 4.3 were obtained when mice with well established tumours were treated with the different mitoxantrone formulations. It can be argued that optimal therapy

Figure 4.3

Efficacy of DSPC/Chol mitoxantrone, DMPC/Chol mitoxantrone and free mitoxantrone in established LS180 and A431 solid tumours in SCID/RAG-2 Mice

SCID/RAG-2 mice were injected bilaterally with 1×10^6 LS180 cells (Panel A) or 2×10^6 A431 cells (Panel B) subcutaneously. Fourteen days after tumour cell inoculation (tumour size of > 0.05 cm^3), mice were injected with a 5 mg/kg dose of free mitoxantrone ($\triangle$), 10 mg/kg drug dose of DSPC/Chol mitoxantrone ($\bullet$), or 10 mg/kg drug dose of DMPC/Chol mitoxantrone ($\blacksquare$) via the lateral tail vein. Control mice were injected with saline (∇). Tumour width and length were measured using calipers and volume was calculated as outlined in Chapter 2, section 14. Points represent average data and the standard error of the mean from at least 6 tumours.
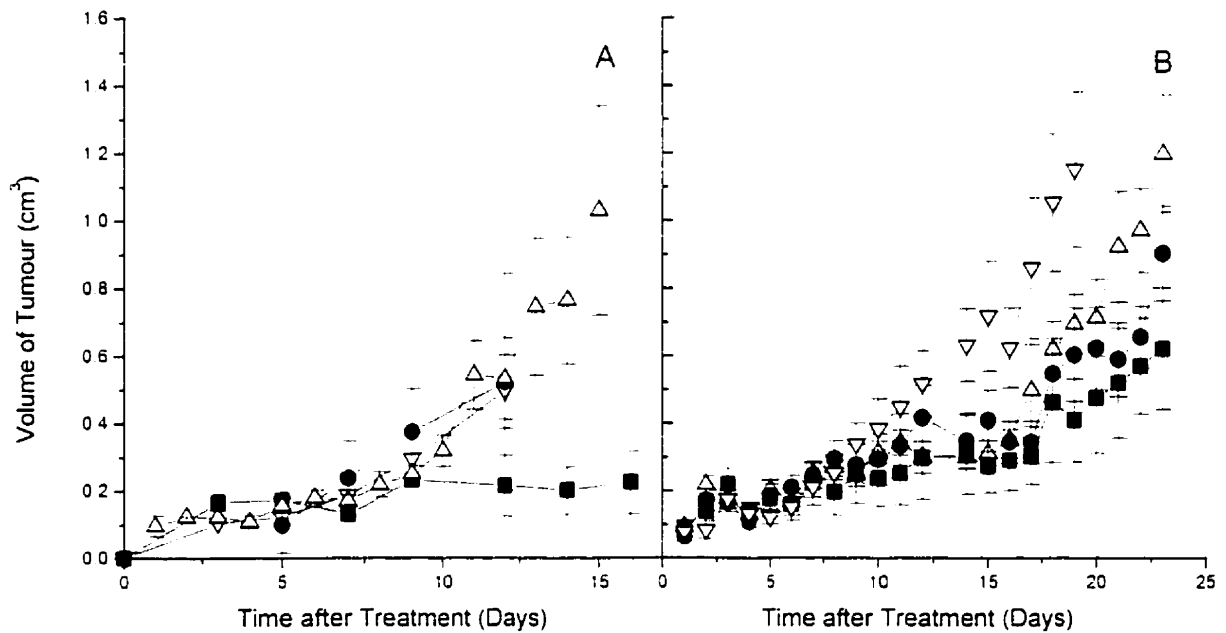
Table 4.2

Attributes of the LS180 and A431 cell lines and their growth characteristics in SCID/RAG-2 mice.

	LS180 cells	A431 cells
Source	Colon Carcinoma	Squamous Cell Carcinoma
Drug Sensitivity (*in vitro*)[a]		
Doxorubicin	99 nM	83 nM
Vincristine	14 nM	3 nM
Mitoxantrone	50 nM	275 nM
Growth Rate (*in vivo*)[b]	0.13 cm^3/day	0.07 cm^3/day
Characteristics	Highly vascularized, poorly metastatic, loosely cohesive, mucin expressing	Poorly vascularized, metastatic, cohesive, EGF receptor positive and VEGF producing

[a] Data refers to IC_{50} concentrations, concentrations of drug that effects 50% growth inhibition or toxicity, determined *in vitro* during a 3-day continuous exposure cytotoxicity assay. Cell viability was determined using the MTT assay as described in the Chapter 2, section 7.

[b] Growth rate was determined for control (untreated) tumours after the size exceeded 0.3 cm^3, a time point where significant increases in tumour size where measurable on a daily basis.

should be observed when treating tumours at a time point prior to formation of a measurable tumour and through use of repeated injections of the drug. To address this, mice were treated with single and multiple doses of free and DMPC/Chol liposomal mitoxantrone two days after tumour cell inoculation. The results of these studies have been summarized in Table 4.3. For simplicity the table reports results as the day of initiation of tumour growth, a parameter determined by taking a linear least-squares analysis of tumour volumes during the rapid growth phase and extrapolating to a tumour volume of zero. The effect of mitoxantrone treatment can then be determined as a delay in initiation of tumour growth. This analysis relies on the assumption that

Table 4.3

Treatment of non-established A431 and LS180 subcutaneous human xenografts in SCID/RAG-2 mice. Treatment is measured by estimations in the Delay in Tumour Growth[a] Initiation.

Dose Schedule	Treatment	Dose (mg/kg)	Day of Tumour Growth	Delay in Tumour Growth (Days)
LS180 tumours				
	Control	-	11	-
Day 2	Free Mitox	5	15	4
	DMPC/Chol Mitox	10	15	4
	Control	-	12	-
Days 2, 3, and 4	Free Mitox	1.5	15	3
	DMPC/Chol Mitox	3.5[b]	23	11
A431 tumours				
	Control	-	11	-
Day 2	Free Mitox	5	11	0
	DMPC/Chol Mitox	10	15	4
	Control	-	17	-
Days 2, 3, and 4	Free Mitox	1.5	21	4
	DMPC/Chol Mitox	3.5	27	10

[a] Determined as the day of initiation of tumour growth, a parameter determined by taking a linear least-squares analysis of tumour volumes during the rapid growth phase and extrapolating to a tumour volume of zero. It should be noted that treatment with mitoxantrone (free or liposomal) did not change the tumour growth rates, rather treatment effected a delay in the time when tumour growth initiated.

[b] One mouse died due to toxic effects.

treatment does not alter the growth rate of the tumour once it is established (i.e. tumour volume in excess of 0.05 cm^3 is attained).

Treatment of non-established tumours with a single injection of DMPC/Chol liposomal mitoxantrone at the maximum tolerated dose did not produce significant delays in tumour growth for the A431 and LS180 tumours. Following a single dose of free mitoxantrone, better therapeutic response was observed for mice bearing LS180 tumours, where delays in tumour

growth of 4 days were observed versus no delay in the A431 tumours. Although a number of different doses schedules were studied, including injections on day 2, 4 and 6 as well as day 2, 6 and 10, optimal therapy was observed for the day 2, 3, and 4 injection schedule reported in Table 4.3. Using this dose schedule, delays in A431 tumour growth of 4 and 11 days were obtained when mice were treated with free and DMPC/Chol liposomal mitoxantrone, respectively. Delays in LS180 tumour growth of 3 and 11 days were obtained when mice were treated (day 2, 3 and 4) with free and DMPC/Chol liposomal mitoxantrone, respectively. In all studies completed, the DMPC/Chol liposomal mitoxantrone formulation was more active than free drug. A comparison of the DMPC/Chol and DSPC/Chol mitoxantrone formulation was made using the more sensitive LS180 tumour model and these results have been summarized in Table 4.4. These data support the conclusion that, regardless of dosing schedule or LS180 tumour burden, the DMPC/Chol formulation of mitoxantrone is therapeutically more active than the DSPC/Chol formulation.

4.2.4 Drug accumulation in the region of tumour cell inoculation

The studies leading to the results summarized in Tables 4.3 and 4.4 raise an important question: Is a liposome-mediated increase in drug delivery achieved at the site of tumour cell inoculation (i.e. prior to significant tumour growth)? This is a relevant question considering that the primary rationale used in the development of liposomal drug formulations is based on observations that demonstrate liposome-mediated increases in drug delivery to established tumours (see Figure 1). This observation has been attributed to the presence of blood vessels that are hyper-permeable to macromolecules in the plasma compartment and it is unlikely that such a vascular structure exists at a time point prior to significant tumour growth. In order to address this question, mitoxantrone delivery to the site of tumour cell inoculation was measured as described in Chapter 2, section 12.

Table 4.4

Treatment of non-established LS180 subcutaneous human xenografts in SCID/RAG-2 mice. Treatment is measured by estimations in the Delay in Tumour Growth[a] Initiation.

	Treatment[b]	Dose (mg/kg)	Day of Tumour Growth	Delay in Tumour Growth (Days)
LS180 tumours	Control	-	12	-
	Free Mitox	1.5	14	2
	DMPC/Chol Mitox	2.5	19	7
	DSPC/Chol Mitox	2.5	14	2

[a] Determined as the day of initiation of tumour growth, a parameter determined by taking a linear least-squares analysis of tumour volumes during the rapid growth phase and extrapolating to a tumour volume of zero. It should be noted that treatment with mitoxantrone (free or liposomal) did not change the tumour growth rates, rather treatment effected a delay in the time when tumour growth initiated.

[b] Mitoxantrone was administered i.v. on days 2, 6 and 10 after tumour cell inoculation

Using a single time point (24 hours after drug administration), drug levels were measured in an area that included and surrounded the site of tumour cell inoculation. To confirm the presence of tumour cells in the site, mice were inoculated with radiolabeled LS180 cells and two days later the injection site was removed. Up to 75% of the injected radioactivity at the injection site could be recovered using this approach. It is recognized that this radioactivity can not be used as an indicator of cell number.

Figure 4.4 shows the amount of mitoxantrone recovered at the site of tumour cell inoculation in comparison to drug levels measured (at the same time point) in established tumours. Although one set of results is obtained from tissue consisting primarily of tumour cells and associated host cells while the other consists of skin and muscle tissue, it does highlight two important points.

Figure 4.4

**Drug accumulation at the site of tumour cell inoculation following *i.v.* administration of
free mitoxantrone or mitoxantrone encapsulated in DMPC/Chol or DSPC/Chol liposomes**

SCID/RAG-2 mice were injected bilaterally with 1 x 10^6 LS180 cells. Mice with established
tumours [tumours with a volume > 0.05 cm^3, (shaded bars)] or non-established tumours [mice
treated 48 hours after tumour cell inoculation, (open bars)] were injected *i.v.* with a 10 mg/kg
drug dose of DSPC/Chol mitoxantrone, DMPC/Chol mitoxantrone, and free mitoxantrone. [^{14}C]
labeled mitoxantrone was used as a tracer. 24 hours after treatment, established tumours were
harvested and for non-established tumours, a 1.5 cm x 1.5 cm area surrounding the tumour cell
injection site was harvested. Tissue was processed as described in Chapter 2. Data shown is the
average of at least 6 tumours ± the standard error. For comparison drug accumulation in
established tumours is provided and these data were obtained from the data set used to generate
Figure 1.
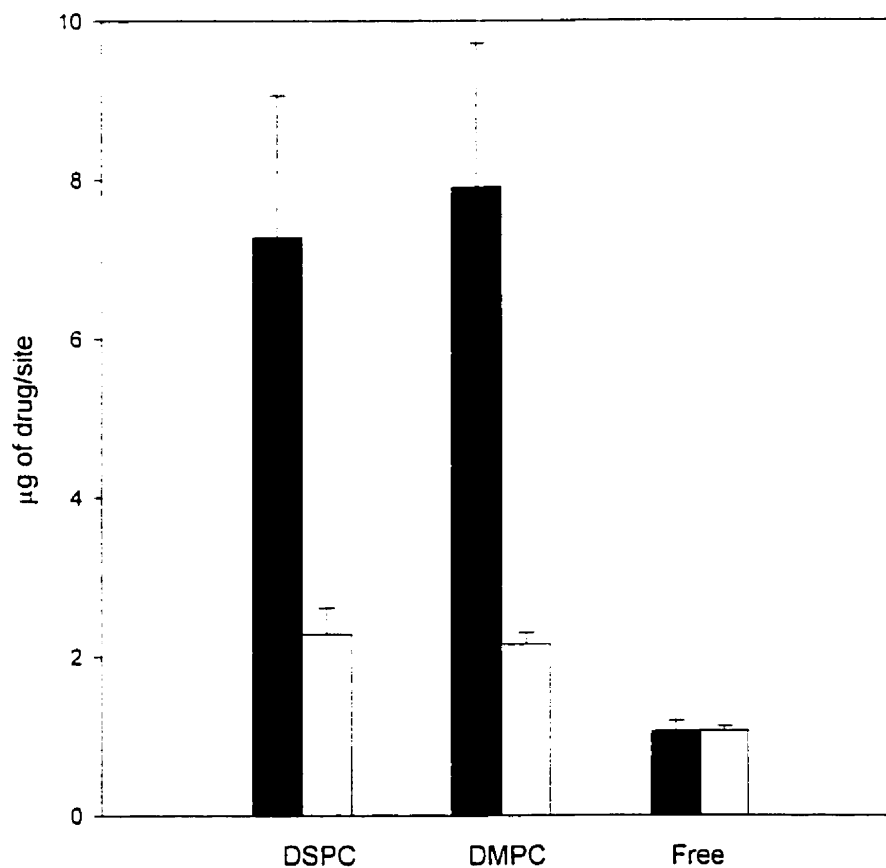
First, following *i.v.* administration of the liposomal mitoxantrone formulations the level of drug obtained in established LS180 tumours is 3-to 4-fold greater than that observed at the site of tumour cell inoculation. This difference in delivery is not observed following administration of free drug. Second, there is a 6-to7-fold increase in mitoxantrone delivered to established tumours when administering either liposomal formulation compared to free drug, however this difference decreases to less than 2-fold if the injection site is evaluated 2 days following tumour cell injection. Since it is established that liposome accumulation in muscle tissue is typically undetectable, it can be suggested that drug delivery to the site of tumour cell inoculation is a consequence of liposome accumulation in the skin (Hwang et al., 1987; Gabizon et al., 1990; Yuan *et al.*, 1994).

4.3 Discussion

A central hypothesis that is guiding the development of lipid-based anti-cancer drug delivery systems in this thesis is that drug release is the most important attribute controlling the therapeutic benefits linked to use of liposomal carriers. Drug release is, of course, an ill-defined term that must take into account the rate at which a drug leaves the liposome. Depending on the drug encapsulated, slow drug release may foster decreases in drug toxicity (Mayer *et al.*, 1989) and/or increases in therapeutic activity (Boman *et al.*, 1994). Slow drug release has, however, also been linked to reduced drug biological availability and an associated decrease in anti-tumour activity (Mayer *et al.*, 1989). Using mitoxantrone as an example, these studies have demonstrated that a slow drug release rate can effect a significant reduction in anti-tumour activity compared to faster-releasing carriers designed to exhibit comparable liposomal plasma elimination rates (Lim *et al.*, 1997). This conclusion was reached by comparing the anti-tumour

activity of mitoxantrone encapsulated in DSPC/Chol and DMPC/Chol liposomes following *i.v.* administration to mice bearing tumours residing primarily in the liver and spleen.

The studies summarized in this chapter were initiated because of concerns that this conclusion was only applicable to liver localized disease, a site where significant and rapid accumulation of liposomes is observed following *i.v.* administration. In order to address this concern, the therapeutic activity of DSPC/Chol and DMPC/Chol liposomal mitoxantrone was measured using two human xenograft models grown as ectopic (s.c.) tumours. The results are considered by focusing this discussion on three important points, all critical if the central hypothesis is to be sustained, including (1) the role of liposome delivery and liposome tumour/host cell interactions, (2) differences in drug targeting efficiencies between free and liposomal drug, and (3) the importance of considering capillary endothelium permeability to circulating macromolecules as well as capillary density within a tumour.

For the anti-cancer drug doxorubicin, where benefits attributed to liposome delivery have been correlated to reductions in cardiotoxicity, reductions in the rate of doxorubicin release have been directly associated with reduced drug accumulation in cardiac tissue. Hence doxorubicin encapsulated in DSPC/Chol liposomes is less toxic than doxorubicin encapsulated in egg PC/Chol liposomes or DMPC/Chol liposomes (Mayer *et al.*, 1994). Interestingly, DMPC/Chol liposomal doxorubicin, which releases 90% of its entrapped drug at a constant rate during the first 24 hours following *i.v.* injection, is approximately 3 times more toxic than free doxorubicin and more than 16 times more toxic than DSPC/Chol liposomal doxorubicin, which releases less than 10% of its entrapped drug *in vivo* in the same period of time (Mayer *et al.*, 1994). The rates of drug release of mitoxantrone are comparable to doxorubicin for these two liposomal lipid compositions. It is noteworthy that the maximum tolerated dose (MTD) of the DSPC/Chol and

DMPC/Chol mitoxantrone are comparable. In BDF1 mice, the MTD of these formulations when given as a single *i.v.* injection was 20 mg/kg mitoxantrone (200 mg/kg lipid). In this study, which used SCID/RAG-2 mice, the MTD of these formulations (single *i.v.* dose) was 10 mg/kg mitoxantrone (100 mg/kg lipid). In contrast to doxorubicin formulated in DSPC/Chol and DMPC/Chol liposomes, both liposomal formulations of mitoxantrone were about half as toxic as free drug. This is an important point considering that the data shown in Figure 4.1 were collected following administration of free and liposomal mitoxantrone at 10 mg/kg. The free drug data were, therefore, obtained at a drug dose that would be toxic within a 30-day time period and the resulting AUC_D values are presumably an overestimate relative to the MTD of 5 mg/kg used for therapeutic studies.

The 3-fold increase in drug exposure achieved using liposomal formulations of mitoxantrone (Table 4.2) resulted in improvements in anti-tumour effects (see Figure 4.3). However, the results presented in this chapter do not support the notion that the greatest therapeutic activity will be obtained using liposome formulations that facilitate the greatest increase in tumour drug AUC. The AUC_D values obtained following administration of DMPC/Chol mitoxantrone were 0.6 and 0.75 of the values obtained for DSPC/Chol for the A431 and LS180 tumours, respectively. The DMPC/Chol liposomal mitoxantrone formulation was therapeutically better than the DSPC/Chol formulation when treating LS180 tumours (Figure 4.3A). Treatment of A431 tumours suggested that the DMPC/Chol was as active as the DSPC/Chol formulation (Figure 4.3B).

Drug AUC values in solid tumours are dependent on the dose of lipid, the liposome plasma elimination rate as well as the drug retention characteristics of the liposome. The latter is illustrated by the data shown in Figure 4.1, where it is demonstrated that comparable liposomal lipid accumulation does not result in comparable drug uptake levels. In this example, reduction in

mitoxantrone uptake is partially a consequence of drug release from the DMPC/Chol liposomes. This, however, is a simplistic analysis that does not account for the accumulation of drug released from liposomes in the plasma compartment or from other tissues that are accumulating and metabolizing liposomes. It has been proposed, for example, that the liver is capable of acting as a drug reservoir where macrophage processing of drug loaded liposomes can result in drug release back into the circulation (Storm *et al.*, 1988). Indications of free (released) drug accumulation in tumours following *i.v.* administration of a liposomal drug have been based on comparisons between the estimated drug-to-lipid ratio in the plasma compartment versus the tumour. As shown in Figure 4.2, the ratio of $tumour_{drug-to-lipid\ ratio}/plasma_{drug-to-lipid\ ratio}$ at the 48 hour time point following administration of the DSPC/Chol mitoxantrone is approximately 0.92 for both tumours. A similar analysis for the DMPC/Chol mitoxantrone formulation results in a ratio of 2.5 for A431 tumours and 3.8 for LS180 tumours. A ratio of greater than 1 suggests that more drug is present in the tissue than would be predicted on the basis of liposome accumulation from the plasma. The higher ratios observed in tumours following administration of DMPC/Chol mitoxantrone are most likely a consequence of released drug accumulation. This can be suggested on the basis of the targeting efficiency (T_e) parameter, a value that is determined by dividing the AUC_D in the tumour by the AUC_D in the plasma compartment (see Table 4.1). The T_e value for free mitoxantrone is at least 8-fold greater than that measured for the liposomal formulations. This is a consequence of differences in size between free drug and the liposomal drug. The free drug is small and readily distributes following *i.v.* administration, hence, the T_e for free drug is large. Since drug is released from DMPC/Chol liposomes while in the plasma compartment it is reasonable to assume that this drug could be efficiently taken into the tumour.

It was demonstrated in this chapter that the rate and extent of liposome accumulation in tumours will also be dependent on the type of tumour and this will likely be a function of the tumour-

specific attributes such as capillary density and structure. In the LS180 tumour model, liposome extravasation occurs rapidly, reaching the C_{max} within four hours after administration (Figure 4.2A). In contrast, in the A431 tumour model the C_{max} is achieved 48 hours after i.v. administration. Almost twice the amount of the liposomal lipid accumulates within the LS180 tumour (AUC values of 10167.32 and 9925.82 µg lipid/g of tumour/hour for the DSPC/Chol and DMPC/Chol formulations, respectively) in comparison to the A431 tumours (AUC values of 5728.22 and 5149.66 µg lipid/g of tumour/hour for the DSPC/Chol and DMPC/Chol formulations, respectively). Gross inspection of the tumours suggests that the LS180 tumour is better vascularized than A431 tumours (Table 4.2) and this may account for differences in rate of accumulation. It can be suggested that liposome extravasation may be dependent on tumour microvascular density as well as capillary endothelium permeability. The increased microvascular density would lead to greater delivery of liposomes to the site of tumour growth. In addition, the extravasation of liposomes is dependent on the permeability of the blood vessel. An increase in the permeability (due to secreted factors such as VEGF) could also result in increased liposome accumulation.

A discussion relating microvascular density and endothelium permeability invites consideration of whether liposome extravasation is a relevant parameter when studying tumours prior to establishment of a significant tumour burden. For the LS180 and A431 tumours studied in this chapter, measurable tumours were obtained 12 to 15 days after tumour cell inoculation. It would be unexpected to see significant vascularization of the tumours shortly after cell inoculation, although no direct measurement of tumour vascularization was made in these studies. It can be suggested from the data shown in Figure 4.4 that liposome extravasation was reduced when the tumour burden was small. This is an indirect measurement and it should be noted that the results in Figure 4.4 compare drug accumulation in a tumour that has been carefully dissected from the

animal to drug levels measured in a large area of tissue that includes the cell inoculation site (as confirmed by recovery of radiolabeled cells) as well as surrounding skin and underlying muscle. Clearly it is important to develop methodologies that can measure liposomal lipid and drug levels in areas where tumour growth is initiating, particularly when considering that most studies evaluating liposome extravasation use large tumours that may have the greatest microvascular density and the most permeable blood vessels. It is also worth noting, however, that extravasation of liposomes into the peritoneal cavity in the absence of disease has been reported and this extravasation process is thought to be across normal vascular endothelium (Bally et al., 1993).

A fundamental element of the central hypothesis is that drug encapsulated inside the liposome is not biologically available. Further, the liposome-encapsulated drug is not therapeutically active unless a feature promoting tumour cell delivery is incorporated. This may involve use of targeting ligands that are known to be internalized, for example the folate acid receptor (Lee and Low 1993; 1994; Wang et al., 1995). In addition, non-internalized targets have also been used in an effort to specifically deliver the drug to tumour and release drug in the vicinity of the tumour cells (Longman et al., 1995; Scherphof et al., 1997). Alternatively, the liposomes can be designed to non-specifically bind and fuse with cells following extravasation into a site of tumour growth. An elegant example of this approach, resulting in a lipid-based delivery system referred to as programmable fusogenic liposomes or PFVs, has recently been described (Holland et al., 1996). In the absence of cell delivery, cell fusion, and/or intracellular processing by phagocytic cells in the site of extravasation; however, the encapsulated drug must be released from the liposomes in order to maximize drug biological availability and therapeutic activity.

In conclusion, in order to fully maximize the benefits of using liposomal carriers, a balance between delivery and drug release must be achieved. It has been argued that the primary source of drug within the tumour is from liposomes that have extravasated into the site (Mayer *et al.*, 1994), an argument that links the rate and extent of liposome accumulation and the rate of drug release to therapeutic activity. However, the possibility that drug release from sites that are distinct from the tumour may contribute to the therapeutic activity can not be excluded. This is perhaps most important when the tumour burden is small and vascularization is low. The results suggest that a conventional (non-targeted, non-fusogenic) formulation of mitoxantrone prepared using DMPC/Chol liposomes is active in treatment of ectopic (s.c.) tumours as well as tumours progressing primarily in the liver and spleen (see Chapter 3). This activity is believed to be a consequence of the rate at which mitoxantrone is released from DMPC/Chol liposomes. The DMPC/Chol formulation of mitoxantrone is particularly well suited for treatment of tumours (or sites of tumour growth) where liposome accumulation is rapid. The next chapter will focus on the anti-tumour effects of DMPC/Chol mitoxantrone when used to treat cancer within the liver.

CHAPTER 5

ROLE OF KUPFFER CELLS AND LIPOSOME MEDIATED DRUG DELIVERY TO LIVER IN GOVERNING THE EFFICACY OF DMPC/CHOL LIPOSOMAL MITOXANTRONE USED TO TREAT LIVER LOCALIZED CANCER

5.1 Introduction

One of the primary reasons for developing a liposomal formulation of an anti-cancer drug is to increase drug exposure at a site of tumour growth. Evidence to support this reasoning has come from many studies documenting that the maximum drug concentration as well as the length of time tumour drug levels are maintained is increased when an anti-cancer drug is administered inside an appropriately designed liposomal carrier (Parr et al., 1997; Bally et al., 1994; Gabizon, 1992; Mayer et al., 1990). Using mice bearing murine or human s.c. tumours, as much as 10% of the injected liposomal drug can be measured in association with an established tumour (Parr et al., 1997). Similar results are shown in Chapter 4 when mitoxantrone levels were evaluated in human xenograft models following i.v. administration of a liposomal formulation of mitoxantrone. The administration of liposomal mitoxantrone resulted in tumour mitoxantrone areas under the curve (AUC_D) that were 4-to 5-fold greater then that observed following injection of free mitoxantrone. This improved delivery has been attributed to the presence of tumour-associated blood vessels that are hyperpermeable to circulating macromolecules (Yuan et al., 1995; Wu et al., 1993; Kohn et al., 1992).

Tumour drug levels are, however, low in comparison to those that can be obtained in the liver following parenteral administration of a liposomal anti-cancer drug. It was established over 20 years ago that liposomes have a tendency to localize in sites containing fenestrated blood vessels and high levels of associated tissue macrophages, such as the liver (Rahman et al., 1982; Hinkle et al., 1978; Caride, 1976). Investigators have shown that liver drug exposure, as measured by

AUC$_D$, can also be at least 5-fold greater than that which can be achieved with free drug (Zou et al., 1993a). Higher drug levels and increased exposure of the liver would imply that liposomal anti-cancer drugs should be well suited for use in the treatment of liver cancer. This has, however, been difficult to demonstrate.

Although there are exceptions (Asao et al., 1992; Gabizon et al., 1983), approaches to treat hepatocellular carcinoma that use liposome-based delivery systems have been clumsy. The methods range from a reliance on immune stimulation (Okuno et al., 1998; Asao et al., 1992), administration via the hepatic artery (Cay et al., 1997; Konno, et al., 1995), the use of liposomes designed to release contents after an external stimulus is provided (Zou et al., 1993b) or on the use of a model that is based on i.v. injection of M5076 cells, a cell line known to actively take up liposomes by phagocytosis (Yachi et al., 1996). There are many possible explanations for why liposomal anti-cancer drugs have not been more successful in treating liver cancer. This would include an inherent insensitivity or resistance to cytotoxic drugs in tumour cells that arise in or metastasize to the liver (Furuya et al., 1997). Alternatively the blood vessels that arise in liver localized disease in response to angiogenesis signals may be less abundant (Toyoda, et al. 1997) and may exhibit altered vascular permeability to circulating macromolecules that is dependent on the microenvironment where the cancer grows (Fukumura et al., 1997). The latter point emphasizes that in the case of anti-cancer drug delivery to the liver, regional and cellular distribution of the drug may be critical if therapeutic activity is to be obtained.

In Chapter 3, a therapeutically active liposomal formulation of mitoxantrone for the treatment of liver localized disease is described. The murine model used in this study was based on i.v. administration of L1210 cells into immune competent BDF1 mice (F1 DBA2/C57-BL6 crosses). The L1210 cells are non-phagocytic and are sensitive to cytotoxic drugs. They have been and

continue to be used for assessing the *in vivo* activity of anti-cancer drugs (Canti *et al.*, 1998;

Perchellet *et al.*, 1997; Gabr *et al.*, 1997; Noda *et al.*, 1997). The results from studies reported

here suggest that the therapeutic activity of liposomal mitoxantrone is unequaled by other

liposomal anti-cancer formulations prepared using comparable methods. In particular, it is

demonstrated that liposomal formulations of doxorubicin and vincristine are only marginally

active in the L1210 *i.v.* tumour model, a model that can be effectively cured when treated with

DMPC/Chol liposomal mitoxantrone (See Chapter 3). Such results provide an opportunity to

address questions about what factors are important when considering development of a liposomal

anti-cancer drug for use in the treatment of liver cancer. More specifically, this chapter

addressed how liposome delivery to the liver may effect therapy in the *i.v.* L1210 tumour model.

Two strategies designed to decrease liposomal delivery to the liver were employed. The first

uses polyethylene glycol (PEG)-modified lipids to decrease serum protein binding (Du *et al.*,

1997; Yuda *et al.*, 1996) and liposome-cell interactions (Du et al, 1997; Yuda *et al.*, 1996). The

second method employs the use of agents (such as clodronate or doxorubicin) known to eliminate

or impair Kupffer cells (Daemen *et al.*, 1995, Parr *et al.*, 1993; Bally *et al.*, 1990; Van Rooijen

and Classen, 1989). The results suggest that the therapeutic activity of liposomal mitoxantrone

used to treat liver localized cancer is not dependent on the presence of Kupffer cells. However,

strategies that non-specifically inhibit liposome-cell interactions (e.g. use of liposomes with

PEG-modified lipids) significantly inhibit the therapeutic benefits achieve with DMPC/Chol

liposomal mitoxantrone.

5.2 Results

5.2.1 Therapeutic activity of free and liposomal anti-cancer drugs given i.v. to mice bearing the L1210 i.v. tumour model

The L1210 i.v. tumour model was used to evaluate the efficacy of mitoxantrone, vincristine and doxorubicin administered i.v in free form or encapsulated in liposomes (Table 5.1). In chapter 3, it was demonstrated that following i.v. injection of 10^4 L1210 cells, tumour development is most evident in the liver and the spleen. The results in Table 5.1 were obtained following a single injection at a drug dose that was either the maximum tolerated dose (free and DSPC/Chol vincristine; free and DSPC/Chol mitoxantrone, EPC/Chol doxorubicin) or at the lowest drug dose required to give maximum therapeutic effect (free doxorubicin and DSPC/Chol doxorubicin and DMPC/Chol mitoxantrone). Untreated and empty liposome (EPC/Chol or DSPC/Chol liposomes with encapsulated citrate buffer and pH 7.5 HBS outside and administered at a lipid dose of 150 mg/kg total lipid) treated animals were terminated as a result of significant tumour related disease within 10 days. The mean of the median survival time (9.8 days) was determined by averaging the median survival time for studies completed in DBA2 mice (vincristine and doxorubicin treated animals, median survival time of 9.5 days) and those completed in BDF1 mice (mitoxantrone treated animals, median survival time of 10 days).

The significant point that can be made from the data in Table 5.1 is that the therapeutic activity of DMPC/Chol liposomal mitoxantrone (100% survival on day 60) is unequaled by the other drugs even when given in liposomal form. This result must, however, be considered in light of four other observations. First, 24 hour cytotoxicity assays measuring the cytotoxic/cytostatic activity of the free drugs (Table 5.2) suggest that L1210 cells are most sensitive to free mitoxantrone. This is consistent with the in vivo results shown in Table 5.1, where free

Table 5.1

Therapeutic activity of free and liposomal formulations of doxorubicin, vincristine and mitoxantrone following a single *i.v.* injection in mice bearing the L1210 *i.v.* tumours.

Treatment	Drug Dose (mg/kg)	Median Survival Time (days)	%ILS[e]	% Survival
Control (saline)		9.8[a]		N/A
Control (EPC/Chol)		11.5[b]	17	0
Control (DSPC/Chol)		10.5[b]	7	0
Free Mitoxantrone	10	17.0[c]	73	0
DSPC/Chol Mitoxantrone	20	25.0[c]	155	0
DMPC/Chol Mitoxantrone	10	>60[c]	ND[f]	100
Free Doxorubicin	10	13.5[b]	38	0
EPC/Chol Doxorubicin	30	18[b,d]	84	0
DSPC/Chol Doxorubicin	30	13[b,d]	33	0
Free Vincristine	2	10[b,d]	2	0
DSPC/Chol Vincristine	3	13.5[b,d]	38	0

[a] Determined in DBA2 and BDF1 mice and the value is based on the mean of the median survival time (days) in these two strains.
[b] Determined in DBA2 mice
[c] Determined in BDF1 mice
[d] Indicates median survival times from one experiment using an n of at least 5 animals
[e] Percentage ILS (Increase in Life Span) Values were determined from mean survival times of treated and untreated control groups. If greater than 50% of the animals survived more than 60 days the ILS% was not determined
[f] Can not be determined because more than half the animals survived past 60 days

mitoxantrone effected a 76% increase in life span (%ILS) compared to 38% ILS and 2% ILS obtained following treatment with doxorubicin and vincristine, respectively. Second, liposomal vincristine and liposomal doxorubicin are very effective when used *i.v.* to treat animals with *i.p.* L1210 tumours (Mayer *et al.*, 1993; Mayer *et al.*, 1989). Treating animals carrying *i.p.* L1210 tumours with DSPC/Chol liposomal vincristine, for example, can result in greater than 50% long

Table 5.2

IC_{50} of doxorubicin, vincristine, and mitoxantrone when incubated with L1210 cells for 24 hours.[a]

Drug	IC_{50} (nM)[a]
Doxorubicin	820
Vincristine	70
Mitoxantrone	55

[a]IC_{50} is defined, based on the MTT assay described in the Chapter 2, as the concentration of drug where cell growth and/or viability is 50% of that observed in control (drug) free cultures.

term (>60 day) survival. Third, DSPC/Chol liposomal mitoxantrone is less active than the DMPC/Chol formulation, a result that has been attributed to differences in the drug release rates from these two liposomes (See Chapter 3). It is important to note that the EPC/Chol doxorubicin formulation (Bally et al., 1990; Harasym et al., 1997) and the DSPC/Chol liposomal vincristine preparation (Mayer et al., 1993) have also been characterized as formulations that support release of entrapped contents following i.v. administration. Fourth, the most significant difference between the liposomal formulations of vincristine, doxorubicin and mitoxantrone is that the vincristine and doxorubicin formulations induce hepatic MPS blockade (Daeman et al., 1995; Bally et al., 1990). It is for this reason that this chapter will evaluate the influence of hepatic MPS avoidance and elimination strategies on the activity of the DMPC/Chol mitoxantrone formulation.

5.2.2 Reducing DMPC/Chol liposomal mitoxantrone delivery to the liver

Two strategies were used to effect reductions in the delivery of DMPC/Chol liposomal mitoxantrone to the liver. One involved incorporation of PEG-modified lipids into the

DMPC/Chol formulation (hepatic MPS avoidance strategy) and the second involved administering a pre-dose of DSPC/Chol doxorubicin (2 mg/kg drug) 24 hours prior to administration of DMPC/Chol liposomal mitoxantrone (hepatic MPS elimination strategy). As illustrated in Figure 5.1A, 5.1B and 5.2A, it was anticipated that both strategies would cause a decrease in the rate of liposomal lipid (Fig. 5.1A) and mitoxantrone (Fig. 5.1B) elimination from the plasma compartment and an associated decrease in drug accumulation in the liver (Figure 5.2A). For example, 24 hours after i.v. administration of DMPC/Chol mitoxantrone, the level of mitoxantrone measured (using a [^{14}C]-labeled drug as a marker) in the liver was 27 µg/g of liver. When mitoxantrone was administered in DMPC/Chol liposomes with 5 mol % PEG$_{2000}$-modified lipids the drug levels in the liver at 24 hours were reduced to 12.2 µg/g of liver. When the mice were given the pre-injection of DSPC/Chol liposomal doxorubicin (2 mg/kg drug), mitoxantrone levels in the liver 24 hours after administration of DMPC/Chol mitoxantrone were below 8 µg/g of liver. The greater than two-fold reduction in liver mitoxantrone levels measured at 24 hours was associated with approximately a 3-fold and 5-fold increase in plasma concentrations of drug and liposomal lipid, respectively. The plasma elimination rates over the first 24 hours after administration were comparable for the PEG-containing liposomes and the DMPC/Chol mitoxantrone formulations given to mice pre-injected with DSPC/Chol liposomal doxorubicin.

5.2.3 Influence of reducing liver mitoxantrone levels on the therapeutic activity of

DMPC/Chol mitoxantrone

Figure 5.2B demonstrates how the two strategies for reducing DMPC/Chol mitoxantrone delivery to the liver affected its therapeutic activity when used to treat the L1210 i.v. tumour model. The results obtained were surprising. Incorporation of PEG$_{2000}$-modified lipids into the DMPC/Chol mitoxantrone resulted in a significant reduction in therapeutic activity. In dramatic contrast, pre-

Figure 5.1

Plasma Elimination of DMPC/Chol Mitoxantrone Liposomes and DMPC/Chol/PEG Mitoxantrone Liposomes.

Mice were pre-treated with 2 mg/kg drug dose of DSPC/Chol Doxorubicin in order to induce MPS blockade. 24 hours later, MPS Blockade mice were injected with DMPC/Chol mitoxantrone (●). Non-MPS Blockade mice were treated with 10 mg/kg dose of DMPC/Chol mitoxantrone (■) or DMPC/Chol/PEG mitoxantrone (▼). Blood was collected as described in Chapter 2. Panel A shows elimination of lipid from the plasma compartment over 24 hours. Panel B shows the elimination of drug from the plasma compartment over 24 hours. Points represent the average and the standard error of at least 8 mice. * signifies p<0.05.
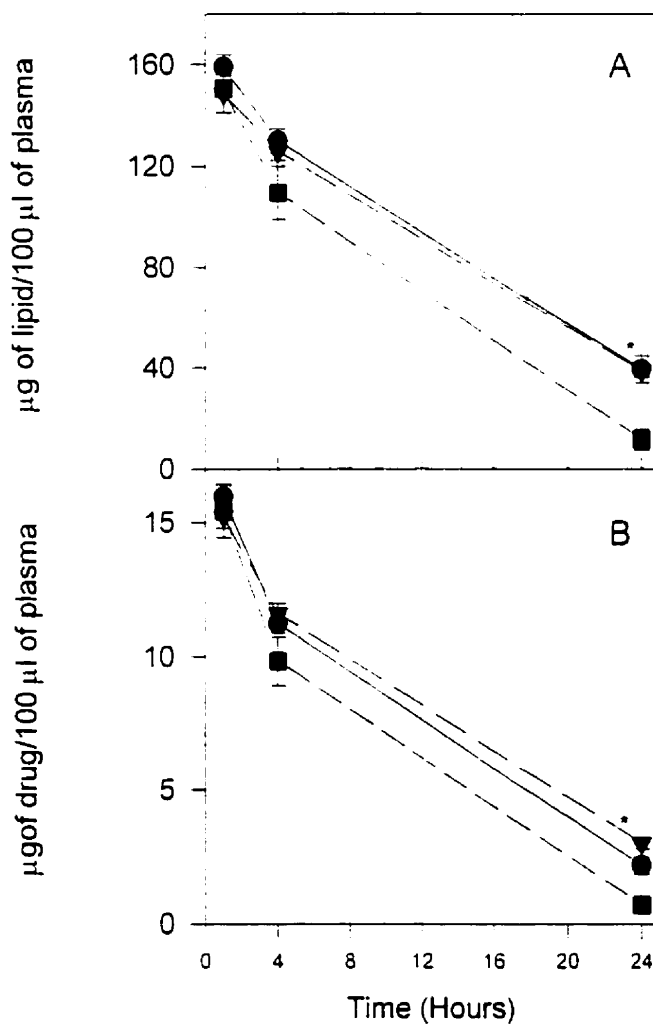
Figure 5.2

Drug accumulation in the liver versus therapeutic activity

In Panel A, drug delivery to the liver was assessed using ^{14}C-mitoxantrone as a tracer. CD1 mice were injected with a 10 mg/kg drug dose of DMPC/Chol mitoxantrone. MPS blockade treated mice were injected with a 2 mg/kg drug dose of DSPC/Chol Doxorubicin 24 hours prior. Livers were harvested and processed as described in Chapter 2. Bars represent the average and standard error collected from 8 mice. * symbolizes significant differences from the DMPC/Chol mitoxantrone group (p < 0.05). In Panel B, therapeutic activity was assessed. BDF1 mice were inoculated with 1 x 10^6 L1210 tumour cells. MPS blockade mice were treated two hours after tumour cell inoculation. 24 hours after tumour cell inoculation, mice were treated with a 10 mg/kg dose of DMPC/Chol mitoxantrone. Dashed line represents the survival time of untreated mice. ** indicates greater than 60 day survival.
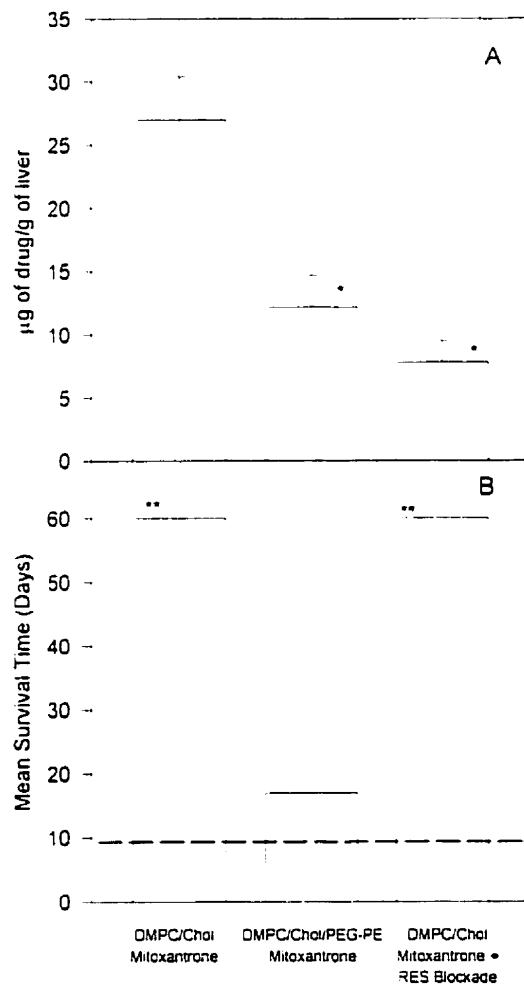
treatment with DSPC/Chol doxorubicin had no impact on the therapeutic activity of the DMPC/Chol mitoxantrone formulation.

Further support of these data is provided in Table 5.3. The rationale for these studies is based on the potential that the pre-dose of DSPC/Chol liposomal doxorubicin may have therapeutic activity. As indicated in Table 5.1, this formulation has minimal activity (< 20 % ILS) when used to treat the L1210 i.v. tumour model at doses of 30 mg/kg. The activity of this formulation, however, could be augmented by mitoxantrone. In order to address this issue, two other approaches to achieve hepatic MPS blockade were used, including a pre-dose of liposomal vincristine or liposomal clodronate. Although vincristine is also an anti-cancer agent, its mechanism of activity is distinct from doxorubicin. As noted in Table 5.1, liposomal vincristine is also not active when treating the L1210 i.v. tumour model. Clodronate is a bisphosphonate that has been developed for treatment of osteoporosis (Fleisch, 1993; Lepore et al., 1991) and is known to deplete macrophages, particularly well when given in liposomal form (Van Rooijen, and Claassen, 1988; Van Rooijen and Van Nieuwmegen, 1984). In addition, the influence of hepatic MPS blockade, achieved using the three different pre-treatment strategies, on the therapeutic activity of the PEG-containing DMPC/Chol mitoxantrone was assessed.

The results presented in Table 5.3 are unambiguous. First, hepatic MPS blockade achieved by pre-treating animals with liposomal doxorubicin, vincristine or clodronate had no impact on the median survival time of mice bearing the i.v. L1210 tumours. Second, the therapeutic activity of the DMPC/Chol/PEG mitoxantrone formulation was not affected by any of the pre-treatment strategies. Third, regardless of what agent was used to achieve hepatic MPS blockade, mice treated with DMPC/Chol mitoxantrone exhibited 100% long term (>60 day) survival.

Table 5.3

Influence of PEG-lipid incorporation and hepatic MPS blockade on the L1210 Anti-tumour Activity of DMPC/Chol Mitoxantrone

Pre-Treatment[a]	Treatment[b]	Median Survival Time	%ILS[c]	% Survival
None	Untreated	9.5		0
Dox Blockade		9	-	0
Vinc Blockade		11	16	0
Clodronate Blockade		9	-	0
None	DMPC/Chol Mito	> 60 days	N.D.[d]	100
Dox Blockade	(10 mg/kg)	> 60 days	N.D.	100
Vinc Blockade		> 60 days	N.D.	100
Clodronate Blockade		> 60 days	N.D.	100
None	DMPC/Chol/PEG Mito	17	79	
Dox Blockade	(10 mg/kg)	20	111	0
Vinc Blockade		15	58	0
Clodronate Blockade		18.5	94	0

[a] Pre-treatment was administered two hours after tumour cell inoculation
[b] Treatment dose was at drug dose of 10 mg/kg at drug to lipid ratio 0.1 (wt:wt)
[c] Percentage Increase in Life Span (ILS) values were determined from median survival times of treated and untreated control groups.
[d] Can not be determined because more than half the animals survived past 60 days

5.2.4 Influence of hepatic MPS avoidance and elimination strategies on mitoxantrone release

In the previous chapters, it was postulated that the therapeutic activity of liposomal mitoxantrone is dependent on the rate of mitoxantrone release from the liposomes following administration. Therefore, it was important to determine whether the hepatic MPS avoidance and elimination strategies affected drug release rates. As shown in Figure 5.3, there was a significantly higher drug-to-lipid ratio observed at 24 hours following injection of DMPC/Chol/PEG mitoxantrone in comparison to DMPC/Chol mitoxantrone, suggesting that the drug release is inhibited in

Figure 5.3

Drug release of mitoxantrone from DMPC/Chol liposomes and DMPC/Chol/PEG liposomes

Mice were pre-treated with 2 mg/kg drug dose of DSPC/Chol Doxorubicin in order to induce MPS blockade. 24 hours later, MPS Blockade mice were injected with DMPC/Chol mitoxantrone (●). Non-MPS Blockade mice were treated with 10 mg/kg dose of DMPC/Chol mitoxantrone (■) or DMPC/Chol/PEG mitoxantrone (▼). Blood was collected as described in Chapter 2, Section 9. Points represent the average and the standard error of at least 8 mice. * signifies $p < 0.05$.
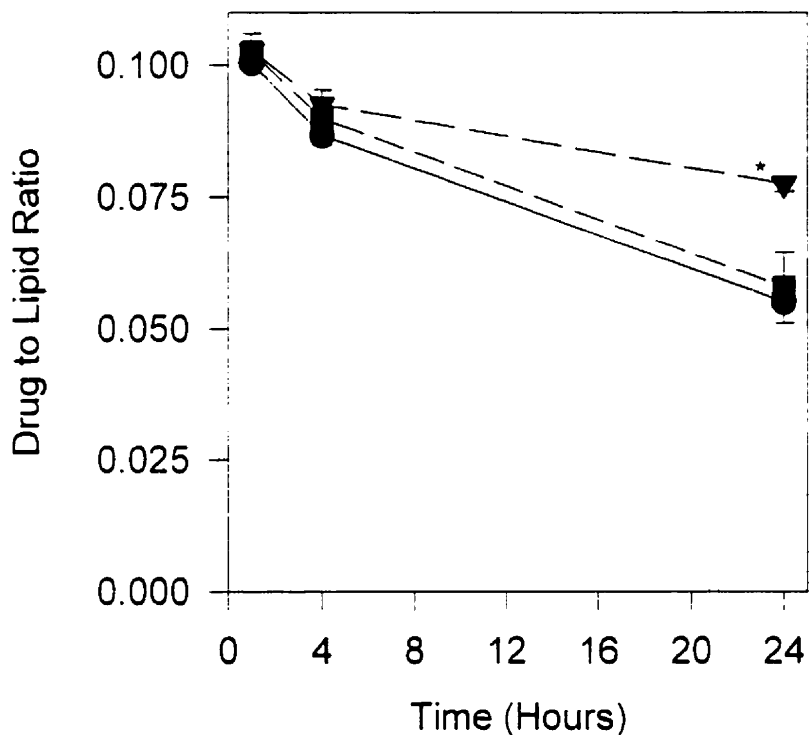
liposomes with the PEG-modified lipid. This was surprising considering results with vincristine suggest that drug release rates are increased when the liposomes used contain PEG-modified lipids (Webb *et al.*, 1998). However, in this case the decrease in protein adsoprtion to the surface of the liposome due to the addition of PEG-modified lipids may have a role in the increased retention of mitoxantrone. It is possible that reduced therapeutic activity is a consequence of reduced drug release from the DMPC/Chol/PEG mitoxantrone formulation. It is important, however, to note that mitoxantrone release from DMPC/Chol/PEG liposomes is faster than that observed for the DSPC/Chol formulation and its therapeutic activity is less than that observed for DSPC/Chol liposomes (Chang *et al.*, 1997). The study reported by Chang *et al.* (1997) also provided data suggesting that the therapeutic activity of a PEG containing formulation was less than that observed for liposomes prepared in the absence of PEG-lipids. As expected, strategies relying on the use of hepatic MPS blockade had no effect on drug release from the DMPC/Chol liposomes (Fig. 5.3).

5.2.5 Influence of hepatic MPS avoidance and elimination strategies on liposome distribution in the liver and on Kupffer cell depletion

Induction of hepatic MPS blockade was achieved by injecting a low dose (2 mg/kg drug) of DSPC/Chol doxorubicin and by the more established technique involving use of liposomal clodronate. Confirmation that these strategies caused depletion of Kupffer cells is provided in the micrographs shown in Figure 5.4. These micrographs were obtained by staining liver cryosections with an antibody (F4/80) that labels mature macrophages (Lee *et al.*, 1985; Hume *et al.*, 1984; Austyn and Gordon, 1981). Sections derived from livers of untreated mice (Panel A) contain many F4/80 positive cells, cells that are presumed to be liver Kupffer cells. The population of labeled cells is reduced significantly when the liver sections are obtained from

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mice that had been injected 24 hours earlier with liposomal doxorubicin (Panel B) or liposomal

clodronate (Panel C). The reduction in F4/80 positive cells was most significant in the

clodronate treated animals.

The data presented in Figure 5.4 is consistent with other reports (Van Rooijen *et al.*, 1990)

however it has not been established how macrophage depletion or macrophage avoidance (PEG-

liposomes) impacts the distribution of liposomal mitoxantrone in the liver. In order to obtain this

information two approaches were taken. First, the liposomal mitoxantrone formulations, either

DMPC/Chol or DMPC/Chol/PEG, were prepared with the fluorescent lipid 1,1'-dioctadecyl-

3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). It has been demonstrated that this

fluorescent lipid does not exchange with neighboring membranes (Claassen, 1992; Honig and

Hume, 1986) and thus it is considered as a useful marker for liposomes *in vivo*. Twenty-four

hours following *i.v.* administration of DiI labeled DMPC/Chol mitoxantrone and

DMPC/Chol/PEG mitoxantrone (10 mg/kg drug dose), livers were removed, crysections were

prepared and the sections were viewed using confocal microscopy. As seen in Figure 5.5,

incorporation of PEG modified lipids caused a reduction in liposome accumulation in the liver

(compare panel A to panel B). Changes in the distribution of DiI labeled DMPC/Chol

mitoxantrone in the liver are more dramatic in livers isolated from mice pre-treated with

liposomal formulations of doxorubicin, vincristine or clodronate (Figure 6). Hepatic MPS

blockade caused a significant reduction in the amount of fluorescently labeled DMPC/Chol

mitoxantrone delivered to the liver (compare Panel A to Panels B-D). In addition to the decrease

in liposome accumulation, the liposome distribution pattern is changed considerably and the

distribution pattern is different when comparing liposomal doxorubicin (Panel B) and vincristine

(Panel C) induced hepatic MPS blockade to that observed with liposomal clodronate (Panel D).

Figure 5.4

F4/80 staining of Kupffer cells in the liver

Livers from CD1 mice were pre-treated with either DSPC/Chol Doxorubicin or EPC/Chol Clodronate. Control liver was left untreated. 24 hours later, livers were extracted and embedded in O.C.T. media. As outlined in the Chapter 2, section 17, livers were then stained with the F4/80 antibody. Magnification is 40x for all panels. Arrows indicate stained Kupffer cells.
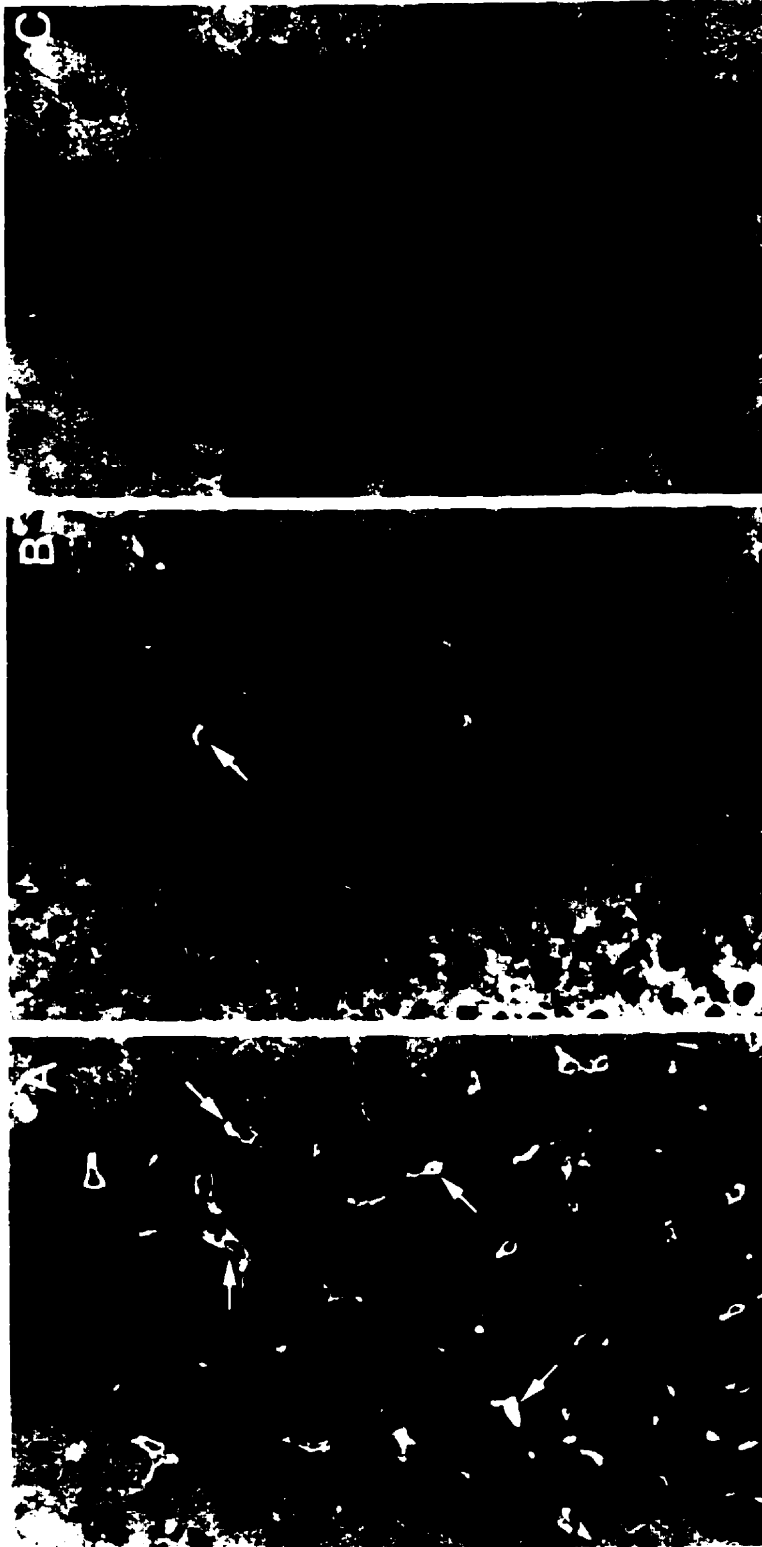
Figure 5.5

Confocal imaging of biodistribution of DiI labeled DMPC/Chol mitoxantrone liposomes and DMPC/Chol/PEG mitoxantrone liposomes in the liver

Mice were injected with a 10 mg/kg drug dose of DiI labeled DMPC/Chol mitoxantrone or DMPC/Chol/PEG mitoxantrone. 24 hours later, mice were terminated via CO_2 asphyxiation, and livers harvested. Livers were processed as outlined in the Chapter 2, section 19 and imaged using a BioRad 6000Z Confocal Imaging System. Panel A represents images from mice injected with DMPC/Chol/DiI mitoxantrone and Panel B represents images from mice injected with DMPC/Chol/PEG/DiI mitoxantrone. Magnification is 10x for all panels.
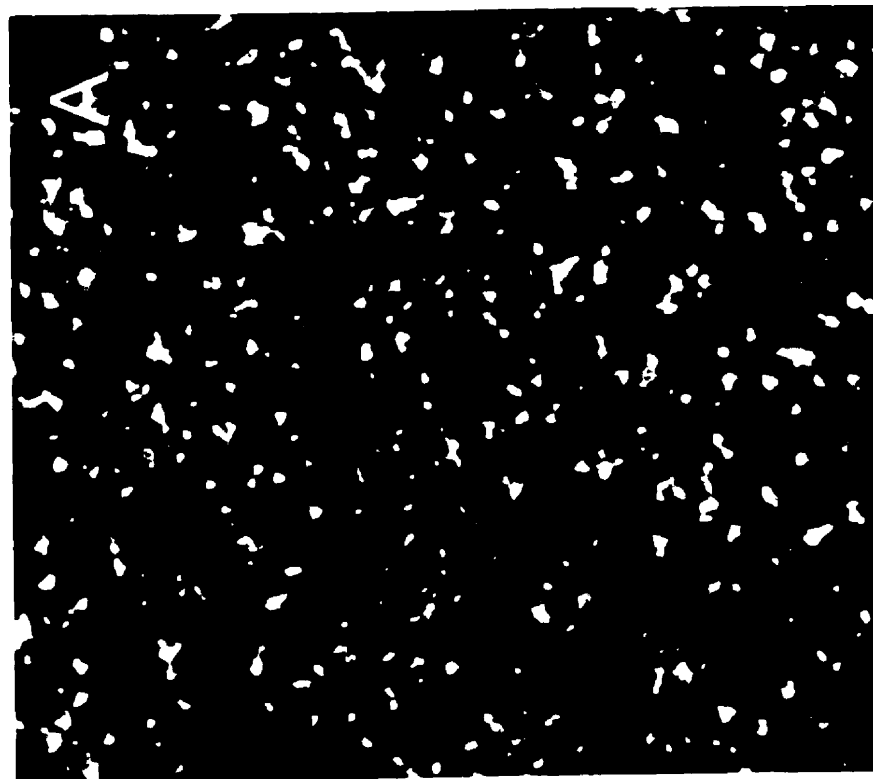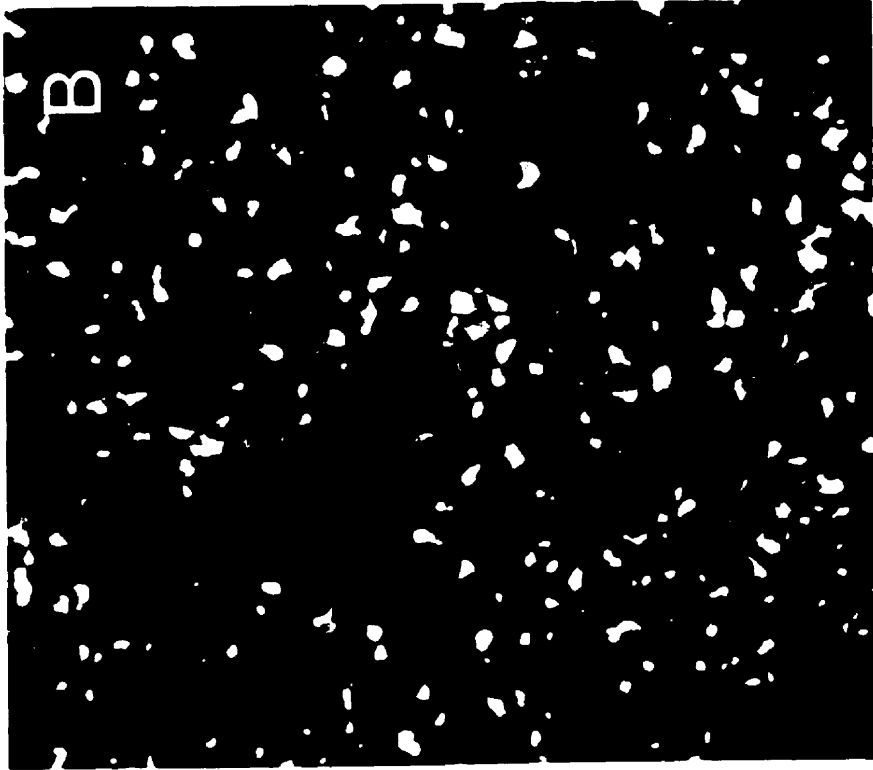
Figure 5.6

Confocal imaging of biodistribution of DiI labeled DMPC/Chol mitoxantrone liposomes with and without MPS blockade.

MPS blockaded mice were pre-treated with a 2 mg/kg drug dose of DSPC/Chol doxorubicin or 1 mg/kg vincristine or EPC/Clodronate. Non-MPS blockaded mice were left untreated. 24 hours later, mice were injected with a 10 mg/kg drug dose of DiI labeled DMPC/Chol mitoxantrone. 24 hours after injection, mice were terminated via CO_2 asphyxiation, and livers harvested. Livers were processed as outlined in Chapter 2, Section 19and imaged using a BioRad 6000Z Confocal Imaging System at 10x. Panel A represents images from non MPS-blockaded mice. Panel B are images from mice with MPS Blockade using DSPC/Chol doxorubicin at a 2 mg/kg drug dose. Panel C are images from mice with MPS Blockade using DSPC/Chol vincristine at a 1 mg/kg drug dose, and Panel D are from mice with MPS Blockade using EPC/Chol clodronate. Magnification is 10x for all panels.
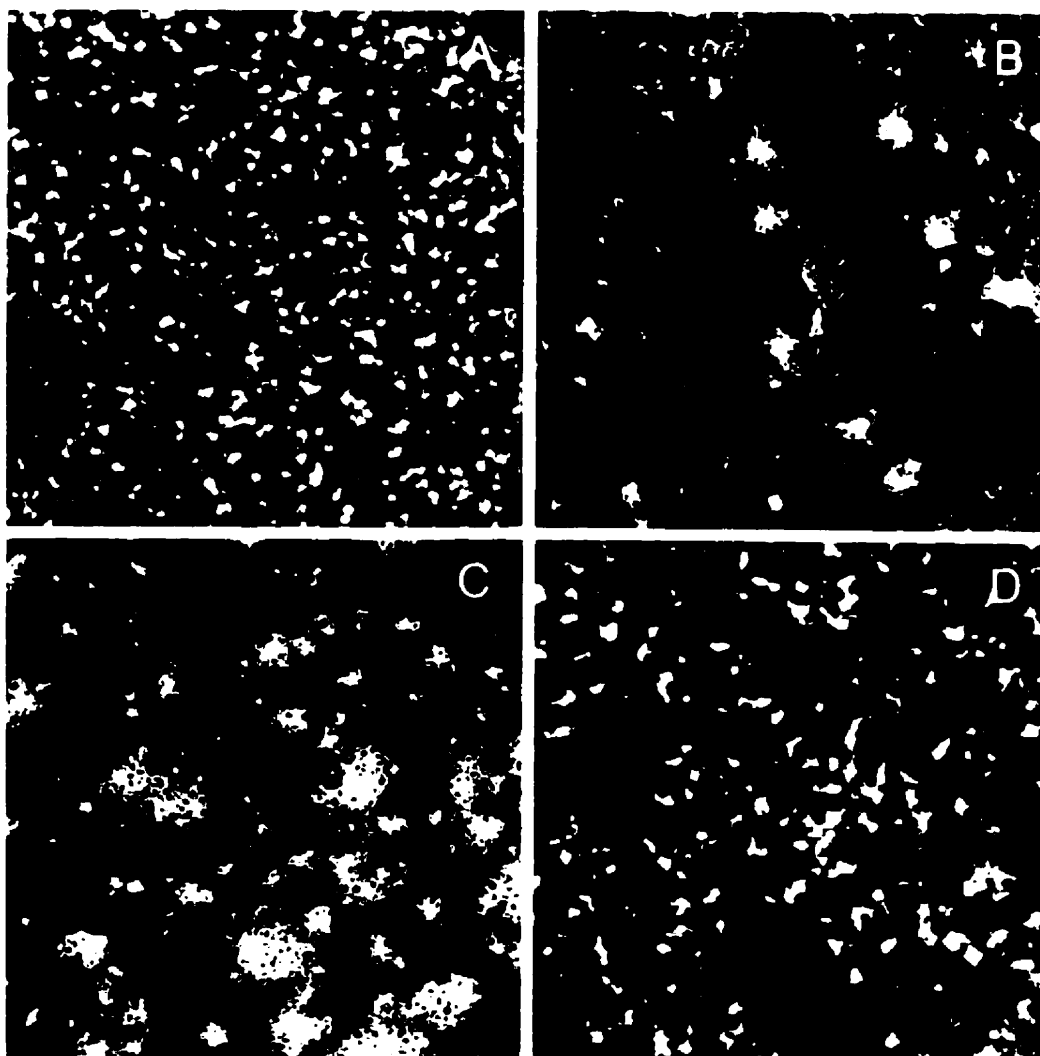
Following hepatic MPS blockade with liposomal doxorubicin and vincristine, DiI labeled DMPC/Chol mitoxantrone distributed in discrete patches. Numerous vacuoles are seen in the micrographs of livers from liposomal vincristine pre-treated mice. These may attributed to vincristine induced autophagocytosis in hepatocytes and the associated appearance of autophagocytic vacuoles (Hirsimaki and Pilstrom, 1982). The distribution pattern observed in animals pretreated with liposomal clodronate (Panel D) is comparable to that observed for DiI labeled DMPC/Chol mitoxantrone, except there are fewer liposomes present.

Mitoxantrone delivery to liver hepatocytes was also measured in an effort to resolve differences between the DMPC/Chol mitoxantrone (in the presence and absence of hepatic MPS blockade population) and the formulation prepared with PEG-modified lipids. Hepatocytes were isolated as described in Chapter 2 and the level of drug was measured using [^{14}C]-mitoxantrone as a marker for drug. Drug levels were standardized to 10^6 hepatocytes. It should be noted that hepatocyte drug levels may be due, in part, to drug that has been taken up during the hepatocyte isolation procedure. Given this analysis, it was anticipated on the basis of the data presented in Figure 5.2A and Figure 5.4, where hepatic MPS blockade affected a 2- to 3-fold reduction in liver mitoxantrone levels and a significant (>90%) reduction in Kupffer cells, that hepatocyte delivery would increase significantly when MPS blockade was used. As shown in Figure 5.7, this was not the case. Liposomal doxorubicin and clodronate pre-treatment effected a 2-fold reduction in liposome delivery to the hepatocytes, a reduction that is comparable to that observed in the whole liver. When hepatocyte mitoxantrone levels were determined in animals given ($i.v.$) DMPC/Chol/PEG mitoxantrone the values also decreased by a factor of 2. It can be suggested that differences in the anti-tumour activity of DMPC/Chol liposomal mitoxantrone due to PEG-lipid incorporation or hepatic MPS blockade can not be attributed to altered drug delivery to hepatocytes or to Kupffer cell processing.

Figure 5.7

Drug delivery to hepatocytes

Non-blockaded female CD1 mice were injected with a 10 mg/kg drug dose DMPC/Chol mitoxantrone (A) or DMPC/Chol/PEG mitoxantrone (D). MPS blockaded mice were pre-treated with either DSPC/Chol Doxorubicin (B) or EPC/Chol Clodronate (C). Twenty four hours later, the mice were then treated with 10 mg/kg drug dose DMPC/Chol mitoxantrone. Livers were extracted and hepatocytes isolated as described in the Chapter 2, Section 18. Lipid and drug concentrations were assessed via scintillation counting for ^3H and ^{14}C. Bars represent the average $\pm$ standard error.
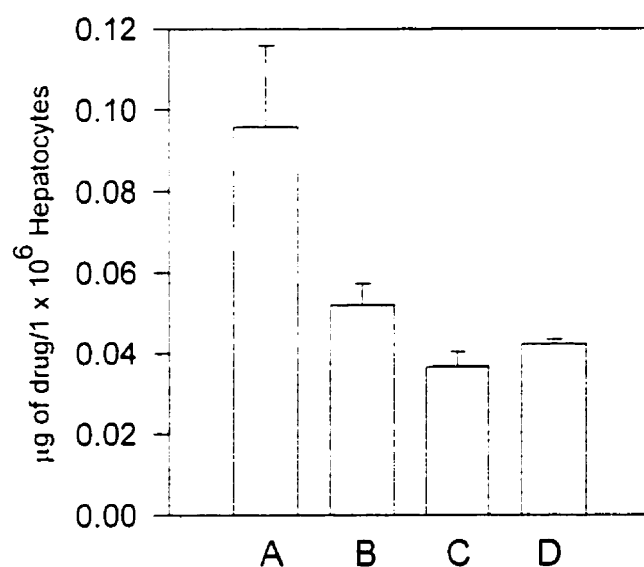
5.3 Discussion

There are two very simple conclusions that can be made on the basis of the data presented in this chapter. First. Kupffer cells do not play a role in governing the therapeutic activity of DMPC/Chol liposomal mitoxantrone. Second. incorporation of PEG-modified lipids significantly inhibits the therapeutic activity of DMPC/Chol liposomal mitoxantrone. The question that needs to be addressed on the basis of these conclusions is equally simple: Why should one strategy designed to reduce drug delivery to the liver inhibit therapy while another. which achieves a similar reduction in drug delivery. have no effect? To address this question it is important to examine the assumptions made when designing the experiments. These assumptions included: 1) drug delivery to the site of disease is critical in defining the therapeutic activity of liposomal mitoxantrone when used to treat liver localized disease: 2) conversely. reduction in drug delivery to the liver would effect reduced therapeutic activity and the related assumption 3) that PEG-lipid mediated reductions in liver delivery would provide similar results when compared to strategies relying on use of hepatic MPS blockade. The three assumptions. in retrospect, seem quite naïve.

The first assumption that drug delivery is critical in defining the therapeutic activity has. in effect. been addressed by previous investigators and confirmed by results shown in Table 5.1. As indicated in the introduction. many liposomal anti-cancer drugs have not been particularly effective in the treatment of liver cancer. This can be attributed to the role of the liver in drug metabolism and detoxification of drugs (Meijer *et al.*, 1990; Erlinger, 1996: Yamazaki *et al.*, 1996) and to inherent drug resistance of colon cancer and hepatocellular carcinomas (Ferry, 1998). It is believed that the latter concern is not really an issue in the present study because the cell line used (murine L1210 cells) was quite sensitive to the drugs selected (see Table 5.2).

Although the cytotoxicity assay would suggest that the L1210 cells are approximately 10-fold less sensitive to doxorubicin, it has been demonstrated that free doxorubicin and liposomal doxorubicin are quite effective in treating animals bearing L1210 tumours in the peritoneal cavity (Mayer *et al.* 1989). For this reason, it can be presumed that difference in therapeutic activity of these drugs, in free or liposomal form, are a consequence of differences in drug metabolism in the liver and elsewhere.

If comparisons are restricted to the anthraquinone mitoxantrone and the anthracycline doxorubicin, then some critical determinants of activity can be identified. The most significant difference in these drugs concerns their ability to generate free-radicals. In the presence of rat liver microsomes and the electron donor NADPH, doxorubicin is reduced to its free radical form and under identical conditions mitoxantrone is not (Vile and Winterbourn, 1989). The cytotoxic properties of doxorubicin have been attributed to generation of semi-quinone radicals that subsequently enter redox cycles with molecular oxygen which, in turn, lead to cation-radical formation (Riley and Hanzlik, 1994). This is associated with doxorubicin mediated stimulation of superoxide anion production that is not observed for mitoxantrone (Basra *et al.*, 1985). It is believed that doxorubicin cardiotoxicity is mediated by free radical production and lipid peroxidation (Vile and Winterbourn, 1989) and differences in generation of reactive oxygen have been used to explain why mitoxantrone exhibits reduced cardiotoxicity. The same argument has been used to explain why liposomal formulations of mitoxantrone do not promote hepatic MPS blockade (Chang *et al.*, 1997; Lim *et al.*, 1997) while formulations of doxorubicin are so effective in depleting non-dividing cells of the MPS (Bally et al, 1990; Daemen *et al.*, 1995).

Part of the rationale used in the hepatic MPS blockade studies was based on the fact that liposomal mitoxantrone does not induce MPS blockade, while liposomal vincristine and

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liposomal doxorubicin do induce MPS blockade. Previous studies have suggested that Kupffer cells can play a role in processing liposomal anti-cancer drugs (Storm et al., 1988), providing a mechanism for drug release back into the systemic circulation and/or within the region of macrophage localization. It was, therefore, convenient to suggest that the reason why liposomal formulations of doxorubicin and vincristine were not active in the treatment of liver localized disease related to hepatic MPS blockade. Conversely liposomal mitoxantrone activity is due, in part, to Kupffer cell processing. The data presented in Figure 5.2B and Table 5.3 clearly demonstrate that this is not the case. The therapeutic activity of liposomal mitoxantrone is not influenced under conditions where Kupffer cells have been eliminated.

There are other attributes of mitoxantrone that may make it better suited for treatment of liver localized cancer. For example, it is established that the cytotoxic activity of mitoxantrone is dependent on functional cytochrome P450-dependent mixed function oxidase (Duthie and Grant, 1989), a result that suggests that a mitoxantrone metabolite may be the primary effector of cytotoxicity (Mewes et al., 1993). Ramirez et al. (1996) has argued that mitoxantrone may be a good agent for treatment of liver disease because its main route of metabolism is within the liver and using a hepatic tumour model in rabbits, this group demonstrated that hepatic artery administration of mitoxantrone provided better therapy then intravenous administration. These data were used to support the conclusion that regional administration of mitoxantrone should be considered for treatment of liver cancer. Perhaps the properties of DMPC/Chol liposomal mitoxantrone that facilitate increases in drug exposure account for the improved activity observed when the liposomal drug is given intravenously. It should be noted that there is a potential concern regarding the use of mitoxantrone to treat liver disease in mice. Schrenk et al. (1996) have suggested that mitoxantrone is not an efficient inducer of mdr1 gene expression in

murine liver, which contrasts results obtained in rats. The mdrl gene encodes for an ABC transporter known to play a role in biliary excretion of certain xenobiotics (Schrenk *et al.*, 1993).

In the *i.v.* L1210 tumour model, the anti-tumour activity between different liposomal drugs can be accounted for by unique attributes of the drug used; however, it is difficult to explain differences between the DMPC/Chol (in the presence and absence of hepatic MPS blockade) and the DMPC/Chol/PEG formulations. Perhaps the most compelling argument is one based on PEG-mediated inhibition of liposome-cell interaction. Conversely, the therapeutic activity of mitoxantrone is dependent on cell processing but the cells involved are not mature liver macrophages. The former argument is supported by data demonstrating that PEG modification inhibits protein and cell binding (Du *et al.*, 1997). Inhibition of cell binding is observed even when targeting ligands are attached to the liposome surface (Harasym *et al.*, 1995) and if cell binding is obtained, the presence of PEG-modified lipids may prevent endocytosis (Ishiwata *et al.*, 1997).

In terms of the counter-argument, that cell processing is required for optimal therapeutic activity, it is essential to expand our discussion beyond the role of Kupffer cells. It is established that several cell types in the liver may be responsible for removal of particles from the blood compartment (Shiratori *et al.*, 1993; Bouwens *et al.*, 1992). Two populations of cells are of particular interest. Sinusoidal endothelial cells are capable of endocytosis and can accumulate particles <200 nm. In addition, Shiratori *et al.* (1993) have shown that when Kupffer cell function is blocked, sinusoidal endothelial cells can provide a compensating role in particle removal. The mechanism of particle removal by endothelial cells is believed to be different then that of Kupffer cells (Dan and Wake, 1985). The second population of interest is monocytes (van Furth, 1980). Bouwens and Wisse (1985) have argued that there are two populations of

phagoctes in the liver, a result that has been confirmed by more recent immunocytochemical analysis (Armbrust and Ramadori, 1996). Further, it has been demonstrated that there can be significant extrahepatic recruitment of monocyte-derived macrophage precursors in liver (Bouwens et al., 1986). The hepatic MPS blockade strategies employed in our studies may have been sufficient to eliminate Kupffer cells, but cell internalization and processing by monocytes that have been recruited to the liver, by immature liver phagocytic cells and by sinusoidal endothelial cells may all contribute to the activity of DMPC/Chol mitoxantrone. Differences in the activity of DMPC/Chol mitoxantrone and the DSPC/Chol formulation could still be attributed to drug release properties following cell uptake.

It is important to note that one can not entirely eliminate the possibility that the reduced activity of DMPC/Chol/PEG mitoxantrone was due to reduced drug release rates (see Figure 5.3). The observation that the PEG-containing formulation released drug slower then the DMPC/Chol formulation was surprising and was contrary to results obtained with liposomal vincristine (Webb et al., 1998). The latter observation was attributed to PEG-mediated changes at the membrane interface that could favor increased partitioning of the drug into the membrane. An alternative model to explain the PEG-induced decreases in mitoxantrone release may involve the influence of serum protein binding on mitoxantrone release from the DMPC/Chol liposomes. Consistent with the drug release argument, we can also not exclude the possibility that drug release from liposomes in the plasma compartment or from a site distinct from the liver may contribute to the therapeutic activity and that cell processing is not important. As indicated in the results, however, it is believed that the rate of drug release from DMPC/Chol/PEG mitoxantrone is sufficient to obtain therapy. This conclusion is based on results obtained with DSPC/Chol formulations of mitoxantrone that are more active in treating the i.v. L1210 tumour model despite having slower (See Chapter 3) or equivalent (Chang et al., 1997) drug release characteristics.

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Therefore, it is concluded that reductions in therapy observed for DMPC/Chol/PEG mitoxantrone were due to inhibition of cell binding and processing. Conversely the activity of the DMPC/Chol mitoxantrone is dependent on cell processing, but Kupffer cells do not play a significant role in this processing step.

CHAPTER 6

SUMMARIZING DISCUSSION

6.1 Summary of results

The objective of the studies presented in this thesis was to outline the importance of drug release in the development of liposomal mitoxantrone. Drug release was evaluated in liver localized disease, a site where rapid liposome accumulation occurs. This was then extended to studies evaluating drug release at a site where liposome accumulation is slow, such as a subcutaneous tumour. Finally, the activity of liposomal mitoxantrone was evaluated in the liver where the effects of drug delivery were assessed.

In Chapter 3, the influence of liposome drug release on the therapeutic activity of encapsulated mitoxantrone was reported. *In vivo* studies demonstrated that DMPC/Chol liposomes released drug faster than DSPC/Chol liposomes. Efficacy studies were conducted in BDF1 mice inoculated *i.v.* with murine P388 cells or L1210 tumour cells. Mice treated with a single dose of 10 mg drug/kg of DMPC/Chol liposomal mitoxantrone resulted in 100% of the treated animals surviving for more than 60 days. In contrast, no long term survivors were obtained in any other treatment group, even when drug doses were escalated to the MTD. Pharmacodynamic studies with DMPC/Chol mitoxantrone and DSPC/Chol liposomal mitoxantrone illustrate the importance of achieving a balance between drug release characteristics and drug delivery to a site of tumour progression.

In Chapter 4, delivery and therapeutic activity of liposomal mitoxantrone formulations exhibiting different drug release characteristics in two human carcinoma xenograft models (A431 and

LS180) that accumulate liposomes at different rates was evulated. When lipid and drug levels were measured in established (> 0.05 cm^3) tumours, accumulation was more rapid in the LS180 tumours (C_{max} 4 hours) when compared to the A431 tumours (C_{max} 48 hours). AUC values for liposomal lipid measured over a 96 hour time course were comparable for both liposomal formulations in A431 and the LS180 tumours, however liposomal lipid AUC values were almost 2-fold higher in LS180 tumours than in A431 tumours. Although drug delivery was less following administration of the DMPC/Chol liposomal mitoxantrone in comparison to the DSPC/Chol formulation, anti-tumour efficacy data suggest that the DMPC/Chol formulation was therapeutically more active in the LS180 tumour model and was as efficacious as the DSPC/Chol formulation when treating A431 tumours. These data place emphasis on the importance of designing liposomal formulations that optimize drug biological availability rather than drug delivery.

In Chapter 5, the role of liposomal drug delivery in the treatment of liver localized cancer was investigated. The therapeutic activity of liposomal formulations of vincristine, doxorubicin and mitoxantrone were tested in a model where L1210 tumour cells seed in the liver and the spleen. Only treatment with DMPC/Chol mitoxantrone at a 10 mg/kg drug dose effected cures as measured by survival beyond 60 days. In order to better understand the activity of mitoxantrone in the liver, the role of drug delivery was assessed. This was modulated through the use of procedures that cause reductions of liposome accumulation in the liver and it was predicted that this would result in decreased therapeutic activity. Reduction in liver accumulation was achieved by either the use of PEG modified lipids or by methods designed to suppress phagocytic cell activity in the liver, referred to as hepatic MPS blockade. Decreases in anti-tumour activity were observed with the PEG formulation; however, the use of MPS blockade failed to reduce the therapeutic activity of DMPC/Chol mitoxantrone, despite lower drug delivery. These data

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demonstrate that although the Kupffer cells play a role in liposome accumulation, this population is not responsible for mediating therapeutic activity of DMPC/Chol mitoxantrone.

6.2 Discussion

The results from this thesis highlight the importance of drug release. Drug encapsulated within the liposome is not biologically available and therefore, does not play a role in the therapeutic activity. Formulations which have focused on drug retention have decreased drug toxicity (Mayer et al., 1989) and improved the therapeutic activity (Boman et al., 1994), whereas other formulations exhibiting rapid drug release in the circulation tend to exhibit increased toxicity and a reduction in the therapeutic activity (Mayer et al., 1994). As demonstrated in Chapter 3, formulations which retain the drug (such as DSPC/Chol mitoxantrone) can be less effective than the free drug, a consequence of reduced drug biological availability. Hence, liposomal formulations must be optimized in terms of the rate of drug release.

It is important to note that stability in the circulation is also a crucial parameter, as drug that is released in the circulation is believed to have a negligible role in the therapeutic activity. Once the liposomes extravasate into the site of tumour development, drug release is required to optimize exposure to the drug. This is in contrast to mechanisms postulated on the basis of slow release of drug from liposomes that reside in the blood compartment. If drug release within the circulation were a crucial parameter, administration of drug via infusion pumps should yield greater increases in therapeutic activity. Often drug infusion procedures result in only marginal improvements in clinical response (Jackson et al., 1989; 1985). It is, of course, easy to stress potential advantages of liposomal delivery systems because the experience with these formulations is far less when compared to studies in humans that have evaluated infusion

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approaches. However, the use of liposomes as drug carriers provides a drug reservoir at the site of tumour development and, if appropriately designed, these systems will decrease systemic exposure of the associated anti-cancer drug.

Triggered release of drug ideally would occur using liposome systems which retain the drug in the circulation but once the liposome extravasates in the disease site, drug release is stimulated by either an external signal or a change in the liposome. These systems would theoretically improve the therapeutic activity but also decrease toxicity since drug release is emphasized at the region where drug is required. The concept of triggered release has been studied through the use of pH sensitive liposomes which exploit the tumour's acidic interior to cause the liposomes to release their contents at the target site (Aicher *et al.*, 1994; Connor *et al.*, 1984). Thermosensitive liposomes are also being developed where liposomes are injected and regional hyperthermia causes release of the liposome contents (Kakinuma *et al.*, 1996; Gaber *et al.*, 1996; Chelvi et al, 1995). In addition, it has been demonstrated that lipids such as unsaturated PE's (which do not normally adopt a bilayer structure) can be stabilized into a bilayer conformation through the use of lipids such as PEG-PE (Holland *et al.*, 1996a). As the PEG moiety leaves the liposome, the liposome destabilizes, releasing the drug or fuses with the tumour cell (See Figure 6.1). It has been established that PEG modified lipids can be designed to exchange out of the liposomal membrane (Holland *et al.*, 1996b) or alternatively, the PEG moiety can be lost due to chemical degradation of the lipid (Kirpotin *et al.*, 1996b; Parr *et al.*, 1994).

As demonstrated in Chapter 3 of this thesis, the DMPC/Chol formulation of mitoxantrone is active in liver localized disease. As noted in Chapter 5, reduction of the therapeutic activity can be attained through the use of PEG-modified lipids, which decreases the accumulation of DMPC/Chol mitoxantrone in the liver. However, reduction achieved through the use of MPS

Figure 6.1

Future design of liposomes

Loss of PEG
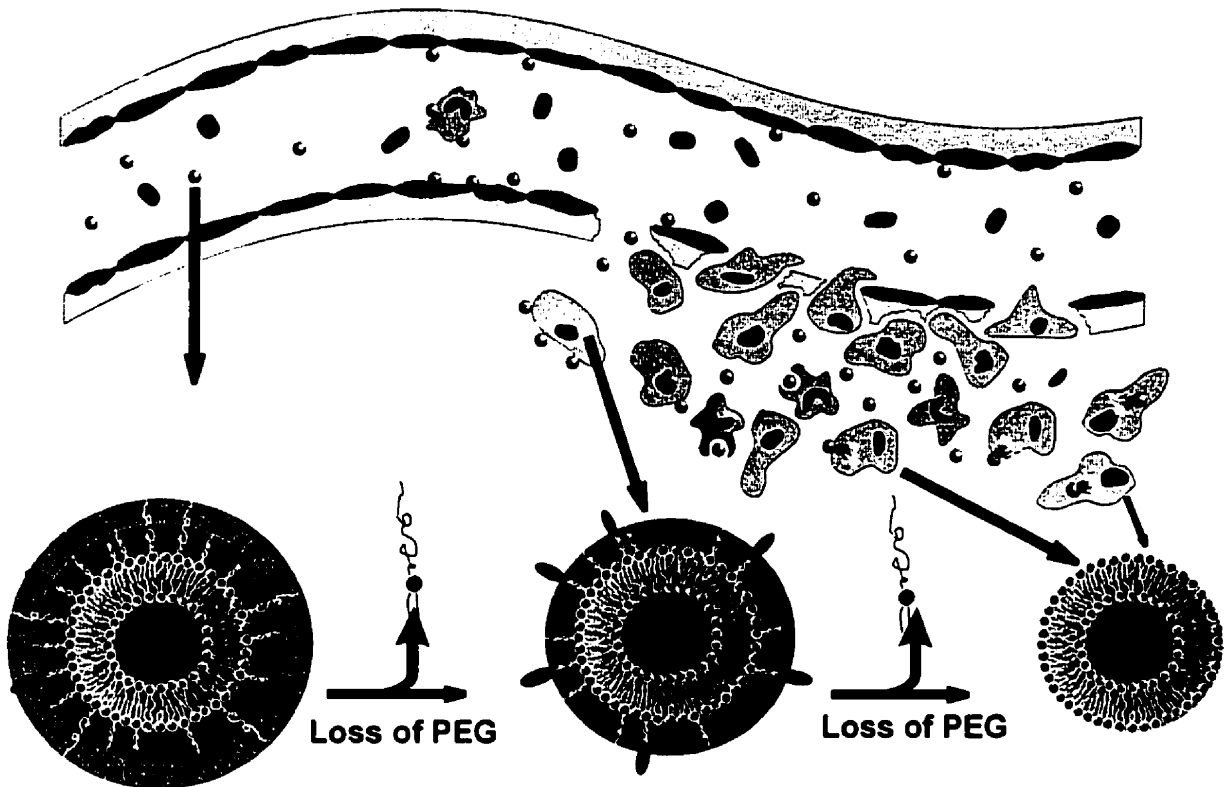
Loss of PEG

Liposomes are designed
to be stable in the circulation.
Incorporation of PEG
increases circulation lifetime
leading to a greater potential
for extravasation into
the tumor site.

Loss of PEG reveals
targeting ligand.
Increases binding
to the target cell.

Further loss of PEG
causes destabilization
of the liposome.
Promotes fusion with
the target cell and
release of drug at
the site.

blockade, did not result in the same effect. It is surprising to find that the Kupffer cells do not play a role in mediating the activity of liposomal mitoxantrone. It has been demonstrated that the Kupffer cells can act as a reservoir for drugs, releasing the free form back into circulation (Storm et al., 1988). In addition, the use of liposomal doxorubicin and vincristine on the L1210 tumour model demonstrated disappointing results. This was thought to be due to the effects of these drugs on the Kupffer cell population thereby reducing drug delivery to the liver. It can be concluded, however, that the Kupffer cells do not play a role in mediating the activity of liposomal mitoxantrone.

The difference between the two methods employed to reduce drug delivery to the liver is specificity. The use of PEG-modified lipids reduces delivery of liposomes to cells due to the steric shielding which inhibits liposome-cell interactions (Du et al., 1997). It is plausible that the use of PEG has altered the distribution of liposomes within the liver, and thus decreasing delivery to the cell population mediating activity. It has been observed that although PEG liposomes can cause an increase in the circulation levels of liposomes, this does not translate to an increase in tumour accumulation (Parr et al., 1997). Similarly in the liver, as the liposome percolate throughout the liver, there are several cell populations which can interact with the liposomes. The use of PEG can inhibit the interactions with these cells; thereby decreasing the therapeutic activity of liposomal mitoxantrone. The use of MPS blockade eliminates only the Kupffer cell population and thus, liposome interaction with this population. Although the use of MPS has excluded the possibility of Kupffer cells, there are still other cell populations which liposomes associate with and this may mediate activity of liposomal mitoxantrone in the liver.

For example, the endothelial cells are also capable of phagocytosis and also play a role in liposomal transport. The use of MPS blockade would not affect this transportation role;

however, the use of PEG lipids inhibits interactions of the liposomes with the endothelial cells, thereby reducing transport. An assessment of delivery to the endothelial cells would be instrumental in determining the involvement of these cells. It has been demonstrated that the Kupffer cells can affect the phagocytic capability of the endothelial cells in the liver (Deaciuc et al., 1994). Thus, the use of MPS blockade may increase the liposome accumulation in the endothelial cell population and in tern, mediate the therapeutic activity of liposomal mitoxantrone.

The MPS affects only the Kupffer cell population and there are still circulating pools of monocytes which are still capable of liposome uptake. Future experiments could examine the monocyte population further by reducing the monocyte population through the use of anti-CD14 antibody and carbonyl iron (Holtrop et al., 1992). This would determine the role of the MPS rather than focusing on the Kupffer cell population. In addition, the mechanisms by which the liver process liposomes are still under investigation. Three pathways have been proposed by Scherphof et al. (1998), suggesting that liposomal elimination is a very complicated process. Two of the pathways involve receptor binding (apoE-mediated receptor and an unknown receptor) and then endocytic internalization into the lysosomal compartment. The third involves an HDL receptor and results in transference of certain bilayer constituents to the bilayer of the hepatocyte. A more thorough examination of how hepatocytes process liposomal mitoxantrone may also explain the therapeutic activity. As seen in Chapter 5, delivery to the hepatocytes was unaffected by MPS blockade or the use of PEG. Although the PEG inhibits cell interactions, the PEG moiety does not provide complete protection and protein binding will eventually overcome these benefits of surface stabilization. Cells that interact with these liposomes may internalize them, however, the remaining PEG may alter the processing of the liposome.

Targeting the liver can also be achieved through the use of charged lipid such as phophatidylserine. The majority of phosphatidylserine containing liposomes accumulate in the liver, and therefore, this would enhance delivery to the site of tumour development. However, the majority of these liposome accumulate in the Kupffer cells (Spanjer et al., 1986) and these cells do not play a role in mediating liposomal mitoxantrone. Thus, it will be interesting to note if the increased accumulation of the liposomes in the liver will result in the same therapeutic activity if the majority of the liposomes are taken up by the Kupffer cells.

In conclusion, drug release is an important parameter when designing liposomal formulations regardless if the liposomes accumulate rapidly at the site of tumour development, such as the liver, or at an extravascular site where liposomes accumulate slowly. In addition, delivery to the cells mediating activity is also critical. Although the Kupffer cells are responsible for liposome uptake in the liver, they do not affect the therapeutic activity of DMCP/Chol mitoxantrone. In closing, the use of liposomal mitoxantrone for the treatment of liver cancer holds much promise and continued studies in the liver's role of processing these carriers could improve liposomal treatment for this disease.

REFERENCES

Abra, R.M. and Hunt, C.A. Liposome disposition in vivo. III. Dose and vesicle-size effects. Biochim Biophys Acta. 666: 493-503, 1981.

Ahmad, I., Longnecker, M., Samuel, J., and Allen, T.M.: Antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes can eradicate lung cancer in mice. Cancer Res. 53: 1484-1488, 1993.

Aicher, A., Miller, K., Reich, E., and Hautmann, R.: Photodynamic therapy of human bladder carcinoma cells in vitro with pH-sensitive liposomes as carriers for 9-acetoxy-tetra-n-propylporphycene. Urol Res. 22: 25-32, 1994.

Allen, T.M., Mehra, T., Hansen, C., and Chin, Y.C.: Stealth liposomes: an improved sustained release system for 1-beta-D-arabinofuranosylcytosine. Cancer Res. 52: 2431-9, 1992.

Allen, T.M., Hansen, C., Martin, F., Redemann, C., and Yau-Young, A.: Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim. Biophys. Acta. 1066: 29-36, 1991.

Allen, T.M., Hansen, C., and Rutledge, J.: Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. Biochim. Biophys. Acta. 981: 27-35, 1989.

Allen, T. M. and Chonn, A.: Large unilamellar liposomes with low uptake into the reticuloendothelial system. FEBS Lett. 223: 42-46, 1987.

Allen, T.M. and Cleland, L.G.: Serum-induced leakage of liposome contents. Biochim Biophys Acta. 597: 418-26, 1980.

Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H., and Boyd, M.R.: Feasibility of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay. Cancer Res. 48: 589-601, 1988.

Amadori, S., Arcese, W., Isacchi, G., Meloni, G., Petti, M.C., Monarca, B., Testi, A.M., and Mandelli, F.: Mitoxantrone, etoposide, and intermediate-dose cytarabine: an effective and tolerable regimen for the treatment of refractory acute myeloid leukemia. J Clin Oncol. 9: 1210-4, 1991.

Archimbaud, E., Leblond, V., Michallet, M., Cordonnier, C., Fenaux, P., Travade, P., Dreyfus, F., Jaubert, J., Devaux, Y., and Fiere, D.: Intensive sequential chemotherapy with mitoxantrone and continuous infusion etoposide and cytarabine for previously treated acute myelogenous leukemia. Blood. 77: 1894-900, 1991.

Armburst, T. and Ramadori, G.: Functional characterization of two different Kupffer cell populations of normal rat liver. J Hepatol. 25: 518-528, 1996.

Asao, T., Shibata, H.R., Batist, G., and Brodt, P.: Eradication of hepatic metastases of carcinoma H-59 by combination chemoimmunotherapy with liposomal muramyl tripeptide, 5-fluoruracil, and leucovorin. Cancer Res. 52: 6254-6257, 1992.

Austyn, J.M. and Gordon, S.: F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur. J. Immunol. 11: 805-815, 1981.

Bailey, A. L. and Cullis, P. R.: Modulation of membrane fusion by asymmetrical transbilayer distributions of amino lipids. Biochemistry 33, 12573-12580, 1994.

Bajetta, E., Buzzoni, R., Valagussa, P., and Bonadonna, G.: Mitoxantrone: an active agent in refractory non-Hodgkin's lymphomas. Am J Clin Oncol. 11: 100-3, 1988.

Bakker-Woudenberg, I.A., Lakerse, A.F., ten-Kate, M.T., and Storm, G. (1992) Enhanced localization of liposomes with prolonged blood circulation time in infected lung tissue. Biochim. Biophys. Acta 1138, 318-326.

Balazsovits, J. A. E., Mayer, L. D., Bally, M. B., Cullis, P. R., McDonnell, M., Ginsberg, R. S., and Falk, R. E.: Analysis of the effect of liposome encapsulation on the vesicant properties, acute and cardiac toxicities, and anti tumour efficacy of doxorubicin. Cancer. Chemother. Pharmacol., 23: 81-86, 1989.

Ballou, R.J. and Tseng, M.T.: In vitro and in vivo responses of a murine transitional cell carcinoma to doxorubicin, mitoxantrone, and acalacinomycin-A. Urological Research 14:217-222, 1986.

Bally, M.B., Masin, D., Nayar, R., Cullis, P.R., and Mayer, L.D.: Transfer of liposomal drug carriers from the blood to the peritoneal cavity of normal and ascitic tumour-bearing mice. Cancer Chemother. Pharamacol., 34: 137-146, 1994.

Bally, M.B., Mayer, L.D., Hope, M.J., and Nayar, R.: Pharmacodynamics of lipsoomal drug carriers: methodological considersations. In Liposome Technology, ed. By G. Gregoriadis, pp. 27-41, CRC Press, 1993.

Bally, M.B., Nayar, R., Masin, D., Hope, M.J., Cullis, P.R., and Mayer, L.D.: Liposomes with entrapped doxorubicin exhibit extended blood residence times. Biochim. Biophys. Acta. 1023: 133-139, 1990

Bangham, A. D., Standish, M. M., and Watkins, J. C., (1965) Diffusion of univalent cations across the lamellae of swollen phospholipids. J. Mol. Biol. 13. 238-252.

Barenholtz, Y., Amselem, S., and Lichtenberg, D.: A new method for preparation for phospholipid vesicles (liposomes) – French press. FEBS Lett. 99:210-214, 1979.

Basra, J., Wolf, C.R., Brown, J.R., and Patterson, L.H.: Evidence for human liver mediated free-radical formation by doxorubicin and mitoxantrone. Anticancer Drug Des. 1: 45-52, 1985.

Baxter, L.T. and Jain, R.K.: Transport of fluid and macromolecules in tumours. I. Role of interstitial pressure and convection. Microvasc Res. 37: 77-104, 1989.

Baxter, L.T. and Jain, R.K.: Transport of fluid and macromolecules in tumours. II. Role of heterogeneous perfusion and lymphatics. Microvasc Res. 40: 246-263, 1990.

Bennett, J.M., Muss, H.B., Doroshow, J.H., Wolff, S., Krementz, E.T., Cartwright, K., Dukart, G., Reisman, A., and Schoch, I.: A randomized multicenter trial comparing mitoxantrone, cyclophosphamide, and fluorouracil with doxorubicin, cyclophosphamide, and fluorouracil in the therapy of metastatic breast carcinoma. J Clin Oncol. 6: 1611-20, 1988.

Berdel, W.E., Von Hoff, D.D., Unger, C., Schick, H.D., Fink, U., Reichert, A., Eibl H., and Rastetter, J.: Ether lipid derivatives: antineoplastic activity in vitro and the structure-activity relationship. Lipids. 21:301-4, 1986.

Bezwoda, W.R., Bernasconi, C., Hutchinson, R.M., Winfield, D.A., de Bock, R., and Mandelli, F.: Mitoxantrone for refractory and relapsed acute leukemia. Cancer. 66: 418-22, 1990.

Bittman, R. and Blau, L.: The phospholipid-cholesterol interaction. Kinetics of water permeability in liposomes. Biochem. 11: 4831-4839, 1972.

Bolotin, E.M., Cohen, R., Bar, L.K., Emanuel, N., Ninio, S., Lasic, D.D., and Barenholz, Y.: Ammonium Sulfate Gradients for efficient and stable remote loading of amphipathic weak bases into liposomes and ligandoliposomes. J. Liposome Res., 4: 455-479, 1994.

Boman, N.L., Tron, V.A., Bally, M.B., and Cullis, P.R.: Vincristine-induced dermal toxicity is significantly reduced when the drug is given in liposomes. Cancer Chemother Pharmacol. 37: 351-5, 1996.

Boman, N.L., Masin, D., Mayer, L.D., Cullis, P.R., and Bally, M.B.: Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumours. Cancer Res., 54: 2830-2833, 1994.

Boman N.L., Mayer, L.D., and Cullis, P.R.: Optimization of the retention properties of vincristine in liposomal systems. Biochim. Biophys. Acta 1152, 253-258, 1993.

Bonte, F. and Julaino, R.L.: Interactions of liposomes with serum proteins. Chem Phys Lipids 40: 359-372, 1986.

Bortoleto, R.K., de Oliveira, A.H., Ruller, R., Arni, R.K., and Ward, R.J.: Tertiary structural changes of the alpha-hemolysin from Staphylococcus aureus on association with liposome membranes. Arch Biochem Biophys. 351(1):47-52, 1998.

Bosworth, M.E. and Hunt, C.A.: Liposome disposition in vivo II: Dose dependency. J Pharm Sci. 71:100-4, 1982.

Bouwens, L., De Bleser, P., Vanderkerken, K., Geerts, B., and Wisse, E.: Liver cell heterogeneity: functions of non-parenchymal cells. Enzyme 46: 155-168, 1992.

Bouwens, L., Baekeland, M., and Wisse, E.: Cytokinetic analysis of the expanding Kupffer-cell population in rat liver. Cell Tissue Kinet. 19: 217-226, 1986.

Bouwens, L. and Wisse, E.: Proliferation, kinetics, and fate of monocytes in rat liver during a zymosan-induced inflammation. J. Leukoc Biol. 37: 531-543, 1985.

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Brambilla, C., Moliterni, A., Codazzi, D., Villani, F., and Crippa, F.: Mitoxantrone as first salvage chemotherapy in relapsed breast cancer. Tumouri. 75: 145-149, 1989.

Brigham, K.L., Meyrick, B., Christman, B., Magnuson, M., King, G., and Berry, L.C. Jr.: In vivo transfection of murine lungs with a functioning prokaryotic gene using a liposome vehicle. Am J Med Sci. 298: 278-81, 1989.

Burkitt, H.G., Young, B., and Heath. J.W.: Wheater's functional hisotlogy. Third edition. Churchill Livingstone, London, p. 147. 1993.

Canti, G., Nicolin, A., Cubeddu, R., Taroni, P., Bandieramonte, G., Valentini, G.: Anti-tumour efficacy of the combination of photodynamic therapy and chemotherapy in murine tumours. Cancer Lett. 125: 39-44, 1998.

Caride, V.J., Taylor, W., Cramer, J.A., and Gottschalk, A.: Evaluation of liposome-entrapped radioactive tracers as scanning agents. Part 1: Organ distribution of liposome (99mTc-DTPA) in mice. J Nucl Med. 17: 1067-72, 1976.

Cay, O., Krushkal, J.P., Nasser, I., Thomas, P., and Clouse, M.E.: Liver metastases from colorectal canter: drug delivery with liposome-encapsulated doxorubicin. Radiology 205: 95-101, 1997.

Chang, C.W., Barber, L., Ouyang, C., Masin, D., Bally, M.B., and Madden, T.D.: Plasma clearance, biodistribution and therapeutic properties of mitoxantrone encapsulated in conventional and sterically stabilized liposomes after intravenous adminstration in BDF1 mice. Br J Cancer. 75: 169-77, 1997.

Chelvi, T.P., Jain, S.K., and Ralhan, R.: Hyperthermia-mediated targeted delivery of thermosensitive liposome-encapsulated melphalan in murine tumours. Oncol Res. 7: 393-8, 1995.

Cheung, B.C.L., Sun, T.H.T., Leenhouts, J.M., and Cullis, PR.: Loading of doxorubicin into liposomes by forming Mn2+-drug complexes. Biochim Biophys Acta. 1414: 205-216, 1998.

Cheng, K.T., Seltzer, S.E., Adams, D.F., and Blau, M.: The production and evaluation of contrast-carrying liposomes made with an automatic high-pressure system. Invest Radiol. 22: 47-55, 1987.

Choice, E., Masin, D., Bally, M.B., Meloche, M., and Madden, T.D.: Liposomal cyclosporine. Comparison of drug and lipid carrier pharmacokinetics and biodistribution. Transplantation. 60: 1006-11, 1995.

Chonn, A., Semple, S.C., and Cullis, P.R.: Beta 2 glycoprotein I is a major protein associated with very rapidly cleared liposomes in vivo, suggesting a significant role in the immune clearance of "non-self" particles. J Biol Chem. 270: 25845-9, 1995.

Chonn, A., Semple, S.C., and Cullis, P.R.: Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes. J Biol Chem. 267: 18759-65, 1992.

Chow, D.D., Essien, H.E., Padki, M.M., and Hwang, K.J.: Targeting small unilamellar liposomes to hepatic parenchymal cells by dose effect. J Pharmacol Exp Ther. 248: 506-13, 1989.

Chowdhry, B.Z. and Dalziel A.W.: Phase transition properties of 1,2- and 1,3-diacylphosphatidylethanolamines with modified head groups. Biochemistry. 24: 4109-17, 1985.

Civalleri, D., Pelliccim R., Decaro, G., Giangaspero, A., Ettorre, G., Decian, F., Mondini, G., and Simoni, G.: Palliative chemoembolization of hepatocellular carcinoma with mitoxantrone, Lipiodol, and Gelfoam. A phase II study. Anticancer Res. 16: 937-41, 1996.

Claassen, E.: Post-formation fluorescent labelling of liposomal membranes. In vivo detection, localisation and kinetics. J Immunol Methods. 147: 231-40, 1992.

Colleoni, M., Nole, F., Di Bartolomeo, M., de Braud, F., and Bajetta E.: Mitoxantrone in patients affected by hepatocellular carcinoma with unfavorable prognostic factors. Oncology. 49: 139-42, 1992.

Connor, J., Yatvin, M.B., and Huang, L.: pH-sensitive liposomes: acid-induced liposome fusion. Proc Natl Acad Sci U S A. 81: 1715-8, 1984.

Corbett, T.H., Roberts, B.J., Trader, M.W., Laster, jr., W.R., and Griswold Jr., D.P.: Response of transplantable tumours of mice to anthracenedione derivatives alone and in combination with clinically useful agents. Cancer Treatment Reports. 66: 1187-1200, 1982.

Crone, C.: Modulation of solute permeability in microvascular endothelium. Fed Proc. 45: 77-83, 1986.

Cullis, P.R., Hope, M.J., and Tilcock, C.P.S.: Lipid polymorphism and the roles of lipids in membranes. Chem. Phys. Lipids 40: 127-144, 1986.

Cullis, P.R., Hope, M.J., Bally, M.B., Madden, T.D., and Mayer, L.D.: Liposomes as pharmaceuticals. In Liposomes From Biophysics to Therapeutics, ed. by M.J. Ostro, pp. 39-72, Marcel Dekkerm New York, NY, 1987.

Daemen, T., Hofstede, G., Ten Kate, M.T., Bakker-Woudenberg, I.A., and Scherphof, G.L.: Liposomal doxorubicin-induced toxicity: depletion and impairment of phagocytic activity of liver macrophages. Int J Cancer. 61: 716-21, 1995.

Dan, C. and Wake, K.: Modes of endocytosis of latex particles in sinusoidal endothelial and Kupffer cells of normal and perfused rat liver. Exp Cell Res 158:75-85, 1985.

David, H.: The hepatocyte. Development, differentiation, and ageing. Exp. Pathol. Suppl. 11: 1-148, 1985.

De Jong, G., Gelmon, K., Bally, M., Goldie, J., and Mayer, L.: Modulation of doxorubicin resistance in P388/ADR cells by Ro44-5912, a tiapamil derivative. Anticancer Res. 15: 911-6, 1995.

De Vries, C., Escobedo, J.A., Ueno, H., Houck, K., Ferrara, N., and Williams, L.T.: The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255: 989-991, 1992.

Deaciuc, I.V., Bagby, G.J., Niesman, M.R., Skrepnik, N., and Spitzer, J.J.: Modulation of hepatic sinusoidal endothelial cell function by Kupffer cells: an example of intercellular communication in the liver. Hepatology 19: 464-470, 1994.

Deamer, D. W. and Nichols, J. W.: Proton-hydroxide permeability of liposomes. Proc. Natl. Acad. Sci., USA 80, 165-168. 1983.

Demel, R.A. and Kruyff, B. de.: The function of sterols in membranes. Biochim. Biophys. Acta 457: 109-132, 1976.

Devine, D.V. and Bradley, A.J.: The complement system in liposome clearance: Can complement deposition be inhibited? Advanced Drug Delivery Reviews. 32: 19-29, 1998.

Devine, D.V., Wong, K., Serrano, K., Chonn, A., and Cullis, P.R.: Liposome-complement interactions in rat serum: implications for liposome survival studies. Biochim Biophys Acta. 1191: 43-51, 1994.

DeVita, V.T. Jr.: Principles of cancer management: chemotherapy. In Cancer: Principles and Practice of Oncology. Fifth Edition, ed. By V.T. Devita, Jr., S. Hellman, and S.A. Rosenberg, pp. 333-347, Lippincott-Raven Publishers, Philadelphia, PA, 1997.

Doerschuk, C.M., Gie, R.P., Bally, M.B., Cullis, P.R., and Reinish, L.W.: Platelet distribution in rabbits following infusion of liposomes. Thromb Haemost. 61: 392-6, 1989.

Du, H., Chandaroy, P., and Hui, S.W.: Grafted poly-(ethylene glycol) on lipid surfaces inhibits protein adsorption and cell adhesion. Biochim Biophys Acta. 1326: 236-48, 1997.

Dukart, G., Iatropoulos, M.J., and Yacobi, A.: Comment on mitoxantrone. Drug Intell. Clin. Pharm. 19: 216-218, 1985.

Dunham, P., Babiarz, P., Israel, A., Zerial, A., and Weissmann, G.: Membrane fusion: studies with a calcium-sensitive dye. arsenazo III, in liposomes. Proc Natl Acad Sci. 74: 1580-4, 1977.

Dunk, A.A., Scott, S.C., Johnson, P.J., Melia, W., Lok, A.S., Murray-Lyon, I., Williams, R., and Thomas, H.C.: Mitozantrone as single agent therapy in hepatocellular carcinoma. A phase II study. J Hepatol. 1: 395-404, 1985.

Durr, F.E.: Biologic and biochemical effects of mitoxantrone. Semin. Oncol. 11: 3-10, 1984.

Duthie, S.J. and Grant, M.H.: The role of reductive and oxidative metabolism in the toxicity of mitoxantrone, adriamycin and menadione in human liver derived Hep G2 hepatoma cells. Br J Cancer 60: 566-571, 1989.

Dvorak, A.M., Kohn, S., Morgan, E.S., Fox, P., Nagy, J.A., and Dvorak, H.F.: The vesiculo-vacuolar organelle (VVO): a distinct endothelial cell structure that provides a transcellular pathway for macromolecular extravasation. J Leukoc Biol. 59: 100-15, 1996.

Dvorak, H. F., Sioussat, T. M., Brown, L. F., Berse, B., Nagy, J. A., Sotrel, A., Manseau, E. J., Van de Water, L., and Senger, D. R.: Distribution of vascular permeability factor (vascular endothelial growth factor) in tumours: concentration in tumour blood vessels. J. Exp. Med., 174: 1275-1278, 1991.

Dvorak, H.F., Nagy, J.A., Dvorak, J.T., and Dvorak, A.M.: Identification and characterization of blood vessels of solid tumours that are leaky to circulating macromolecules. Amer. J. Path. 133: 95-109, 1988.

Ehninger, G., Schuler, U., Proksch, B., Zeller, K.P., and Blanz, J.: Pharmacokinetics and metabolism of mitoxantrone. A review. Clin Pharmacokinet. 18: 365-80, 1990.

Elias, A.D., Ayash, L., Tepler, I., Wheeler, C., Schwartz, G., Mazanet, R., Schnipper, L., Frei, E. 3d., and Antman, K.: The use of G-CSF or GM-CSF mobilized peripheral blood progenitor cells (PBPC) alone or to augment marrow as hematologic support of single or multiple cycle high-dose chemotherapy. J Hematother. 2: 377-82, 1993.

Erlinger, S.: Mechanisms of hepatic transport and bile secretion. Acta Gastroenterol Belg. 59: 159-162, 1996.

Felgner, P.L. and Ringold, G.M.: Cationic liposome-mediated transfection. Nature. 337: 387-8, 1989.

Fenske, D.B., Wong, K.F., Maurer, E., Maurer, N., Leenhouts, J.M., Boman, N., Amankwa, L., and Cullis, P.R.: Ionophore-mediated uptake of ciprofloxacin and vincristine into large unilamellar vesicles exhibiting transmembrane ion gradients Biochim Biophys Acta. 1414: 188-204, 1998.

Ferry, D.R.: Testing the role of P-glycoprotein expression in clinical trials: applying pharmacological principles and best methods for detection together with good clinical trails methodology. Int J Clin Pharmacol Ther 36: 29-40, 1998.

Fielding, R.M. and Abra, R.M.: Factors affecting the release rate of terbutaline from liposome formulations after intratracheal instillation in the guinea pig. Pharm Res. 9: 220-3, 1992.

Fielding, R.M.: Liposomal Drug Delivery - Advantages and limitations from a clinical pharmacokinetic and therapeutic perspective. Clin. Pharmacokinet., 21: 155-164, 1991.

Fisher, G.R. and Patterson, L.H.: Oxidative activation of the anti-tumour agent mitoxantrone. Journal of Pharmacy and Pharmacology. 41: 150P, 1989.

Fisher, G.R., Maine, J.E., Cairns,D., and Patterson, L.H.: Comparison of free radical generation by doxorubicin and mitoxantrone in MCF-7 breast cancer cells. Journal of Pharmacy and Pharmacology. 41: 23P, 1989.

Fleisch, H.: New bisphosphonates in osteoporosis. Osteoporos Int. 3: S15-22, 1993.

Folkman, J. and Shing, Y.: Angiogenesis. J Biol Chem. 267: 10931-10934, 1992.

Folkman, J: Tumour angiogenesis. Adv Cancer Res. 43: 175-203, 1985.

Fornari, F.A., Randolph, J.K., Yalowich, J.C., Ritke, M.K., and Gewirtz, D.A.: Interference by doxorubicin with DNA unwinding in MCF-7 breast tumour cells. Mol Pharmacol. 45: 649-56, 1994.

Fujimoto, S., and Ogawa, M.: Anti-tumour activity of mitoxantrone against murine experimental tumours: comparative analysis against various antitumour antibiotics. Cancer Chemtherapy and Pharmacology. 8:157-162, 1982.

Fukumura, D., Yuan, F., Monsky, W.L., Chen, Y., and Jain, R.K.: Effect of host microenvironment on the microcirculation of human colon adenocarcinoma. Am J Pathol. 151: 679-88, 1997.

Furuya, K.N., Bradley, G., Sun, D., Schuetz, E.G., and Scheutz, J.D.: Identification of a new P-glycoprotein-like ATP-binding cassette transporter gene that is overexpressed during hepatocarcinogenesis. Cancer Res. 57:3708-3716, 1997.

Gaber, M.H., Wu, N.Z., Hong, K., Huang, S.K., Dewhirst, M.W., and Papahadjopoulos, D.: Thermosensitive liposomes: extravasation and release of contents in tumour microvascular networks. Int J Radiat Oncol Biol Phys. 36: 1177-87, 1996.

Gabizon, A. and Martin, F.: Polyethylene glycol-coated (pegylated) liposomal doxorubicin. Rationale for use in solid tumours. Drugs. 54 Suppl 4: 15-21, 1997.

Gabizon, A. and Papahadjopoulos, D.: Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumours. Proc Natl Acad Sci U S A. 85: 6949-53, 1988.

Gabizon, A., Chemla, M., Tzemach, D., Horowitz, A.T., and Goren D.: Liposome longevity and stability in circulation: effects on the in vivo delivery to tumours and therapeutic efficacy of encapsulated anthracyclines. J Drug Target. 3: 391-8, 1996.

Gabizon, A., Price, D.C., Huberty, J., Bresalier, R.S., and Papahadjopoulos, D.: Effect of liposome composition and other factors on the targeting of liposomes to experimental tumors: biodistribution and imaging studies. Cancer Res. 50: 6371-6378, 1990.

Gabizon, A., Dagan, A., Goren, D., Barenholz, Y., and Fuks, Z.: Liposomes as in vivo carriers of adriamycin: reduced cardiac uptake and preserved anti-tumour activity in mice. Cancer Res. 42: 4734-4739, 1982.

Gabizon, A., Goren, D., Barenholz, Y., Dagan, A., and Meshorer, A.: Enhancement of adriamycin delivery to liver metastatic cells with increased tumouricidal effect using liposomes as drug carriers. Cancer Res. 43: 4730-4735, 1983.

Gabizon, A.A.: Selective tumour localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. Cancer Res., 52: 891-896, 1992.

Gabr, A., Kuin, A., Aalders, M., El-Gawly, H., and Smets, L.A.: Cellular pharmacokinetics and cytotoxicity of camptothecin and topotecan at normal and acidic pH. Cancer Res. 57: 4811-6, 1997.

Gall, J.A. and Bhathal, P.S.: A quantitative analysis of the liver following ligation of the common bile duct. Liver 10:116-125, 1990.

Goormaghtigh, E., Brasseur, R., Huart, P., and Ruysschaert, J.M.: Study of the adriamycin-cardiolipin complex structure using attenuated total reflection infrared spectroscopy. Biochemistry. 26: 1789-94, 1987.

Grant, C.W., Hamilton, K.S., Hamilton, K.D., and Barber, K.R.: Physical biochemistry of a liposomal amphotericin B mixture used for patient treatment. Biochim Biophys Acta. 984: 11-20, 1989.

Grayhill, J.R., Craven, P.C., Taylor, R.L., Williams, D.M., and Magee, W.E.: Treatment of murine cryptococcosis with liposome-associated amphotericin B. J Infect Dis. 145: 748-52, 1982.

Haran, G., Cohen, R., Bar, L. K., and Barenholz, Y.: Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim. Biophys. Acta 1151: 201-215, 1993.

Harasym, T.O., Tardi, P., Longman, S.A., Ansell, S.M., Bally, M.B., Cullis, P.R., and Choi, L.S.L.: Poly(ethylene glycol)-modified phospholipids prevent aggregation during covalent conjugation of proteins to liposomes. Bioconj. Chem. 6, 187–194, 1995.

Harasym, T.O., Cullis, P.R., and Bally, M.B.: Intratumour distribution of doxorubicin following i.v. administration of drug encapsulated in egg phosphatidylcholine/cholesterol liposomes. Cancer Chemother Pharmacol. 40: 309-17, 1997a.

Harasym, T.O.: Antibody targeted liposomal systems. Ph.D. Thesis, U.B.C., 1997b.

Harris, A.L., Cantwell, B.M.J., Carmichael, J., Wilson, R., and Farndon, J.: Comparison of short-term and continuous chemotherapy (mitoxantrone) for advanced breast cancer. Lancet 335: 186-190, 1990.

Harrison, M. Tomlinson, D., and Stewart, S. (1995) Liposomal-entrapped doxorubicin - an active agent in AIDS-related Karposi's sarcoma. J. Clin. Oncol. 13, 914-920.

Herman, E. H., Rahman, A., Ferrans, V. J., Vick, J. A., and Schein, P. S.: Prevention of chronic doxorubicin cardiotoxicity in beagles by liposomal encapsulation. Cancer Res. 43: 5427-5432, 1983.

Hernandez-Caselles, T., Villalain, J., and Gomez-Fernandez, J.C.: Influence of liposome charge and composition on their interaction with human blood serum proteins. Mol Cell Biochem. 120: 119-26, 1993.

Heuser, L.S. and Miller, F.N.: Differential macromolecular leakage from the vasculature of tumours. Cancer 57: 461-464, 1986.

Hiddemann, W., Aul, C., Maschmeyer, G., Schonrock-Nabulsi, R., Ludwig, W.D., Bartholomaus, A., Bettelheim, P., Becker, K., Balleisen, L., Lathan, B. et al.: High-dose versus intermediate-dose cytarabine combined with mitoxantrone for the treatment of relapsed and refractory acute myeloid leukemia: results of an age-adjusted randomized comparison. Semin Hematol. 28: 35-8, 1991.

Hinkle, G.H., Born, G.S., Kessler, W., and Shaw, S.M.: Preferential localization of radiolabeled liposomes in liver. J Pharm Sci. 67: 795-8, 1978.

Hirsimaki, P. and Pilstrom, L.: Studies on vinblastine-induced autophagocytosis in mouse liver. III A quantitative study. Virchows Arch B Cell Pathol Incl Mol Pathol. 41: 51-66, 1982.

Ho, A.D., Del Valle, F., Engelhard, M., Hiddemann, W., Ruckle, H., Schlimok, G., Haas, R., Thiel, E., Andreesen, R., Fiedler W., et al.: Mitoxantrone/high-dose Ara-C and recombinant human GM-CSF in the treatment of refractory non-Hodgkin's lymphoma. A pilot study. Cancer. 66: 423-30, 1990.

Holland, J.W., Cullis, P.R., and Madden, T.D.: Poly(ethylene glycol)-lipid conjugates promote bilayer formation in mixtures of non-bilayer-forming lipids. Biochemistry. 35: 2610-7, 1996a.

Holland, J.W., Hui, C., Cullis, P.R., and Madden, T.D.: Poly(ethylene glycol)--lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. Biochemistry. 35: 2618-24, 1996b.

Holtrop, S., Rijke-Schilder, G.P., Tamboer, W.P., Koene, R.A., and Tax, W.J.: Removal of monocytes from cell suspensions with anti-CD14 antibody and carbonyl-iron, using Fc gamma R-dependent accessory function as a sensitive measure of monocyte presence. J Immunol Methods. 156: 217-22, 1992.

Honig, M.G. and Hume, R.I.: Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. J Cell Biol. 103: 171-87, 1986.

Hope, M. J., Bally, M. B., Mayer, L. D., Janoff, A. S., and Cullis, P. R.: Generation of multilamellar vesicles. Chem. Phys. Lipids 40, 89-107, 1986.

Hope, M.J., Bally, M.B., Webb, G., and Cullis, P.R.: Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochim. Biophys. Acta. 812: 55-65, 1985.

Hu, Q. and Liu, D.: Co-existence of serum-dependent and serum-independent mechanisms for liposome clearance and involvement of non-Kupffer cells in liposome uptake by mouse liver. Biochim Biophys Acta. 1284: 153-61, 1996.

Huang, C., Li, S., Wang, Z.Q., and Lin, H.N.: Dependence of the bilayer phase transition temperatures on the structural parameters of phosphatidylcholines. Lipids. 28: 365-70, 1993.

Hubbell, W.L. and McConnell, H.M.: Molecular motion in spin-labeled phospholipids and membranes. J. Am. Chem. Soc., 93: 314-326, 1971.

Hume, D.A., Perry, H., and Gordon, S.: The mononuclear phagocyte system of the mouse defined by immunohistochemical localisation of antigen F4/80: Macrophages associated with epithelia. The Anatomical Record. 210: 503-512, 1984.

Hwang, K.J. and Beaumier, P.L.: Kinetic evidences of enhancing interaction of small liposomes with hepatic parenchymal cells mediated by large liposomes blockade. Res Commun Chem Pathol Pharmacol. 54: 417-20, 1986.

Hwang, K.J.: Liposome Pharmacokinetics. In Liposomes From Biophysics to Therapeutics, ed. by M. J. Ostro, pp 109-156, Marcel Dekker, New York, NY, 1987.

Hwang, K.J., Padki, M.M., Chow, D.D., Essisn, H.E., Lai, J.Y., and Beaumier, P.L.: Uptake of small liposomes by nonreticuloendothelial tissues. Biochim, Biophys. Acta. 901: 88-96, 1987.

Ishiwata, H., Sato, S.B., Vertut-Doi, A., Hamashima, Y., and Miyajima, K.: Cholesterol derivative of poly(ethylene glycol) inhibits clathrin-independent, but clathrin-dependent endocytosis. Biochim Biophys Acta. 1359: 123-135, 1997.

Jackson, D.V., Case, L.D., Pope, E.K., White, D.R., Spurr, C.L., Richard, F., Stuart, J.J., Muss, H.B., Cooper, M.R., Black, W.R., et al.: Single agent vincrisitine by infusion in refractory multiple myeloma. J. Clin Oncol. 3: 1508-12, 1985.

Jackson, D.V., Powell, B.L., Cruz, J.M., Spurr, c.L., and Muss, H.B.: Infusion of etoposide and vincristine in non-Hodgkin's lymphoma. Sel Cancer Ther. 5: 129-136, 1989.

Jain, R.K. and Baxter, L.T.: Mechanisms of heterogeneous distribution of monoclonal antibodies and other macromolecules in tumours: significance of elevated interstitial pressure. Cancer Res. 48: 7022-7032, 1988.

Jain, R.K.: Transport of molecules across tumour vasculature. Cancer Metastasis Rev. 6: 559-93, 1987.

Janoff, A.S., Boni, L.T., Popescu, M.C., Minchey, S.R., Cullis, P.R., Madden, T.D., Taraschi, T., Gruner, S.M., Shyamsunder, E., Tate, M.W. et al.: Unusual lipid structures selectively reduce the toxicity of amphotericin B. Proc Natl Acad Sci U S A. 85: 6122-6, 1988.

Johnson, R.K., Zee-Cheng, R.K.-Y., Lee, W.W., Acton, E.M., Henry, D.W.: Experimental antitumour activity of aminoanthraquinones. Cancer Treatment Reports. 63: 425-439, 1979.

Kakinuma, K., Tanaka, R., Takahashi, H., Watanabe, M., Nakagawa, T., and Kuroki, M. Targeting chemotherapy for malignant brain tumour using thermosensitive liposome and localized hyperthermia. J Neurosurg. 84: 180-4, 1996.

Kanter, P. M., Klaich, G.M., Bullard, G.A., King, J.M., Bally, M.B., and Mayer, L.D.: Liposome encapsulated vincristine: preclinical toxicologic and pharmacologic comparison with free vincristine and empty liposomes in mice, rats, and dogs. Anti-Cancer Drugs. 5: 579-590, 1994.

Kim, S.: Liposomes as Carriers of Cancer Chemotherapy: current status and future prospects. Drugs, 46: 618-638, 1993.

Kirby, C., Clarke, J., and Gregoriadis, G. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. Biochem. J. 186, 591-598, 1980.

Kirpotin, D., Hong, K.L., Mullah, N., Papahadjopoulos, D., and Zalipsky, S.: Liposomes with detachable polymer coating - destabilization and fusion of dioleoylphosphatidylethanolamine vesicles triggered by cleavage of surface-grafted poly(ethylene glycol) FEBS Lett. 388: 115-118, 1996.

Klaunig, J.E., Goldblatt, P.J., Hinton, D.E., Lipsky, M.M., Chacko, J., and Trump, B.F.: Mouse liver cell culture. I. Hepatocyte isolation. In Vitro. 17: 913-25, 1981.

Kling, D., Holzschuh, T., Strohschneider, T., and Betz, E.: Enhanced endothelial permeability and invasion of leukocytes into the artery wall as initial events in experimental arteriosclerosis. Int Angiol. 6: 21-8, 1987.

Kohn, S., Nagy, J.A., Dvorak, H.F., and Dvorak, A.M.: Pathways of macromolecular tracer transport across venules and small veins. Structural basis for the hyperpermeability of tumour blood vessels. Lab Invest. 67: 596-607, 1992.

Konno, H., Maruo, Y., Matsuda, I., Nakamura, S., and Baba, S.: Intra-arterial liposomal adriamycin for metastatic adenocarcinoma of the liver. Eur. Surg. Res. 27: 301-3016, 1995.

Krause, H.J. and Juliano, R.L.: Interactions of liposome-incorporated amphotericin B with kidney epithelial cell cultures. Mol Pharmacol. 34: 286-97, 1988.

Kruyjf, B. de, Demel, R.A., Slotboom, A.J., Deenen, L.L. van, and Rosenthal, A.F.: The effect of the polar headgroup on the lipid-cholesterol interactions: a monolayer and differential scanning calorimetry study. Biochim. Biophys. Acta. 307: 1-19, 1973.

Lai, K.H., Tsai, Y.T., Lee, S.D., Ng, W.W., Teng, H.C., Tam, T.N., Lo, G.H., Lin, H.C., Lin, H.J., Wu, J.C. et al.: Phase II study of mitoxantrone in unresectable primary hepatocellular carcinoma following hepatitis B infection. Cancer Chemother Pharmacol. 23: 54-6, 1989.

Lasic, D.D., Frederik, P.M., Stuart, M.C., Barenholz, Y., and McIntosh, T.J.: Gelation of liposome interior. A novel method for drug encapsulation. FEBS Lett. 312: 255-8, 1992.

Law, S.L., Ho, C.K., Jang, T.F., Chang, P., and Lin, F.M.: Anti-tumour effect of mitoxantrone-containing liposomes. Int J of Pharm. 128: 139-143, 1996.

Lee, C.L. and Ko, Y.C.: Hepatitis B vaccination and hepatocellular carcinoma in Taiwan. Pediatrics. 99: 351-3, 1997.

Lee, R.J. and Low, P.S.: Delivery of liposomes in cultured KB cells via folate receptor mediated endocytosis. J. Biol. Chem. 25:24847-24854, 1993.

Lee, R.J. and Low, P.S.: Folate mediated tumour cell targeting of liposome entrapped doxorubicin in vitro. Biochim. Biophys. Acta. 1233:134-144, 1994.

Lee, S., Starkey, P.M., and Gordon, S.: Quantitative analysis of total macrophage content in adult mouse tissues: Immunochemical studies with monoclonal antibody F4/80. J. Exp. Med. 161: 475-489, 1985.

Lepore, L., Pennesi, M., Barbi, E., and Pozzi, R.: Treatment and prevention of osteoporosis in juvenile chronic arthritis with disodium clodronate. Clin Exp Rheumatol. 9: 33-5, 1991.

Leserman, L.D., Machy, P., and Barbet, J.: Cell-specific drug transfer from liposomes bearing monoclonal antibodies. Nature (London) 293:, 226-228, 1981.

Lewis, R.N.A.H., Mak, N., and McElhaney, R.: A differential scanning calorimetric study of the thermotropic phase behavior of model membranes composed of phosphatidylcholines containing linear saturated fatty acyl chains. Biochemistry, 26: 6118-6126, 1987.

Li, Y.P., Mold, C., and Du Clos, T.W.: Sublytic complement attack exposes C-reactive protein binding sites on cell membranes. J. Immunol. 152: 2995-3005, 1994.

Lichtenberg, D. and Barenholz, Y.: Liposomes: preparation, characterization, and preservation. Methods Biochem Anal. 33: 337-462, 1988.

Lichtenberg, D. and Markello, T. Structural characteristics of phospholipid multilamellar liposomes. J Pharm Sci. 73: 122-5, 1984.

Lim, H.J, Masin, D., Madden, T.D., and Bally, M.B.: Influence of drug release characteristics on the therapeutic activity of liposomal mitoxantrone. J. Pharmacol. Exp. Ther. 281: 566-573, 1997.

Liu, S., Ishida, T., and Kiwada, H.: Effect of serum components from different species on destabilizing hydrogenated phosphatidylcholine-based liposomes. Biol Pharm Bull. 20: 874-80, 1997.

Livingston, R. B.: Dose intensity and high dose therapy. Cancer 74: 1177-1183, 1994.

Longman, S.A., Tardi, P.G., Parr, M.J., Choi, L., Cullis, P.R., and Bally, M.B.: Accumulation of protein-coated liposomes in an extravascular site: influence of increasing carrier circulation lifetimes. J Pharmacol Exp Ther. 275: 1177-84, 1995.

Madden, T.D., Harrigan, P.R., Tai, L.C.L., Bally, M.B., Mayer, L.D., Redelmeier, T.E., Loughrey, H.C., Tilcock, C.P.S., Reinsih, L.W., and Cullis, P.R.: The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: A survey. Chem. Phys. Lipids. 53: 37-46, 1990.

Madden, T.D., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P., and Janoff, A.S.: Protection of large unilamellar vesicles by trehalose during dehydration: retention of vesicle contents. Biochim Biophys Acta. 817: 67-74, 1985.

Magin, R.L., Hunter, J.M., Niesman, M.R., and Bark, G.A.: Effect of vesicle size on the clearance, distribution, and tumour uptake of temperature-sensitive liposomes. Cancer Drug Deliv. 3: 223-237, 1986.

Malinski, J.A. and Nelestuen, G.L.: Membrane permeability to macromolecules mediated by the membrane attack complex. Biochemistry 28: 61-70, 1989.

Mayer L.D., Cullis, P.R. and Bally, M.B.: The use of transmembrane pH gradient-driven drug encapsulation in the pharmacodynamic evaluation of liposomal doxorubicin. J. Liposome Res. 4: 529-553, 1994.

Mayer, L.D., Bally, M.B., Cullis, P.R., Wilson, S.L., and Emerman, J.T.: Comparison of free and liposome encapsulated doxorubicin tumour drug uptake and antitumour efficacy in the SC115 murine mammary tumour. Cancer Lett. 53: 183-90, 1990.

Mayer, L.D., Tai, L.C.L., Ko, D.S.C., Masin, D., Ginsberg, R.S., Cullis, P.R., and Bally, M.B.: Influence of vesicle size, lipid composition, and drug to lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Res., 49: 5922-5930, 1989.

Mayer, L.D., Dougherty, G., Harasym, T.O., Bally, M.B.: The role of tumour associated macrophages in the delivery of liposomal doxorubicin to solid murine fibrosarcoma tumours. J Pharmaol Exp Ther 280: 1406-1414, 1997.

Mayer, L.D., Nayar, R., Thies, R.L., Boman, N.L., Cullis, P.R., and Bally, M.B.: Identification of vesicle properties that enhance the antitumour activity of liposomal vincristine against murine L1210 leukemia. Cancer Chemother Pharmacol. 33: 17-24, 1993.

Mayer, L.D., Hope, M.J., and Cullis, P.R.: Vesicles of variable sizes produced by a rapid extrusion procedure. Biochim Biophys Acta. 858: 161-8, 1986.

Mayer, L., D., Hope, M. J., Cullis, P. R., and Janoff, A. S.: Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. Biochim. Biophys. Acta 817, 193-196, 1985a.

Mayer, L.D., Bally, M.B., Hope, M.J., and Cullis, P.R.: Uptake of antineoplastic agents into large unilamellar vesicles in response to a membrane potential. Biochim. Biophys. Acta. 816: 294-302, 1985b.

Mayhew, E.G., Goldrosen, M.H., Vaage, J., and Rustum, Y.M.: Effects of liposome-entrapped doxorubicin on liver metastases of mouse colon carcinomas 26 and 38. J Natl Cancer Inst. 78:707-13, 1987.

McElhaney, R.N. The use of differential scanning calorimetry and differential thermal analysis in studies of model and biological membranes. Chem. Phys. Lipids 30: 229-259, 1982.

Meijer, D.K., Mol, W.E., Muller, M., and Kurz, G.: Carrier-mediated transport in the hepatic distribution and elimination of drugs, with special reference to the category of organic cations. J Pharacokinet Biopharm 18: 35-70, 1990.

Mewes, K., Blanz, J., Ehninger, G., Gebhardt, R., and Zeller, K.P.: Cytochrome P-450 induced cytotoxicity of mitoxantrone by formation of electrophilic intermediates. Cancer Res. 53: 5135-5142, 1993.

Minow, R.A., Benjamin, R.S., and Gottlieb, J.A.: Adriamycin (NSC-123127) cardiomyopathy - an overview with determination of risk factors. Cancer Chemother. Rep., 6: 195-201, 1975.

Moghimi, S. M. and Patel, H. M. Serum opsonins and phagocytosis of saturated and unsaturated phospholipid liposomes. Biochim. Biophys. Acta 984, 384-387, 1989.

Mold, C., Rodgers, C.P., Richards, R.L., Alving, C.R., and Gewurz, H.: Interaction of C-reactive protein with liposomes. III. Membrane requirements for binding. J Immunol. 126: 856-60, 1981.

Morrow, M.R., Whitehead, J.P., and Lu. D.: Chain-length dependence of lipid bilayer properties near the liquid crystal to gel-phase transition. Biophys J. 63:18-27, 1992.

Mui, B.L., Cullis, P.R., Pritchard. P.H., and Madden, T.D.: Influence of plasma on the osmotic sensitivity of large unilamellar vesicles prepared by extrusion. J Biol Chem. 269: 7364-70, 1994.

Mulder, P.H. and de Wit, R.: Experience with dose and schedule variations in germ cell tumours. Anticancer Drugs. 6 Suppl 5: 43-52, 1995.

Nagle, J.F. Theory of lipid monolayer and bilayer phase transitions: effect of headgroup interactions. J Membr Biol. 27: 233-50, 1976.

Nagy, J.A., Herzberg, K.T., Masse, E.M., Zientara, G.P., and Dvorak, H.F.: Exchange of macromolecules between plasma and peritoneal cavity in ascites tumour-bearing, normal, and serotonin-injected mice. Cancer Res., 49:5448-5458, 1989.

Neidhart, J.A., Gochnour, D., Roach, R., Hoth, D., and Young, D.: A comparison of mitoxantrone and doxorubicin in breast cancer. J. Clin. Oncol. 4: 672-677, 1986.

Noda, Y., Kaiya, T., Kohda, K., and Kawazoe, Y.: Enhanced cytotoxicity of some triterpenes toward leukemia L1210 cells cultured in low pH media: possibility of a new mode of cell killing. Chem Pharm Bull. 45: 1665-70, 1997.

Northfelt, D.W., Dezube, B.J., Thommes, J.A., Levine, R., Von Roenn, J.H., Dosik, G.M., Rios, A., Krown, S.E., DuMond, C., and Mamelok, R.D.: Efficacy of pegylated-liposomal doxorubicin in the treatment of AIDS-related Kaposi's sarcoma after failure of standard chemotherapy. J Clin Oncol. 15: 653-9, 1997.

Ogihara-Umeda, I., Sasaki, T., Toyama, H., Oda, K., Senda, M., and Nishigori, H.: Rapid tumour imaging by active background reduction using biotin-bearing liposomes and avidin. Cancer Res., 54: 463-467, 1994.

Ogiwara, M.: Clearance and maximum removal rate of liposomes in normal and impaired liver of rat. Gastroenerol. Jpn. 19: 34-40, 1984.

Ohsawa, T., Miura, H., and Harada, K.: Evaluation of a new liposome preparation technique, the freeze-thawing method, using L-asparaginase as a model drug. Chem Pharm Bull. 33: 2916-23, 1985.

Oja, C.D., Semple, S.C., Chonn, A., and Cullis, P.R.: Influence of dose on liposome clearance: critical role of blood proteins. Biochim Biophys Acta. 1281: 31-7, 1996.

Oku, N., Tokudome, Y., Namba, Y., Saito, N., Endo, M., Hasegawa, Y., Kawai, M., Tsukada, H., and Okada, S.: Effect of serum protein binding on real-time trafficking of liposomes with different charges analyzed by positron emission tomography. Biochim Biophys Acta. 1280: 149-54, 1996.

Okuno, K.Nakamura, K., Tanaka, A., Yachi, K., and Yasutomi, M.: Hepatic immunopotentiation by galactose-entrapped liposomal IL-2 compound in the treatment of liver metastases. Surg. Today 28: 64-69, 1998.

Olson, F., Meyhew, E., Maslow, D., Rustums, Y., and Szoka, F.: Characterization, toxicity, and therapeutic activity of adriamycin encapsulated in liposomes. Eur J Cancer. 18: 167-175. 1982.

Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J., and Papahadjopoulos, D.: Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim Biophys Acta. 557: 9-23, 1979.

Ostro, M.J. and Cullis, P.R.: Use of liposomes as injectable-drug delivery systems. Am J Hosp Pharm. 46: 1576-87, 1989.

O'Sullivan, M.M., Powell, N., French, A.P., Williams, K.E., Morgan, J.R., and Williams. B.D.: Inflammatory joint disease: a comparison of liposome scanning, bone scanning and radiography. Ann. Rheum. Dis. 47: 485-491, 1988.

Ouyang, C., Choice, E., Holland, J., Meloche, M., and Madden, T.D.: Liposomal cyclosporine. Characterization of drug incorporation and interbilayer exchange. Transplantation. 60: 999-1006, 1995.

Papahadjopoulos, D., Allen, T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K., Lee, K.D., Woodle, M.C., Lasic, D.D., Redemann, C., and Martin, F.J.: Sterically stabilized liposomes: improvements in pharmacokinetics and anti-tumour activity. Proc. Natl. Acad. Sci., U.S.A. 88: 11460-11464, 1991.

Papahadjopoulos, D., Jacobson, K., Nir, S., and Isac, T.: Phase transitions in phospholipid vesicles. Fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. Biochim Biophys Acta. 311: 330-48, 1973.

Parr, M.J., Masin, D., Cullis, P.R., and Bally, M.B.: Accumulation of liposomal lipid and encapsulated doxorubicin in murine Lewis lung carcinoma: the lack of beneficial effects by coating liposomes with poly(ethylene glycol). J Pharmacol Exp Ther. 280: 1319-27, 1997.

Parr, M.J., Ansell, S.M., Choi, L.S., and Cullis, P.R.: Factors influencing the retention and chemical stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles. Biochim Biophys Acta. 1195: 21-30, 1994.

Parr, M.J.: Circulation lifetimes and tumour accumulation of liposomal drug delivery systems. Ph.D. Thesis, U.B.C., 1995.

Parr, M.J., Bally, M.B., and Cullis, P.R.: The presence G_{M1} in liposomes with entrapped doxorubicin does not prevent RES blockade. Biochim Biophys Acta 1168: 249-252, 1993.

Patel, H.M., Tuzel, N.S., and Ryman. B.E.: Inhibitory effect of cholesterol on the uptake of liposomes by liver and spleen. Biochim Biophys Acta. 761: 142-51, 1983.

Paulsen, D.F.: Basic histology. Third edition. Appleton and Lange. Stamford. 1996.

Pearson, J.D.: Endothelial cell biology. Radiology. 179: 9-14, 1991.

Perchellet, J.P., Newell, S.W., Ladesich, J.B., Perchellet. E.M., Chen, Y., Hua, D.H., Kraft, S.L., Basaraba, R.J., Omura, S., and Tomoda, H.: Anti-tumour activity of novel tricyclic pyrone analogs in murine leukemia cells in vitro. Anticancer Res. 17: 2427-34, 1997.

Perez-Soler, R., Lopez-Berestein, G., Lautersztain, J., al-Baker, S., Francis, K., Macias-Kiger, D., Raber, M.N., and Khokhar, A.R.: Phase I clinical and pharmacological study of liposome-entrapped cis-bis-neodecanoato-trans-R.R-1,2-diaminocyclohexane platinum(II). Cancer Res. 50: 4254-9, 1990.

Perez-Soler, R.: Liposomes as carriers of antitumour agents: toward a clinical reality. Cancer Treat. Rev. 16: 67-82, 1989.

Perkins, W.R., and Minchey, S.R., Ostro, M.J., Taraschi, T.F., and Janoff, A.S.: The captured volume of multilamellar vesicles. Biochim. Biophys. Acta. 943: 103-107, 1988.

Pestalozzi, B.C., Vass, A., Adam, H., Horber, D.H., Schwendener, R.A., and Sauter, C.: Phase II study of liposome-complexed mitoxantrone in patients with advanced breast cancer. Eur J Cancer. 31A: 1024, 1995.

Pluschke, G. and Overath P.: Function of phospholipids in Escherichia coli. Influence of changes in polar head group composition on the lipid phase transition and characterization of a mutant containing only saturated phospholipid acyl chains. J Biol Chem. 256: 3207-12, 1981.

Proffitt, R.T., Williams, L.E., Presant, C.A., Tin, G.W., Uliana, J.A., Gamble, R.C., and Baldeschwieler, J.D.: Tumour-imaging potential of liposomes loaded with In-111-NTA: biodistribution in mice. J. Nuc. Med. 24, 45–51, 1983.

Rahman, Y.E., Cerny, E.A., Patel, K.R., Lau, E.H., and Wright, B.J.: Differential uptake of liposomes varying in size and lipid composition by parenchymal and kupffer cells of mouse liver. Life Sci. 31: 2061-71, 1982.

Rajagopalan, S., Politi, P.M., Sinha, B.K., and Meyers, C.E.: Adriamycin-induced free radical formation in the perfused rat heart: applications for cardiotoxicity. Cancer Res., 48: 4766-4769, 1988.

Rammirez, L.H., Zhao, Z., Rougier, P., Bognel, C., Dzodic, R., Vassal, G., Ardouin, P., Gouyette, A., and Munck, J.N.: Pharmacokinetics and anti-tumour effects of mitoxantrone after intratumoural of intraarterial administration in rabbits. Cancer Chemother Pharmacol 37: 371-376, 1996.

Reinish, L.W., Bally, M.B., Loughrey, H.C., and Cullis, P.R.: Interactions of liposomes and platelets. Thromb Haemost. 60: 518-23, 1988.

Richards, R.L., Bewurz, H., Siegel, J., and Alving, C.R.: Interactions of C-reactive protein and complement with liposomes. II. Influence of membrane composition. J. Immunol, 122:1185-1189, 1979.

Richards, R.L., Gewurz, H., Osmand, A.P., and Alving, C.R.: Interactions of C-reactive protein and complement with liposomes. Proc. Natl. Acad. Sci. U.S.A. 74: 5672-5676, 1977.

Richardson, V.J., Ryman, B.E., Jewkes, R.F., Jeyasingh, K., Tattersall, M.N., Newlands, E.S., and Kaye, S.B.: Tissue distributio and rumor localization of 99m-technetium labeled liposomes in cancer patients. Br. J. Cancer 40, 35–43, 1979.

Riley, P. and Hanzlik, R.P.: Electron transfer in P450 mechanisms. Microsomal metabolism of cyclopropylbenezene and p-cycloporpylanisole. Xenobiotica. 24: 1-16, 1994.

Robertson, J.F.R., Williams, M.R., Todd, J.H., and Blamey, R.W.: Mitoxantrone – a useful palliative therapy in advanced breast cancer. American Journal of Clinical Oncology – Cancer Clinical Trials. 12: 393-396, 1989.

Rogers, M.J. and Strittmatter ,P.: The interaction of NADH-cytochrome b5 reductase and cytochrome b5 bound to egg lecithin liposomes. J Biol Chem. 250: 5713-8, 1975.

Ryan, U.S.: Phagocytic properties of endothelial cells. In Endothelial cells. Ed. U.S. Ryan pp. 33-49, Boca Raton, Florida, 1988.

Safa, A.R., Chegini, N., and Tseng, M.T.: Influence of mitoxantrone on nucleic acid synthesis on the T47D breast tumour cell line. J. Cell. Biochem. 22: 111-120, 1983.

Schabel Jr., F.M., Corbett, T.H., Griswold Jr., D.P., Laster Jr., W.R., and Trader, M.W.: Therapeutic activity of mitoxantrone and ametantrone against murine tumours. Cancer Treatment Reviews 10 Suppl. B: 13-21, 1983a.

Schabel Jr., F.M., Skipper, H.E., Trader, M.W., Laster Jr., W.R., and Griswold Jr., D.P.: Establishment of cross-resistance profiles for new agents. Cancer Treatment Reports. 67:905-922, 1983b.

Scherphof, G.L. and Kamps, J.A.A.M.: Receptor versus non-receptor mediated clearance of liposomes. Advanced Drug Delivery Reviews 32:81-97, 1998.

Scherphof, G.L., Spanjer, H. H., Derksen, J.T.P., Kuipers, F., Vonk, R.J., Daemen, T., and Roerdink, F.H.: Delivery of liposome-associated drugs to liver cells. Biochemical Society Transactions. 15: 345-348, 1987.

Scherphof, G.L., Kamps, J.A.A.M., and Koning, G.A.: In vivo targeting of surface-modified liposome to metastatically growing colon carcinoma cells and sinusoidal endothelial cells in the rat liver. J. Liposome Res. 7:419-432, 1997.

Scherphof, G., Morselt, H., Regts, J., and Wilschut, J.C.: The involvement of the lipid phase transition in the plasma induced dissolution of multilamellar phosphatidylcholine vesicles. Biochim Biophys Acta. 556: 196-207, 1979.

Scherphof, G., Roerdink, F., Waite, M., and Parks, J.: Disintegration of phosphatidylcholine liposomes in plasma as a result of interaction with high-density liposomes. Biochim Biophys Acta. 542: 296-307, 1978.

Schrenk, D., Michalke, A., Grant, T.W., Brown, P.C., Silverman, J.A., and Thorgeirsson, S.S.: Multidrug resistance gene expression in rodents and rodent hepatocytes treated with mitoxantrone. Biochem Pharmacol 52: 1453-1460, 1996.

Schrenk, D., Grant, T.W., Presegger K.H., Silverman, J.A., Marino, P.A., and Thorgeirsson, S.S.: Induction of multidrug resistance gene expression during cholestasis in rats and nonhuman primates. Hepatology 17: 854-860, 1993.

Schwendener, R.A., Horber, D.H., Rentsch, K., Hanseler, E., Pestalozzi, B., and Sauter, C.: Preclinical and clinical experience with liposome-encapsulated mitoxantrone. J. Liposome Res.. 4: 605-639, 1994.

Schwendener, R.A., Fiebig, H.H., Berger, M.R., and Berger, D.P.: Evaluation of incorporation characteristics of mitoxantrone into unilamellar liposomes and analysis of their pharmacokinetics properties, acute toxicity, and antitumour efficacy. Cancer Chemother. Pharmacol., 27: 429-439, 1991.

Seltzer, S.E., Gregoriadis, G., and Dick, R.: Evaluation of the dehydration-rehydration method for production of contrast-carrying liposomes. Invest. Radiol. 23:131-138, 1988.

Semple, S.C., Chonn, A., and Cullis, P.R.: Influence of cholesterol on the association of plasma proteins with liposomes. Biochemistry. 35: 2521-5, 1996.

Senger, D.R., Van De Water, L., Brown, L.F., Nagy, J.A., Yeo, K.T., Yeo, T.K., Berse, B., Jackman, R.W., Dvorak, A.M., and Dvorak, H.F.: Vascular permeability factor (VPF, VEGF) in tumour biology. Cancer and Metastasis Reviews 12: 303-324, 1993.

Shapiro, A.B. and Ling, V.: Reconstitution of drug transport by purified P-glycoprotein. J Biol Chem. 270: 16167-75, 1995.

Shew, RL. and Deamer, D.W.: A novel method for encapsulation of macromolecules in liposomes. Biochim Biophys Acta. 816: 1-8, 1985.

Shinozawa, S., Araki, Y., and Oda, T.: Tissue distribution and antitumour effect of liposome-entrapped doxorubicin (adriamycin) in Ehrlich solid tumour-bearing mouse. Acta Med Okayama. 35: 395-405, 1981.

Shiratori, Y., Tananka, M., Kawase, T., Shiina, S., Komatsu, Y., and omata, M.: Quantification of sinusoidal cell function in vivo. Semin Liver Dis. 13: 39-49, 1993.

Shiver, J.W., Dankert, J.R., and Esser, A.F.: Formation of ion-conducting channels by the membrane attack complex proteins of complement. Biophys. J. 60: 761-769, 1991.

Silversmith, R.E. and Nelsestuen, G.L.: Assembly of the membrane attack complex of complement on small unilamellar phospholipid vesicles. Biochemistry 25: 852-860, 1986a.

Silversmith, R.E. and Nelsestuen, G.L.: Interaction of complement proteins C5b-6 and C5b-7 with phospholipid vesicles: effects of phospholipid structural features. Biochemistry 25: 7717-7725, 1986b.

Simionescu, M., et al. (1987) News. Physiol. Sci. 2, 97-100.

Simionescu, N.: Cellular aspects of transcapillary exchange. Physiol. Rev. 63:1536-1579, 1993.
Smith, I.E.: Mitoxantrone (novantrone): a review of experimental and early clinical studies. Cancer Treat Rev. 10: 103-15, 1983.

Smith, I.E., Stuart-Harris, R., Pavlidis, N., Bozek, T.: Mitoxantrone (Novantrone) as a single agent and in combination chemotherapy in the treatment of advanced breast cancer. Cancer Treat. Rev. 10(Suppl. B): 37-40, 1983.

Sorace, J.M., Rollins, S., Aniagolu, J.U., Mergner, W.J., Cole, K., Swartz, G.M. Jr., and Green, S.J.: Role of atheroma liposomes and malondialdehyde-modified low-density lipoproteins in complement activation. Pathobiology 64: 73-78, 1996.

Sogor, B.V. and Zull, J.E.: Studies of a serum albumin-liposome complex as a model lipoprotein membrane. Biochim Biophys Acta. 375: 363-80, 1975.

Spanjer, H.H., van Galen, M., Roerdink, F.H., Regts, J. and Scherphof, G.L.: Intrahepatic distribution of small unilamellar liposomes as a function of liposomal lipid composition. Biochim Biophys Acta. 863: 224-30, 1986.

Stein, Y., Halperin, G. and Stein, O.: Biological stability of [^{3}H] cholesteryl oleyl ether in cultured fibroblasts and intact rat. FEBS Lett., 111: 104-106, 1980.

Stein, I., Neeman, M., Shweiki, D., Itin, A., and Keshet, E.: Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregualtion with other ischemia-induced genes. Mol Cell Biol 15: 5363-5369, 1995.

Storm, G., Steerenberg, P.A., Emmen, F., van Borssum Waalkes, M., and Crommelin, D.J.: Release of doxorubicin from peritoneal macrophages exposed in vivo to doxorubicin-containing liposomes. Biochim Biophys Acta. 965:136-45, 1988.

Sugarman, S.M. and Perez-Soler, R.: Liposomes in the treatment of malignancy: a clinical perspective. Critical Reviews in Oncology/Hematology, 12: 231-242, 1992.

Swenson, C.E., Popescu, M.C., and Ginsberg, R.S.: Preparation and use of liposomes in the treatment of microbial infections. Crit Rev Microbiol. 15 Suppl 1: S1-31, 1988.

Szoka, F., and Papahadjopoulos. D.: Comparative properties and methods of preparation of lipid vesicles (liposomes). Ann. Rev. Biophys. Bioeng. 9, 467-508, 1980.

Szoka, F. and Papahadjopoulos. D.: Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. U. S. A. 75, 4194-4198, 1978.

Takagi, H., King, G.L., Ferrara, N., and Aiello. L.P.: Hypoxia regultes vascular endothelial growth factor receptor KDR/Flk gene expression through adenosine A2 receptors in retinal capillary endothelial cells. Invest Opth Visual Sci. 37: 1311-1321, 1996.

Tannock, I.F., Osoba. D., Stockler. M.R., Ernst. D.S., Neville. A.J., Moore. M.J., Armitage. G.R., Wilson, J.J., Venner, P.M., Coppin, C.M., and Murphy, K.C.: Chemotherapy with mitoxantrone plus prednisone or prednisone alone for symptomatic hormone-resistant prostate cancer: a Canadian randomized trial with palliative end points. J Clin Oncol. 14: 1756-64, 1996.

Taylor, K.M.G., Taylor, G., Kellaway, I.W., and Stevens, J.: Int. Journal of Pharmaceutics 58: 49-55, 1990.

ten Bokkel Huinink. W.W., Clavel, M., Rodenhuis. S., Guastalla, J.P., Franklin, H.R., Koier, J.J., Vlasveld, T., Dalesio, O., van Tinteren, H., and Pinedo. H.M.: Mitoxantrone and GM-CSF: a phase I study with an escalated dose of mitoxantrone in breast cancer. Pathol Biol (Paris). 39: 962, 1992.

Thureson-Klein, A., Hedqvist. P., and Lindbom. L.: Leukocyte diapedesis and plasma extravasation after leukotriene B4: lack of structural injury to the endothelium. Tissue Cell. 18: 1-12, 1986.

Tomayko, M.M. and Reynolds, C.P.: Determination of subcutaneous tumour size in athymic (nude) mice. . Cancer Chemother. Pharmacol. 24: 148-154, 1989.

Toyoda, H., Fukada, Y., Hayakawa. T., Kumada, T., and Nakano, S.: Changes in blood supply in small hepatocellular carcinoma: correlation of angiographic images and immunohistochemical findings. J. Hepatol. 27: 654-660. 1997.

Tse, M.Y. and Singer, M.: Permeability of bilayers composed of mixtures of saturated phospholipids: evidence for compositional segregation of lipids at phase boundries. Can J Biochem Cell Biol. 62: 72-77, 1984.

Uchiyama, K., Nagayasu, A., Yamagiwa, Y., Nishida, T., Harashima. H., and Kiwada, H.: Effects of the size and fluidity of liposomes on their accumulation in tumours: A presumption of their interaction with tumours. Int. J. Pharm.. 121: 195-203, 1995.

Vail, D.M., Kravis, L.D., Cooley, A.J., Chun, R., and MacEwen, E.G.: Preclinical trial of doxorubicin entrapped in sterically stabilized liposomes in dogs with spontaneously arising malignant tumours. Cancer Chemother Pharmacol. 39: 410-6, 1997.

Van der Meer, B.W., Fugate. R.D., and Sims, P.J.: Complement proteins C5b-9 induce transbilayer migration of membrane phospholipids. Biophys. J. 56: 935-946, 1989.

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Van Furth, R.: Monocyte origin of Kupffer cells. Blood Cells. 6: 87-92, 1980.

Van Meer, G., Davoust, J., and Simons, K. (1985) Parameters affecting low-pH-mediated fusion of liposomes with the plasma membrane of cells injected with influenza virus. Biochemistry 24, 3593-3602.

Van Rooijen, N. and Sanders. A.: Liposome mediated depletion of macrophages: mechanism of action, preparation of liposmes and applications. J. Immunol. Methods 174: 83-93, 1994

Van Rooijen, N., Kors, N., v.d. Endo, M., and Dijkstra, C.D.: Depletion and repopulation of macrophages in spleen and liver of rat after intravenous treatment with liposome-encapsulated dichloromethylene diphosphonate. Cell Tissue Res. 260: 215-222, 1990.

Van Rooijen, N. and Claassen, E.: In vivo elimination of macrophages in spleen and liver, using liposome-encapsulated drugs: Methods and applications. In: Liposomes as Drug Carriers ed. G. Gregoriadis, Wiley, London. p. 131, 1989.

Van Rooijen, N. and Van Nieuwmegen, R.: Elimination of phagocytic cells in the spleen after intravenous injection of liposome-encapsulated dichloromethylene-disphosphonate. An enzyme-histochemical study. Cell Tissue Res. 238: 355-358, 1984.

Viero, J. A. and Cullis, P. R. (1990) A novel method for the efficient entrapment of calcium in large unilamellar phospholipid vesicles. Biochim. Biophys. Acta 1025, 109-115.

Vile, G.F. and Winterbourn, C.C.: Microsomal lipid peroxidation induced by adriamycin, epirubicin, daunorubicin, and mitoxantrone: a comparative study. Cancer. Chemother. Pharamcol. 24: 105-108, 1989.

Vuillemard, J.C.: Recent advances in the large-scale production of lipid vesicles for use in food products: microfluidization. J Microencapsul. 8: 547-62, 1991.

Wahlin, A., Hornsten, P., Hedenus, M., and Malm, C.: Mitoxantrone and cytarabine versus daunorubicin and cytarabine in previously untreated patients with acute myeloid leukemia. Cancer Chemother Pharmacol. 28: 480-3, 1991.

Wang, G., Li, S., Lin, H.N., and Huang, C.: Influence of cis double bonds in the sn-2 acyl chain of phosphatidylethanolamine on the gel-to-liquid crystalline phase transition. Biophys J. 73: 283-92, 1997.

Wang, S., Lee, R.J., Cauchon, G., Gorenstein, D.G., and Low, P.S.: Delivery of antisense oligodeoxynucleotides against the human epidermal growth factor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol. Proc. Natl. Acad. Aci. USA. 92: 3318-3322, 1995.

Webb, M.S., Saxon, D., Wong, F.M.P., Lim, H.J., Wang, Z., Bally, M.B., Choi, L.S.L., Cullis, P.R., and Mayer, L.D.: Comparison of different hydrophobic anchors conjugated to poly(ethyleneglycol): effects on the pharmacokinetics of liposomal vincristine. Biochim Biophys Acta. 1372: 272-282, 1998.

Webb, M.S., Harasym, T.O., Masin, D., Bally, M.B., and Mayer, L.D.: Sphingomyelin-cholesterol liposomes significantly enhance the pharmacokinetic and therapeutic properties of vincristine in murine and human tumour models. Br J Cancer. 72: 896-904, 1995.

Weinstein, J.N., Magin, R.L., Cysyk, R.L., and Zaharko, D.S.: Treatment of solid L1210 murine tumours with local hyperthermia and temperature-sensitive liposomes containing methotrexate. Cancer Res. 40: 1388-1395, 1980.

Weiss, R.B.: Mitoxantrone: its development and role in clinical practice.: Oncology 3:135-48, 1989.

Witzke, N.M. and Bittman, R.: Dissociation kinetics and equilibrium binding properties of polyene antibiotic complexes with phosphatidylcholine/sterol vesicles. Biochemistry. 23: 1668-74, 1984.

Wu, N.Z., Da, D., Rudoll, T.L., Needham, D., Whorton, A.R., and Dewhirst, M.W.: Increased microvascular permeability contributes to preferential accumulation of Stealth liposomes in tumour tissue. Cancer Res., 53: 3765-3770, 1993.

Yachi, K., Suzuki, N., Tanaka, N., Okada, K., Mitsui, I., Kawato, Y., Komagata, Y., Komiyama, K., and Kikuchi, H.: The effect of adriamycin against a liver metastatic model by encapsulation in liposomes. Biopharm. Drug Dispos 17: 699-715, 1996.

Yamaji, A., Sekizawa, Y., Emoto, K., Sakuraba, H., Inoue, K., Kobayashi, H., and Umeda, M.: Lysenin, a novel sphingomyelin-specific binding protein. J Biol Chem. 273: 5300-6, 1998.

Yamazaki, M., Suzuki, H., and Sugiyama, Y.: Recent advances in carrier-mediated hepatic uptake and biliary excretion of xenobiotics. Pharm Res. 13: 497-513, 1996.

Yoshida, T., Okazaki, N., Yoshino, M., Ohkura, H., Miyamoto, K., and Shimada, Y.: Phase II trial of mitoxantrone in patients with hepatocellular carcinoma. Eur J Cancer Clin Oncol. 24: 1897-8, 1988.

Yuan, F., Dellian, M., Fukumura, D., Leunig, M., Berk, D.A., Torchilin, V.P., and Jain, R.K.: Vascular permeability in a human tumour xenograft: molecular size dependence and cutoff size. Cancer Res. 55: 3752-6, 1995.

Yuan, F., Leunig, M., Huang, S.K., Berk, D.A., Papahadjopoulos, D., and Jain, R.K.: Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumour xenograft. Cancer Res. 54: 3352-6, 1994.

Yuda, T., Maruyama, K., and Iwatsuru, M.: Prolongation of liposome circulation times by various derivatives of polyethyleneglycols. Biol Pharm Bull. 19: 1347-1351, 1996.

Zou, Y., Horishi, I., Kasagi, T., Gu, X., and Perez-Soler, R.: Organ distribution and antitumour of free and lipsomal doxorubicin injected into the hepatic artery. Cancer Chemother. Pharmacol. 31: 313-318, 1993a.

Zou, Y., Yamagishi, M., Horioski, I., Ueno, M., Gu, X., and Perez-Soler, R.: Enhanced therapeutic effect against liver W256 carcinoma with temperature-sensitive liposomal adriamycin administered into the hepatic artery. Cancer Res. 53: 3046-3051, 1993b.