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UNIVERSITY OF ALBERTA

# A LIPOSOMAL SUSTAINED RELEASED SYSTEM FOR CYTOSINE ARABINOSIDE

by

TARUN MEHRA (C)

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **A LIPOSOMAL SUSTAINED RELEASE SYSTEM FOR CYTOSINE ARABINOSIDE**, submitted by **TARUN MEHRA** in partial fulfilment of the requirements for the degree of Master of Science in Pharmacology.

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Date: Sept. 19, 1990

# DEDICATION

To the memory of my grandparents,

Mrs Dhan Devi Nanda and Mr Ratan Chand Mehra

## ABSTRACT

recent years, the use of liposomes as drug carriers ..ned considerable interest. New liposome formulations ..g long circulation half-lives, called stealth liposomes, are excellent candidates for use in the slow release of drugs which are normally degraded rapidly *in vivo*. Administration of 1-β-D-arabinofuranosylcytosine (araC), a schedule dependent antineoplastic drug useful in the treatment of leukemia, is complicated by its short half-life *in vivo*, necessitating administration of this drug by continuous infusion. Experiments were done to test the utility of stealth liposomes as sustained release systems for araC in the treatment of mice bearing L1210 leukemia.

Two different formulations of liposomes, one with long circulation half-lives (stealth liposomes) and one with short circulation half-lives (non-stealth liposomes), were compared to free araC and to araC administered by 24 hour infusion for their ability to prolong the survival times of mice injected with either intravenous (i.v.) or intraperitoneal (i.p.) L1210 leukemia. Stealth liposomes, composed of sphingmyelin: egg phosphatidylcholine:cholesterol:monosialylganglioside $G_{M1}$ (SM:PC:CH:$G_{M1}$) in a molar ratio of 1:1:1:0.14 were less susceptible to plasma-induced leakage of entrapped araC than were non-stealth liposomes composed of PC:CH, at a molar ratio of 2:1. Stealth liposomes had longer half-lives *in vivo* and reduced uptake by cells of the mononuclear phagocyte system,

also termed the reticuloendothelial (RE) system, as compared to non-stealth liposomes. Stealth liposomes were also found in higher concentrations in carcass tissues than non-stealth liposomes.

Therapeutic studies showed that, regardless of the route of drug administration or leukemia implantation, for the treatment of leukemic mice, single injections of stealth liposomes containing entrapped araC had an antileukemic effect that was significantly greater than seen for araC entrapped in non-stealth liposomes, or for single injections of free araC. Treatment of leukemic mice with araC entrapped in SM:PC:CH:$G_{M1}$ liposomes, approached or was as effective as 24 hour infusion of the free drug at low doses, and was a superior treatment to infusion when administered at higher doses. Liposome-entrapped araC was protected from rapid enzymatic degradation and, particularily when entrapped in stealth liposomes, was released at therapeutically effective concentrations for extended periods of time. Liposomes with prolonged circulations half-lives, such as those described here, may be clinically useful sustained release systems for drugs that are rapidly degraded <u>in vivo</u>.

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## ABBREVIATIONS

| | |
|---|---|
| ALL | acute lymphoblastic leukemia |
| AML | actue myelocytic leukemia |
| araC | 1-β-D-arabinofuranosylcytosine |
| araU | 1-β-D-arabinofuranosyluracil |
| CH | cholesterol |
| CR | complete remission |
| DSPC | distearoylphosphatidylcholine |
| FAC | single injections of free araC |
| FAT | freeze and thaw multilamellar vesicles |
| $G_{M1}$ | monosialogangliosides ($II^3$NeuAc-GgOse$_4$Cer) |
| HDL | high density lipoproteins |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| LCL | long circulating liposomes |
| LUV | large unilamellar liposomes |
| MST | mean survival times |
| MLV | multilammelar vesicles |
| PC | egg phosphatidylcholine |
| PG | phosphatidylglycerol |
| PS | phosphatidylserine |
| RE | reticuloendothelial |
| REV | reverse phase evaporation vesicle |
| SCL | short circulating liposomes |
| SM | bovine brain sphingomyelin |
| SUV | small unilammelar vesicles |

| | |
|---|---|
| Tc | gel to liquid crystalline transition temperature |
| TES | N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid |
| $T_{\frac{1}{2}}\alpha$ | half-time for the first leakage phase |
| $T_{\frac{1}{2}}\beta$ | half-time for the second leakage phase |

# CHAPTER I INTRODUCTION

Liposomes have been of interest as drug carriers for several reasons. Liposomes are composed of phospholipids which are constituents of cell membranes. Liposomes have low toxicity and are degradable when administered in vivo (Rustum et al. 1979, Allen et al., 1984). Numerous studies have shown that many different drugs can be entrapped in liposomes in a quantifiable and reproducible manner. Polar drugs, such as methotrexate, fluorodeoxyuridine or 1-β-D-arabinofuranosylcytosine (araC), may be entrapped in the internal aqueous compartment of liposomes and are generally released from liposomes more readily than nonpolar drugs when the liposome membrane has been breached. Non-polar drugs such as actinomycin D or vinblastine are bound within the liposomal membrane and may also be present in the aqueous compartment of the liposome. Liposomes appear to prevent degradation of water-soluble drugs that are rapidly degraded in vivo such as araC (Juliano and Stamp, 1978 and 1979).

**A) Liposome size and composition**

**Liposomes were first described about 25 years ago by Bangham and Horne (1964), who observed that the addition of aqueous media to dried phospholipid films deposited on the wall of glass containers resulted in the spontaneous formation of phospholipid bilayers. Simple agitation of these bilayers caused the formation of structures that had several concentric**

bilayers of phospholipids, that were named multilamellar vesicles (MLV) to reflect their physical structure. It soon became apparent that biologically active substances trapped within liposomes may be treated differently in cells and tissues than the same substances in free form. As a result liposomes have become popular tools for biochemists, cell physiologists, and for investigators interested in drug delivery systems (Juliano and Stamp, 1979; Kimbelberg and Mayhew, 1979; Juliano and Layton, 1980; Weinstein, 1984; Poznansky and Juliano, 1984).

Other types of liposomes, such as small unilamellar vesicles (SUV) or large unilamellar vesicles (LUV) can be prepared by other methods. Multilamellar liposomes, if sonicated give rise to SUV (Papahadjopoulos and Miller, 1967). Both SUV and MLV have the disadvantage of high ratios of lipid to internal aqueous volumes and, therefore, have poor trapping efficiencies for water-soluble drugs. Trapping efficiencies of MLV have been improved by rapidly freezing the MLV preparation in liquid nitrogen and then thawing the frozen MLV at 22°C several times (Mayer et al. 1985). Szoka and Papahadjopoulos (1978) developed the reverse phase evaporation (REV) method for preparation of LUV which substantially decreased the ratios of lipid to internal aqueous volumes and increased the trapping efficiency of drugs. Heterogeneously-sized liposomes, after formation by either the REV or the MLV method, may be made more homogeneous in size by passing them

several times under pressure through unipore polycarbonate filters (Olson et al., 1979; Mayer et al., 1986).

Liposomes can be prepared with a variety of different phospholipids and other membrane constituents that can alter the surface charge, membrane rigidity or specific recognition characteristics. Based on this knowledge, it is possible to optimize drug carriers for given applications by altering the physical parameters of liposomes.

B) Physical characteristics of liposomes

The phospholipids in liposomes can be arranged in either a gel phase (solid phase) or a liquid crystalline phase (fluid phase). In the gel phase, the fatty acyl chains are tightly packed in ordered structures, whereas in the liquid crystalline phase there is a high degree of disorder and a greater degree of mobility among the fatty acyl chains. The physical state of the phospholipids in liposomal membranes is temperature-dependent and the temperature at which the phospholipids change from one phase to another is called the gel to liquid crystalline transition temperature (Tc). The composition of the liposomal membrane can alter the membrane rigidity, which in turn can affect leakage rates of the liposomal contents. Phospholipids such as sphingomyelin (SM) or distearoylphosphatidylcholine (DSPC), or cholesterol (CH) have a tendency to increase membrane rigidity (Kimelberg and Mayhew;

1979)

C) Fate of liposomes *in vivo*

The utility of liposomes as drug carriers *in vivo* may be limited by the reticuloendothelial (RE) system also termed the mononuclear phagocyte system. The RE system removes foreign particulate matter from the circulation. Liposomes introduced into the circulation are rapidly removed by the RE system, despite their resemblance to cell membranes. The RE system consists of circulating macrophages and resident macrophages, found in liver (Kupffer cells), spleen, bone marrow, lymph nodes and lungs. When liposomes of conventional formulations are administered intravenously, they rapidly accumulate in RE cells. Two major sites of liposome accumulation are the liver and spleen. The adminstration of liposomes by intraperitoneal (i.p.) or subcutaneous routes may reduce uptake by the liver and spleen, but the RE system remains the major site of liposome accumulation (Hwang, 1987; Allen et al. 1989).

Among the many factors affecting the residence times of liposomes in circulation, the surface charge of liposomes is an important determinant of liposomal behaviour *in vivo* and *in vitro*. Positively- or negatively-charged liposomes were found to be more stable against aggregation and fusion than uncharged liposomes (Hsu and Juliano, 1982). It is postulated that neutral liposomes are stabilized by repulsive hydration

forces which counteract the attractive van der Waal forces (Van Dalen et al., 1988). It is also recognised that positively-charged liposomes and those without net charge circulate in blood for longer times than negatively-charged liposomes of similar size (Hsu and Juliano, 1982).

The total amounts of liposomal lipid administered will affect rates of uptake and clearance of liposomes from the circulation. Administration of high doses of liposomes will impair the functioning of the RE system and the severity of that impairment will depend upon liposome composition size and dosage (Allen et al. 1984). Impairment of the RE system will also result in the redistribution of liposomes in vivo and increases in the circulation half-times of liposomes. Upon saturation of the hepatic uptake of liposomes, uptake by the spleen increases. Liposomes may spill over into bone marrow tissue when hepatic and splenic uptake become saturated (Poste, 1983).

Another factor affecting liposomes in the circulation is their interaction with high density lipoproteins (HDL), which may destabilize liposomal membranes by a mechanism involving phospholipid exchange and transfer to HDL, which results in the loss of entrapped contents at rates determined by liposome composition (Allen and Cleland, 1980; Allen, 1981; Damen et al., 1980; Senior et al., 1983). HDL phospholipids exchange with phospholipid molecules present in the outer monolayer of the vesicle membrane (Scherphof et al., 1983) is a process

mediated by HDL apolipoproteins. Other lipoproteins such as very low density lipoprotein, intermediate density lipoprotein and low density lipoprotein have lesser effects than HDL in destabilizing liposomal membranes (Senior et al., 1983). Fluid vesicles such as egg phosphatidylcholine (PC) have been shown to be very susceptible to disintegration by plasma constituents such as lipoproteins (Allen, 1981). Thus, the utility of this and similar liposome formulations for the sustained release of rapidly degraded drugs in vivo is limited by rapid drug release rates triggered by liposomal interactions with plasma components.

Numerous studies have shown that the presence of particular constituents in membrane bilayers may influence bilayer properties, for example by tightening the phospholipid packing, increasing membrane rigidity, or reducing the exchange or transfer of phospholipids from liposomes to HDL. The addition of cholesterol to liquid-crystalline liposome compositions, by tightening bilayer packing, reduces serum-induced leakage of entrapped contents (Finkelstein and Weissmann, 1979; Allen and Cleland, 1980; Allen, 1981; Kirby et al., 1980). The inclusion of cholesterol and phospholipids with high phase-transition temperatures reduced leakage of contents by decreasing the number of membrane defects or possible insertion sites for proteins within the lipid matrix (Allen, 1981).

In the circulation, liposomes are exposed to plasma

proteins. Alpha and beta globulins, immunoglobulins, lipoproteins, components of the clotting system, complement factors and albumin are known to bind and interact with the lipid surface of liposomes (Swaney, 1980; Poznansky and Juliano, 1984; Juliano and Layton 1980; Hsu and Juliano, 1982 and Allen, unpublished results). It is hypothesized that bound plasma constituents lead to the removal of liposomes from circulation by cells of the RE system (Gabizon and Papahadjopoulos, 1988).

Experiments by Allen et al. (1985) with mono-, di- and tri-sialogangliosides incorporated into liposomes demonstrated that (i) the retention of entrapped contents in liposomes is correlated with increasing levels of ganglioside sialic acid residues, and (ii) that gangliosides and cholesterol were synergistic in stabilizing the lipid bilayer. The sialic acid residue(s) of gangliosides are negatively charged. Other lipids that impart negative charges to the surface of the liposome membrane, such as phosphatidylserine (PS), phosphatidylglycerol (PG), or sulfatides were not as effective as gangliosides in stabilizing liposomes in the presence of plasma. The protective effect of gangliosides against plasma-induced leakage of liposome contents has, in part, been attributed to inhibition of binding of apolipoproteins to liposome surfaces (Allen et al., 1985; Allen et al., 1989).

By altering the composition of liposomes it is possible to alter their circulation half-lives. Vesicles formulated

with phospholipids having high Tc values, such as SM or DSPC, are removed more slowly from circulation than those formulated with "fluid" phospholipids (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen, 1989; Allen and Mehra, 1989). The presence of gangliosides in liposomes not only improves their membrane stability in the presence of plasma, but also prolongs their circulation half-lives in blood as a result of decreasing their uptake by liver and spleen (Allen and Chonn, 1987). Several different types of gangliosides were examined for their ability to increase circulation times in vivo (Allen and Chonn, 1987). It was observed that liposomes containing $G_{M1}$, but not other gangliosides, have the ability to remain in circulation for prolonged periods. Both membrane rigidity and the presence of carbohydrate-containing lipid, which, in case of $G_{M1}$ tends to shield surface negative charges, were shown to work synergistically in prolonging circulation half-lives of liposomes and reducing the uptake of liposomes by cells of the RE system (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen, et al. 1989). A postulated mechanism for the effects of $G_{M1}$ on circulation half-lives has been attributed in part to interactions between the sialic acid residue and the terminal galactose of $G_{M1}$, which may create an internal chelation complex. As a result, the sialic acid residue would not be available for interaction with opsonizing proteins (Sillerud et al., 1978). Thus, the presence of gangliosides has two important effects. The

presence of $G_{M1}$ reduces the leakage of liposome-entrapped contents induced by the presence of plasma and furthermore, its presence tends to reduce the ability of the RE system to clear liposomes from the plasma.

Liposomes with long circulation half-lives that avoid uptake by the RE system, also called stealth liposomes (Allen, 1989), have an increased ability to accumulate in tissues where there normally is little uptake of liposomes. It is hypothesized that the long half-lives of stealth liposomes may be partly attributed to mimicry of the outer leaflet of erythrocyte membranes. Because stealth liposomes avoid RE uptake, it is now possible to explore new therapies and treatments using stealth liposomes. It is postulated that the mechanism of the decreased uptake of these liposomes by RE cells possibly results due to reduced opsonization by plasma constituents (Gabizon and Papahadjopoulos, 1988; Allen et al., 1988; Allen and Mehra 1989). These improved formulations of liposomes now make it possible to utilize liposomes as a sustained release system within the vasculature (Allen and Mehra 1989).

**D) Cytosine arabinoside**

The development of a cure for cancer could easily be accomplished by designing a drug that is toxic only to neoplastic cells. Unfortunately, no such drug has been

discovered and the use of antileukemic or other antineoplastic drugs is plagued with numerous complications such as: (i) the lack of drug specificity for the leukemic or other cells, (ii) the tendency for tumor cell populations to develop resistance to the administered drug over extended periods of time, and (iii) the tendency for different classes of tumor cells to respond differently when exposed to the same drug.

Effective cancer chemotherapy for the treatment of metastatic or disseminated malignant neoplastic diseases primarily depends upon (i) whether the disease is localized or widely disseminated, (ii) the origin of the primary tumor from where the spread of neoplastic cells began and (iii) the pathological state of the disease at the time of treatment. In order to obtain cures, the entire neoplastic cell population must be eradicated, therefore, effective chemotherapy treatments are generally administered at the maximum tolerated dose near the target site for an extended period of time to achieve total cell kill. The concept of "total cell kill" is a fundamental principle of antineoplastic chemotherapy, where cytotoxic agents are employed to reduce the total number of viable neoplastic cells. The cell kill concept still dominates the stratigical thinking of clinical chemotherapists. Some important principles, that have been long established and are applied for the use of modern cancer chemotherapy, are: (i) a single clonogenic malignant neoplastic cell has the potential to give rise to a sufficient number of progeny that

can result in the death of the host, (ii) the contribution of the immune system has a negligible role in the treatment of neoplastic cell and (iii) cell death of neoplastic cells from treatment with antineoplastic agents follows first-order kinetics. Therefore, a patient with advanced acute leukemia may have $10^{12}$ leukemic cells and treatment with antineoplastic agents that will kill 99.99% of the leukemic cells will reduce the population to $10^{8}$ cells. The remaining viable leukemic cells can lead to the relapse of the disease. It is suggested that the failure of chemotherapy may be attributed to intrinsic drug resistance by the neoplastic cells or that these cells develop resistance to individual drugs; normal tissues do not develop drug resistance (Calabresi and Parks, 1985).

The antineoplastic drug, 1-β-D-arabinofuranosylcytosine (araC), is a schedule-dependent analogue of 2'-deoxycytidine synthesized in 1959 (Walwick et al., 1959). Studies in the 1960s revealed that araC had the capacity to inhibit the growth of several mouse tumors (Evans et al., 1961). By the late 1960s, araC was shown to be very effective against acute myeloblastic leukemia and is used today for the treatment of adult acute leukemias (Ellison et al., 1968; Robinson and Nesbit, 1985; Peters et al., 1988).

Administration of araC by the oral route is ineffective for the treatment of cancers because the drug is rapidly degraded. Deamination by cytidine deaminase converts araC to the therapeutically inactive product, uracil arabinoside

(araU). Cytidine deaminase is a ubiquitous enzyme found in high concentrations in the intestine, intestinal flora, kidney and liver (Creasey et al., 1966; Camiener and Smith, 1965). In order for araC to be therapeutically effective it must be parentally administered. Free araC leaves the circulation rapidly with an initial half-life of 10 to 15 minutes followed by a second slower half-life of 2 to 3 hours (Ho and Frei, 1971). Twenty-four hours after i.v. administration of araC, approximately 80 per cent of the administered dose is found in the urine in the form of araU. In order to maintain prolonged concentrations of araC in plasma the drug must be intravenously administered by continuous infusion. The simultaneous administration of tetrahydrouridine, a potent inhibitor of cytidine deaminase, will prolong the half-life of araC in the circulation (Mulligan and Mellet, 1968).

AraC enters cells by a nucleoside transport mechanism. After entering cells, araC is converted to araC monophosphate (araCMP), araC diphosphate (araCDP) and then to araC triphosphate (araCTP) by kinases (Monparler and Fischer, 1968). Recent studies have demonstrated that araCTP, within the cell competitively inhibits DNA polymerase and consequently inhibits DNA synthesis in mammalian cells (Chu and Fischer, 1962; Graham and Whitemore, 1970; Reddy et al., 1971; Rashbaum and Cozzarelli, 1976; Townsend and Cheng, 1987). The therapeutic activity of araC within the cell is closely related to the conversion of araC to araCTP (Chu and Fischer, 1965).

More recent studies have demonstred that araC also inhibits polymerase responsible for DNA repair (Fram and Kufe, 1985 (a); Fram and Kufe, 1985 (b)). It has been shown that araCTP can be incorporated into DNA, and is not readily exised form the DNA strand. The incorporation of araCTP into the DNA strand causes unusual reiterations of DNA segments and also inhibits template function and chain elongation (Woodcock et al., 1979; Major et al., 1982; Kufe et al., 1984.; Mikita and Beardsley, 1988). It has been suggested that the unusual DNA segments increases the possibility of recombination, crossover and gene amplification (Woodcock et al., 1979). AraCTP can also be incorporated into RNA which could lead to malfunctions in the production of proteins therefore leading to cell death (Karon et al., 1972; Chuang and Chuang, 1976). AraC has several effects on DNA sysnthesis but its incorporation into DNA seems to have the greatest effect on cell growth (Chabner, 1990).

The cytotoxic effects of araC are more pronounced during late S phase and early $G_2$ phase of the cell cycle (Benidict et al., 1970). Therefore, anticancer therapies with single injections of araC will be expected to kill only a fraction of the treated cell population randomly distributed around the cell cycle. Infusion of araC over an interval longer than the replicative cycle of the treated cells will increase the proportion of the cell population exposed to araC during the S phase of the cell cycle, resulting in greater cell kill.

Acute leukemia is a term used to describe a malignant hematolgical disorder involving blood leukocytes. Acute lymphoblastic leukemia (ALL) is a disorder that is prevelent in childrern under the age of 15 in approximately 32% of all malignant cases. It has been recognised since 1950 that combination chemotherapy is the most effective treatment for ALL. Antileukeimic drugs such as vincristine used in combination with prednisone for the treatment of patients with ALL have achieved complete remission (CR) rates as high as 83 to 93%. Current treatments for patients, with ALL have achieved remission rates of 90 to 95%, using therapies consisting of vincristine, prednisone and a third agent, usually L-asparginase (Robinsen and Nesbit, 1985). Some other valuable drugs used to treat ALL are daunomicin, carmustine, doxorubicin and araC (Salmon and Sartorelli, 1984). However, approximately 17% of the ALL cases found in childeren are classified as acute non-lymphoblastic leukemia (ANLL). AraC is one of the most effective drugs currently available for the treatment of patients with ANLL. The highest CR rate for ANLL patients may be achieved when araC therapy is combined with an anthracycline antibiotic such as daunomicin. Patients with ANLL frequently relapse and die from leukemia despite continued therapy with araC. Children that survive longer than 5 years after chemotherpy have a 50% chance of remaining in permenant CR or cured from the disease (Salmon and Sartorelli, 1984).

Acute myelocytic leukemia (AML) is the predominant type

of leukemia found in adults. Patients with AML have a mean survival time of 3 months and sucessful treatment of this disorder has posed some difficulty (Salmon and Sartorelli, 1984). Two effective antileukemic agent used to treate AML are araC and daunorubicin (Omura, 1985). When araC was used as a single drug to treat previously untreated patients with AML at a dose of 10-30 $mg/m^2$, a CR rate of 10 to 15% was obtained. When the dose of araC administered was increased to 100 to 200 $mg/m^2$ the CR rate was increased to 30%. Combinations of araC, with thioguanine and anthracycline antibiotics (such as daunomicin) or drug combinations of araC with vincristine and prednisone can increase the CR rate to 65 to 75% (Salmon and Sartorelli, 1984; Peters et al., 1988). In recent studies, high dose (1 to 4.5 $g/m^2$) therapies with araC alone or in combination with L-asparginase, vincristine, anthracycline antibiotics or prednisone have been examined for their effect on AML or ALL. Unfortunately, these treatments have not shown a significant improvement over conventional therapies. However, studies have shown that the treatment of myelodysplastic syndromes with low doses of araC, 3 $mg/m^2$, were as effective as short treatments with 10 $mg/m^2$ and better tolerated (Chomienne et al., 1987).

Bone marrow suppression is the major toxic effect of araC, some other hematological consequences are megaloblastosis of erythrocyte precursors, reticulopenia, leukopenia and thrombopenia. Some toxic effects as a result of treatment

with araC include skin reactions such as erythema, maculo-papular exanthema and palmo-plantar erythrodysesthesia and ocular complications include keratoconjunctivitis, pain, tearing, photophobia and blurred vision. Treatment with high doses of araC can result in central nervous toxicity and non-cardiogenic pulmonary edema, also called "araC lung". It is also known that araC can also cause immunosuppression, therefore the potential risk of immunosuppression must be weighed against the benefits before using this drug (Frei and Laszlo, 1982; Peters et al., 1988).

E) Therapeutic applications of liposome-entrapped drugs

The capacity of liposomes to entrap both hydrophillic and hydrophobic antineoplastic drugs make them excellent candidates for drug delivery systems (Juliano and Stamp, 1978 and 1979). Administration of antineoplastic drugs in entrapped liposomes may alter rates of drug metabolism and excretion, and the pharmacokinetics of the drug relative to the same drug administered in free form (Gabizon et al., 1982; Rahaman et al., 1985; Gabizon et al., 1989).

A potential clinical use of liposomes as drug carriers is for the treatment of Leishmaniasis infection. In the host, the Leishmania parasite will localize within the lysozomes of phagocytic cells, such as Kupffer cells, which is also the site of localization of several liposome formulations.

Intravenous delivery of liposome-entrapped antimonial drugs as opposed to i.v. delivery of the same agents in the free form, resulted in a 700-fold increase in the treatment of Leishmaniasis (Alving et al., 1978; Croft, 1986).

Another promising clinical use of liposome-entrapped drug is for the treatment of systemic fungal infections (Lopez-Berestein et al., 1987). Systemic fungal infections frequently occur in patients that undergo antineoplastic drug therapy (Bodey, 1969). Liposome-entrapped amphotericin B results in a 16-fold improvement in the therapeutic index of the drug, while reducing the toxic side effects associated with this drug (Lopez-Berestein et al., 1987).

**F) AraC in liposomes**

Early in vivo studies of tumor treatment with liposome-entrapped araC showed that the MLVs tended to localize in the lung. The localization was a size-dependent phenomenon, evidently due to entrapment of large-sized liposomes, such as MLVs, as pulmonary microemboli (McCullough and Juliano, 1979; Hunt et al., 1979). Thus, it was postulated that liposome-entrapped araC may be therapeutically useful for the treatment of lung tumors. Mayhew and Rustum (1983) showed that the treatment of tumors with liposome-entrapped araC had some selective action against lung and liver tumors. Based on these experiments, it was proposed that araC leaked out of the

liposome and was taken up by the organ at high local concentrations that were sufficient to exert their cytotoxic effects on tumor cells present in those organs.

AraC-loaded MLV composed of PS,PC and CH (1:1:2), and administered by the i.p. route for the treatment of mice with L1210 leukemia, had a greater anti-leukemic activity than free araC at the same total dose. The present study and several similar studies have shown that single injections of liposome-entrapped araC inhibited proliferation of L1210 leukemia cells in vivo, and did so at araC dosages substantially lower than free araC dosages required to achieve a similar effect (Kobayashi et al., 1975; Mayhew et al. 1976; Kobayashi et al., 1977; Kataoka and Kobayashi, 1978). Recent studies by Kim et al. (1987), and Hong and Mayhew (1989) have demonstrated that liposome-entrapped araC is more efficacious than administration of free araC at the same total dose in the treatment of intracranial tumors. It was also shown that MLV were more therapeutically effective in curing leukemic-bearing mice than were REV or SUV liposomes. AraC-loaded REV composed of DPPC:CH were more effective in curing leukemic mice than liposomes composed of PS:PC or PC:CH (Hong and Mayhew, 1989). Therapeutic studies with liposome-entrapped araC used for the treatment of tumor-bearing mice, demonstrated that positively-charged liposomes were more efficacious than were similar negatively-charged liposomes (Kataoka and Kobayashi, 1978; Rustum et al., 1979). More extensive treatment conditions

further showed that araC entrapped in liposomes was a superior form of treatment when compared to administrations of free drug at the same dose regardless of the treatment schedule or administration route (Kobayashi et al., 1975; Kobayashi et al., 1977; Mayhew et al., 1979, Allen and Mehra, 1989).

The addition of membrane constituents that increase membrane rigidity, such as CH, SM or DSPC, increase the retention of liposome-entrapped contents (Kobayashi et al., 1977; Mayhew et al., 1979; Allen, 1981; Ganapathi et al.,1980). The antileukemic activity of liposome-entrapped araC was shown to be inversely proportional to the permeability properties of the liposomal membrane (Mayhew et al., 1979; Ganapathi et al.,1980). It was suggested that liposome-entrapped araC probably acted as a drug depot from which araC was slowly released thereby enhancing leukemic cell exposure to araC during the S-phase of the cell cycle (Mayhew et al.1976; Kobayashi et al., 1977; Mayhew et al.1978; Kataoka and Kobayashi, 1978; Mayhew et al. 1979; Rustum et al. 1979; Rustum et al., 1981; Kim et al., 1987; Hong and Mayhew, 1989).

The toxic effects of araC *in vivo* are related to the length of exposure to the drug. The $LD_{50}$ of single injections of free araC administered by the i.v. route in NMR1 mice was found to be greater than 4 g/kg weight, whereas, the $LD_{50}$ values of araC in mice infused for 1 or 2 days, with free araC, were 500 and 135 mg/kg respectively (Menten et al., 1985). Toxic effects of single injections of liposome-

entrapped araC were seen at doses of 100 mg/kg weight (Kobayashi et al., 1975; Kataoka and Kobayashi, 1978; Mayhew et al., 1979; Allen, unpublished results). The $LD_{50}$ of single injections of liposome-entrapped (PS:PC:CH, 1:4:5) araC was shown to be 350-400 mg/kg weight (Mayhew et al., 1979). Multiple doses of liposome-entrapped (PC:CH, 1:1 or PC:CH, 1:0.5) araC administered every 4.5 hours (three times) at a dose of 40 mg/kg weight resulted in the death of at least half of the animals treated (Ganapathi et al., 1980). From these experiments, it was concluded that (i) administered doses of liposome-entrapped araC are protected from rapid enzymatic degradation, and (ii) the slow release of araC substantially increases the toxicity of araC toward leukemic cells in vivo.

Early therapeutic studies that utilized liposome-entrapped drugs to treat cancers were limited to liposomal formulations that were easily recognised and removed from circulation by the RE system. The advent of stealth liposome formulations, with their low leakage rates and extended circulation times in vivo, appear to have improved the prospects of liposomal drug slow release systems for treatment of neoplastic disease by liposome entrapped agents (Allen and Chonn, 1987; Gabizon and Papahadjopoulos 1988; Allen et al., 1989).

## G) Thesis objectives

The principal objective of this study was to examine the therapeutic efficacy of liposomes with long circulation half-lives containing entrapped araC in the treatment of L1210/C2 leukemia in male B6D2F$_1$ hybrid mice. Before undertaking therapeutic experiments, it was necessary to evaluate araC permeability in the long-circulating liposomes. The following experiments were performed:

(1) The influence of plasma constituents and the effects of osmolarity variations on the kinetics of araC leakage from liposomes were examined. Leakage kinetics in buffer with and without 25 % human plasma were examined for different concentrations of araC entrapped in liposomes with long (SM:PC:CH:GM$_1$, 1:1:1:0.14) or short (PC:CH, 2:1) circulation half-lives.

(2) The effect of route of administration (i.p. versus i.v.) on tissue distribution of liposomes with long (SM:PC:CH:GM$_1$, 1:1:1:0.14) or short (PC:CH, 2:1) circulation half-lives was examined. Similar experiments were performed in leukemic mice.

(3) The therapeutic efficacy of araC entrapped either in liposomes with long (SM:PC:CH:GM$_1$, 1:1:1:0.14) or short (PC:CH, 2:1) circulation half-lives was examined in mice implanted with either $10^5$ or $10^6$ L1210/C2 leukemia cells.

Survival times of leukemic mice that received liposomal therapies were compared with those achieved in leukemic mice treated with single injections of free araC or with slow infusion of the drug at the same total dose. The effects of different routes of drug administration (i.p. versus i.v.) and tumor inoculations (i.p. versus i.v.) were also examined.

# CHAPTER II MATERIALS and METHODS

A) Liposome preparation

Purified egg phosphatidylcholine (PC), and bovine brain sphingomyelin (SM) (Avanti Polar Lipids, Birmingham, AL) and cholesterol (CH) (Sigma Chemicals, St Louis, MO) were dissolved in chloroform (American Burdick and Jackson: high purity solvent, Fisher Chemicals, Edmonton, Alta). Individual lipids in chloroform (10 µmole/ml) were sealed under nitrogen (99.7% pure) in glass ampoules and stored at -80°C. Bovine brain monosialogangliosides ($G_{M1}$) (Makor Chemicals, Jerusalem) were dissolved in chloroform:methanol, 2:1 at a concentration of 4 µmoles/ml and stored at -20°C until use.

Large unilamellar liposomes (LUV) composed of either PC:CH with a molar ratio of 2:1 or SM:PC:CH:$G_{M1}$ with a molar ratio of 1:1:1:0.14, were prepared by either the reverse phase evaporation (REV) method (Szoka and Papahadjopoulos, 1978) or the freeze and thaw multilamellar vesicles (FAT) method (Mayer et al., 1985). In the REV method 10 µm of phospholipids are dried under high vacuum and dissolved in 1.0 ml of hydrated diethyl ether. 1-β-D-Arabinofuranosylcytosine (araC) (Sigma Chemicals, St Louis, MO), was dissolved in distilled water and was added to the either-lipid solution in two stages. First, one-third of the buffer was added and the mixture was sonicated (sonicator, Laboratory Supplies Company, Hicksville, N.Y.) at 22°C until a stable emulsion was obtained. The ether was slowly removed from the emulsion in a rotary evaporator

maintained at 25°C, under vacuum (300-400 mm Hg) until an opaque gel resulted. The remaining two third of the buffer was added and the preparation was evaporated under vacuum for 15 minutes at which point LUVs had formed. In the FAT method, MLVs were made first and then frozen in liquid nitrogen and thawed at room temperature, this process was repeated 5 times. Liposomes prepared by either the REV or the FAT method, were subsequently extruded (Extruder™, Lipex Biomembranes, Vancouver, BC) 10 times at room temperature (20-23°C) through two stacked 0.4 µm Nuclepore polycarbonate filters (Nuclepore Corp., Pleasanton, CA) using compressed nitrogen at 100 p.s.i. (250 KPa) (Olson et al., 1979; Mayer et al., 1986). Liposomes were prepared in 246.9 µM araC in distilled water which containted [$^3$H]araC, specific activity, 0.55-1.1 TBq/mmol (Amersham, Oakville, Ont.) such that the specific activity of the final araC solution was 37 kBq/ml.

Two methods were used to separate liposome-entrapped araC from free araC. Liposomes were chromatographed using 1 mM N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) (Sigma Chemicals, St Louis, MO) buffer (pH of 7.4, 22°C) in 145 mM NaCl (TES buffer), on an 8 x 1.5 cm fine grade Sephadex G-50 column (Pharmacia, Canada, Dorval, Que.). Alternatively, liposomes were dialyzed for 12 hours through Spectrapore dialysis tubing (molecular weight cut off of 12,000 daltons, Spectrum Medical Instruments, Los Angeles, CA) in 1 l of 0.145 M NaCl, at 5 °C. The buffer was changed every three hours.

Portions of the LUV preparations were collected before and after separation of free drug and were assayed for $^{3}H$ content in ACS counting fluid (Amersham, Oakville, Ont.) using a Beckman LS 7500 scintillation counter. The concentration of araC entrapped in liposomes was determined from the percentage of the total drug used, using the following formulae.

1) % of entrapped araC =

$$\frac{\text{total cpm of liposome-entrapped araC}}{\text{total cpm of free + entrapped araC}} \times 100\ \%$$

2) Amount of araC entrapped in liposomes = (mg araC/ ml of liposome solution)

percentage of entrapped araC X concentration of araC solution [60 mg/ml]

Extruded vesicles prepared by either the REV or the FAT method were approximately uniform in size with a trapped volume of about 4.0 l of water/mole of phospholipid. The liposomes were sized by quasielastic light scattering (Nicomp Instruments, Santa Barbara, CA), or by a BI-90 particle sizer (Brookhaven Instruments, Holtsville, NJ). The phosphate content of each liposome preparation was determined (Bartlett, 1959) and from the phospholipid molarity the trapped volume was determined by the following formula:

$$\text{Trapped volume} = \frac{\dfrac{\text{total cpm of liposome-entrapped araC}}{\text{total cpm of liposome-entrapped araC + free araC}}}{\text{Molarity of phospholipid}}$$

Once concentrations of liposome-entrapped araC were established, it was possible to administer the preparation to mice on the basis of an araC dosage (mg/kg body weight).

B) In vitro leakage of liposome contents

AraC was entrapped in liposomes either at 60 mg/ml, with a measured osmolarity of 290 mOsm, or at 150 mg/ml, corresponding to an osmotic pressure of 850 mOsm. The osmolarity of the araC solutions and buffers were determined by using a Westcor vapour pressure osmometer Model 5500 (Logan, UT). Either SM:PC:CH:$G_{M1}$, 1:1:1:0.14, or PC:CH, 2:1, liposomes were employed at each concentration of araC. Leakage of liposome-entrapped araC was determined for both concentrations of araC and both types of liposomes at 37°C in TES buffer (290 mOsm) and also in TES buffer containing 25% pooled whole human plasma (Edmonton Red Cross Blood transfusion service).

A LUV preparation (1 ml, 10 µmoles of phospholipid/ml) was prepared by the REV method and extruded 10 times through

0.4 μm Nuclepore filters at room temperature (20-23°C) and liposome-entrapped araC was separated from free araC by chromatography as described above. The elution fraction containing liposomes was collected in the void volume of the column (approximately 5 ml) and diluted to a final volume of 6 ml with TES buffer. A portion of the liposome suspension was diluted with TES buffer and human plasma, to achieve a final plasma content of 25%. The liposomes were then incubated in a water bath maintained at 37°C. Portions (1 ml) were taken at 1, 3, 6 and 24 hours and chromatographed over Sephadex G-50 as described above: eluate fractions were assayed for $^{3}H$-content. Concentrations of liposome-entrapped araC were calculated for each time point. Half-times for leakage were estimated with the Graphpad software (ISI, Philadelphia, PA).

C) Distribution of liposomes in vivo

Time courses for the elimination of liposomes from the circulation and their tissue distribution in vivo were determined for either SM:PC:CH:$G_{M1}$, 1:1:1:0.14 or PC:CH, 2:1 liposomes in male D6D2$F_1$ mice (3-4 months old, weighing 25-30 grams) (Laboratory Animal services, Faculty of medicine, University of Alberta). Liposomes were LUV prepared by the REV method, extruded 10 times through 0.4 μm Nuclepore filters as described above. [$^{125}I$]Tyraminylinulin ([$^{125}I$]TI) was

prepared as described by Sommerman et al. (1984) and used as an aqueous space marker in following the elimination of liposomes from the circulation. It was shown by Sommerman et al. (1984), and confirmed in this laboratory (Allen and Chonn, 1987), that free [$^{125}$I]TI is rapidly cleared from circulation in mice within 30 minutes after intravenous (i.v.) injection. Therefore, the label remaining *in vivo* is a marker for intact liposomes. The LUVs were chromatographed over AcA-34 (Pharmacia, Canada, Dorval, Que.) on a 20 x 1.5 cm column to separate free from entrapped label and the radioactivity of entrapped [$^{125}$I]TI was measured in a Beckman 8000 gamma counter. Each mouse received either an intraperitoneal (i.p.) or an i.v. injection of ($\leq$ 0.5 µmol) phospholipid in 0.2 ml volume containing $10^5$-$10^6$ cpm of entrapped [$^{125}$I]TI. Similar experiments were performed with mice implanted 24 hours earlier, either i.p. or i.v., with $10^6$ L1210/C2 leukemia cells. At various times after the administration of labelled liposomes, groups of 3 to 6 mice were killed by cervical dislocation and the following tissues were sampled for radioactivity assay: blood, liver, spleen, lung, heart, kidney, thyroid, and the remaining carcass tissues. Radioactivity in 100 µl blood samples were multiplied by 7.7% of body weight to give the total radioactivity in blood (Allen, 1989). In addition, the radioactivity that was detected in each organ or the remaining carcass tissues, was corrected for the amount of blood present in the organ or tissues using a

set of correction factors determined by Allen (1989). The radioactivity measured in the blood and the tissues was expressed as a percentage of total radioactivity remaining in the mouse at various times after liposome injections.

D) Passage of L1210/C2 leukemia cells in mice

The L1210 mouse leukemia cell line originated as an isolate from spleen and lymph nodes from a leukemic DBA/2 mouse following treatment with methylcholantrene by skin painting (Law et al. 1949; Dunham and Stewart, 1953). The L1210/C2 clone was established in 1972 from a cultured line of L1210 cells (Cass and Au-Yeung, 1976). Our original stock of L1210/C2 leukemia cells was obtained from Dr Alan R.P. Paterson of this department. These cells may be propagated in culture or by in vivo passage; in this study, the L1210/C2 clonal line was passaged in vivo by weekly i.p. transplants of $10^6$ cells in either male or female B6D2$F_1$ hybrid mice. Stocks of L1210/C2 cells were stored under liquid nitrogen by conventional methods. After 25 weeks of in vivo passage, the passage line was restarted from frozen stock in order to preclude the possibility of genetic drift that might alter the leukemic cell response to araC. In the weekly passage of L1210/C2 cells, ascitic fluid was taken from a mouse on the seventh day after i.p. implantation of $10^6$ L1210/C2 cells and cells from that fluid were suspended in sterile 0.145 M NaCl

for i.p. transplantation of $10^6$ cells per mouse in the next transplant generation. Leukemia cell concentrations were determined with an electronic particle counter after lysis of erythrocytes in the ascitic fluid samples using Zap-Oglobin® II (Coulter Counter Burlington, Ont.)

E) Therapeutic studies

Groups of 7-21 $B6D2F_1$ hybrid mice of either sex (3-4 months old, weighing 25-30 grams) were injected with either $10^5$ or $10^6$ L1210/C2 leukemia cells. In these studies both the i.p. and the i.v. routes of administration were examined, both for transplantation of L1210/C2 cells and for the administration of araC. This resulted in four different experimental groups:

| | Route of Leukemia transplantation | Route of treatment |
|---|---|---|
| i) | i.v. | i.v. |
| ii) | i.v. | i.p. |
| iii) | i.p. | i.p. |
| iv) | i.p. | i.v. |

Twenty-four hours after implantation of leukemic cells, mice were treated with single injections of free araC, or with single injections of araC entrapped in either of two types of liposomes (SM:PC:CH:$G_{M1}$, 1:1:1:0.14, or PC:CH, 2:1) and the

mean survival times of mice in each treatment group were observed. Control mice received single injections of sterile 0.145 M NaCl solution, other groups of mice were treated by tail-vein infusion of free araC over 24 hours (see below). Three doses of araC were used in each treatment group. AraC was entrapped in liposomes by the REV method of preparation for experiments at the two lower doses of 10 and 20 mg/kg while the FAT method was used to trap the drug at the highest concentration of 80 mg/kg.

Leukemic mice were observed until death appeared imminent, at which time they were killed. Leukemic mice treated with araC were monitored for a minimum of 30 days, after which the experiment was terminated (Schabel et al., 1977). Survival times of mice in the various treatment groups were compared by an analysis of variance (Sokal and Rohlf, 1981).

F) Intravenous infusion procedure: tail-vein cannulation

B6D2$F_1$ hybrid male mice (25-30 grams) were obtained from the University of Alberta, Health Sciences Laboratory Animal Services. The mice were maintained as previously described. The tail-vein was easier to identify and cannulate if the mouse was placed under a heat lamp for 5 minutes. The mouse was then restrained in a plastic conical container (60 x 27 mm). The conical end of the container was cut off, creating

an opening that was 10 mm in diameter which provided the mouse with sufficient ventilation. A small piece of adhesive cloth tape was fastened to the entrance to prevent the mouse escaping from the container. The tail was cleaned with ethanol and the tail-vein was punctured with a 26 gauge sterile needle (Precisionguide™ Needles, Becton Dickson, Rutherford, NJ). The initial puncture of the tail-vein was required for the insertion of 5-10 mm of polyethylene tubing (inner diameter 0.28 mm, outer diameter 0.61 mm Intramedic® Polyethylene tubing, Clay Adams division of Beckton and Dickson, Parsippany, NJ) which was filled with sterile 0.145 M NaCl. The opposite end of the tubing was attached to a 10 ml syringe (Beckton Dickson, Rutherford, NJ) by a 30 gauge needle. Eight such syringes were attached to a Harvard Infusion Pump (model 940). Proper insertion of the tubing into the vein was determined by immediate withdrawal of a small volume of blood into the tubing, or by the lack of edema when 0.05 ml of saline was infused over about half an hour at a flow rate of 0.00206 ml/minute. The tubing was held in place by spraying the tail liberally with an aerosol adhesive dressing (Aeroplast Dressing®, Park, Davis and Company, Brockville, Ont.). Once the dressing was dry, the tail of the animal was secured to a metallic splint (200 x 6 x 1 mm) using three strips of adhesive cloth tape. The tail was then encased in a plastic cylindrical tube (175 mm in length, inside diameter 9 mm, outside diameter 12 mm) which was

anchored to the top of the mouse cage with a length of copper wire so that the tail was suspended at a minimum angle of 45° with respect to the base of the cage. Complete immobilization of the tail was necessary to prevent the mouse from chewing its tail or the cannula during the 24 hour infusion. While this procedure limited the mobility of the mouse, the animal was able to eat and drink during the 24 hour infusion process. This procedure is similar to that reported by Paul and Dave (1975) and Danhauser and Rustum (1979).

All drugs were administered in a total volume of 3 ml by slow infusion at a flow rate of 0.00206 ml/minute over a 24 hour period. Once the drug had been successfully administered the mouse was placed in the restraining container and the cannula was carefully removed. The cannulation site was cleaned and held firmly until the bleeding had stopped. The plastic cylindrical tube was left on the tail for a minimum of 48 hours to facilitate healing of the tail and to prevent the mouse from gnawing its tail. Each mouse was housed separately during this recovery period.

# CHAPTER III RESULTS

A) Liposome characterization

After each liposome preparation was extruded 10 times through 0.4 μm Nuclepore filters, the phosphate content was determined and the trapped volume within the liposomes was calculated. Trapped volume measurements from numerous liposome preparations of either PC:CH or SM:PC:CH:$G_{M1}$ (stealth), indicated that trapped volumes were about 4.0 l of water/mole of phospholipid. When possible, liposome size was determined by quasi-elastic light scattering. Representative size distribution analysis of liposomes composed of SM:PC:CH:-$G_{M1}$ and PC:CH are shown in Tables 1 and 2 respectively.

B) AraC leakage from liposomes

AraC leakage from liposomes was evaluated in experiments with $^{3}$H-araC entrapped in both PC:CH, 2:1 and stealth liposomes (0.4 μm LUV). The liposomes were prepared with $^{3}$H-araC at two concentrations of 60 mg/ml (290 mOsm) and 150 mg/ml (850 mOsm) which were, respectively, isotonic and hypertonic. The latter concentration of araC was chosen to examine the effect of an osmotic gradient on araC leakage to determine if the use of hypertonic concentrations of entrapped araC in liposomes was an option. The liposomes were incubated in either TES buffer or TES buffer containing 25% human plasma.

Table 1 Size distribution analysis of liposomes for SM:PC:CH:$G_{M1}$ LUV (0.4 μm)

| Diameter (nm) | Cumulative intensity |
|---|---|
| 103 | 1 |
| 110 | 3 |
| 117 | 7 |
| 124 | 12 |
| 132 | 20 |
| 141 | 30 |
| 150 | 40 |
| 159 | 49 |
| 170 | 57 |
| 181 | 62 |
| 192 | 65 |
| 205 | 66 |
| 218 | 66 |
| 232 | 67 |
| 247 | 70 |
| 263 | 73 |
| 279 | 78 |
| 297 | 84 |
| 317 | 89 |
| 337 | 94 |
| 359 | 97 |
| 382 | 99 |
| 406 | 100 |

**Mean diameter of SM:PC:CH:$G_{M1}$ liposomes = 204 nm**

**Trapped volume of SM:PC:CH:$G_{M1}$ liposomes = 3.8 litres/mole**

Table 2 Size distribution analysis of liposomes for PC:CH LUV (0.4 μm)

| Diameter (nm) | Cumulative intensity |
|---|---|
| 77 | 0 |
| 82 | 2 |
| 87 | 5 |
| 92 | 8 |
| 104 | 19 |
| 110 | 26 |
| 117 | 29 |
| 124 | 31 |
| 132 | 32 |
| 140 | 33 |
| 149 | 33 |
| 158 | 33 |
| 168 | 37 |
| 178 | 43 |
| 189 | 52 |
| 200 | 62 |
| 213 | 72 |
| 226 | 82 |
| 239 | 89 |
| 254 | 94 |
| 270 | 97 |
| 286 | 99 |
| 304 | 100 |

**Mean diameter of PC:CH liposomes** = 179 nm

**Trapped volume of PC:CH liposomes** = 3.3 litres/mole

Liposomes were incubated in plasma-containing buffer to assess their stability in a system that approximated *in vivo* conditions (Allen and Cleland, 1980).

PC:CH liposomes prepared with entrapped araC under isotonic or hypertonic conditions, when incubated at 37°C in TES buffer (290 mOsm pH 7.4), lost approximately 25% of the entrapped contents over a 24-hour period (Figure 1). Under both conditions of osmolarity the leakage of araC from these liposomes occurred in a biphasic fashion with a half-time for the first leakage phase ($T_{\frac{1}{2}}\alpha$) of 0.4 hours, and for the longer second phase of leakage ($T_{\frac{1}{2}}\beta$) of 442 hours (Table 3A and 4A). When PC:CH liposomes containing isotonic concentrations of araC were incubated in TES buffer containing human plasma, the liposomes had a high initial rate of leakage during the first 5 hours of incubation, during which time approximately 60% of entrapped contents leaked from the liposomes. There was also a further loss of 15% of entrapped contents in the following 19 hours (Figure 1). The initial rapid phase of leakage, $T_{\frac{1}{2}}\alpha$, had a half-time of 1.3 hours, while the second phase of leakage had an apparent half-time of 126 hours (Table 3B). PC:CH liposomes, loaded with hypertonic araC solution and incubated in 25% plasma in buffer, leaked faster than liposomes loaded with an isotonic araC solution; the araC loss was about 80% within the first 5 hours of incubation with a further loss of 10% during the following 19 hours (Figure 1). The initial leakage phase had a half-time of 0.5 hours and the

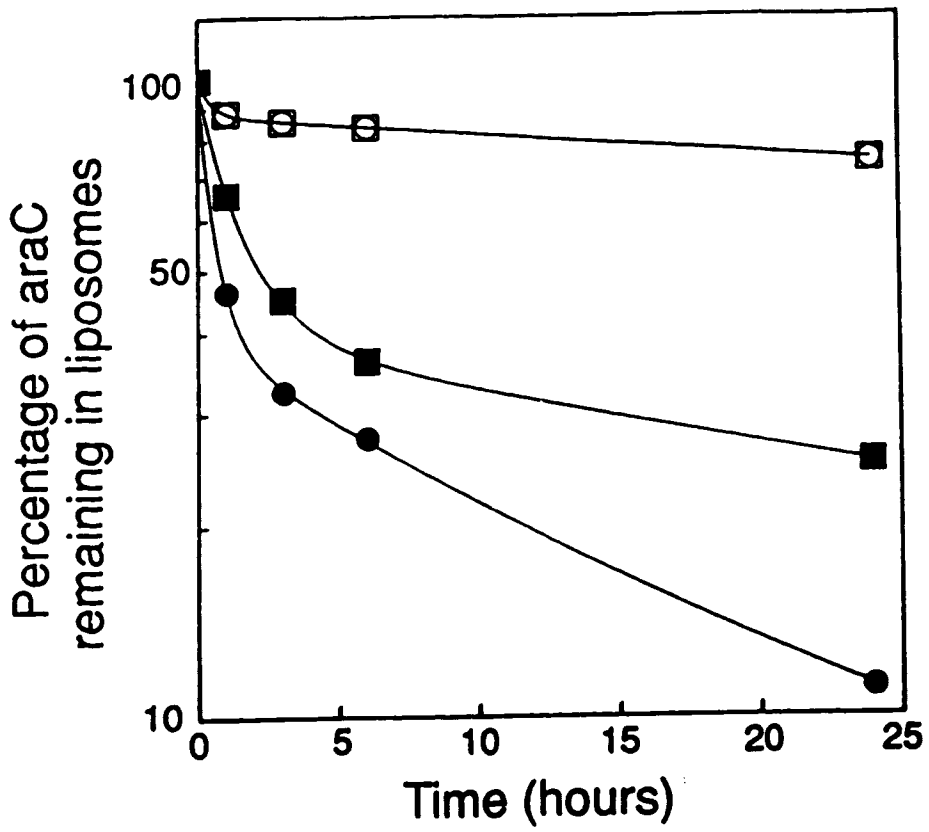


Figure 1

Leakage of araC from PC:CH, 2:1 liposomes.

Liposomes composed of PC:CH were prepared with either isotonic araC (60 mg/ml) (squares) or hypertonic araC (150 mg/ml) (circles) and incubated at 37°C in TES buffer (open symbols) or in 25% human plasma in TES buffer (filled symbols).

Table 3

The half-times for araC (60 mg/ml) leakage at 37°C for LUV composed of PC:CH, 2:1 or SM:PC:CH:$G_{M1}$, 1:1:1:0.14

The liposomes were prepared with isotonic araC concentration (60 mg/ml) and the half-times for araC leakage was estimated from the loss of initial entrapped contents, in the presence of TES buffer (A) or in TES buffer containing 25% human plasma (B).

A

| Liposome composition | $T_{1/2}\alpha$ | $T_{1/2}\beta$ |
|---|---|---|
| SM:PC:CH:$G_{M1}$ | 0.4 | 424 |
| PC:CH | 0.4 | 442 |

B

| Liposome composition | $T_{1/2}\alpha$ | $T_{1/2}\beta$ |
|---|---|---|
| SM:PC:CH:$G_{M1}$ | 0.8 | 204 |
| PC:CH | 1.3 | 126 |

Table 4

The half-times for araC (150 mg/ml) leakage at 37°C for LUV composed of PC:CH, 2:1 or SM:PC:CH:$G_{M1}$, 1:1:1:0.14

The liposomes were prepared with hypertonic araC concentration (150 mg/ml) and the half-times for araC leakage was estimated from the loss of initial entrapped contents, in the presence of TES buffer (A) or in TES buffer containing 25% human plasma (B).

A

| Liposome composition | $T_{\frac{1}{2}}\alpha$ | $T_{\frac{1}{2}}\beta$ |
|---|---|---|
| SM:PC:CH:$G_{M1}$ | 0.5 | 545 |
| PC:CH | 0.4 | 442 |

B

| Liposome composition | $T_{\frac{1}{2}}\alpha$ | $T_{\frac{1}{2}}\beta$ |
|---|---|---|
| SM:PC:CH:$G_{M1}$ | 0.3 | 379 |
| PC:CH | 0.5 | 40 |

second phase had a half-time of more than 40 hours (Table 4B).

When vesicles composed of SM:PC:CH:$G_{M1}$, containing an isotonic solution of araC, were incubated in an isotonic TES buffer, the araC loss was about 20% during a 24-hour period (Figure 2), with a $T_{\frac{1}{2}}\alpha$ of 0.4 hours and a $T_{\frac{1}{2}}\beta$ of 424 hours (Table 3A). Under the same conditions, vesicles containing hypertonic araC solution lost about 40% of their araC contents within the same time span (Figure 2). Under these conditions the $T_{\frac{1}{2}}\alpha$ was 0.5 hours and the $T_{\frac{1}{2}}\beta$ was 545 hours (Table 4A). When stealth liposomes containing isotonic araC solutions, were incubated in TES buffer containing human plasma, the loss of entrapped contents over 24 hours was approximately 50% (Figure 2). The leakage also occurred in a biphasic fashion, with a $T_{\frac{1}{2}}\alpha$ of 0.8 hours and a $T_{\frac{1}{2}}\beta$ of 204 hours (Table 3B). However, when stealth liposomes containing hypertonic solution of araC, were incubated under identical conditions, a biphasic leakage pattern resulted with a substantial loss of contents (about 60%) within the first hour, and a further loss of 10% of the entrapped contents over the next 23 hours (Figure 2). Under these conditions the $T_{\frac{1}{2}}\alpha$ was 0.3 hours and the $T_{\frac{1}{2}}\beta$ was 379 hours (Table 4B).

In summary, araC leakage in isotonic buffer was similar in liposomes of both compositions, whether loaded with isotonic or hypertonic araC solutions. However, stealth liposomes were less susceptible to leakage induction by human

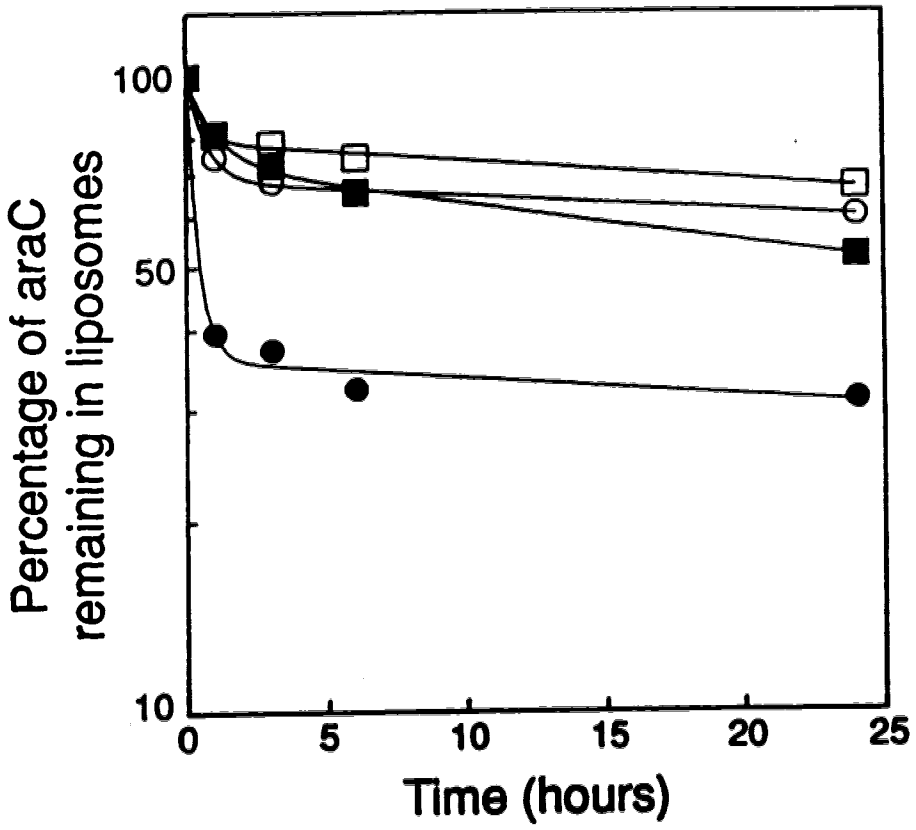


Figure 2

Leakage of araC from SM:PC:CH:$G_{M1}$, 1:1:1:0.14 liposomes.

Liposomes composed of SM:PC:CH:$G_{M1}$ were prepared with either isotonic araC (60 mg/ml) (squares) or hypertonic araC (150 mg/ml) (circles) and incubated at 37°C in TES buffer (open symbols) or in 25% human plasma in TES buffer (filled symbols).

plasma than were PC:CH liposomes and maintained entrapped araC over longer time periods.

C) Tissue distribution of liposomes *in vivo*

Time courses for the *in vivo* elimination and the distribution of liposomes of both types were determined in mice following administration by both the i.p. and i.v. routes. Liposomes were radioactively labelled with [$^{125}$I]TI. In addition, similar distribution studies were conducted with leukemic mice 24 hours after i.v. or i.p. implantation of $10^6$ L1210/C2 cells.

C 1) Pharmacokinetics and distribution of liposomes following i.v. administration

LUVs composed of SM:PC:CH:$G_{M1}$ remained in the circulation longer than did LUVs composed of PC:CH (compare Figures 3, 4A,4B with Figures 5A,5B) and were cleared from circulation in a biphasic fashion. More than 10% of the liposomes remained in circulation 24 hours after i.v. administrations (Figure 3). Fifty percent of liposome-entrapped [$^{125}$I]TI was present within the vasculature 4 hours after liposomal administration. The clearance of liposomes from blood coincided with uptake of liposomes by the liver and spleen. Thirty-three to 39% of liposomes remaining *in vivo* 24 hours

after injections were detected in the liver and 19 to 25% were present in the spleen. In addition, 20 to 30% of liposomes remaining *in vivo* were present in carcass tissues (Figure 4A,B). The pharmacokinetic behaviour of $G_{M1}$-containing liposomes, when administered by the i.v. route, were similar in leukemic and non-leukemic mice (compare Figure 4A and 4B).

When PC:CH liposomes were injected intravenously, the liposomes were removed from circulation in a biphasic fashion, but much more rapidly than stealth liposomes. Less than 1% of the liposomes remained in the vasculature 24 hours after administered (Figure 3). Fifty percent of PC:CH liposomes were cleared from circulation in about 0.5 hours after administration. It was also observed that PC:CH liposomes accumulated more rapidly than stealth liposomes in the liver and spleen; 2 hours after mice received injections of PC:CH liposomes, 45 to 50% of the liposomes remaining *in vivo* were detected in the liver and 11 to 21% were in the spleen. After 24 hours, 60 to 65% of the liposomes had accumulated in the liver and 20 to 23% were present in the spleen while 10 to 15% of liposomes remaining *in vivo* were present in carcass tissues (Figure 5A). As observed for the $G_{M1}$-containing liposomes, the time-dependent distribution of PC:CH liposomes was not significantly different in leukemic mice from that of non-leukemic mice (Figure 5B).

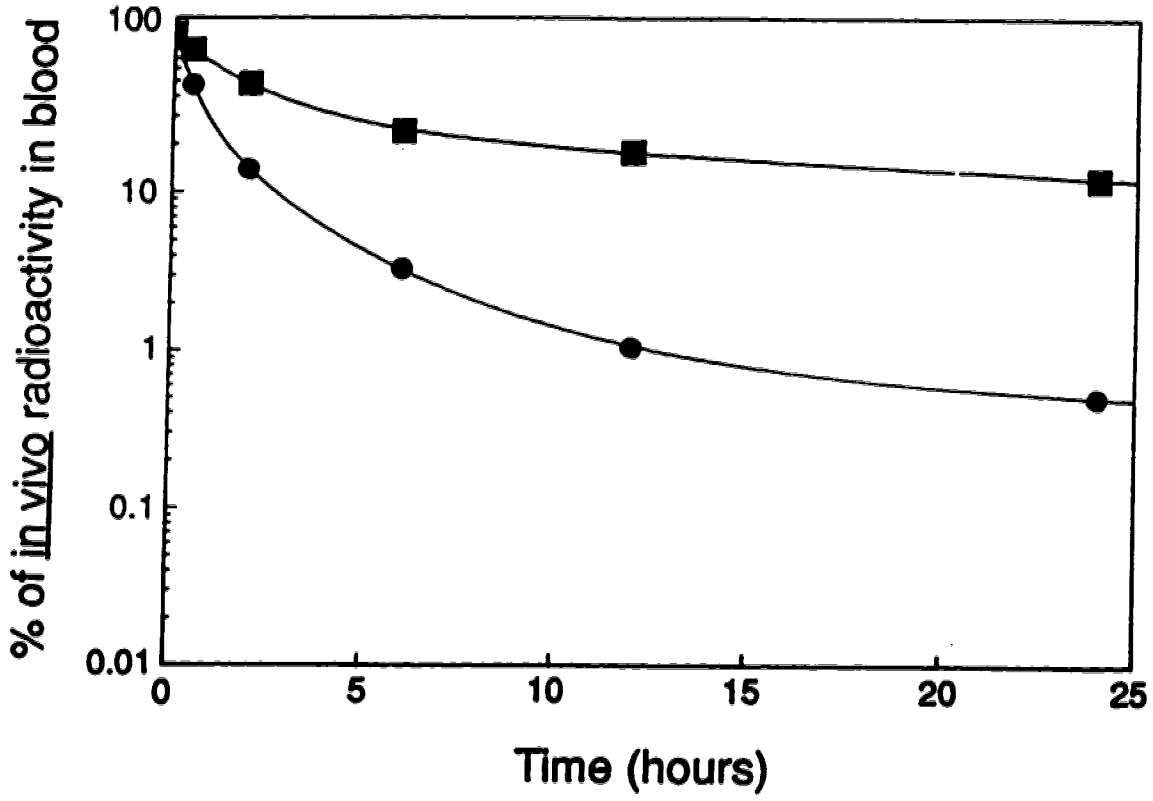


Figure 3

Clearance of [$^{125}$I]TI-containing liposomes (SM:PC:CH:$G_{M1}$, 1:1:1:0.14 and PC:CH, 2:1) from the circulation of mice following i.v. administration.

After receiving i.v. injections (0.5 µmol of phospholipid) of liposomes containing [$^{125}$I]TI, mice were killed at the times shown and blood samples were assayed for $^{125}$I-content. The results are expressed as the percent of <u>in vivo</u> radioactivity remaining in blood with respect to time. SM:PC:CH:$G_{M1}$, 1:1:1:0.14 (squares); PC:CH, 2:1 (circles).

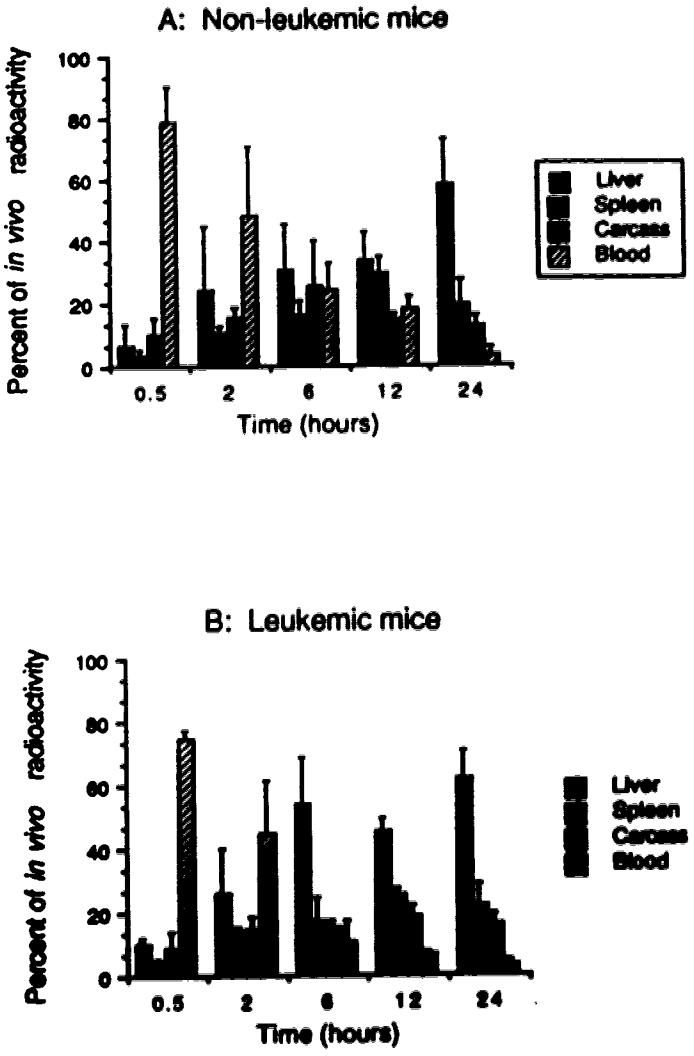


Figure 4

Retention of SM:PC:CH:$G_{M1}$ liposomes in tissues at different time intervals after i.v. injections.

The percent of injected radioactivity in liver, spleen, blood and carcass tissues was determined in non-leukemic mice (A) or leukemic mice that were transplanted 24 hours earlier with $10^6$ L1210/C2 cells, by the i.v. route (B) (mean $\pm$ standard deviation, n=3).

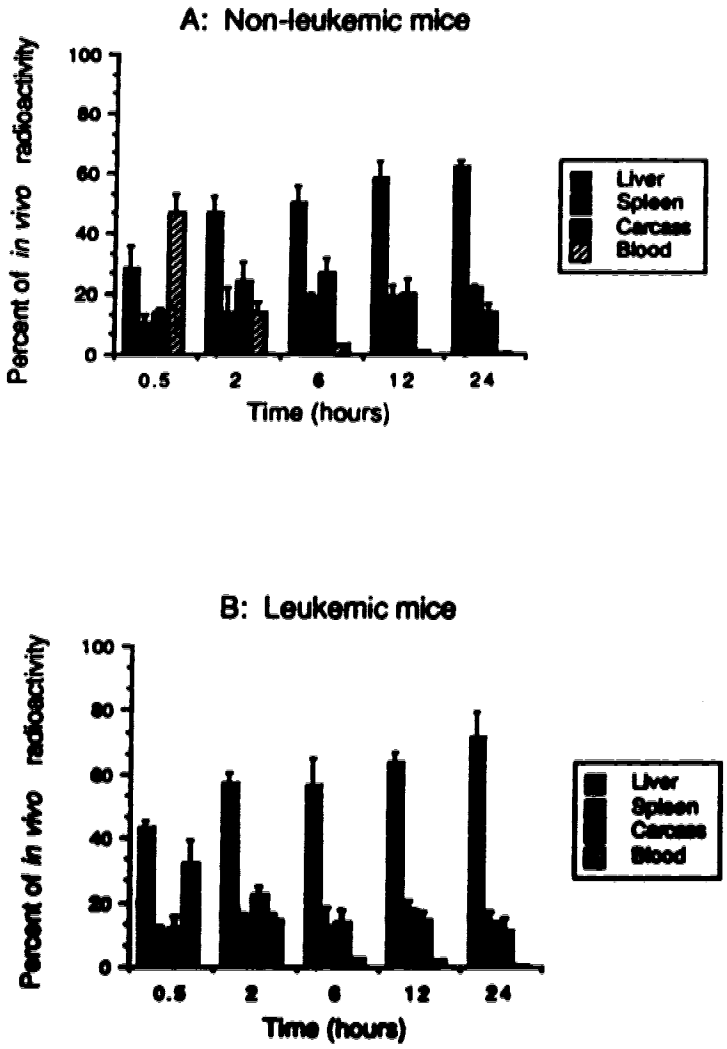


**Figure 5**

**Retention of PC:CH liposomes in tissues at different time intervals after i.v. injections.**

**The percent of injected radioactivity in liver, spleen, blood and carcass tissues was determined in non-leukemic mice (A) or leukemic mice that were transplanted 24 hours earlier with $10^6$ L1210/C2 cells, by the i.v. route (B) (mean $\pm$ standard deviation, n=3).**

C 2) Pharmacokinetics and distribution of liposomes following i.p. administration

When liposome administration was by the i.p. route, $G_{M1}$ containing liposomes persisted in the blood at higher levels and over longer intervals than PC:CH liposomes (compare Figures 6, 7A,7B with 8A,8B). Administration of stealth liposomes by the i.p. route resulted in a delayed exit of liposomes from the peritoneal cavity into the vasculature (Figure 7A and 7B). The movement of liposomes from the peritoneal cavity into the vasculature was apparent during the first two hours after injection, at which time maximum blood concentrations of liposomes were reached. This was followed by a slow decrease of liposomes in blood over the next 22 hours. Two hours after injections of stealth liposomes, maximum blood liposome content of 42 to 52% was observed and more than 10% of the liposomes remained in the blood 24 hours after administration. Twenty-four hours after liposomes were injected i.p., 11 to 35% of the liposome dose was found in the liver and 7 to 23% was in the spleen. Because of the delayed exit of liposomes from the peritoneal cavity, there was also a reduced accumulation of liposomes in the liver and spleen when compared to the results of similar studies in which the i.v. route was employed.

Similar experiments were performed using PC:CH liposomes (Figures 8A and 8B). Two hours after mice received liposomes

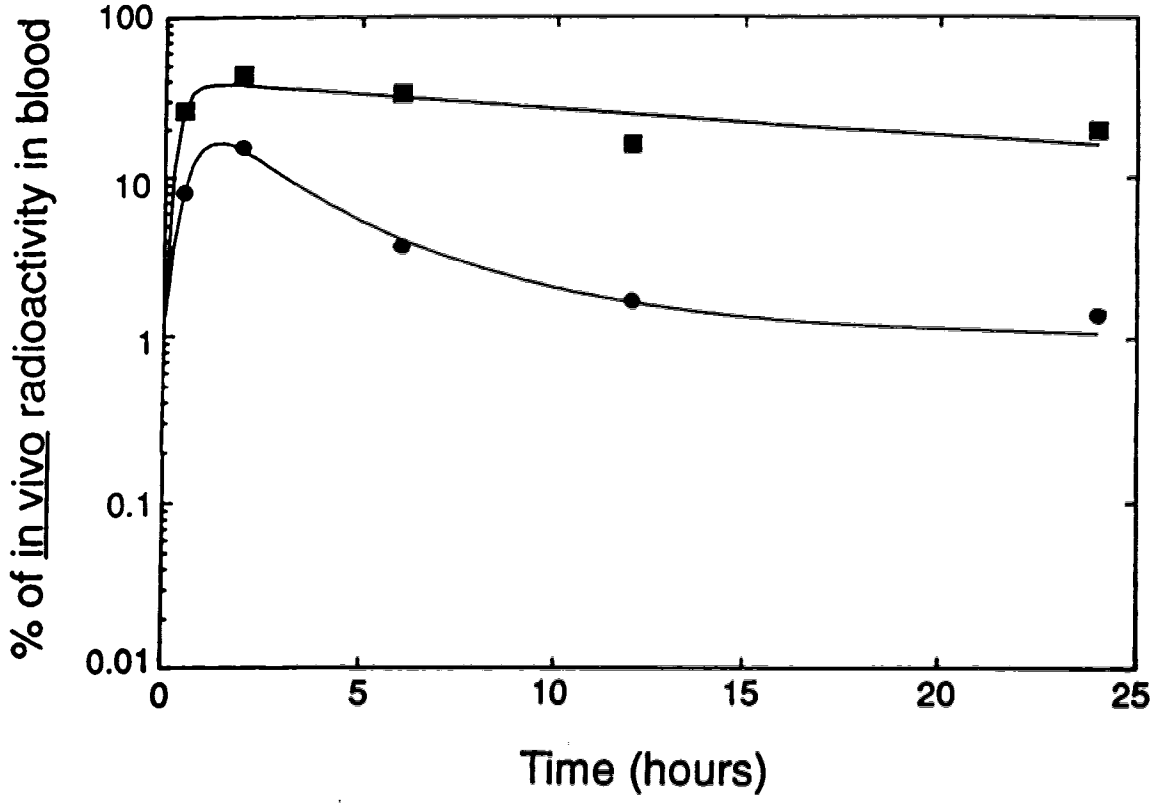


Figure 6

Clearance of [$^{125}$I]TI-containing liposomes (SM:PC:CH:$G_{M1}$, 1:1:1:0.14 and PC:CH, 2:1) from the circulation of mice following i.p. adminstration.

After receiving i.p. injections (0.5 µmol of phospholipid) of liposomes containing [$^{125}$I]TI, mice were killed at the times shown and blood samples were assayed for $^{125}$I-content. The results are expressed as the percent of in vivo radioactivity remaining in blood with respect to time. SM:PC:CH:$G_{M1}$, 1:1:1:0.14 (squares); PC:CH, 2:1 (circles).

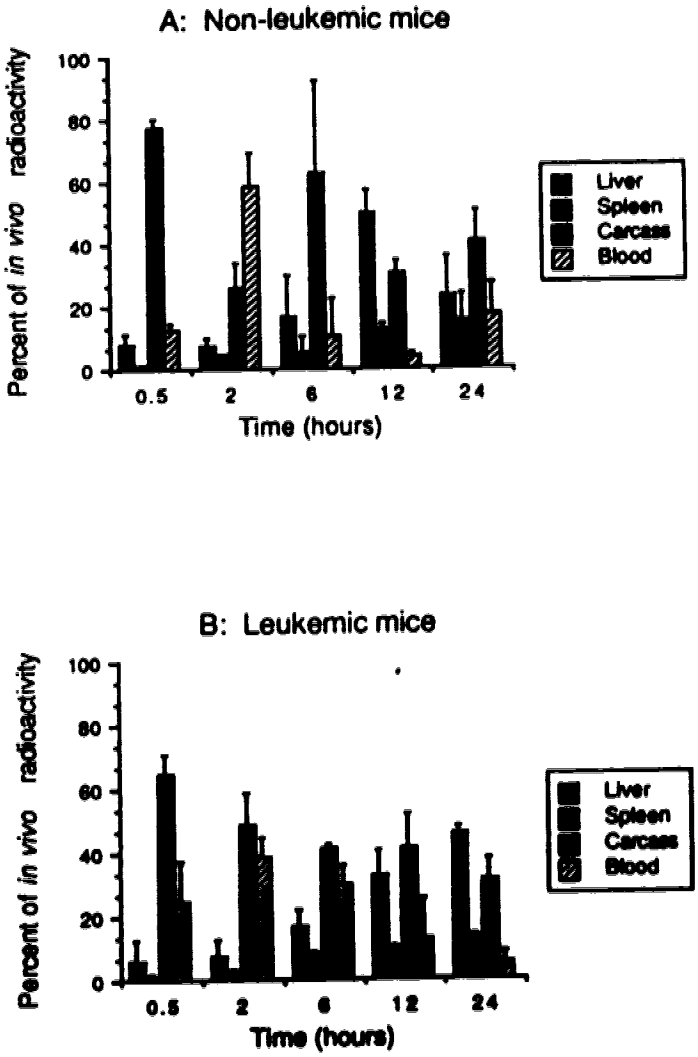


Figure 7

Retention of SM:PC:CH:$G_{M1}$ liposomes in tissues at different time intervals after i.p. injections.

The percent of injected radioactivity in liver, spleen, blood and carcass tissues was determined in non-leukemic mice (A) or leukemic mice that were transplanted 24 hours earlier with $10^6$ L1210/C2 cells, by the i.p. route (B) (mean $\pm$ standard deviation, n=3).

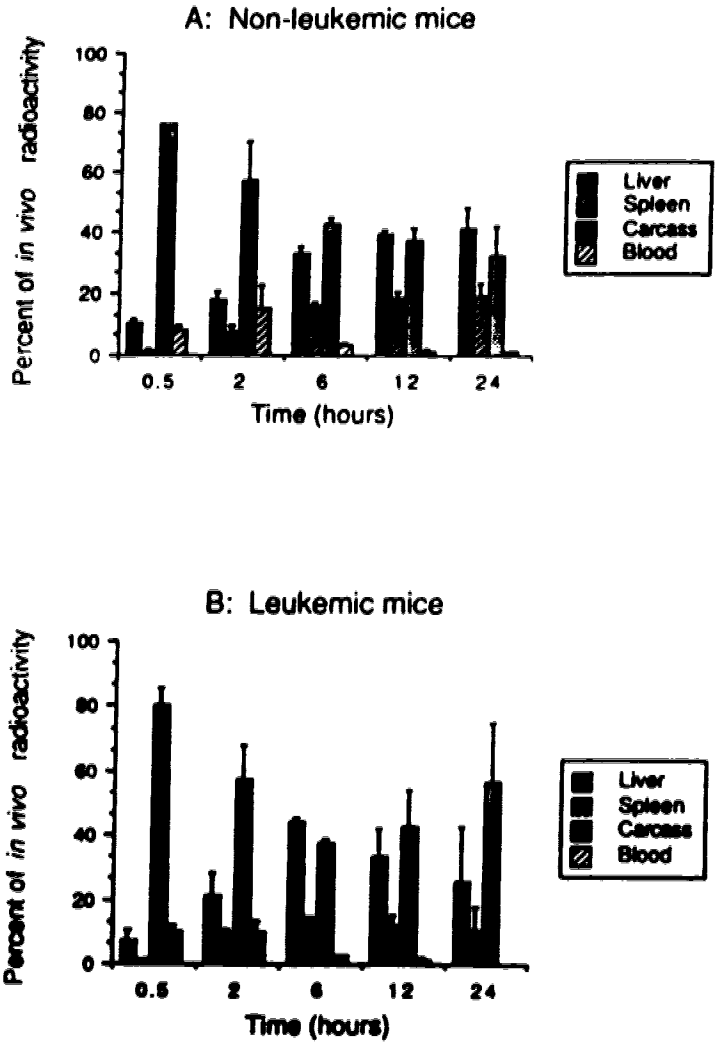


Figure 8

Retention of PC:CH liposomes in tissues at different time intervals after i.p. injections.

The percent of injected radioactivity in liver, spleen, blood and carcass tissues was determined in non-leukemic mice (A) or leukemic mice that were transplanted 24 hours earlier with $10^6$ L1210/C2 cells, by the i.p. route (B) (mean $\pm$ standard deviation, n=3).

composed of PC:CH by the i.p. route, maximum blood liposome levels of 8 to 22% were observed and 24 hours after liposome injections approximately 0.02 to 1% of the liposomes remained within the vasculature. Twenty-four hours after the injection of these liposomes by the i.p. route, 34 to 48% of liposomes remaining in vivo had accumulated in the liver and 15 to 23% were in the spleen. The pharmacokinetics of either liposome composition injected by the i.p. route, was not significantly affected for mice implanted with $10^6$ L1210/C2 cells, 24 hours before the liposomes injections.

**D) Effects of L1210/C2 leukemia cells on mean survival times of mice**

Before examining different cancer treatments using liposomes as a sustained release drug delivery system for antineoplastic agents, survival times of untreated mice implanted with graded numbers of L1210/C2 leukemia cells were investigated (Schabel et al., 1977). An exponential relationship was observed between the number of L1210/C2 cells implanted, by either the i.p. or the i.v. route, and the number of days mice survived after implantation (Figure 9). Implantation by the i.v. route reduced MST realative to that by the i.p. route. Extrapolation of the (survival times versus cells implanted) relationship indicated that i.v. inoculation of one leukemic cell per mouse would lead to the

death of the animal in approximately 17.5 days. Similar calculations for i.p. inoculations suggests that the animals would survive for approximately 20 days (Figure 9). This relationship provides a better understanding of the growth kinetics of the L1210/C2 cell implanted into mice.

In order to cure leukemic mice it is necessary to eradicate all leukemic cells. Leukemic mice that are treated with antileukemic drugs, such as araC, may experience an increased MST. Based on the survival times of mice it is possible to estimate the fraction of leukemic cells killed by a therapeutic regimen (Schabel et al., 1977).

**E) Therapeutic studies in leukemic-bearing mice**

The therapeutic efficacy of varied doses of araC entrapped in liposomes was examined for: (i) the two liposome types, (ii) i.v. or i.p. leukemic cell implantation and (iii) i.v. or i.p. routes of liposome administration. In addition, the administration of araC (that is not entrapped in liposomes) served as a control condition. Free araC was administered by continuous infusion over 24 hours, or by single i.p. or i.v. injections. In another set of controls, mice received single injections of drug-free liposomes, or drug-free liposomes plus free araC.

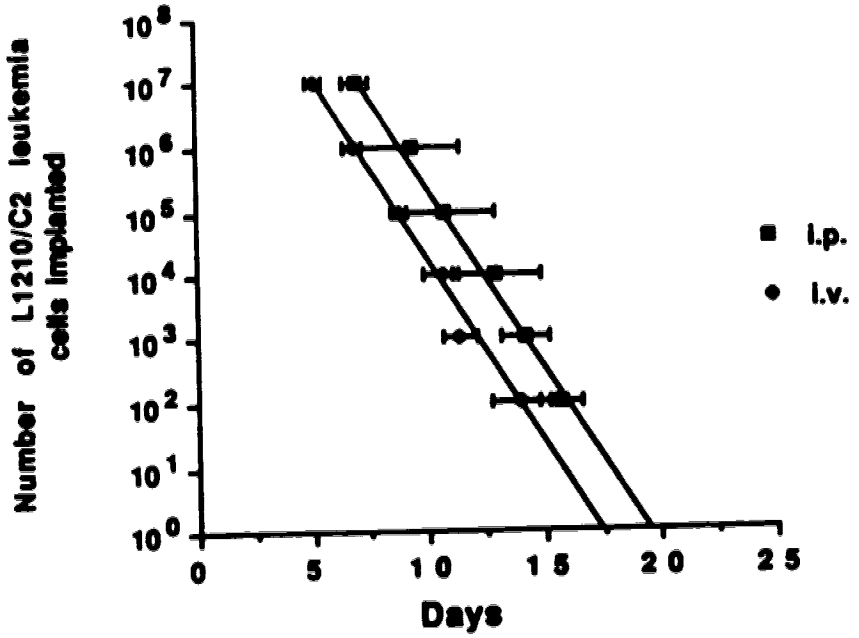


Figure 9

Mean survival times of mice receiving inoculations of L1210/C2 leukemia cells.

Groups of mice (10 mice per group) were inoculated by either the i.p. or the i.v. route with different L1210/C2 cell burdens and the mean survival times ($\pm$ standard deviation) of mice were determined.

**E 1) Influence of drug-free liposomes on the mean survival times of leukemic mice**

The effects of treatment with liposomes containing only buffer (empty liposomes) on survival of mice inoculated with $10^6$ L1210/C2 cells, are shown for i.v. administration (Table 5), and for i.p. administration (Table 6). Single injections of empty PC:CH liposomes given alone, or given with 10 mg/kg araC by the i.v. route, had no significant effect on the growth of L1210/C2 leukemia (Table 5). Similarly, when leukemic mice were treated with i.v. single injections of empty stealth liposomes or with empty liposomes plus 10 mg/kg araC, no significant effects on the growth of L1210/C2 leukemia were observed (Table 5). Similar experiments in which both the leukemia inoculations and treatments were given by the i.p. route, were also without significant effect on the survival times of leukemic mice (Table 6).

**E 2) Influence of inoculation route and treatment route on mean survival times of mice transplanted with $10^5$ L1210/C2 leukemia cells**

Initial studies of the therapeutic efficacy of liposomes containing entrapped araC were conducted with mice implanted with $10^5$ leukemic cells 24 hours before treatment started and at a drug dose of 10 mg/kg. With i.p. implanted leukemia

Table 5

Effects of empty liposomes or free araC administered by the i.v. route on the MST of mice implanted with L1210/C2 leukemic cells by the i.v. route

The effect of empty liposomes composed of either PC:CH, 2:1 or SM:PC:CH:$G_{M1}$, 1:1:1:0.14 (LUV, 0.4 μm), injected by the i.v. route, on the mean survival times of B6D2$F_1$ hybrid mice implanted with $10^6$ L1210/C2 leukemia cells (10 mice/group), by the i.v. route, were examined.

| Treatment | Route of L1210/C2 implantation | Route of treatment | Days survived ± S.E. |
|---|---|---|---|
| Control[a] | i.v. | i.v. | 6.0 ± 0.00 |
| Free araC[b] + PC:CH[c] | i.v. | i.v. | 6.3 ± 0.15 |
| Free araC[b] + SM:PC:CH:$G_{M1}$[c] | i.v. | i.v. | 6.3 ± 0.15 |
| PC:CH[c] | i.v. | i.v. | 6.0 ± 0.00 |
| SM:PC:CH:$G_{M1}$[c] | i.v. | i.v. | 6.0 ± 0.00 |

a = injections of sterile 0.145 M NaCl
b = 10 mg/kg araC
c = empty liposomes

Table 6

Effects of empty liposomes or free araC administered by the i.p. route on the MST of mice implanted with L1210/C2 leukemia cells by the i.p. route

The effect of empty liposomes composed of either PC:CH, 2:1 or SM:PC:CH:$G_{M1}$, 1:1:1:0.14 (LUV, 0.4 μm), injected by the i.p. route, on the mean survival times of B6D2$F_1$ hybrid mice implanted with $10^6$ L1210/C2 leukemia cells (10 mice/group), by the i.p. route, were examined.

| Treatment | Route of L1210/C2 implantation | Route of treatment | Days survived ± S.E. |
|---|---|---|---|
| Control[a] | i.p. | i.p. | 9.9 ± 0.31 |
| Free araC[b] + PC:CH[c] | i.p. | i.p. | 9.2 ± 0.13 |
| Free araC[b] + SM:PC:CH:$G_{M1}$[c] | i.p. | i.p. | 9.3 ± 0.26 |
| PC:CH[c] | i.p. | i.p. | 10.2 ± 0.25 |
| SM:PC:CH:$G_{M1}$[c] | i.p. | i.p. | 11.1 ± 0.48 |

a = injections of sterile 0.145 M NaCl
b = 10 mg/kg araC
c = empty liposomes

cells, the mean survival time (MST) of control mice (without araC treatment) was 10.3 ± 0.26 days (Tables 7 and 8). Single treatments with free araC, by either the i.p. or the i.v route, did not significantly prolong the survival of leukemic mice (Tables 7 and 8). Continuous infusion, by the i.v. route, of free araC increased the MST to 16.9 ± 0.88 days, a significant prolongation of survival times relative to control animals, or compared to animals treated with (i) free araC or (ii) PC:CH liposomes containing araC. Single i.v. treatments with stealth liposomes containing araC increased survival times of leukemic mice to 15.7 ± 0.80 days, a result similar to that obtained with treatment by continuous i.v. infusion of free araC.

Single doses of araC entrapped in PC:CH liposomes, when administered by the i.v. route, were not more effective in killing leukemia cells than single doses (10 mg/kg) of free araC (Table 8). When the liposomes were administered by the i.p. route, however, a moderate increase in the MST of leukemic mice was observed for PC:CH liposomes with entrapped araC (MST= 13.0 ± 0.46). The greatest increase in the MST in this set of experiments was achieved with single i.p. injections of stealth liposomes, which yielded survival times of 19.2 ± 0.83 days (Table 7).

Similar results were obtained in the treatment of mice implanted with leukemia cells by the i.v. route (Tables 9 and 10). Single treatments with free araC by either route were

Table 7

Effect of i.p. araC (10 mg/kg) treatment on L1210/C2 leukemia ($10^5$ cells), implanted by the i.p. route, on the MST of mice.

The effect of single injections of free araC or single injections of liposomes containing entrapped araC, injected by the i.p. route (10 mg/kg), on the mean survival times of $B6D2F_1$ hybrid mice (7 to 21 mice/group) were examined. The mice were implanted with $10^5$ L1210/C2 leukemia cells, by the i.p. route 24 hours before treatment.

| Treatment | Route of L1210/C2 implantation | Route of treatment | Days survived ± S.E. AraC 10 mg/kg |
|---|---|---|---|
| Control[a] | i.p. | i.p. | 10.3 ± 0.26 |
| Free araC[b] | i.p. | i.p. | 11.4 ± 0.17 |
| PC:CH[c] | i.p. | i.p. | 13.0 ± 0.46 |
| SM:PC:CH:$G_{M1}$[c] | i.p. | i.p. | 19.2 ± 0.83 |

a = injections of sterile 0.145 M NaCl
b = single injection of free araC
c = single injection of liposome entrapped araC

Table 8

Effect of araC (10 mg/kg) administered by the i.v. route and L1210/C2 leukemia ($10^5$ cells) implanted by the i.p. route, on the MST of mice.

The effect of single injections of free araC, 24 hour infusions of free araC or single injections of liposomes with entrapped araC, administered by the i.v. route (10 mg/kg), on the mean survival times of B6D2$F_1$ hybrid mice (7 to 21 mice/group) were examined. Mice were implanted with $10^5$ L1210/C2 leukemia cells, by the i.p. route 24 hours before treatment.

| Treatment | Route of L1210/C2 implantation | Route of treatment | Days survived ± S.E. AraC 10 mg/kg |
|---|---|---|---|
| Control[a] | i.p. | i.v. | 10.3 ± 0.26 |
| Free araC[b] | i.p. | i.v. | 11.1 ± 0.36 |
| Free araC[c] | i.p. | i.v. | 16.9 ± 0.88 |
| PC:CH[d] | i.p. | i.v. | 11.4 ± 0.20 |
| SM:PC:CH:$G_{M1}$[d] | i.p. | i.v. | 15.7 ± 0.80 |

a = injections of sterile 0.145 M NaCl
b = single injection of free araC
c = 24 hour infusion of free araC
d = single injection of liposome entrapped araC

ineffective whereas either i.p. or i.v. administration of araC entrapped in stealth liposomes containing araC prolonged survival of leukemic mice (Tables 9 and 10). However, treatment of leukemic-bearing mice by the i.p. route, but not by the i.v. route, with PC:CH liposomes moderately improved the MST of leukemic-bearing mice (Table 8 and 10). Again, stealth liposomes, given by the i.p. route, resulted in the greatest survival of leukemic mice (13.0 $\pm$ 0.43 days, Table 10).

**E 3) Influence of inoculation route and treatment route on mean survival times of mice transplanted with $10^6$ L1210/C2 leukemia cells**

The therapeutic efficacy of liposomes containing entrapped araC was evalulated for mice implanted with $10^6$ leukemic cells 24 hours before treatment started. In these studies three doses of araC were employed; 10, 20 and 80 mg/kg. In some instances data were not obtained at the highest concentration of araC, since the results obtained at the lower concentrations were unequivocal and the additional data would not be expected to contribute substantially to the overall conclusions.

**E 3 i) Drug dose: 10 mg/kg**

The therapeutic efficacy of araC entrapped in liposomes

Table 9

Effect of i.v. araC (10 mg/kg) treatment on L1210/C2 leukemia ($10^5$ cells), implanted by the i.v. route, on the MST of mice.

The effect of single injections of free araC, 24 hour infusions of free araC or single injections of liposomes containing entrapped araC, injected by the i.v. route (10 mg/kg), on the mean survival times of B6D2F$_1$ hybrid mice (7 to 21 mice/group) were examined. Mice were implanted with $10^5$ L1210/C2 leukemia cells, by the i.v. route 24 hours before treatment.

| Treatment | Route of L1210/C2 implantation | Route of treatment | Days survived ± S.E. AraC 10 mg/kg |
|---|---|---|---|
| Control[a] | i.v. | i.v. | 7.5 ± 0.11 |
| Free araC[b] | i.v. | i.v. | 8.0 ± 0.00 |
| Free araC[c] | i.v. | i.v. | 12.5 ± 0.17 |
| PC:CH[d] | i.v. | i.v. | 8.9 ± 0.26 |
| SM:PC:CH:$G_{M1}$[d] | i.v. | i.v. | 11.2 ± 0.18 |

a = injections of sterile 0.145 M NaCl
b = single injection of free araC
c = 24 hour infusion of free araC
d = single injection of liposome entrapped araC

Table 10

Effect of araC (10 mg/kg) administered by the i.p. route and L1210/C2 leukemia ($10^5$ cells) implanted by the i.v. route, on the MST of mice.

The effect of single injections of free araC or single injections of liposomes containing entrapped araC, injected by the i.p. route (10 mg/kg), on the mean survival times of B6D2$F_1$ hybrid mice (7 to 21 mice/group) were examined. Mice were implanted with $10^5$ L1210/C2 leukemia cells, by the i.v. route 24 hours before treatment.

| Treatment | Route of L1210/C2 implantation | Route of treatment | Days survived ± S.E. AraC 10 mg/kg |
|---|---|---|---|
| Control[a] | i.v. | i.p. | 7.5 ± 0.11 |
| Free araC[b] | i.v. | i.p. | 9.7 ± 0.23 |
| PC:CH[c] | i.v. | i.p. | 10.5 ± 0.19 |
| SM:PC:CH:$G_{M1}$[c] | i.v. | i.p. | 13.0 ± 0.43 |

a = injections of sterile 0.145 M NaCl
b = single injection of free araC
c = single injection of liposome entrapped araC

was determined at an inoculum of $10^6$ L1210/C2 cells per mouse and at a drug dose of 10 mg/kg. When the leukemia was administered by the i.p. route, the MST was 8.8 ± 0 34 days in control animals (Tables 11 and 12). Single injections of free araC, by either the i.p. or the i.v route, did not significantly prolong the survival times (MST were 10.1 ± 0.34, and 9.3 ± 0.33 days respectively) of leukemic mice as when compared to control animals. Continuous i.v. infusion of free araC resulted in a MST of 13.6 ± 0.33 days, a significant improvement of survival relative to control animals, or to mice treated by single injections of free araC or of PC:CH liposomes containing araC (Table 12). Single i.v. injections of stealth liposomes containing araC, increased survival to 13.1 ± 0.41 days, a result similar to that obtained with continuous i.v. infusion of free araC. However, treatment with PC:CH liposomes containing araC yielded no significant therapeutic benefit relative to treatment with single injections of free araC (Table 12). When araC, entrapped in PC:CH liposomes was administered by the i.p. route, a slight increase in the MST (11.0 ± 0.67 days) was observed relative to the survival times of control animals. The greatest increase in survival of leukemic mice in this set of experiments was achieved with single i.p. injections of stealth liposomes, which yielded survivals of 15.8 ± 0.50 days in leukemic mice (Table 11).

Similar results were obtained in mice implanted with

L1210/C2 cells by the i.v. route (Tables 13 and 14). Single injections of free araC by either route were without any noticible effect, whereas, either i.p. or i.v. administration of stealth liposomes containing araC prolonged the survival of leukemic mice more than treatment with PC:CH liposomes containing araC. Treatment of leukemic mice by the i.p. route, with PC:CH liposomes moderately improved the survival of leukemic mice (Table 14).

**E 3 ii) Drug dose: 20 mg/kg**

The therapeutic efficacy of liposomes with entrapped araC was studied at the araC dose of 20 mg/kg. Untreated mice with i.p. implanted L1210/C2 cells survived for 8.0 days, and when treated with single injections of free araC by the i.p. or i.v route, survival times of leukemic mice remained unchanged (Tables 11 and 12). Continuous i.v. infusion of free araC significantly prolonged the survival times of leukemic mice, relative to controls, giving a MST of 13.8 $\pm$ 0.35 days (Table 12). Single injections by the i.v. route of stealth liposomes with entrapped araC gave a similar MST to the infusion group (13.5 $\pm$ 0.56 days), while i.v. injections of PC:CH liposomes with entrapped araC had a modest effect in improving the MST of leukemic-bearing mice (11.4 $\pm$ 0.16 days) as compared to either controls or single injections of free araC (Table 12). When the liposomes were administered by the i.p. route, the

Table 11

Effect of different doses of araC administered by the i.p. route and L1210/C2 leukemia ($10^6$ cells) implanted by the i.p. route, on the MST of mice.

The effect of single injections of free araC or single injections of liposomes containing entrapped araC, injected by the i.p. route, on the mean survival times of $B6D2F_1$ hybrid mice (7 to 21 mice/group) were examined. Mice were implanted with $10^6$ L1210/C2 leukemia cells, by the i.p. route 24 hours before treatment.

| Treatment Group | Route of L1210/C2 Implantation | Route of Treatment | Days Survived ±S.E. AraC Doses mg/kg 10 | 20 | 80 |
|---|---|---|---|---|---|
| Control Mice[a] | i.p. | i.p. | 8.8 ±0.34 | 8.0 ±0.00 | 7.9 ±0.11 |
| Free araC[b] | i.p. | i.p. | 10.1 ±0.35 | 9.1 ±0.23 | n.d. |
| PC:CH[c] | i.p. | i.p. | 11.0 ±0.67 | 12.8 ±0.38 | 17.5 ±1.32 |
| SM:PC:CH:$GM_1$[c] | i.p. | i.p. | 15.8 ±0.50 | 15.2 ±0.50 | 18.6 ±0.33 |

a=injections of sterile 0.145 M NaCl
b=single injection of free araC
c=single injection of liposome entrapped araC
n.d.=not determined

Table 12

Effect of different doses of araC administered by the i.v. route and L1210/C2 leukemia ($10^6$ cells) implanted by the i.p. route, on the MST of mice.

The effect of single injections of free araC, 24 hour infusions of free araC or single injections of liposomes containing entrapped araC, injected by the i.v. route, on the mean survival times of B6D2F$_1$ hybrid mice (7 to 21 mice/group) were examined. Mice were implanted with $10^6$ L1210/C2 leukemia cells, by the i.p. route 24 hours before treatment.

| Treatment Group | Route of L1210/C2 Implantation | Route of Treatment | Days Survived ± S.E. AraC Doses, mg/kg 10 | 20 |
|---|---|---|---|---|
| Control[a] | i.p. | i.v. | 8.8 ± 0.34 | 8.0 ± 0.00 |
| Free araC[b] | i.p. | i.v. | 9.3 ± 0.33 | 9.8 ± 0.13 |
| Free araC[c] | i.p. | i.v. | 13.6 ± 0.33 | 13.8 ± 0.35 |
| PC:CH[d] | i.p. | i.v. | 9.2 ± 0.15 | 11.4 ± 0.16 |
| SM:PC:CH:GM$_1$[d] | i.p. | i.v. | 13.1 ± 0.41 | 13.5 ± 0.56 |

a=injections of sterile 0.145 M NaCl
b=single injection of free araC
c=24 hour infusion of free araC
d=single injection of liposome entrapped araC

MST increased to 12.8 ± 0.38 days for PC:CH liposomes containing araC. The best survival in this series was achieved with single i.p. administration of stealth liposomes, resulting in MST of 15.2 ± 0.50 days (Table 11).

Similar results were obtained in mice implanted with L1210/C2 by the i.v. route (Tables 13 and 14). Single injections of free araC by either route of administration again produced little benefit while either i.p. or i.v. administration of single injections of stealth liposomes containing araC were more successful in increasing the survival of leukemic mice than treatments with PC:CH liposomes containing araC. Treatment of mice by the i.p.route with liposomes composed of PC:CH containing entrapped araC had a modest effect in improving the survival of leukemic mice (Table 14).

E 3 iii) Drug dose: 80 mg/kg

A further series of experiments on the therapeutic efficacy of liposomes containing araC were conducted at a drug dose of 80 mg/kg (Tables 11 and 13). When the tumor was administered by the i.p. route, the MST was 7.9 ± 0.11 days for control animals that were not treated with araC. When PC:CH liposomes containing araC were administered as a single i.p. injections, survival of leukemic mice increased to 17.5 ± 1.32 days. Administration of single injections of stealth

Table 13

Effect of different doses of araC administered by the i.v. route and L1210/C2 leukemia ($10^6$ cells) implanted by the i.v. route, on the MST of mice.

The effect of single injections of free araC, 24 hour infusions of free araC or single injections of liposomes containing entrapped araC, injected by the i.v. route, on the mean survival times of B6D2F$_1$ hybrid mice (7 to 21 mice/group) were examined. Mice were implanted with $10^6$ L1210/C2 leukemia cells, by the i.v. route 24 hours before treatment.

| Treatment Group | Route of L1210/C2 Implantation | Route of Treatment | Days Survived ±S.E. AraC Doses mg/kg 10 | 20 | 80 |
|---|---|---|---|---|---|
| Control Mice[a] | i.v. | i.v. | 6.3 ±0.13 | 8.0 ±0.00 | 7.9 ±0.11 |
| Free araC[b] | i.v. | i.v. | 7.2 ±0.13 | 9.1 ±0.23 | n.d. |
| Free araC[c] | i.v. | i.v. | 10.8 ±0.20 | 12.4 ±0.14 | 14.0 ±0.18 |
| PC:CH[d] | i.v. | i.v. | 7.5 ±0.20 | 8.6 ±0.24 | 15.2 ±0.13 |
| SM:PC:CH:GM$_1$[d] | i.v. | i.v. | 8.8 ±0.30 | 12.5 ±0.50 | 18.4 ±0.24 |

a=injections of sterile 0.145 M NaCl
b=single injection of free araC
c=24 hour infusion of free araC
d=single injection of liposome entrapped araC
n.d.=not determined

Table 14

Effect of different doses of araC administered by the i.p. route and L1210/C2 leukemia ($10^6$ cells) implanted by the i.v. route, on the MST of mice.

The effect of single injections of free araC or single injections of liposomes containing entrapped araC, injected by the i.p. route, on the mean survival times of B6D2$F_1$ hybrid mice (7 to 21 mice/group) were examined. Mice were implanted with $10^6$ L1210/C2 leukemia cells, by the i.v. route 24 hours before treatment.

| Treatment Group | Route of L1210/C2 Implantation | Route of Treatment | Days Survived ± S.E. AraC Doses, mg/kg 10 | 20 |
|---|---|---|---|---|
| Control[a] | i.v. | i.p. | 6.3 ± 0.13 | 6.3 ± 0.12 |
| Free araC[b] | i.v. | i.p. | 7.0 ± 0.00 | 7.2 ± 0.13 |
| PC:CH[c] | i.v. | i.p. | 7.9 ± 0.46 | 10.5 ± 0.22 |
| SM:PC:CH:$GM_1$[c] | i.v. | i.p. | 10.0 ± 0.21 | 11.6 ± 0.25 |

a=injections of sterile 0.145 M NaCl
b=single injection of free araC
c=single injection of liposome entrapped araC

liposomes containing araC resulted in a similar MST of 18.6 ± 0.33 days for leukemic mice (Table 11).

When both leukemic inoculations and injections of liposomes were by the i.v. route, administration of stealth liposomes containing araC was much more successful in prolonging the survival of leukemic mice than treatments with PC:CH liposomes containing araC, or tail-vein infusion of free araC (Table 13). Treatment of mice by the i.v. route with PC:CH liposomes containing araC had a moderate effect in improving the survival of leukemic mice (Table 11).

F) Statistics

Results in treatment groups in each series of experiments were compared by an analysis of variance as described by Sokal and Rohlf (1981). The results from the statistical analysis are presented in Table 15. Trends in these results indicate that infusion of free araC (INF) was superior to single doses of long-circulating liposomes (LCL) at the low araC drug dose, equivalent at the middle drug dose but at the highest drug dose, treatment with LCL was superior to treatment with INF. In most cases, treatment with LCL was superior to treatment with short-circulating liposomes (SCL). This finding was independent of the route of treatment or leukemia inoculation. Treatment of mice with SCL, by the i.p. route, was superior to single injections of free araC. Treatment of mice with SCL

by the i.v. route, was not significantly different from that with free araC at low dosages. As increasing doses of SCL with entrapped drug were administered, treatments became significantly superior to treatment with free araC (Table 15).

Table 15

A statistical comparison of therapeutic response of treatment groups that differ in route of leukemia cell implantation, cell numbers implanted and treatment routes.

Analysis of variance was done by comparing the MST of leukemic mice for each group against the MST for other individual groups within each treatment series. The degree of significance is indicated in the table; probabilities of significance are indicated as follows:

*** = $P < 0.001$

** = $P < 0.01$

* = $P < 0.05$

NS = not significant

Treatment groups

INF= tail-vein infusion of free araC over 24 hours

LCL= single injections of long-circulating liposomes (SM:PC:CH:$G_{M1}$, 1:1:1:0.14 LUV, 0.4 μm) with entrapped araC

SCL= single injections of short-circulating liposomes (PC:CH, 2:1 LUV, 0.4 μm) with entrapped araC

FAC= single injections of free araC

Table 15 (continued)

| L1210/C2 Cells Implanted (Route, Number) | | AraC Dosage (mg/kg) | Therapeutic response: relative efficacy of delivery form. I.V. AraC | I.P. AraC |
|---|---|---|---|---|
| i.v. | $10^5$ | 10 | $INF^{***} > LCL^{***} > SCL^{***} > FAC$ | $LCL^{***} > SCL^{***} > FAC$ |
| i.v. | $10^6$ | 10 | $INF^{***} > LCL^{***} > SCL^{NS} = FAC$ | $LCL^{**} > SCL^{**} > FAC$ |
| i.v. | $10^6$ | 20 | $INF^{NS} = LCL^{***} > SCL^{***} > FAC$ | $LCL^{*} > SCL^{***} > FAC$ |
| i.v. | $10^6$ | 80 | $LCL^{***} > SCL^{***} > INF$ | Not Determined |
| i.p. | $10^5$ | 10 | $INF^{NS} = LCL^{***} > SC>^{***} = FAC$ | $LCL^{***} > SCL^{***} > FAC$ |
| i.p. | $10^6$ | 10 | $INF^{NS} = LCL^{***} > SCL^{NS} = FAC$ | $LCL^{**} > SCL^{***} > FAC$ |
| i.p. | $10^6$ | 20 | $INF^{NS} = LCL^{**} > SCL^{*} > FAC$ | $LCL^{***} > SCL^{***} > FAC$ |
| i.p. | $10^6$ | 80 | Not Determined | $LCL \overset{NS}{=} SCL$ |

# CHAPTER IV DISCUSSION

The objectives of the work described here were (i) to examine the leakage kinetics of entrapped contents from liposomes of two compositions *in vitro*, (ii) to examine the pharmacokinetics and tissue distribution of liposomes *in vivo* and (iii) to measure therapeutic efficacy of liposomes with entrapped araC. The response of mouse leukemia L1210/C2 to treatment with araC provides an opportunity to test the hypothesis that liposomes may serve as a sustained release drug delivery system within the vasculature. As described in the introduction, araC is an effective anticancer drug, but its therapeutic value is compromised by its short *i. vivo* half-time in the circulation (because of degradation and rapid urinary excretion). Consequently, araC is administered to patients by i.v. infusion for maximal efficacy.

**A) Leakage of araC from liposomes**

Studies of the araC leakage characteristics from liposomes *in vitro* employed two different liposome compositions (SM:PC:CH:$G_{M1}$, 1:1:1:0.14 and PC:CH, 2:1) at two different osmotic strengths of araC. Given that we desire to keep liposome size and trapped volumes constant in our therapeutic experiments, there are two ways by which we can increase the dose of araC administered in mice. The dose of liposome-entrapped araC administered in mice may be increased or the concentration of araC inside the liposomes may be increased.

There are problems with each approach. In the first case, if the pharmacokinetics were dose-dependent (i.e. if RE uptake was saturated), then drug availability would vary with drug dose. In the second case, treatment of mice with liposomes containing high drug concentrations may allow administration of non-saturating, liposome doses (Abra and Hunt, 1981; Allen et al., 1984), but will result in an osmotic gradient (hypertonic concentrations in the internal aqueous compartment of the liposome). Thus, the in vitro leakage kinetics of araC from liposomes prepared with high concentrations of entrapped araC (hypertonic araC conditions) were examined to ascertain if these liposomes could maintain their entrapped contents in the presence of an osmotic gradient across the bilayer and in the presence of 25% human plasma.

Liposomes prepared with isotonic or hypertonic araC concentrations experienced a 20-40 % loss of entrapped contents when incubated for 24 hours in TES buffer. This slow leakage of araC from liposomes in buffer has also been observed for other liposome compositions by Kim and Howell, (1987), Mayhew, et al., (1978) and Juliano and Stamp, (1978). It is postulated that minor perturbations or flaws in the liposome bilayer lead to the loss of contents. The extent of loss of entrapped contents can be influenced by the number of lipid bilayers. Small unilamellar vesicles (SUV, 50-80 µm in diameter) have higher leakage rates of contents than multilamellar vesicles (MLV, 1000-2000 µm in diameter) (Mayhew et

al.,1978; Kim and Howell, 1987). LUV prepared by the extrusion of REV have fewer lamellae than MLV, as evidenced by their increased trapped volumes (Mayer et al., 1986; Jousma et al., 1987; Pidgeon and McNeely, 1987, Liposomes prepared by the REV method have content leakage rates slower than those of SUV (Kirby and Gregoriadis, 1984), but would be expected to be leakier than MLV. Some of the liposome preparations used in this study contained small populations of large vesicles (5-10% of the liposomes), which, if multilamellar, would tend to decrease the overall rate of contents leakage from the liposomes.

It was observed that PC:CH liposomes containing isotonic or hypertonic solutions of araC, experienced a greater loss of contents than similar-sized stealth liposomes, when incubated in TES buffer containing 25% human plasma (Figures 1 and 2). Stealth liposomes containing hypertonic solutions of araC, experienced a rapid burst of leakage upon incubation in plasma-containing buffer. This initial rapid loss of contents during the first hour may be attributed to an "osmotic shock", as a result of the osmotic gradient (higher drug concentration inside the liposomes than outside the liposomes). The osmotic gradient could swell the liposomes, which could lead to the formation of pores or defects in the liposome bilayer that are large enough for araC to pass through. Alternatively, a population of liposomes may burst under these conditions releasing all of their contents.

Studies by Gregoriadis and Davis (1979) on the leakage of different sizes of molecules from liposomes, suggest that the latter hypothesis is the predominant mechanism of leakage in isotonic conditions, while the former hypothesis is favoured under hypertonic conditions. Such flaws might reasonably be expected to be more frequent when the liposomes were prepared under conditions where there is an osmotic gradient. Thus, it would appear that liposomes loaded under hypertonic conditions have decreased ability to retain the entrapped drug over long periods of time. Liposomes containing hypertonic araC solutions leaked substantially when exposed to plasma proteins and were therefore, considered unsuitable for our therapeutic experiments; in all other studies, isotonic concentrations of araC in liposomes were employed.

Since liposomes composed of SM:PC:CH:$G_{M1}$, were less leaky in plasma than were liposomes composed of PC:CH and were capable of retaining entrapped contents in the presence of plasma over long periods of time, it would appear that these liposomes may be suitable for use as a sustained drug release system. It is our hypothesis that slow release liposomal formulations will be more effective as sustained release systems for rapidly degraded drugs than very leaky liposomal formulations. In order to test this hypothesis araC entrapped in both liposome formulations was employed to treat leukemic mice.

B) Fate of liposomes *in vivo*

One of the major barriers for the *in vivo* use of liposomes as a sustained release drug systems is the RE system. One of the functions of the RE system is to remove foreign particulate matter from circulation. Liposomes are treated as foreign matter by the RE system despite their similarities to natural cell membranes (Allen, 1988). As a result, following *in vivo* liposome administration, liposomes are rapidly removed from circulation in a size-dependent manner and accumulated in the cells of the RE system (Allen and Everest, 1983). The avid uptake of liposomes by the RE system greatly limits their use as a sustained release system within the vasculature and also limits the prospect of targeting the liposomes to tissues other than the liver and spleen. Pharmacokinetic studies were performed on liposomes of two compositions in order to study the *in vivo* fate of each composition.

The pharmacokinetic studies for liposomes administered by the i.v. route, clearly demonstrate that stealth liposomes had much longer circulation half-lives in blood than PC:CH liposomes (Figures 3 to 5). Both compositions of liposomes were removed from circulation in a biphasic fashion, with an initial rapid phase followed by a longer slower phase of liposome removal (Figure 3). However, the initial rapid phase of removal of the stealth liposomes was very brief compared

to that of PC:CH liposomes. It is suspected that the liposomes prepared in this study were not homogeneous in size after extrusion. The liposome size distribution data suggested that 5-10% of the liposomes of our liposome preparations may be in the form of with large vesicles, which would be removed more rapidly from circulation than the smaller liposomes. Unpublished work from this laboratory, has shown that small unilamellar vesicles composed of SM:PC:CH:$G_{M1}$ (0.1 μm in size), that are homogeneous in size, are removed from circulation in a monophasic fashion.

In addition, the tissue distribution studies have shown that PC:CH liposomes accumulated more rapidly in the liver and spleen than did the liposomes containing $G_{M1}$ (compare Figures 4A,4B with 5A,5B). Stealth liposomes had significantly reduced accumulation in the RE tissues and were present in greater proportions in carcass tissues than PC:CH liposomes. It is postulated that the RE uptake of liposomes is triggered by rapid opsonization of liposomes by plasma proteins (Allen, 1989). The proteins involved in the recognition and uptake process have not been identified.

Similar tissue distribution studies were performed for i.p. administration of liposomes and demonstrated that LUV (0.4 μm in size) experienced a delayed exit from the peritoneal cavity into the vasculature. It is interesting to note that two hours after i.p. liposome administrations in mice, maximum blood-liposome concentrations were achieved for each

of the liposome compositions examined (Figure 6). Similar conclusions were obtained from pharmacokinetic studies conducted by Ellens et al., (1981) and Senior et al., (1983). In addition, the extent of vesicle accumulation in RE tissues by this route of injection was lower than that for liposomes injected by the i.v. route. The present studies showed that, liposomes containing $G_{M1}$ achieved greater absolute blood-liposome concentrations and remained in circulation longer than did PC:CH liposomes. Liposomes of the latter composition were more susceptible to plasma-induced leakage of contents than were the liposomes containing $G_{M1}$. Consequently, these liposomes were not able to achieve as high concentrations of liposomes in blood as stealth liposomes. Since the liposomes were able to escape the peritoneal cavity and enter the vasculature, the peritoneal cavity may serve as a depot for slow release of free drugs or for liposomes containing entrapped drug.

**C) Therapeutic applications of liposome-entrapped araC**

In assessing the antileukemic effects of liposome-entrapped araC, it was necessary to examine the therapeutic effects of empty liposomes. Liposomes have been shown to have toxic effects on cultured cells, particularly at high doses (Allen et al., 1981; Allen, 1988). Furthermore, the presence of murine L1210/C2 leukemia cells could influence the

distribution and/or stability of liposomes. It has been reported that, in mice, the presence of $10^6$ A-B-lymphosarcoma cells implanted 24 hours before liposome (SM:PS:CH, 4:1:5) injections, altered the blood clearance and tissue distribution of the liposomes. This effect was attributed to impairment of the RE system as a result of the tumor inoculation (Ellens et al.,1983). In contrast, in the present experiments, inoculation of $10^6$ L1210/C2 leukemia cells by either the i.v. or the i.p. route 24 hours before liposomes administration did not appreciably affect the pharmacokinetics of liposomes composed of either SM:PC:CH:$G_{M1}$ or PC:CH, injected by i.p. or i.v. routes. This apparent discrepancy may arise from differences in the type of neoplastic cells studied and in liposome composition.

The utility of long-circulating liposomes as a sustained drug release systems within the vasculature has been examined. Initial therapeutic studies were performed on mice that had a burden of $10^5$ L1210/C2 cells. Significant improvement of the MST of mice was not observed when mice were injected with araC-free liposomes showing that the empty vesicles were without significant effect on proliferation of the leukemic cells. It was also found that coadministration of empty liposomes and free araC (single dose, 10 mg/kg) had no significant effect on the MST of leukemic mice. Thus, araC was no more effective in the presence of empty phospholipid vesicles than in its absence (Tables 5 and 6).

To determine whether i.p. or i.v. routes of administration of araC-containing liposomes and leukemic cells affected the therapeutic outcomes, variables were systematically explored. Animals with i.p. implants of leukemic cells developed 5-7 ml ascitic fluid by the sixth or seventh day after leukemic cell implantation. While ascitic fluid was rich with leukemic cells, the leukemia was generalized (Kim and Howell, 1987). Mice implanted with leukemic cells by the i.p. route tended to live longer than mice similarly implanted by the i.v. route.

A reproducible observation from the therapeutic experiments was that leukemic mice treated with single injections of free araC, at doses of 10 or 20 mg/kg, by either route, experienced little or no improvement in survival times as compared to untreated controls. This observation was not influenced by the leukemic cell burden nor by the route by which the L1210/C2 cells are implanted (Tables 5 to 14). Since, araC exerts its antineoplastic activity during S phase of the cell replicative cycle and the <u>in vivo</u> half-life of the araC is 10-15 minutes, the drug was not present in its active form long enough to have any therapeutic effect against the leukemia. However, when the same total drug dose was administered in a final volume of 3 ml by tail-vein infusion over a 24 hour period, the survival of leukemic mice was substantially improved (Tables 8,9,12 and 13).

Mice implanted with leukemic cells by the i.p. route,

experienced modest improvements in their MST when treated by single i.p. injections of araC entrapped within PC:CH liposomes, as compared to the MST of mice treated with free araC. As higher drug doses of araC contained in PC:CH liposomes were administered to leukemic mice survival times increased. It is likely that araC contained in PC:CH liposomes is protected from enzymatic degradation and is released from liposomes at rates that sustain plasma concentrations of free araC within the therapeutic range.

In similar studies, leukemic mice treated with stealth liposomes, survived significantly longer than did mice treated with (i) single injections of either araC entrapped in PC:CH liposomes or (ii) free araC at a total dose of 10 and 20 mg/kg. The in vitro leakage studies demonstrated that stealth liposomes were not as susceptible to the effects of plasma as were the PC:CH liposomes and appeared to release their entrapped contents over longer periods in vivo than did PC:CH liposomes.

Treatment of leukemic mice with the highest dose of araC entrapped in either type of liposome significantly improved survival times which were independent of liposome composition. The in vivo pharmacokinetic studies demonstrated that stealth liposomes remained in circulation longer than PC:CH liposomes at liposome doses that did not saturate the RE system. The high doses of araC in PC:CH liposomes greater than 10 μm of phospholipid are phospholipid doses that saturated the RE

system (Abra and Hunt, 1981). The latter condition increases the circulation half-life of PC:CH liposomes which would, in turn tend to decrease the half-life differences between the two compositions at the higher doses.

In the experiments in which mice were inoculated with leukemic cells by the i.v. route and treatment was administered by the i.p. route, some improvement in MST of mice was observed when araC was entrapped in liposomes composed of PC:CH, relative to untreated animals, or to animals treated by single injections of free araC. Similar experiments in which leukemic mice were treated with stealth liposomes with entrapped araC, the survivals were increased compared to those mice treated with (i) single injections of araC in PC:CH liposomes (ii) or with free araC. It would appear that the liposomes administered by the i.p. route are able to exert a significant effect on the growth of i.v. implanted leukemic cells.

Liposomes administered intraperitonealy may enter the circulation and release their entrapped contents slowly over several hours. It is also possible that the liposomes remaining within the peritoneal cavity may release their entrapped contents over several hours and the free drug may diffuse into the circulatory system where it becomes accessible to the neoplastic cells, although this is less likely due to their rapid breakdown of the free drug. Thus, the peritoneal cavity may be, in part, functioning as a depot for

the release of intact liposomes into the circulation and/or for the release of free drug into the circulation or directly into tissues via the lymph.

When mice were inoculated i.p. with either a $10^5$ or $10^6$ leukemic cells and were treated by single, i.v. injections of PC:CH liposomes with entrapped araC at a dose of 10 mg/kg, survival of mice was not significantly different from that of mice that were treated by single injections of free araC. The poor therapeutic activity of araC entrapped in PC:CH liposomes at the lower drug doses likely arises from the rapid disintegration of these liposomes in plasma and by their rapid uptake by RE tissues. In contrast, similar treatment with araC entrapped within stealth liposomes resulted in significant improvements in the MST of leukemic mice as compared to the MST of animals treated with either single injections of free araC or PC:CH liposome with entrapped araC. Treatment of animals with stealth liposomes with entrapped araC was as therapeutically effective as treatment with 24 hour tail-vein infusion of araC at the same total dose. The $G_{M1}$-containing liposomes are not as susceptible to plasma induced leakage and RE uptake, as a result liposomal release of araC is slower.

It is interesting to note that liposomes administered by the i.v. route have a significant therapeutic effect against the growth of leukemic cells that are in the peritoneal cavity. This observation could imply that the liposomes may enter the peritoneal cavity where they can slowly release

their entrapped contents or that free drug is diffusing into the peritoneal cavity.

In the series of experiments, where mice were inoculated with leukemia, and were treated with drug by the i.v. route, the survival of leukemic mice improved as doses of araC contained in PC:CH liposomes were increased. When leukemic mice were treated with stealth liposomes with entrapped araC, the MST of mice were clearly longer than the MST of mice treated with either single injections of free araC or PC:CH liposomes with entrapped araC. When leukemic mice were treated with PC:CH liposomes with entrapped araC at the highest dose (80 mg/kg), the animals survived longer than mice treated by tail-vein infusion of free araC at the same total dose. The survival of leukemic mice that were treated with stealth liposomes, containing entrapped araC, became significantly longer than the MST of mice that were treated with tail-vein infusion of the drug at the same total dose of araC, indicating that this liposomal formulation was releasing drug in therapeutically effective concentrations for longer than 24 hours.

A log-linear relationship exists between the number of L1210/C2 leukemia cells implanted (either by the i.p. route or the i.v. route) and the mean survival times of mice (Figure 9). The fractional cell kill of leukemia cells as a result of treatment of the mice with free araC or liposome-entrapped araC can be determined from this relationship.

In the series of experiments in which mice were implanted with $10^5$ leukemia cells by the i.p. route and treatment was i.v. with either single injections of stealth liposomes containing araC or by slow infusion of free araC, an increase in the mean survival times of leukemic mice corresponding to a three log reduction in leukemic cells was observed (Table 8). Treatment with single injections of non-stealth liposomes of free araC resulted in less than a one log reduction of leukemic cells (Table 8).

When the leukemic cells were implanted, and the treatment with araC, were both by the i.v. route, treatment with single injections of stealth liposomes with entrapped araC or by slow infusion of araC resulted in a two and a half log reduction of leukemic cells. Treatment with single injections of araC in either non-stealth liposomes or as free araC resulted in less than a one log reduction of leukemia cells.

When cell implantation and treatment were both by the i.p. route (Table 7), treatment with araC in stealth liposomes resulted in a four log reduction of leukemia cells. Treatments with araC in non-stealth liposomes resulted in only a one log reduction, and treatment with free ara C resulted in less than a one log resuction of leukemia cells.

When mice were implanted with leukemia cells by the i.v. route and treated by the i.p. route, treatment with araC in stealth liposomes resulted in a three log reduction of leukemic cells (Table 10). Treatment with non-stealth

liposomes containing araC led to a one and a half log reduction, and treatment with free araC led to a one log reduction in the numbers of leukemia cells.

When the tumor burden was increased to $10^6$ cells (Tables 11 to 14) treatment of the mice with single injections of araC in non-stealth liposomes or with free ara C at doses of 20 mg/kg did not result in substantial reductions in the number of leukemia cells, although stealth liposomes and infusion of free araC were more effective, each resulting in a three log reduction in the numbers of leukemic cells. The most effective treatments in mice bearing a tumor burden of $10^6$ cells were araC in stealth liposomes (80 mg/kg), resulting in close to a six log reduction in i.v. implanted cells and a five log reduction in i.p. implanted cells. These treatments reduced the tumor burden to such low levels that another injection of stealth liposomes might lead to cures in the mice.

In summary, these studies demonstrate that treatment of leukemia-bearing mice with araC entrapped in stealth liposomes resulted in greater fractional cell kill of leukemia cells than treatments with either non-stealth liposomes or with free araC. The log cell kill seen with stealth liposomes was equivalent to that seen with 24 hour infusions of free araC at the lower doses, and was superior to 24 hour infusions at the higher dose.

## D) Conclusion

In summary, this research has demonstrated that liposomes composed of SM:PC:CH:$G_{M1}$, with a molar ratio of 1:1:1:0.14 (0.4 μm LUV) containing entrapped araC, are less susceptible to plasma-induced leakage of entrapped contents as compared to liposomes composed of PC:CH, with a molar ratio of 2:1 (0.4 μm LUV). The *in vivo* pharmacokinetic studies have shown that stealth liposomes have longer circulation times *in vivo* and reduced uptake by cells of the RE system, as compared to PC:CH liposomes. Liposomes containing $G_{M1}$ were also present in greater proportions in carcass tissues than PC:CH liposomes. The therapeutic studies have demonstrated that stealth liposomes, with entrapped araC, have an antileukemic effect that is significantly greater than that seen for PC:CH liposomes, also with entrapped araC, or that seen for single injections of free araC. Antileukemic treatment with stealth liposomes, with entrapped araC, approaches or is not significantly different from, slow infusion of the free drug at the doses of 10 and 20 mg/kg, but is a superior treatment when administered at high liposome doses. AraC entrapped in liposomes is protected from enzymatic degradation and these liposomes are thus providing a sustained release of active araC when administered *in vivo*. However, when PC:CH liposomes, with entrapped araC are injected by the i.v. route, it is speculated that these liposomes are rapidly opsonized and

broken down in plasma, resulting in the release of their entrapped contents and these liposomes are also rapidly removed from circulation by RE uptake. Therefore, PC:CH liposomes containing araC has an antileukemic effect that is, at best, a modest improvement over injections of free drug at the low doses of araC.

Based on the in vitro and in vivo studies we can try to determine the approximate concentrations of free araC in vivo (Figure 10 A,B and C) for each therapeutic group. In order to have an effective treatment, sufficiently high extracelluar concentrations of araC must be maintained for at least one cell cycle. When free araC is administered by a single injection, therapeutically effective concentrations of the drug are maintained for a very short time and those L1210/C2 cells that are not in the S phase of the cell cycle will not be killed and will continue to grow and kill the animal (Figure 10 A). Since infused free araC maintains therapeutically effective concentrations of araC over a 24 hour period this treatment is more effective than single injections of free araC (Figure 10 B). The therapeutic effect of liposome-entrapped araC could be explained by two different mechanisms. Liposomes maintain a sustained release of araC within the circulation, maintaining therapeutically effective concentrations of araC over several hours (Figure 10 C). It is speculated that the higher the dose of araC, the longer the time over which therapeutically effective concentrations of

free drug are maintained (Figure 10 C). In addition araC may gain access to neoplastic cells through internalization of liposomal contents via endocytosis and/or fusion mechanisms (Poznansky and Juliano, 1984).

It can be concluded that the use of liposomes composed of SM:PC:CH:$G_{M1}$ may be a clinically useful as a sustained release system for drugs that are rapidly degraded *in vivo*.

# CHAPTER V REFERENCES

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