AN INVESTIGATION OF THE THERAPEUTIC POTENTIAL OF LIPOSOME-ENCAPSULATED N-(PHOSPHONACETYL)-L-ASPARTIC ACID

by

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A dissertation submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

(Pharmacy)

at the

UNIVERSITY OF WISCONSIN-MADISON

1994

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DATE: 12/9/94

To My Family, Sujin and Andrew

Acknowledgments

I sincerely thank Professor Timothy D. Heath for his guidance, encouragement, and support throughout my graduate study at UW-Madison. I also thank Professor Robert M. Straubinger, Ms. Ninfa Straubinger, and Mr. Niels Gothegen, all at SUNY-Buffalo School of Pharmacy, for their help with animal study for my dissertation. I am very grateful for Professor Joseph R. Robinson and other faculty members, who always kept the door open for invaluable discussions.

Financial support during my graduate study was provided by the Rotary International Foundation (Evanston, IL), University of Wisconsin School of Pharmacy, and Chiron Inc. (Emeryville, CA).

I dedicate this thesis to my parents and parents in law who showed profound understanding during my study abroad, and also to my wonderful wife who always encouraged, convinced, and loved me.

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Chapter 1. INTRODUCTION

In 1965, A. D. Bangham wrote:

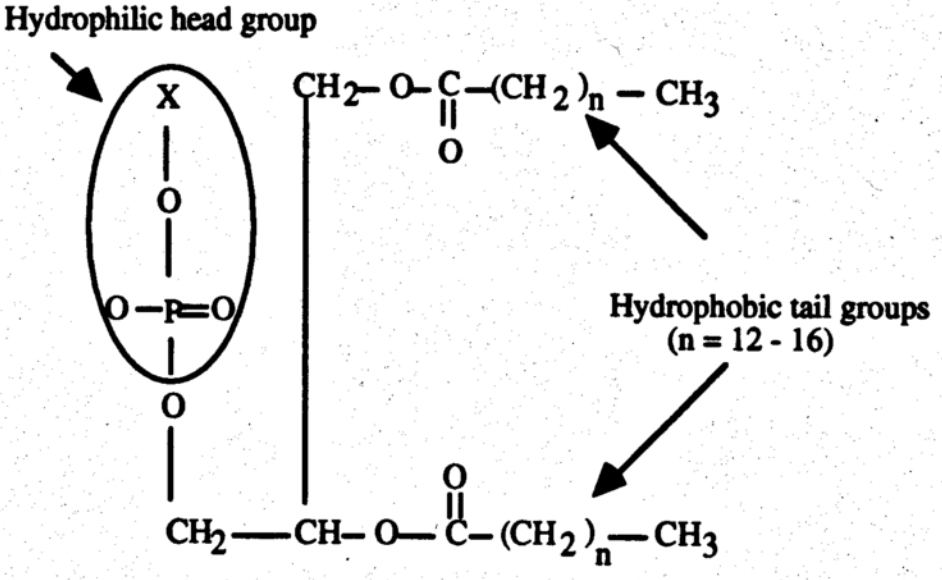
"It is probable that at equilibrium each and every lipid bilayer forms an unbroken membrane—— there being no exposed hydrocarbon / water interfaces —— from which it follows that every aqueous compartment would be discrete and isolated from its neighbour, including a complete separation of the outermost compartment of the whole structure from the continuous phase in which it is suspended..." (Bangham 1965).

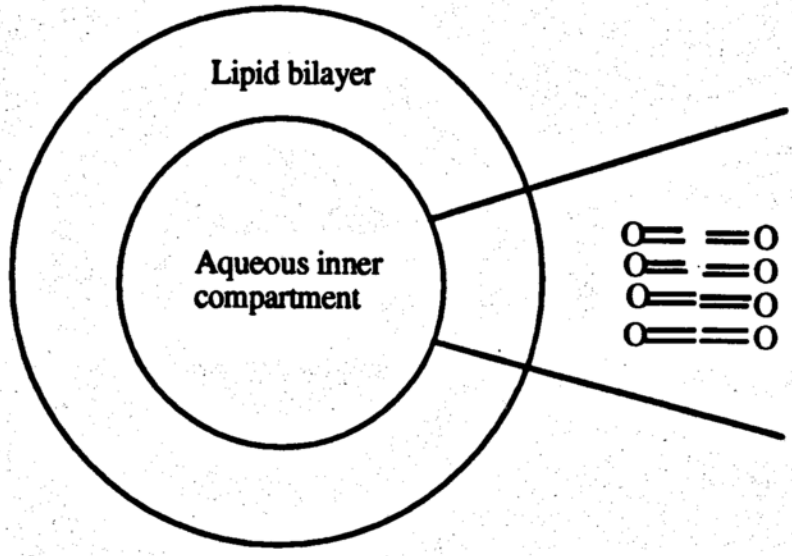
This "unbroken membrane" structure, rather than the "bimolecular leaflet" structure suggested by others, was later on termed a "*liposome*".

According to the International Dictionary of Medicine and Biology, a liposome is defined as " a small vesicular structure which forms spontaneously when phospholipids are placed in water ..... with the hydrophobic portion of the molecules facing toward the middle of the layer and the hydrophilic portions of the molecules facing outward ..... the phospholipid bilayer being separated by an aqueous phase ".

This finding made possible numerous model membrane studies on such diverse topics as the diffusion of ion molecules through membranes, the fluidity or microviscosity of membranes, and the reconstitution of membrane proteins. However, it was not until the 1970s that this "unbroken membrane" or "liposome" was extensively studied as a possible means of controlled drug delivery (Gregoriadis 1971; Gregoriadis 1973). Liposomes employed for drug delivery are typically composed of various kinds of phospholipids as shown in Figure 1.1 and in many cases cholesterol is added. Liposomes range in diameter from 0.2 micron to several microns (the diameter of a red blood cell is roughly 10 μm). They can be either "onion-skinned" multilamellar vesicles (MLV) made up of several concentric lipid bilayers separated by fluid, or unilamellar vesicles, consisting of a single bilayer surrounding a single aqueous core.

Figure 1.1. Phospholipid molecule is the typical constituent of liposomes. The head group can be any of several hydrophilic molecules (X), such as choline (PC), serine (PS), and ethanolamine (PE). Liposome is a spherical vesicle composed of lipid bilayers of phospholipids with aqueous compartment inside of bilayers.





Unilamellar Liposome

Unilamellar vesicles are typically characterized as being small (SUV) or large vesicles (LUV). However, both multilamellar and unilamellar preparations initially exhibited a relatively small encapsulation volume, and their utility was restricted to the encapsulation of small molecules (molecular weight up to 97,000) only (Adrian and Huang 1979). Attempts to overcome these problems with techniques such as the ethanol injection method (Batzri and Korn 1973), and the ether infusion technique (Deamer and Bangham 1976) were only partially successful. Liposomes made by the reverse-phase evaporation method (REV) became the most advantageous method for making liposomes, because they encapsulate a large percentage of the aqueous material presented, have a high aqueous space-to-lipid ratio, and can be prepared from a wide range of different lipid components (Szoka and Papahadjopoulos 1978). The name 'reverse-phase evaporation' was used because the process involves the formation of a reverse, or 'water-in-oil' emulsion, which is then converted to a liposome suspension when the ether is evaporated.

An alternative approach to liposome preparation and the control of their size involves the use of extrusion through membranes with well defined pores. Olson et al. first showed that liposomes of defined size distribution could be produced by sequential extrusion of the usual MLV through polycarbonate membranes (Olson, Hunt et al. 1979). This process proved to be easy, reproducible, produced no detectable degradation of the phospholipids, and could double the encapsulation efficiency of the liposome preparation. Subsequently, Szoka et al. showed that extrusion of REV also modulated their size (Szoka, Olson et al. 1980). The intermediate-size (0.1-0.2 μm) vesicles formed by this process had an aqueous-to-lipid ratio of 3 : 5 and captured between 12 and 23% of the aqueous phase. Mayer and co-workers have developed these techniques further by showing that the use of higher pressure for extrusion,

between 200 and 500 psi, permits the use of the process at high lipid concentrations (400 mg/ml) (Mayer and Hope 1986). Extrusion of MLV in this way, often by multiple passes through polycarbonate membranes, produces LUV of well defined size. Production of liposomes on a commercial scale has either sought to adapt membrane extrusion techniques, or to use high pressure homogenization devices such as the microfluidizer (Cheng and Seltzer 1987).

Initially, liposomes had some disadvantages as a tool for drug delivery. These included low encapsulation efficiency, uptake by the body's own defense system, called reticuloendothelial system (RES), and difficulties in mass production. Many of these disadvantages, as indicated above, have been overcome by the development of new technologies and new fabrication methodologies, which have allowed the production of several liposome formulations that are now on the market. These include liposomal formulation of doxorubicin and amphotericin B. There are many advantages of using the liposomes as a drug delivery system. Their naturally occurring components, including phospholipids and cholesterol, are biocompatible and non-immunogenic. Moreover, encapsulation of labile molecules in liposomes protects them from inactivation during their circulation in the body. However, the most fascinating advantage of liposomes as a drug delivery system is their possible use for targeting of drug molecules to desired sites of the body, thereby minimizing or avoiding toxicity, and improving therapeutic efficacy of drugs. To this end, many investigators have been attracted by the possibility that liposomes could be targeted to specific organs or tissues of the body by attaching to the liposomes cell recognizing ligands. Such molecules could interact selectively with specific determinants on the surface of the target cells. In popular reviews of this concept, terms like "magic bullet", "molecular zip codes" or "guided missiles" became familiar to the general public (Anon 1982).

In Vivo Disposition of Liposomes

A) Intravenous administration of liposomes

The disposition of liposomes administered intravenously (i.v.) is dominated mainly by their localization in the cells of the mononuclear phagocytic system (MPS), also known as the reticuloendothelial system (RES), in the liver, spleen, and blood. In experiments in mice , about 80-85% of i.v. injected large MLV composed of sphingomyelin : cholesterol (2:1 molar ratio) are taken up by liver, and another 10% by spleen and blood after 1 hr (Hwang 1987). Various factors, including liposome size (Allen and Everest 1983), composition (Maruyama 1990), and amount of lipid administered (Allen, Murray et al. 1984), affect the rate of removal of liposomes from the blood. Experimental efforts to induce a significant redistribution of liposomes away from the RES to other tissues by blocking the ability of mononuclear phagocytes to take up the circulating liposomes have not been successful. However, efforts to reduce RES uptake of liposomes by modification of the liposome composition has proved effective in prolonging the half-life of liposomes within the circulation.

One of the most successful approaches has been the use of sterically-stabilized liposomes. Sterically-stabilized liposomes are mainly composed of conventional phospholipids plus a sterically-stabilized composition, which is usually monosialo ganglioside (G_{M1}) (Allen and Chonn 1987) or polyethylene glycol derivatized distearoyl phosphatidylethanolamine (PEG-DSPE) (Klibanov, Maruyama et al. 1990). The term steric stabilization refers to the use of specific glycolipids or polymer-derivatized phospholipids which partially evade rapid recognition and uptake by cells of the RES. The main advantage of using sterically-stabilized liposomes includes their prolonged circulation half-life ($t_{1/2}$) *in vivo*, which results from the decrease of uptake of the liposomes by the RES. This effect is believed to be caused by steric hindrance of

the surface of the liposomes by hydrophilic polymers that are a component of steric-stabilizing lipids. Recently, it was reported that LUV composed of distearoyl phosphatidylcholine (DSPC) : cholesterol (1:1 molar ratio, 200 nm in mean diameter) containing 6 mol% of PEG-DSPE, for which the PEG group had a 1000 to 2000 molecular weight, largely remained circulating in the blood for over 24 hr after i.v. injection in mice (Maruyama, Yuda et al. 1992). The PEG-DSPE containing, sterically-stabilized liposomes have also been reported to circulate in the bloodstream of mice and rats for up to several days following i.v. injection (Allen 1991). Doxorubicin administered in sterically-stabilized liposomes has been reported to exhibit improved therapeutic efficacy, as compared to doxorubicin in normal liposomes, for treatment of the mouse colon carcinoma C-26-bearing mice (Papahadjopoulos 1991; Maruyama, Unezaki et al. 1994).

B) Intraperitoneal administration of liposomes

Intraperitoneal (i.p.) administration of pharmacological agents is the most common route of injection for animal experiments, although this route is not usually used for human administration. Generally, the i.p. injected liposomes exit largely via the lymphatic lacunae (or cavity) in the diaphragm, thereby passing into lymphatic vessels and lymph nodes, and finally entering the bloodstream via the thoracic duct (Parker, Sieber et al. 1981). For example, it has been shown that following i.p. injection into rats, the egg PC liposomes were absorbed from the peritoneal cavity and distributed through the circulation to other organs and tissues (McDougall, Dunnick et al. 1975). DPPC : Chol (2 : 1 molar ratio) SUV were also shown to be rapidly removed from the intraperitoneal cavity via the abdominal lymphatics with subsequent drainage into the venous system and distribution throughout the mouse through the blood circulation, with an absorption phase of about 3-4 hr (Hwang and Mauk 1977). PC : Chol : sphingomyelin (SM) : PEG-DSPE (1 : 1 : 1 : 0.2 molar ratio) MLVs of 0.1 μm in diameter, after i.p, injection into mice, were released from the peritoneal cavity into the blood over a 6hr time period, with very little uptake by the liver and spleen (Allen 1991). For doxorubicin-encapsulated liposomes, by 24hr after i.p. injection in mice, approximately 30% of the injected dose was present in the peritoneal cavity, compared to 1% for free doxorubicin (Mayhew 1990).

Having reached the blood stream, the i.p. injected liposomes appear to distribute to the various organs as if injected intravenously, being taken up mainly by the liver and spleen. Because of this passage through the lymphatics after i.p. injection, liposomes have been considered for possible treatment of metastatic tumors in lymph nodes draining the peritoneum (Parker, Hartman et al. 1981). However, particles also remain in the peritoneum for some period of time and, depending on the liposome type,

the volume injected, and other variables, some fraction will end up being bound to the serosal surface of the peritoneum. In general, large liposomes and liposomes with positive surface charge are absorbed more slowly than are small or negatively charged liposomes from peritoneal sites. It has been suggested that the retarded absorption of positive liposomes may be caused by an interaction with negatively-charged cell surfaces (Hirano, Hunt et al. 1985). As liposomes do not appear to pass through the serosa to enter local blood vessel, they can act as sustained release vesicles in the peritoneal space. This could be useful in treatment of ovarian and some colorectal carcinomas, in which early spread can involve malignant cells in the peritoneal space or tumor implants in the serosal surface.

C) Therapeutic potentials of liposomes

A variety of drug molecules and other therapeutic or diagnostic agents have shown improved therapeutic efficacy in liposomes in many animal model studies, and in some human clinical studies, as compared to the free drug form. These include liposome-based vaccines, liposome-mediated treatment of viral and bacterial infections, and the diagnosis and treatment of cancer.

Liposomes have been proposed as a tool for the delivery of vaccines. In contrast to the often-cited disadvantages associated with delivery of liposomes to phagocytic cells, delivery of substances to the RES does not represent a disadvantage in the field of immunology. On the contrary, macrophages, and particularly Kuppfer cells, are the major sites for immunological processing of certain antigens, and macrophages are the principal sites for expression of the activities of certain adjuvants (for review see Alving 1987).

The treatment of infection with the protozoan parasite *Leishmania donovani* in mice with liposome-encapsulated concanavalin A and muramyl dipeptide (MDP) (Reed et al. 1984), or recombinant mouse interferon-γ (IFN-γ) (Schroit et al. 1982) has been attempted and shown to be promising (for review see Reed 1988).

The use of liposomes as drug carriers in cancer chemotherapeutics has been successful in various tumor models, and examples include doxorubicin (or adriamycin) (Mayhew, Cimino et al. 1990; Ahmad, Longenecker et al. 1993; Vaage 1993; Williams 1993; Maruyama, Unezaki et al. 1994), cisplatin (Sur, Ray et al. 1983), and methotrexate (Kosloski, Rosen et al. 1978).

More recently, gene delivery using cationic liposomes has resulted in very promising results both in *in vitro* and in *in vivo* studies. It is now hoped that some genetic diseases can be cured by use of gene transfection technology using liposomes as

the delivery system (Stribling 1992).

Targeting of Liposomes

Drug targeting using liposomes is generally divided into passive targeting, and active targeting. Passive targeting usually refers to the natural localization of liposomes in the RES, mainly in the liver and spleen. This distribution pattern can be exploited for certain therapies if these organs are the sites of choice for drug targeting. It is also possible to modify this passive distribution pattern to a certain degree by changing lipid charge, liposome size, and other physical characteristics of the liposomes . Active targeting, on the other hand, attempts to avoid uptake of the liposomes by the RES, and alters the passive distribution pattern so that liposomes can accumulate in the desired tissues other than the liver and spleen. This can be achieved by constructing the ligand-conjugated liposomes which recognize surface determinants of the target cells (Heath et al. 1981)

Several classes of ligands have been found useful in liposome targeting, which include sugars, lectins, peptides, hormones, small haptens, and other proteins (for a review see Weinstein and Lesermam 1984; Torchilin 1985). However, the use of antibodies as a 'homing device' is the most attractive and powerful technique employed so far, simply because of their high selectivity and specificity for a wide range of different antigens. The binding of ligands to liposomes can be either direct or indirect. In direct methods, the ligand that is coupled to the liposome recognizes a cell surface component. Hence a cell surface antigen can be recognized by a liposome-conjugated antibody (Heath 1983). In indirect methods, an intervening molecule is added between liposomes and ligands. This intervening molecule may be a cross-linking molecule that binds to similar sites on liposomes and the target cell. An example would be the crosslinking of biotinylated-antibody to biotinylated-liposomes via an avidin linker, which bridges the two by binding to the biotin (Urdal and Hakomori 1980). This

approach has also been achieved by using lectins to cross-link liposomes to cells via sugars on the cell surface and on glycolipids in the liposome membrane (Szoka et al. 1981). A further indirect method has been to conjugate *Staphylococcus aureus* Protein -A to liposomes, which are then subsequently able to attach to cells via their ability to bind to the Fc portion of cell surface bound immunoglobulin molecules (Leserman et al. 1980).

Techniques of conjugation of monoclonal antibodies or other ligands to liposomes have attracted a great deal of attention, mainly through the potential use of liposomes as a targeted drug delivery system (Heath 1983), or as a diagnostic tool (O'Connel, Campbell et al. 1985). In the late 1970s, some early methods were developed for conjugation, where amino-reactive homobifunctional reagents such as glutaraldehyde and diethyl suberimidate were used (Torchilin, Goldmacher et al. 1978). Water-soluble carbodiimides have also been used to catalyze the formation of an amide linkage between amino groups in phosphatidylethanolamine of liposomes with carboxyl group in proteins (Dunnick, McDougall et al. 1975). However, these conjugation methods were inefficient, and were capable of producing protein-protein conjugates (for review see Heath and Martin 1986).

The conjugation method has been revolutionized by the development of efficient and selective reactions that can be performed in aqueous media. Typically a heterobifunctional reagent is used, which is first conjugated to a phosphatidylethanolamine in organic solvent via a reaction with N-succinimidyl carboxylate to produce an amide linkage. The second arm of the agent contains either a pyridyldithiol which reacts with a sulfhydryl group to produce a disulfide bond, or a maleimide group which reacts with a sulfhydryl group to produce a thioether bond (Martin, Heath et al. 1990). Both of these reactions occur rapidly at neutral pH in

aqueous solution, making them ideal for use with proteins, since the denaturation of protein due to high or low pH can be prevented. Factors affecting conjugation efficiency include the number of reactive ligands per protein molecule, the concentration of the reduced protein and lipid (Shek and Heath 1983), liposome size (Machy and Leserman 1983), and the time course of coupling (Martin and Papahadjopoulos 1982). For most cases, the protein concentration rather than lipid concentration in the conjugation mixture determines the protein : lipid ratio of the product.

A) *In vitro* targeting of liposomes

Since efficient methods for conjugation of protein to liposomes were developed by Heath et al. (Heath, Macher et al. 1981), the use of such liposomes for delivery of cytotoxic agents has been proposed because targeted liposomes bind to the appropriate cells with high efficiency. Subsequently, *in vitro* drug targeting using liposomes has been studied extensively using either Protein A-conjugated antibody-directed liposomes, or antibody-conjugated liposomes.

Heath and co-workers have shown that liposomes conjugated with anti-$H2K^k$ antibody associated with L929 murine fibroblasts in 6- to 20-fold greater amount than did nonspecific liposomes (Heath, Montgomery et al. 1983). The ability of methotrexate-γ-aspartate to inhibit L929 growth was shown to increase 10-fold when encapsulated in targeted liposomes but to decrease by 50% when encapsulated in liposomes with no specificity for the target cells. Ammonium chloride was shown to inhibit the effects of the encapsulated but not the free drug, suggesting that the inhibitory effects of encapsulated methotrexate-γ-aspartate involved the endocytosis of the liposomes. These results demonstrate the potential of antibody-targeted liposomes and the importance of selecting liposome-dependent cytotoxic agents.

Matthay and co-workers have shown that liposomes conjugated to *Staphylococcus aureus* Protein A were more potent than liposomes conjugated to either rabbit or affinity purified goat anti-mouse immunoglobulin (Ig) when incubated with AKR/J SL2 cells sensitized with specific antibody (Matthay 1986). It was also shown that when methotrexate-γ-aspartate was encapsulated in antibody-directed Protein A-conjugated liposomes, the cytotoxicity for AKR/J SL2 cells increased up to 150-times compared to free drug, and 50-times, compared to drug in liposomes conjugated to a non-specific antibody. The large difference between the free drug and the drug

encapsulated in antibody-directed Protein A liposomes was maintained even with short incubation times, thus providing a system which might be useful for eradication of tumor cells from bone marrow *in vitro*.

Straubinger and co-workers have examined *in vitro* the cytotoxicity of drugs in antibody-conjugated liposomes for OVCAR-3, a human ovarian cancer cell line (Straubinger et al. 1988). OC-125, a monoclonal antibody recognizing an antigen common to a number of human ovarian cancers (CA-125), was coupled covalently to the liposome surface. Liposomes bearing OC-125 and containing methotrexate-γ-aspartate showed an 8-fold increase in potency against OVCAR-3 cells in a 96hr growth inhibition assay. Brief exposure of tumor cells to treatment increased the difference between the potency of targeted liposomes and free drugs.

Leonetti et al. have shown that oligodeoxyribonucleotide-containing liposomes, conjugated with monoclonal antibodies specific for the major histocompatibility complex (MHC)-encoded $H2K^k$ molecule showed more than 95% reduction of viral multiplication, when the oligomer was a 15-mer complementary to the 5'-end region of the mRNA encoding the N-protein of vesicular stomatitis virus (Leonetti 1990). Protection from viral multiplication was not seen, however, for empty liposomes or liposomes containing a random oligomer sequence in mouse L929 cells. These results demonstrate the possible use of antibody-conjugated oligodeoxyribonucleotide-containing liposomes in treatment of viral infections in human including AIDS.

B) *In vivo* targeting of liposomes

There have been very few reports on the *in vivo* use of liposomes for active targeting of therapeutic agents. One of the first attempts for using antibody-conjugated liposomes in tumor therapy with an animal model was reported by Hashimoto et al. (Hashimoto, Sugawara et al. 1983). A single intraperitoneal (i.p.) injection of actinomycin D-containing liposomes, conjugated with subunits of an IgM antibody against a mouse mammary tumor-associated antigen (MM antigen), in mice with intraperitoneally transplanted tumor (MM46) resulted in the cure of some mice and a prolonged survival time for the rest of the mice. In this study, either free actinomycin D or BSA-coated actinomycin D containing liposomes were marginally effective or ineffective. They also examined potential systemic effects by intravenous (i.v.) administration of liposomes targeted against a subcutaneous MM46 tumor. A single i.v. dose of liposomes for treatment of tumors implanted 4 days earlier was found to be moderately effective in reducing tumor weights in mice, compared to the effects of control liposomes.

Hughes et al. have shown that liposomes conjugated to monoclonal antibody 273-34A, which specifically binds the epitope expressed almost exclusively on capillary endothelial cells of the lung, localize in the lung 20- and 15-times better than did liposomes conjugated to the non-specific monoclonal antibody at 15min and 24hr post injection in BALB/c mice respectively (Hughes, Kennel et al. 1989). This result demonstrates that this antibody-conjugated liposome system could be used as a model for enhanced drug delivery to the lung.

Sing et al. demonstrated that monoclonal antibody DAL K29, specific for a human renal cell carcinoma-associated cell surface antigen, could be covalently linked to liposomes containing methotrexate with full retention of antibody activity.

These antibody-conjugated methotrexate liposomes were tested in an ascites tumor model produced by i.p. injection of the human kidney cancer cell line, Caki-1, in nude mice (Singh 1991). The results showed that methotrexate-containing DAL K29-conjugated SUV were a more potent tumor inhibitor ($P < 0.0005$) than the free drug, monoclonal antibody alone, methotrexate containing SUV, a mixture of DAL K29 and methotrexate-containing SUV, or methotrexate-containing SUV linked to an isotype matched non tumor specific IgG.

Ahmad et al. showed that doxorubicin encapsulated in long circulating liposomes conjugated with specific antibody (174 H.64 IgG_1) targeted to KLN-205 squamous cell carcinoma of the lung eradicated the tumor in DBA/2 mice (Ahmad, Longenecker et al. 1993). In Ahmad's study, the monoclonal antibody was biotinylated and attached to the surface of liposomes containing biotinylated phosphatidylethanolamine by means of an avidin linker.

Active targeting of chemotherapeutic agents in antibody-conjugated liposomes *in vivo* has also been examined for treatment of a solid tumor model using nude mice. Anti-human alpha-fetoprotein (AFP) monoclonal antibody was conjugated to adriamycin containing SUV for targeting to AFP-positive human hepatoma cells (Li-7) maintained in nude mice as xenografted subcutaneous tumors (Konno 1987). When the treatment was initiated by administration of three i.v. injections of liposomes 10-14 days after tumor inoculation, when tumor weight exceeded 100 mg, reduction in tumor weight and histopathological evidence indicated that the drug-encapsulated antibody-conjugated liposomes were therapeutically more effective than the unconjugated liposomes.

Other targeting related approaches for chemotherapy are the use of pH- or temperature-sensitive liposomes. It is known that at their phase transition temperature

liposomes become more leaky to encapsulated drug molecules (Papahadjopoulos and Kimelberg 1973). It is also known that the pH in the vicinity of some tumors may be lower than that in normal tissues. Administration of pH-sensitive immunoliposomes intraperitoneally to mice carrying RDM-4 lymphoma cells as an ascites tumor resulted in accumulation of approximately 30% of the injected drug dose in the ascites cells (Wang and Huang 1987). Temperature-sensitive liposomes were also investigated for delivery of methotrexate in liposomes to L1210 solid tumor cells following local hyperthermia, and showed improved treatment of solid L1210 tumor (Weinstein, Magin et al. 1980).

Mechanisms of Liposomal Drug Delivery

The mechanism of drug delivery using liposomes has been proposed to occur by two different mechanisms. It has been argued that increased therapeutic efficacy of drug molecule when encapsulated in liposomes can be caused by the controlled (or delayed) release of drug from the lamellar structure of liposomes. It has also been suggested that the increased local accumulation (or targeting) of drug molecule within the site of action may be an important factor. If controlled release improves drug efficacy, it is not strictly necessary for the liposomes to localize in the target organs or tissues. Targeting, on the other hand, depends on the ability of liposome to reach and remain in the target organs or tissues. In some cases, it is possible that both targeting and controlled release will contribute to the overall increased therapeutic efficacy of the drugs. The extent to which these two mechanisms are important for delivery depends on the properties of the drug being used. Heath et al. have proposed the terms liposome-dependent and liposome-independent (Heath, Lopez et al. 1985).

A) Liposome-dependent drug delivery

Liposome-dependent drug delivery is a process by which liposomes deliver a drug to intracellular compartments more efficiently than occurs when a drug is delivered in the free form (Heath, Lopez et al. 1985). In this situation, delivery of drug is dependent upon uptake of liposomes by the cell but not dependent upon conventional passage of the drug molecule through plasma membranes. *In vivo* liposome-dependent drug delivery would be dependent on targeting to or localization of liposomes in the target cells.

Drug molecules, which are delivered to cells by this mechanism, are defined as liposome-dependent drugs. Experimentally, liposome-dependent drugs are easily

identified with an *in vitro* endpoint analysis method (Heath 1985). Briefly, cultured cells are exposed to various concentrations of free or liposome-encapsulated drug for a defined growth period. The live cells are counted, and the drug concentration of 50% inhibition of the cell growth or IC_{50} is determined. The IC_{50} bears an inverse relationship to drug potency; the greater the IC_{50}, the less potent the drug. A dimensionless potency increase factor (PIF) is often used to express the degree of increase in drug potency. The PIF is the ratio of the IC_{50} of the free drug over the IC_{50} of the encapsulated drug.

All known liposome-dependent drugs are soluble in water, unable to pass readily through cell membranes, and active in cell growth inhibition if delivered to the cytoplasm of a cell. Examples of liposome-dependent drugs include methotrexate-γ -aspartate (Heath 1983), 5-fluoroorotic acid (Heath, Lopez et al. 1985), clodronate (Monkkonen and Heath 1993), gallium-NTA (Monkkonen, Brown, et al. 1993), and N-(phosphonacetyl)-L-aspartic acid (PALA) (Heath 1989). Liposome-dependent drugs rely on adsorptive endocytosis of the liposome for their efficient delivery to cells. Consequently, the extent to which liposomes retain their contents in culture medium, their efficiency of binding to the cell surface, and the efficiency with which the cell internalizes the surface-bound liposomes all contribute to the potency of the encapsulated drug. Therefore, targeted delivery of drugs by liposomes to specific sites in the body is likely to benefit from the use of liposome-dependent drugs.

Methotrexate-γ -aspartate is a derivative of methotrexate which is equipotent in its inhibition of dihydrofolate reductase but has an influx Km at least 100-fold greater than that of the parent compound (Piper et al. 1982). Methotrexate-γ -aspartate, when encapsulated in anti-$H2K^k$ antibody-conjugated liposomes, was 20-fold more potent for the growth inhibition of L929 cells than free drug (Heath 1983). When encapsulated in

unconjugated (non-targeted) neutral liposomes or liposomes conjugated to a non-reactive antibody, methotrexate-γ-aspartate was no more effective for growth inhibition than it was in the free form.

Fluoroorotic acid, a fluorouracil derivative known to be ineffective in the free form, is up to 30-fold more potent for growth inhibition of cells when encapsulated in negatively charged liposomes composed of high phase-transition temperature lipid, compared to its free form (Heath, Lopez et al. 1985). Gallium is known to prevent the activation of macrophages and might be useful as an immunosuppresive agent. When encapsulated in DSPG liposomes, gallium nitrilotriacetate is 16-fold more potent for the growth inhibition of RAW 264 than free drug (Monkkonen, Brown et al. 1993). Clodronate is known to inhibit the activity of osteoclasts, thereby preventing bone resorption in disorders characterized by excessive bone loss. Clodronate encapsulated in DSPG liposomes was shown to inhibit the growth of RAW 264 cells 50-fold and CV1P cells 350-fold more than free drug (Monkkonen and Heath 1993).

PALA exhibited a considerable increase in its *in vitro* growth inhibitory potency when it was encapsulated in liposomes. Encapsulation in negatively charged liposomes increased the growth inhibitory potency of PALA for CV1P cells by up to 360 times regardless of the size of liposomes used, if between 0.1 and 1 μm in diameter (Heath 1989). Encapsulated PALA in negatively charged liposomes was also shown to be up to 570-fold more potent for growth inhibitory effect on human ovarian tumor cells *in vitro* (Sharma 1993).

Even though PALA has been shown to exhibit increased growth inhibitory potency in negatively-charged liposomes, it does not automatically mean that it can be delivered to the target cells in antibody-conjugated liposomes which could enhance the potency of PALA much more by targeting to the cells. So it is important to study

whether PALA can be delivered to the target cells in antibody-conjugated liposomes or not. Furthermore, it will be of interest to see whether PALA-liposomes can cause a prolonged life span in tumor bearing animal models as compared to free drug.

B) Liposome-independent drug delivery

Relatively few drugs exhibit increased potency when encapsulated in liposomes. Drugs that do not exhibit increased potency in this way have been called liposome-independent drugs. Liposome-independent drugs are compounds that freely penetrate cell membranes. The failure of liposomes to improve their potency arises from two factors. First, they will rapidly gain entry into cells after leakage from liposomes. Second, their ability to penetrate cell membranes will lead to their rapid loss from cells after delivery. Liposome-independent drugs can exhibit increased efficacy as a result of controlled release from liposomes and localization of liposomes in the target organ.

One example of liposome-independent drugs is doxorubicin (or adriamycin) whose cytotoxic potency in an *in vitro* system is not significantly increased when it is encapsulated in liposomes, compared to free doxorubicin. However, doxorubicin was recently studied in antibody-conjugated liposomes, and a 15-fold increase in its growth inhibitory potency against KLN-205 squamous lung cancer cells *in vitro* as compared to free doxorubicin or doxorubicin encapsulated in antibody-free liposomes was observed (Ahmad 1992). In most of the *in vitro* growth inhibition studies, the comparison of drug potencies between its free form and encapsulated form is made on the basis of longer time continuous exposure of the cells to the drug treatment, usually 48 hr or 72 hr (Matthay et al. 1986; Straubinger et al. 1988). However, it is noteworthy that, in Ahmad's study, cells were exposed to drug treatment for 1hr only, and washed with PBS and cultured in growth media for another 24 hr. If, as in Ahmad's study, only

a brief exposure is used, there is a possibility that the difference in potency will arise from the difference in the amount of drug retained by the cells. For free drug this amount will be small, whereas for targeted liposomes it will be large. Subsequent processing of the liposomes will release the drug into the medium, from where it will act upon the cells. Based on other studies (Matthay et al. 1986; Straubinger et al. 1988), it is very doubtful whether doxorubicin could exhibit increased cytotoxicity in antibody-conjugated liposomes to the same degree if the cells were exposed to drug treatment continuously for a longer period than 1hr.

Although doxorubicin is a liposome-independent drug, there are therapeutic benefits that arise from liposomal formulation of doxorubicin. Among the benefits from liposomal formulation of doxorubicin is the altered pharmacokinetics of tissue distribution of doxorubicin so that its accumulation in heart tissue is minimized, thereby reducing cardiotoxicity of doxorubicin. As doxorubicin is known to cause congestive heart failure when given chronically, avoiding drug accumulation in heart tissue is clinically very desirable.

Doxorubicin was also studied in antibody-conjugated long circulating liposomes using KLN-205 squamous lung cancer bearing DBA/2 mice and shown to increase the life span of tumor mouse significantly compared to free drug and non-antibody conjugated doxorubicin liposomes (Ahmad 1993). This result demonstrates that a liposome-independent drug, like doxorubicin, can exhibit selective effects on the target cells by a controlled release effect due to the prolonged circulation time of the sterically-stabilized liposomes, or by simple localization of liposomes in the target cells due to the antibody-antigen specificity, or by both.

It is noteworthy that many of the currently developed liposomal formulations, such as encapsulated amphotericin B or doxorubicin, involve liposome-independent

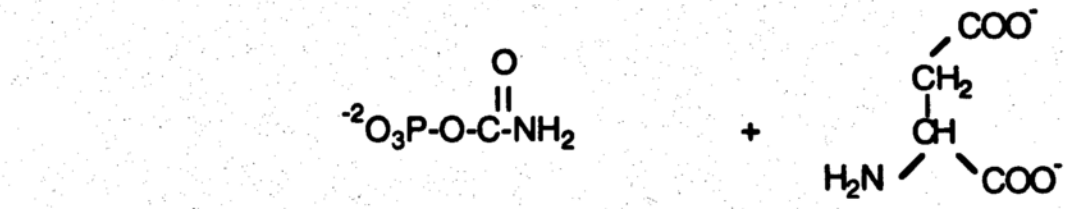
delivery and the therapeutic benefits from these liposomal formulations arise from reduced side effects to specific organs and controlled release of drugs over a longer time period. Therefore, it is of considerable importance that drug targeting must be attempted using a liposome-dependent drug. Therapeutic benefits from the use of liposome-dependent drugs should be much greater than those from the use of liposome-independent drugs.

C) Mechanisms of Action of PALA

N-(Phosphonacetyl)-L-aspartic acid (PALA) is a rationally synthesized compound, designed by Collins and Stark (Collins and Stark 1971) to be a stable inhibitor of acetyl-carbamyl transferase (ACTase). This enzyme is involved in the second step of de novo pyrimidine biosynthesis (see Figure 1.2). PALA combines structural features of the two natural substrates of this enzyme and appears to put the enzyme into a conformation closely resembling that associated with the transition state (Collins and Stark 1971). Uptake of PALA appears to be mediated by fluid phase endocytosis, with fusion of the endocytic vesicle with the lysosome (White and Hines 1984). Upon passage through the lysosomal membrane into the cytosol, PALA may become highly charged, and thus trapped intracellularly, permitting prolonged contact with the target enzyme, ACTase.

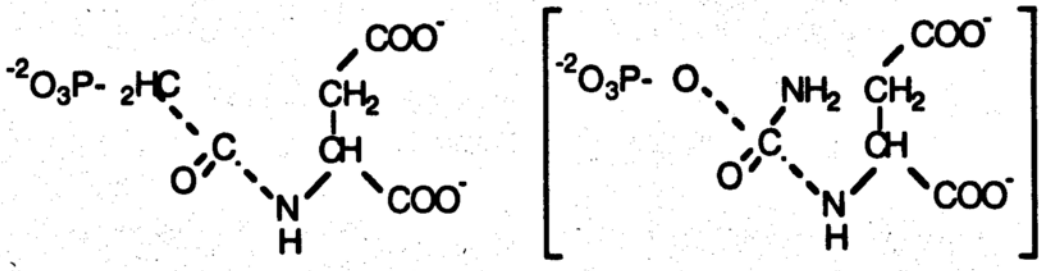
Studies of the acute toxicity of a single intraperitoneal (i.p.) injection of PALA in C57BL x DBA/2 mice have been performed. These studies revealed that a dose of 1079 mg/kg was lethal to 10% of the mice, a dose of 1587 mg/kg was lethal to 50% of the mice, and a dose of 2333 mg/kg was lethal to 90% of the mice (Clinical Brochure 1977). Radiolabeled ^{14}C-PALA administered intravenously (i.v.) to mice was found to have a rapid initial half-life ($t_{1/2}$) of 46 min with approximately 80% of the drug excreted unchanged in the urine by 24 hr (Chadwick, Silveira et al. 1982). The clinical pharmacokinetics of PALA have shown that, following i.v. administration, plasma levels decayed in a bi-or tri-exponential manner with a terminal $t_{1/2}$ between 4.8 and 12.7 hr. The volume of distribution (V_d = 309 ml/kg) was found to be slightly larger than the extracellular fluid volume, suggesting limited access of PALA to tissue compartments (Loo 1980). This is to be expected in light of the poor ability of PALA to penetrate cells (White and Hines 1984).

Figure 1.2. De novo synthesis of pyrimidine and the relationship of PALA to the possible transition-state intermediate. The structure of PALA is very similar to that of the transition-state intermediate, being a competitive inhibitor to the synthesis of pyrimidine. The ACTase is an acetyl-carbamyl transferase.

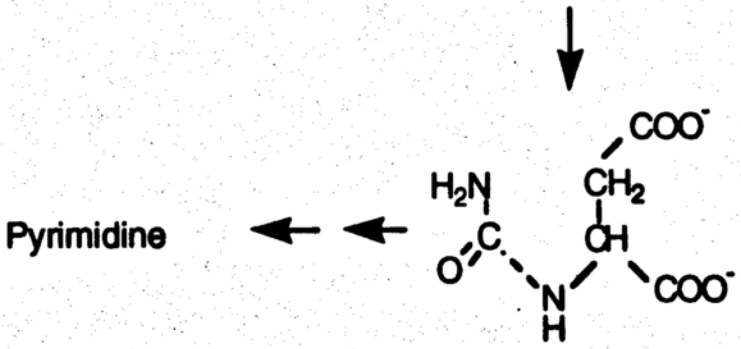


carbamyl phosphate + L-aspartate

ACTase



PALA possible transition-state intermediate



Pyrimidine

carbamyl-L-aspartate

Tumor-Specific Targets

It is not possible to prove the existence of a completely tumor-specific antigen in human neoplasms. Rather, tumor-specific antigens have also been found in normal tissues, although they are only present in trace amounts (Hellstrom and Hellstrom 1984). In other words, these tumor-specific antigens have relative rather than absolute specificity. For example, glycoprotein p97, which is a phosphorylated sialoglycoprotein of 97,000 mol. wt., is one of the best studied tumor-specific antigens and is expressed in approximately 50% of melanomas at a density of over 50,000 p97 molecules per tumor. Normal adult tissues, on the other hand, express less than about 8,000 p97 molecules per cell, and generally only a few hundred molecules per cell (Brown, Woodbury et al. 1981). Thus, about half of all melanomas have approximately a 10-fold greater amount of p97 than the most highly expressing normal cells, and some 100 times more p97 than normal cells in organs such as blood, bone marrow, kidney, gut, and brain. It is interesting to note that the difference in the expression of oncogene products, between neoplastic and normal cells, is also quantitative and of a similar magnitude (Slamon, DeKernion et al. 1984).

A) Oncogenes and oncogene products

An oncogene is a viral gene that produces a substance (or an oncogene product) capable of transforming a cell to the malignant state. A proto-oncogene, the normal cellular counterpart from which the viral oncogene is derived, represents a family of normal cellular genes that were identified on the basis of their similarity to genetic sequences with known tumorigenic or transforming potential (Bishop 1983; Varmus 1984). Considerable circumstantial evidence now exists to show that alteration in either the structure, copy number, or expression of one or another of these genes may play an important role in the pathogenesis of some human malignancies (Slamon 1987). Of the more than 24 known oncogenes (see Table 1.1), the three that have been correlated with known cellular proteins are related to either a growth factor or a growth factor receptor. The first is the c-*sis* oncogene, which encodes the B-chain of platelet-derived growth factor (PDGF). The second is the *erb*B oncogene, whose product, gp68, is a truncated form of the epidermal growth factor receptor (EGFR). The third is the c-*fms* proto-oncogene, which may be related to, or identical to, the receptor for macrophage colony-stimulating factor (CSF-1^R) (Sherr 1985).

The *erb*B oncogene was first identified by transfection studies in which NIH 3T3 cells were transformed with DNA from chemically induced rat neuroglioblastomas (Shih, Padhy et al. 1981). Though differences exist between the*erb*B proto-oncogene and the neu/HER-2 proto-oncogene, which encodes a 185 kd cell surface protein, they are often considered the same due to structural and functional similarity, and are referred to as neu/HER-2/*erb*B oncogen. The *erb*B oncogene encodes a protein (gp68) that has extracellular, transmembrane, and intracellular domains, which is consistent with the structure of a growth factor receptor (Coussens 1985). Gp68 was later found to be overexpressed in one third of human breast and ovarian cancers (Slamon, Clark et

al. 1987; Slamon, Godolphin et al. 1989) and at higher levels in salivary gland carcinomas (Semba, Kamata et al. 1985), gastric carcinomas (Falck and Gullick 1989), and colon carcinomas (Yokota, Yamamoto et al. 1988). The overexpression of *erb*B has been shown to correlate with poor survival of the patients (Paik, Hazan et al. 1990). It is thought that overexpression of *erb*B/neu/HER-2 oncogene may confer a selective advantage to cells through augmentation of pathways involved in stimulation of proliferation.

Monoclonal antibodies that bind *erb*B/neu and down-regulate the cell surface level of *erb*B/neu can inhibit the growth of cells that overexpress this proto-oncogene product. Actually, a monoclonal antibody specific for c-*erb*B-2 (TAB 250) has been shown to act synergistically with cis-diaminedichloroplatinum, both *in vitro* and *in vivo*, to kill tumor cells overexpressing this proto-protein (Hancock, Langton et al. 1991). Immunotoxins were made using five different murine monoclonal antibodies to the human *erb*B gene product, and LysPE40, a 40-kDa recombinant form of *Pseudomonas* exotoxin (PE) lacking its cell-binding domain. The resultant immunotoxins were shown to be cytotoxic to cancer cell lines overexpressing *erb*B protein (Batra, Kasprzyk et al. 1992). The same idea could be applied to the delivery of a chemotherapeutic agent in liposomes, to which are attached monoclonal antibodies specific for the *erb*B-2 oncogene products.

Table 1.1. Oncogenes originally identified through their presence in transforming retrovirus (Alberts 1989). The *erb*-B oncogene is one of the most studied growth factor receptor related oncogenes.

Oncogene	Proto-oncogene Function	Source of Virus	Virus-induced Tumor
abl	protein kinase (tyrosine)	mouse cat	pre-B-cell leukemia sarcoma
akt	?	chicken	T-cell lymphoma
crk	activator of tyrosine-specific protein kinase(s)	chicken	sarcoma
erb-A	thyroid hormone receptor	chicken	(supplements action of *v-erb*-B)
***erb*-B**	**protein kinase (tyrosine): epidermal growth factor (EGF)receptor**	**chicken**	**erythrolukemia; fibrosarcoma**
ets	nuclear protein	chicken	(supplements action of *v-myb*)
fes/fps	protein kinase (tyrosine)	cat/chicken	sarcoma
fgr	protein kinase (tyrosine)	cat	sarcoma
fms	macrophage colony-stimulating factor (M-CSF) receptor		
fos	nuclear transcription factor	mouse	osteosarcoma
**jun*	nuclear protein: AP-1 transcription factor	chicken	fibrosarcoma
kit	protein kinase (tyrosine)	cat	sarcoma
mil/raf	protein kinase (serine/threonine)	chicken / mouse	sarcoma
mos	protein kinase (serine/threonine)	mouse	sarcoma
myb	nuclear protein	chicken	myeloblastosis
myc	nuclear protein	chicken	sarcoma; myelocytoma; sarcoma
H-*ras*	G-protein	rat	sarcoma; erythrolukemia
K-*ras*	G-protein	rat	sarcoma; erythrolukemia
rel	nuclear protein	turkey	reticuloendotheliosis
ros	protein kinase (tyrosine)	chicken	sarcoma
sea	protein kinase (tyrosine)	chicken	sarcoma; leukemia
**sis*	platelet-derived growth factor, B-chain	monkey	sarcoma
ski	nuclear protein	chicken	carcinoma
src	protein kinase (tyrosine)	chicken	sarcoma
yes	protein kinase (tyrosine)	chicken	sarcoma

* three oncogenes related to either growth factor or growth factor receptor (see text for detailed explanation)

B) Transferrin receptor

Transferrin (Tf) is an abundant serum glycoprotein with the capacity to bind and transport iron. The main function of this 80-kDa glycoprotein (MacGillivary, Mendez et al. 1983) consists of providing cells with iron which they need in the process of DNA synthesis for the activity of ribonucleotide reductase, and for specialized functions such as the synthesis of hemoglobin in reticulocytes and iron transport to the fetus in syncytial trophoblasts. The transferrin receptor, a dimeric transmembrane glycoprotein of 180 kDa (Schneider, Sutherland et al. 1982), is expressed on the surface of most proliferating cells, and in elevated numbers on erythroblasts and on many kinds of tumor cells.

Receptor-mediated endocytosis is a natural cellular mechanism, which cells utilize for the uptake of proteins or peptides such as low-density lipoprotein, asialoglycoproteins, epidermal growth factor, transferrin, insulin, and small vitamins such as folic acid. This process is a very efficient delivery process, and many scientists have examined whether this mechanism could be exploited for the targeted delivery of drugs to cells, especially to tumor cells (Wagner, Curiel et al. 1994). Synthesis of anti-transferrin receptor antibody conjugates of the plant toxin ricin A, or the bacterial diphtheria toxin A subunit has produced potential therapeutic agents that are up to 1000-fold more effective than non-conjugated toxin fragments alone for the killing of human tumor cells *in vitro* (Trowbridge and Domingo 1981).

Intraperitoneal Tumor Model

The relevance of any given animal model to clinical application is a constant question in every area of therapeutics, but probably more so in cancer treatment. Cell lines *in vitro*, ascitic tumors, implanted syngeneic tumors, xenografts, and intravenous metastases can all be instructive, but can all be misleading as well. Intraperitoneal tumor models are the most useful when the corresponding human disease site is on or around the peritoneal area. This is the case for ovarian cancer (Dedrick, Myers et al. 1978) and some forms of colorectal cancer (Mayhew, Cimino et al. 1990). Especially, since ovarian cancer remains confined to the peritoneal cavity virtually throughout its entire clinical course, even when far advanced, this type of cancer is an attractive candidate for intraperitoneal chemotherapy.

The C-26 mouse colon carcinoma is one of the best studied mouse tumor cell lines for cancer therapy. The doubling time of this tumor in mice is about 4 days when the tumor is two weeks old, which makes the C-26 carcinoma a very useful mouse tumor model. The*in vitro* growth rate of the C-26 carcinoma is also fast in RPMI 1640 growth medium, so that it can be grown easily in large amounts for injection into mice. Therefore, the C-26 carcinoma has been successfully used to create several types of *in vivo* tumor models in rodents, including subcutaneous tumors, liver tumors (Huang 1992), and intraperitoneal tumors (Mayhew, Cimino et al. 1990).

I.P. Therapy for I.P. Tumor Model

The pharmacokinetic rationale for i.p. chemotherapy was developed by Dedrick in 1978 (Dedrick, Myers et al. 1978). Subsequent clinical trials confirmed that chemotherapeutic drugs can be safely administered by the i.p. route, and i.p. drug concentrations 1-3 log higher than peak plasma levels can be achieved (Markman 1986). Thereafter, i.p. chemotherapy has been investigated as an adjunct to surgery to kill residual cancer cells, inhibit cancer cell seeding, and prevent local recurrence and metastases. This approach has been attempted in cases of ovarian, gastric, and colon cancer (Sugarbarker, Gianola et al. 1985). This approach is desirable, because some of these tumors are not visible at the time of surgery, or because there are unavoidable and undesirable consequences of surgical removal. That is why, following surgery for these cancers, invasive and metastatic involvement of peritoneal and serosal surfaces are the most frequent modes of treatment failure (Gunderson and Sosin 1982; Willett, Tepper et al. 1984; Gunderson, Sosin et al. 1985; Minsky, Mies et al. 1988).

Mouse colon carcinoma C-26 has been studied for a subcutaneous or liver implanted tumor model (Huang 1992) and i.p. tumor model (Mayhew, Cimino et al. 1990) in mice with i.v. and i.p. injection of doxorubicin, respectively. In Mayhew's study (Mayhew, Cimino et al. 1990), the therapeutic effects of doxorubicin and liposome-encapsulated doxorubicin against i.p. mouse C-26 tumor were compared, and both free doxorubicin and liposome-doxorubicin were found to be effective in prolonged survival time of tumor mice when therapy was initiated 1 day after tumor inoculation.

Evaluation of Therapeutic Efficacy in Animal Tumor Model

Before the therapeutic efficacy of drugs can be evaluated in a diseased animal, the maximum tolerated dose (MTD) should be determined first using healthy animals. The MTD has various definitions, among them is one from the National Toxicology Program's (NTP) Bioassay Program which defines MTD as the dose that suppresses body weight no more than a specified amount in a 90-day subchronic study, although the duration of exposure can last for shorter period of times, such as 3 weeks. The amount specified can be as low as 10% of total body weight, though higher figures are often used. There are two main principles in determining the MTD for a chemotherapeutic agent in experimental animals. The first is that the effects produced by the compound in laboratory animals, when properly qualified, are applicable to humans. On the basis of dose per unit body surface area, toxic effects in humans are usually in the same range as those in experimental animals. On the body weight basis, humans are generally more vulnerable than experimental animals, probably by a factor of about 10. With an awareness of these quantitative differences, appropriate safety factors can be applied to calculate relatively safe doses for humans. The second main principle is that exposure of experimental animals to toxic agents in high doses is a necessary and a valid method of discovering possible hazards in humans. This principle is based on the quintal dose-response concept that the incidence of an effect in a population is greater as the dose or exposure increases.

Evaluation of therapeutic efficacy of drugs is usually done by following the survival of tumor animals for a given time period after appropriate doses were given. Dose selection is based on the MTD. Typically, fractions like one half, one third, or one quarter of the MTD are usually used for injection. During the experimental period, animals are to be carefully observed for any sign of abnormality like weight loss, loss

of activity, and morbidity as well as death. Usually, the experiment is terminated when the last mouse dies, or when there are any humane reasons to terminate the experiments.

Statement of Purpose

As a liposome-dependent drug, the chemotherapeutic agent N-(phosphonacetyl)-L-aspartic acid (PALA) has previously proven to be more cytotoxic *in vitro* in negatively-charged liposomes than free drug (Heath and Brown 1989). However, its efficacy in antibody-targeted liposomes has not previously been studied. As not every liposome-dependent drug whose potency was increased in negatively-charged liposomes can show an improved cytotoxicity in antibody-targeted liposomes, a study was undertaken to examine whether PALA can be targeted in antibody-conjugated liposomes. Previously, no *in vivo* studies have been carried out with liposome-dependent drugs. Consequently, whether PALA can exhibit better therapeutic efficacy in liposomes in a tumor-bearing animal model *in vivo* compared to free drug has also been evaluated. The results from this work are expected to lay a foundation for the possible therapy of human ovarian cancer using antibody-conjugated PALA liposomes.

In order to achieve this goals, eight different kinds of tumor-specific monoclonal antibodies, specific for both the c-*erb*B-2 oncogene product and transferrin receptor, were examined for their ability to deliver PALA to the cells *in vitro* using a growth inhibition assay. For the *in vivo* animal tumor study, the C-26 murine colon carcinoma-bearing BALB/c mice were tested with PALA in different liposome formulations administered either 1 day (immediately after tumor seeding) or 8 days (after tumor was well established) after tumor implantation.

Chapter 2. MATERIALS AND METHODS

Materials

N-(phosphonacetyl)-L-aspartic acid was a generous gift from Dr. K. Groover at the National Cancer Institute (Bethesda, MD). Bovine serum albumin (Fraction IV) and Protein A (from Staphylococcus aureus) were bought from Sigma (St. Louis, MO). Monoclonal antibodies against the c-*erb*B2 oncogene product, the transferrin receptor, and a high molecular weight mucin, respectively, were a gift from Chiron Inc. (Emeryville, CA). Calcein was purchased from the Molecular Probes Inc. (Eugene, OR). Egg phosphatidylcholine (PC) and transphosphatidylated egg phosphatidylethanolamine (TPE) were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol (Chol), dithiothreitol (DTT), 4-(p-maleimidophenyl)-butyric acid N-hydroxy succinimide ester (SMPB), and N-succimidyl 3-(2-pyridyldithio) propionate (SPDP) were purchased from Sigma Co. (St. Louis, MO). All organic solvents were reagent or HPLC grade. PC, Chol, and synthesized MPB-PE were stored ampouled in chloroform under argon at -20°C until use.

Polyethylene glycol derivatized DSPE (PEG 1900-DSPE) was synthesized and provided by Dr. F. Martin (Liposome Technology Inc., Menlo Park, CA). Protease (Type IX), collagenase (Type IV), and DNase were bought from Sigma (St. Louis, MO). Female BALB/c mice weighing 16-21 g were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and maintained with normal feeding and water throughout the experimental period. All the animal facilities used were at the State University of New York-Buffalo School of Pharmacy.

Cell Culture

A murine fibroblast cell line, L929, was originally obtained from Dr. L.B. Epstein (University of California, San Francisco) and grown in DME medium with 5% fetal bovine serum, penicillin, and streptomycin. The HEY 1B and SKOV-3 human ovarian cancer cell lines were obtained from Prof. R. M. Straubinger at SUNY at Buffalo (Buffalo, NY). HEY 1B cells were grown in RPMI 1640 medium with L-glutamine, and SKOV-3 cells were grown in McCoy's 5A medium with L-glutamine(Gibco, Grand Island NY). Growth medium for HEY1B and SKOV-3 cells was supplemented with 10 % fetal bovine serum and 100 units/ml of penicillin and 100 µg/ml of streptomycin. Cells were normally grown in 25 cm^2 polystyrene tissue culture flasks (Corning, NY) in a humidified incubator (Queue, WV) at 37°C with a 4% CO_2 atmosphere until they became confluent. Confluent flasks were split by treatment with 0.05% trypsin / 1 mM EDTA for 20-30 min to remove the cell monolayer. Serum was added to stop the action of the trypsin, and the cells were harvested by centrifugation at 300 g for 10 min. The cells were resuspended in fresh medium and used for experiments, or setting up new flasks.

A murine colon tumor cell line, C-26, was obtained from Prof. R. Straubinger (SUNY at Buffalo, NY) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units / ml of penicillin and 100 µg/ml of streptomycin. The C-26 cells were grown in 25 cm^2 polystyrene tissue culture flask (Corning, NY) in a humidified incubator (Queue, WV) at 37°C with a 4% CO_2 atmosphere. Confluent cells were harvested in the same manner as HEY 1B or SKOV-3 cells, and new flasks were set up for future experiments.

Monoclonal antibody (mAb)

The monoclonal antibodies used in this study include 8 murine immunoglobulins which were developed at Chiron Inc. (Emeryville, CA) and generously supplied for experiments. Some of the characteristics of these monoclonal antibodies are indicated in Table 2-1. Three mAbs (454C11, 520C9, and 741F8) are anti-*erb*B2-antibodies. The c-*erb*B2 antigen is overexpressed by many tumor cell lines, including HEY1B and SKOV-3. Another three (260F9, 317G5, and 454A12) are anti-transferrin receptor antibodies. One (2G3) is an antibody that binds to high molecular weight mucin, and one (113F1) binds to a series of 40-200 kDa tumor-associated cell surface proteins. All of these monoclonal antibodies are considered to be tumor-specific because of their binding specificity for the surface antigens of tumor cells.

All of the antibodies were stored at 4°C in buffered solution until use. The concentration of the monoclonal antibodies was determined from the Bradford protein assay using immunoglobulin G as a standard.

Table 2-1. Selected characteristics of the monoclonal antibodies used for this study from Chiron Inc. (Emeryville, CA).

mAb	Class	Mol. Weight	Corresponding antigen
454C11	IgG_{2A}	200 kDa	c-*erb*B2 shed antigen (epitope B)
520C9	IgG_1	200 kDa	c-*erb*B2 shed antigen (epitope A)
741F8	IgG_1	185 kDa	c-*erb*B2 shed antigen (epitope A)
260F9	IgG_1	55 kDa	transferrin receptor
317G5	IgG_1	42 kDa	transferrin receptor
454A12	IgG_1	180 kDa	transferrin receptor
2G3	IgG_1	n/a	high MW mucin
113F1	IgG_3	40-400 kDa	40-200 kDa protein

Experimental Methods

Synthesis of maleimidophenyl butyl-phosphatidyl ethanolamine (MPB-PE)

Synthesis of MPB-PE was performed using the method of Martin et al. (Martin 1982). Briefly, 200 µmol transphosphatidylated egg phosphatidylethanolamine (TPE) and 100 mg of 4-(p-maleimidophenyl)-butyric acid N-hydroxy succinimide ester (SMPB) were dissolved in 4 ml of freshly distilled lutidine and 10 ml of anhydrous methanol. After 2 hours of reaction with stirring under argon at room temperature, the formation of MPB-PE was monitored by thin layer chromatography (TLC). Briefly, the sample was developed by thin layer chromatography (TLC) using Merck silica gel 60 F-254 (0.2 mm thickness) glass-baked TLC plates, which were developed in chloroform : methanol : H_2O (65 : 25 : 4), and the spots were visualized by spraying with ethanol solution of phosphomolybdic acid and heating at 200°C for 10 min. The R_f values from the phosphomolybdate staining were 0.7 and 0.6 for TPE and MPB-PE, respectively. As the TPE stains blue by ninhydrin spray because of the free amino groups, whereas MPB-PE gives no coloration by ninhydrin spray, the formation of MPB-PE was further monitored by the disappearance of ninhydrin staining with TPE as a standard. Evaporation of solvents (methanol and lutidine) and redissolving MPB-PE in chloroform were followed by column separation using silica gel (230-400 mesh, 60 Å). The column was eluted with mixtures of chloroform and methanol of increasing polarity (chloroform : methanol = 40 : 1, 30 : 1, 25 : 1, 20 : 1, 15 : 1, and 10 : 1 volume ratio) to purify the product. Pure MPB-PE was further identified by thin layer chromatography (chloroform : methanol : acetic acid = 60 : 20 : 3 and chloroform : methanol : ammonium hydroxide : water =69 : 27 : 1.5 : 2.3), phosphomolybdate staining, and NMR spectroscopy. The concentration of MPB-PE was determined by measuring the content of phosphorus using Bartlett's method (Bartlett 1958). The yield

of MPB-PE was over 90%, and it was stored in chloroform ampouled at -20°C until use.

Preparation of liposomes for antibody conjugation

All reagents and glassware used for liposome preparation were sterile and all open tube manipulations, except rotary evaporation, were carried out in a biosafety hood. Liposomes were prepared by the reverse-phase evaporation method (Szoka and Papahadjopoulos 1978). Typically, a dry film of PC : Chol : MPB-PE at a molar ratio of 20 : 20 : 1 was suspended in 2 ml of freshly hydrated diethyl ether, to which was added 0.8 ml of aqueous drug solution. The mixture was then sonicated (Laboratory Supply Co., Hicksville, NY) for 3 min to produce a water-in-ether emulsion, and ether was then eliminated by rotary evaporation (Rotavapor RE-111, Buchi Laboratoriums, Switzerland) to produce a suspension of large unilamellar vesicles (LUV). The liposomes were downsized by extrusion through 0.2 μm pore size polycarbonate membranes (Nuclepore, Pleasanton, CA) 5 to 10 times. Extrusion was carried out with an anodized aluminum extruder (Lipex Biomembranes, Vancouver, Canada) fitted with a 1.5 ml capacity chamber and 13 mm diameter membranes. Liposomes were forced through the membrane using the pressure of 150 - 200 psi. from an argon gas cylinder (Badger Welding Supplies, Madison, WI) The whole extruder unit was also autoclaved prior to use. The size of vesicles was confirmed by quasi elastic laser light scattering analysis (Nicomp, Santa Barbara, CA).

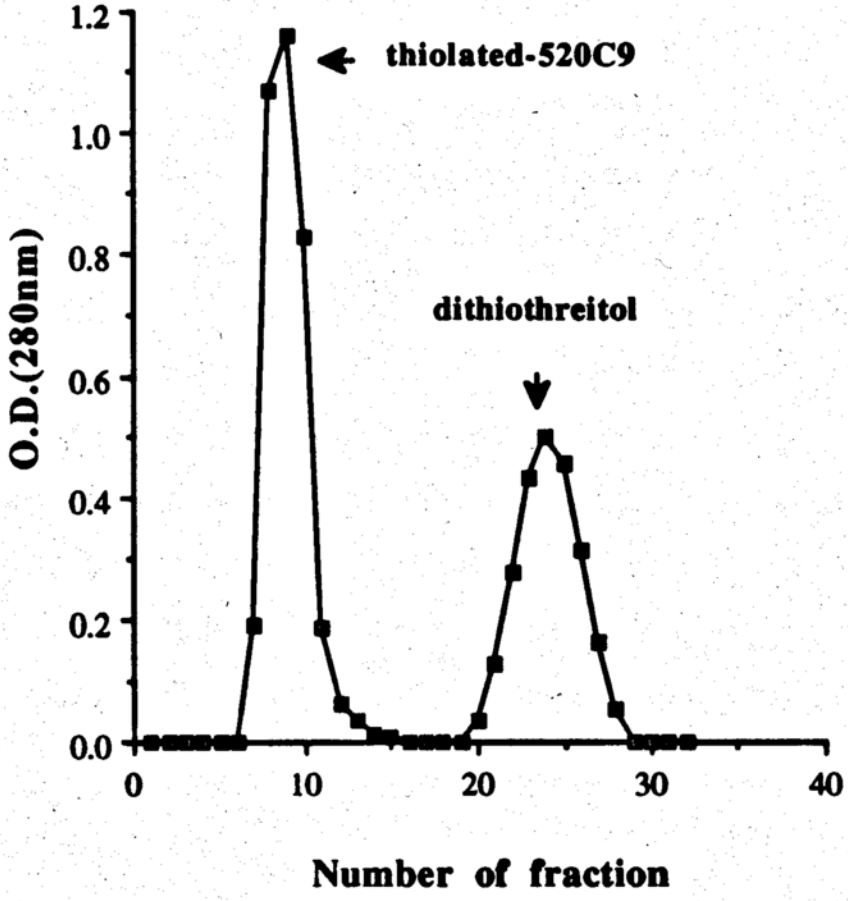
N-phosphonacetyl-L-aspartic acid (PALA) solution was prepared for encapsulation at 60 mM (pH 6.7) in a buffer containing 50 mM MES, 50 mM HEPES, and 80 mM NaCl. Calcein was also included in the PALA solution at a concentration of 2 mM, and was used as an indicator for measuring encapsulation of PALA. It was assumed that encapsulation of PALA and calcein in liposomes followed passive aqueous capture and that the ratio of 30 mol PALA per mol calcein in the original solution was maintained after encapsulation in liposomes. The tonicity of drug solution

was measured using an osmometer (Wescor, Logan, UT) and, if necessary, was adjusted to 290 mmol/kg by addition of NaCl (Mallinckrodt) before use. Liposomes were separated from unencapsulated materials by gel chromatography using a sterile 1 x 10 cm Sephadex G-75 column (Pharmacia) eluted with a buffer containing 50 mM MES, 50 mM HEPES, and 80 mM NaCl at pH 6.7. The concentration of phospholipid was determined by a phosphorus assay (Bartlett 1958) on triplicate samples, which were extracted by the method of Bligh and Dyer (Bligh and Dyer 1959) to eliminate PALA. As the lower chloroform phase of extraction mixture contained the phospholipid, the lipid content was determined from the phosphorus assay of the lower phase. The concentration of PALA was determined indirectly from the concentration of co-encapsulated calcein after the liposomes were solubilized using 0.1% Triton X-100 detergent. The concentration of calcein was determined spectrophotometrically assuming a molar extinction coefficient at 493 nm in a MES / HEPES buffer, pH 6.7 of 18,258 L $mole^{-1}$ cm^{-1}.

Conjugation of antibody to liposomes

The monoclonal antibodies (mAbs) for conjugation were dissolved in 0.1M phosphate buffered saline (PBS) solution at a concentration of 2 mg/ml. The concentration of mAb was determined spectrophotometrically assuming that the absorbance value of 0.1% (w/w) immunoglobulin at 278 nm is 1.38 (Sober 1968). The SPDP solution was freshly prepared at 20 mM in methanol. Typically, 1ml of mAb (2 mg/ml concentration) solution was reacted with 15 µl of fresh SPDP to give a 15 : 1 molar ratio of SPDP : mAb at room temperature. After 30 min reaction with stirring, the pyridyl-dithio propionate derivatized mAb (PDP-mAb) was separated from reactants by gel chromatography on a Sephadex G-75 column, eluted with a buffer containing 50 mM citrate, 50 mM phosphate, and 50 mM NaCl at pH 7.0. The PDP : mAb ratio was determined by the method of Carlsson et al. (Carlsson, Drevin et al. 1978) and typically found to be between 7 - 9 PDP groups per mAb molecule. The PDP-mAb solution was adjusted to pH 4.5 and reduced to give thiolated-mAb (SH-mAb) by treating with dithiothreitol (DTT) in citrate buffer at a final concentration of 25 mM. After 30 min of reaction, the SH-mAb was separated from reactants by gel chromatography on a Sephadex G-75 column, eluted with a buffer containing 50 mM MES/HEPES at pH 6.7. A typical separation of SH-mAb (SH-520C9 antibody) from other reactants is shown in Figure 2.2. Special care is required to prevent the oxidation of sulfhydryl group on the mAb. For this reason, the eluting buffer was purged with argon to remove oxygen, eluant was collected in an argon purged tube, which was capped tightly when full, and tubes were repurged whenever the caps were opened.

Figure 2.2. Separation of SH-520 C9 from dithiothreitol (DTT) on a Sephadex G-75 column under argon to prevent oxidation of the thiol group on the mAb.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
O.D.(280nm)
thiolated-520C9
dithiothreitol
0
10
20
30
40
Number of fraction

The SH- mAb solution was sterilized by filtering through a 0.2 μm pore size Nalgene membrane and conjugation was initiated by mixing equal amounts of MPB-PE-liposomes with SH-mAb overnight at room temperature. The mAb-conjugated liposomes were then separated from free mAb by flotation on a discontinuous metrizamide gradient. Briefly, 0.5 ml of mixture of SH-mAb and MPB-PE liposomes was mixed with 1 ml of 10% metrizamide (275 mmol/kg) in a 13 x 51 mm Ultra-clear ™ tube (Beckman Instruments, Palo Alto, CA). The second layer was made by overlaying 3 ml of 5% metrizamide (290 mmol/kg) on top of the first layer, and 0.5 ml of MES/HEPES buffer was also overlayered as a third layer. After the sample tubes were loaded into a SW 55Ti Rotor (Beckman Instruments), centrifugation was performed at 45,000 rpm for 45 min at 10°C with minimum acceleration and deceleration (Beckman L8-60M Ultracentrifuge, Beckman Instruments). The band of mAb-conjugated liposomes, which was formed underneath the buffer layer, was removed from each tube by careful aspiration with a Pasteur pipette in a volume as little as 0.5 ml.

Preparation of liposomes for in vivo study

The DSPC-PALA (DSPC:Chol, 20:10 molar ratio), DSPG-PALA (DSPG:Chol, 20:10 molar ratio), and sterically stabilized PEG-DSPE-PALA liposomes (DSPC:Chol:PEG-DSPE, 20:10:1 molar ratio) were prepared for *in vivo* animal study using the reverse phase evaporation method (Szoka and Papahadjopoulos 1978). The method of preparation was similar to that described above, except that higher temperatures were maintained in order that all processes occurred above the gel to liquid crystalline phase transition temperature of the phospholipid. Briefly, the lipid mixture in chloroform was placed in a 20 x130 mm sterile test tube inside of a sterile boiling tube, and chloroform was evaporated in a rotary evaporator at 55°C, slightly above the phase transition temperature of the lipids being used. The lipid film was then dissolved in isopropyl ether, and aqueous PALA solution was added. The mixture was then sonicated for 3 min at 55°C. After the isopropyl ether was evaporated, the liposome suspension was vortexed vigorously, and was extruded through a 0.2 μm pore size polycarbonate membrane 5-10 times using an extruder at 55°C. The liposomes were then fractionated using a Sephadex G-75 column to remove unencapsulated drug and residual ether.

Determination of liposome size by laser light scattering analysis

The liposome size was determined using a Nicomp quasi electric laser light scattering system (QUELS) (Nicomp Inc., Santa Barbara, CA). Typically, an appropriate dilution of freshly made liposomes was run for 10 min at a room temperature with a count rate of 300-326 kHz. The wave length of the light source was 632.8 nm. Using a volume-weighted Gaussian analysis, the sizes were found to be 215 ± 112 nm (mean ± s.d.), 132 ± 62 nm, and 181± 57 nm for DSPC-PALA liposomes, DSPG-PALA liposomes, and sterically stabilized PEG-DSPE-PALA liposomes, respectively.

Protein Assay

A) Lowry Protein Assay

The amount of bovine serum albumin (BSA) after conjugation to liposomes was determined by the Lowry protein assay (Lowry, Rosebrough et al. 1951). The solutions for the assay were made as follows: Folin A solution was 2% (w/v) Na_2CO_3 in distilled water, Folin B1 solution was 1% (w/v) $CuSO_4 \cdot 5\ H_2O$ in distilled water, and Folin B2 solution was 2% (w/v) sodium potassium tartrate in distilled water. Typically, to 0.2 ml of sample or standard solution was added 1 ml of Folin C solution (0.5 ml Folin B1 + 0.5 ml Folin B2 + 50 ml Folin A) in a clean 13 mm x 250 mm glass tube. After 10 min, 0.1 ml Folin D solution (Folin & Ciocalteu's phenol reagent : H_2O = 1 : 1 mixture) was added to above sample mixture and immediately mixed using a vortex mixer. The mixture was allowed to stand for 30 min at room temperature, and the concentration of BSA was determined spectrophotometrically after reading absorbance (O.D.) of the sample at 720 nm using BSA as a standard.

B) Bradford Protein Assay

The amounts of mAb attached to the liposomes were assayed by the Bradford protein assay (Bradford 1976). Five dilutions of an immunoglobulin G (IgG) standard solution were used in the assay. The standard was prepared by redydrating lyophilized bovine plasma gamma globulin (Bio-Rad Laboratories, Richmond, CA) with deionized water to give a 1.54 mg/ml IgG concentration, which was stored at -20°C until use. Typically, 100 µl of a 1 : 100 dilution of an antibody-conjugated liposome solution was used as a sample. Portions containing 800 µl of either standard or sample were prepared in triplicate by diluting with buffer in clean, dry test tubes. To each tube was added 200 µl of Bio-Rad protein assay dye concentrate with mixing. The mixture was

incubated at room temperature for 15 min. Absorbance was measured at 595 nm using a spectrophotometer (DU-64 Spectrophotometer, Beckman Instruments, Fullerton CA).

Lipid Assay

A) Phospholipid extraction for lipid assay

The Bligh and Dyer extraction method (Bligh and Dyer 1959) was used to extract phospholipid from the liposome preparations, which contained PALA. To each 160 µl sample of liposomes in a glass tube was added 400 µl of methanol and 200 µl of chloroform, with mixing to form a homogeneous solution. To this homogeneous solution was added 200 µl of chloroform with mixing, followed by adding another 200 µl of deionized water. This mixture was thoroughly mixed, and centrifuged using a table top centrifuge (IEC Model CL Centrifuge, Needham Heights, MA). After centrifugation for 5min at room temperature, the mixture was separated into two clean layers. The bottom layer contained phospholipid and other hydrophobic materials, and the top layer contained PALA and other hydrophilic materials. The bottom chloroform layer was carefully aspirated out from underneath the upper layer using a Pasteur pipette, and was used for the phosphorus assay.

B) Bartlett's phosphorus assay

The concentration of phospholipid was determined by phosphorus assay (Bartlett 1958). The standard solution for the phosphorus assay was a KH_2PO_4 solution. Standards containing between 0.1 and 0.4 µmole of phosphorus were prepared in triplicate by adding 50-200 µl of a 2 µmole/ml dilution of a stock standard (2.195 g KH_2PO_4 in 500 ml deionized water) to clean tubes. Sample was typically 100 µl from the chloroform layer of the Bligh and Dyer extraction. To each standard and sample solution in 20 x 150 mm borosilicate glass tubes (Pyrex®, Corning) was added 0.4 ml of 10 N sulfuric acid, and the tubes were placed in a heating block (Thermolyne, Dubuque, IA) at 165°C for 30 min. The tubes were cooled down, and 30 µl of 30%

hydrogen peroxide (J.T. Baker, Phillipsburg, NJ) was added to each tube, which was then heated for another 30 min. After further cooling, 4.6 ml of 22% (w/w) ammonium molybdate solution was added to each tube, followed by 0.2 ml of Fiske-Subba Row reagent (15 g sodium metabisulfite, 0.25 g 1-amino-2-naphthol-4-sulfonic acid, and 0.5 g sodium sulfite dissolved in 100 ml water, or ANSA solution) with immediate mixing. These tubes are then heated in a boiling water bath for 10 min, and, after cooling, the absorbance was measured at 830 nm using a Beckman DU®-64 spectrophotometer (Beckman, Fullerton, CA).

Growth Inhibition Assay

A) Growth inhibition assay with Protein A-conjugated PALA liposomes

A murine fibroblast cell line, L929, was used to test the cytotoxic potency of Protein A-conjugated PALA liposomes *in vitro*. Cells were plated at a density of 4×10^4 cells/ml in 24-well plates and allowed to adhere to the bottom of wells overnight. On the next day, 40 µl (or 20 µl) of 11-4.1 mAb (1 mg/ml concentration) solution in 0.1 M PBS was added to each cell well, and the plates were incubated for 30 min at 4°C. The growth medium containing non-bound or excess 11-4.1 mAb was then removed, and fresh, warm growth medium added. Cells were then exposed to either free PALA or Protein A-PALA liposomes and returned to an incubator at 37°C with 7% CO_2 for an additional 48 hr . The percent growth (% Growth) was calculated according to the following equation:

$$\% \text{ Growth} = \frac{[\text{control count} - \text{original count}]}{[\text{sample count} - \text{original count}]} \times 100$$

where the control count is the cell count in the wells treated only with buffer, the sample count is the cell count from the wells treated with the drugs, and the original count is the cell count at the time of drug treatment. The mean percent growth was plotted against the $\log_{10}$ of the drug concentration. The drug concentration required to produce 50 % inhibition of growth (or IC_{50}) was determined graphically from the plot.

B) Growth inhibition assay with antibody-conjugated PALA liposomes

The HEY 1B and SKOV-3 , both human ovarian cancer cell lines, were used to test the sensitivity to free PALA and mAb-conjugated PALA liposomes. Typically, cells were grown in 25 cm^2 tissue culture treated flask (Corning, NY) in appropriate growth medium and plated at 2 x 10^4 cells/ml density in a 24-well plate (Corning, NY) After attachment to the bottom of the plate overnight, triplicate wells were treated with either free PALA or antibody-conjugated PALA liposomes. Control wells were treated with buffer alone. Live cells from three wells were counted at the time of drug treatment to give the original cell count. After continuous exposure of the cells to the treatment for 72 hr, dead cells and growth medium were removed and 1 ml of 0.05% trypsin in phosphate-buffered saline/1 mM EDTA solution was added for 20 min at 37°C to harvest the cells from cell wells. Cells were then counted using a Coulter Counter Model ZM (Coulter Electronics, UK) after appropriate dilution. The IC_{50} was calculated as described previously.

C) Growth inhibition assay for C-26 cells with PALA liposomes

The C-26 murine colon carcinoma cells were used to test the sensitivity to free PALA, DSPC-PALA, DSPG-PALA, and sterically stabilized PEG-DSPE-PALA liposomes. Cells, normally grown in 25 cm^2 tissue culture treated flasks (Corning, NY) in RPMI 1640 growth medium, were plated at 2 x 10^4 cells/ml density in a 24-well plate (Corning, NY), and allowed to attach to the bottom of the plate overnight. Triplicate wells were then treated with either free PALA or PALA-liposome formulations after overnight incubation. Control wells were treated with MES/HEPES buffer alone. Three wells were counted at the time of drug treatment to give the

original cell count. After continuous exposure to the drug or buffer for 72 hours, dead cells and growth medium were removed and 1 ml of 0.05% trypsin in phosphate-buffered saline supplemented with 1 mM EDTA solution was added and incubated for 20 min at 37°C to harvest the cells from the wells. Cells were then counted using a Coulter Counter Model ZM(Coulter Electronics, UK) after 1/50 dilution. The percent growth (% Growth) was calculated as described previously.

D) 1hr Wash Experiment with C-26 cells

To investigate the effect of short period exposure of drug on the growth inhibitory effect of PALA using the C-26 cells *in vitro*, cells were exposed for 1 hr to free or encapsulated PALA liposomes and washed with 1 ml of warm Ca^{++}/Mg^{++} /PBS solution twice and allowed to grow for an additional 71hr in fresh growth medium. At the end of the growth inhibition assay, cells were counted using a Coulter Counter at appropriate dilutions, and the IC_{50} was determined graphically as explained previously.

Determination of MTD for PALA

The maximum tolerated dose (MTD) for free PALA or PALA-liposomes after a single i.p. injection was determined using healthy female BALB/c mice. Experimental mice were randomly divided into 20 groups of 6. The groups included a non-treated control group, a buffer-injected control group, and 18 groups which received various PALA containing treatments. Treatments consisted of 6 different doses of free PALA, DSPC-PALA, and DSPG-PALA liposomes as detailed in the results section. Each mouse was ear coded by punching, and injected intraperitoneally with 1.4 ml of free PALA, or PALA-liposome, or buffer solution, using a 25G5/8" Precision Glide® needle (Becton-Dickinson & Co., Rutherford, NJ). Mice were housed, six per cage, and the groups were randomized among the various cages. Mice were weighed daily and survival was recorded for up to three weeks. The lowest dose which did not show 15% weight loss or more was chosen as the MTD. Animals showing more than 20% weight loss were sacrificed for humane reasons, because such animals were close to death.

C-26 Tumor Model with BALB/c Mice

Female BALB/c mice were used as hosts for the C-26 murine colon carcinoma. The C-26 cells, which had been grown in RPMI 1640 medium plus 10% FBS, were harvested and resuspended at an appropriate density for injection. Typically, $1x10^6$ C-26 cells were inoculated subcutaneously on each flank of a donor mouse, and two to three weeks after inoculation, when tumor size has reached about 1000 mm^3, a tumor inoculum was prepared by a protease-collagenase tumor isolation method (Corbett, Griswold et al. 1975). Briefly, tumors were surgically removed from the animal aseptically, scraped from their capsules, and minced as finely as possible. The minced tumor was transferred to a sterile Gibco bottle with 20 ml of 0.25% protease/collagenase solution, to which 0.2 ml of 0.02% DNase was added dropwise, and incubated for 45 min at 37°C with stirring. Supernatant was collected and filtered through a 40 µm pore size Nalgene membrane, and centrifuged for 3 min in a table top centrifuge at 300 rpm (IEC Clinical Centrifuge, MA). After centrifugation, 0.2 ml of 0.02% DNase was added to the pelleted tumor cells, which were resuspended, washed, and diluted to $1x10^6$ cells / 0.2 ml in sterile saline solution. The viability of this cell suspension was tested by the Trypan blue exclusion method using a Hemocytometer (SPotlite™ Hemocytometer, American Scientific Products, MaGraw Park, IL). For this purpose, a 0.2 ml portion of the cell suspension was diluted with 0.8 ml PBS solution, and 1 ml of 0.1% Trypan Blue solution was then added. After 15 min, 10 µl of this solution was placed in a Hemocytometer, and viable non-stained cells were counted. The average of three different counts was used for the calculation of viability, which was over 95%.

The mice were injected intraperitoneally with 1×10^6 tumor cells per mouse and they were randomized into 13 different groups, each group having 10 mice. Treatment

was initiated either eight days or one day after i.p. tumor inoculation . Control mice were injected with MES/HEPES buffer only. Animals had free access to water and food throughout the experiments. After initiation of drug treatment, the mice were weighed three times a week and were inspected daily during the experimental period. Deaths were recorded daily, and mice whose weight was less than 80% of the starting weight, or mice that were obviously in discomfort were humanely sacrificed. In cases where mice were humanely sacrificed, the day of sacrifice was recorded as the day of death.

Chapter 3. *IN VITRO* RESULTS

Characterization of Modified Liposomes

A) Characterization of BSA-Conjugated Liposomes

As a preliminary step for successful conjugation of monoclonal antibody to liposomes, bovine serum albumin (BSA Fraction V, Sigma, St. Louis, MO) was first used as a model protein for conjugation to liposomes. It was assumed that the molecular weight of BSA was 66 kDa and absorbance of 0.1% aqueous solution at 278 nm was 0.67. The conjugation procedure for BSA was same as that for monoclonal antibody, followed by metrizamide flotation separation.

The starting molar ratio of -SH group per BSA before conjugation to liposome was shown to be between 15 to 22, based on the Equation 6 in Chapter 3. The maximum workable ratio of BSA : lipid after conjugation was found to be between 100 and 150 μg protein / μmole lipid, which was determined using the Equation 6 in Chapter 3 after phosphorus assay and Lowry protein assay. Higher ratios of BSA : lipid often caused difficulties in the metrizamide flotation procedure for separation of BSA-conjugated liposomes from free BSA, presumably because of the higher density of the product. The protein : lipid ratio of the product was also found to be dependent upon the starting concentration of PDP-BSA, however, when the starting concentration of PDP-BSA was above 0.15 mg/ml, the ratio of BSA :lipid remained almost constant around 150 μg / μmole. This suggested that at approximately 150 μg BSA/μmole lipid, the BSA may saturate the outer liposome surface, thereby inhibiting further significant attachment of BSA to liposomes. Under these conditions, there was no difficulty in flotation separation of BSA-conjugated liposomes.

B) Characterization of Protein A-conjugated PALA Liposomes

Protein A is a surface protein of many strains of *Staphylococcus aureus* that binds the F_c portion of many immunoglobulins. Protein A can also be used as a powerful tool for *in vitro* targeted drug delivery when conjugated to liposomes, because Protein A-conjugated liposomes can serve as a universal tool for the binding of the Fc portion of any kind of immunoglobulins present in experimental environment. In this study, Protein A from the cell wall of Cowan strain of *S. aureus* (Sigma, St. Louis, MO) was conjugated to PALA encapsulated in PC : Chol : MPB-PE liposomes using the same conjugation protocol as antibody conjugation. As Protein A is known to contain no endogenous sulfhydryl bond, reduction of PDP-Protein A to SH-Protein A with DTT can be carried out at pH 7.5.

It was assumed that the molecular weight of Protein A was 43,000 and the absorbance of a 1mg/ml aqueous solution at 280 nm was 0.2. The starting molar ratio of -SH : Protein A before conjugation with liposomes was determined to be between 10 and 16. The drug to lipid molar ratio for PALA containing Protein A- liposome was typically found to be 0.8. Throughout the experiments this value remained almost the same, suggesting modification of proteins and conjugation of modified proteins to liposomes could be successfully performed under these conditions.

C) Characterization of Antibody-Conjugated PALA Liposomes

Before monoclonal antibodies were conjugated to liposomes, they were first modified by N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) to produce PDP-mAb. The PDP-mAb was further treated with dithiothreitol (DTT) to produce sulfhydryl derivatized mAb (SH-mAb) and the SH-mAb were separated from reactants using a Sephadex G-75 column under argon gas to prevent oxidation of sulfhydryl group to disulfide. The typical separation of SH-mAb is shown in Figure 2.3 in Chapter 2. Conjugation of SH-mAb to MPB-PE liposomes was done by mixing SH-mAb with liposomes overnight and mAb-conjugated liposomes were separated from free mAb by metrizamide gradient separation. Table 3.1 shows typical physical properties of antibody-conjugated PALA liposomes used for this study. Both the molar ratio of PDP group to protein (PDP : mAb) and sulfhydryl group to protein (-SH : mAb) determine the amount of attached mAb to each liposome in the final liposome product. The PDP : mAb ratio was calculated by the method of Carlsson et al. (Carlsson, Drevin et al. 1978). The absorbance (O.D.) of PDP-mAb solution was measured at 280 nm (A_{280}) after appropriate dilution. Another sample of the same material was treated with 50 µl of 50 mM DTT solution for 30 min at room temperature to produce SH-mAb and pyridine-2-thione at 1 : 1 molar ratio and the absorbance at 343 nm (A_{343}) was measured. As the production of SH-mAb and pyridine-2-thione was 1 : 1 molar ratio, the concentration of SH-mAb can be calculated from the concentration of pyridine-2-thione, which is

$$\text{Conc. of -SH moiety} = (A_{343}) / (8.08 \times 10^3) \quad \text{----------} \quad \text{(Eq. 2)}$$

where, 8.08×10^3 is the molar extinction coefficient of pyridine-2-thione at 343 nm.

As the 2-pyridyl disulfide structure of PDP-mAb has its own absorbance at 280 nm, an erroneously high protein concentration will be obtained when calculated on the basis of A_{280}. Therefore, it is necessary to correct the A_{280} by the following equation:

Corrected $A_{280} = A_{280} - A_{343} \times [(5.1 \times 10^3)/(8.08 \times 10^3)]$ --------- (Eq. 3)

where, 5.1×10^3 is the molar extinction coefficient of the 2-pyridyl disulfide group at 280 nm. It is also known that absorbance of 0.1 % (w/w) aqueous solution of gamma globulin at 280 nm is 1.38 and molecular weight of IgG is about 170,000 daltons (Sober 1968). Therefore, the exact concentration of IgG in SH-mAb is calculated from the following equation:

Conc. of mAb (mole / liter) = (corrected A_{280}) /(1.38 x 170,000) ----- (Eq. 4)

The ratio of PDP group per mAb can be calculated by the following equation:

PDP group : mAb = [(A_{343}) / (8.08×10^3)] / (Eq. 4) --------- (Eq. 5)

The number of -SH groups per protein molecule is important because it determines the degree of binding of antibody (or protein)-conjugated liposomes to a corresponding epitope of antigen on the surface of cells. In all of the experiments performed in this study the number of PDP groups per antibody molecule was between 7 and 9. Among the factors that affect this ratio were presumably the freshness of SPDP solution, the accuracy of setting up the ratio of mAb : SPDP, and slow but complete reaction of DTT with PDP-mAb.

It was also possible to calculate the actual number of mAb per liposome. In order to calculate this, the following assumptions were made. The thickness of lipid bilayer of the liposomes was 40 Å, the area occupied by each phospholipid was 70 $Å^2$, and the size of liposomes was 0.2 μm. Using these values, there are 1.8×10^{12} liposomes of 0.2 μm size in diameter per 1 μmole lipid. As the molecular weight of IgG is approximately 170,000 daltons, each mAb weighs 2.728×10^{-19} g. The IgG :

lipid ratio in g/mol can be converted to the number of mAb per liposome by multiplying by 1.98. Therefore, if the IgG : lipid ratio is 100 g/mol, the actual number of mAb molecules attached to one 0.2 μm liposome is 198.

The drug : lipid molar ratio (PALA : lipid) is also an important factor in the evaluation of a liposomal drug delivery system. Factors affecting this ratio include the concentration of the stock drug solution used for encapsulation, and the diameter and lamellarity of the liposomes. In this study the ratio was found to be between 0.2 and 0.4, which is similar to the average drug : lipid ratio of the same liposomes without monoclonal antibody attached.

Table 3.1. Selected physical properties of antibody-conjugated PALA liposomes used for *in vitro* study.

mAb-IL (immunoliposomes)	PALA : Lipid (mol / mol)	IgG : Lipid (g / mol)	Number of IgG molecule per liposome
454C11-IL	1.03/4.41 = 0.23	624/4.41 = 142	281
520 C9-IL	1.32/5.91 = 0.22	704/5.91 = 119	236
741F8-IL	1.18/6.52 = 0.18	992/6.52 = 153	303
260 F9-IL	1.03/5.73 = 0.18	860/5.73 = 151	299
317G5-IL	0.90/4.82 = 0.19	668/0.19 = 139	275
454A12-IL	0.62/3.99 = 0.16	636/3.99 = 158	313
2G3-IL	0.62/4.21 = 0.15	576/4.21 = 137	271
113F1-IL	0.76/4.61 = 0.17	616/4.61 = 134	265

Growth Inhibition of Cells By Modified Liposomes

A) Growth Inhibition of Protein A-PALA Liposomes on L929 Cells

The murine fibroblast L929 was used to test the cytotoxic effect of PALA in Protein A conjugated liposomes with monoclonal antibody 11-4.1 which binds specifically to these cells via an interaction with $H2K^k$, one of the antigens of the mouse major histocompatibility complex. The L929 cells were preincubated with 11-4.1 antibody for 30 min and exposed to free PALA or Protein A-conjugated PALA liposomes for 48 hr continuously. At the end of the experiments dead cells were aspirated out and only live cells were counted, and the 50% growth inhibition concentration (or IC_{50}) was determined based on Equation 1 in Chapter 2. The results are shown in Figure 3.1 and Table 3.2.

The IC_{50} of free PALA and PC : Chol : MPB-PE (20:20:1 molar ratio)-PALA liposomes (no Protein A conjugated) on L929 were shown to be 18 μM and 0.2 μM, respectively. When 40 μg of 11-4.1 mAb was preincubated with the L929 cells for 30 min prior to the addition of either free PALA or PC :Chol : MPB-PE-PALA liposomes or Protein A-conjugated PALA liposomes, the IC_{50} for Protein A-conjugated PALA liposomes was 0.04 μM but the IC_{50} of either free PALA or PC : Chol-PALA liposomes remained unchanged. Therefore, Protein A-conjugated PALA liposomes were over 400-fold more potent than free PALA and 5-fold more potent than non-targeted PC : Chol : MPB-PE-PALA liposomes in the cytotoxicity of L929 cells *in vitro* when directed with 11-4.1 mAb. The preincubation of various amounts of 11-4.1 mAb (between 20 and 40 μg) with L929 cells did not affect the degree of increase of potency of Protein A-conjugated PALA liposomes. There was no substantial lipid toxicity on

L929 up to 500 μM lipid concentration which was much higher than the lipid concentration used for a growth inhibition assay.

It is worthy of note that the cytotoxicity of PALA for L929 cells was increased by over 400 times by encapsulating in Protein A-conjugated liposomes targeted by the use of 11-4.1 antibody. PALA has previously exhibited an increase in cytotoxicity for L929 cells of up to 50 times, when encapsulated in DSPG liposomes (Heath and Brown 1989-1990). By previously established criteria (Heath et al. 1985), this shows that PALA is a liposome-dependent drug. However, not all drugs, whose potency is increased in negatively charged liposomes, will also be more potent in Protein A-conjugated, antibody-directed liposomes. In this regard, this finding here,which proved for the first time the increase of cytotoxicity of PALA in Protein A-conjugated liposomes, is very significant, and suggests the possibility of targeting PALA in antibody-conjugated liposomes to a specific tissue in the body.

Figure 3.1. Growth inhibition curve of Protein A-conjugated PALA liposomes for L929 cells *in vitro*.

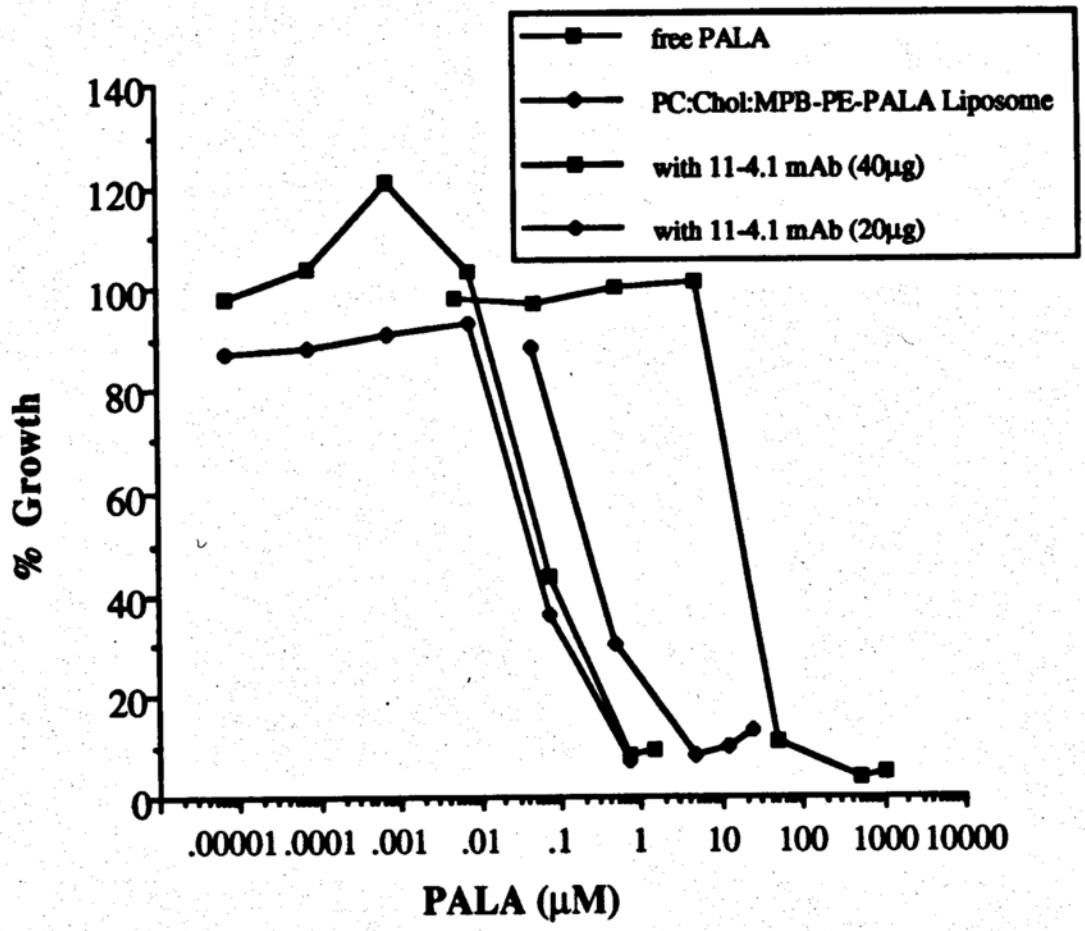

free PALA
PC:Chol:MPB-PE-PALA Liposome
with 11-4.1 mAb (40µg)
with 11-4.1 mAb (20µg)
% Growth
140
120
100
80
60
40
20
0
.00001 .0001 .001 .01 .1 1 10 100 1000 10000
PALA (µM)

Table 3.2. Potency of PALA in 11-4.1 monoclonal antibody directed Protein A-conjugated liposomes in L929 cells *in vitro*.

Liposome	IC_{50} (μM)[a]	Potency Increase Factor (PIF)[b]
free PALA	18	1
PC : Chol-PALA	15	1.2
MPB-PE : PC : Chol-PALA	0.2	90
Protein A-MPB-PE : PC : Chol-PALA	0.041 (20 μg of mAb)[c]	440
	0.044 (40 μg of mAb)	410

a ; mean IC_{50} values from 5 experiments

b ; PIF = (IC_{50} of free drug) / (IC_{50} of encapsulated drug)

c ; 20 μg of 11-4.1 mAb was pre-incubated for 30 min at 4°C before drug treatment

B) Growth Inhibition of Antibody-PALA Liposomes on HEY 1B Cells

Tumor specific antibodies were directly conjugated to PALA-containing liposomes and tested using HEY 1B human ovarian cancer cells *in vitro*. The c-*erb*B2 oncogene product and transferrin receptor were used as tumor markers and monoclonal antibodies against these tumor markers were conjugated to PALA liposomes. Three monoclonal antibodies (454C11, 520C9, and 741F8) were specific for c-*erb*B2 oncogene product, another three monoclonal antibodies (260F9, 317G5, and 454A12) were specific for human transferrin receptors, and two others (2G3 and 113F1) were specific for a high molecular weight mucin and a series of 40-200 kDa protein respectively. The HEY1B cells are known to express abundant c-*erb*B2 oncogene product, and transferrin receptors. All of the growth inhibition assays were of 72hr duration and at the end of the period cells were counted, and the IC_{50} was calculated.

Figures 3.2-3.4 show the typical growth inhibition curves for HEY 1B cells *in vitro*, from which the IC_{50} value is determined. The 50% inhibitory concentration (or IC_{50}) of free PALA on HEY1B cells was 11.5 ± 4.4 μM, whereas the IC_{50} of 454C11-PALA liposome was 0.6 ± 0.3 μM and the IC_{50} of 454A12-PALA liposome was 0.2 ± 0.06 μM showing about 20-fold and 60-fold increases in their cytotoxicity, respectively, when compared to free PALA. The overall order of potency was as follows; 454A12 (0.2 μM) > 454C11 (0.6 μM) > 317G5 (1.4 μM) > 260F9 (1.42 μM) > 741F8 (1.6 μM) > 520C9 (1.7 μM) > 2G3 (2.4 μM) > 113F1 (2.6 μM). The PALA liposomes composed of PC : Chol : MPB-PE (20 : 10 : 1 molar ratio), but lacking antibody, did not show a significant growth inhibitory effect on this cell line. Lipid toxicity was not seen at lipid concentrations as high as 1 mM.

It is important to note that the increase in cytotoxicity of PALA in antibody-conjugated liposomes was caused by the targeting of PALA containing antibody-conjugated liposomes and the potency increase of up to 60-fold was a significant increase. This also shows that the monoclonal antibodies retain their binding activities after conjugation to the liposomes, which also validates the use of SPDP as a heterobifunctional conjugatiing agent. It also seems that the increased potency of PALA in antibody-conjugated liposomes is antibody-dependent and antibody-specific.

From the HEY 1B study, among the monoclonal antibodies tested, anti-c-*erb*B2 antibody 454C11 and anti-transferrin receptor antibody 454A12 were the best in delivering PALA to the target cells when conjugated to liposomes. As these two antibodies are clinically relevant to the immunotherapy of human ovarian and breast cancer, it is expected that this result could serve as a basis for the possible immunotherapy of human ovarian and breast cancer using antibody-conjugated PALA-liposomes in the future.

Figure 3.2. Growth inhibition curve of HEY 1B cells *in vitro* by free PALA, 2G3-conjugated PALA-liposomes, and 113F1-conjugated PALA-liposomes.

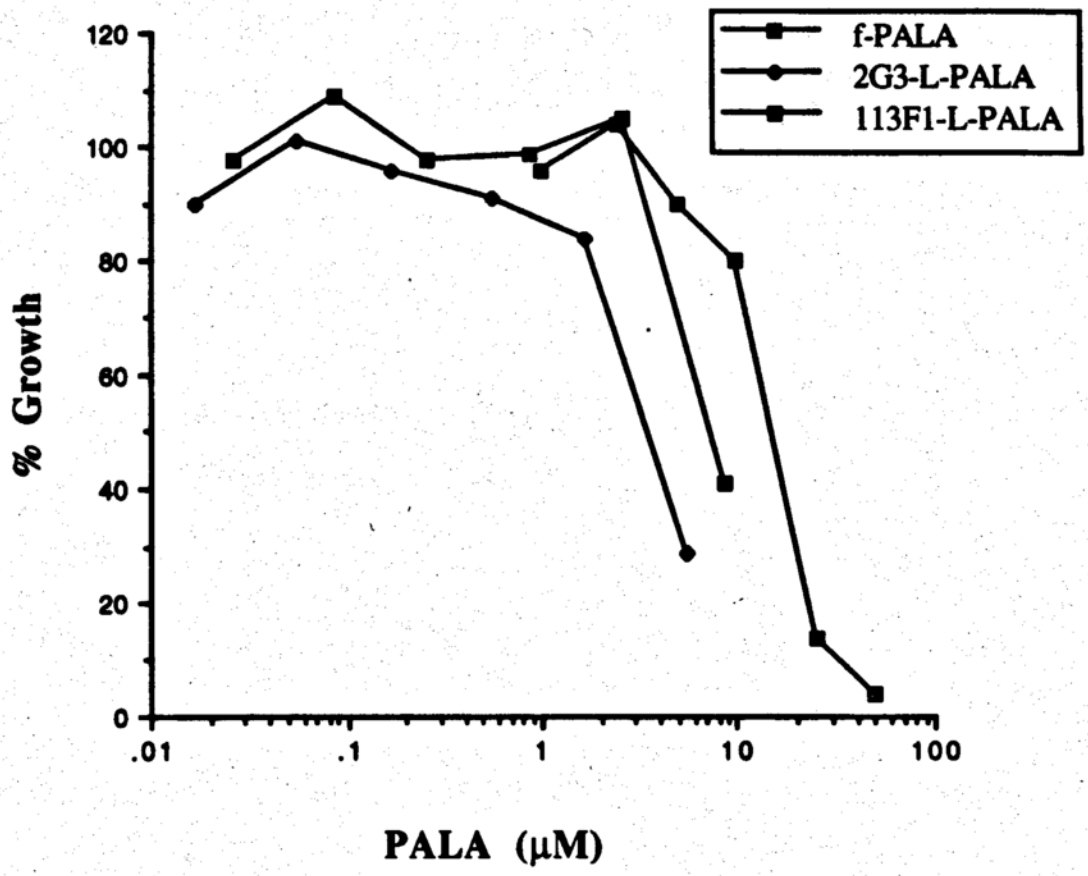

f-PALA
2G3-L-PALA
113F1-L-PALA
% Growth
120
100
80
60
40
20
0
.01
.1
1
10
100
PALA (μM)

Figure 3.3 Growth inhibition curve of HEY 1B cells *in vitro* by anti-c-*erb*B2 antibody-conjugated PALA liposomes.

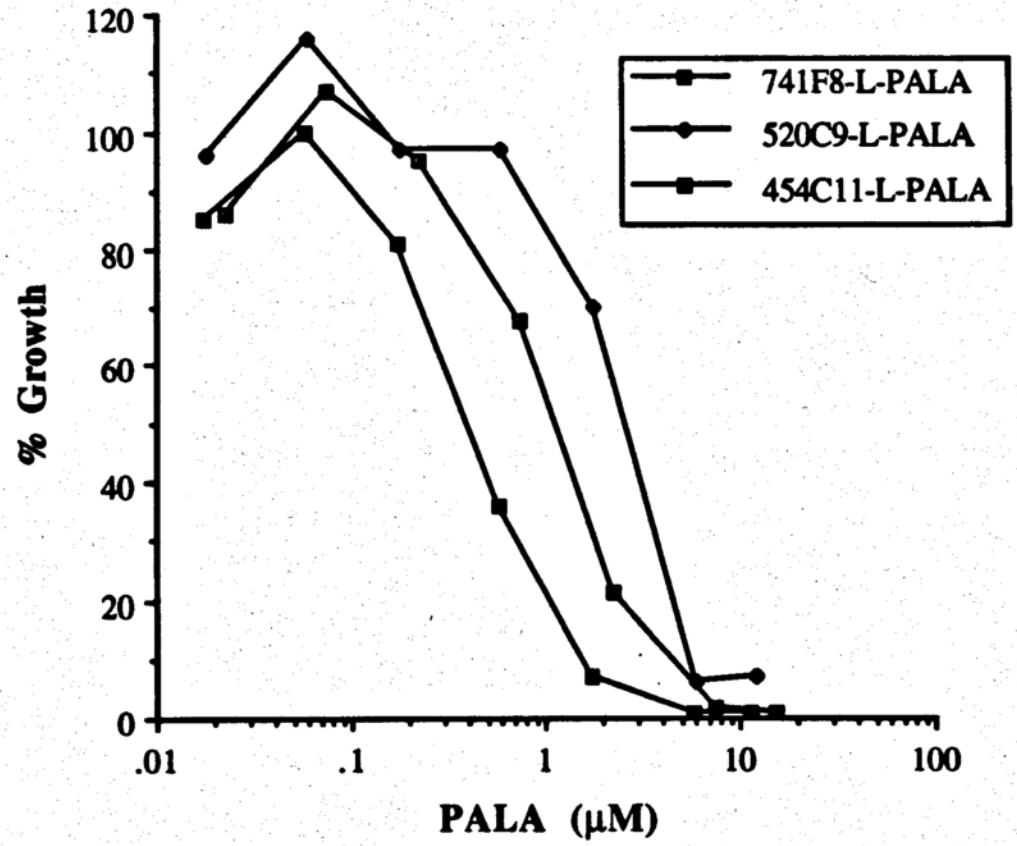
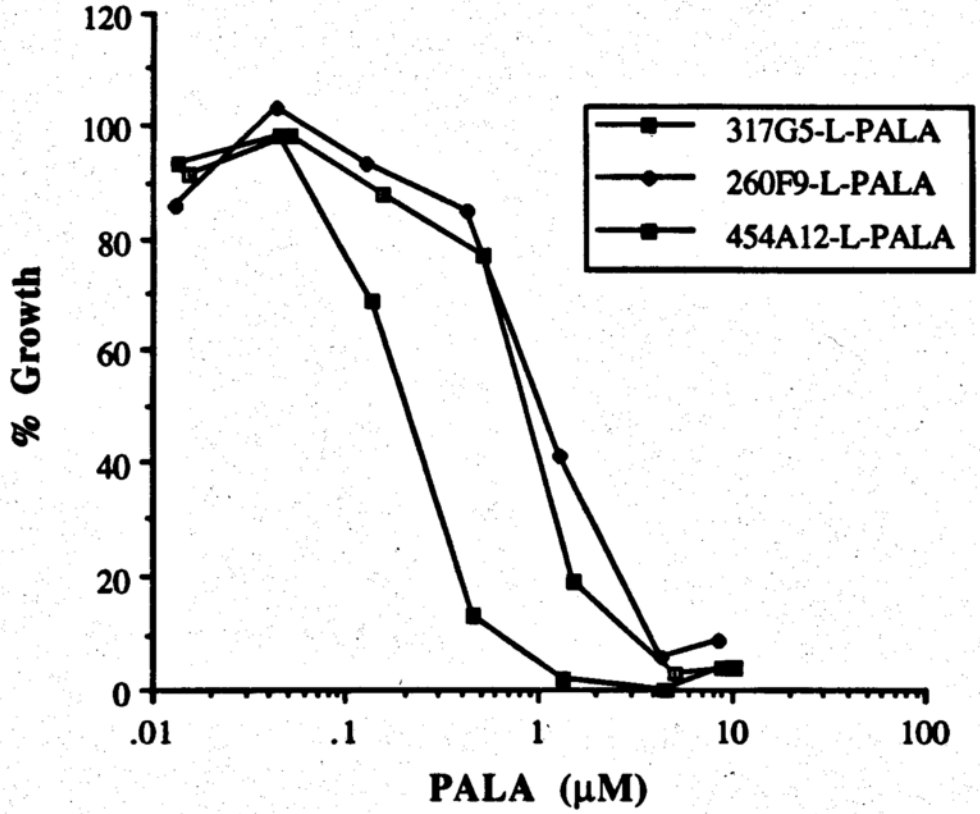

120
100
80
60
40
20
0
% Growth
.01
.1
1
10
100
PALA (μM)
741F8-L-PALA
520C9-L-PALA
454C11-L-PALA

Figure 3.4. Growth inhibition curve of HEY 1B cells *in vitro* by anti-transferrin receptor antibody-conjugated PALA-liposomes.


120
100
80
60
40
20
0
% Growth
.01
.1
1
10
100
PALA (μM)
317G5-L-PALA
260F9-L-PALA
454A12-L-PALA

C) Growth Inhibition of Antibody-PALA Liposomes on SKOV-3 Cells

Tumor-specific antibody-conjugated PALA liposomes were tested using another human ovarian cancer cell line, SKOV-3. The experimental protocols were the same as were used with HEY 1B cells. The growth rate of SKOV-3 cells was much slower than that of HEY 1B cells, which made it more difficult to perform the growth inhibition assay.

The overall growth inhibitory pattern of mAb-PALA liposomes on SKOV-3 cells was similar to that on HEY 1B cells as shown in Figures 3.5-3.7. However, SKOV-3 cells were less sensitive to free PALA than HEY1B cells. The results are shown in Table 3.4. The IC_{50} for free PALA was 50 μM and for 454C11-PALA liposomes, it was 0.9 μM, showing a 55-fold increase of PALA potency by 454C11-PALA liposomes. The IC_{50} of 2G3-PALA liposomes was about 0.8 μM, which is about 60-fold more potent than free drug. The overall order of potency was as follows; 2G3 (~0.5 μM) > 454C11 (0.9 μM) > 454A12 (3.2 μM) > 520C9 (4.9 μM) and 260F9 (4.9 μM) > 741F8 (5.8 μM) > 317G5 (6.8 μM) > 113F1 (23 μM).

The results from SKOV-3 cells were found to be more variable those that from HEY 1B cells, which seems to be connected with slow growth rate for SKOV-3 cells. Previous experience with other slow growing cells also showed a greater variation in the IC_{50} values. Therefore, data analysis with SKOV-3 cells was done with the IC_{50} values obtained in the two experiments that were the most reproducible, even though 7 experiments were performed in total. Both 2G3, a high molecular weight mucin, and 454C11, an anti-*erb*B2 antibody, were the best of those tested for targeted delivery of PALA in antibody-conjugated liposomes to SKOV-3 cells *in vitro*. The results from both HEY 1B and SKOV-3 experiments suggest that anti-*erb*B-2 antibody and anti-

Figure 3.5. Growth inhibition curve of SKOV-3 cells *in vitro* by free PALA, 2G3-conjugated PALA-liposomes, and 113F1-conjugated PALA-liposomes.

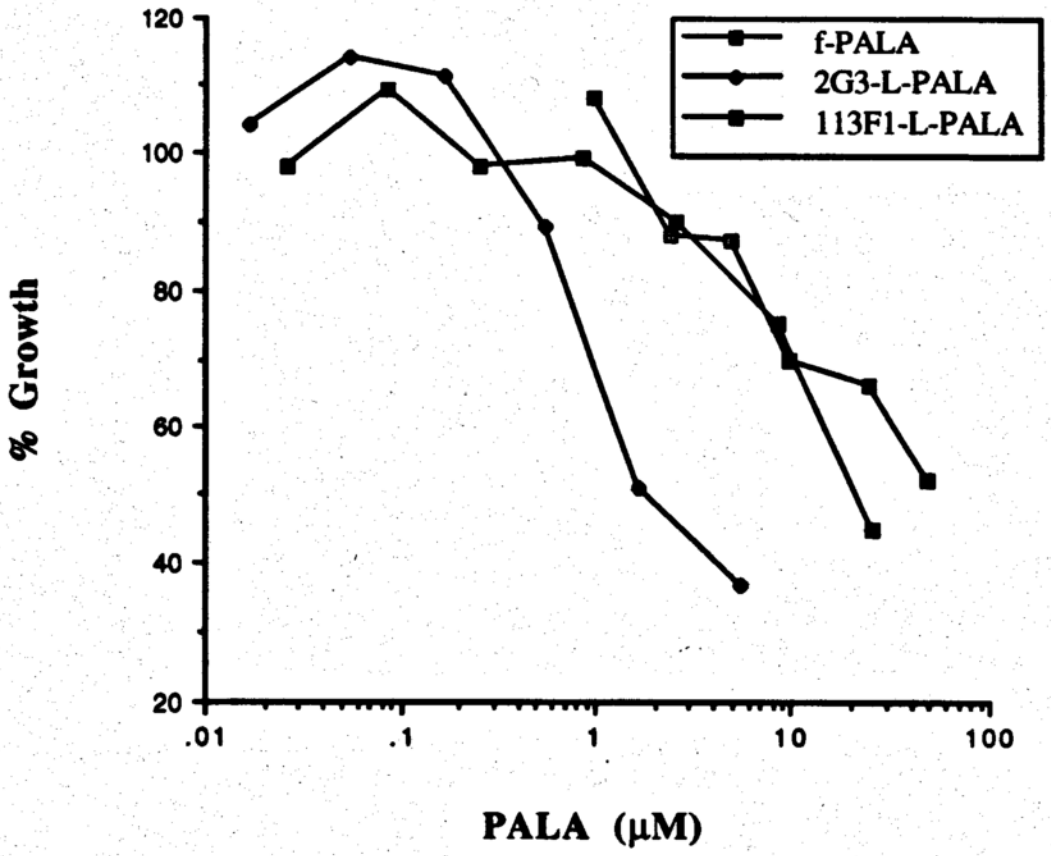

f-PALA
2G3-L-PALA
113F1-L-PALA
% Growth
120
100
80
60
40
20
.01
.1
1
10
100
PALA (μM)

Figure 3.6. Growth inhibition curve of SKOV-3 cells *in vitro* by anti-c-*erb*B2 antibody-conjugated PALA-liposomes.

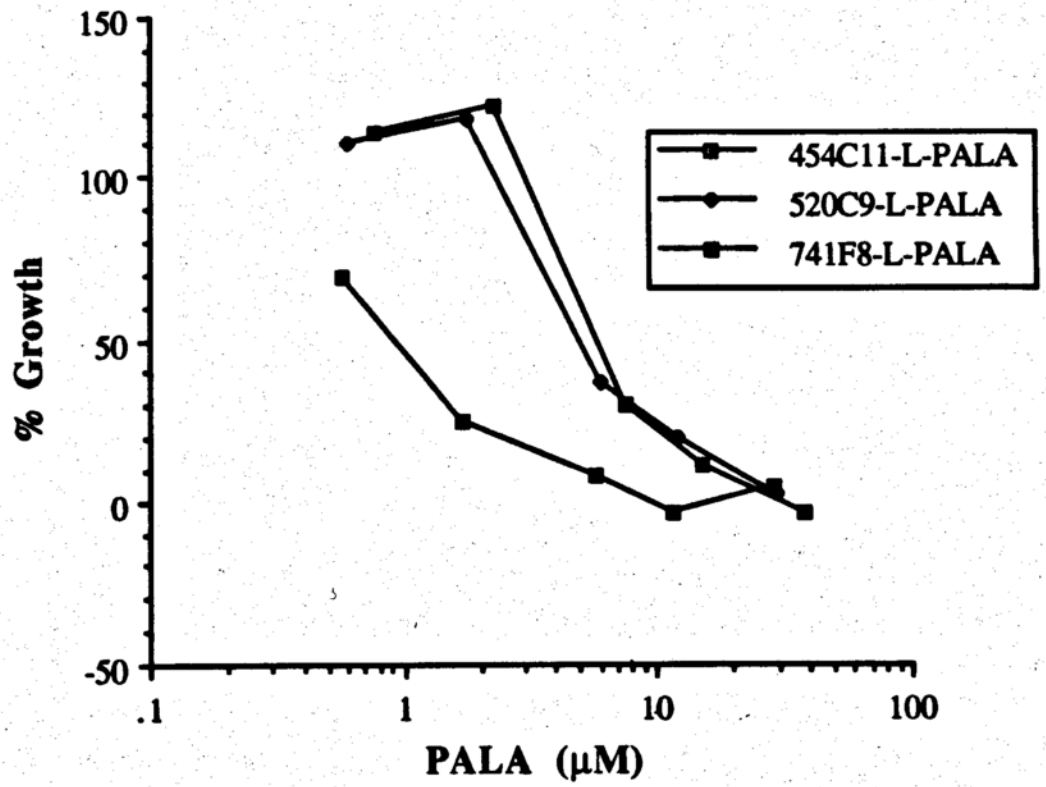

150
100
50
0
-50
% Growth
.1
1
10
100
PALA (μM)
454C11-L-PALA
520C9-L-PALA
741F8-L-PALA

Figure 3.7. Growth inhibition curve of SKOV-3 cells *in vitro* by anti-transferrin receptor antibody-conjugated PALA-liposomes.

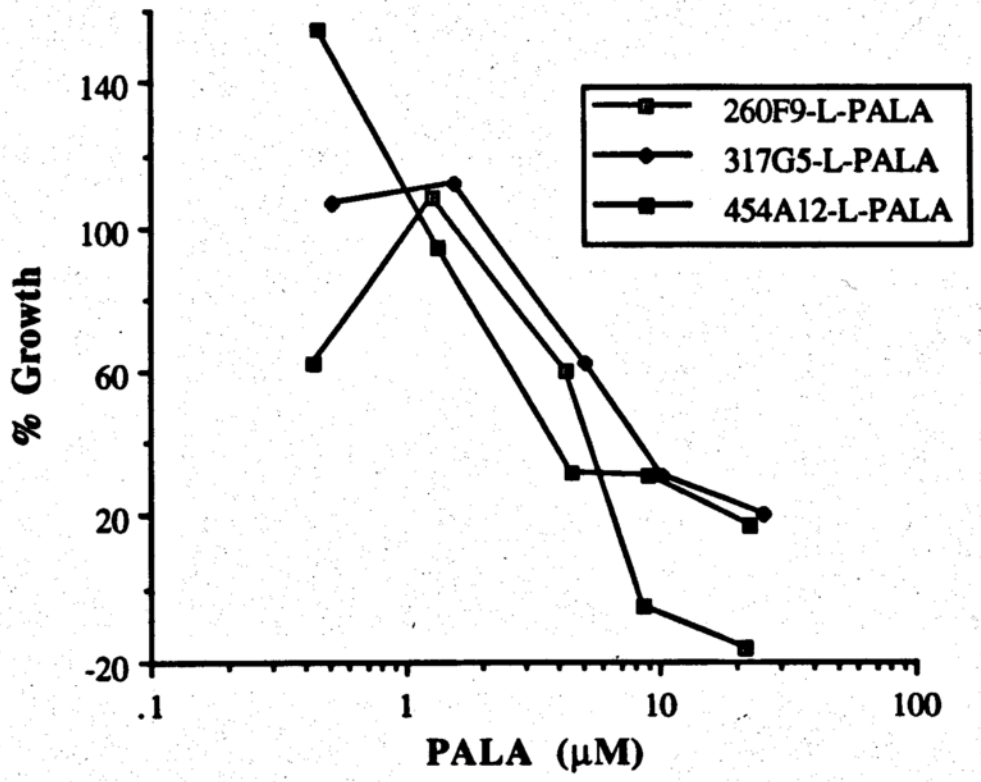

140
100
60
20
-20
% Growth
.1
1
10
100
PALA (μM)
260F9-L-PALA
317G5-L-PALA
454A12-L-PALA

Table 3.3. Potency of monoclonal antibody-conjugated PALA-liposomes in two human ovarian cancer cell lines *in vitro*.

mAb-conjugated-PALA immunoliposomes	IC_{50} (μM) [a] (72 hr continuous exposure of cells to drug) [b]	
	HEY1B	SKOV-3
free PALA	11.5 ± 4.4 [c]	50[d] (>50, 52[e])
454C11-PALA-IL	0.6 ± 0.3	0.9 (0.7, 1.5)
520C9-PALA-IL	1.7 ± 1.3	4.9 (12, > 6.0)
741F8-PALA-IL	1.6 ± 0.8	5.8 (7.9, n/d[f])
260F9-PALA-IL	1.9 ± 1.4	4.9 (> 4.3, 8.5)
317G5-PALA-IL	1.4 ± 1.0	6.8 (> 5.2, 43)
454A12-PALA-IL	0.2 ± 0.06	3.2 (> 4.5, 23)
2G3-PALA-IL	2.4 ± 1.2	0.8 (1.8, 0.5)
113F1-PALA-IL	2.6 ± 1.9	23 (n/d)

[a] ; 50% growth inhibitory concentration of PALA
[b] ; 2 x 10^4 cells / ml in triplicates
[c] ; mean ± s.d. of thirteen experiments
[d] ; typical value from two experiments
[e] ; values from different experiments
[f] ; not detected

Leakage of Drug from antibody-conjugated PALA liposomes in storage

The physical stability of liposomes during the storage significantly affects the potency of encapsulated drug. It is more important when a liposome-dependent drug is being used, because the intact structure of the liposomes is crucial in efficient delivery of encapsulated drug to the cells. It has been shown that the stability of liposomes on storage, or the leakage of drug from liposomes, is mainly a function of the charge of the lipids used, the degree of packing of the lipid bilayers, and the size of the liposomes. One of the *in vitro* testing methods to determine the degree of drug leakage from liposomes is to compare the IC_{50} values of the same liposome batch of different ages.

The potency of antibody-conjugated PALA-liposomes was determined over a month period using HEY 1B cells in order to examine the extent of drug leakage. The results are shown in Table 3.4. The data shows that antibody-conjugated PALA-liposomes leaked substantially over this relatively short time period. After a month of storage at 4°C, the IC_{50} increased 2- to 3-fold for most of the liposome formulations when tested in HEY 1B cells. Assuming that the potency of liposome-encapsulated PALA is constant with respect to drug : lipid ratio, the % leakage can be calculated by [IC_{50} (old) - IC_{50} (fresh)] / IC_{50} (old) x 100. From this calculation, about 50-67 % leakage of PALA occurred during one month storage.

Table 3.4. Change in the potency of antibody-conjugated PALA-liposomes for growth inhibition of HEY 1B cells during one month storage at 4°C.

Antibody-conjugated PALA-liposomes	IC_{50} (μM) (fresh)	IC_{50} (μM) (1 month old)
free PALA	13	15
454C11-PALA	0.39	1.1
520C9-PALA	2.60	> 5.98
741F8-PALA	1.15	3.0
260F9-PALA	1.05	2.6
317G5-PALA	0.86	3.0
454A12-PALA	0.2	0.25
2G3-PALA	1.2	3.5
113F1-PALA	2.9	3.0

Chapter 4. *IN VIVO* RESULTS

Growth Inhibition of PALA-Liposomes on C-26 Cells

A) 72hr Growth Inhibition Assay with the C-26 Cells

Before initiating the therapy of C-26 murine colon carcinoma-bearing mice with PALA-liposomes, free PALA and PALA-liposomes were tested for their cytotoxicity using C-26 cells *in vitro*. With a continuous 72hr exposure of C-26 cells to drug treatment, the sensitivity of C-26 cells to the PALA formulations was studied. The liposome formulations studied were DSPG-PALA liposomes, DSPC-PALA liposomes, and PEG(1900)-DSPE : DSPC-PALA. Some of the selected physical properties of these liposome formulations are listed in Table 4.1.

The IC_{50} for free PALA, DSPG-PALA, DSPC-PALA, and PEG-DSPE-PALA liposomes were shown to be 5.1 μM, 0.09 μM, 11.8 μM, and 10.7 μM, respectively as shown in Table 4.2. Negatively charged DSPG-PALA was 60-fold more potent than free drug, but both DSPC-PALA and PEG-DSPE-PALA were 50% less potent than free drug. This result was consistent with previous findings from other studies where negatively charged liposomes delivered PALA to CV1-P and J-774 cells *in vitro* more efficiently than neutral liposome (Heath and Brown 1989; Sharma 1993). PEG-DSPE-liposomes, which are composed of DSPC:Chol:PEG-DSPE (20:10:1 molar ratio), have a similar composition to DSPC-PALA liposomes (DSPC:Chol 20:10 molar ratio) except that they contain 5 mol% PEG-DSPE as a sterically stabilizing composition, showing slightly negative charge on its surface. Even though PEG-DSPE-PALA-liposomes are negatively charged, they are as ineffective as DSPC-PALA liposomes in delivering PALA to C-26 cells *in vitro*, and less effective than free PALA. From this experiment, it is expected that both DSPC- and PEG-DSPE-PALA liposomes will exhibit their drug potency *in vivo* only through controlled release, and not from targeted delivery.

Table 4.1. Selected physical properties of PALA liposomes used for the *in vivo* study using C-26 murine colon carcinoma cells.

Liposomes	PALA concentration (mg/ml) [a]	PALA encapsulation (%)	Lipid concentration (mM) [b]	drug : lipid ratio	Liposome size in diameter (μm) [c]
DSPC-PALA	3.3	16	17.2	0.55	215 ± 112
DSPG-PALA	2.1	11	20.6	0.3	132 ± 62
PEG-DSPE-PALA	1.8	7	18.8	0.28	181 ± 57

[a] ; determined indirectly from co-encapsulated calcein
[b] ; determined by phosphorus assay
[c] ; determined by laser light scattering method

Experiments were also undertaken to determine the inherent growth inhibitory effect of the liposome carrier itself without drug encapsulation. It was found that there was no significant lipid toxicity up to 2 mM lipid concentrationin a cell well, which is much higher than the concentration used for a growth inhibition experiment.

B) 1hr Wash Experiment with C-26 Cells In Vitro

Continuous exposure of cells to a therapeutic agent is one of the most frequently used methods to test the efficacy of this agent *in vitro*, but this condition does not simulate the real situation which likely prevails *in vivo*. The PALA, when injected intraperitoneally, is cleared from the injection site and eventually from the body. This clearance process reduces the exposure time of tumor cells to the drug within the body. In order to mimic this kind of *in vivo* situation in a cell culture system, the potency of PALA-liposomes in 1hr short-term exposure of cells to drug solution was examined.

As shown in Table 4.4, the IC_{50} of free PALA, DSPG-PALA, DSPC-PALA, and PEG-DSPE-PALA liposomes for 1 hr exposure were 823 μM, 14.3 μM, 2040 μM, and 1400 μM, respectively. All the IC_{50} values were increased by over 150 times, compared to 72 hr exposure, but the rank-order of efficacy remained the same and the potency increase factor (Free drug IC_{50} / Encapsulated drug IC_{50}) remained almost the same as in the 72 hr exposure experiment. There was no substantial lipid toxicity from empty liposome up to 2 mM lipid concentration. This is similar to a previous observation using short term exposure in other cell lines (Heath and Brown 1989).

Table 4.2. Potency of PALA in liposomes in different exposure time length using C-26 murine colon carcinoma cells *in vitro*.

Exposure time	IC_{50} on C-26 cells (μM)			
	free PALA	DSPG-PALA liposomes	DSPC-PALA liposomes	PEG-DSPE-PALA liposomes
1hr wash[a]	823	14.3	2040	1400
PIF[b]	1	58	0.4	0.6
72hr continuous[c]	5.1	0.09	11.8	10.7
PIF	1	57	0.4	0.5

a; cells were exposed to drug for 1hr and washed three times with buffer, followed by incubation for another 71hr for growth.

b; PIF is a potency increase factor (= IC_{50} of free PALA / IC_{50} of encapsulated PALA)

c; cells were exposed to drug for 72hr continuously

Determination of the MTD

In order to examine the pharmacological efficacy of PALA in tumor-bearing mice, the maximum tolerated dose (or MTD) of PALA in healthy mice should first be determined. Healthy BALB/c mice were used to determine the MTD of PALA for a single i.p. injection over a three-week period by weighing individual mice once a day. The % weight change on day n for individual mice was calculated as follows:

$$\%\ \text{weight change on day n} = \frac{(\text{weight on day n} - \text{weight on day 0})}{(\text{weight on day 0})} \times 100 \quad \text{--- (Eq. 7)}$$

As shown in Figures 4.1-4.3, the MTD was taken to be either the highest dose given or the highest dose that at no time caused a weight loss greater than 15% of the original weight. The MTD of free PALA, DSPC-PALA, and DSPG-PALA in BALB/c mice were found to be 750 mg/kg, >150 mg/kg, and >150 mg/kg, respectively, for a single i.p. injection of drug. As none of the DSPC-PALA and DSPG-PALA doses injected showed more than 3 % weight loss in this study, the MTD for these two formulations was higher than 150 mg/kg. This dose of PALA was the largest possible for a liposome formulation, because the maximum possible injection volume was 1.4 ml.

Figure 4.1. Determination of the maximum tolerated dose (MTD) for a single i.p. injection of free PALA using healthy BALB/c mice.

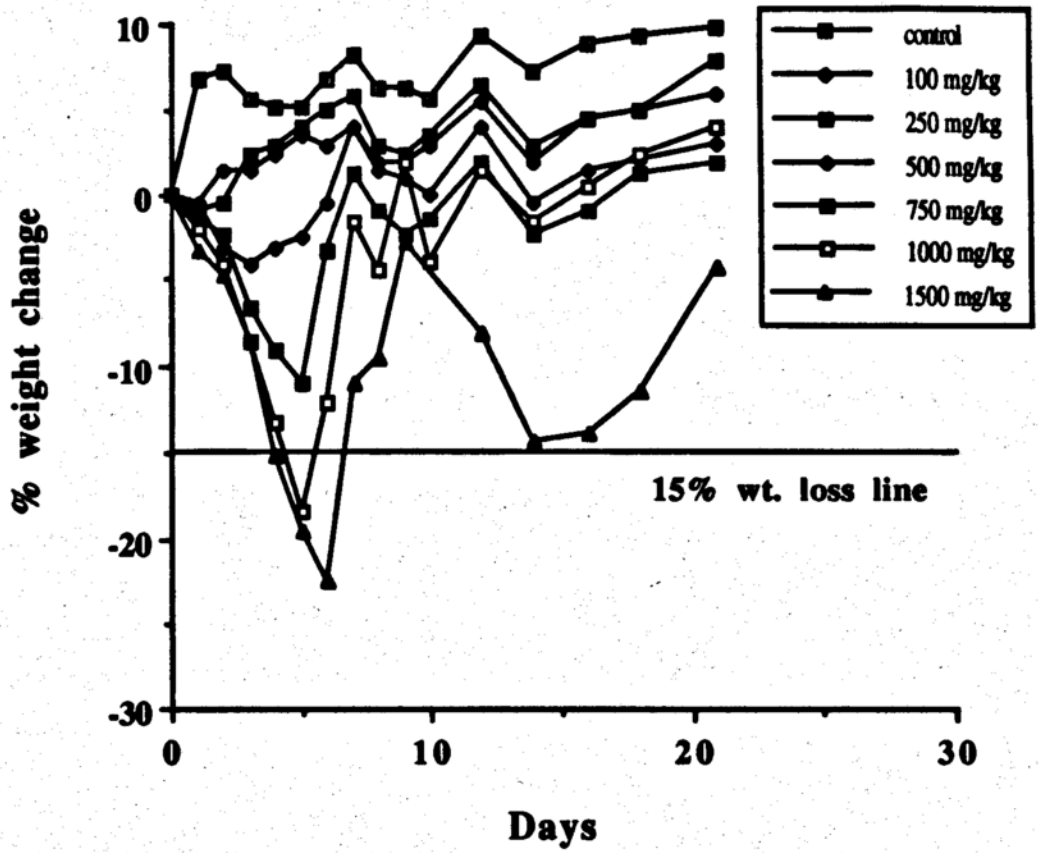

10
0
-10
-20
-30
0
10
20
30
% weight change
Days
15% wt. loss line
control
100 mg/kg
250 mg/kg
500 mg/kg
750 mg/kg
1000 mg/kg
1500 mg/kg

Figure 4.2. Determination of the maximum tolerated dose (MTD) for a single i.p. injection of DSPC-PALA liposomes using healthy BALB/c mice.

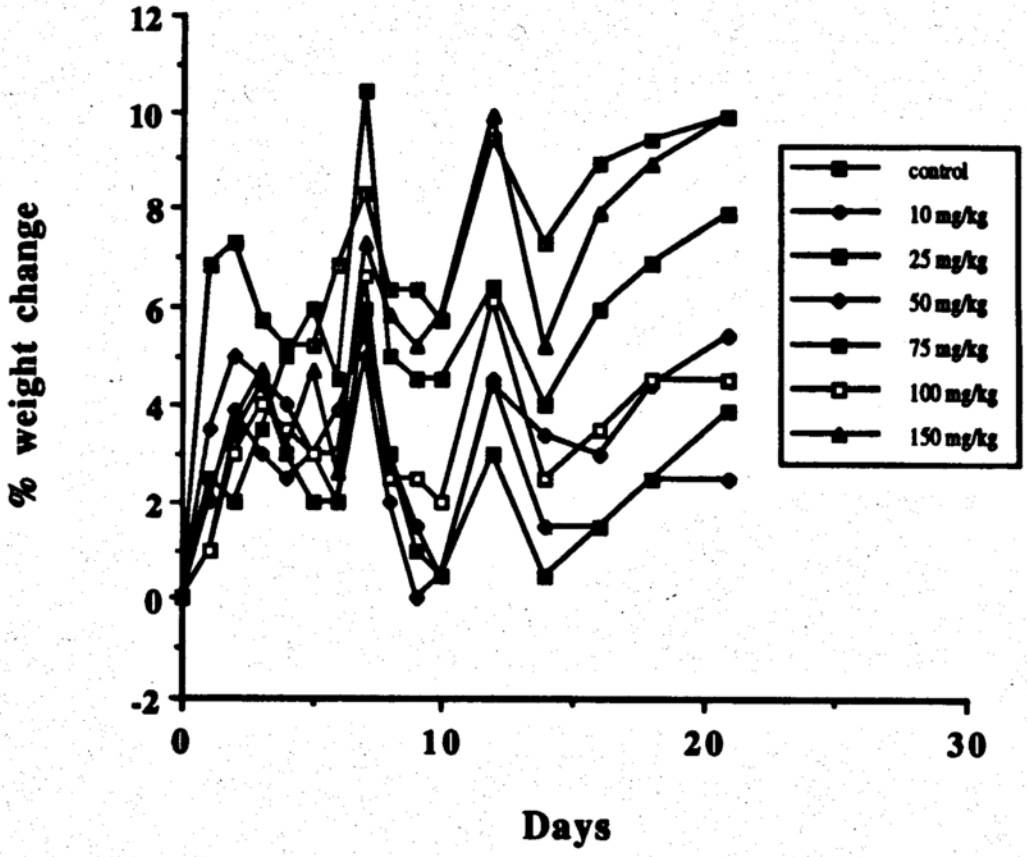
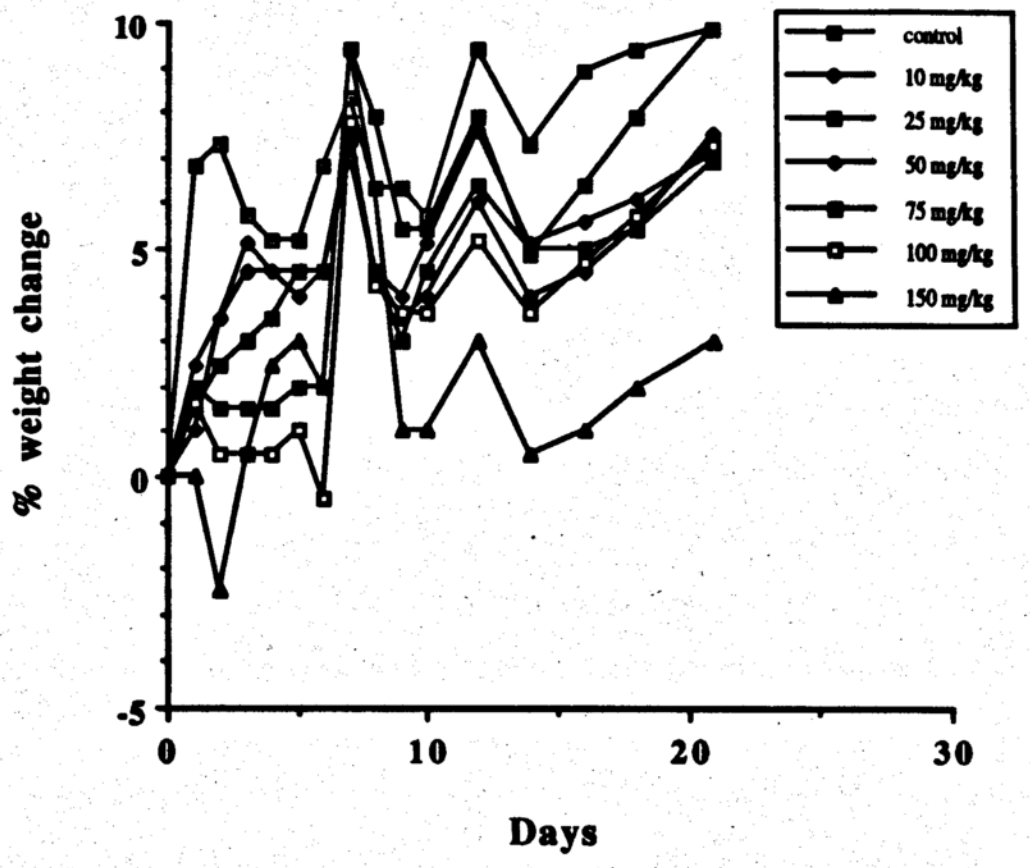

12
10
8
6
4
2
0
-2
0
10
20
30
% weight change
Days
control
10 mg/kg
25 mg/kg
50 mg/kg
75 mg/kg
100 mg/kg
150 mg/kg

Figure 4.3. Determination of the maximum tolerated dose (MTD) for a single i.p. injection of DSPG-PALA liposomes using healthy BALB/c mice.


10
5
0
-5
0
10
20
30
% weight change
Days
control
10 mg/kg
25 mg/kg
50 mg/kg
75 mg/kg
100 mg/kg
150 mg/kg

8-Day Therapy of C-26 Tumor Bearing Mice

Considering that the average life span of BALB/c mice is 1-3 years and that of human is 70 years, 8 days for mice is almost equivalent to one year for humans and 1 day for mice is almost equivalent to two months in humans. It is not uncommon for cancer patients to start chemotherapy only after tumor has been well established, which in many cases takes one year or longer. In order to simulate such a time course of tumor in human, therapy for tumor mice was initiated 8 days after tumor implantation.

Eight days after C-26 tumor cells were implanted into BALB/c mice, intraperitoneal (i.p.) therapy was initiated using free or liposome-encapsulated PALA formulations. The dose of PALA to be injected was chosen based on the result of MTD experiments. Specifically, the doses used were the MTD, and one half and one third or one fifth of the MTD. For free PALA, 750 mg/kg, 375 mg/kg, and 150 mg/kg were chosen. For DSPC-PALA and DSPG-PALA liposomes, 150 mg/kg, 75 mg/kg, and 50 mg/kg were chosen for each formulation. Injection volume was kept at 1.4 ml throughout the experiment to minimize the variation due to the volume change.

As shown in Figures 4.4-4.6 and Table 4.3, treatment with DSPC-PALA at 150 mg/kg resulted in a significant increase in median survival time (MST) of 56% over the control group which received MES/HEPES buffer alone. However, none of the free PALA and DSPG-PALA liposomes doses caused a statistically significant increase in median survival time (or MST) over control group at the 95% confidence level. At 750 mg/kg dose, free PALA caused a marginally significant improvement in MST by 34%, but both 375 mg/kg and 150 mg/kg doses of free PALA caused only a 2% and a 4% increase in MST, respectively. The DSPG-PALA liposomes did not cause a significant increase in MST either.

It seems likely that the i.p. injected C-26 tumor may have advanced too much to respond to a single injection of PALA in any of the formulations used, though DSPC-PALA liposomes at a 150 mg/kg dose caused a slight increase in MST over control group. It is probable that the DSPC-PALA liposomes are taken up by tumor cells much more than any other PALA formulations, because they remain at the tumor sites longer. They may also deliver drugs in a controlled release, because the *in vitro* cytotoxicity of DSPC-PALA liposomes on C-26 cells was even less than free PALA. It is not clearly known currently why DSPG-PALA liposomes did not show the same effect as DSPC-PALA liposomes but it is postulated that the lower stability of DSPG-PALA liposomes in body fluid and the shorter residence time in body cavity might have affected the uptake of PALA by tumor cells.

Figure 4.4. Survival of C-26 tumor bearing mice by a single i.p. injection of free PALA 8 days after C-26 tumor implantation.

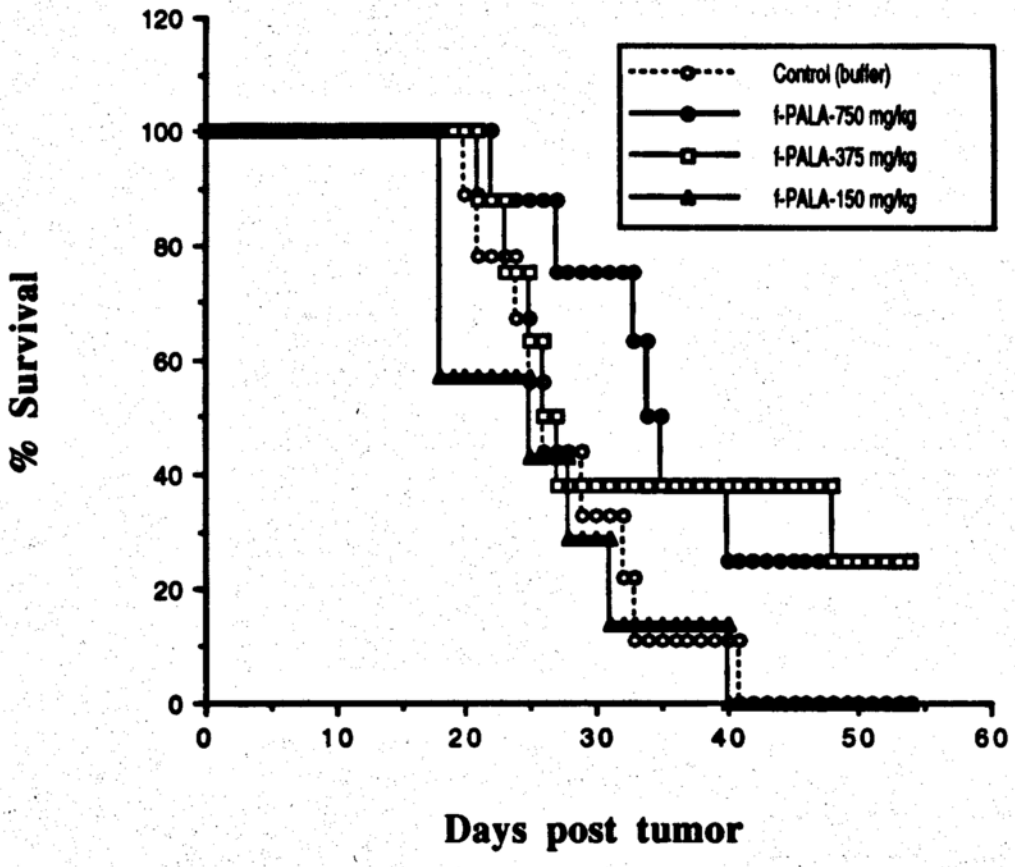

Control (buffer)
f-PALA-750 mg/kg
f-PALA-375 mg/kg
f-PALA-150 mg/kg
% Survival
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Days post tumor

Figure 4.5. Survival of C-26 tumor bearing mice by a single i.p. injection of DSPC-PALA liposomes 8 days after tumor implantation.

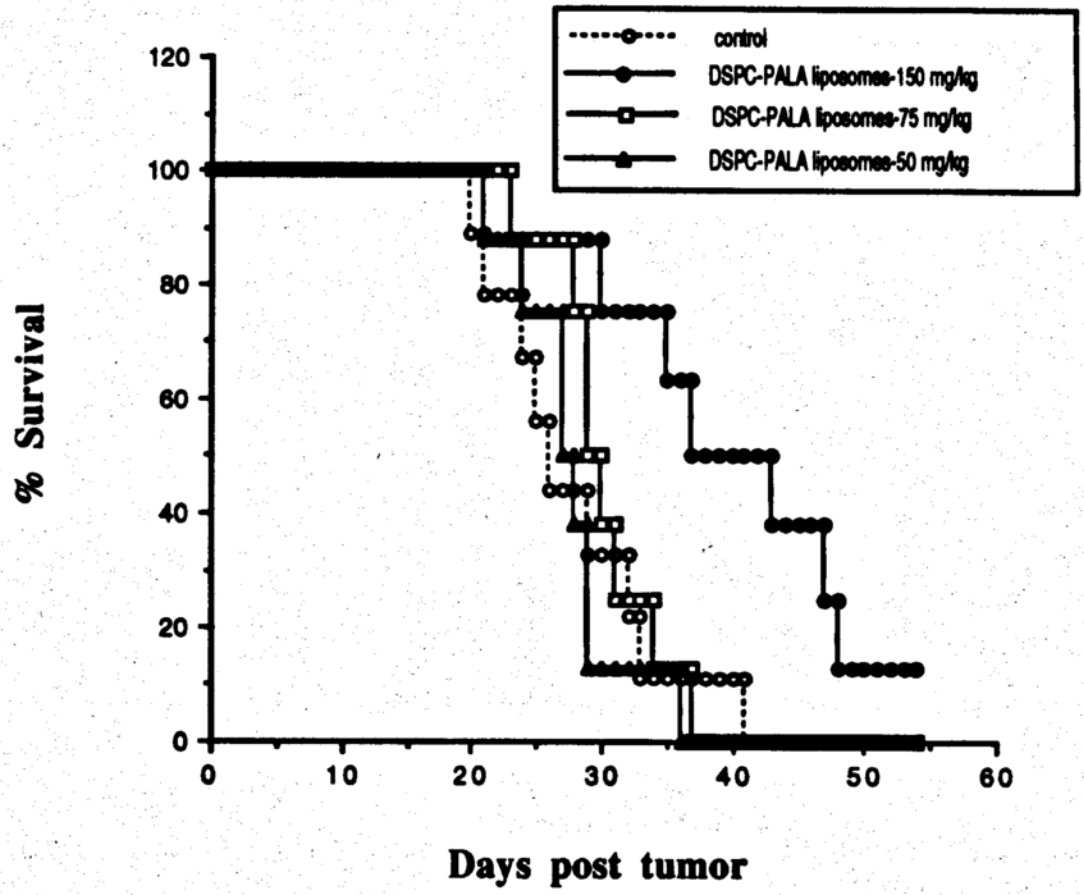

control
DSPC-PALA liposomes-150 mg/kg
DSPC-PALA liposomes-75 mg/kg
DSPC-PALA liposomes-50 mg/kg
% Survival
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Days post tumor

Figure 4.6. Survival of C-26 tumor bearing mice by a single i.p. injection of DSPG-PALA liposomes 8 days after tumor implantation.

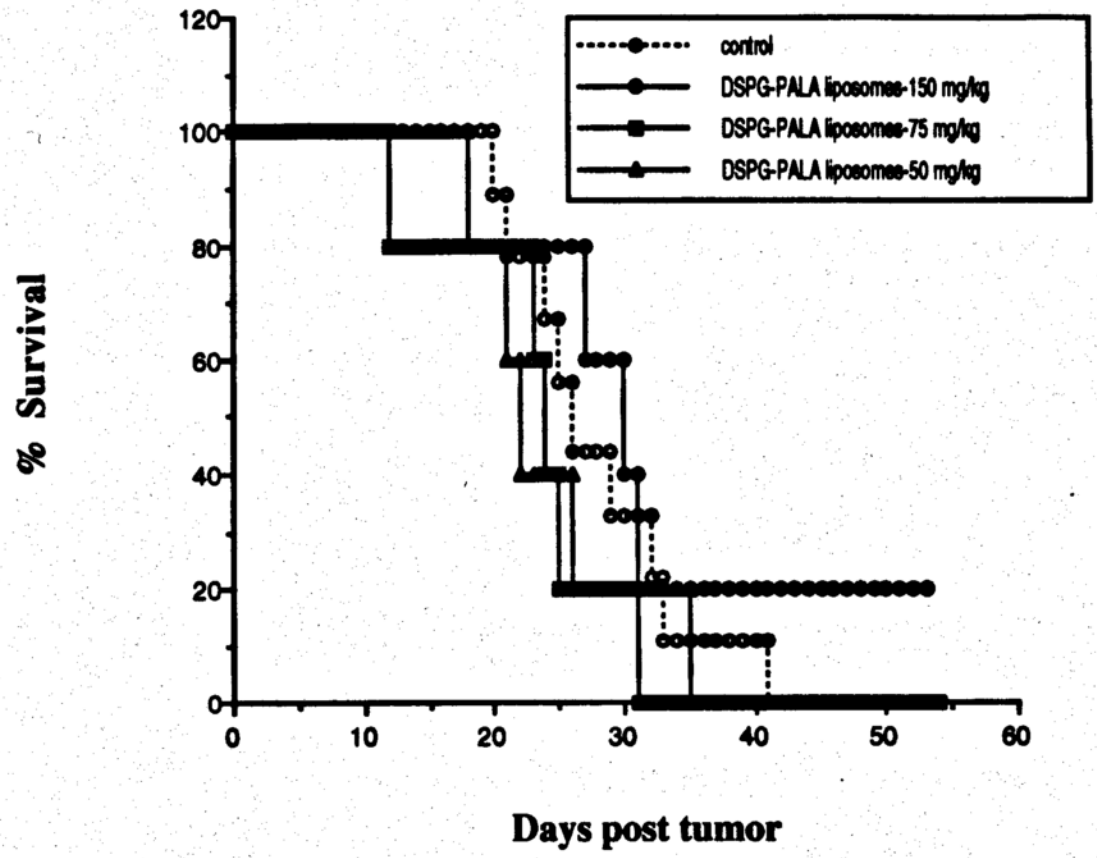

control
DSPG-PALA liposomes-150 mg/kg
DSPG-PALA liposomes-75 mg/kg
DSPG-PALA liposomes-50 mg/kg
% Survival
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Days post tumor

Table 4.3. Summary of the survival of C-26 tumor-bearing mice with the treatment of PALA in various formulations 8 days after tumor implantation.

Treatment	# mice/group	PALA (mg/kg)	Median Survival Time (days)	Increase in life span (%)[a]
control	9	none	25 ± 6.6 [b]	
free PALA	8	750	33.5 ± 12	+ 34
	8	375	25.5 ± 14.1	+ 2
	7	150	24 ± 8.3	- 4
DSPG -PALA Lipo.	5	150	29 ± 15.4	+ 16
	5	75	23 ± 6.9	- 8
	5	50	21 ± 6.5	- 16
DSPC -PALA Lipo.	8	150	39 ± 11	+ 56
	8	75	28.5 ± 4.2	+ 14
	8	50	26.5 ± 4.4	+ 6

[a] % increase in life span = (median survival time of treated / median survival time of control x 100) - 100

[b] median ± s.d.

1-Day Therapy of C-26 Tumor Bearing Mice with PALA

As discussed in the 8-day therapy study, treatment was started too late for PALA to cause a significant increase in MST in the eight day therapy schedule, suggesting a shortened initiation time of therapy after tumor implantation. So it was proposed to initiate the therapy one day after tumor was implanted into BALB/c mice using the same free or liposome-encapsulated PALA as previously. Moreover, an additional liposome formulation, sterically stabilized PEG-DSPE-PALA liposomes, was examined to see if liposomes known to have a prolonged residence time in the body cavity would be effective. The PEG-DSPE-liposomes are composed of the same lipids as DSPC-PALA liposomes, plus 5 mol% of polyethylene glycol (1900) derivatized DSPE. Doses for each group were chosen based on the result of MTD as before. Free PALA was given in three doses; 750 mg/kg (the MTD), 375 mg/kg (one half the MTD), and 150 mg/kg (one fifth the MTD). The PALA-liposomes were also given in three doses; 150 mg/kg, 75 mg/kg, and 50 mg/kg except for the PEG-DSPE-PALA liposome formulation. For PEG-DSPE-PALA liposomes, the PALA dose used were 100 mg/kg, 50 mg/kg, and 30 mg/kg. Injection volume was kept at 1.4 ml throughout the experiment to minimize the variation due to the volume change. Usually, each group had 8 mice, 4 housed per cage. A control group of 12 tumor-bearing mice was given MES/HEPES buffer alone, and 6 non-tumor bearing mice were given buffer alone, and followed to monitor the environment for animal room facility.

It should be noted that the 1-day therapy experiment had to be terminated at day 36 after tumor implantation because of a regulatory problem, and the % survival at day 36 from various treatments was compared, whereas the median survival time (MST) was compared in the 8-day therapy experiment.

As shown in Figures 4.7-4.10 and in Table 4.4, one half of the tumor-bearing mice of the control group had died by 20 days after tumor implantation.
At day 36, there was only an 8% survival from a tumor-bearing control group, whereas all of the mice which received free PALA at the 750 mg/kg dose had died by 31 days. There was a 25% survival from the free PALA at both the 375 mg/kg and 150 mg/kg doses, however, these doses of free PALA also caused high toxicity of severe weight loss and immediate death of some animal. Therefore, it appears that therapeutic effects of free PALA was limited by the balance between the toxicity to normal organs and the cytotoxic effects on tumor cells.

The DSPC-PALA liposomes caused a 50% survival at the 150 mg/kg dose and a 25% survival at both the 75 mg/kg and 50 mg/kg doses. PALA in DSPC liposomes caused a slightly significant increase in % survival at day 36 at all doses used, with no evidence of toxicity. The increase in % survival was almost commensurate with dose, being 50% for the 150 mg/kg dose, 25% for the 100 mg/kg and 50 mg/kg doses. The increase in % survival at day 36 at 150 mg/kg dose of DSPC-PALA liposomes was greater than that seen in free PALA. As such PALA in DSPC liposomes is marginally more effective than free PALA, the best PALA formulation tested was found to be DSPG-PALA liposomes, where a significant increase in % survival at day 36 was observed at all of the doses given. When DSPG-PALA liposomes were given at the 150 mg/kg dose, only 1 death occurred by the end of the experiment, showing an 88% survival at day 36. At doses of 75 mg/kg and 50 mg/kg, 2 deaths occurred during the entire experimental period showing 75% survival at day 36. Owing to the limited number of deaths it is not possible to assign a value to the MST, nor to calculate its increase. However, a comparison of the curve from the DSPG-PALA liposomes to that from control (MST for control is 22.5 days) gives estimate of MST of 43 days for 75

and 50 mg/kg doses, and 45 days for the 150 mg/kg dose, hence increases of 95% and 100 % respectively.

The PEG-DSPE-PALA liposomes, which were as ineffective as DSPC-PALA liposomes in the *in vitro* cytotoxicity experiment, caused a significant increase in % survival at day 36, showing a 38% survival at 100 mg/kg dose, a 78% survival at 75 mg/kg dose, and a 56% survival at 50 mg/kg dose. The increase in % survival at day 36 for the highest dose of PALA in PEG-DSPE-liposomes (38%) is not as great as was seen for the two lower doses (56% and 78%). Based on the % survival at the time of termination, the MST is estimated to be 38 days for the 30 mg/kg dose, and 40 days for the 50 mg/kg dose, increases of 67% and 78% respectively. Hence, the PEG-DSPE-liposome formulation is significantly more effective than the DSPC liposome formulation, though not as effective as the DSPG liposome formulation. However it also caused significant toxicity at the highest dose, resulting in the observed reduction in % survival at that dose.

When the experiments were terminated, all of the surviving mice were examined for any sign of i.p. or subcutaneous (s.c.) tumor. It was found that all of the mice still had either i.p. or s.c. tumors, except for two mice, which received the 75 mg/kg dose of DSPG-PALA liposomes, which showed no visible tumor at all. This suggests that by a single i.p. injection of DSPG-PALA liposomes, the C-26 i.p. tumor can be either eradicated or can at least prolong the survival time of tumor-bearing mice.

Figure 4.7. Survival of C-26 tumor-bearing mice by a single i.p. injection of free PALA 1 day after tumor implantation.

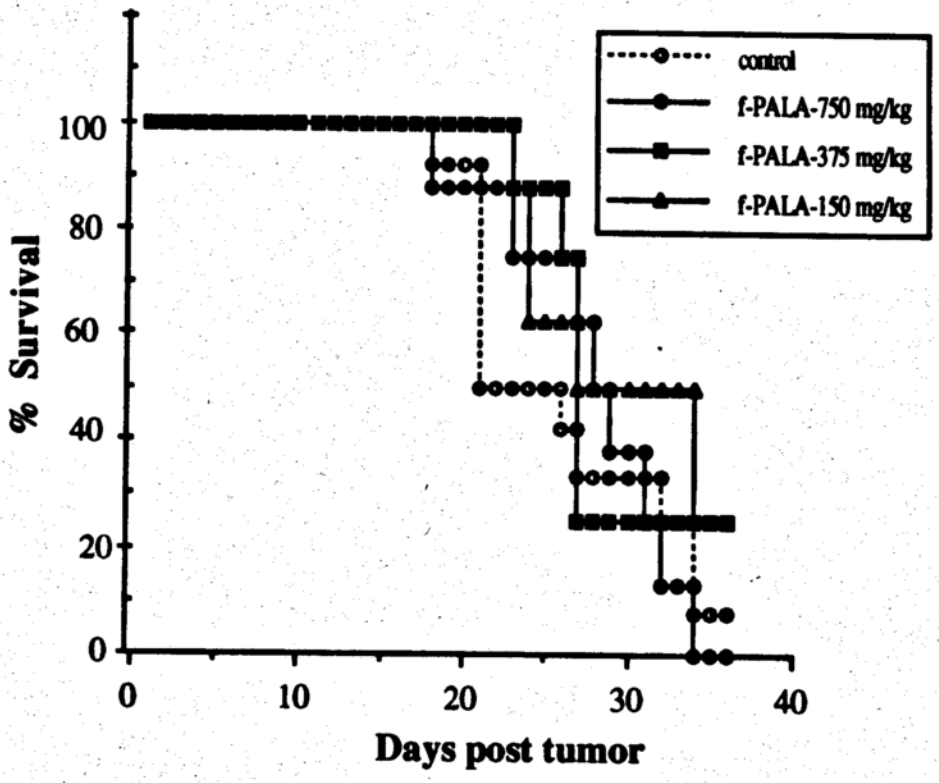

control
f-PALA-750 mg/kg
f-PALA-375 mg/kg
f-PALA-150 mg/kg
% Survival
100
80
60
40
20
0
0
10
20
30
40
Days post tumor

Figure 4.8. Survival of C-26 tumor-bearing mice by a single i.p. injection of DSPC-PALA liposomes 1 day after tumor implantation.

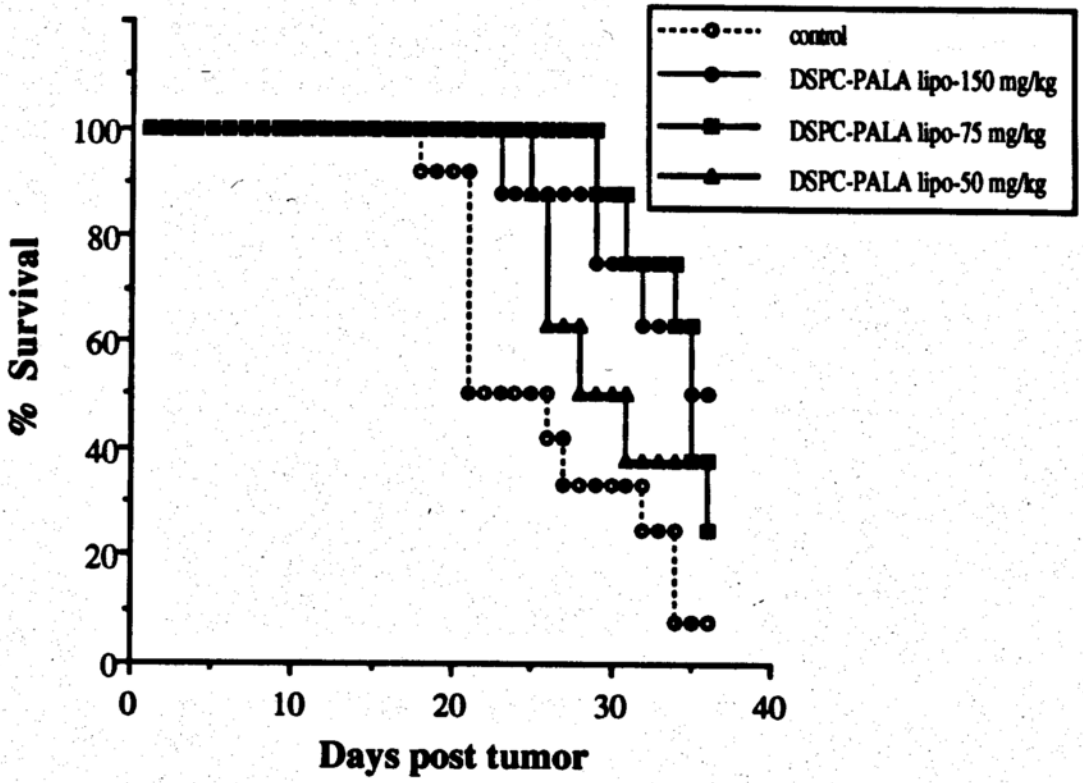

control
DSPC-PALA lipo-150 mg/kg
DSPC-PALA lipo-75 mg/kg
DSPC-PALA lipo-50 mg/kg
% Survival
100
80
60
40
20
0
0
10
20
30
40
Days post tumor

Figure 4.9. Survival of C-26 tumor-bearing mice by a single i.p. injection of DSPG-PALA liposomes 1 day after tumor implantation.

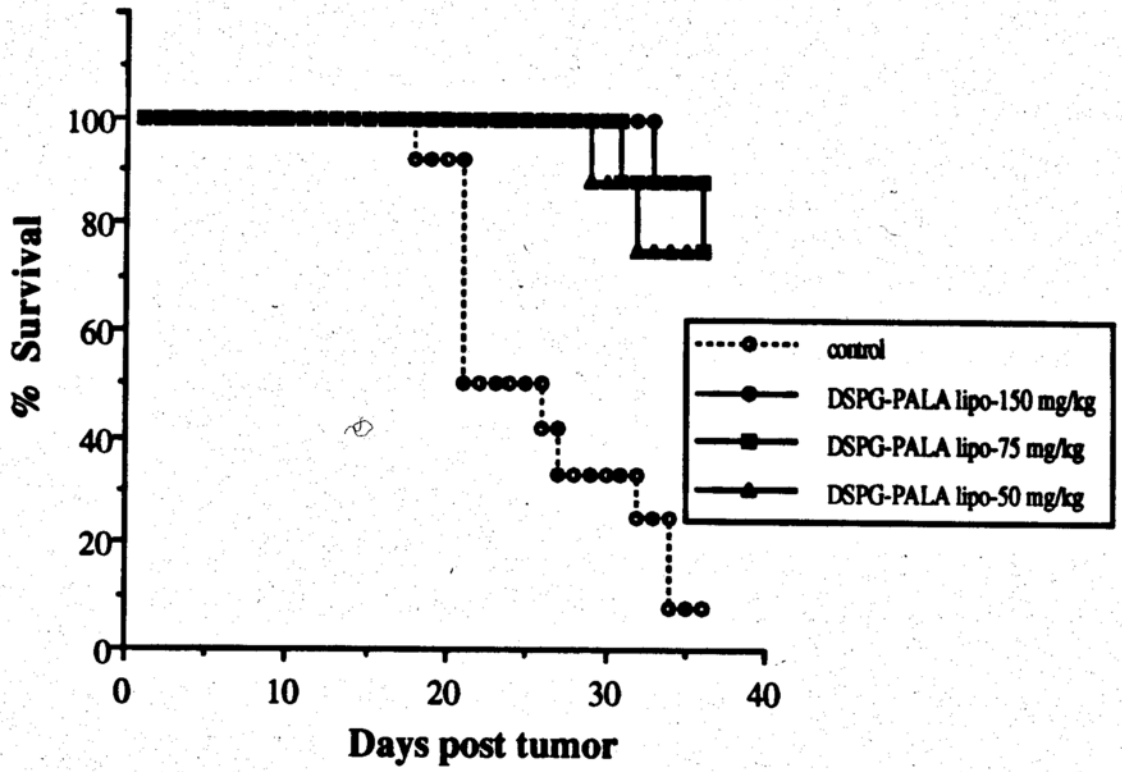

% Survival
100
80
60
40
20
0
0
10
20
30
40
Days post tumor
control
DSPG-PALA lipo-150 mg/kg
DSPG-PALA lipo-75 mg/kg
DSPG-PALA lipo-50 mg/kg

Figure 4.10. Survival of C-26 tumor bearing mice by a single i.p. injection of PEG-DSPE-PALA liposomes 1 day after tumor implantation.

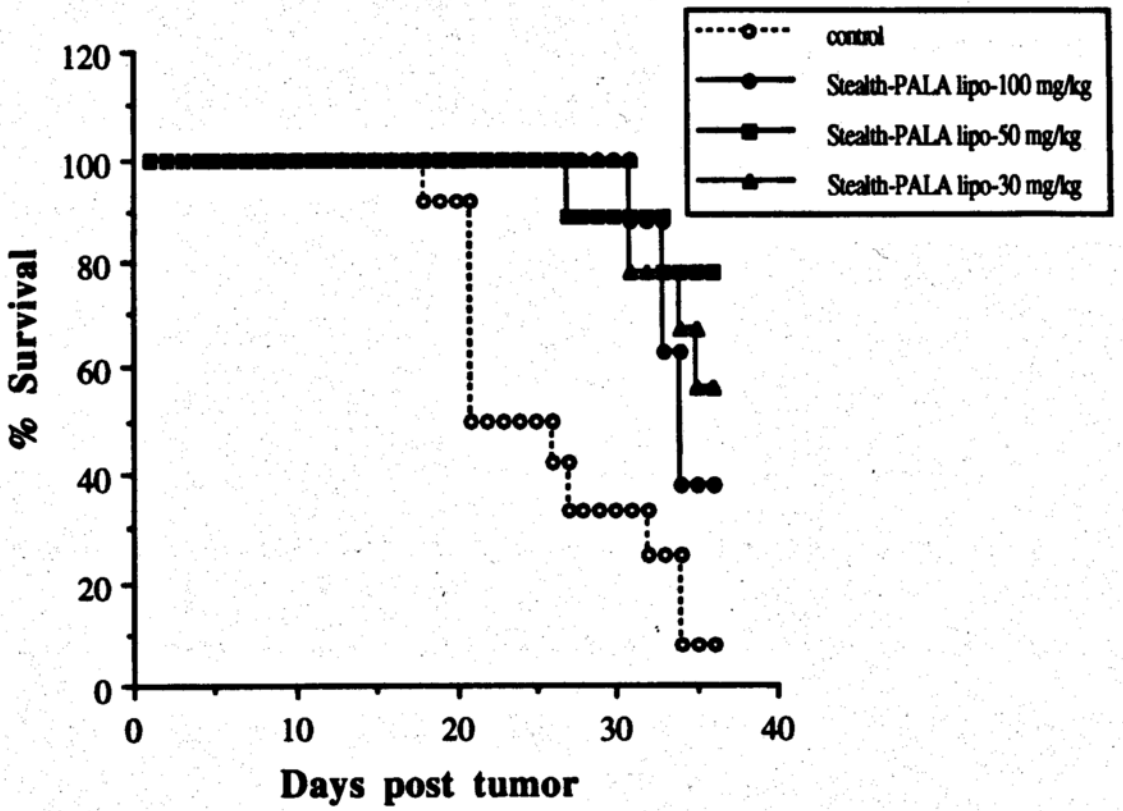

control
Stealth-PALA lipo-100 mg/kg
Stealth-PALA lipo-50 mg/kg
Stealth-PALA lipo-30 mg/kg
% Survival
120
100
80
60
40
20
0
0
10
20
30
40
Days post tumor

Table 4.4. Summary of the % survival of C-26 tumor-bearing mice by a single i.p. injection of various PALA formulations 1 day after tumor implantation.

Treatment	# mice/group	PALA (mg/kg)	% Survival at day 36
control	12	buffer	8
free PALA	8	750	8
	8	375	25
	8	150	25
DSPG-PALA Lipo.	8	150	88
	8	75	75
	8	50	75
DSPC-PALA Lipo.	8	150	50
	8	75	25
	8	50	25
PEG-DSPE-PALA Lipo.	8	100	38
	9	50	78
	9	30	56

In summary, the results described in this thesis demonstrate;

1) that PALA in antibody-conjugated liposomes is up to 60-fold more effective than free PALA.

2) that anti-c-*erb*B2 and anti-transferrin receptor antibodies are particularly effective in this regard.

3) that human ovarian carcinoma lines are valid targets for this form of drug delivery.

4) that PALA is well tolerated at 150 mg/kg in liposomes, but toxic at doses as low as 750 mg/kg in free form.

5) that free PALA increases % survival at day 36 from 8% (control) up to 25%.

6) that DSPC-PALA liposomes increases the % survival at day 36 from 8% up to 50%.

7) that PEG-DSPE-PALA liposomes increases the % survival at day 36 from 8% up to 78%.

8) that DSPG-PALA liposomes increases % survival at day 36 from 8% up to 88%.

Chapter 5. DISCUSSION

Drug delivery to desired sites in the body has been proposed as an ideal mode of therapy, but has proved to be difficult to achieve. It has evolved from the need to preferentially deliver pharmacological agents to desired tissues or organs so that the toxicity to normal tissues can be avoided. Drug targeting to a specific tissue of the body has always been hampered by the reticuloendothelial system (RES) predominantly present in the liver and spleen, unless the macrophages of the RES constitute the intended target.

Attempts have been made to achieve targeting in various ways, among which is the use of antibodies as a homing device specific for tissue-specific antigens. Direct conjugation of drug molecules to this homing device is one way of achieving drug targeting. Immunoconjugates, direct conjugates of drugs to antibodies, are good example of direct conjugation for drug targeting. Doxorubicin, for example, was conjugated directly to a chimeric monoclonal antibody, BR-96, which binds an antigen related to Lewis Y that is abundantly expressed at the surface of cells from many human carcinomas . The BR-96-Doxorubicin immunoconjugate was shown to induce complete regressions and cures of xenografted human lung, breast, and colon carcinomas growing subcutaneously in athymic mice and cured 70% of mice bearing extensive metastases of a human lung carcinoma (Trail, Willner et al. 1993). However, it is not always possible to produce immunoconjugates, which fully maintain the pharmacological activity of the drug in the final product, because of the relatively harsh conjugation conditions used.

Drug molecules can be indirectly conjugated to a homing device via a carrier system like liposomes. Targeting of drugs via carrier systems to the sites of the body in need of pharmacological intervention has proved to be very effective in the treatment

of cancer, bacterial infection, and enzyme disorders. Because the chemical modification of a drug molecule for conjugation, which is necessary for the direct conjugation method, is not required for this approach, full pharmacological activity of a drug can still be maintained after conjugation.

The use of antibody-conjugated liposomes for drug delivery is a unique method of active targeting of drug molecules to specific tissues or organs in the body. Among the prerequisites for this approach are the development of tissue-specific antibodies and the production of stable antibody-conjugated drug encapsulated liposomes. As tumor-specific monoclonal antibodies or other therapeutically useful antibodies are found to be so important in understanding of the basic pathology of cancer and other human diseases, they play a critical role in finding possible therapeutic modalities for treatment of various diseases. Moreover, the methods of conjugation of tumor-specific antibody to liposomes are simple and efficient as a result of development of heterobifunctional cross-linking reagents, such as SPDP and SMPB.

The choice of drug molecule is also crucial to the success of drug targeting. As discussed previously, the degree of increase in the potency of liposome-dependent drugs (such as PALA and methotrexate-γ-aspartate) is very substantial, whereas that of liposome-independent drugs (such as doxorubicin) is very marginal, when encapsulated in liposomes. Even though doxorubicin was shown to exhibit improved delivery in antibody-conjugated liposomes in a 1hr wash experiment *in vitro* (Ahmad and Allen 1992), it can't exhibit a similar improvement in continuous exposure.

In this study, Protein A was conjugated to PALA encapsulated liposomes and tested in L929 mouse fibroblast cells after pretreatment of the cells with a monoclonal antibody 11-4.1 which specifically binds the $H2K^k$ protein of L929 cells. As Protein A is known to bind the Fc portion of any kinds of the antibodies, the Protein A-conjugated PALA liposomes can bind the free Fc portion of 11-4.1 monoclonal antibody which binds the $H2K^k$ protein of L929 cells. As shown in Table 3.2, Protein A-conjugated PALA liposomes were 400-fold more cytotoxic to L929 cells than free drug and 360-fold more potent than PC-PALA (non Protein A-conjugated) liposomes, when the cells were pretreated with 40 μg of 11-4.1 monoclonal antibody. It was also found that no more than 20 μg of 11-4.1 antibody per 4×10^4 L929 cells was needed to achieve the above result. This suggests that PALA can be targeted to cells *in vitro* using Protein A-conjugated liposomes with the pretreatment of the target cells with antibody which is specific to the cells to be targeted. Even though the Protein A-conjugated drug delivery system is only useful in an *in vitro* study because of the non-specificity of the binding of Protein A to the Fc portion of any immunoglobulins in the body, the results from this study led to the following experiments, where the targeting of PALA in antibody-conjugated liposomes was explored.

Tumor-specific antibody-conjugated PALA-liposomes were tested for their cytotoxicity in 72hr continuous growth inhibition experiment using human ovarian cancer cell lines (HEY 1B and SKOV-3) which were screened for the abundant presence of antigens for the antibodies to be conjugated. The conjugation resulted in 280 monoclonal antibodies on the average attached to each liposome of 0.2 μm in diameter. The stability test over a month period of storage at 4°C showed that the leakage of PALA from antibody-conjugated liposomes was substantial (50-70%

leakage). For further studies, the lipid composition PC : Chol : MPB-PE (20 : 10 : 1 molar ratio), will need to be changed to an alternative lipid composition that is more stable.

As summarized in Table 3.3, the 454A12-(anti-transferrin receptor antibody) and the 454C11-(anti-c-*erb*B2 antibody) conjugated PALA liposomes proved to be the two best formulations of those tested for delivering PALA to HEY1B cells *in vitro*, though all of the other antibody-conjugated PALA liposomes also showed a significant improvement in this regard, compared to free PALA. When tested in SKOV-3 cells, the 454C11-(anti-c-*erb*B2 antibody) and the 2G3-(specific to a high molecular weight mucin) conjugated PALA liposomes proved to be the best two formulations of those tested. The SKBR-3 cells, a human breast cancer cell line, were also tested for the delivery of PALA in antibody-conjugated liposomes. However, due to the very slow growth rate of this cell, it was not possible to obtain reproducible IC_{50} values, though an improvement in PALA delivery was seen in some antibody-conjugated PALA liposomes.

This clearly demonstrates that PALA, when encapsulated in antibody-conjugated liposomes, can be delivered to the target cells more efficiently and exhibit greater cytotoxicity than it does in the free form. As both HEY 1B and SKOV-3 are human ovarian cancer cell lines, and the c-*erb*B2 oncogene products are overexpressed in one third of human ovarian cancer patients, the results here from antibody-conjugated PALA liposomes are very important in assessing the possibility of using PALA for the treatment of human ovarian cancer in antibody-conjugated liposome formulation and possibly for the treatment of human breast cancer as well.

It can be argued that if only one antibody-conjugated PALA liposome kills a cell in *in vitro* test system, potency difference in antibody conjugated liposomes between different antibody conjugation degree will be affected by homogeneous distribution of liposomes, concentration gradient of PALA, and aggregation of antibody-conjugated liposomes in test system. However, under the circumstances this study was done where more than 1,000 liposomes are to be bound to a cell, the number of liposomes per cell in test system are far more than the critical number of liposomes for exhibiting cytotoxic effects, so that the possibility that those factors described above will affect the validation of this test system was minimum.

PALA, when encapsulated in negatively-charged DSPG liposomes, has been shown previously to exhibit up to 360- and 412-fold increase in its *in vitro* growth inhibitory potency for CV1-P and RAW 264 cells, respectively, as compared to free PALA (Heath and Brown 1989). However, it is important to note that not every liposome-dependent drug, whose potency was increased in negatively-charged liposomes, can show an improved efficacy in antibody-conjugated liposomes . In this regard, PALA has proved for the first time to exhibit up to 60-fold increase in potency in antibody-conjugated liposomes . This *in vitro* study points to the possibility that PALA could be used for targeting to desired tissues *in vivo* when encapsulated in antibody-conjugated liposomes.

In *in vivo* studies, where the C-26 murine colon carcinoma-bearing BALB/c mice were used as a tumor model, PALA was tested in various liposome formulations. The DSPC-PALA and DSPG-PALA liposomes were well tolerated in healthy mice at up to 150 mg/kg, whereas free PALA showed a severe acute toxicity at the 750 mg/kg dose by a single i.p. injection. Due to the volume limit for an i.p. injection (1.4 ml /

injection), the 150 mg/kg dose was the highest dose used here for the PALA-liposome formulations. As no severe toxicity was seen from the PALA-liposomes at the 150 mg/kg dose, the actual MTD (maximum tolerated dose) for these formulations should have been higher than this dose.

Almost 90% of the mice from the control groups died by Day 36, though the majority of the deaths occurred by Day 21. DSPC-PALA and DSPG-PALA liposomes increased the median life span (ILS) by 56% and 16%, respectively, when the therapy was initiated eight days after tumor implantation. Free PALA at 750 mg/kg caused a significant increase in life span (34% ILS), though this dose caused severe toxicity. A Student's t-test revealed that only the 34% ILS caused by 750 mg/kg free PALA and the 56% ILS caused by 150 mg/kg DSPC-PALA liposomes were statistically significant over the control group at the 95% confidence level. When liposomes are injected intraperitoneally, they will be removed from the intraperitoneal cavity via the abdominal lymphatics with subsequent drainage into the venous system for the circulation. It is conceivable that DSPC-PALA liposomes remain longer in the peritoneal cavity due to their higher stability than the DSPG-PALA liposomes, and free PALA is released from liposomes in a controlled-release fashion, and exhibited a better therapeutic efficacy than the DSPG-PALA liposomes.

When the treatment was initiated one day after tumor implantation, the DSPG-PALA liposomes at the 150 mg/kg dose increased the % survival at Day 36 by 350%, compared to free PALA at the same dose (Table 4.4). Moreover, the DSPG-PALA liposomes at this dose caused only 1 death by the end of the experiment, showing an 88% survival at Day 36, whereas free PALA at this dose caused severe toxicity of weight loss and immediate death of some animals. At doses of 75 mg/kg and 50 mg/kg,

groups receiving the DSPG-PALA liposomes exhibited 2 deaths during the entire period of the experiment, showing a 75% survival at Day 36. The DSPC-PALA liposomes at these doses increased the % survival at Day 36 by 299%, compared to free PALA. This result can be interpreted with respect to the *in vitro* cytotoxicity of the DSPG-PALA and the DSPC-PALA liposomes to the C-26 cells (IC_{50} for the DSPG-PALA liposomes was 0.09 μM; IC_{50} for the DSPC-PALA liposomes was 11.8 μM).

The sterically stabilized PEG-DSPE-PALA liposomes at the 100 mg/kg dose seem to be very toxic, though the 50 mg/kg dose and the 30 mg/kg dose showed a 310% and a 220% increase in % survival at Day 36, respectively, compared to the free PALA groups on the average. The PEG-DSPE-PALA liposomes at the 50 mg/kg dose was almost as effective as the DSPG-PALA liposomes at the same dose, though the *in vitro* cytotoxicity of the DSPG-PALA liposomes was greater than that of the DSPC-PALA liposomes (IC_{50} for the DSPG-PALA liposomes was 0.09 μM; IC_{50} for the PEG-DSPE-PALA liposomes was 10.7 μM). As the sterically stabilized PEG-DSPE-liposomes are known not to intensively interact with many opsonins in body fluid, the above results are probably caused by prolonged residence time of the PEG-DSPE-PALA liposomes in the peritoneal cavity and sustained release of PALA into the peritoneal area. Sterically stabilized PEG-DSPE-liposomes are believed to have prolonged residence times, because they have the bulky hydrophilic 1900 mol. wt. PEG group attached to the DSPE. Overall, PEG-DSPE-PALA liposomes are significantly more effective than the DSPC-PALA liposomes, though not as effective as the DSPG-PALA liposomes.

When the *in vivo* experiments were finished, the surviving animals were sacrificed according to the IACUC protocol and a post-mortem examination was performed for a sign of any tumors in the body. There were six survivals from the 8-day therapy experiment; two from the free PALA at the 750 mg/kg dose group, two from the free PALA at the 375 mg/kg dose group, one from the DSPG-PALA liposomes at the 150 mg/kg dose group, and one from the DSPC-PALA liposomes at the 150 mg/kg dose group. All of the survivors from the 8-day therapy experiment showed no sign of visible tumors. On the other hand, most of the survivors from the 1-day therapy experiment (46 survivals in total by Day 36) showed visible sign of either s.c. tumors or i.p. tumors or both at Day 36, though two from the DSPG-PALA liposomes at the 75 mg/kg dose group and one from the DSPC-PALA liposomes at the 150 mg/kg dose group did not show any visible tumors. It should be noted that the post-mortem examinations for the 8-day therapy group was performed at Day 54, whereas that for the 1-day therapy group was done at Day 36. Therefore, the degree of tumor dissemination would be different, which makes it very hard to compare these two post-mortem data as they are. However, it is conceivable that the i.p. injection in a large volume (1.4 ml) into a relatively small peritoneal cavity of mice one day after tumor implantation might have spread tumors into the adjacent tissues, resulting in the visible s.c. tumors. On the other hand in the 8-day therapy group, the tumors would be firmly fixed before injection of the therapeutic agent so that only killing of tumor cells specifically in the peritoneal cavity would be required for cure.

Even though PALA has been shown to increase its potency in negatively charged liposomes in L929 and Raw 264 cells *in vitro* previously (Heath and Brown 1989), it does not automatically mean that PALA can be targeted in antibody-

conjugated liposomes to human cancer cells, and that PALA-liposomes can exhibit an improved therapeutic efficacy *in vivo* tumor models. In this regard, the significance of this study lies in that it demonstrates, for the first time, that a liposome-dependent drug like PALA can be targeted to the human tumor cells in anti-c-*erb*B2 antibody-conjugated liposomes *in vitro*. Moreover, PALA exhibited a significant improvement in its therapeutic efficacy both in the DSPG-liposome and PEG-DSPE-liposome formulations for the C-26 murine colon carcinoma-bearing BALB/c mice with a single i.p. injection.

As is obvious from both the antibody-conjugated PALA liposome study and the C-26 tumor-bearing animal study that PALA can be targeted to specific tissues in the body under certain conditions, it will be desirable in the future to examine if antibody-conjugated PALA-liposomes can cure human carcinoma-bearing athymic mice. Any human ovarian carcinoma cells, including HEY 1B and SKOV-3, which overexpress the c-*erb*B2 oncogene products and the transferrin receptors, can be candidates for human tumor models in athymic mice. The anti-c-*erb*B2-antibody 454C11 and the anti-transferrin receptor antibody 454A12 are reasonable antibody candidates for conjugation to the PALA-encapsulated liposomes. It would also be nice to develop different tumor / therapy models, such as an i.v. tumor / i.v. therapy model, if the effect of prolonged circulation time of the sterically stabilized liposomes on the cure of the tumor-bearing mice is to be studied.

Chapter 6. CONCLUSIONS

CONCLUSIONS

i) PALA in Protein A-conjugated antibody directed liposomes is over 400-fold more effective than free drug for growth inhibition of L929 cells *in vitro*.

ii) PALA in antibody-conjugated liposomes is up to 60-fold more effective than its free form for growth inhibition of HEY 1B and SKOV-3 cells *in vitro*. Anti-c-*erb*B2 antibodies and anti-transferrin receptor antibodies are particularly effective in this regard.

iii) PALA is well tolerated at 150 mg/kg in liposomes, but toxic at doses as low as 750 mg/kg in its free form by a single i.p. injection in BALB/c mice.

iv) DSPC-PALA liposomes increase the median survival time (MST) of the C-26 tumor-bearing mice up to 56% when given 8 days after tumor implantation.

v) DSPG-PALA liposomes increase % survival (at Day 36) of the C-26 tumor-bearing mice from 8% (control group) to 88% when given 1 day after tumor implantation. This formulation is found to be the best of those tested.

vii) Sterically stabilized PEG-DSPE-PALA liposomes increase the % survival (at Day 36) of the C-26 tumor-bearing mice from 8% (control group) to 78% when given 1 day after tumor implantation.

viii) DSPC-PALA liposomes increase the % survival (at Day 36) of the C-26 tumor-bearing mice from 8% (control group) to 50% when given 1 day after tumor implantation.

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