DEVELOPMENT OF SECRETORY PHOSPHOLIPASE A_2 RESPONSIVE LIPOSOMES AND TRACKING SYNTHETIC NOVEL LIPIDS IN BIOLOGICAL SAMPLES

By

GUODONG ZHU

(Under the Direction of Robert D. Arnold)

ABSTRACT

Lipid based nanoparticulate drug-carriers, such as long-circulating sterically-stabilized liposomes (SSL), can alter a drugs pharmacokinetic profile and improve its antitumor activity. However, mechanisms to "tune" their drug release kinetics and ability to track drug-carrier disposition *in vivo* are limited.

The goals of this dissertation were to develop lipid based nanoparticulate drug carriers that are responsive to elevated expression of secretory phospholipase A_2 ($sPLA_2$) in malignant tissues, to modulate drug release and track the distribution and metabolism of these formulations from biological samples. In **Chapter 1** we reviewed lipid-based nanoparticulate drug-carriers and contemporary approaches to modulate *in vivo* drug release. In **Chapter 2** we developed an electrospray ionization-mass spectrometry

(ESI-MS) method to determine $sPLA_2$ selectivity on individual and combinations of lipids. Studies in **Chapters 2** and **3** demonstrated that in the presence of $sPLA_2$, incorporation of lipids like distearoylphosphatidylglycerol (DSPG), distearoylphosphatidylethanolamine (DSPE) and synthetic odd-chain (C:15:0/C:16:0) lipids: 1-*O*-hexadecyl-2-pentadenoyl-s*n*-glycerol-3-phosphocholine (C31PC) and 1-*O*-hexadecyl-2-pentadenoyl-s*n*-glycerol-3-phosphomethanol (C31PM) in drug carriers could significantly change the release profiles of 6-carboxyfluoresein (6-CF, a fluorescent probe). These studies supported our hypothesis that $sPLA_2$–mediated drug release could be modulated by altering lipids' acyl chain length and use of head groups with different physicochemical properties.

In **Chapters 3** and **4**, we demonstrated that odd chain acyl lipids, *i.e.*, C31PC, could be used to track the deposition of liposomes *in vivo*. Interference of endogenous lipids in biological samples to C31PC was negligible. C31PC was extracted from biological samples and identified using ESI-MS. This method may be used to gain mechanistic insight into the disposition, degradation and release kinetics of lipid-nanoparticles. In **Chapter 4** we also demonstrated that acidification of samples during a Bligh and Dyer extraction improved the extraction efficiency of anionic lipids.

This dissertation reports a deviation from the status quo where the focus is on encapsulating the greatest quantity of drug stably and using external physiological stimuli to trigger "burst" release from particles. Development of $sPLA_2$-targeted formulations by

incorporating $sPLA_2$-preferred lipids has the potential to enhance drug carrier deposition in tumors, optimize exposure profiles to maximize antitumor activity and may be extended to additional drugs, targeting ligands or contrast agents.

INDEX WORDS: Liposomes, Nanoparticles, Drug carriers, Phospholipids, Secretory phospholipase A_2, ESI-MS, Bligh and Dyer method, Synthesis

DEVELOPMENT OF SECRETARY PHOSPHOLIPASE A_2 RESPONSIVE LIPOSOMES AND TRACKING SYNTHETIC NOVEL LIPIDS IN BIOLOGICAL SAMPLES

By

GUODONG ZHU

B.S., Guangdong Pharmaceutical University, China, 1996

M.S. China Pharmaceutical University, China, 2004

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2012

DEVELOPMENT OF SECRETARY PHOSPHOLIPASE A_2 RESPONSIVE LIPOSOMES AND TRACKING SYNTHETIC NOVEL LIPIDS IN BIOLOGICAL SAMPLES

By

GUODONG ZHU

Major Professor: Robert D. Arnold

Committee: Brian S. Cummings

Catherine A. White

Michael Bartlett

Michael Pierce

Electronic Version Approved:

Maureen Grasso

Dean of the Graduate School

The University of Georgia

May 2012

DEDICATION

To my mother Ming Lu, my deceased father Wenjin Chen and my late grandmother Zhaodi Wu.

ACKNOWLEDGEMENTS

I would like to thank my mother Ming Lu. Since my father passed away when I was 10 years old, she alone managed to raise me and paid tuitions for me until I finished studies in the college. Without her sacrifice and supports, I would never be able to stand on USA soil to pursue my Ph.D. degree.

I would like thank to my advisor Doctor Robert D Arnold for his patience, useful instructions and positive supports during the course of my Ph.D. study. All my achievements during 5 years in experimentations, posters, presentations, writings and publications came from his guidance. Also I would like to thank to Doctor Brian Cummings for providing enormous help in my experiment design and writings.

I am grateful to other committee members: Doctor Bartlett, White and Pierce for provisions of informative, instructive, thought-provoking comments and ideas to my research from their professional perspectives during committees meetings. I would like to express my sincere appreciation to them.

Next, I am thankful to my colleagues and lab members: Jason, Ibrahim, Yahya, Jeong-yeon, Ha, Leah, and other undergraduate students and people in chemistry department in UGA: Jeremy, Jennifer, Rong and Dr. Yang. They offered help to me at some points of my research.

Finally I would like to thank department of pharmaceutical and biomedical sciences for providing teaching assistantship on the course of my study.

TABLE OF CONTENTS

Page

APPENDIX

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Guodong Zhu, Brian S. Cummings and Robert D. Arnold. To be submitted to Journal of Controlled Release.

ABSTRACT

Chemotherapy is the primary approach used to the treat metastatic cancers, but pharmacological and pathophysiological factors limit their activity. Drug carriers, such as sterically stabilized liposomes (SSLs), can stably entrap drug, enhance distribution and intratumor accumulation of drug, decrease toxicity to normal non-target cells and improve efficacy. However, our ability to control drug release kinetics at pathological sites limits the full clinical potential of lipid based nanoparticulate drug carriers. A variety of mechanical and physiological approaches to control drug release have been explored. One such approach utilizes secretory phospholipase A_2 ($sPLA_2$), a lipase that's expression is elevated in a variety of tumors and inflammatory sites. In this chapter, we review the barriers to effective chemotherapy, application of lipid based drug carriers, approaches to modulate drug release from lipid based nanoparticles and factors that can affect $sPLA_2$–mediated degradation and drug release from liposomes. We also describe the development and use of a lipid marker and modification of the traditional Bligh and Dyer (BD) method to improve extraction efficiency, selectivity, and analysis of lipids from complex biological samples.

Cancer and chemotherapy

Cancer is the second leading cause of death and is responsible for about 1 in 4 deaths in the United States.[1] The lifetime incidence of developing cancer is 1 in 2 for males and 1 in 3 for females. In 2011 it was estimated that there were 1,596,670 new cancer cases and 571,950 cancer-caused deaths in the United States.[2]

Conventional chemotherapy is one of three primary strategies for the treatment of cancer in addition to surgical resection and sterilizing radiation. According to a report by Milliman Inc, 22% of patients with cancer in the United States received chemotherapy.[3] Conventional chemotherapy typically involves the use of antineoplastic agents to stop tumor growth by disrupting DNA synthesis or cell division. In comparison to localized treatments such as surgical resection and radiation, the use of systemic chemotherapy is the only effective treatment strategy for metastatic disease. Further, metastatic spread of tumors is the primary cause of mortality in patients.[2] In contrast, surgical and radiation therapy may be effective in the treatment of localized or regional tumors, but they are not suitable when metastasis occurs due to the potential for increased non-target tissue toxicity. The use of chemotherapy is also limited by its cytotoxic effects on healthy, fast-growing cells, *e.g.*, bone marrow, the digestive tract and hair follicles, and its limited activity against tumors with slow rates of growth.

Barriers to affect chemotherapy

The clinical response of chemotherapy is controlled by the pharmacological factors of the anti-tumor agents. To achieve clinically significant effects in the treatment of cancer, chemotherapeutic agents need to accumulate within tumors in sufficient quantities and remain effective in the *in vivo* microenvironment of the tumor.[4-6] Delivery of chemotherapeutic agents to solid tumors is a complex process: after administration into the systemic circulation, drug molecules need to be stable (resistant to metabolism and excretion), distribute to regions of disease, extravasate tumor endothelial wall, move through interstitial space *via* diffusion and/or convection, interact with the surface of tumor cells and/or undergo intracellular uptake by tumor cells to exert their cytotoxic effects.[7] The pharmacokinetic (PK) characteristics, *i.e.*, absorption, distribution, metabolism and excretion (ADME) profiles, determine drug exposure at the tumor site. Unfavorable PK characteristics, *e.g.*, poor distribution to the tumor, rapid metabolism and/or fast excretion can result in inadequate access of a drug to the tumor site. Furthermore, distribution of therapeutic agents to non-target tissues can result in toxicity to normal, reactive non-target cells/organs, thus limiting treatment effectiveness. Strategies to improve tumor targeting and control of drug exposure are needed.

Pathophysiological factors of tumors also play an important role regulating clinical response to chemotherapy. Solid tumors are composed of cancer cells (parenchyma) and stroma cells like fibroblasts and inflammatory cells.[8] Tumor heterogeneity has profound

effects on the response to chemotherapy. The heterogeneity includes existence of parenchyma cells in different phases of the cell cycle, irregular distribution, growth and formation of vasculature, and differences in oxygen tension and pH values in tumor microenvironment. For some antitumor agents, specifically cell cycle specific agents, they are only effective at killing malignant cells that are within a specific phase of the cell cycle.

Extravasation of drug from vasculature into tumors is enhanced by hyperpermeability of the tumor microvasculature. Fenestrations and defects in vasculature are due to rapid and incomplete angiogenesis, however, this enhanced permeability is not uniform along blood vessels. Leakiness in some regions along with irregular distribution of blood vessels in tumor tissues results in temporal and spatial differences in access to drug.

Heterogeneity in tumor vasculature also results in poor delivery of oxygen and nutrients to all regions within a tumor. These differences lead to the creation of hypoxic and acidic regions. Tumor cells in these regions have much slower rates of growth, and genotypic and phenotypic differences that reduce their sensitivity to radiation treatment and chemotherapy.[9] Further, higher mutation rates and non-uniform or incomplete drug exposure can lead to the development of drug resistance phenotypes and reduce effectiveness and sensitivity to chemotherapy.[10]

Drug carriers and liposome nanoparticles

The application of conventional chemotherapeutic agents has been beset with problems like toxic effects to non-target tissues/organs, unfavorable PK characteristics and insufficient delivery to the site of action. The use of drug carries to encapsulate therapeutic compounds, alter their ADME profiles, and facilitate delivery to target sites is one approach to overcome these challenges. Liposomal drug delivery systems are one of most commonly used drug carries for such purposes.

Liposome are a self-assembling microscopic spheres in which an aqueous core encloses one or more concentric lipid bilayer membrane(s).[11] Their sizes range from 20 nm to several micrometers. The basic building blocks of liposomes are lipids, which are amphiphilic molecules with a polar head group and two hydrophobic acyl chains. In the aqueous phase, when hydrated, phospholipids spontaneously aggregate by sequestering their acyl chains interiorly while facing polar head groups toward aqueous solution to minimize unfavorably water-hydrocarbon interactions. This results in the creation of a lipid bilayer which is the basic structure of liposomes.

Liposomes have long been evaluated as drug carriers since their structures were elucidated. Since then, some important achievements related to the application of liposomes as drug carriers have been made, *e.g.*, development of efficient drug loading procedures (*e.g.*, gradient remote loading), improved stability (introduction of cholesterol and high phase-transition temperature neutral lipids to liposome membranes) and use of

hydrophilic polymers, such as polyethylene glycol (PEG) to protect the surface of liposomes from opsonization of circulating proteins.

A variety of loading methods have been used to encapsulate drugs in liposomes. Drugs can be encapsulated into the aqueous core or lipid bilayers according to their hydrophilicity or hydrophobicity. Hydrophobic drugs can partition into the lipid membrane while hydrophilic drugs can be encapsulated within the aqueous core. Hydrophobic drugs like paclitaxel have been loaded in liposome carriers by hydration of compounds with lipid films.[12] In most situations, encapsulation efficiency of hydrophobic drugs can achieve 100%.[13] For hydrophilic drugs, there are two common ways to load drug molecules in the aqueous core of liposomes, passive loading and remote loading. Passive loading strategies entrap drug as liposome formulations are hydrated. The encapsulation efficiency of passive loading depends on the volume of the buffer liposomes can encapsulate and the drug solubility in the buffer. Encapsulation efficiencies are typically less than 30%.[13] Remote loading is a process that occurs after liposomes have been prepared. This approach uses drug concentration, pH[14] or other ionic[15] gradients as a driving force to induce accumulation or even precipitation of hydrophilic molecules inside liposomal aqueous cores. Depending on the physicochemical properties of the drug, encapsulation efficiencies for remote loading may reach 100%.

To achieve stable loading of therapeutic agents in liposomes, long saturated acyl chain neutral lipids[16] in combination with cholesterol (CHOL) (>30% molar ratio) have been used. Both contribute to minimize the membrane defects and reduce membrane fluidity and permeability. With respect to lipids, long saturated acyl chains provide thicker barriers to restrict drug leakage when compared to short and unsaturated phospholipids.[17,18] Neutral lipids in membranes also reduce lipid-protein interactions in systemic circulation, which may destabilize the lipid membranes as compared to anionic lipids.[19,20] CHOL, on the other hand, homogenously intercalates among lipids, reduces the free volume in the membranes and thus makes bilayer membranes more compact which are resistant to drug leakage and serum induced instability.[17,18]

To increase the stability and circulation time of liposome systems *in vivo*, hydrophilic polymers, such as polyethylene glycol (PEG) have been attached to the membrane surface. Conventional liposomes composed only of lipids are cleared rapidly by the reticuloendothelial system (RES). The RES is comprised of circulating and fixed (lung and liver) macrophages. It is responsible for the elimination of foreign particles, and is the primary route of elimination of liposomes and particulate based drug carriers. It is believed that PEG provides a hydrophilic coating on the liposome membrane surfaces, which reduces the binding of serum proteins including opsonins to liposomes,[21] thus reducing liposome recognition and clearance by the RES. The introduction of PEG

greatly increased the circulating half-life of liposomes *in vivo*, and is considered a seminal breakthrough in the liposome field.

Long-circulating liposome drug carriers have been shown to exploit the enhanced permeability and retention effects (EPR)[22] in cancerous tissues to achieve selective and increased deposition. By manipulating the sizes of liposomes and improving their circulation time, *i.e.*, reducing RES clearance, these formulations have been shown to be effective for a variety of cancers. The combination of abnormal leaky vasculature and defective lymphatic drainage in tumor tissues result in EPR. Tumor growth beyond 1 mm^3 initiates angiogenesis, *i.e.*, development of new blood vessels in cancer.[23] This process is rapid and often incomplete, which leads to abnormal leaky vasculature in tumor tissues. Pore sizes in leaky tumor endothelial walls range from 100 nm to several micrometers [24,25] (dependent on tumor types) while only 1-3 nm in normal endothelium. Liposomes engineered to approximately 100-200 nm in size extravasate through these gaps into tumor sites and accumulate at perivascular regions due to lack of functional lymphatic vessels in tumor tissues.[26] Studies showed long circulation time of drug carriers is required for this slow deposition process in tumor sites (peaking at 3-7 days after injection).[27]

As described previously, formulations containing only lipids have short half-lives *in vivo*, *e.g.*, several minutes and do not accumulate well in tumors. Liposomes formulated with high (>30% molar ratio) CHOL and saturated long acyl chain lipids have much

greater half-lives from several hours to days[28] Coating liposomal membrane surface with hydrophilic polymer, such as PEG, produces so called sterically stabilized liposomes (SSL) (**Figure 1-1**) or pegylated liposomes.[29] Practical use of SSL formulations takes advantage of combining long saturated acyl chain neutral lipids and high percentages of CHOL (>30% molar ratio) in their membranes because the use of these two decreases membrane fluidity and restricts membrane permeability, which ensures stable loading of drugs. The half-life of some SSL formulations is greater than 2 days.[27,30] Studies showed advantages from using SSL as drug carriers for chemotherapy. Compared to free drugs, liposome-encapsulated anti-cancer drugs have lower adverse effects and in some cases showed better efficacy in animal models and clinical studies.[31] To date, the ability to stably encapsulate and alter deposition PK with SSLs has been investigated for the majority of anticancer drugs that are used clinically.[32] Many are undergoing clinical evaluation and some have been approved by FDA, such as DOXIL™.

Modulation of liposome drug release

The formulation of liposomes with greater stability and enhanced circulation time has increased drug carrier deposition in solid tumors. However, the ability to control release kinetics *in vivo* is limited. Furthermore, the process of drug release is not well understood. The substantial stability of liposome membranes, which benefits drug loading, and the stability of drug carriers when in systemic circulation may create a scenario that the majority of drug molecules are trapped within drug carriers, rendering them inaccessible

to malignant cells. This scenario compromises drug bioavailability to target tissues and must be overcome for broader and more effective application of liposome drug carriers[33]. Moreover, tumors have a dense and viscous interstitial matrix that is high in collagen and sulfated glycosaminoglycan content restricts large particles, like the penetration of liposome drug carriers inside tumors after extravasation.[34] This barrier results in inadequate cytotoxic effects of therapeutic agents to tumor tissues. Drug carriers with active release mechanisms may improve drug exposure and thus efficacy. Small drug molecules released from carriers should have better diffusion than carriers and uniform delivery of therapeutic agents to whole tumor tissues may be attained.

Studies have shown a positive correlation between drug release mechanisms possessed by SSL drug carriers at pathological sites and the therapeutic efficacy of the formulations.[33] SSL drug carriers substantially reduce the severe toxic effects of anticancer agents. However, these formulations do not always improve efficacy compared to free drug. In some instance efficacy may be reduced because the release kinetics are not optimal. The passive-loading cisplatin-SSL formulation known as SPI-077 is a good example of how improved formulation stability hampered the efficacy of the encapsulated drug.[35] Though a prolonged circulation time and enhanced tumor uptake for SPI-077 in different tumor-bearing mice models was observed, SPI-077 didn't show superior antitumor activity to free cisplatin. It was hypothesized that the improved stability and extremely slow release kinetics of SPI-077 at tumor sites is the likely

explanation for this phenomenon. Some *in vitro* release experiments and cytotoxicity assays for SPI-077 indirectly support this hypothesis.[35] On the other hand, DOXIL which is a SSL formulation loaded with doxorubicin using remote loading technique, demonstrates that possession of a passive release mechanism grants better efficacy in some cancer models when compared to free doxorubicin. Unlike SPI-077, it is believed that DOXIL likely possesses a drug release mechanism at tumor sites. A slow drug release from SSL formulation could be achieved in tumor interstitial fluids by disruption of the ammonium sulfate gradient used to load and retain drugs within the SSL.[15,36] In animal models and clinical trials, DOXIL showed enhanced therapeutic efficacy as compared to the free drug.[37] These two examples above demonstrate that drug release kinetics possessed by SSL formulations are an important determinant of their efficacy.

Though liposome formulations like DOXIL could use a passive drug release mechanism, the release profile may still not be sufficient to achieve optimal drug exposure. Increases in efficacy and therapeutic effects (survival rate) of DOXIL compared to free doxorubicin were modest, or in some clinical studies, not statistically significant.[38,39] Moreover, the passive drug release mechanism of DOXIL cannot be applied for other drugs like SPI-077, where gradients used to entrap drugs do not exist. Lack of active control over drug release in liposome carriers compromises efficacy of doxorubicin and limits the wide applications of SSLs to other anticancer drugs.

Developing liposome carriers with active drug release mechanisms are needed to improve efficacy and therapeutic effects.

At least two strategies can be used to engineer liposomes with active drug release mechanisms. The first is to make the liposomes sensitive to external physical stimuli *e.g.*, heat[40] or light[41]. Another strategy is to prepare liposomes to be responsive to endogenous factors uniquely or differently expressed in tumor microenvironments, *e.g.*, acidic pH, elevated enzyme levels or over expressed cell surface receptors. This strategy involves exploiting differences between normal and tumor tissues, and then determining if these differences can be used to trigger and/or control drug release from liposomes. The focus of my dissertation has been to engineer liposomes, lipid based nanoparticulate drug carriers, to be responsive to secretory phospholipase A_2 ($sPLA_2$), a lipase that is over expressed in many tumors.

Secretory phospholipase A_2

Phospholipase A_2's (PLA_2) are a class of enzymes that cleave phospholipids preferentially at *sn-2* ester bonds, producing a lysophospholipid (LP) and a fatty acid (FA). There are five types of PLA_2: cytosolic phospholipase A_2 ($cPLA_2$), calcium-independent phospholipase A_2 ($iPLA_2$), lysosomal PLA_2, platelet-activating factor hydrolases (PAF-AH) and secretory phospholipase A_2 ($sPLA_2$).[42] The first three are located intracellularly and PAF-AH is found in the blood or organs such as the brain, kidney and liver while $sPLA_2$ exists in extracellular fluids.

In humans, there are 15 subtypes in the $sPLA_2$ family**,** including IA, IB (GIB), IIA, IIB, IID, IIE, IIF, III, V, X, IX, XI, XII, XIII and XIV**.**[43,44] They are all low MW (13-16 KDa) enzymes with 20-50% identity in their amino acid sequences. They share some common characteristics: Usually there are 5-8 disulfide bonds in their structure, which confers high stability. They have the same active center, which consists of a histidine/arginine catalytic dyad and a conserved Ca^{2+}-binding loop[45]. They are all Ca^{2+}-dependent enzymes. However, they show variations in tissue distribution, hydrolytic activity and substrate specificity. $sPLA_2$ are interfacial enzymes, that only act on membrane surfaces or aggregate lipids. In contrast, they have no catalytic ability on monomeric lipids.

The various $sPLA_2$ subtypes have different preferences to phospholipids either negatively or neutral charged, which are determined by charges of amino acid residues in their interfacial binding surface (IBS)[46]. Electrostatic attractions play a role in it. Group IIA is known to have affinity to negative charged lipid membranes while Group X $sPLA_2$ prefer neutral lipid membranes.[43]

Expression and/or activity of $sPLA_2$ increases under pathological conditions, including bacterial infection, arthritis, cerebral ischemia or stroke, atherosclerosis and cancers[47]. Group IIA and X $sPLA_2$s are particularly of interest because they are related to human cancers.[48] Elevated levels of Group IIA $sPLA_2$ have been found in various tumor

tissues including colon, breast, liver, skin, stomach, pancreatic and prostate cancers[49] while increased levels of Group X $sPLA_2$ have been found in colon carcinomas.[48]

Secretory phospholipase A_2 responsive liposomes

Based on that fact that elevated levels of $sPLA_2$ in various pathological sites, including tumor tissues, together with $sPLA_2$ ability to destabilize lipid membranes/degrade lipids, we hypothesized that liposome drug carriers with an active drug release mechanism could be designed. In theory, this type of drug carrier would be superior to conventional SSL liposome carriers in that the rate and extent of drug release can be controlled, thus improving and optimizing exposure profiles and therapeutic efficacy. Furthermore, liposome drug carriers can be engineered to be responsive to a specific subtype of $sPLA_2$ elevated in a specific disease. This is based on the fact that $sPLA_2$ subtypes show differences in their distribution, hydrolytic activity, substrate/membrane preference and different diseases express different subtypes. This strategy could lead to site specific trigger release and more effective drug release from drug carriers.

The design of effective $sPLA_2$ responsive liposomes are dependent on the membrane composition (lipid, CHOL and PEG) of the liposome, $sPLA_2$ species (catalytic ability and substrate preference) and effect of microenvironment, *i.e., "*media effects" (Ca^{2+}, buffer and serum). Modifications and optimization of membrane composition to increase

sensitivity to $sPLA_2$ was the focus of my dissertation research. We also determined the effect of media on $sPLA_2$–mediated drug release profiles from liposomal carriers.

Sensitivity to $sPLA_2$ and $sPLA_2$–mediated drug release can be controlled by manipulation of the liposome membrane. In our studies, different combinations of two phospholipids, which are different in head groups and/or charge in different ratios, were used in SSL-like formulations. Although there are numerous studies examining the selectivity of $sPLA_2$ on different classes of lipids,[43] the effects/activity of different $sPLA_2$ isoforms on combinations of lipids has not been studied.

Different subtypes of $sPLA_2$ have differences in affinity to negatively charged or neutral lipid membranes. Based on the specific $sPLA_2$ subtype, negative or neutral lipids can be introduced into SSL formulations to increase enzyme affinity. Furthermore, degradation of lipids by $sPLA_2$ at membranes produces LP and fatty acid FA. In theory, increased production of these two species will decrease membrane rigidity and increase permeability.

To identify $sPLA_2$–preferred lipids, a rapid screen was necessary to determine how lipid charges, head group, length and saturated level of *sn-1*, *sn-2* acyl chain affect $sPLA_2$–mediated degradation and leakage. The goal is to identify lipids that are responsive to $sPLA_2$, but that whose incorporation does not alter the long-circulating and stable entrapment properties of SSLs. For example, $sPLA_2$ Group IIA has a preference for lipids with short acyl chains and negative charged lipid membranes. However, lipids with

shorter acyl chain in liposome membranes have been shown poor drug loading and anionic charge lipids are more easily recognized by the RES.[19,50] So with regard to design of $sPLA_2$ Group IIA responsive liposomes, a balance between membrane sensitivity to $sPLA_2$ and membrane stability in drug loading and systemic circulation needs to be manipulated. Our goal was to develop optimal SSL formulations that were sensitive to $sPLA_2$ Group IIA without losing their drug carrier functions.

It is unclear how CHOL composition affects $sPLA_2$ ability to destabilize liposomal membranes. Previous studies have examined the effect of CHOL, but the literature is contradictory. For example, Bezzine S *et.al* (2002) suggest that cholesterol in lipid membranes do not significantly alter the kinetics of $sPLA_2$ catalysis[46], while several other studies suggest that CHOL has an inhibitory effect of on $sPLA_2$ activity.[51] Also a theory known as CHOL superlattices[52,53] proposed that CHOL membrane content at or near certain critical concentrations (*i.e.*, 14.3, 15.4, 20.0, 25.0, 33.3, 40, 50 molar mol% CHOL), formed either hexagonal or centered rectangular super lattices in membrane which reduced $sPLA_2$ activity to a local minimum.

The effect of PEG on activity of $sPLA_2$ is unclear. PEG reduces opsonization of proteins to the surface of liposomes *in vivo*. However, it may also reduce the ability of $sPLA_2$ to interact with surface of lipid membranes and cleave lipids. Effects of lipids, CHOL and PEG on $sPLA_2$–mediated lipid degradation and leakage will be examined and discussed in **Chapter 2**.

$sPLA_2$'s ability to destabilize lipid membrane is also affected by other factors, *e.g.*, calcium ion concentration and the presence of blood/plasma/serum in the media. Some studies have already addressed some of these issues. For example, studies suggest that $sPLA_2$ activity increases in a calcium dependent manner,[54] and other studies have found that high concentrations (up to 2mM) of calcium ions inhibit $sPLA_2$ interfacial binding to lipid membranes, thus decreased its activity[46]. In our experiments, we determine the effect of calcium concentrations on $sPLA_2$–mediated liposome release profiles. However, since free calcium ion concentrations in body fluids are about 1mM, we chose to use this concentration for our studies**.** The presence of blood/plasma/serum has also been shown to reduce $sPLA_2$ activity[55-57]. The mechanism is not well understood though it was proposed that there are certain endogenous inhibitors to $sPLA_2$ contained in them. In our experiments, we examined the effect of 10% serum on $sPLA_2$–mediated drug release from liposomes.

Developing odd-chain lipid marker with choline head group

Lipids may be used as markers to study the fate of liposomal carriers *in vitro* and *in vivo*, like their distribution, deposition, receptor-mediated intracellular uptake and lipid degradation. Interference from endogenous lipids in biological samples makes quantification of lipids used in liposome formulations difficult. Fluorescent markers like rhodamine-labeled lipids or radiolabeled markers like [^{3}H] cholesteryl hexadecyl ether (CHE) can be used for these purposes; however, they run a risk of altering lipid

membrane properties like permeability or fluidity or even alter degradation. Second, they don't distribute uniformly into all phospholipid bilayers. Furthermore, many are not suitable for human use. Finally, these types of markers could not be used for tracking lipid degradation in liposomal membrane since they are not representatives of real lipids used in carriers.

Natural (endogenous) lipids contain even number carbon atoms in their acyl chain. Therefore, we designed and synthesized lipids containing an odd number of carbon atoms and determined if they can be used as tracers to study the liposome fate and lipid degradation in biological samples as seen in **Chapters 3** and **4**.

A variety of methods have been developed for the quantification of lipid compounds. Traditional lipid measurement techniques include the gravimetric method, phosphorus analysis and thin-layer chromatography (TLC), while modern techniques commonly use gas/liquid chromatography combined with mass spectrometry (MS). Traditional techniques, *e.g*, TLC methods, usually have lower sensitivity and specificity and poorer reproducibility compared to MS, *e.g*, electrospray ionization-mass spectrometry (ESI-MS).[58] In my studies, ESI-MS was used to determine $sPLA_2$–mediated lipid degradation and quantify/track the synthetic odd-chain lipid marker in biological samples. Different lipid classes show differences in ESI-MS sensitivity. This probably results from their differences in ionization efficiency. Lipid classes with choline head groups have the

highest sensitivity.[59] This was the reason I designed the lipid marker with choline head group (**Chapter 3**).

Investigation of extraction efficiency of Bligh and Dyer procedures on individual lipids

Systematic study on the extraction efficiency of Bligh and Dyer lipid extraction on individual lipids has never been done. Lipid classes, *e.g*, phosphatidic acid (PA) and phosphatidylserine (PS), and both of their lysophospholipids (LP), were found to be recovered poorly by the traditional BD method in our studies. Further studies showed that extraction efficiencies of 1,2-distearoyl-*sn*-glycero-3-phosphatidic acid (DSPA) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylserine (DSPS) were less than 30%. Efficiency of the traditional BD method to PS, PA and their LPs were improved by acidification of the media. By identifying the defects of traditional Bligh and Dyer method to certain lipid classes and providing an acidification step to overcome these defects, our studies will extend its applications to anionic lipids like PS and PA. (**Chapter 4**)

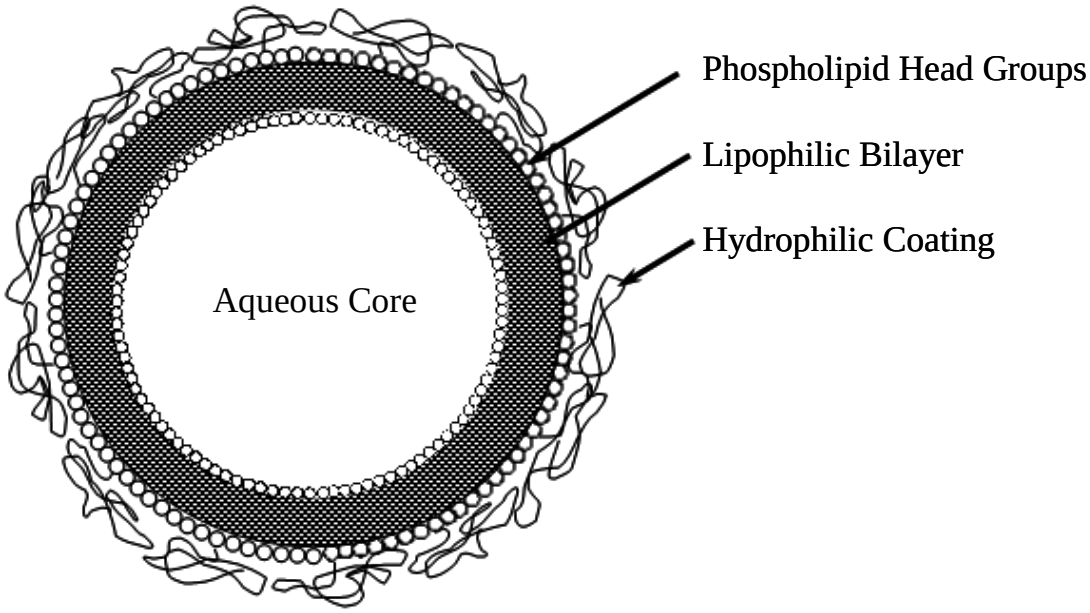


Figure 1-1. A diagram depicting the major structural features of long-circulating (SSL) liposomes.

REFERENCES:

1. Sherry L. Murphy JX, and Kenneth D. Kochanek 2012. Deaths: Preliminary Data for 2010. National Vital Statistics Reports 60(4).
2. Rebecca Siegel EW, Otis Brawley, and Ahmedin Jemal 2012. Cancer Statistics, 2011. CA: A Cancer Journal for Clinicians.
3. Kate Fitch BP. 2010. Cancer patients receiving chemotherapy:opportunities for better managemnt. ed.: Milliman Inc.
4. Jain RK 1996. 1995 Whitaker Lecture: delivery of molecules, particles, and cells to solid tumors. Ann Biomed Eng 24(4):457-473.
5. Jain RK 1998. Delivery of molecular and cellular medicine to solid tumors. J Control Release 53(1-3):49-67.
6. Jain RK 1999. Transport of molecules, particles, and cells in solid tumors. Annu Rev Biomed Eng 1:241-263.
7. Jain RK 1989. Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. J Natl Cancer Inst 81(8):570-576.
8. Dvorak HF, Nagy JA, Dvorak AM 1991. Structure of solid tumors and their vasculature: implications for therapy with monoclonal antibodies. Cancer cells (Cold Spring Harbor, NY : 1989) 3(3):77-85.

9. Trédan O, Galmarini CM, Patel K, Tannock IF 2007. Drug Resistance and the Solid Tumor Microenvironment. Journal of the National Cancer Institute 99(19):1441-1454.
10. Chen KG, Sikic BI Molecular Pathways: Regulation and Therapeutic Implications of Multidrug Resistance. Clin Cancer Res.
11. Bangham AD, Standish MM, Watkins JC 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol 13(1):238-IN227.
12. Crosasso P, Ceruti M, Brusa P, Arpicco S, Dosio F, Cattel L 2000. Preparation, characterization and properties of sterically stabilized paclitaxel-containing liposomes. Journal of Controlled Release 63(1–2):19-30.
13. Chonn A, Cullis PR 1995. Recent advances in liposomal drug-delivery systems. Curr Opin Biotechnol 6(6):698-708.
14. Madden TD, Harrigan PR, Tai LCL, Bally MB, Mayer LD, Redelmeier TE, Loughrey HC, Tilcock CPS, Reinish LW, Cullis PR 1990. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. Chem Phys Lipids 53(1):37-46.
15. Haran G, Cohen R, Bar LK, Barenholz Y 1993. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochimica et Biophysica Acta (BBA) - Biomembranes 1151(2):201-215.

16. Lian T, Ho RJY 2001. Trends and developments in liposome drug delivery systems. J Pharm Sci 90(6):667-680.
17. Corvera E, Mouritsen OG, Singer MA, Zuckermann MJ 1992. The permeability and the effect of acyl-chain length for phospholipid bilayers containing cholesterol: theory and experiment. Biochimica et Biophysica Acta (BBA) - Biomembranes 1107(2):261-270.
18. Senior J, Gregoriadis G 1982. Stability of small unilamellar liposomes in serum and clearance from the circulation: The effect of the phospholipid and cholesterol components. Life Sciences 30(24):2123-2136.
19. Senior JH 1987. Fate and behavior of liposomes in vivo: a review of controlling factors. Crit Rev Ther Drug Carrier Syst 3(2):123-193.
20. Felnerova D, Viret J-Fo, GlÃ¼ck R, Moser C 2004. Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs. Current Opinion in Biotechnology 15(6):518-529.
21. Moghimi SM, Muir IS, Illum L, Davis SS, Kolb-Bachofen V 1993. Coating particles with a block co-polymer (poloxamine-908) suppresses opsonization but permits the activity of dysopsonins in the serum. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1179(2):157-165.

22. Matsumura Y, Maeda H 1986. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 46(12 Pt 1):6387-6392.

23. Hanahan D, Folkman J 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86(3):353-364.

24. Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP, Jain RK 1998. Regulation of transport pathways in tumor vessels: Role of tumor type and microenvironment. Proceedings of the National Academy of Sciences 95(8):4607-4612.

25. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM 2000. Openings between Defective Endothelial Cells Explain Tumor Vessel Leakiness. The American Journal of Pathology 156(4):1363-1380.

26. Ishida O, Maruyama K, Sasaki K, Iwatsuru M 1999. Size-dependent extravasation and interstitial localization of polyethyleneglycol liposomes in solid tumor-bearing mice. International Journal of Pharmaceutics 190(1):49-56.

27. Gabizon A, Catane R, Uziely B, Kaufman B, Safra T, Cohen R, Martin F, Huang A, Barenholz Y 1994. Prolonged Circulation Time and Enhanced Accumulation in Malignant Exudates of Doxorubicin Encapsulated in Polyethylene-glycol Coated Liposomes. Cancer Res 54(4):987-992.

28. Heinemann V, Bosse D, Jehn U, Kahny B, Wachholz K, Debus A, Scholz P, Kolb H, Wilmanns W 1997. Pharmacokinetics of liposomal amphotericin B (Ambisome) in critically ill patients. Antimicrobial Agents and Chemotherapy 41(6):1275-1280.
29. Klibanov AL, Maruyama K, Torchilin VP, Huang L 1990. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett 268(1):235-237.
30. Arnold RD, Mager DE, Slack JE, Straubinger RM 2005. Effect of repetitive administration of Doxorubicin-containing liposomes on plasma pharmacokinetics and drug biodistribution in a rat brain tumor model. Clin Cancer Res 11(24 Pt 1):8856-8865.
31. Sharma US, Sharma A, Chau RI, Straubinger RM 1997. Liposome-mediated therapy of intracranial brain tumors in a rat model. Pharm Res 14(8):992-998.
32. Hofheinz R-D, Gnad-Vogt SU, Beyer U, Hochhaus A 2005. Liposomal encapsulated anti-cancer drugs. Anti-Cancer Drugs 16(7):691-707.
33. Yechezkel B 2001. Liposome application: problems and prospects. Current Opinion in Colloid & Interface Science 6(1):66-77.
34. Jain RK, Stylianopoulos T 2010. Delivering nanomedicine to solid tumors. Nat Rev Clin Oncol 7(11):653-664.

35. Bandak S, Goren D, Horowitz A, Tzemach D, Gabizon A 1999. Pharmacological studies of cisplatin encapsulated in long-circulating liposomes in mouse tumor models. Anti-Cancer Drugs 10(10):911-920.

36. Mouritsen OG, Jørgensen K 1998. A New Look at Lipid-Membrane Structure in Relation to Drug Research. Pharm Res 15(10):1507-1519.

37. Gordon AN, Fleagle JT, Guthrie D, Parkin DE, Gore ME, Lacave AJ 2001. Recurrent Epithelial Ovarian Carcinoma: A Randomized Phase III Study of Pegylated Liposomal Doxorubicin Versus Topotecan. J Clin Oncol 19(14):3312-3322.

38. Northfelt DW, Dezube BJ, Thommes JA, Miller BJ, Fischl MA, Friedman-Kien A, Kaplan LD, Du Mond C, Mamelok RD, Henry DH 1998. Pegylated-liposomal doxorubicin versus doxorubicin, bleomycin, and vincristine in the treatment of AIDS-related Kaposi's sarcoma: results of a randomized phase III clinical trial. J Clin Oncol 16(7):2445-2451.

39. O'Brien MER, Wigler N, Inbar M, Rosso R, Grischke E, Santoro A, Catane R, Kieback DG, Tomczak P, Ackland SP, Orlandi F, Mellars L, Alland L, Tendler C 2004. Reduced cardiotoxicity and comparable efficacy in a phase III trial of pegylated liposomal doxorubicin HCl (CAELYX™/Doxil®) versus conventional doxorubicin for first-line treatment of metastatic breast cancer. Ann Oncol 15(3):440-449.

40. Yatvin M, Weinstein J, Dennis W, Blumenthal R 1978. Design of liposomes for enhanced local release of drugs by hyperthermia. Science 202(4374):1290-1293.

41. Bondurant B, Mueller A, O'Brien DF 2001. Photoinitiated destabilization of sterically stabilized liposomes. Biochimica et Biophysica Acta (BBA) - Biomembranes 1511(1):113-122.
42. Hooks SB, Cummings BS 2008. Role of Ca2+-independent phospholipase A2 in cell growth and signaling. Biochemical Pharmacology 76(9):1059-1067.
43. Boyanovsky B, Webb N 2009. Biology of Secretory Phospholipase A2. Cardiovascular Drugs and Therapy 23(1):61-72.
44. Balsinde Js, Winstead MV, Dennis EA 2002. Phospholipase A2 regulation of arachidonic acid mobilization. FEBS Letters 531(1):2-6.
45. Singh N, Somvanshi RK, Sharma S, Dey S, Kaur P, Singh TP 2007. Structural elements of ligand recognition site in secretory phospho-lipase A2 and structure-based design of specific inhibitors. Curr Top Med Chem 7(8):757-764.
46. Bezzine S, Bollinger JG, Singer AG, Veatch SL, Keller SL, Gelb MH 2002. On the Binding Preference of Human Groups IIA and X Phospholipases A2 for Membranes with Anionic Phospholipids. J Biol Chem 277(50):48523-48534.
47. Lambeau G, Gelb MH 2008. Biochemistry and Physiology of Mammalian Secreted Phospholipases A2. Annu Rev Biochem 77(1):495-520.
48. Tribler L, Jensen LT, Jorgensen K, Brunner N, Gelb MH, Nielsen HJ, Jensen SS 2007. Increased expression and activity of group IIA and X secretory phospholipase

A2 in peritumoral versus central colon carcinoma tissue. Anticancer Res 27(5A):3179-3185.

49. Abe T, Sakamoto K, Kamohara H, Hirano Y-i, Kuwahara N, Ogawa M 1997. Group II phospholipase A2 is increased in peritoneal and pleural effusions in patients with various types of cancer. Int J Cancer 74(3):245-250.
50. Semple SC, Chonn A, Cullis PR 1998. Interactions of liposomes and lipid-based carrier systems with blood proteins: Relation to clearance behaviour in vivo. Advanced Drug Delivery Reviews 32(1-2):3-17.
51. Heiner AL, Gibbons E, Fairbourn JL, Gonzalez LJ, McLemore CO, Brueseke TJ, Judd AM, Bell JD 2008. Effects of Cholesterol on Physical Properties of Human Erythrocyte Membranes: Impact on Susceptibility to Hydrolysis by Secretory Phospholipase A2. Biophys J 94(8):3084-3093.
52. Liu F, Chong PL-G 1999. Evidence for a Regulatory Role of Cholesterol Superlattices in the Hydrolytic Activity of Secretory Phospholipase A2 in Lipid Membranes†. Biochemistry (Mosc) 38(13):3867-3873.
53. Parasassi T, Giusti AM, Raimondi M, Gratton E 1995. Abrupt modifications of phospholipid bilayer properties at critical cholesterol concentrations. Biophys J 68(5):1895-1902.
54. Singer AG, Ghomashchi F, Le Calvez C, Bollinger J, Bezzine S, Rouault M, Sadilek M, Nguyen E, Lazdunski M, Lambeau G, Gelb MH 2002. Interfacial Kinetic and

Binding Properties of the Complete Set of Human and Mouse Groups I, II, V, X, and XII Secreted Phospholipases A2. J Biol Chem 277(50):48535-48549.

55. Cunningham T, Yao L, Lucena A 2008. Product inhibition of secreted phospholipase A2 may explain lysophosphatidylcholines' unexpected therapeutic properties. J Inflammation 5(1):17.

56. Samoilova E, Pirkova A, Prokazova N, Korotaeva A 2006. Effect of serum from cardiovascular patients on catalytic activity of secretory phospholipase A2 (IIA). Bulletin of Experimental Biology and Medicine 142(5):581-582.

57. Billy D, Speijer H, Zwaal RFA, Hack EC, Hermens WT. 2002. Anticoagulant and membrane-degrading effects of secretory (non-pancreatic) phospholipase A2 are inhibited in plasma. ed., Stuttgart, Allemagne: Schattauer.

58. Peterson BL, Cummings BS 2006. A review of chromatographic methods for the assessment of phospholipids in biological samples. Biomed Chromatogr 20(3):227-243.

59. Kim H-Y, Wang T-CL, Ma Y-C 1994. Liquid Chromatography/Mass Spectrometry of Phospholipids using Electrospray Ionization. Analytical Chemistry 66(22):3977-3982.

ABBREVIATIONS

ADME	absorption, distribution, metabolism and excretion
Bligh-Dyer	BD
C31PC	1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphocholine
C3b	complement component 3b
CHE	cholesteryl hexadecyl ether
CHOL	cholesterol
EPR	enhanced permeability and retention
ESI-MS	electrospray ionization-mass spectrometry
PC	phosphatidylcholine
PG	phosphatidylglycerol
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PA	phosphatidic acid
PS	phosphatidylserine
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine
DSPA	1,2-distearoyl-*sn*-glycero-3-phosphatidic acid
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine
D_{70}-DSPC	1,2-distearoyl(deuterated 70)-*sn*-glycero-3-phosphatidylcholine
DSPE	1,2- distearoyl-*sn*-glycero-3-phosphatidylethanolamine
DSPG	1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol

DSPS	1,2-distearoyl-*sn*-glycero-3-phosphatidylserine
ESI-MS	electrospray ionization - mass spectrometry
FA	fatty acid
IBS	interfacial binding surface
LP	lysophospholipid
MS	mass spectrometry
PLA_2	phospholipases A_2
RES	reticuloendothelial system
$sPLA_2$	secretory phospholipase A_2
SSL	sterically-stabilized liposome
TLC	thin-layer chromatography

CHAPTER 2

SECRETORY PHOSPHOLIPASE A_2 RESPONSIVE LIPOSOMES

Guodong Zhu, Jason N. Mock, Ibrahim Aljuffali, Brian S. Cummings and Robert D. Arnold. 2011. *The AAPS Journal Pharm Sci*. 100(8):3146-3159.
Reprinted here with permission of the publisher.

ABSTRACT

Secretory phospholipase A_2 ($sPLA_2$) expression is increased in several cancers and can trigger release from some lipid-carriers. This study used electrospray ionization-mass spectrometry (ESI-MS) and release of 6-carboxyfluorescein (6-CF) to determine the effects of $sPLA_2$ on various liposome formulations. Different combinations of zwitterionic (DPPC, DSPC and DSPE) and anionic (DSPA, DSPG, DSPS and PEG-DSPE) phospholipids were examined. DSPG and DSPE were most susceptible to $sPLA_2$-mediated degradation compared to other phospholipids. Increased 6-CF release was observed after inclusion of 10 mole% DSPE and anionic lipids into different liposome formulations. Group IIa $sPLA_2$ mediated 6-CF release was less than Group III and relatively insensitive to cholesterol (CHOL), whereas CHOL reduced $sPLA_2$-mediated release. Inclusion of PEG-DSPE increased $sPLA_2$-mediated 6-CF release, whereas serum reduced lipid degradation and 6-CF release significantly ($p \leq 0.05$). These data demonstrate that ESI-MS and 6-CF release were useful at determining the selectivity of $sPLA_2$ and release from liposomes, that differences in the activity of different $sPLA_2$ isoforms exist, and that PEG-DSPE enhanced $sPLA_2$-mediated release of liposomal constituents. These findings will aid in the selection of lipids and optimization of the kinetics of drug release for the treatment of cancers and diseases of inflammation where $sPLA_2$ expression is over expressed.

INTRODUCTION

Lipid based nanoparticulate drug carriers, such as long-circulating sterically-stabilized liposomes (SSL), can encapsulate drugs stably and alter their pharmacokinetics radically compared to free drug and confer new pharmacological activity.[1-3] Differences in drug-carrier circulation half-life and tissue/tumor biodistribution are believed responsible for their improved antitumor activity and reduced toxicity.[4,5] The clinical advantage of nanoparticles, such as SSL, was established with the approval of Doxil® and other liposome formulations in the United States. Following administration, SSL can accumulate passively in solid tumors due to the enhanced permeability and retention (EPR) effect, mediated by defects in the vasculature and lack of functional lymphatics.[6,7] Despite increased accumulation of SSL into tumors, for many drugs the rate of drug release is not optimal and clinical utility is limited.[8] To overcome these barriers a variety of physical and physiological approaches are being examined to facilitate and control drug release; these include exposure to light,[9-11] heat[12-14] and use of ultrasound,[15,16].

In this study we choose to exploit pathophysiological differences in enzyme expression in normal and malignant tissues, *i.e.*, differences in secretory phospholipase A_2 ($sPLA_2$) expression,[17-19] to modulate drug release. Previous studies examined the effect of porcine pancreatic and bee-venom $sPLA_2$ on phosphatidylcholine vesicles with different physical attributes.[20] These studies suggested that $sPLA_2$ present in some

pathologies stimulate drug release from lipid based drug-carriers, such as liposomes (**Figure 2-1**).[21-23] In contrast, reduced expression of $sPLA_2$ in non-diseased tissues would limit lipid degradation and drug release, and is hypothesized to reduce toxicity (**Figure 2-1**).

Recent studies demonstrated that $sPLA_2$ expression and activity is increased in prostate[24-27], breast[28-30], and pancreatic[31-33] cancers. In prostate cancers Group IIa $sPLA_2$ is reported to be expressed at levels 22-fold greater than disease-free paired controls.[26,27,34] Increased $sPLA_2$ expression in cancer tissues correlates to increased immunohistochemical staining at the plasma membrane.[26] This coincides with the proposed mechanism of $sPLA_2$ action and suggests these increases are localized to the site of injury. Typically there is limited $sPLA_2$ in the systemic circulation, except in the case of septic shock or inflammation.

$sPLA_2$ are esterases that cleave glycerophospholipids, such as phosphatidylcholine, at the *sn*-2 ester bond, releasing a fatty acid and a lysophospholipid.[35] Many investigators have examined the effect of $sPLA_2$ on cellular membranes, lipid vesicles and lipid-based drug-carriers, but a majority of these studies were limited to bee venom (Group III), snake venom (Group Ia) or porcine pancreas (Group Ib) $sPLA_2$.[20,36,37] In contrast, there are few published studies that examined the effect of human $sPLA_2$ on the degradation of lipid-based drug carriers[23] or made comparisons between the different isoforms.

The overall goal of this research was to determine the effect of $sPLA_2$ on lipid profiles and on the rate and extent of drug release from lipid nanoparticulate drug-carriers. This was accomplished by examining the effect of different $sPLA_2$ on individual and mixed-lipid degradation using ESI-MS. This information was then used to formulate prototype $sPLA_2$ responsive liposomes (SPRL). The functional activity of $sPLA_2$ was determined by assessing the release of 6-carboxyfluorescin (6-CF), an aqueous soluble fluorescent marker, encapsulated in conventional, SSL, and SPRL formulations in buffer and serum. Understanding the time-course of $sPLA_2$-mediated lipid degradation and 6-CF release will accelerate the rational development of drug-carriers to achieve optimal drug exposure selectively, thus enhancing drug efficacy and minimizing non-target tissue toxicity.

MATERIALS AND METHODS

Chemical and reagents

Phospholipids, DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine), DSPC (1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine), DSPG (1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol), DSPS (1,2-distearoyl-*sn*-glycero-3-phosphatidylserine), DSPE (1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine), DSPA (1,2-distearoyl-*sn*-glycero-3-phosphatidic acid), DSPE-PEG (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000), and D_{70}-DSPC (deuterated DSPC)

were purchased from Avanti Polar Lipids Inc (Alabaster, AL). A listing of their structures, acyl-chain lengths, phase transition temperatures and charges under physiological conditions are provided in **Table 2-1**. 6-Carboxyfluorescein (6-CF) was purchased from Acros Organics (Geel, Belgium). Group III and Group IIa $sPLA_2$ were purchased from Cayman Chemical Company (Ann Arbor, MI) and Genway Biotech Inc (San Diego, CA). F-12K cell culture media and fetal bovine serum (FBS) were purchased from Hyclone (Rockford, Il). MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) reagent and cholesterol (CHOL) were purchased from Sigma (St. Louis, MO). Acetonitrile and methanol were of HPLC grade from Fisher Scientific (Pittsburgh, PA). All other chemicals and solvents were of analytical grade, obtained from commercial sources and used without further purification. All experiments used ultrapure water (> 3MΩ) obtained from a Millipore Milli-Q synthesis system (Billerica, MA).

Preparation of liposomes

Liposomes were prepared by hydration of thin-films followed by a freeze-thaw and a high-pressure extrusion process. A listing of all formulations prepared is presented in **Table 2-2**. Briefly, phospholipids, CHOL and/or DSPE-PEG in chloroform were mixed together, dried under vacuum at 55-65°C (water bath) for 25 min using a rotary evaporator (Büchi) and the thin-film was hydrated using normal saline or Tris buffer (5 mM, pH 7.4) for 15-20 min to achieve a final lipid concentration of 10 mM. The formulation then underwent seven liquid nitrogen freeze–thaw cycles above the phase

transition temperature of the primary lipid prior to extrusion (n=5) through double-stacked polycarbonate membranes (80 nm, Osmonics Inc.) using a Lipex extruder (Northern Lipids Inc.) at 65°C. All liposome formulations had a mean particle diameter of 80-110 nm, as determined using a Nicomp™ model 370 dynamic light scattering particle size analyzer (Santa Barbara, CA). Samples were stored under a nitrogen atmosphere at 4ºC and protected from light and used within 24 hr of preparation. Total phospholipid concentration of each formulation was quantified using an assay for inorganic phosphate following acid hydrolysis.[38]

sPLA$_2$–mediated degradation and lipid extraction

Phospholipid degradation was determined by combining each lipid sample (1 µmol lipid/mL), sPLA$_2$ (0-10 µg/mL), $CaCl_2$ (0-10 mM) in phosphate buffered saline, followed by incubation at 37°C for 0 to 24 hr. Phospholipids and metabolites were isolated using Bligh and Dyer extraction for ESI-MS analysis.[39] Briefly, following incubation, 100 µL of methanol was added to samples followed by the addition of 300 µL chloroform, vortexing and centrifugation at 2,500 × g for 5 min. The organic layer was removed and transferred to a clean test tube. The above extraction was repeated twice and the final organic phase was evaporated under nitrogen, and reconstituted in 1 mL chloroform and methanol (3:1 v/v). A 100 µL aliquot of the resultant organic solution was diluted in 900 µL chloroform and methanol (3:1 v/v) for ESI-MS analysis.

Profiling lipid degradation by ESI-MS

The stability of phospholipids treated with $sPLA_2$ was assessed by the loss of intensity of parent-ions and the appearance and increased intensity of the primary metabolites, *i.e.*, lysophospholipids and fatty acids. Analysis was performed on an Agilent high performance liquid chromatography – mass spectrometer (LC/MSD-Trap XCT Ultra Plus) system (Santa Clara, CA). The mobile phase was acetonitrile, methanol, and 0.1% ammonium formate (2:3:1 v/v/v). The flow rate was 0.15 mL/min and the injection volume was 5 µL. Nitrogen was used as a nebulizing gas at 25 psi and a drying gas at 8 psi. The drying temperature was 350°C. The capillary, capillary exit and skimmer potentials were 3,500, 150.3 and 40.0 V, respectively. The *m/z* range for scanning was 200 to 2,200. The positive-ion (+MS) mode was used to measure the intensity of the parent-ions, whereas the negative-ion (-MS) mode was used to measure the intensity of anionic lipids and fatty acid-ions (FA). Differences in ionization and extraction efficiencies of phospholipids and their FA metabolites prevent a simple comparison of signal intensities between individual lipids, however changes within a specific lipid can provide insights into its relative sensitivity to $sPLA_2$. Further, these differences were greatest in the anionic lipids (DSPA, DSPS, DSPG) compared to zwitterionic lipids (DPPC, DSPC and DSPE).

Quantification of lipid degradation by ESI-MS

The effect of $sPLA_2$ on individual and multi-lipid formulations was determined as above with the following alterations. Calibration curves of individual lipids, *e.g.*, DSPC (0.78-100 nmol) were prepared by diluting lipids in organic solvents (chloroform and methanol; 3:1 v/v) and spiked with internal standard D_{70}-DSPC (m/z 872.1). Liposome samples of the DSPC SSL formulation (0.05 µmol/mL) were prepared and then incubated in the presence or absence of $sPLA_2$ as described previously. Internal standard, D_{70}-DSPC, was added to each sample prior to Bligh and Dyer extraction, as described above. Calibration samples were prepared fresh by serial dilution of lipid standards in mobile phase or media containing serum. Standard curves were constructed by calculating the ratio of the analyte peak area to that of the internal standard, and plotting the ratio (ordinate) *versus* the theoretical concentration (abscissa); data was fit using weighted least squares; the inverse of the variance ($1/x^2$) of the observed data was used as the weighting factor. The standard curve was considered acceptable if greater than 90% of the standards had calculated accuracies within 15% of their theoretical value and no systematic deviations over the linear range were observed, precision had a coefficient of variation (CV) of $\leq$ 20%. The limit of quantification was determined experimentally as minimum concentration whose response was greater than five times baseline value, with a CV $\leq$ 20% and accuracy ± 20%. For multi-lipid formulations, calibration curves were prepared by mixing DSPC and DSPE or DSPC and DSPG at approximately the same

molar ratio as the formulations, *i.e.*, DSPC and DSPE or DSPG at 8:1 (lipid molar ratio). This was necessary because of the ion-suppression effect of the primary lipid, DSPC.

Preparation of 6-CF-loaded liposomes

Aliquots containing 100 mM 6-CF solution were prepared by dissolving 37.7 mg of 6-CF in 1 mL 5 mM Tris-HCl buffer (pH 7.4, adjusted with NaOH 1 M). The liposomes were prepared as explained above, except a solution of 100 mM 6-CF was used to hydrate the lipid thin-film instead of PBS or TRIS buffer. Free 6-CF was removed by size exclusion chromatography (Sephadex G-75, Pharmacia). The mobile phase for these separations consisted of 5 mM TRIS-HCl buffer (pH 7.4, adjusted with NaOH 1 M). Phosphate assays were performed prior to conduction of enzymatic reactions to determine the concentration of total phospholipids.[38]

Determination of $sPLA_2$–mediated 6-CF leakage and analysis

Release of 6-CF from liposome samples (10 μmol/mL diluted to 0.05 μmol/mL) was determined in the presence and absence of Group IIa or III $sPLA_2$ (0-10 μg/mL), $CaCl_2$ (0 to 10 mM) and Tris-HCl buffer (5 mM, pH 7.4). Fluorescent intensity of 6-CF was measured using a Synergy HT spectrofluorometer (Bio-Tek Instruments Inc.) at excitation and emission wavelengths of 480 and 510 nm, respectively. Fluorescence was detected at 10, 30 and 45 min, and 1, 2, 4, 8, 12, 24, 36, 48, 72 and 108 hr. After measurement at each time point, 10% (v/v) of Triton X-100 was added to the samples to calculate total 6-CF. Percentage of 6-CF leakage was calculated using the equation:

$$\text{Percentage} = [(F_t - F_0)/(F_{Triton} - F_0)] \times 100\%;$$

where, F_t represents the fluorescent intensity (FI) at a specific time point, F_0 represents FI at time zero, and F_{Triton} represents total fluorescent intensity after addition of Triton X-100.

Effect of serum on sPLA$_2$–mediated leakage

The effect of serum on the release of 6-CF and lipid metabolism was determined following incubation with F-12K with/without 10% FBS (v/v). Samples were kept at 37 °C and protected from light. At 0, 24 and 108 hr samples were removed and 6-CF fluorescence and lipid degradation by ESI-MS were quantified as described above.

Statistics

The mass spectrometry data are presented as the mean ± the standard deviation (STD), the 6-CF release are shown as the mean ± the standard error (SEM) of at least 3 separate experiments (n=5/study). Differences were determined following an analysis of variance for each data set using SAS software (SAS Institute, Cary, NC) followed by a Dunnett's t-test or a Student's t-test when comparisons involved a control and single variable. Differences were considered significant if the *p*-value ≤ 0.05.

RESULTS

ESI-MS profiling of phospholipids and sPLA$_2$-mediated metabolites

To demonstrate that ESI-MS can be used to profile sPLA$_2$ induced lipid degradation we exposed individual phospholipids and multi-lipid formulations to sPLA$_2$. Group III sPLA$_2$ was chosen for the initial studies as it is readily available, been used widely in the literature and known to be over expressed in select cancers and in arteriosclerosis.[40] The concentrations of sPLA$_2$ used in these studies ranged from 0.5 to 10 μg/mL. The lower range of these concentrations corresponds to the activity of sPLA$_2$ in tissues under normal physiological conditions (0.025 to 0.5 μg/mL). The higher range of these concentrations correspond to the activity of sPLA$_2$ in cancer and inflamed tissues, where sPLA$_2$ levels are increased 2- to 1,000-fold.[41-44]

DPPC, a zwitterionic lipid with two C-16 acyl chains, was exposed to solvent control and increasing concentrations of sPLA$_2$ for 1 hr and analyzed by ESI-MS in the positive-ion mode (**Figure 2-2**). The primary peak identified in control samples corresponded to un-degraded DPPC (734.5 *m/z*). A concentration-dependent decrease in the intensity of DPPC, parent peak at *m/z* 734.5, resulted in a greater than 50% decrease after exposure to 10 μg/mL sPLA$_2$ for 1 hr (**Figure 2-2**) and a concentration-dependent increase in the intensity of peaks corresponding to DPPC's 16:0-lyosphosphatidylcholine (LPC) at 496.3 *m/z* and the 16:0 fatty acid (FA) at 255.5 *m/z* (**Figure 2-2**). These data

demonstrated that $sPLA_2$ degrades DPPC in a concentration-dependent manner and that ESI-MS can be used to track $sPLA_2$-mediated degradation of phospholipids.

Selectivity of $sPLA_2$

The selectivity of Group III $sPLA_2$ (2.5 μg/mL) for individual zwitterionic and anionic phospholipids using ESI-MS was determined (**Table 2-3**). Although its established in the literature that shorter acyl chain and anionic lipids are more susceptible to $sPLA_2$-mediated degradation, it was unclear if this could be determined by ESI-MS and what was the relative selectivity for each of these lipids.[20] Further, it's unclear if these differences in lipid metabolism correlated to increased release of entrapped drug. As described above, degradation was indicated by the loss of intensity of *m/z* values corresponding to the parent phospholipid, in parallel with an increase in the intensity of the corresponding fatty acid.

Table 2-3 shows the change in signal intensity of different species of phospholipids exposed to solvent control or 2.5 μg/mL $sPLA_2$ for 6 and 24 hr. The presence of $sPLA_2$ decreased the signal intensity for parent phospholipids significantly ($p \leq 0.05$), and increased the signal intensity for each of their metabolites. This was observed for all phospholipids except DSPA.

Typically, phospholipids with a shorter chain length were more susceptible to $sPLA_2$-mediated degradation, *e.g.*, DPPC (C:16) *vs.* DSPC (C:18), **Table 2-3**. Among the phospholipids with 18 carbons (C:18) acyl chain length, DSPE and DSPG were the most

susceptible to $sPLA_2$-mediated degradation (**Table 2-3**). It should be noted that intensities of parent phospholipids between different groups (*i.e.*, zwitterionic and anionic) and their lysophospholipids and fatty acids are not directly comparable due to differences in ionization efficiency that exist using ESI-MS. These data agree with the published literature that $sPLA_2$ shows greater activity towards anionic than neutral phospholipids.[45,46]

$sPLA_2$-mediated release of 6-CF

Increased degradation of phospholipids does not indicate increased release of constituents entrapped in the aqueous core of liposomes. The effect on release was assessed by encapsulating 6-CF, which typically has low levels of fluorescence when encapsulated at high concentrations, but significantly higher levels when released and diluted in aqueous solutions.[47-49] As shown in **Figure 2-3A**, we assessed 6-CF release induced by Group III $sPLA_2$ (2.5 μg/mL) for 0-108 hr. Release of 6-CF from control samples increased slightly over time, less than 20% after 108 hr. Treatment of zwitterionic formulations (DSPC and DSPC:DSPE at 9:1 mole ratio) with Group III $sPLA_2$ induced a rapid and significant ($p \leq 0.05$) increase in the release of 6-CF, compared to non-treated controls. The addition of 10 mole% anionic phospholipids such as DSPA, DSPG to basic DSPC formulations accelerated $sPLA_2$-mediated 6-CF release compared to formulations using neutral lipids alone **Figure 2-3B**.

Effect of Ca^{2+} on $sPLA_2$ activity

The effect of Ca^{2+} on the release of 6-CF from plain DSPC liposome formulations was determined (**Figure 2-4**). In the absence of Ca^{2+}, 6-CF release increased linearly over time, reaching 50% release after about 30 hr. In contrast, the addition of Ca^{2+} (0.1-10 mM) resulted in a rapid, and concentration-dependent, increase in 6-CF release. For example, 50% 6-CF release was observed after 6 hr exposure with the addition of 1 mM Ca^{2+}, which is the physiological requirement for this enzyme. These data agree with previous studies demonstrating the Ca^{2+} requirement of $sPLA_2$.[35]

Effect of $sPLA_2$ on release of 6-CF from conventional liposomes and SSL formulations

The above data suggest that Group III $sPLA_2$ can degrade formulations containing anionic phospholipids and increase the release of 6-CF; however, SSL formulations used clinically contain polyethylene glycol (PEG) as well as CHOL. Thus, we determined the effect of CHOL and PEG on Group III $sPLA_2$-mediated (2.5 μg/mL) release of 6-CF from DSPC formulations (**Figure 2-5A**) and those containing 10 lipid mole% DSPG (**Figure 2-5B**). The concentration of CHOL and PEG chosen were similar to those used in Doxil® and other formulations that exhibit long-circulating properties.[50,51]

Control samples were stable over the time course measured as indicated by a low percentage of 6-CF release. Addition of CHOL tended to decrease 6-CF release approximately 20% after the initial release (0-5 hr), compared to formulations containing phospholipid alone and consistent with the literature.[45,46] Interestingly, addition of

PEG-DSPE increased the percentage of 6-CF release for all formulations. Inclusion of CHOL into PEGylated formulations did not alter the percentage of 6-CF release, but did decrease 6-CF release in the absence of PEG. These data demonstrate that Group III $sPLA_2$ enhances 6-CF release from PEGylated and CHOL -containing formulations.

Selectivity of group III vs. IIa $sPLA_2$

Figure 2-5 demonstrated the effects of CHOL and PEG on Group III $sPLA_2$-mediated release of 6-CF. However, Group III $sPLA_2$ is not the only $sPLA_2$ isoform involved in human diseases. In fact, Group IIa $sPLA_2$ is increased 5 to 20-fold in human breast and prostate cancer.[34] Thus, we tested the ability of human Group IIa $sPLA_2$ to release 6-CF from these formulations.

As demonstrated previously, incubation of liposomes with Group III $sPLA_2$ resulted in concentration-dependent release of 6-CF (**Figure 2-6A**). 6-CF release was reduced in formulations containing CHOL only, but was enhanced by the presence of PEG. Similar to Group III $sPLA_2$, Group IIa $sPLA_2$ also induced 6-CF release (**Figure 2-6B**). The level of 6-CF release was lower than that observed after Group III $sPLA_2$ treatment, but was still concentration-dependent. Further, the presence of PEG increased 6-CF release. In contrast, CHOL did not have as large of an inhibitory effect, compared to Group III $sPLA_2$. These data show that different isoforms of $sPLA_2$ display distinct selectivity with respect to release of 6-CF from these formulations.

Effect of group IIa sPLA$_2$ on 6-CF release

To test the ability of Group IIa sPLA$_2$ to mediate 6-CF release from SPRL formulations, we incubated 2.5 µg/mL Group IIa sPLA$_2$ with DSPC alone or in combination with CHOL, PEG or 10 mole% DSPE (**Figure 2-7**). The addition of Group IIa sPLA$_2$ increased 6-CF release from all formulations tested in a time-dependent manner (**Figures 2-7A** and **B**), including those containing cholesterol and PEG. The greatest increase was found in SSL (DSPC: CHOL:DSPE-PEG) and SPRL (DSPC:DSPE: CHOL:DSPE-PEG) formulations. The inclusion of CHOL did not reduce Group IIa sPLA$_2$ mediated release of 6-CF, as observed with Group III (**Figure 2-6**).

Effect of serum on sPLA$_2$–mediated lipid degradation and 6-CF release

The association of serum proteins with drug-carriers is known to alter their disposition and release profiles. The effect of serum, *i.e.,* FBS 10% (v/v), on sPLA$_2$-mediated lipid degradation and 6-CF release was determined. Pegylated (SSL), zwitterionic (+10 mol% DSPE), and anionic (+10 mol% DSPG) were examined. The presence of serum decreased sPLA$_2$-mediated degradation of all lipids after 24 and 108 hr exposure (**Table 2-4**). In all formulations the majority (>78%) of the lipids were degraded by 108 hr in the absence of serum. Evidence of FA and LPC formation was observed in all samples, suggesting lipid degradation, but not quantified. In the presence of serum DSPC degradation was reduced to 55-62%, while DSPE degraded 26% and DSPG 3%. Likewise, the presence of serum altered the release of 6-CF (**Figure 2-8**) compared to

release profiles in buffer alone (**Figures 2-3** and **2-5**). The formulation containing DSPG had the greatest release, followed by inclusion of DSPE compared to the SSL formulation. In the absence of serum (data not shown) release profiles were similar to those conducted in $sPLA_2$ buffer (**Figures 2-3** and **2-5**). Control samples in the absence of $sPLA_2$ were found to have limited (<2%) release over 108 hr.

DISCUSSION

Recent studies demonstrate that spatial and temporal differences in $sPLA_2$ expression exist in several diseases, including breast and prostate cancer and arteriosclerosis.[24,34,40] $sPLA_2$ expression appears to be localized to the diseased tissue, and not expressed systemically,[26,52] whereas in arteriosclerosis Group III $sPLA_2$ was expressed primarily in the arteriosclerotic lesion.[40] In this study we used ESI-MS to profile $sPLA_2$-medated degradation of various phospholipids. This information was then used to formulate various liposome formulations that displayed enhanced 6-CF release kinetics compared to conventional and long-circulating (SSL) formulations.

While studies have assessed the effect of phospholipases on drug release from liposomes,[21,45,53-55] few described approaches that rapidly determine the selectivity of different $sPLA_2$ isoforms for different phospholipids. The use of ESI-MS to rapidly profile phospholipid degradation directly to assist in the selection of lipids susceptible to $sPLA_2$-mediated degradation has not been described in the literature.

These studies focused on Group IIa and III $sPLA_2$. While other $sPLA_2$ exist, and are

worthy of study, both Group IIa and III are over expressed in several human diseases. The source for the Group III $sPLA_2$ was bee venom, which shares high sequence homology and structural similarities with human Group III $sPLA_2$. In fact, their central active site domains are essentially equal.[40] The use of non-human $sPLA_2$ for such studies is not unprecedented as other studies have used snake venom PLA_2, structurally similar to human $sPLA_2$, to evaluate $sPLA_2$ mediated effect on drug release.[54,56]

The goal of this study was not to directly compare Group IIa and III $sPLA_2$, rather to demonstrate that differences exist and that they could be distinguished in our assays and could be exploited for controlling drug release to optimize drug exposure. We showed marked differences in substrate preference and activity of $sPLA_2$ Group III and IIa against both SSL and SPRL liposomes. Group III $sPLA_2$ was more effective in inducing lipid degradation and 6-CF leakage than Group IIa under *in vitro* conditions. This finding is relevant because several studies investigating the use of $sPLA_2$ for nanoparticle degradation use Group III $sPLA_2$, which may be more specific for treating arteriosclerosis, but not optimal for treatment of cancer where Group IIa $sPLA_2$ is over expressed. Further, these findings suggest that liposome degradation and drug release *in vivo* is dependent on the $sPLA_2$ isoform expressed.

Model formulations were based on conventional SSL drug-carriers. We modified a variety of lipid-based formulations, including SSL, to enhance their degradation rates by incorporating zwitterionic and anionic phospholipids that would be responsive to specific

forms of $sPLA_2$–mediated degradation. Concentrations of anionic lipids greater than 10 mole% were not assessed in these formulations because they have been shown to have limited circulation time.[57,58] Further, liposomes with high content of anionic phospholipids may also activate the reticular endothelial system,[57,59] which reduces retention time in the circulation and lowers therapeutic efficacy. Other factors assessed included calcium concentration, CHOL and PEG content. The goal was to find a combination of these factors that displayed enhanced degradation in the presence of $sPLA_2$, compared to standard SSL.

ESI-MS was used to profile lipid susceptibility and to quantify the degradation of multiple phospholipids. In contrast to traditional lipid analysis, such as thin-layer chromatography and phosphorus analysis, ESI-MS has increased sensitivity, specificity, and speed of analysis. In this study we quantified and profiled individual and multiple phospholipids using a deuterated internal standard. It should be noted that it is possible to quantify FA and LPC from these formulations, with the use of deuterated standards, but it is difficult to do this in multi-phospholipid containing formulations due to differences in extraction efficiency and ion-suppression effects (data not show). Although the quantification of each FA and LPC was beyond the scope of this paper, we used ESI-MS to identify their formation, thus supporting our conclusion that the primary lipids were being degraded by $sPLA_2$.

Studies using ESI-MS showed that the acyl chain length, the polar head group and the organized structure of phospholipid species all alter $sPLA_2$-mediated degradation of liposomes. For example, DPPC (C:16) degradation was greater than that of DSPC (C:18). Further, phospholipids with anionic polar head groups tended to display greater amounts of degradation compared to those with neutral head groups. This agrees with the reported specificity of $sPLA_2$ for phospholipids[45,46] and suggests that changes in the rates of degradation are directly related to $sPLA_2$. This suggests that degradation profiles obtained by ESI-MS correlated to 6-CF release following $sPLA_2$-exposure.

It is important to note that degradation of phospholipids as assessed by ESI-MS may not always indicate that the lipid in question is a good candidate for formulations. For example, DPPC was highly susceptible to $sPLA_2$-medaited degradation due to its short chain length (C:16). However, it was not used for further study because of the greater permeability of DPPC, relative to DSPC (C:18) liposome formulations, which results from both its short acyl chain and relatively low phase-transition temperature (44°C).

With the above limitation in mind, data reported in this study demonstrate that ESI-MS is suitable for profiling lipid degradation rapidly and quantifying the degradation of individual lipids within multi-lipid formulations. ESI-MS is also excellent for identifying differences in structures of chemicals containing radiolabels or isotopes. We previously used it in biological tissues to distinguish differences in degradation of phospholipids labeled with deuterated standards.[60] Addition of such labels may permit the

use of ESI-MS to determine the time-course of lipid-based carrier degradation *in vivo*. This could significantly advance the study of mechanisms surrounding drug-release from liposomes.

$sPLA_2$-mediated lipid degradation and 6-CF release in serum supplemented media was greater in formulations containing anionic phospholipids, compared to those that contained neutral phospholipids. Furthermore, 6-CF release from SPRL formulations was Ca^{2+}-dependent, which agrees with the Ca^{2+}-dependence of $sPLA_2$ and supports the hypothesis that degradation of the formulations is $sPLA_2$-dependent. However, these data do not directly demonstrate that 6-CF release is mediated by lipid degradation alone. **Table 2-4** and **Figure 2-8** support this hypothesis. A significant increase in 6-CF release was observed in formulations containing DSPG, but ESI-MS suggested only 3 to 8% of this lipid was degraded after 24 and 108 hr exposure. Although it has been shown that anionic lipids are most susceptible to $sPLA_2$-mediated degradation, with multiple lipid formulations a greater percentage of DSPC was degraded compared to DSPG or DSPE. Interestingly, the release of 6-CF was greatest in formulations containing DSPG and DSPE, suggesting carrier-$sPLA_2$ interactions may also have a destabilizing effect and contribute to release kinetics. These data suggest that addition of small quantities of these "helper-lipids" could be used to modulate release.

An interesting finding from this study is that the effect of CHOL was dependent on the isoform of $sPLA_2$ involved. For Group III $sPLA_2$, CHOL reduced 6-CF release 20 to

50% in the PEG-free DSPC formulations. In contrast, inclusion of CHOL into PEG-free DSPG formulations increased 6-CF release approximately 30% when Group IIa $sPLA_2$ was used. This may result from differences in interactions of these individual $sPLA_2$ with CHOL itself, or from differences in the phospholipids used. These differences suggest that inclusion of membrane stabilizing CHOL and/or other constitutes, may impact $sPLA_2$ function significantly.

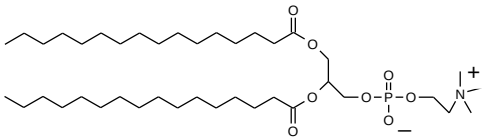
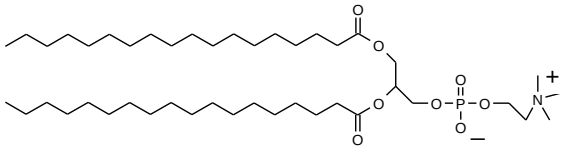
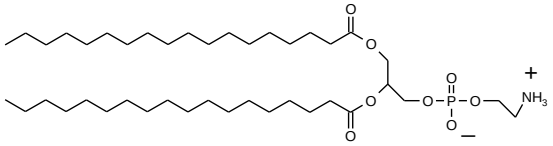
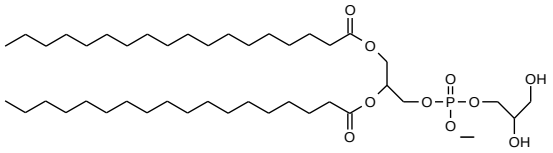
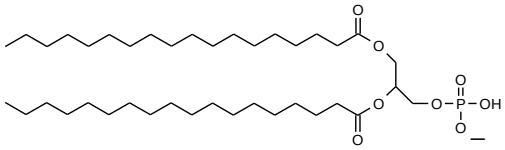
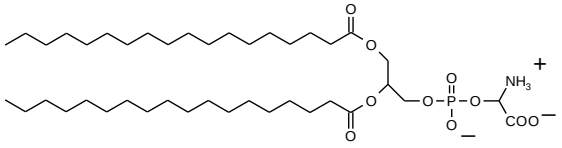
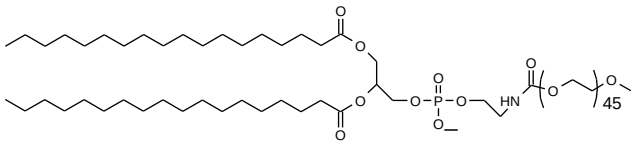
PEG enhanced $sPLA_2$-mediated 6-CF release independently of the phospholipid or $sPLA_2$ isoform used. This is somewhat surprising as PEG is hypothesized to alter the interaction of liposomes with serum proteins decreasing opsonization *in vivo*, thus enhancing their circulation half-life. Other studies showed decreased release of the fluorescent model drug calcein when PEG (10 lipid-mol%) was included in DPPC (C:16) formulations.[54] Data from this study suggest that PEGylation using PEG-DSPE inhibits neither $sPLA_2$ interaction, nor activity at liposome membranes. DSPE is zwitterionic under normal physiological conditions, but is anionic when bound covalently to PEG. We believe that the anionic nature of PEG-DSPE is responsible for this enhanced activity. It is possible that PEG alters the structure of the membrane surface permitting enhanced binding or activity of $sPLA_2$; however further studies are needed to test these hypotheses. Independent of the mechanism of action, this finding suggest formulations may be developed that incorporate the benefits of long-circulation half-lives (long, saturated acyl chains, CHOL, and PEG) with the use of $sPLA_2$-sensitive lipids to control drug release.

Pathological based control of drug release has the potential to increase drug efficacy and decrease overall systemic toxicity. Differences in the expression of different $sPLA_2$ isoforms may be exploited to selectively degrade and modulate the rate and extent of drug release from lipid based nanoparticulate drug-carriers. In this study we used ESI-MS to profile and quantify lipid degradation, and 6-CF release to engineer prototype SPRL formulations. Additional studies are needed to gain mechanistic insights into $sPLA_2$ activity to optimize their *in vivo* activity and further their clinical potential.

ACKNOWLEDGEMENTS

This research was funded in part by Georgia Cancer Coalition Distinguished Scholar Grants and a NIH NIBIB (EB08153) to BSC and RDA, a University of Georgia Graduate Fellowship Stipend Award to GZ, a University of King Saud Fellowship to IA, and an American Foundation for Pharmaceutical Education–Wyeth New Investigator Award to RDA.

Table 2-1. Phospholipid characteristics

Lipid (MW)	HG & ACL[a]	PT[b] (°C)	Charge[c]	Structure
DPPC (734.5)	choline C16:0	44.1	Z	
DSPC (790.9)	choline C18:0	55.1	Z	
DSPE (748.1)	ethanolamine C18:0	74.2	Z	
DSPG (779.1)	glycerol C18:0	54.2	A	
DSPA (705.0)	acid C18:0	75.4	A	
DSPS (792.1)	serine C18:0	79.0	A	
DSPE-PEG (2803.8)	ethanolamine (PEG_{2000}] C18:0	12.8	A	

[a] HG & ACL: head group and acyl chain length;

[b] PT**:** phase transition;

[c] Z**:** zwitterionic; A: anionic; PEG: polyethylene glycol

Table 2-2. Liposome formulations

Formulation	Lipid Mole Ratio
DPPC	1
DSPC	1
DSPA	1
DSPG	1
DSPE	1
DSPS	1
DSPC:DSPA	9:1
DSPC:DSPG	9:1
DSPC:DSPE	9:1
DSPC:DSPS	9:1
DPPC: CHOL	10:5
DSPC: CHOL	10:5
DSPC:DSPE: CHOL	9:1:5
DSPC:DSPG: CHOL	9:1:5
DSPC: CHOL:DSPE-PEG[a]	9:5:1
DSPC:DSPE: CHOL:DSPE-PEG[b]	8:1:5:1
DSPC:DSPG: CHOL:DSPE-PEG[c]	8:1:5:1

[a]This formulation was used as our prototypical long-circulating, sterically-stabilized liposome (SSL) formulation;

[b]This formulation was used as a prototypical SPRL formulations containing a zwitterionic lipid (DSPE);

[c]This formulation was used as a prototypical SPRL formulations containing an anionic lipid (DSPG).

Table 2-3. Effect of $sPLA_2$ on lipid signal intensity[a]

Lipid (m/z)	(hr)	Control Sample Intensity ($\times 10^6$)	+ $sPLA_2$ (2.5 μg/mL) Intensity ($\times 10^6$)	Percent Change
DPPC	**6**	74.8±1.6	29.1±8.4	38.9*
(734.5)	**24**	73.6± 0.7	28.7±0.5	39.0*
DSPC	**6**	81.0±2.8	70.6±3.4	87.2*
(790.9)	**24**	66.6±7.4	51.9±1.8	77.9*
DSPE	**6**	13.1±1.0	6.87±1.67	52.4*
(748.1)	**24**	12.1±0.1	2.73±0.07	22.6*
DSPG	**6**	2.70±0.30	1.74±0.19	37.0*
(779.1)	**24**	2.09±0.04	1.11±0.19	53.1*
DSPA	**6**	0.792±0.041	0.685±0.111	86.5
(705.0)	**24**	0.735±0.077	0.624±0.135	84.9
DSPS	**6**	0.769±0.055	0.592±0.093	77.0*
(792.1)	**24**	0.779±0.015	0.645±0.078	83.0*

Fatty Acid *(m/z)*	(hr)	Control Intensity ($\times 10^4$)	+ $sPLA_2$ (2.5 μg/mL) Intensity ($\times 10^4$)	Percent Change
DPPC-FA	**6**	1.08±0.13	20.0±1.9	1,850
(255.5)	**24**	0.739±0.134	19.8±4.4	2,670
DSPC-FA	**6**	1.26±0.19	8.32±3.7	659
(283.3)	**24**	1.11±0.46	18.6±2.5	1,680
DSPE-FA	**6**	1.42±0.16	28.6±2.4	2,010
(283.3)	**24**	1.56±0.07	35.3±0.9	2,260
DSPG-FA	**6**	1.18±0.21	18.4±2.0	1,560
(283.3)	**24**	1.16±0.26	41.7±4.6	3,600
DSPA-FA	**6**	1.47±0.05	1.87±0.15	127
(283.3)	**24**	1.06±0.11	1.48±0.15	140
DSPS-FA	**6**	0.763±0.034	3.62±0.17	475
(283.3)	**24**	0.926±0.238	9.61±0.95	1,040

[a]Data are represented as the mean intensity ± STD (n=3);

*Indicates a significant ($p \leq 0.05$) difference compared to control.

Table 2-4. Effect of serum on lipid degradation of different liposome formulations[a]

		0 hr		24 hr		108 hr	
	Lipid	**nM ± STD**	**FBS**	**nM ± STD**	**%[b]**	**nM ± STD**	**%[b]**
"SSL" DSPC: CHOL:PEG (9:5:1 mol ratio)	DSPC	21.1±0.3	-	8.99±0.63	-57%	0.344±0.010	-98%
			+	14.4±0.0	-32%*	9.54±0.20	-55%*
"Zwitterionic" DSPC:DSPE: CHOL:PEG (8:1:5:1 mole ratio)	DSPC	19.9±0.3	-	9.19±0.07	-58%	0.99±0.05	-95%
			+	16.9±0.1	-15%*	8.84±0.10	-56%*
	DSPE	1.60±0.08	-	1.02±0.06	-36%	ND	ND
			+	1.16±0.05	-28%*	1.19±0.03	-26%
"Anionic" DSPC:DSPG: CHOL:PEG (8:1:5:1 mole ratio)	DSPC	16.0±1.0	-	7.08±0.40	-56%	0.71±0.03	-96%
			+	11.3±0.5	-29%*	6.11±0.42	-62%*
	DSPG	0.91±0.04	-	0.54±0.02	-41%	0.202±0.029	-78%
			+	0.83±0.11	-8.8%*	0.886±0.124	-3%*

[a]FBS, fetal bovine serum 10% (v/v);

ND, not detectable;

[b]Represents percent change relative to initial (0 hr);

Data are represented as the mean concentration ± STD (n=3);

*Indicates a significant ($p<0.05$) difference compared to control.

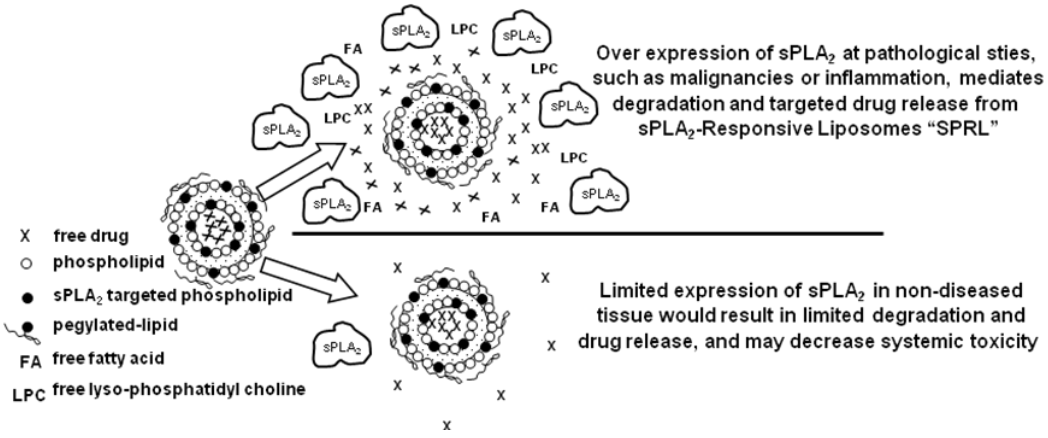


Figure 2-1. $sPLA_2$ facilitated drug release. This illustration describes the proposed mechanism of $sPLA_2$ mediated degradation and drug release from liposomes. Increased expression of $sPLA_2$ in solid tumors or other diseased tissue would enhance the degradation of phospholipids resulting in increased membrane permeability and drug release. Increased lipid degradation would be evidenced by increases in fatty acid (FA) and lysophospholipid lipid (LPC) levels. Limited expression of $sPLA_2$, in non-cancerous/diseased tissues would result in reduced lipid degradation and drug release.

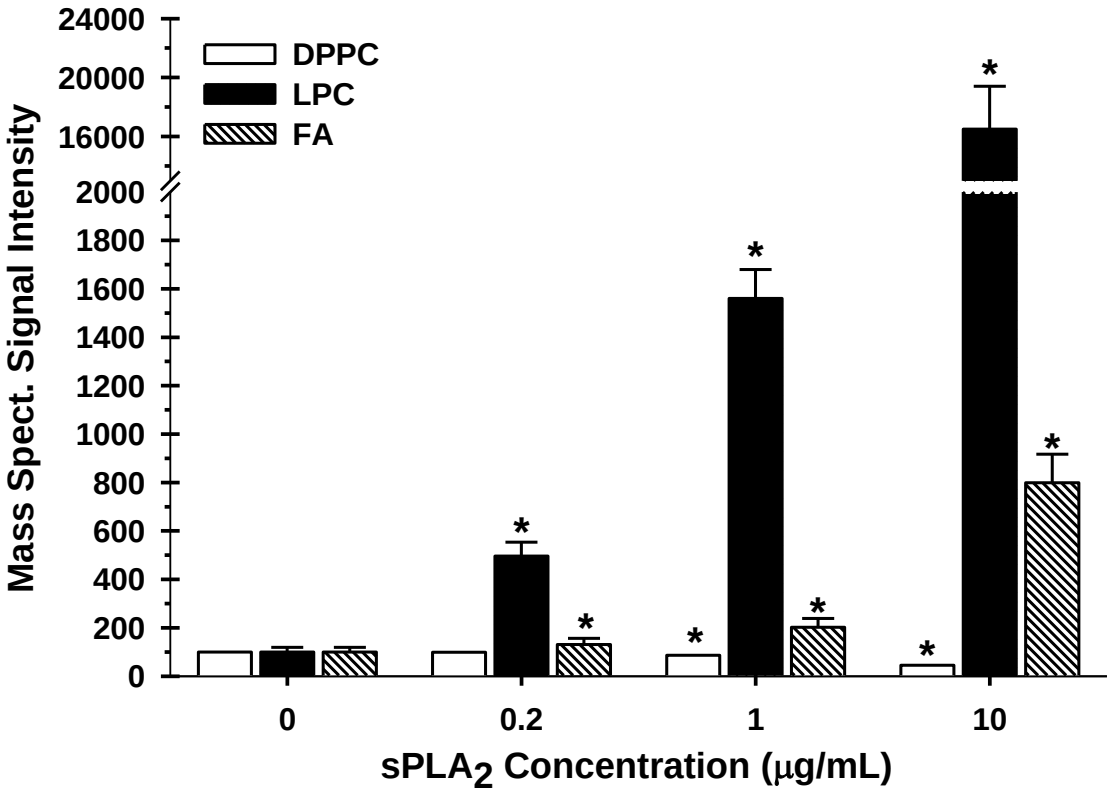


Figure 2-2. Concentration-dependence of $sPLA_2$ mediated degradation of DPPC. The effect of $sPLA_2$ on degradation of DPPC (734.5 *m/z*) and formation of 16:0-lyosphosphatidylcholine (LPC) at 496.3 *m/z* and the 16:0 fatty acid (FA), at 255.5 *m/z*, were determined by ESI-MS. Data were normalized to signal intensity of DPPC, LPC and FA in control samples. A concentration-dependent decrease in signal intensity of DPPC and increases in LPC and FA were observed. Data are represented as the mean intensity ± STD (n=3). *Indicates a significant ($p \leq 0.05$) difference compared to control.

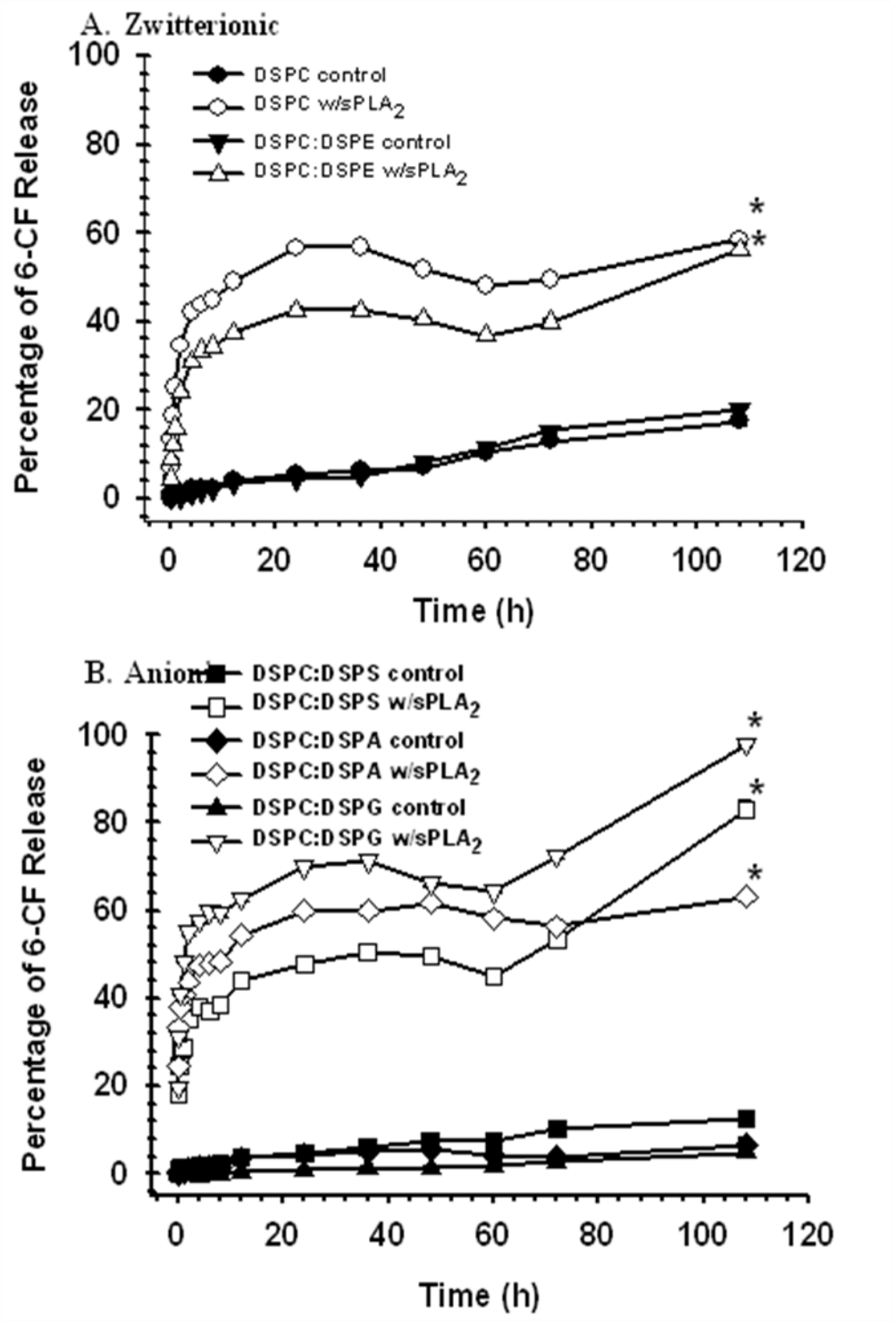

A. Zwitterionic
DSPC control
DSPC w/sPLA2
DSPC:DSPE control
DSPC:DSPE w/sPLA2
Percentage of 6-CF Release
Time (h)
B. Anionic
DSPC:DSPS control
DSPC:DSPS w/sPLA2
DSPC:DSPA control
DSPC:DSPA w/sPLA2
DSPC:DSPG control
DSPC:DSPG w/sPLA2
Percentage of 6-CF Release
Time (h)

Figure 2-3. Effect of Group III sPLA$_2$-mediated 6-CF release from A) zwitterionic (neutral) formulations and B) anionic formulations. **A)** Percentage of 6-CF release from DSPC and DSPC:DSPE (9:1 mole ratio) formulations was assessed by fluorescent intensity changes in the media. Liposome samples (total phospholipid: 0.05 µg/mL) were treated with 0 (control) or 2.5 µg/mL Group III sPLA$_2$ for 0-108 hr at 25ºC. Fluorescence intensity was obtained by measuring the fluorescence at excitation and emission wavelengths of 480 and 510 nm, respectively. **B)** Percentage of 6-CF release from anionic formulations containing DSPC with anionic phospholipids DSPA, DSPS or DSPG (at 9:1 mole ratio). Data are represented as the mean ± SEM of a least 3 separate experiments (n=5/study). *Indicates significant ($p \leq 0.05$) difference compared to control.

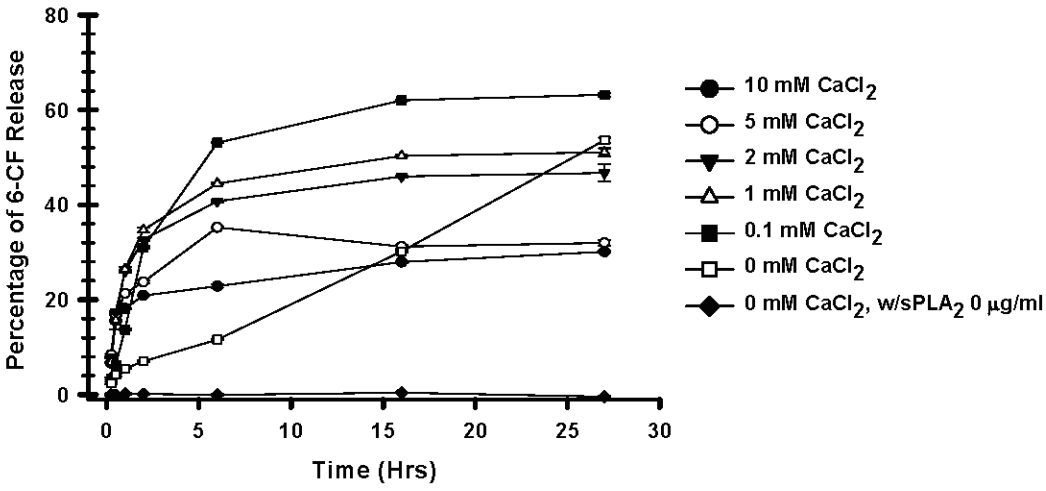


Figure 2-4. Ca^{2+}-dependence of Group III $sPLA_2$-mediated 6-CF release from plain DSPC formulations. Percentage of 6-CF release from plain DSPC liposome formulations was assessed by fluorescent intensity changes in the media. Liposome samples (total phospholipid: 0.05 µg/mL) were treated with 0 or 2.5 µg/mL Group III $sPLA_2$ for 0-27 hr at 25ºC at the presence of 0-10mM $CaCl_2$. Fluorescence intensity was obtained by measuring the fluorescence at excitation and emission wavelengths of 480 and 510 nm, respectively. Data are represented as the mean ± SEM of a least 3 separate experiments. *Indicates significant ($P \leq 0.05$) difference compared to control.

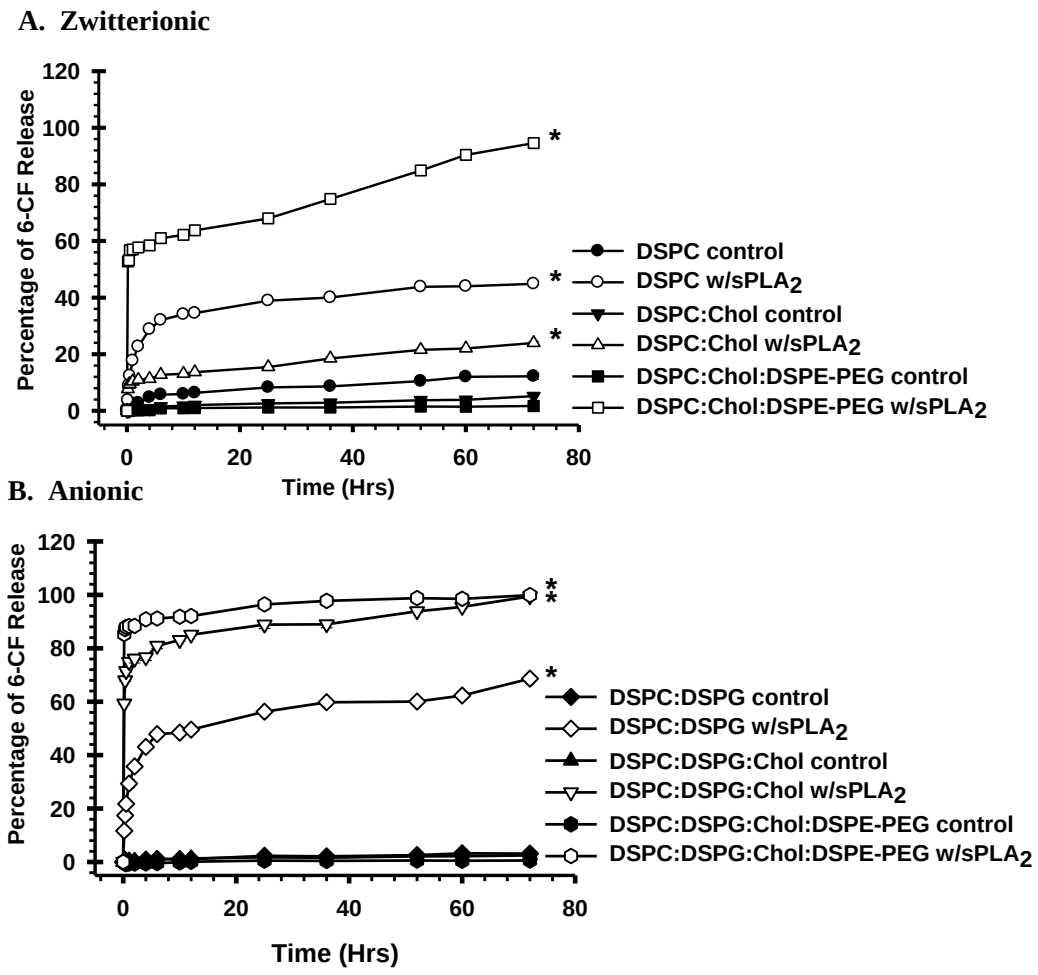


Figure 2-5. Effect of PEG and CHOL on Group III $sPLA_2$-mediated 6-CF release from modified SSL liposome formulations. The effect of CHOL and DSPE-PEG on $sPLA_2$-mediated (2.5 µg/mL Group III) release of 6-CF from DSPC liposomes (total phospholipid: 0.05 µg/mL) without **(A)** or with **(B)** incorporation of 10 mole% DSPG was determined by fluorescent intensity changes in the media for 0-72 hr at 25ºC. Fluorescence intensity was obtained by measuring the fluorescence at excitation and emission wavelengths of 480 and 510 nm, respectively. Data are represented as the mean ± SEM of a least 3 separate experiments (n=5/study). *Indicates significant ($p \leq 0.05$) difference compared to control.

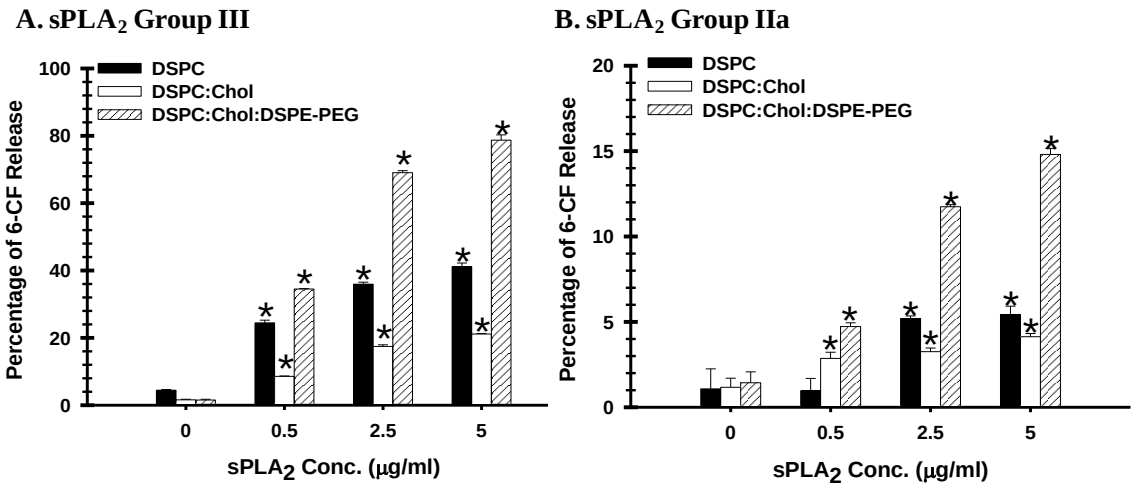


Figure 2-6. Comparison of Group IIa and III $sPLA_2$-mediated release of 6-CF from liposomes. The effect of CHOL and DSPE-PEG on DSPC formulations (total phospholipid: 0.05 μg/mL) on 6-CF release was determined after exposure to either Group III (**A**) or IIa (**B**) $sPLA_2$ (0 to 5 μg/mL) for 6 hr at 25ºC. 6-CF release was assessed by fluorescent intensity changes in the media at excitation and emission wavelengths of 480 and 510 nm, respectively. Data are represented as the mean ± SEM of at least 3 separate experiments (n=5/study). *Indicates significant ($p \leq 0.05$) difference compared to control.

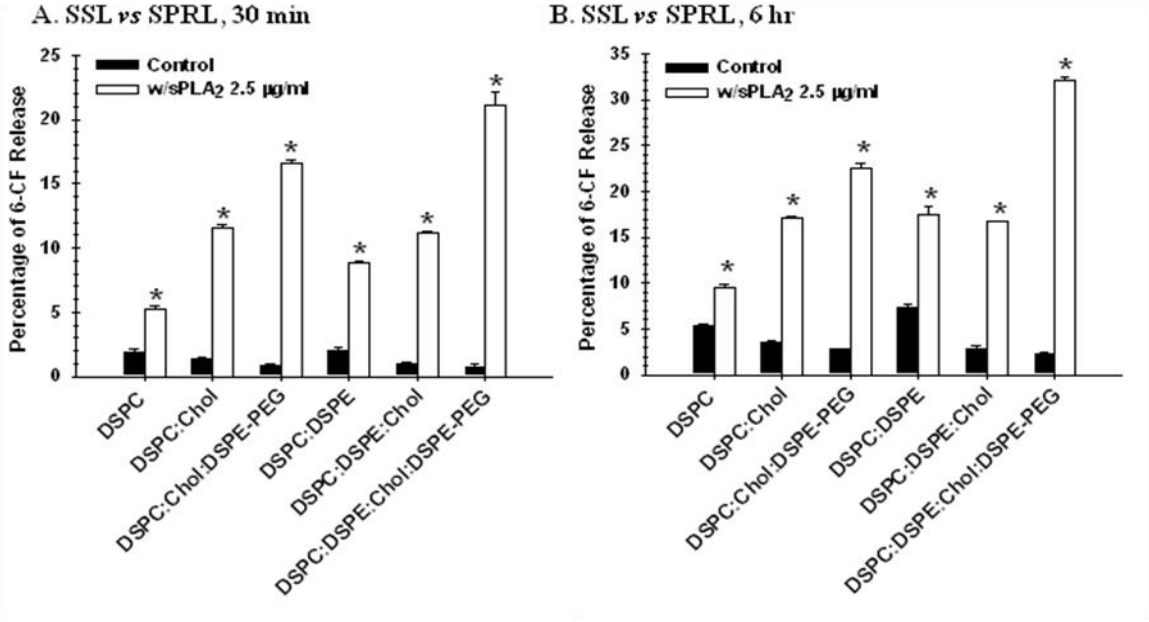


Figure 2-7. Comparison of Group IIa sPLA$_2$-mediated release of 6-CF from standard liposome *vs.* SPRL preparations. Formulations (total phospholipid: 0.05 µg/mL) consisting of DSPC, DSPC:DSPE alone, DSPC or DSPC:DSPE plus CHOL, or DSPC or DSPC:DSPE with CHOL and DSPE-PEG were exposed to 2.5 µg/mL of Group IIa sPLA$_2$ for 30 min (**A**) or 6 hr (**B**) at 25ºC. 6-CF was assessed by fluorescent intensity changes in the media at excitation and emission wavelengths of 480 and 510 nm, respectively. Data are represented as the mean ± SEM of at least 3 separate experiments (n=5/study). *Indicates significant ($p \leq 0.05$) difference compared to control.

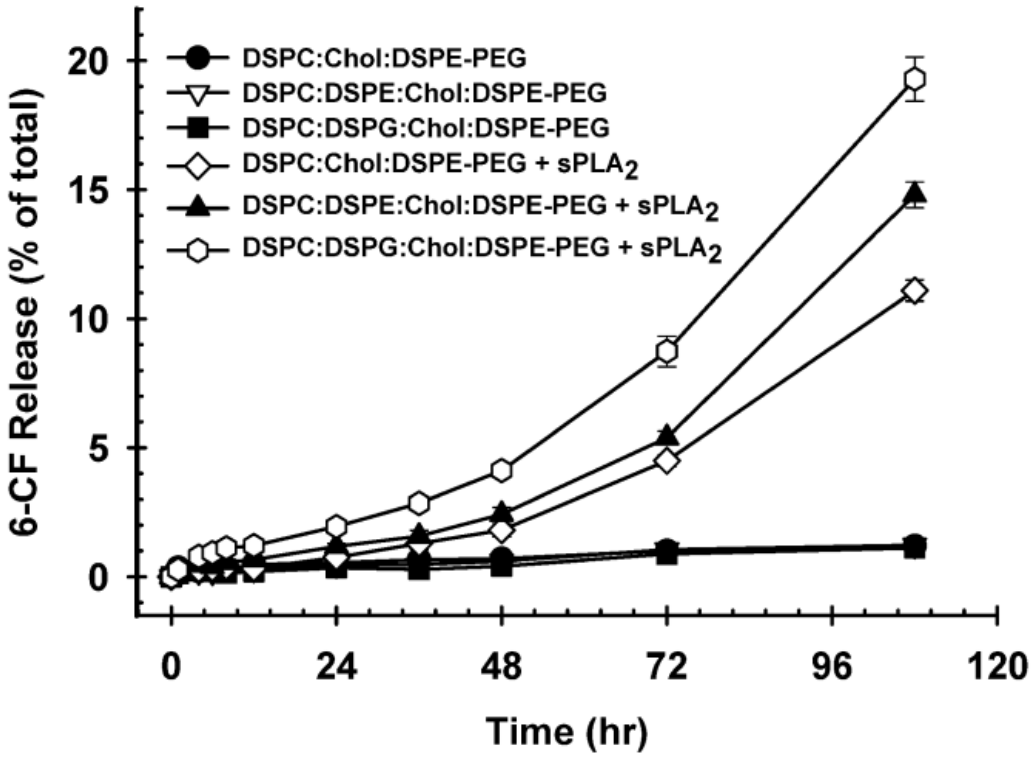


Figure 2-8. Effect of serum on 6-CF release. The effect of FBS and $sPLA_2$ on the release of 6-CF from SSL (DSPC:CHOL:DSPE-PEG), zwitterionic (DSPC:DSPE: CHOL:DSPE-PEG) and anionic (DSPC:DSPG: CHOL:DSPE-PEG) formulations at 37 ^{o}C. Fluorescence intensity was obtained by measuring the fluorescence at excitation and emission wavelengths of 480 and 510 nm, respectively. Data are represented as the mean ± SEM of a least 3 separate experiments (n=5/study). *Indicates significant ($p \leq 0.05$) difference compared to DSPC: CHOL:DSPE-PEG (SSL) formulation.

REFERENCES

1. Gabizon A 1989. Liposomes as a drug delivery system in cancer chemotherapy. Horizons in Biochemistry & Biophysics 9:185-211.
2. Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, Redemann C 1991. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci USA 88(24):11460-11464.
3. Muggia FM 2001. Liposomal encapsulated anthracyclines: new therapeutic horizons. Current Oncol 3:156-162.
4. Lasic DD, Martin FJ, Gabizon A, Huang SK, Papahadjopoulos D 1991. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. Biochim Biophys Acta 1070(1):187-192.
5. Sharma US, Sharma A, Chau RI, Straubinger RM 1997. Liposome-mediated therapy of intracranial brain tumors in a rat model. Pharm Res 14(8):992-998.
6. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K 2000. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release 65(1-2):271-284.
7. Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D, Jain RK 1994. Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. Cancer Res 54(13):3352-3356.

8. Straubinger RM, Arnold RD, Zhou R, Mazurchuk R, Slack JE 2004. Antivascular and antitumor activities of liposome-associated drugs. Anticancer Res 24(2A):397-404.
9. Bondurant B, Mueller A, O'Brien DF 2001. Photoinitiated destabilization of sterically stabilized liposomes. Biochim Biophys Acta 1511(1):113-122.
10. Shum P, Kim JM, Thompson DH 2001. Phototriggering of liposomal drug delivery systems. Adv Drug Deliv Rev 53(3):273-284.
11. Spratt T, Bondurant B, O'Brien DF 2003. Rapid release of liposomal contents upon photoinitiated destabilization with UV exposure. Biochim Biophys Acta 1611(1-2):35-43.
12. Yatvin MB, Weinstein JN, Dennis WH, Blumenthal R 1978. Design of liposomes for enhanced local release of drugs by hyperthermia. Science 202(4374):1290-1293.
13. Kong G, Anyarambhatla G, Petros WP, Braun RD, Colvin OM, Needham D, Dewhirst MW 2000. Efficacy of liposomes and hyperthermia in a human tumor xenograft model: importance of triggered drug release. Cancer Res 60(24):6950-6957.
14. Needham D, Anyarambhatla G, Kong G, Dewhirst MW 2000. A new temperature-sensitive liposome for use with mild hyperthermia: characterization and testing in a human tumor xenograft model. Cancer Res 60(5):1197-1201.

15. Huang SL, MacDonald RC 2004. Acoustically active liposomes for drug encapsulation and ultrasound-triggered release. Biochim Biophys Acta 1665(1-2):134-141.

16. Schroeder A, Avnir Y, Weisman S, Najajreh Y, Gabizon A, Talmon Y, Kost J, Barenholz Y 2007. Controlling liposomal drug release with low frequency ultrasound: mechanism and feasibility. Langmuir 23(7):4019-4025.

17. Hafez IM, Cullis PR 2001. Roles of lipid polymorphism in intracellular delivery. Adv Drug Deliv Rev 47(2-3):139-148.

18. Goni FM, Alonso A 2000. Membrane fusion induced by phospholipase C and sphingomyelinases. Biosci Rep 20(6):443-463.

19. Ruiz-Arguello MB, Goni FM, Alonso A 1998. Vesicle membrane fusion induced by the concerted activities of sphingomyelinase and phospholipase C. J Biol Chem 273(36):22977-22982.

20. Wilschut JC, Regts J, Westenberg H, Scherphof G 1978. Action of phospholipases A2 on phosphatidylcholine bilayers. Effects of the phase transition, bilayer curvature and structural defects. Biochim Biophys Acta 508(2):185-196.

21. Andresen TL, Davidsen J, Begtrup M, Mouritsen OG, Jorgensen K 2004. Enzymatic release of antitumor ether lipids by specific phospholipase A2 activation of liposome-forming prodrugs. J Med Chem 47(7):1694-1703.

22. Andresen TL, Jensen SS, Kaasgaard T, Jorgensen K 2005. Triggered activation and release of liposomal prodrugs and drugs in cancer tissue by secretory phospholipase A2. Curr Drug Deliv 2(4):353-362.

23. Jensen SS, Andresen TL, Davidsen J, Hoyrup P, Shnyder SD, Bibby MC, Gill JH, Jorgensen K 2004. Secretory phospholipase A2 as a tumor-specific trigger for targeted delivery of a novel class of liposomal prodrug anticancer etherlipids. Mol Cancer Ther 3(11):1451-1458.

24. Dong Q, Patel M, Scott KF, Graham GG, Russell PJ, Sved P 2006. Oncogenic action of phospholipase A2 in prostate cancer. Cancer Lett 240(1):9-16.

25. Kallajoki M, Alanen KA, Nevalainen M, Nevalainen TJ 1998. Group II phospholipase A2 in human male reproductive organs and genital tumors. Prostate 35(4):263-272.

26. Jiang J, Neubauer BL, Graff JR, Chedid M, Thomas JE, Roehm NW, Zhang S, Eckert GJ, Koch MO, Eble JN, Cheng L 2002. Expression of Group IIA Secretory Phospholipase A2 Is Elevated in Prostatic Intraepithelial Neoplasia and Adenocarcinoma. Am J Pathol 160(2):667-671.

27. Graff JR, Konicek BW, Deddens JA, Chedid M, Hurst BM, Colligan B, Neubauer BL, Carter HW, Carter JH 2001. Expression of group IIa secretory phospholipase A2 increases with prostate tumor grade. Clin Cancer Res 7(12):3857-3861.

28. Yamashita S, Yamashita J, Ogawa M 1994. Overexpression of group II phospholipase A2 in human breast cancer tissues is closely associated with their malignant potency. Br J Cancer 69(6):1166-1170.

29. Yamashita S, Ogawa M, Sakamoto K, Abe T, Arakawa H, Yamashita J 1994. Elevation of serum group II phospholipase A2 levels in patients with advanced cancer. Clin Chim Acta 228(2):91-99.

30. Yamashita S, Yamashita J, Sakamoto K, Inada K, Nakashima Y, Murata K, Saishoji T, Nomura K, Ogawa M 1993. Increased expression of membrane-associated phospholipase A2 shows malignant potential of human breast cancer cells. Cancer 71(10):3058-3064.

31. Kiyohara H, Egami H, Kako H, Shibata Y, Murata K, Ohshima S, Sei K, Suko S, Kurano R, Ogawa M 1993. Immunohistochemical localization of group II phospholipase A2 in human pancreatic carcinomas. Int J Pancreatol 13(1):49-57.

32. Kuopio T, Ekfors TO, Nikkanen V, Nevalainen TJ 1995. Acinar cell carcinoma of the pancreas. Report of three cases. Apmis 103(1):69-78.

33. Oka Y, Ogawa M, Matsuda Y, Murata A, Nishijima J, Miyauchi K, Uda K, Yasuda T, Mori T 1990. Serum immunoreactive pancreatic phospholipase A2 in patients with various malignant tumors. Enzyme 43(2):80-88.

34. Sved P, Scott KF, McLeod D, King NJ, Singh J, Tsatralis T, Nikolov B, Boulas J, Nallan L, Gelb MH, Sajinovic M, Graham GG, Russell PJ, Dong Q 2004. Oncogenic

action of secreted phospholipase A2 in prostate cancer. Cancer Res 64(19):6934-6940.

35. Cummings BS, McHowat J, Schnellmann RG 2000. Phospholipase A(2)s in cell injury and death. J Pharmacol Exp Ther 294(3):793-799.
36. Wilschut JC, Regts J, Scherphof G 1979. Action of phospholipase A2 on phospholipid vesicles. Preservation of the membrane permeability barrier during asymmetric bilayer degradation. FEBS Lett 98(1):181-186.
37. Menashe M, Romero G, Biltonen RL, Lichtenberg D 1986. Hydrolysis of dipalmitoylphosphatidylcholine small unilamellar vesicles by porcine pancreatic phospholipase A2. J Biol Chem 261(12):5328-5333.
38. Bartlett GR 1959. Phosphorus assay in column chromatography. J Biol Chem 234(3):466-468.
39. Bligh EG, Dyer WJ 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37(8):911-917.
40. Sato H, Kato R, Isogai Y, Saka G-i, Ohtsuki M, Taketomi Y, Yamamoto K, Tsutsumi K, Yamada J, Masuda S, Ishikawa Y, Ishii T, Kobayashi T, Ikeda K, Taguchi R, Hatakeyama S, Hara S, Kudo I, Itabe H, Murakami M 2008. Analyses of Group III Secreted Phospholipase A2 Transgenic Mice Reveal Potential Participation of This Enzyme in Plasma Lipoprotein Modification, Macrophage Foam Cell Formation, and Atherosclerosis. J Biol Chem 283(48):33483-33497.

41. Faas FH, Dang AQ, Pollard M, Hong XM, Fan K, Luckert PH, Schutz M 1996. Increased phospholipid fatty acid remodeling in human and rat prostatic adenocarcinoma tissues. J Urol 156(1):243-248.

42. Nevalainen TJ, Eskola JU, Aho AJ, Havia VT, Lovgren TN, Nanto V 1985. Immunoreactive phospholipase A2 in serum in acute pancreatitis and pancreatic cancer. Clin Chem 31(7):1116-1120.

43. Birts CN, Barton CH, Wilton DC 2008. A Catalytically Independent Physiological Function for Human Acute Phase Protein Group IIA Phospholipase A2: CELLULAR UPTAKE FACILITATES CELL DEBRIS REMOVAL. J Biol Chem 283(8):5034-5045.

44. Groeneveld AB, Tacx AN, Bossink AW, van Mierlo GJ, Hack CE 2003. Circulating inflammatory mediators predict shock and mortality in febrile patients with microbial infection. Clin Immunol 106(2):106-115.

45. Leidy C, Linderoth L, Andresen TL, Mouritsen OG, Jorgensen K, Peters GH 2006. Domain-induced activation of human phospholipase A2 type IIA: local versus global lipid composition. Biophys J 90(9):3165-3175.

46. Buckland AG, Wilton DC 2000. Anionic phospholipids, interfacial binding and the regulation of cell functions. Biochim Biophys Acta 1483(2):199-216.

47. Bajoria R, Sooranna SR, Contractor SF 1997. Endocytotic uptake of small unilamellar liposomes by human trophoblast cells in culture. Hum Reprod 12(6):1343-1348.

48. Kokkona M, Kallinteri P, Fatouros D, Antimisiaris SG 2000. Stability of SUV liposomes in the presence of cholate salts and pancreatic lipases: effect of lipid composition. Eur J Pharm Sci 9(3):245-252.

49. Weinstein JN, Yoshikami S, Henkart P, Blumenthal R, Hagins WA 1977. Liposome-cell interaction: transfer and intracellular release of a trapped fluorescent marker. Science 195(4277):489-492.

50. Gabizon A, Shiota R, Papahadjopoulos D 1989. Pharmacokinetics and tissue distribution of doxorubicin encapsulated in stable liposomes with long circulation times. J Natl Cancer Inst 81(19):1484-1488.

51. Arnold RD, Mager DE, Slack JE, Straubinger RM 2005. Effect of repetitive administration of Doxorubicin-containing liposomes on plasma pharmacokinetics and drug biodistribution in a rat brain tumor model. Clin Cancer Res 11(24 Pt 1):8856-8865.

52. Putz T, Ramoner R, Gander H, Rahm A, Bartsch G, Thurnher M 2006. Antitumor action and immune activation through cooperation of bee venom secretory phospholipase A2 and phosphatidylinositol-(3,4)-bisphosphate. Cancer Immunol Immunother 55(11):1374-1383.

53. Andresen TL, Jensen SS, Jorgensen K 2005. Advanced strategies in liposomal cancer therapy: problems and prospects of active and tumor specific drug release. Prog Lipid Res 44(1):68-97.

54. Davidsen J, Jorgensen K, Andresen TL, Mouritsen OG 2003. Secreted phospholipase A(2) as a new enzymatic trigger mechanism for localised liposomal drug release and absorption in diseased tissue. Biochim Biophys Acta 1609(1):95-101.

55. Davidsen J, Mouritsen OG, Jorgensen K 2002. Synergistic permeability enhancing effect of lysophospholipids and fatty acids on lipid membranes. Biochim Biophys Acta 1564(1):256-262.

56. Davidson FF, Dennis EA 1990. Amino acid sequence and circular dichroism of Indian cobra (Naja naja naja) venom acidic phospholipase A2. Biochim Biophys Acta 1037(1):7-15.

57. Drummond DC, Meyer O, Hong K, Kirpotin DB, Papahadjopoulos D 1999. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacol Rev 51(4):691-743.

58. Gabizon A, Papahadjopoulos D 1988. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc Natl Acad Sci U S A 85(18):6949-6953.

59. Papahadjopoulos D, Gabizon A 1990. Liposomes designed to avoid the reticuloendothelial system. Prog Clin Biol Res 343:85-93.

60. Peterson BL, Cummings BS 2006. A review of chromatographic methods for the assessment of phospholipids in biological samples. Biomed Chromatogr 20(3):227-243.

ABBREVIATIONS

6-CF	6-carboxyfluorescein
CHOL	cholesterol
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine
DSPA	1,2-distearoyl-*sn*-glycero-3-phosphatidic acid
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine
D_{70}-DSPC	1,2-distearoyl(deuterated 70)-*sn*-glycero-3-phosphatidylcholine
DSPE	1,2- distearoyl-*sn*-glycero-3-phosphatidylethanolamine
DSPE-PEG	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000
DSPG	1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol
DSPS	1,2-distearoyl-*sn*-glycero-3-phosphatidylserine
ESI-MS	electrospray ionization - mass spectrometry
FA	fatty acid
FBS	fetal bovine serum
LPC	lysophospholipid
PEG	polyethylene glycol
PLA_2	phospholipase A_2
SPRL	secretory phospholipase A_2 responsive liposomes
SEM	standard error of the mean
SSL	sterically-stabilized liposome
$sPLA_2$	secretory phospholipase A_2

CHAPTER 3

SYNTHESIS OF LIPIDS FOR DEVELOPMENT OF MULTIFUNTIONAL LIPID-BASED DRUG CARRIERS

Guodong Zhu, Yahya Alhamhoom, Brian S. Cummings and Robert D. Arnold. 2011. *Bioorganic and Medicinal Chemistry Letters*. 21(21):6370-6375.
Reprinted here with permission of the publisher.

ABSTRACT

A simple approach to steroselectively synthesize phospholipids to modulate drug release and track lipid-based particulate drug-carriers is described. We synthesized two ether lipids, **1** *1-O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3- phosphocholine (C31PC) and **2** *1-O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphomethanol (C31PM), and examined their ability to alter enzymatically triggered release of 6-carboxyfluorescein from liposomes incubated in TRIS buffer or fetal bovine serum solutions. Further, we demonstrated that odd-chain lipids, *e.g.*, C31PC, could be identified in rat plasma without interference of endogenous lipids. This approach can be adapted to synthesize a variety of lipids for use in developing and optimizing multifunctional drug-carriers**.**

INTRODUCTION

Lipid-based drug carriers, such as liposomes, can alter the pharmacokinetics and improve the efficacy of a variety of therapeutic agents[1]. However, mechanisms to "control" or "tune" their drug release kinetics and track drug-carrier disposition *in vivo* are limited. A variety of physical and physiological approaches have been examined to control drug release from drug carriers, including exposure to light[2-4], heat[5-7]and use of ultrasound[8,9]. However, the clinical use of these strategies has been limited because of their ability to induce rapid "burst" drug release profiles and the inaccessibility of some tissue to light or heat. Further, these strategies are targeted to primary tumors, whereas metastatic lesions may be undetectable.

Enzymatic approaches exploiting elevated expression of enzymes, such as esterases, in some disease states have the potential to modulate drug-release selectively. Secretory phospholipase A_2 ($sPLA_2$) is an esterase that preferentially hydrolyzes glycerophospholipids, such as phosphatidylcholine, at the *sn*-2 ester bond, releasing a fatty acid (FA) and a lysophospholipid (LP)[10] (**Figure 3-1**). Recent studies demonstrated that the expression and catalytic activity of $sPLA_2$ is increased in prostate[11-14], breast[15-17] and pancreatic[18-20] cancers.

We, and others, have developed $sPLA_2$ responsive liposomes (SPRL) with enhanced drug release[21-24]. $sPLA_2$-mediated degradation of phospholipids results in the formation of FA and LP; these increase membrane fluidity and result in a transition of the lipid bilayer from a gel-like to a liquid-crystalline phase. Alterations in membrane fluidity are believed responsible for the enhanced diffusion and release of contents entrapped within the aqueous-core of liposomes, however, the complete mechanism is not fully known.

Our recent studies have used electrospray ionization-mass spectrometry to examine the selectivity of $sPLA_2$ for various lipids and quantify $sPLA_2$-mediated degradation of prototype liposomes formulations[24]. Lipid degradation was correlated to enhanced release of 6-CF, a fluorescent probe. We, and others, have hypothesized that slight modifications of existing lipids or synthesis of novel lipids and lipid-prodrugs could be used to further tune drug release and improve $sPLA_2$-activity[21].

The specificity and activity of $sPLA_2$ is greatest for phospholipids with anionic head groups and shorter FA acyl chains[25-27]. However, optimal formulations need to balance drug retention properties with their circulation half-life, tissue distribution, and drug release kinetics. The use of high-phase transition, saturated phospholipids with neutral head groups have been shown, in combination with cholesterol (CHOL) and hydrophilic coatings (*e.g.*, polyethylene glycol), to produce long-circulating, sterically-stabilized liposomes (SSL). Unfortunately, the clinical utility of these formulations is limited, in part, because some drugs, such as doxorubicin or vincristine, are entrapped stably and the rate of drug release is slow, whereas for other drugs, such as topotecan or paclitaxel, the rate of release is fast[28]. The synthesis of novel lipids, based on existing lipids, may allow for the modulation, or tuning, of drug release.

Another challenge associated with evaluating $sPLA_2$-mediated liposome degradation and drug release is the ability to quantify the degradation of lipids from *in vivo* samples. The majority of lipids used to prepare drug-carriers cannot be separated from similar lipids found endogenously. Therefore, we sought to synthesize and use lipids containing odd FA acyl chain lengths, not commonly found *in vivo*, thus, permitting direct assessment of liposome tissue distribution and their degradation.

The aim of this study was to develop a simple and rapid approach to synthesize glycerophospholipids with *sn-1* ether and *sn-2* ester linked FA (**Figure 3-2**) that could be used to tune drug-release and be identified in biological samples without interference from endogenous lipids. Specifically we synthesized two ether lipids, **1** *1-O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphocholine (C31PC) and **2** *1-O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphomethanol (C31PM), and examined their ability to alter enzymatically triggered release of 6-carboxyfluorescein (6-CF) from liposomes incubated in TRIS buffer or fetal bovine serum (FBS) solutions, and evaluated their ability to be identified from lipids found endogenously in plasma.

MATERIALS AND METHODS

Synthesis, equipment, chemicals and reagents

A syringe-septum technique was used for moisture-sensitive reactions. THF was prepared freshly by distilling with sodium and benzophenone ketyl. Other solvents like DFM, DCM, Et_3N, and pyridine were mixed with 3 Å molecular sieves before use. Reagents were purchased from Sigma-Aldrich Chemical Co. and used without further purification. Silica gel (35-70μm, 200-430 mesh) was used for column chromatography. Products were visualized on TLC using either iodine vapor or Hanessian's stain method. Low resolusion MS was obtained on a HPLC-LC/MSD trap XCT Ultra Plus system (Agilent), high resolution MS was obtained on a LCT Premier Orthogonal Acceleration TOF Mass Spectrometer and 400 MHz 1H NMR spectra were used for chemical identifications. A Mettler Toledo DSC 1 Star System was used to determine melting temperature of DSPC, DPPC, and products 1 and 2. DSPC, DSPG, DSPE, and

DSPE-PEG were purchased from Avanti Polar Lipids Inc. (Alabaster, USA). $sPLA_2$ was purchased from Cayman Chemical Company (Ann Arbor, MI) and Genway Biotech Inc (San Diego, CA). F-12K cell culture media and FBS were purchased from Hyclone (Rockford, Illinois). CHOL was purchased from Sigma-Aldrich (St. Louis, Missouri). Acetonitrile and methanol were of HPLC grade from Fisher Scientific (Pittsburgh, PA). All other chemicals and solvents were of analytical grade, obtained from commercial sources and used without further purification. All experiments used ultrapure water (>3MΩ) obtained from a Millipore Milli-Q synthesis system (Billerica, MA)

Synthesis of 1-O-Hexadecyl-3-Benzyl-sn-Glycerol (4)

Pure NaH (0.0765 g, 3.20 mmol) was obtained by washing a NaH dispersion (60% mineral oil) with dry petroleum ether (20 mL×3) in a 50mL flask under N_2 protection.[28] All the petroleum ether was removed from the flask and then cetyl alcohol (0.74 g, 3.05 mmol) in dry THF (10 mL) solvent was added at 0°C. The reaction mixture was refluxed at 80°C for 1 h. Then (*R*)-*O*-benzyl glycidol (**3**) (0.25 g, 1.53 mmol) in DMF (25 mL) was added drop-wise over 5 min. The reaction mixture was stirred overnight at 80°C. The mixture was cooled to room temperature and the reaction was stopped by adding water (2.5 mL). Solvents were removed by vacuum. The residue was dissolved with diethyl ether, washed by brine 3 times and dried over Na_2SO_4. The product was purified using two rounds of column chromatography using ether/DCM (1:4), then ether/hexane (1:1). Product yield was 58.3%. ***1-O-hexadecyl-3-Obenzyl-sn-glycerol (4)*** has a *Rf* 0.31 (ether/hexane 1:4), ^1H NMR (400 MHz, $CDCl_3$): σ7.35 (m, 5H, Ph), σ4.57(s, 2H, CH_2Ph), σ4.00 (quintet, 1H, CH), σ3.52 (m, 6H, CH_2CHCH_2, O-CH_2), σ1.73(s, OH),

σ1.57 (quintet, 2H, β-CH_2), σ1.26 (br, s, 26H, 13×CH_2), σ0.887 (t, 3H, CH_3) (**Figure 3-3**) and ESI-MS+ of 407.1 *m/z* (MW=406.3).

Synthesis of 1-O-Hexadecyl-2-Pentadenoyl-3-O-Benzyl-sn-Glycerol (5)

Three g of 4 (7.4 mmol), 2.7 g pentadecanoic acid (11.1 mmol), 0.17 g DMAP (1.48 mmol), 150 mL DCM and 3.1 g DCC (14.8 mmol) were added to a flame-dried, 250 mL flask under N_2 protection. The reaction was cooled to 0°C for 15 min and allowed to stand at room temperature for 24 hr. The reaction was stopped by adding 5 mL acetic acid and stirred for 0.5 hr. The sample was stored at -20°C overnight and the resulting precipitate was removed by filtering. The solvent was evaporated and the resulting residues were dissolved with EtOAC. This resulting solvent was washed with 1 M HCl (30 mL×3), sat. $NaHCO_3$ (30 mL×3), brine(30 mL×3) and dried over Na_2SO_4. The intermediate 5 was purified using column chromatography using petroleum ether/DCM (1:4) as a mobile phase. Product yield was 69%. ***1-O-Hexadecyl-2-Pentadenoyl-3-O-Benzyl-Glycerol (5)*** has a *Rf* 0.25 (petroleum ether/DCM 1:4), ^{1}H NMR (400 MHz, $CDCl_3$): σ7.33(m, 5H, Ph), σ5.18 (quintet, 1H, CH) σ4.54-4.56 (AB, 2H, CH_2Ph), σ3.63 (d, 2H, $CH_2CH\mathit{CH_2}$), σ3.58 (d, 2H, $\mathit{CH_2}CHCH_2$), σ3.42 (m, 2H, $OCH_2C_{15}H_{31}$), σ2.34 (t, 2H, CH_2COO), σ1.62 (quintet, 2H,β-CH_2), σ1.53 (quintet, 2H, β-CH_2), σ1.26 (br, s, 48H, 24×CH_2), σ0.887 (t, 6H, CH_3×2) (**Figure 3-4**) and ESI-MS+ of 631.3 m/z (MW=630.5).

Synthesis of 1-O-Hexadecyl-2-Pentadenoyl-sn-Glycerol (6)

Intermediate 5 (0.2 g, 0.32 mmol) was added to a flame-dried 250 mL flask under N_2 protection with 200 mL DCM. The flask was bathed into a mixture of dry ice and acetone that produced temperature as -78°C. BBr_3 (0.64 mL 1M BBr_3 in DCM, 0.64 mmol) was injected into the flask and allowed to stand for 5 min. The reaction was quenched with 20

mL sat. $NaHCO_3$ and 20 mL diethyl ethyl. The solvent was then washed with saturated $NaHCO_3$ (30 mL×3), water (30 mL×3), brine (30 mL×3) and dried over Na_2SO_4. Intermediate 6 was concentrated under vacuum yielding a crude white solid. It was unstable resulting from its continuous acyl group migration, in order to avoid more loss of 6 during the workout procedure, this crude product was subjected to next reaction without further purification. To identify intermediate 6, the crude product was purified on a short column (DCM/diethyl ether=10:1). ***1-O-Hexadecyl-2-Pentadenoyl-sn-Glycerol (6)*** which has a *Rf* 0.71 (DCM/diethyl ether 10:1), ^{1}H NMR (400 MHz, $CDCl_3$): σ5.00 (quintet, 1H, CH), σ3.81 (d, 2H, $CH_2CH\mathit{CH_2}$), σ3.63 (m, 2H, $\mathit{CH_2}CHCH_2$), σ3.45 (m, 2H, $O\mathit{CH_2}C_{15}H_{31}$), σ2.36 (t, 2H, CH_2COO), σ1.63 (quintet, 2H,β-CH_2), σ1.56 (quintet, 2H, β-CH_2), σ1.26 (br, s, 48H, 24×CH_2), σ0.886 (t, 6H, CH_3×2) (**Figure 3-5**) and ESI-MS+ of 541.3 m/z (MW=540.9).

Synthesis of C31PC (1)

To a flame-dried, 50 ml flask under N_2 protection, first 18 μL $POCl_3$ (0.19 mmol) and 1.5mL DCM were added. Then 3 ml DCM with 27 μL Et_3N (0.19 mmol) and 6 (0.16 mg, 0.14 mmol) was injected into the flask drop-wise over 20 min. This mixture was stirred for 1 hr at room temperature. After this, 0.2 mL pyridine (1.2 mmol) and 128 mg choline tosylate (0.223 mmol) was added. The reaction was allowed to stand for 24 hr at room temperature. Water (0.2 ml) was added to quench the reaction. The reaction mixture was concentrated and then passed through a TMD-8 resin column using THF/H_2O (9:1) as a mobile phase. The resulting crude product was purified on a silica gel column using three mobile phases in a row (100 mL DCM: MeOH=85:15, 200 mL MeOH and then 100 mL DCM:MeOH:H_2O=65:25:4). Product yield (from 5 to 1, two steps) was 42%. C31PC has

a *Rf* 0.35 (DCM:MeOH:H_2O=65:25:4), ^{1}H NMR (400 MHz, $CDCl_3$): σ5.13 (quintet, 1H, CH), σ4.36 (s, 2H, PO*CH_2*CH_2N^+), σ3.98 (m, 2H, CH_2CH*CH_2*), σ3.88 (s, 2H, $POCH_2$*CH_2*N^+), σ3.55 (m, 4H, *CH_2*$CHCH_2$, $OCH_2C_{15}H_{31}$), σ3.44 (s, 9H, $N^+(CH_3)_3$), σ2.32 (t, 2H, CH_2COO), σ1.60(quintet, 2H,β-CH_2), σ1.53 (quintet, 2H, β-CH_2), σ1.26 (br, s, 48H, 24×CH_2), σ0.887 (t, 6H, CH_3×2) (**Figure 3-6**) and HRMS via TOF ES MS+ 706.5803 m/z (MW=706.0)

Synthesis of C31PM (2)

To a solution of $MOPOCL_2$ (3.4 mmol) and TMP (1.82 mmol) in dried toluene (2 mL) under N_2 protected at -20°C, 0.4 g of intermediate 6 (0.74 mmol) in toluene (10 mL) was added drop wise. This mixture was stirred for 24 hr at room temperature. After this, 2 mL sat. $NaHCO_3$ was added and stirred for 2 hr. This reaction mixture was concentrated by azeotropic distillation (with ethanol and toluene). The resulting crude product was purified on a silica gel column (DCM:MeOH=4:1). Product yield (from 5 to 2, two steps) was 40%. C31PM has a *Rf* 0.4 (DCM:MeOH=4:1), ^{1}H NMR (400 MHz, $CDCl_3$): σ5.18 (quintet, 1H, CH), σ4.00 (d, 2H, CH_2CH*CH_2*), σ3.61-3.57 (m, 5H, *CH_2*$CHCH_2$, $POCH_3$), σ3.40 (m, 2H, $OCH_2C_{15}H_{31}$), σ2.32 (t, 2H, CH_2COO), σ1.60 (quintet, 2H,β-CH_2), σ1.52 (quintet, 2H, β-CH_2), σ1.26 (br, s, 48H, 24×CH_2), σ0.887 (t, 6H, CH_3×2) (**Figure 3-7**) and TOF MS+, 657.5034 m/z, M+Na^+ adduct (MW=633.5).

Preparation of 6-CF-loaded liposomes

Aliquots containing 100 mM 6-CF solution were prepared by dissolving 37.7 mg of 6-CF in 1 mL 5 mM Tris-HCl buffer (pH 7.4, adjusted with NaOH 1 M). Liposomes were prepared by hydration of thin-films followed by a freeze-thaw and a high-pressure extrusion process. Briefly, phospholipids, cholesterol and DSPE-PEG in chloroform were

mixed together, dried under vacuum at 55-65°C (water bath) for 25 min using a rotary evaporator (Büchi) and the thin-film was hydrated using a solution of 6-CF (100 mM, pH 7.4) for 15-20 min to achieve a final lipid concentration of 10 mM. The formulation then underwent seven liquid nitrogen freeze–thaw cycles above the phase transition temperature of the primary lipid prior to extrusion (n=5) through double-stacked polycarbonate membranes (80 nm, Osmonics Inc.) using a Lipex extruder (Northern Lipids Inc.) at 65°C. Free 6-CF was removed by size exclusion chromatography (Sephadex G-75, Pharmacia). The mobile phase for these separations consisted of 5 mM TRIS-HCl buffer (pH 7.4, adjusted with NaOH 1 M). Phosphate assays were performed prior to conduction of enzymatic reactions to determine the concentration of total phospholipids. All liposome formulations had a mean particle diameter of 80-110 nm, as determined using a Nicomp™ model 370 dynamic light scattering particle size analyzer (Santa Barbara, CA). Samples were stored under a nitrogen atmosphere at 4ºC and protected from light and used within 24 hr of preparation.

Determination of $sPLA_2$–mediated 6-CF leakage and analysis

The effect of incorporation of C31PC or C31PM on the $sPLA_2$–mediated release of 6-CF from prototypical SSL samples was determined as previously described by us[24]; SSL formulations contained 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC), CHOL and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)$_{2000}$] (DSPC:CHOL:PEG) in a 9:5:1 mole ratio. Briefly, release of 6-CF from liposome samples (0.05 µmol/mL) was determined in the presence and absence of Group III $sPLA_2$ (2.5 µg/mL), $CaCl_2$ (1 mM) and Tris-HCl buffer (5 mM, pH 7.4). Fluorescent intensity of 6-CF was measured using a Synergy HT spectrofluorometer (Bio-Tek

Instruments Inc.) at excitation and emission wavelengths of 480 and 510 nm, respectively. Fluorescence was detected from 0 to 48 hr. After measurement at each time point, 10% (v/v) of Triton X-100 was added to the samples to calculate total 6-CF. Percentage of 6-CF leakage was calculated using the equation:

$$\text{Percentage} = [(F_t - F_0)/(F_{\text{Triton}} - F_0)] \times 100\%;$$

where, F_t represents the fluorescent intensity (FI) at a specific time point, F_0 represents FI at time zero, and F_{Triton} represents total fluorescent intensity after addition of Triton X-100.

Effect of serum on sPLA$_2$–mediated 6-CF leakage

The effect of serum on release of 6-CF from liposome samples (0.05 μmol/mL) was determined in the presence and absence of Group III sPLA$_2$ (2.5 μg/mL), $CaCl_2$ (1 mM) using F-12K with 10% FBS (v/v) as media. Fluorescent intensity of 6-CF was measured using a Synergy HT spectrofluorometer (Bio-Tek Instruments Inc.) at excitation and emission wavelengths of 480 and 510 nm, respectively. Fluorescence was detected from 0 to 218 hr. The rest of the steps were the same as mentioned above.

Tracking C31PC in rat plasma samples using LC-MS/MS method

Lipids from blank rat plasma or rat plasma spiked with DSPC (10 nmol/mL) or C31PC (10 nmol/mL) or SSL formulation [DSPC:C31PC (9:1 mol%, total 10 nmol/mL)] were extracted using a Bligh-Dyer assay[29], and lipids were identified *via* their unique ion-pairs by LC/MS/MS using mixed reaction monitoring (MRM) mode as previously described by us[24]. Briefly, 100 μL of methanol was added to samples (200 μL) followed by the addition of 300 μL chloroform, vortexing and centrifugation at 2,500 × g for 5 min. The organic layer was removed and transferred to a clean test tube. The above extraction

was repeated twice and the final organic phase was evaporated under nitrogen, and reconstituted in 1 mL chloroform and methanol (3:1 v/v). A 500 µL aliquot of the resultant organic solution was for ESI-MS measurement. Analysis was performed on an Agilent high performance liquid chromatography – mass spectrometer (LC/MSD-Trap XCT Ultra Plus) system (Santa Clara, CA). The mobile phase was acetonitrile, methanol, and 0.1% ammonium formate (2:3:1 v/v/v). The flow rate was 0.15 mL/min and the injection volume was 5 µL. Nitrogen was used as a nebulizing gas at 25 psi and a drying gas at 8 psi. The drying temperature was 350°C. The capillary, capillary exit and skimmer potentials were 3,500, 150.3 and 40.0 V, respectively. The *m/z* range for scanning was 200 to 2,200. DSPC was detected using MRM based on a fragmentation of *m/z* 791 to 608 while C31PC was identified based on a fragmentation of C31PC from *m/z* 707 to 523.

Statistics

The mass spectrometry data are presented as the mean ± the standard deviation (STD), the 6-CF release are shown as the man ± the standard error (SEM) of at least 3 separate experiments (n=5/study). Differences were determined following an analysis of variance for each data set using SAS software (SAS Institute, Cary, NC) followed by a Dunnett's t-test or a Student's t-test when comparisons involved a control and single variable. Differences were considered significant if the *p*-value ≤ 0.05.

RESULTS AND DISCUSSION

Using *R-(O)*-benzyl glycidol as a starting material, intermediate **6** (**Figure 3-8**) was synthesized by a three-step reaction, *i.e.* regioselective opening of epoxide ring with ether,

ii. Steglich esterification to its *sn*-2 position with fatty acid and *iii.* deprotection reaction of benzyl group using Lewis acid boron tribromide[25].

Intermediate **6** was used as the starting material for the synthesis of both *1-O* lipids **1** and **2** (**Table 3-1**) by conjugating either phosphocholine or phosphomethanol (**Figure 3-9**). H2/Pd-C has been used successfully to deprotect the benzyl group, resulting in high product yield. However, this method is limited in that it can only be applied for saturated lipid synthesis. Therefore, we used alternative methods to remove the benzyl group. First chlorosulfonyl isocyanate (CSI) was tried[30], but the result was not desirable. The failure of this reaction may have resulted from the low selectivity of CSI for ether bonds or the instability of intermediate **6** in the resulting harsh chemical environment, *i.e.,* use of sodium hydroxide and the long reaction time (up to 21 hr). The instability of intermediate **6** is most likely the result of acyl migration that is accelerated at elevated temperatures and the non-neutral pH environment of this reaction. With this in mind, we adapted an existing method using boron tribromide (BBr_3)[30]. The reaction was performed at -78°C and was completed in 5 min. BBr_3 displayed high selectivity over the benzyl ether group, resulting in an almost complete (~100%) formation of **6** from **5**. Furthermore, the short reaction time has also been shown to reduce acyl migration[31]. The primary drawback of this reaction is the use of large quantities of DCM solvent, *i.e.,* 0.1 g of **5** needs 100 mL of DCM to dissolve at -78°C.

Synthesis of C31PC **(1)** and C31PM (**2**) were completed using the conditions developed by Hirth, G[32]. Initial purification of C31PM by chromatography using a TMD-8 resin column and a silica gel column according to the literature did not produce a pure product. However, purity was achieved using a gradient method in flash

chromatography. It should be mentioned in the preparation of C31PM, $NaHCO_3$ was used in place of water because of the resulting formation of the sodium salt form of C31PM facilitated purification, compared to its free acid form.

The effect of incorporation of C31PC or C31PM on the release of 6-CF from prototypical SSL samples was determined in the presence and absence of $sPLA_2$. Incorporation of 10 or 30 lipid mol% of C31PM resulted in enhanced $sPLA_2$-mediated release of 6-CF compared to SSL formulations (**Figure 3-10A**). Preparations using 90% or 30 lipid mol% C31PC had greater and equal release to those incorporating 10 lipid mol% DSPG (**Figure 3-10B**). DSPG is an anionic lipid that is degraded rapidly by $sPLA_2$, but results in liposome formulations that display poor drug retention[24]. Together, these data suggest that shortening of FA acyl chains from C18:0/18:0 to C15:0/16:0, using an ester/ether linked acyl-chains and controlling the percentage of C31PM or C31PC can be used to tune the release from SSL-like formulations. This represents an advancement over the use of other anionic lipids, such as DSPG, that are very sensitive to $sPLA_2$, but have bulky head groups that decrease their stability and an anionic charge that can reduce the circulation half-life of liposomes.

Serum can alter release from SSL and SPLR formulations[24]. The effect of 10% (v/v) fetal bovine serum (FBS) on $sPLA_2$-mediated 6-CF release was determined (**Figure 3-11**). The addition of 10 or 30 mol% of C31PM to SSL formulations reduced the overall rate of 6-CF release compared to SSL formulations in the presence and absence of 10 mol% DSPE (**Figure 3-11A**). This is not unexpected given the higher phase transition (T_m 52.2 °C, **Table 3-1**) of hydrated C31PM compared to DSPC (49.6°C). Inclusion of DSPE (10 mol%) was similar to the C31PM (10 mol%). DSPE had the greatest phase transition

temperature (74 °C, **Table 3-1**), that would be expected to improve membrane stability, but also has a bulkier head group relative the phosphomethanol head group on C31PM. Further, C31PM is anionic, its relatively small head group may be protected from $sPLA_2$-mediated degradation, relative to other bulkier anionic lipids such as DSPE and DSPG. SSL formulations containing 10 mol% DSPG or 10 mol% C31PC had similar release profiles (**Figure 3-11B**). Whereas, incorporation of C31PC at 30 and 90 mol% resulted in a stepwise decrease in 6-CF release, relative to 10 mol% DSPG (**Figure 3-11B**). Although the mechanisms underlying these effects are not fully known, DSPG is an anionic lipid with a phase transition of 54.8°C (**Table 3-1**), whereas C31PC is zwitterionic in nature. Most importantly is that the shorter acyl-chain FA used in **1** and **2** showed concentration dependent alterations in 6-CF release, suggesting that the rate and extent of release may be modulated.

These studies suggest that this approach can be used to synthesize lipids and modulate the release of intra-luminal constituents from liposomes. Although each formulation will have to be tailored to the physicochemical properties of a target drug(s), this approach provides a platform for making lipid modifications based on drug-carrier release kinetics.

Another challenge optimizing lipid based drug-carriers is our ability to track their disposition *in vivo* due to the presence of endogenous lipids. To overcome this challenge we synthesized odd chain lipids, not normally found in nature. Although a variety of fluorescent and radio-labeled probes exist, their effect on membrane fluidity and particulate disposition is not well known and these probes are generally not approved for human use. This is one reason why a goal of this work was to prepare lipids that were

multifunctional, *i.e.*, ability to tune release and be identified in biological samples.

As expected, analysis of blank plasma demonstrated background levels of ion-pairs corresponding to DSPC (791→608 *m/z*) and C31PC (707→523 *m/z*). Spiking of plasma with DSPC (**Figure 3-12B**), C31PC (**Figure 3-12C**) and SSL formulations containing DSPC and C31PC in a 9:1 mole ratio (**Figure 3-12D**) resulted in an increased intensity for peaks corresponding to both ion-pairs. These data suggest that odd-chain lipids, *e.g.,* C31PC, can be identified in rat plasma without interference of endogenous lipids. Further, the presence of a C15:0 FA on C31PC may facilitate the tracking of its degradation and metabolism *in vivo*. Currently, determining liposome degradation *in vivo* is difficult without the use of tracers or radiolabels due to the high endogenous levels of even-chained lipids and their fatty acid metabolites found in biological tissues.

In conclusion, a simple approach is presented that permits synthesis of a variety of ether phospholipids from a few intermediates while decreasing acyl migration. We demonstrated the synthesis of two odd-chain ether lipids with a PC or PM head-group could be used to alter drug release. We also demonstrated that the odd chain lipid was able to be identified after extraction from complex biological samples, *e.g.,* plasma. These data suggest that this approach may be used to make slight modifications to existing or creation of novel lipids to tune release of therapeutic agents from lipid based particulate carriers and track their lipids *in vivo*. Further, we believe this intra-luminal approach can be extended to use incorporation of other head groups with functional groups suitable for attaching targeting species (*e.g.,* antibodies or peptides) or imaging probes.

ACKNOWLEDGEMENT

We thank Jeremy L Grove, Jennifer A Haley, and Dr. Yang Geng for their technical assistance related to the synthesis. We also thank Dr. Timothy Long for helpful discussions. This research was funded in part by Georgia Cancer Coalition Distinguished Scholar Grants to RDA/BSC, an NIH NIBIB (EB08153) to RDA/BSC, a University of Georgia Graduate Fellowship Stipend Award to GZ, a King Khalid University Fellowship to YA, and an American Foundation for Pharmaceutical Education–Wyeth New Investigator Grant to RDA.

Table 3-1. Structures of existing and novel $sPLA_2$-targeted ether lipids.

Compound	R^1 (linkage)	R^2 (linkage)	R^3	T_m°C*
DSPC	18:0 (ester)	18:0 (ester)	Choline	49.6
DPPC	16:0 (ester)	16:0 (ester)	Choline	38.9
DSPE	18:0 (ester)	18:0 (ester)	Ethanolamine	74.3
DSPG	18:0 (ester)	18:0 (ester)	Glycerol	54.8
1	16:0 (ether)	15:0 (ester)	Choline	34.4
2	16:0 (ether)	15:0 (ester)	Methanol	52.2

(**1**) *1-O*-C31PC;

(**2**) *1-O*-C31PM;

*T_m was determined using individual lipids hydrated in dd-water at 40 mM.

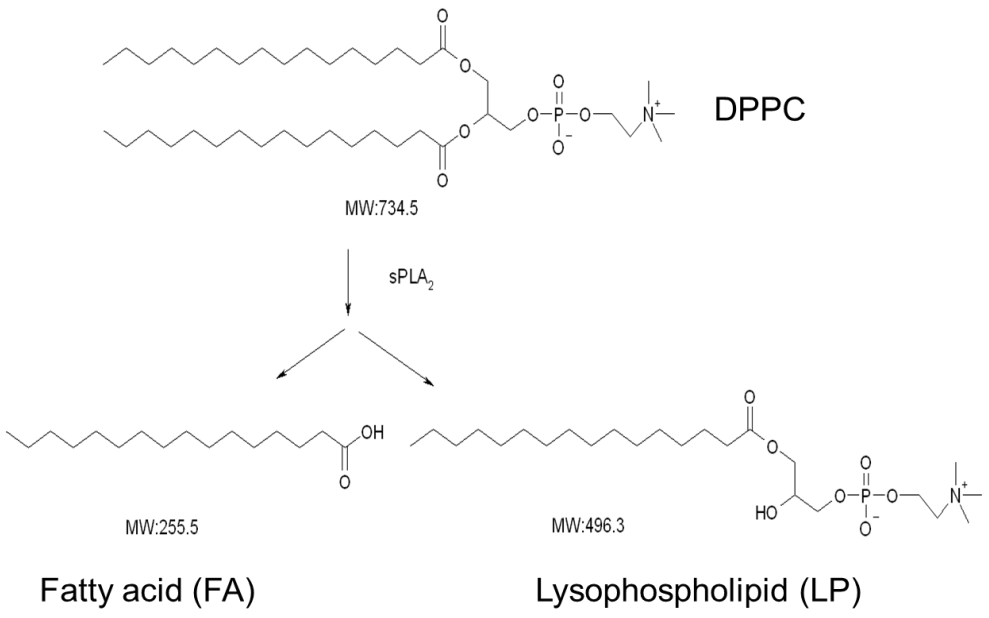


Figure 3-1. Phospholipid degradation by $sPLA_2$ gives lysophospholipid and free fatty acid

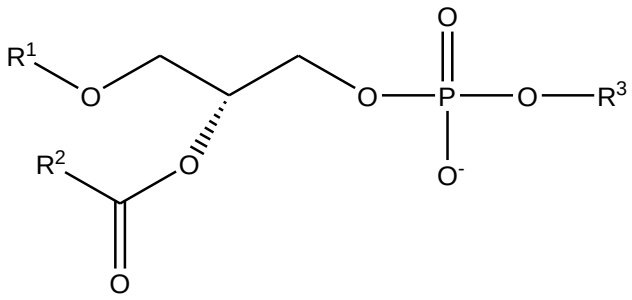


Figure 3-2. Structure of ether lipids

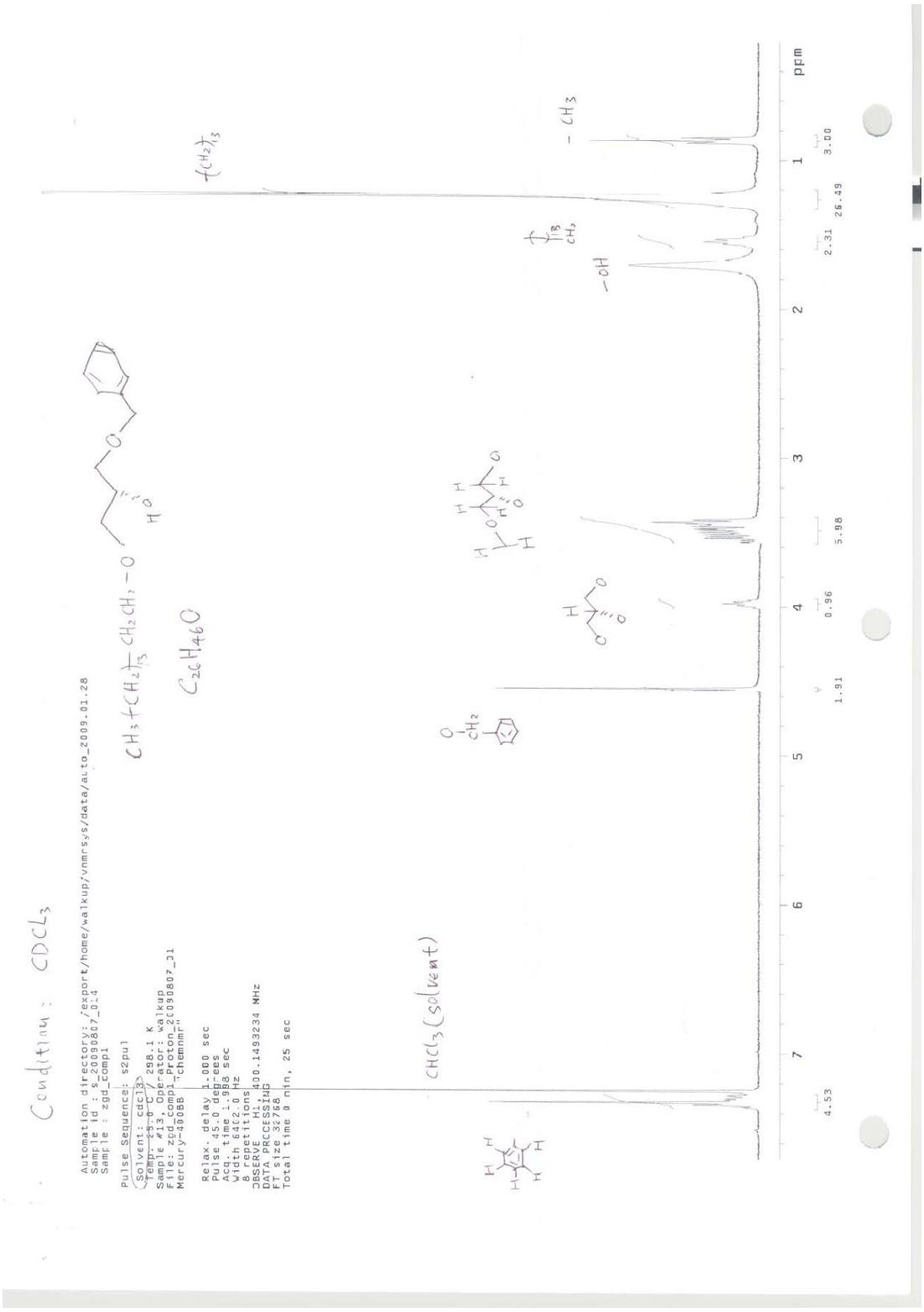


Figure 3-3. ^{1}H NMR spectrum of intermediate 4

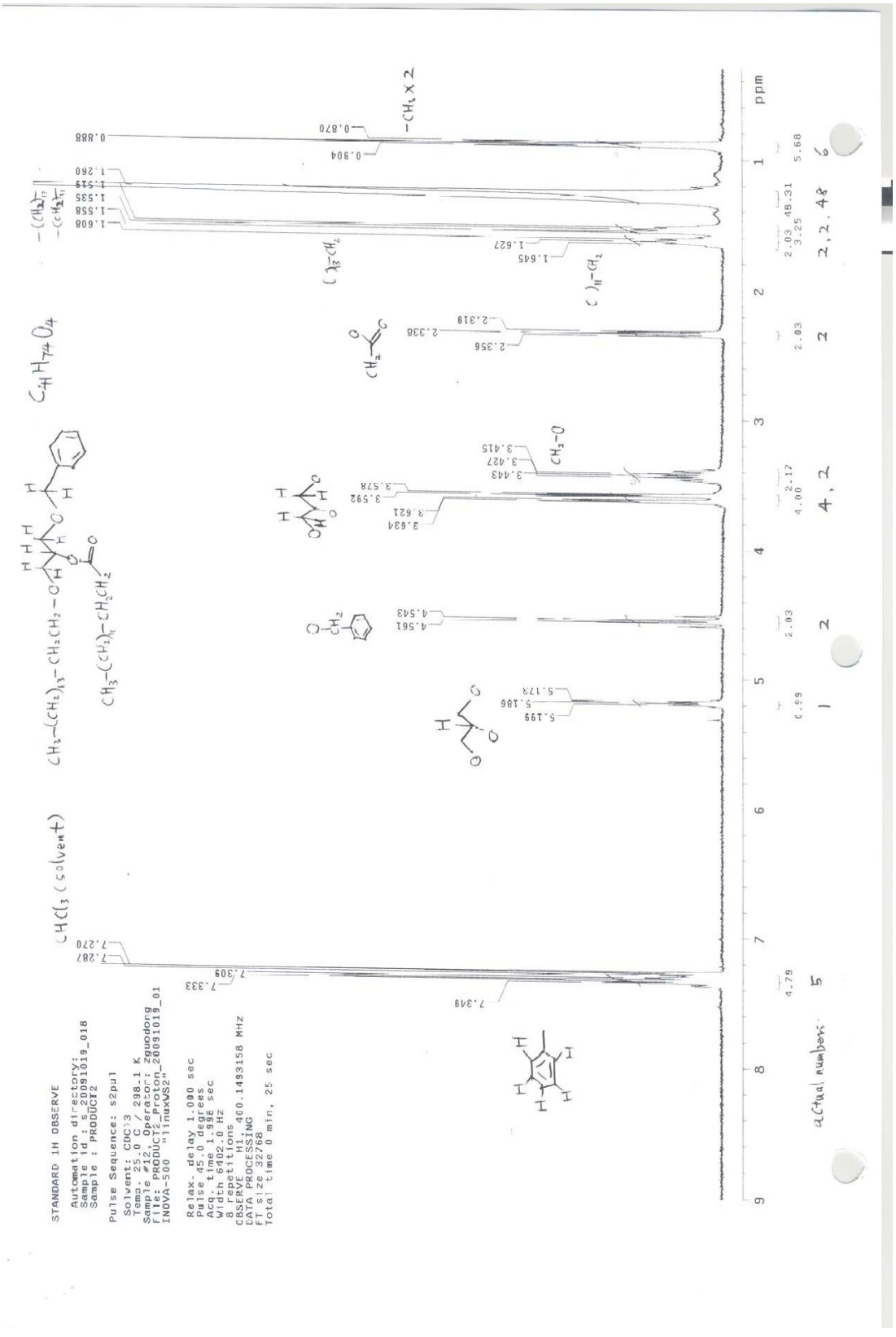


Figure 3-4. ^{1}H NMR spectrum of intermediate 5

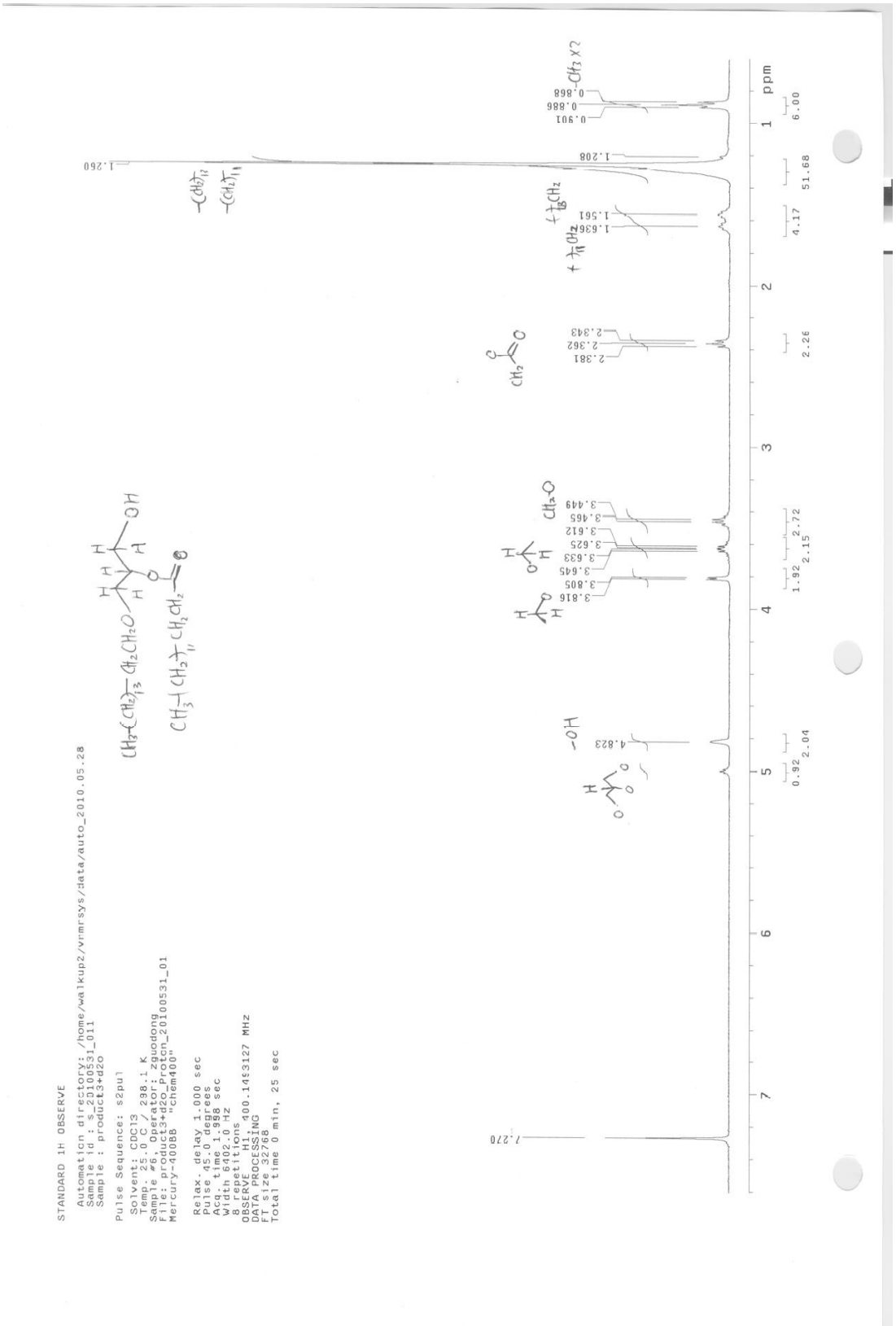


Figure 3-5. ^{1}H NMR spectrum of intermediate 6

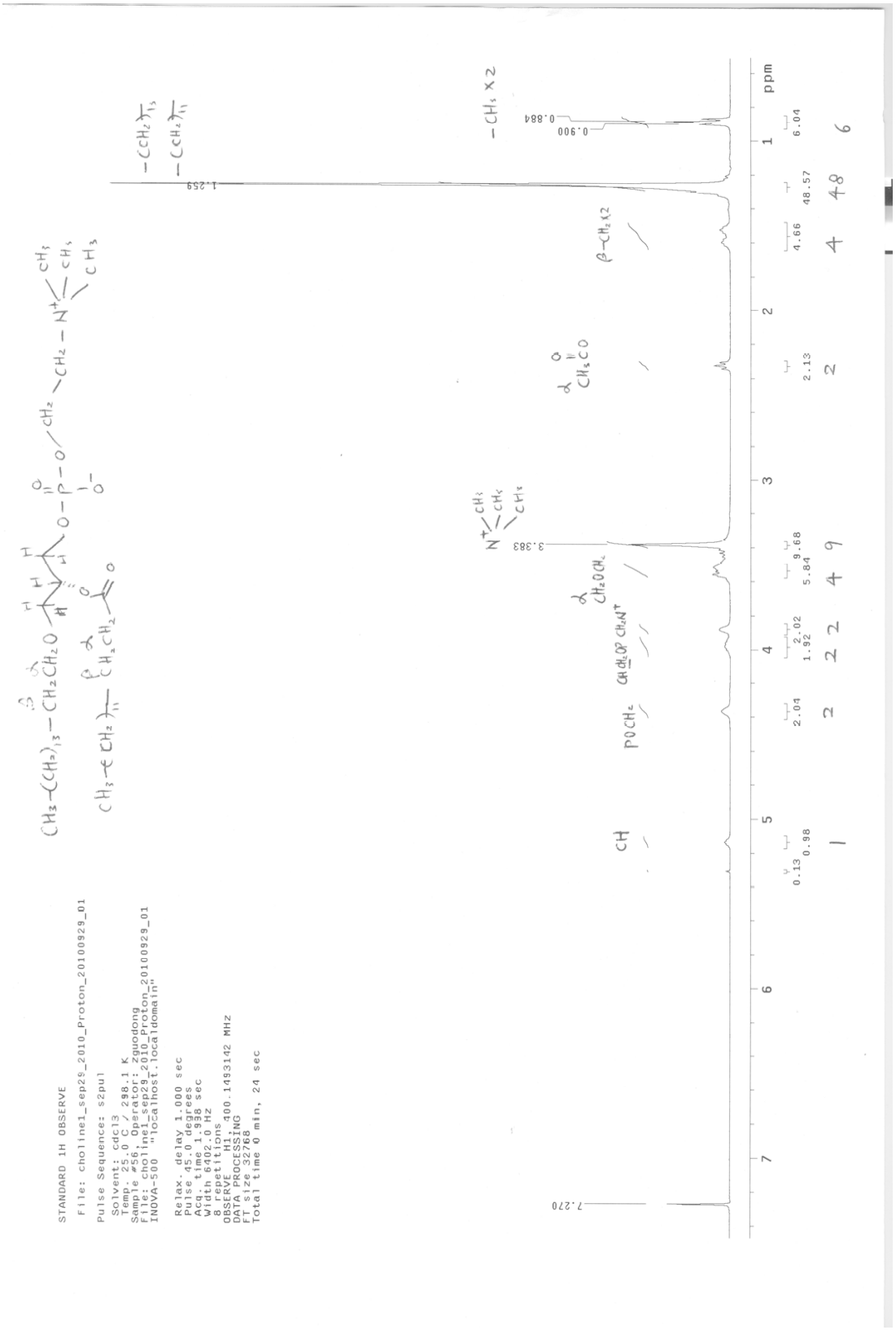


Figure 3-6. ^{1}H NMR spectrum of C31PC

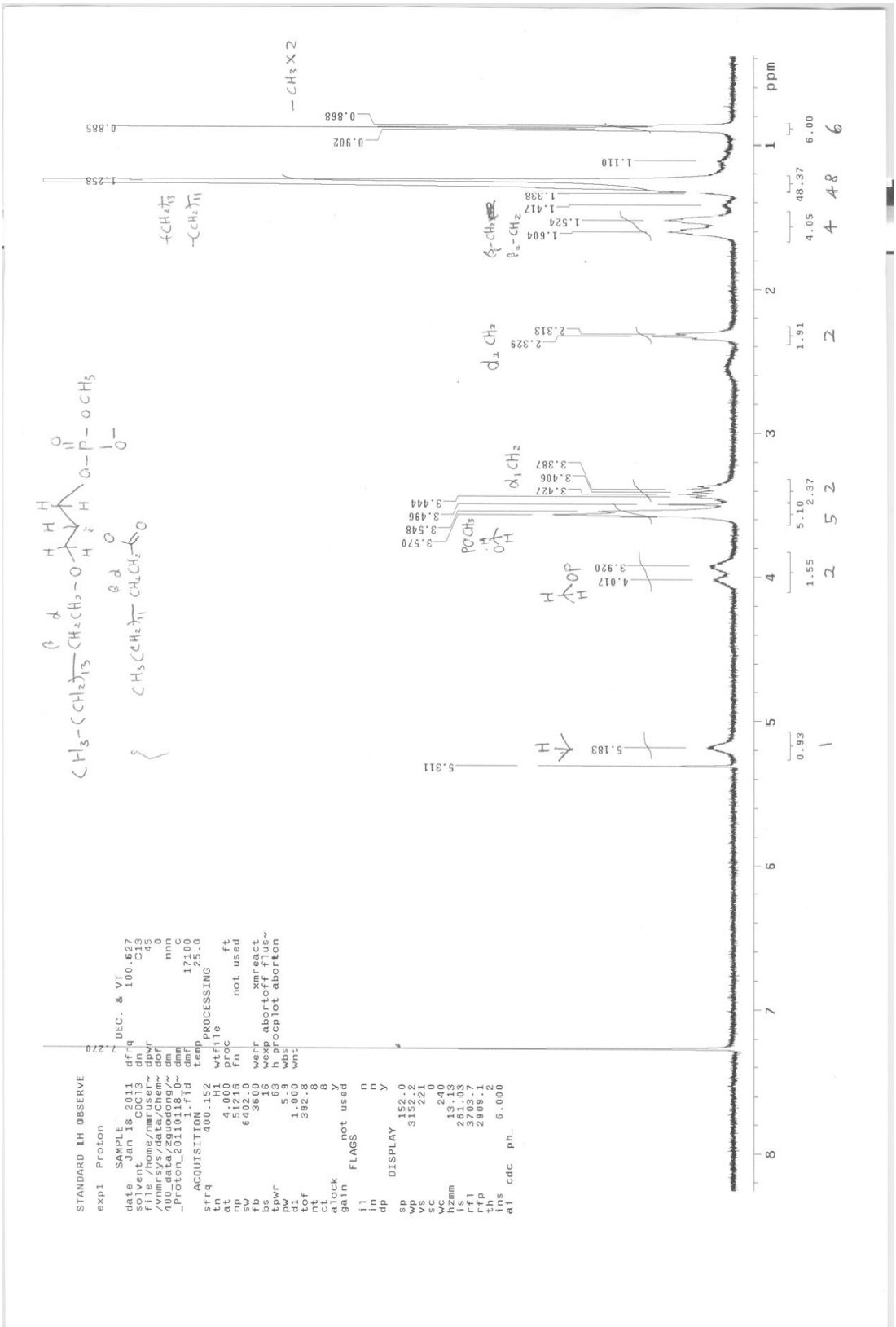
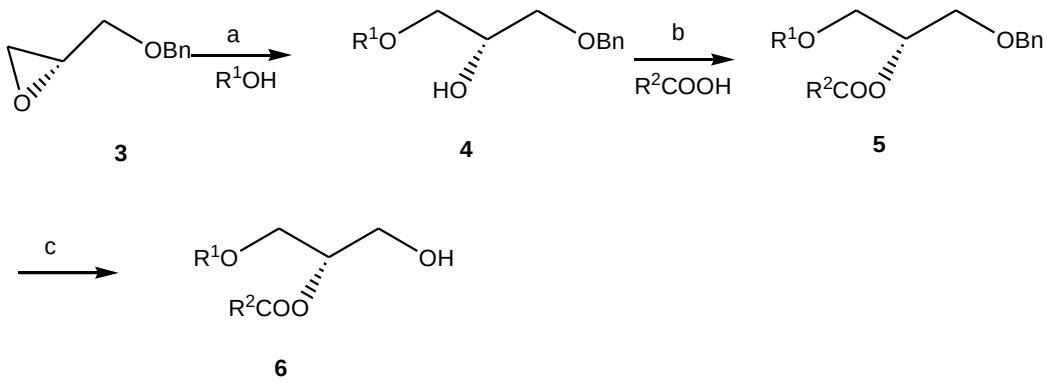


Figure 3-7. ^{1}H NMR spectrum of C31PM

Figure 3-8. Synthesis of intermediate 6

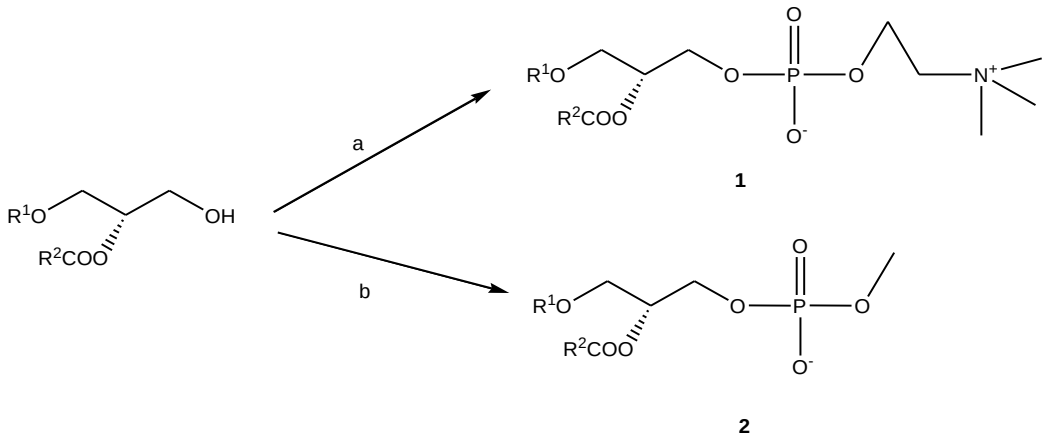


(a) R^1OH, NaH, THF, DMF, 16 hr, 80°C

(b) R^2COOH, DCC, DMAP, DCM, 24 hr, room temperature

(c) BBr_3, DCM, 5 min, -78°C

Figure 3-9. Synthesis of products 1 and 2



(a) (i) $POCl_3$, Et_3N, DCM, 1 hr, room temperature; (ii) pyridine, choline tosylate, 16 hr, room temperature; (iii) H_2O,1 hr

(b) (i) $MeOPOCl_2$, TMP, 24 hr, room temperature; (ii) sat. $NaHCO_3$, 2 hr

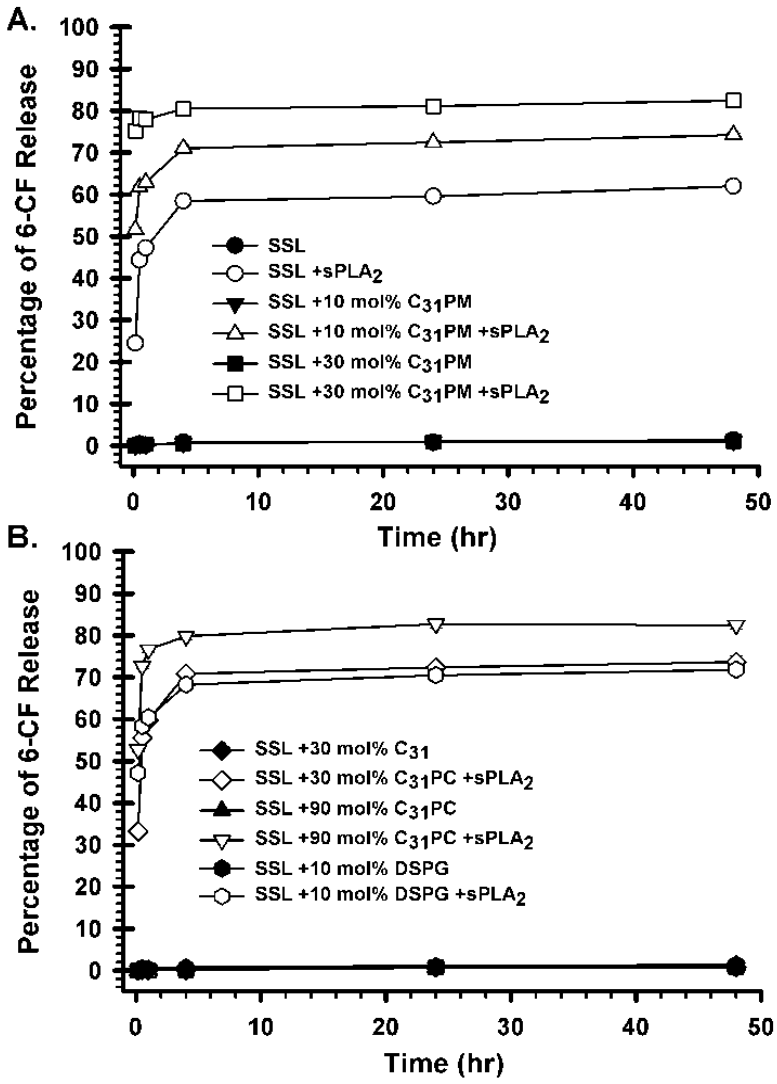


Figure 3-10. $sPLA_2$-mediated release of 6-CF from **A.** prototype SSL and C31PM (10 and 30 mol%) modified SSL and **B.** C31PC (90 or 30 mol%) and DSPG (10 mol%) modified SSL formulations. Liposome samples were treated with 0 (control) or 2.5 µg/mL $sPLA_2$ for 0 to 48 hr at 25ºC in TRIS buffer (pH 7.4) and 6-CF release was determined by quantifying fluorescence at an excitation wavelength of 480 nm and an emission intensity at 510 nm. Data are represented as the mean ± SEM of a least 3 separate experiments, (n=5/study).

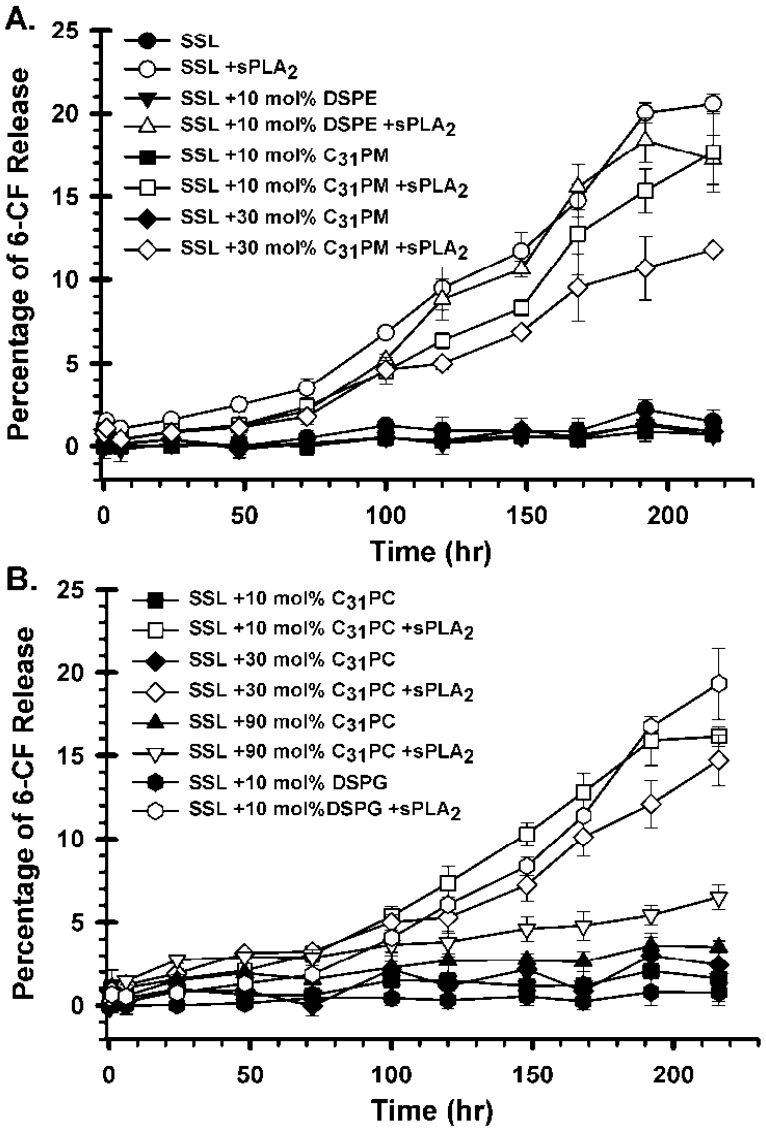


Figure 3-11. Effect of 10% fetal bovine serum on $sPLA_2$-mediated 6-CF release from **A.** SSL, and DSPE (10 mol%) and C31PM (10 and 30 mol%) modified SSL formulations and **B.** C31PC (10, 30, and 90 mol%) and DSPG (10 mol%) modified SSL. The effect of serum on $sPLA_2$-mediated release of 6-CF from formulations was determined over 216 hr at 37ºC by quantifying fluorescent at an excitation wavelength of 480 nm and an emission intensity at 510 nm. Data are represented as the mean ± SEM of a least 3 separate experiments.

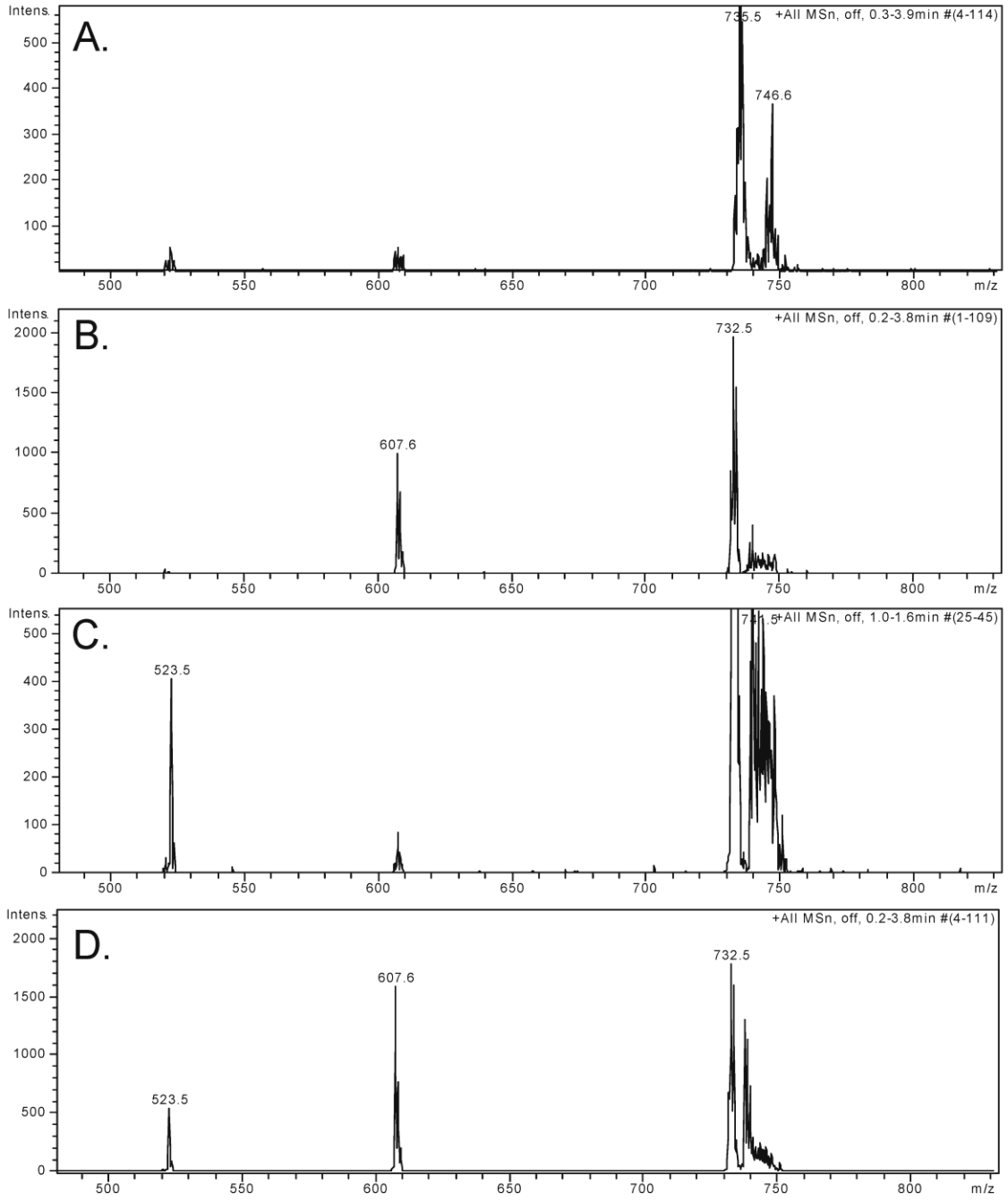


Figure 3-12. Mass spectra of blank rat plasma A. or plasma spiked DSPC **B**., C31PC **C**. or SSL formulation containing DSPC and C31PC in a 9:1 mole ratio **D**. DSPC (791→608 *m/z*) and C31PC (707→523 *m/z*) were identified based on their ion-pair as determined using MRM.

REFERENCES

1. Drummond DC, Meyer O, Hong KL, Kirpotin DB, Papahadjopoulos D 1999. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacological Reviews 51(4):691-743.
2. Bondurant B, Mueller A, O'Brien DF 2001. Photoinitiated destabilization of sterically stabilized liposomes. Biochim Biophys Acta 1511(1):113-122.
3. Shum P, Kim JM, Thompson DH 2001. Phototriggering of liposomal drug delivery systems. Adv Drug Deliv Rev 53(3):273-284.
4. Spratt T, Bondurant B, O'Brien DF 2003. Rapid release of liposomal contents upon photoinitiated destabilization with UV exposure. Biochim Biophys Acta 1611(1-2):35-43.
5. Yatvin MB, Weinstein JN, Dennis WH, Blumenthal R 1978. Design of liposomes for enhanced local release of drugs by hyperthermia. Science 202(4374):1290-1293.
6. Kong G, Anyarambhatla G, Petros WP, Braun RD, Colvin OM, Needham D, Dewhirst MW 2000. Efficacy of liposomes and hyperthermia in a human tumor xenograft model: importance of triggered drug release. Cancer Res 60(24):6950-6957.
7. Needham D, Anyarambhatla G, Kong G, Dewhirst MW 2000. A new temperature-sensitive liposome for use with mild hyperthermia: characterization and testing in a human tumor xenograft model. Cancer Res 60(5):1197-1201.
8. Huang SL, MacDonald RC 2004. Acoustically active liposomes for drug encapsulation and ultrasound-triggered release. Biochim Biophys Acta 1665(1-2):134-141.

9. Schroeder A, Avnir Y, Weisman S, Najajreh Y, Gabizon A, Talmon Y, Kost J, Barenholz Y 2007. Controlling liposomal drug release with low frequency ultrasound: mechanism and feasibility. Langmuir 23(7):4019-4025.
10. Cummings BS, McHowat J, Schnellmann RG 2000. Phospholipase A(2)s in cell injury and death. J Pharmacol Exp Ther 294(3):793-799.
11. Dong Q, Patel M, Scott KF, Graham GG, Russell PJ, Sved P 2006. Oncogenic action of phospholipase A2 in prostate cancer. Cancer Lett 240(1):9-16.
12. Kallajoki M, Alanen KA, Nevalainen M, Nevalainen TJ 1998. Group II phospholipase A2 in human male reproductive organs and genital tumors. Prostate 35(4):263-272.
13. Jiang J, Neubauer BL, Graff JR, Chedid M, Thomas JE, Roehm NW, Zhang S, Eckert GJ, Koch MO, Eble JN, Cheng L 2002. Expression of Group IIA Secretory Phospholipase A2 Is Elevated in Prostatic Intraepithelial Neoplasia and Adenocarcinoma. Am J Pathol 160(2):667-671.
14. Graff JR, Konicek BW, Deddens JA, Chedid M, Hurst BM, Colligan B, Neubauer BL, Carter HW, Carter JH 2001. Expression of group IIa secretory phospholipase A2 increases with prostate tumor grade. Clin Cancer Res 7(12):3857-3861.
15. Yamashita S, Yamashita J, Ogawa M 1994. Overexpression of group II phospholipase A2 in human breast cancer tissues is closely associated with their malignant potency. Br J Cancer 69(6):1166-1170.
16. Yamashita S, Ogawa M, Sakamoto K, Abe T, Arakawa H, Yamashita J 1994. Elevation of serum group II phospholipase A2 levels in patients with advanced cancer. Clin Chim Acta 228(2):91-99.

17. Yamashita S, Yamashita J, Sakamoto K, Inada K, Nakashima Y, Murata K, Saishoji T, Nomura K, Ogawa M 1993. Increased expression of membrane-associated phospholipase A2 shows malignant potential of human breast cancer cells. Cancer 71(10):3058-3064.
18. Kiyohara H, Egami H, Kako H, Shibata Y, Murata K, Ohshima S, Sei K, Suko S, Kurano R, Ogawa M 1993. Immunohistochemical localization of group II phospholipase A2 in human pancreatic carcinomas. Int J Pancreatol 13(1):49-57.
19. Kuopio T, Ekfors TO, Nikkanen V, Nevalainen TJ 1995. Acinar cell carcinoma of the pancreas. Report of three cases. Apmis 103(1):69-78.
20. Oka Y, Ogawa M, Matsuda Y, Murata A, Nishijima J, Miyauchi K, Uda K, Yasuda T, Mori T 1990. Serum immunoreactive pancreatic phospholipase A2 in patients with various malignant tumors. Enzyme 43(2):80-88.
21. Jensen SS, Andresen TL, Davidsen J, Hoyrup P, Shnyder SD, Bibby MC, Gill JH, Jorgensen K 2004. Secretory phospholipase A(2) as a tumor-specific trigger for targeted delivery of a novel class of liposomal prodrug anticancer etherlipids. Molecular Cancer Therapeutics 3(11):1451-1458.
22. Davidsen J, Vermehren C, Frokjaer S, Mouritsen OG, Jorgensen K 2001. Drug delivery by phospholipase A(2) degradable liposomes. International Journal of Pharmaceutics 214(1-2):67-69.
23. Linderoth L, Peters GH, Jorgensen K, Madsen R, Andresen TL 2007. Synthesis of sn-1 functionalized phospholipids as substrates for secretory phospholipase A(2). Chemistry and Physics of Lipids 146(1):54-66.

24. Zhu G, Mock JN, Aljuffali I, Cummings BS, Arnold RD 2011. Secretory phospholipase A(2) responsive liposomes. J Pharm Sci 100(8):3146-3159.
25. Andresen TL, Davidsen J, Begtrup M, Mouritsen OG, Jorgensen K 2004. Enzymatic release of antitumor ether lipids by specific phospholipase A2 activation of liposome-forming prodrugs. J Med Chem 47(7):1694-1703.
26. Andresen TL, Jensen SS, Kaasgaard T, Jorgensen K 2005. Triggered activation and release of liposomal prodrugs and drugs in cancer tissue by secretory phospholipase A2. Curr Drug Deliv 2(4):353-362.
27. Jensen SS, Andresen TL, Davidsen J, Hoyrup P, Shnyder SD, Bibby MC, Gill JH, Jorgensen K 2004. Secretory phospholipase A2 as a tumor-specific trigger for targeted delivery of a novel class of liposomal prodrug anticancer etherlipids. Mol Cancer Ther 3(11):1451-1458.
28. Drummond DC, Noble CO, Hayes ME, Park JW, Kirpotin DB 2008. Pharmacokinetics and In Vivo Drug Release Rates in Liposomal Nanocarrier Development. Journal of Pharmaceutical Sciences 97(11):4696-4740.
29. Bligh EG, Dyer WJ 1959. A Rapid Method of Total Lipid Extraction and Purification. Canadian Journal of Biochemistry and Physiology 37(8):911-917.
30. Kim JD, Han G, Zee OP, Jung YH 2003. Deprotection of benzyl and p-methoxybenzyl ethers by chlorosulfonyl isocyanate-sodium hydroxide. Tetrahedron Letters 44(4):733-735.
31. Pluckthun A, Dennis EA 1982. Acyl and phosphoryl migration in lysophospholipids: importance in phospholipid synthesis and phospholipase specificity. Biochemistry 21(8):1743-1750.

32. Hirth G, Barner R 1982. Synthesis of Glyceryletherphosphatides .1. Preparation of 1-O-Octadecyl-2-O-Acetyl-Sn-Glyceryl-3-Phosphorylcholine (Platelet Activating Factor), of Its Enantiomer and of Some Analogous Compounds. Helvetica Chimica Acta 65(3):1059-1084.

ABBREVIATIONS

6-CF	6-carboxyfluorescein
BBr_3	boron tribromide
C31PC	1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphocholine
C31PM	1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphomethanol
CSI	chlorosulfonyl isocyanate
DCM	dichloride methylene
DMF	dimethylformamide
DPPC	1,2-dipalmityl-*sn*-glycero-3-phosphatidylcholine
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine
DSPG	1,2-distearoyl-*sn*-glycerol-3-phosphatidylglycerol
DSPE	1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine
DSPE-PEG	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)$_{2000}$]
ESI-MS	electrospray ionization-mass spectrometry
FA	fatty acid
HPLC	high performance liquid chromatography
LP	lysophospholipid
MRM	mixed reaction monitoring
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PM	phosphatidylmethanol
PG	phosphatidylglycerol

$sPLA_2$	secretory phospholipase A_2
SSL	sterically-stabilized liposome
SPRL	$sPLA_2$ responsive liposome
THF	tetrahydrofuran

CHAPTER 4

SYSTEMATIC STUDY OF EXTRATION EFFICIENCY OF BLIGH AND DYER PROCEDURE ON INDIVIDUAL PHOSPHOLIPID AND ITS METABOLITES BY ELECTROSPRAY IONIZATION-MASS SPECTROMETRY AND QUANTIFICATION OF A SYNTHETIC LIPID WITH A *SN*-2 ODD-CARBON-NUMBER ACYL CHAIN IN BIOLOGICAL SAMPLES

Guodong Zhu, Brian S. Cummings and Robert D. Arnold. To be submitted to *Journal of Pharmaceutical Technology and Drug Research*.

ABSTRACT

Combining Bligh-Dyer (BD) extraction and electrospray ionization-mass spectrometry (ESI-MS) techniques is useful for examination and quantification of lipids. Unfortunately, this methods typically does not allow for simultaneously monitoring of some negatively charged lipids, *e.g.*, 1,2-distearoyl-*sn*-glycero-3-phosphatidic (DSPA) acid and 1,2-distearoyl-*sn*-glycero-3-phosphatidylserine (DSPS) and their metabolites [lysophospholipid (LP) and fatty acid (FA)]. Further, data showed that the extent in loss of precursor ions did not correspond to the formation of metabolite ions and, in some cases, no detectable signals of LP. This suggests that precursor lipids and LP are not fully recovered from aqueous phases using the BD extraction or accurately measured. Optimizing the conditions by altering pH by addition of hydrochloric acid into media, we showed that signal intensity of precursor ions and LP ions of these negative lipids increased about 6 to 36 fold. We also determined the recovery rates for DSPA, DSPS, 1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol (DSPG) and neutral lipids, 1,2-dipalmitoyl-*sn*-glycero-3 phosphatidylcholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphatidyl ethanolamine (DSPE) from aqueous media (in the presence and absence of secretory phospholipase A_2, calcium ion and TRIS buffer) using the traditional BD method. Not surprisingly, traditional BD extraction methods did not result in efficient extraction of DSPA and DSPS. These studies suggest that acidification of media improves the efficiency of BD methods to extract negative-charged lipids like DSPA and DSPS and their metabolites.

To extend these studies to novel lipids we investigated the ability of BD extraction methods to extract a synthetic lipid 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-

phosphocholine (C31PC), which we recently developed as a probe to track entry of nanoparticles into cancer cells. Our data showed that C31PC, which is distinct from natural lipids because of its odd numbered its *sn*-2 acyl chain, could be identified and quantified from human prostate carcinoma (PC-3) cells without interference of endogenous lipids. In this study, lipids from PC-3 cells were extracted by BD methods and mixed with C31PC. C31PC could be detected by ESI-MS method to concentrations as low as 1.25 pmol/mL without interference from endogenous lipids extracted from PC-3 cells. Our study suggested that C31PC is a suitable lipid probe to investigate liposome-cancer cell interaction and the modified BD extraction methods are amicable to this approach.

INTRODUCTION

Bligh-Dyer (BD) extraction methods,[1] are well-established for extraction of total lipids, but their efficiency with regard to recovery of individual lipids and their metabolites from aqueous samples have rarely been investigated. Traditionally, many studies using BD methods were focused on the extraction efficiency of total lipid content, not of individual lipids from complex samples.[2-5] Additionally, at the time of development (1950s) of BD methods, the decreased sensitivity and specificity of traditional lipid measurement techniques like gravimetric methods, thin-layer chromatography and phosphorus analysis, made it difficult to accurately track any specific lipid. A review of the literature indicated that only a few studies addressed the extraction efficiency of BD methods for phosphatidic acids (PAs) or lysophospholipids in the same sample.[6-8] No studies could be found that have systematically assessed the extraction efficiency of BD methods of specific lipid classes and/or their metabolites.

Electrospray ionization-mass spectrometry (ESI-MS) has significantly advanced the study of lipids in biological samples. Compared to traditional lipid detection techniques, ESI-MS has increased sensitivity, specificity, and speed of analysis.[9] In a previous study investigating the specificity of secretory phospholipase A_2 ($sPLA_2$) against various lipids using BD extraction and ESI-MS,[10] we found that the extent of degradation of neutral lipids, like DPPC, in the presence of $sPLA_2$ could be monitored by the loss of precursor ions followed by formation of metabolite ions. However, with some negative lipids, *e.g.* DSPA and DSPS, the loss of precursor ions was not proportional to formation of metabolite ions. For example, the percentages of signal intensity of LP and FA ions were

significantly increased indicating degradation; however, the percentage of signal intensity of the precursor ions did not decrease significantly.

We recently used ESI-MS to assess the use of synthetic lipids as markers of liposome degradation in vitro after BD extraction.[10] One challenge this presented was the interference of endogenous lipids. This is also a problem if synthetic lipids found in nanoparticles, such as liposomes, are to be tracked in biological samples. Synthetic lipids offer an advantage over current markers, such as fluorescent and radio-labeled lipid as the later are indirect markers and not always suitable for human use as they can alter membrane morphology and properties of liposomes, *e.g.* bilateral microstructure and fluidity. In addition, these lipids do not always distribute uniformly in lipid membranes. As we have previously discussed in **Chapter 3**, lipids with odd carbon chains do not typically exist in nature, thus have the potential to be used as lipid probes that can be identified by ESI-MS method.

In this study, we used ESI-MS as a tool to investigate efficiency of BD methods on neutral and negatively charged lipids and developed a modified BD method to improve the recovery of lipids and metabolites with poor extraction efficiency. This technique was also applied to biological samples to identify the presence of a synthetic lipid used in nanoparticle formulations for the treatment of cancer. The results suggest that BD extraction techniques can be used in conjunction with ESI-MS to assess the delivery of specific phospholipids to biological tissues.

MATERIALS AND METHODS

Chemical and reagents

Phospholipids, DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine), DSPC (1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine), DSPG (1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol), DSPS (1,2-distearoyl-*sn*-glycero-3-phosphatidylserine), DSPE (1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine), DSPA (1,2-distearoyl-*sn*-glycero-3-phosphatidic acid), and D_{70}-DSPC (deuterated DSPC) were purchased from Avanti Polar Lipids Inc (Alabaster, AL). *1-O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphocholine (C31PC) was synthesized in our lab as previously decribed.[11] Group III $sPLA_2$ was purchased from Cayman Chemical Company (Ann Arbor, MI) and Genway Biotech Inc (San Diego, CA). Acetonitrile and methanol were of HPLC grade from Fisher Scientific (Pittsburgh, PA). All other chemicals and solvents were of analytical grade, obtained from commercial sources and used without further purification. All experiments used ultrapure water (> 3MΩ) obtained from a Millipore Milli-Q synthesis system (Billerica, MA).

$sPLA_2$–mediated degradation and BD lipid extraction

Phospholipid degradation was carried out by combining each lipid sample (1 µmol lipid/mL), $sPLA_2$ (0-10 µg/mL), $CaCl_2$ (1 mM) in phosphate buffered saline (total volume: 200 µL) , followed by incubation at 37°C for 1-24 hr. Phospholipids and metabolites were isolated using traditional Bligh and Dyer extraction for ESI-MS analysis.[1] Briefly, following incubation, 100 µL of methanol was added to samples followed by the addition of 300 µL chloroform, vortexing and centrifugation at 2,500 × g for 5 min. The organic layer was removed and transferred to a clean test tube. The above

extraction was repeated twice by adding 300 µL chloroform and the final organic phase was evaporated under nitrogen, and reconstituted in 1 mL chloroform and methanol (3:1 v/v). A 100 µL aliquot of the resultant organic solution was diluted in 900 µL chloroform and methanol (3:1 v/v) for ESI-MS analysis.

Modified BD lipid extraction for DSPA and DSPS

A modified BD method was developed specially for DSPA and DSPS. The procedure was the same as mentioned above except for adding 5 µL hydrochloric acid (HCl) solution to aqueous media (0.06N HCl to DSPA, 0.3N HCl to DSPS) after incubation.

Measurement of lipids and their metabolites by ESI-MS

The extent of phospholipid degradation in samples treated with $sPLA_2$ was assessed by the loss of intensity of precursor ions and the appearance and increased intensity of the primary metabolites, that is, LPs and FAs. Analysis was performed on an Agilent high performance liquid chromatography – mass spectrometer (LC/MSD-Trap XCT Ultra Plus) system (Santa Clara, CA). The mobile phase consisted of acetonitrile, methanol and 0.1% ammonium formate (2:3:1 v/v/v). The flow rate was 0.15 mL/min and the injection volume was 5 µL. Nitrogen was used as a nebulizing gas at 25 psi and a drying gas at 8 psi. The drying temperature was 350°C. The capillary, capillary exit and skimmer potentials were 3,500, 150.3 and 40.0 V, respectively. The *m/z* range for scanning was 200 to 2,200. The positive-ion (+MS) mode was used to measure the intensity of the precursor- and lysophospholipid-ions (LP), whereas the negative-ion (-MS) mode was used to measure the intensity of anionic lipids and fatty acid-ions (FA). Percentages of signal loss in phospholipids and signal gain in their FA and LP metabolites were

compared between individual lipids using the traditional BD extraction methods. For a specific lipid, such as DSPA or DSPS, actual signal intensity of precursor lipid and metabolites was compared between traditional and modified BD extraction methods.

Extraction efficiency of lipids by traditional BD method

Calibration curves for individual lipids (0.78-100 nmol) were prepared by diluting lipids in organic solvents (chloroform and methanol; 3:1 v/v) and spiked with internal standard (IS) D_{70}-DSPC (m/z 872.1). Standard curves were constructed by calculating the ratio of the analyte peak area to that of the internal standard, and plotting the ratio (ordinate) *versus* the theoretical concentration (abscissa); data was fit using weighted least squares; the inverse of the variance ($1/y^2$) of the observed data was used as the weighting factor. The standard curve was considered acceptable if greater than 90% of the standards, had calculated accuracies within 15% of their theoretical value, no systematic deviations over the linear range were observed, and precision had a coefficient of variation (CV) of $\leq$ 20%. The limit of quantification was determined experimentally as minimum concentration whose response was greater than five times baseline value, with a CV $\leq$ 20% and accuracy ± 20%.

Control samples were prepared by spiking 10 µL lipid (10 µmol/mL) and 20 µL IS into a 400 µL solution of chloroform and methanol (3:1 v/v). The resulting solvent was transferred to a clean new vial. The old vial was washed with 300 µL chloroform twice, and all the organic solvent also was transferred to the new vial. Buffer samples were prepared by spiking 10 µL lipid (10 µmol/mL) into a 400 µl of chloroform and methanol (3:1 v/v), then 200 µL TRIS buffer (0.05 M, including 2.5 µg/mL $sPLA_2$, 1 mM Ca^{2+}) and 20 µL IS was mixed with them and vortexed vigorously. The mixture was

centrifuged at 2,500 × g for 5 min and the organic layer was removed and transferred to a clean new vial. The above extraction was repeated twice by adding 300 µL chloroform and the resulting organic layers were transferred to the new test vial.

Organic solvent in vials was evaporated completely using a steady stream of nitrogen gas. Residues were reconstituted with 1 mL chloroform and methanol (3:1 v/v) and a 500 µL aliquot was used for ESI-MS detection. The concentrations (CONC) of control and buffer samples were calculated using calibration curves. Extraction efficiency was calculated using equation (EQ) 1:

EQ1. Extraction efficiency = $(CONC_{buffer}/CONC_{control}) \times 100\%$.

Tracking C31PC in lipid extractions from PC-3 cells

PC-3 cells were maintained in a T-75 flask until they reached confluence. Then media was removed from the flask. Cells were collected using a rubber policeman and suspended in methanol:water (2:1, 2 mL), which was transferred to a glass tube using a pipette.

Lipid content in cancer cells was extracted by using a traditional BD extraction method. A modified BD method with an acidification step was not used because C31PC is a neutral phosphatidylcholine (PC). Traditional BD methods have high extraction efficiencies for PC based on our previous studies. Briefly, chloroform (3 mL) was spiked into the glass tube and the resulting mixture vortexed for 2-3 min and centrifuged at 2,500 × g for 5 min. The organic layer was transferred to a new tube using a glass pipette and the above procedure was repeated twice. The organic layer (about 9 mL) was collected and evaporated using a steady stream of nitrogen gas to obtain lipid residues, which were then diluted with methanol:chloroform (1:3 v/v). An aliquot (10 µL) was

then subjected to a modified Bartlett phosphate assay procedure to estimate lipid concentration.[12]

For studies with C31PC, this lipid was diluted to create a series of concentrations of 80, 40, 20, 10, 5, 2.5 and 1.25 pmol/mL in lipids isolated from PC-3 cell lipids (20 pmol/mL) as a matrix. Briefly, 10µL of samples were subjected to ESI-MS. Parameters of ESI-MS system were as described above. C31PC was identified using MRM based on a fragmentation of C31PC from *m/z* 707 to 523. The standard curve C31PC of was created and assessed as described above.

Statistics

The mass spectrometry data are presented as the mean ± the standard error of the mean (SEM) of at least 3 separate experiments (n=5/study). Differences were determined following an analysis of variance for each data set using SAS software (SAS Institute, Cary, NC) followed by a Dunnett's t-test or a Student's t-test when comparisons involved a control and single variable. Differences were considered significant if the *p*-value $\leq$ 0.05.

RESULTS

BD and ESI-MS methods for measurement of DPPC degradation

To demonstrate that a combination of BD and ESI-MS can be used to track the extent of phospholipid degradation of individual lipids, we exposed DPPC to a series of concentration of Group III $sPLA_2$ (0-10 µg/mL). $sPLA_2$ are esterases that readily degrade phospholipids at *sn*-2 position, producing lysophospholipids and fatty acids. In some disease states, levels of $sPLA_2$ are elevated from several nanogram per mL in control

tissue to several microgram per mL at pathological sites.[13,14] DPPC is a neutral phospholipid with two C-16 acyl chains and a choline group, was exposed to solvent control and increasing concentrations of $sPLA_2$ for 1 hr at 37°C. Lipids and possible metabolites were extracted using traditional BD methods and analyzed by ESI-MS (**Table 4-1**). The primary peak identified in control samples corresponded to un-degraded DPPC (734.5 m/z). A concentration-dependent decrease in the intensity of DPPC, precursor peak at m/z 734.5, resulted in a greater than 50% decrease after exposure to 10 μg/mL $sPLA_2$ for 1 hr (**Table 4-1**) and a concentration-dependent increase in the intensity of peaks corresponding to DPPC's 16:0-lyosphosphatidylcholine (LP) at 496.3 m/z and the 16:0 fatty acid (FA) at 255.5 m/z (**Table 4-1**). These data demonstrated that $sPLA_2$ degrades DPPC in a concentration-dependent manner and that combination of BD and ESI-MS can be used to track $sPLA_2$-mediated degradation.

Another important finding is that metabolites are more sensitive indicators of lipid degradation than the precursor-ions. Changes in the signal intensities for LP and FA were much greater than the precursor lipid itself. This suggested that small changes in lipid signals could be observed by large changes in LP and FA. This hypothesis is supported the fact that exposure of DPPC to 0.2 μg/mL $sPLA_2$ resulted in signal intensities for DPPC that remained statistically unchanged. In contrast, signal intensities for LP and FA signals significantly increased (**Table 4-1**). These data highlight the importance of assessing both precursor phospholipids and its metabolites when assessing degradation as opposed to simply monitoring the precursor lipid itself and the difficulty in measuring changes in lipids due to differences in their extractability and ionization efficiency.

Abnormal data on DSPA and DSPS using traditional BD method

A combination of traditional BD extraction methods and ESI-MS was used to study selectivity of Group III $sPLA_2$ for specific phospholipids. **Table 4-2** shows the change in signal intensity of different species of phospholipids exposed to 2.5 μg/mL $sPLA_2$ for 24 hr compared to solvent control. For DPPC, DSPC, DSPE and DSPG, the presence of $sPLA_2$ decreased the signal intensity for precursor phospholipids and significantly increased the signal intensity for each of their metabolites ($p<0.05$). Based on these data we concluded that phospholipids with a shorter chain length were most susceptible to $sPLA_2$-mediated degradation, e.g., DPPC (C:16) vs. DSPC (C:18) and PE and PG head groups were the more susceptible to $sPLA_2$-mediated degradation.[10]

Two specific lipids whose formation of metabolites did not correlate to loss of precursor metabolites using traditional BD extraction methods were DSPA and DSPS (**Table 4-2**). For example, for DSPA, the intensity of the precursor lipid ions decreased about 75%. In contrast, there was not a corresponding increase in signal intensity of LP and FA ions, and the signal intensity for the LP. Similarly, the signal intensity for DSPS precursor lipid didn't significantly change while signals for the LPC and FA ions increased significantly ($p\leq0.05$). The increase in signal intensities for LPC and fatty acids in the presence of DSPC and $sPLA_2$ suggest that DSPS is being degraded significantly. Thus, we hypothesized that this discrepancy was related to extraction efficiency.

Traditional BD methods vs modified BD methods on DSPA and DSPS

Since phosphatidic acid (PA) and phosphatidylserine (PS) are anionic in aqueous media, addition of an inorganic acid would shift their equilibrium from ionized molecules to more unionized molecules, thus making them easier to be extracted to organic phase.

Based on this rationale, we determined the effect of acidifying the media, using hydrochloric acid, on the extraction efficiency of the traditional BD procedure. **Table 4-3** shows signal intensities of DSPA, DSPS and their metabolites after exposure to 0-2.5 μg/mL $sPLA_2$ for 24 hr using the acid BD extraction method *versus* the traditional BD method.

Acidification of media significantly ($p \leq 0.05$) increased the signal intensity of precursor lipid ions and their metabolites (**Table 4-3**). For DSPA, use of acidified BD extraction methods increased lipid signal intensity of $sPLA_2$–treated samples about 36-fold in control sample, compared to samples extracted using standard BD extraction methods. Use of the acidified BD extraction methods also resulted in about 10-fold increases in signal intensities in samples dosed with $sPLA_2$, compared to traditional BD extraction methods. For DSPS, compared to traditional BD extraction methods, lipid signal intensities of $sPLA_2$–treated samples increased about 6-fold in control samples and about 7-fold using the acidified BD extraction methods. Increases of 3- to 4-fold for LP and FA signal intensities were seen in DSPS samples exposed to $sPLA_2$. These data suggest that using an acidified BD extraction method increased the recovery or signal intensity of DSPA, DSPS and their metabolites from aqueous media.

Extraction efficiency of various lipids by traditional BD method

To determine the actual extraction efficiency for all lipids tested using standard BD extraction methods we used deuterated DSPC, D_{70}-DSPC, as an internal standard and determined the extraction efficiency of DSPC, DPPC, DSPE, DSPG, DSPA and DSPS (**Table 4-4**). The extraction efficiencies of all lipids, beside DSPA and DSPS, were above 94%. In contrast, the extraction efficiencies of DSPA and DSPA were less than 30%,

supporting the hypothesis that low signal intensities of these lipids results, in part, from their low extraction efficiencies.

Identification C31PC in PC-3 cell lipids

C31PC (m/z 707→523) could be detected in lipid samples extracted from PC-3 cells with little inference (**Figure 4-1**). Compared to solvent sample**,** signal intensity at *m/z* 523 for blank PC-3 cell lipids only had very slight increase. This suggests that interference from PC-3 cell lipids was negligible. The limit of detection was 1.25 pmol/mL (5 × background). There was a concentration-dependent increase in the intensity of the 523.5 m/z fragment. The linear range was 2.5 to 40 pmol/mL with an accuracy of 89.4 to 104% and CV $\leq$15%. (**Figure 4-2**)

DISSCUSION

This study represents a systematic investigation assessing the ability of traditional BD extraction methods to recover individual lipids for analysis by ESI-MS. Surprisingly, little information on this subject could be found in the literature despite the fact that this BD extraction methods have been in use for over 50 years. Lipid content in biological samples includes phospholipids, lysophospholipids, fatty acids and cholesterols. Theoretically, BD extraction methods should be able to extract all these species with high extraction efficiencies. Yet, others have reported decreased effectiveness against certain lipids species. For example, Iverson et al., reported that BD extraction methods were ineffective for extracting samples with high lipid content (>2%).[2] Bjerve *et.al*, also reported that BD extraction methods resulted in less than optimal recoveries of select LPs from aqueous media.[7] Despite these studies, no data could be found investigating the

efficiency of traditional BD extraction methods on common lipid classes and their metabolites. Even fewer studies could be found combining BD extraction methods in combination with ESI-MS to track phospholipid degradation. One commonality of data presented in this study is that assessment of loss of precursor phospholipids alone cannot be used to assess degradation. For example, if we assessed degradation of DSPA only by examining the precursor phospholipid, we would have erroneously concluded that DSPA was highly susceptible to $sPLA_2$-mediated degradation. This fact is important as many studies in the expanding field of lipidomics typically examine only the precursor phospholipids, resulting in the possibility of an incomplete interpretation and inaccurate conclusions.

Our data clearly show that traditional BD extraction methods yield excellent extraction efficiencies for neutral lipids PC, PE and the anionic lipid PG. In contrast, we show that this approach exhibits poor recovery for PA and PS and their LPs. Some of these data are consistent with Bjerve's data (1974) where they used a radiolabeled lipid method to determine extraction efficiencies of the traditional BD methods on different classes of LPs and found low efficiency of the traditional BD procedure on acidic LPs; however, our data demonstrate that low extraction efficiencies for these lipids can be overcome by the acidification of media. This suggests that acidification of media may be needed to recover total lipid content, including anionic lipids, from biological samples. In addition, this study showed that ESI-MS, in combination with modified extraction methods, can be used to study phospholipid classes with traditionally lower ionization efficiency, such as DSPS and PA. Further research is needed to determine the effects of these approaches in more complicated mixtures of lipids as found in biological samples.

We also used traditional BD extraction methods to assess the level of a synthetic lipid we used in nanoparticle formulations used for treatment for cancer. Our studies with C31PC once again prove that this odd-chain lipid C31PC, could be identified in PC-3 cells without interference of endogenous lipids. However, unlike our previous studies we quantified the level of C31PC in the presence and absence of biological matrices. More importantly we demonstrated that C31PC could be tracked in lipid matrices isolated from PC-3 cells. This is important because PC-3 cells are prostate cancer in origin and represent the target of nanoparticles containing C31PC. These studies further substantiate the potential therapeutic use of C31PC being as a lipid probe for assessing in *vivo* liposome deposition and lipid exchanges during endocytosis.

ACKNOWLEDGEMENTS

This research was funded in part by Georgia Cancer Coalition Distinguished Scholar Grants and an NIH NIBIB (EB08153) to BSC and RDA, a University of Georgia Graduate Fellowship Stipend Award to GZ and an American Foundation for Pharmaceutical Education–Wyeth New Investigator Award to RDA.

Table 4-1. Concentration-dependence of Group III $sPLA_2$-mediated degradation of DPPC by ESI-MS using traditional BD methods (Percent of Control)

Species (*m/z*)	$sPLA_2$ Concentration (μg/mL)			
	0	**0.2**	**1.0**	**10**
DPPC (734.5)	100 ± 3	98.6 ± 3.6	86.6 ± 2.8*	45.7 ± 7.4*
Lysophospholipid (496.3)	100 ± 17	496 ± 34*	1,560 ± 70*	16,500 ± 1,700*
Fatty acid (255.5)	100 ± 11	131 ± 14*	202 ± 21*	799 ± 68*

Data represented as the mean intensity ± SEM (n=3);

*Indicates a significant ($p \leq 0.05$) difference compared to control as observed using a one-way analysis of variance followed by a Dunnett's t-test.

Table 4-2. Traditional BD *vs* modified BD methods: Percent changes of signal intensity of precursor lipids and their metabolites after exposure to Group III $sPLA_2$ in 24hr

Lipids	Lipid ions	LP ions	FA ions
DPPC (t)	39.0±0.7*	18,300±1,040*	2,670±600*
DSPC (t)	78.0±2.7*	10,500±1,700*	1,680±230*
DSPE (t)	22.6±0.6*	13,400±540*	2,260±56*
DSPG (t)	72.1±2.2*	4,210±680*	3,510±320*
DSPA (t)	26.4±11.7*	ND	114±10
DSPS (t)	105±7.0	2130±510*	1,330±260*
DSPA (m)	84.9±18.4	100±4	139±13*
DSPS (m)	82.8±10.0*	2,378±400*	1,040±100*

t: traditional BD method;

m: modified BD method;

ND: not detectable;

Data represented as the mean intensity ± SEM (n=3);

*Indicates a significant ($p \leq 0.05$) difference compared to control as observed using a one-way analysis of variance followed by a Dunnett's t-test.

Table 4-3. Modified BD methods increased signal intensity of precursor lipids and LPs

	Lipid ions		LP ions		FA ions	
Lipids	**sPLA$_2$** $\times 10^4$	**Controls** $\times 10^4$	**sPLA$_2$** $\times 10^4$	**Controls** $\times 10^4$	**sPLA$_2$** $\times 10^4$	**Controls** $\times 10^4$
DSPA (t)	1.69±0.74	6.37±1.6	ND	ND	1.16±0.11	1.03±0.12
DSPA (m)	62.4±1.4	73.5±7.7	2.79±0.10	2.79±0.13	1.47±0.14	1.06±0.11
DSPS (t)	10.1±0.67	10.3±1.0	7.26±1.7	0.34±0.13	11.7±2.3	0.88±0.03
DSPS (m)	64.5±7.8	77.9±1.5	23.2±3.9	0.98±0.15	9.61±0.95	0.93±0.23

t: traditional BD method

m: modified BD method

ND: not detectable

Data represented as the mean intensity ± SEM (n=3).

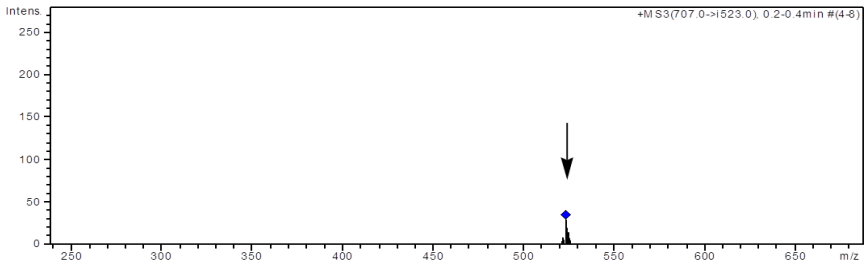
Table 4-4. Extraction efficiency of various lipids by traditional BD method (nmol/ml)

Lipids	Controls CONC	Buffer CONC	Extraction Efficiency (%)
DSPC	105±2.4	108±3.5	103
DPPC	122±3.8	115±11	94.3
DSPE	70.5±2.3	74.4±5.6	106
DSPG	83.7±4.3	83.0±3.2	99.2
DSPA	88.1±3.7	16.6±2.4*	18.8
DSPS	98.8±11	29.0±1.3*	29.4

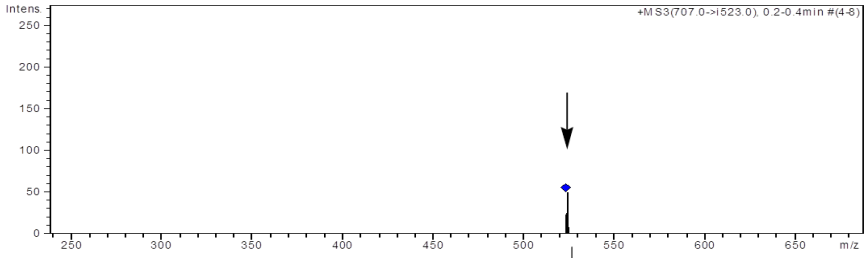
Data represented as the mean intensity ± SEM (n=5);

*Indicates a significant ($p \leq 0.05$) difference compared to control as observed using a one-way analysis of variance followed by a Dunnett's t-test.

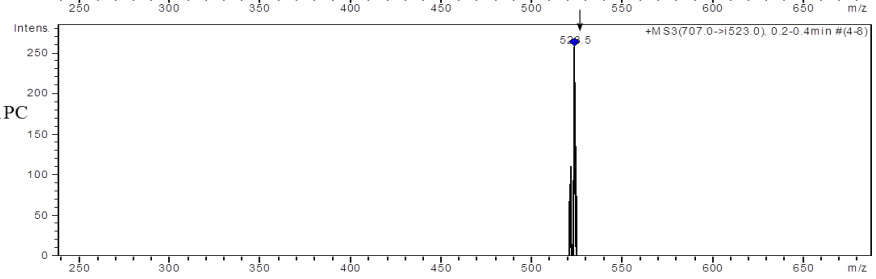
A: solvent only



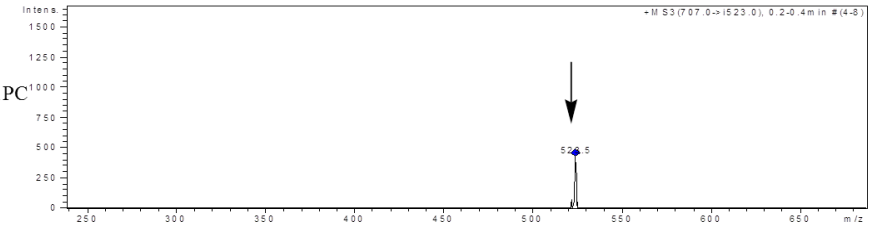
B: PC-3 only



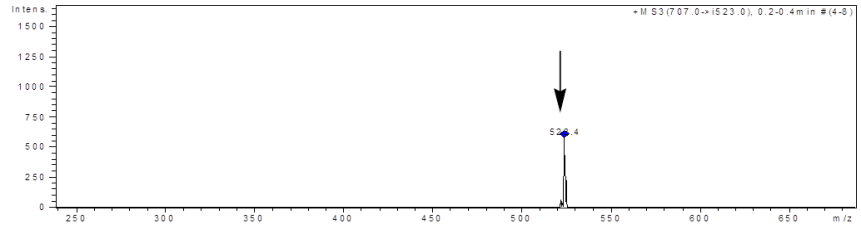
C: 1.25 pmol/ml C31PC with PC-3



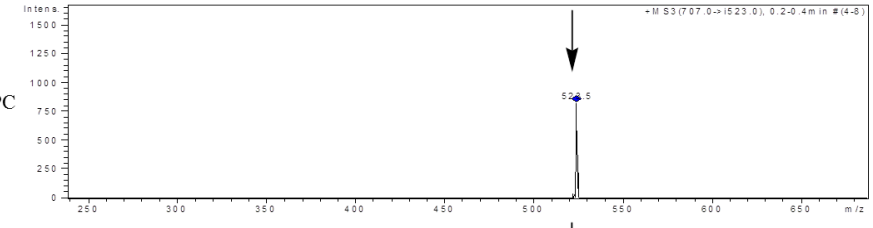
D: 2.5 pmol/ml C31PC with PC-3



E: 5 pmol/ml C31PC with PC-3



F: 10 pmol/ml C31PC with PC-3



G: 20 pmol/ml C31PC with PC-3

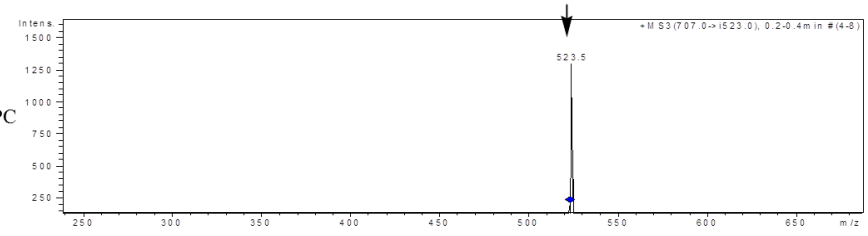


Figure 4-1. Mass spectra of solvent A, blank PC-3 cell lipids 20 pmol/mL **B**. or PC-3 cell lipids spiked C31PC 1.25 pmol/mL **C**. or 2.5 pmol/mL **D**. or 5 pmol/mL **E.** or 10 pmol/mL **F** and 20 pmol/mL **G.** C31PC (707→523 *m/z*) were identified based on their ion-pair as determined using MRM.

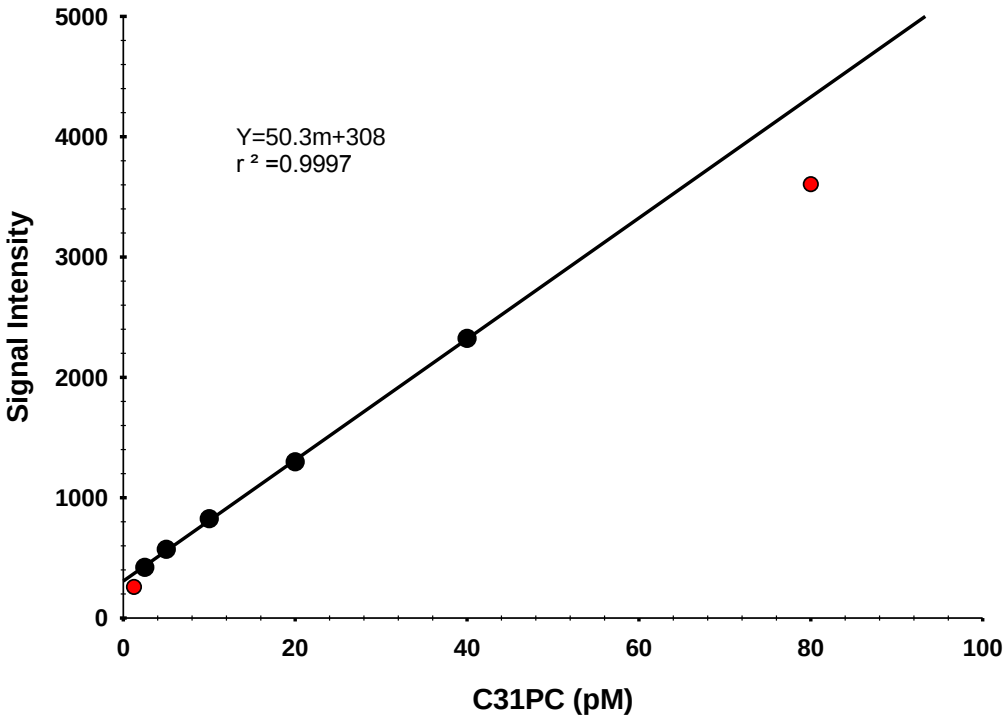


Figure 4-2. Standard curve of C31PC (707→523 *m/z*) in lipid extraction from PC-3 cells. Red dots represent standards that were detectable but outside the linear range. Black dots represent C31PC concentrations in linear range. The linear range was 2.5 to 40 pmol/mL with an accuracy ranged from 89.4 to 104%.

REFERENCES

1. Bligh EG, Dyer WJ 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37(8):911-917.
2. Iverson SJ, Lang SL, Cooper MH 2001. Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. Lipids 36(11):1283-1287.
3. Smedes F, Askland Tk 1999. Revisiting the Development of the Bligh and Dyer Total Lipid Determination Method. Marine Pollution Bulletin 38(3):193-201.
4. Manirakiza P, Covaci A, Schepens P 2001. Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh & Dyer Extraction Methods. Journal of Food Composition and Analysis 14(1):93-100.
5. Smedes F, Thomasen TK 1996. Evaluation of the Bligh & Dyer lipid determination method. Marine Pollution Bulletin 32(8-9):681-688.
6. N.W. Daae L, Bremer J 1970. The acylation of glycerophosphate in rat liver A new assay procedure for glycerophosphate acylation, studies on its subcellular and submitochondrial localization and determination of the reaction products. Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism 210(1):92-104.
7. Bjerve KS, Daae LN, Bremer J 1974. The selective loss of lysophospholipids in some commonly used lipid-extraction procedures. Anal Biochem 58(1):238-245.
8. Gerrard JM, Robinson P 1989. Identification of the molecular species of lysophosphatidic acid produced when platelets are stimulated by thrombin. Biochim Biophys Acta 1001(3):282-285.

9. Peterson BL, Cummings BS 2006. A review of chromatographic methods for the assessment of phospholipids in biological samples. Biomed Chromatogr 20(3):227-243.
10. Zhu G, Mock JN, Aljuffali I, Cummings BS, Arnold RD Secretory phospholipase A responsive liposomes. J Pharm Sci 100(8):3146-3159.
11. Zhu G, Alhamhoom Y, Cummings BS, Arnold RD Synthesis of lipids for development of multifunctional lipid-based drug-carriers. Bioorganic & Medicinal Chemistry Letters 21(21):6370-6375.
12. Bartlett GR 1959. Phosphorus assay in column chromatography. J Biol Chem 234(3):466-468.
13. Abe T, Sakamoto K, Kamohara H, Hirano Y, Kuwahara N, Ogawa M 1997. Group II phospholipase A2 is increased in peritoneal and pleural effusions in patients with various types of cancer. Int J Cancer 74(3):245-250.
14. Nevalainen TJ, Graham GG, Scott KF 2008. Antibacterial actions of secreted phospholipases A2. Review. Biochim Biophys Acta 1781(1-2):1-9.

ABBREVIATIONS

Bligh-Dyer	BD
C31PC	1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphocholine
CHOL	cholesterol
CONC	concentration
PC	phosphatidylcholine
PG	phosphatidylglycerol
PE	phosphatidylethanolamine
PA	phosphatidic acid
PS	phosphatidylserine
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine
DSPA	1,2-distearoyl-*sn*-glycero-3-phosphatidic acid
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine
D_{70}-DSPC	1,2-distearoyl(deuterated 70)-*sn*-glycero-3-phosphatidylcholine
DSPE	1,2- distearoyl-*sn*-glycero-3-phosphatidylethanolamine
DSPG	1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol
DSPS	1,2-distearoyl-*sn*-glycero-3-phosphatidylserine
ESI-MS	electrospray ionization - mass spectrometry
FA	fatty acid
FBS	fetal bovine serum
LP	lysophospholipid
SEM	standard error of the mean

$sPLA_2$	secretory phospholipase A_2
SSL	sterically-stabilized liposome

CHAPTER 5

RESEARCH SUMMARY

The primary goal of my dissertation research was to develop lipid based nanoparticulate drug carriers that are responsive to elevated expression of secretory phospholipase A_2 ($sPLA_2$) in malignant tissues to modulate drug release. This pathological-based control of release has the potential to increase drug efficacy and reduce systemic toxicity.

In **Chapters 2** and **3**, we developed a platform to evaluate $sPLA_2$-mediated lipid degradation and release kinetics from prototype formulations. We then demonstrated that we were able to prepare sterically-stabilized liposome (SSL)-like formulations with different release rates at the presence of $sPLA_2$. In **Chapter 2**, we found incorporating small amounts (10 mol%) of $sPLA_2$-preferred 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine (DSPE) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol (DSPG) in liposome formulations significantly increased in $sPLA_2$-mediated 6-carboxyfluorescein (6-CF) release in TRIS media. In **Chapter 3**, we demonstrated that $sPLA_2$-mediated 6-CF release was enhanced by including lipids with shorter acyl chains and/or methanol head group [1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphocholine (C31PC) and 1-*O*-hexadecyl-2-pentadenoyl-*sn*-glycerol-3-phosphomethanol (C31PM)] in formulations in TRIS media. The presence of serum (10%, v/v) greatly reduced $sPLA_2$-mediated 6-CF release from nanoparticles. In this media, compared to prototype SSL, inclusion of DSPE and DSPG in formulations led to

increased $sPLA_2$-mediated 6-CF release, while presence of C31PC and C31PM reduced $sPLA_2$-mediated 6-CF release in a concentration-dependent manner. These data suggest that the rates of $sPLA_2$-mediated 6-CF release from SSL-like formulations can be controlled, *i.e.* increasing or decreasing kinetics by alteration the lipid composition.

Significant differences among $sPLA_2$ isoforms in triggering 6-CF release from liposome formulations were observed. Group III was much more effective at inducing 6-CF release from liposome carriers than Group IIa (**Chapter 2**). Furthermore, Group III was more sensitive to presence of cholesterol in lipid membranes. Difference in their ability in triggering 6-CF release suggests difference in catalytic ability and substrate preference. Since Group III and IIa are elevated in different human diseases and pathological sites, our findings reveals the potential to formulate $sPLA_2$ isoform-specific drug carriers. This represents a novel approach at controlling drug delivery based on expression of different isoforms of same enzyme depending on pathophysiology.

A procedure combining Bligh and Dyer lipid extraction and electrospray ionization-mass spectrometry (ESI-MS) method was developed for determination of $sPLA_2$ specificity on individual lipid (**Chapter 4)**. In our research, we identified lipid classes like phosphatidic acid (PA), phosphatidylserine (PS) and both of their lysophospholipids (LPs) that could not be well-extracted by traditional Bligh and Dyer method. We optimized traditional Bligh and Dyer method by acidifying samples with hydrochloric acid. This resulted in an increase in unionized forms of anionic lipids and LPs thus increasing their partitioning into organic phase. This approach helped to overcome a pitfall in traditional Bligh and Dyer methods. ESI-MS provides a good platform to study lipid degradation. It is superior to traditional lipid measurement like

phosphorus assay and thin layer chromatography in its high sensitivity, specificity and is less time-consuming. We monitored the percentage change of lipid ions, LP and fatty acid (FA) ions together because they were correlated. In $sPLA_2$-mediated lipid degradation, reduction of one phospholipid resulted in formation of one LP and one FA stochastically. Tracking all three ions simultaneously reduces the risk in erroneously estimating the extent of $sPLA_2$-mediated lipid degradation. Using this platform, we determined $sPLA_2$ preferred shorter acyl chain lipids and lipids with phosphatidylglycerol (PG) or phosphatidylethanolamine (PE) head groups. This procedure is effective for screening $sPLA_2$-preferred lipids and to study lipid degradation.

An analytical method was developed to quantify $sPLA_2$-mediated lipid degradation in SSL-like formulations (**Chapter 3**). Using 1,2-distearoyl(deuterated 70)-*sn*-glycero-3-phosphatidylcholine (D_{70}-DSPC) as an internal standard, formulations containing 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC), DSPE and DSPG were extracted and quantified by ESI-MS. Data showed that the presence of fetal bovine serum (10%, v/v) greatly reduced $sPLA_2$-mediated lipid degradation. This was correlated strongly with a reduction in $sPLA_2$-mediated 6-CF release in these formulations. Yet at the same media conditions, the 6-CF release profiles among 3 formulations didn't agree with their observed lipid degradation profiles. This study suggested that $sPLA_2$-mediated lipid degradation may not be only factor controlling drug-release from liposome.

A synthetic approach to prepare novel lipids with different head groups and side chains for laboratory use was developed in **Chapter 3**. Using *R-(O)*-benzyl glycidol as starting material, we were able to regioselectively conjugate an ether chain to its *sn*-1 position and an ester chain to its *sn*-2 position. We developed an optimal method using

boron tribromide to remove benzyl group. This method was quick and carried out at low temperature (78°C, 5 min), which greatly reduced the possibility of acyl chain migration. After this, various phosphate head groups could be added to *sn*-3 position.

Odd-carbon-number acyl chain lipids could be used as lipid probes for tracking liposomes. Though fluorescent and radio-labeled lipid markers are in use, they are often not suitable for human use, have the potential to alter membrane fluidity of liposomes and do not distribute uniformly in lipid membranes. Odd-carbon-number acyl chain lipids are distinct from endogenous lipids and do not typically exist in nature, thus can be used as lipid probes. We demonstrated that C31PC could be extracted from biological samples like rat serum and human prostate carcinoma (PC-3) cells and identified by ESI-MS. Further we showed that we were able to quantify lipids from PC-3 cells with a detection limit as low as 1.25 pmol/mL.

In conclusion, we showed that $sPLA_2$-medated drug release could be controlled using different combinations of lipids. An optimized Bligh and Dyer extraction and ESI-MS method was developed and provided a rapid and sensitive platform to study lipid degradation. A synthetic approach was developed to steroselectively design novel lipids for modulating $sPLA_2$-medated drug release and to be used as lipid probes. These tools were used to assay, evaluate, synthesize and optimize the development of $sPLA_2$-responsive liposome. Our success in modulating $sPLA_2$-mediated release from drug carriers can be extended for the treatment of diseases associated with increased $sPLA_2$ level (*e.g.* cancer, arteriosclerosis and infection). We hypothesize that this approach also can be used to develop diagnostics or drug carriers that can be individualized to a patient's disease and its stage.

Future studies will determine the effects of $sPLA_2$-targeted SSL formulations *versus* prototype SSL on efficacy of specific chemotherapeutic agent *e.g.* doxorubicin in a nude mouse model of implanted tumors with high level of $sPLA_2$ (*e.g.* human prostate cancer cells, PC-3). The goal will be to determine if carriers with active release mechanism are superior to prototype carriers in the term of better efficacy. Using companion pet (*i.e.,* dog and cat) and human patients' samples, the temporal and spatial distribution and level of expression of individual $sPLA_2$-isoforms and cancer stage will be determined. These data will aid in determining optimal application of these carriers to improve treatment outcomes. The development of C31PC, as a lipid marker, will be used to examine tissue distribution of carriers and the effect of $sPLA_2$-mediated degradation. For example, C31PC and/or its metabolites (*e.g.* odd carbon chain fatty acid, C15:0 FA) will be incorporated into formulations and liposome-cancer cell interactions (*in vitro)* or determining liposome distribution/degradation (*in vivo*) will be determined. Overall I have developed lipid-based nanoparticulate drug carriers that are responsive to $sPLA_2$-mediated degradation and tools to examine the mechanistic and functional effect of this interaction. This research will serve as the foundation to develop individualized targeted therapies and diagnostics that may aid in treatment of human malignancies and other diseases that differentially express phospholipases.

APPENDIX A

MODIFIED BARTLETT PHOSPHATE ASSAY[1]

A. Procedure for total phosphorus assay

Spike phospholipid samples into 20 × 150 mm glass test tubes in triplicate. Remember buffers to suspend samples should not contain any inorganic phosphate salts. If samples are in organic solvent, remove solvent by evaporation before the procedure.

1. Heat metal blocks to 180-200ºC
2. Spike 400 µL H_2SO_4 (10 N) solution into each test tubes. Put test tubes into heated metal blocks. Digest for 1 hr at 180-200ºC
3. Cool the tubes. Spike 100 µL hydrogen peroxide (30%) solution into each tubes. Vortex after each addition. Digest again at 180-200ºC for 1.5 hr
4. Turn on water bath machine, set temperature to 100ºC
5. Pull out tubes from metal blocks. Cool the tubes to room temperature. Spike 4.6 mL fresh Molybdate reagent into each tube. Vortex after each addition
6. Spike 100 µL ascorbic acid (15%) solution into each tube. Vortex after each addition
7. Boil tubes at 100ºC for 10-15 min
8. Cool to room temperature
9. Sampling 200 µL solution from each tube and spike into a 96-well round bottom plate. Read at 830 nm at a plate reader.

B. Preparation of standard curve

Standard phosphate solution from Sigma (#661-9), concentration: 20 µg/ml.

1. Spike 50 µL (1 µg), 100 µL (2 µg), 150 µL (3 µg) and 225 (4.5 µg) of standard phosphate solution into 20 × 150 mm glass test tubes in triplicate.

2. Spike 150 distilled water into 20 × 150 mm glass test tubes in triplicate as blanks

3. Follow the procedure describe above at part A

4. Create the standard curve

5. Use the standard curve to calculate phospholipid concentrations in samples

C. Preparation of reagents

1. Molybdate Reagents

   Dissolve 1.1 gm Ammonium Molybdate and12.5 mL 10 N H_2SO_4 into 500 mL double distilled water

2. Ascorbic acid solution

   Make up 15% ascorbic acid water solution (w/v). Store in refrigerator at 4ºC. Protected from light. Use no more than 2 monthes.

REFERENCES

1. Bartlett GR 1959. Phosphorus assay in column chromatography. J Biol Chem 234(3):466-468.