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Design, Development, Characterization and Testing of CD22 Targeted Long Circulating Liposomal Drug Delivery Systems for B Cell Malignancies

Abstract

Hematological malignancies of the B cells affect almost 130,000 people in the United States every year of which approximately 44,000 lose their lives. Therapies for B cell malignancies such as doxorubicin, have limitations due to dose related adverse effects such as neutropenia and cardiomyopathy. AD 198 is a novel PKC-delta activating agent that has cytotoxic superiority over doxorubicin by being able to circumvent resistance mechanisms developed by the cancer cells towards doxorubicin and being cardioprotective from the damage caused to cardiomyocytes by doxorubicin. Targeted delivery of AD 198 is crucial to moderate the non-specific interactions of the chemotherapeutic agent with healthy cells. The objective of this work was to design, develop and evaluate AD 198 loaded liposomal formulations for targeted delivery to CD22 overexpressing B cell cancers. Liposomes were prepared by the classical Bangham method followed by size reduction by extrusion. Anti-CD22 Fab' were generated and conjugated to the long circulating AD 198 liposomes by thioether bonds. Physicochemical parameters of the CD22 targeted liposomal formulation such as size, zeta-potential, drug encapsulation, drug release and drug targeting were optimized to maximize efficacy and stability of the nanoparticles. In vitro studies established that uptake of the targeted liposomes was via an endocytotic pathway independent of the conventional proteins involved in clathrin and caveolae dependent endocytosis. Uptake was preferentially higher in CD22 overexpressing malignant B cells (Daudi) compared to cells devoid of CD22 (Jurkat). Intracellular localization of the targeted liposomes was observed to be in endolysosomes which suggests that drug release was via lysosomal enzymatic breakdown of the liposomal structure which would release encapsulated AD 198. Cytotoxicity was also observed to be higher in Daudi cells compared to Jurkat cells whereas free solution AD 198 had comparable cytotoxicity in both cell types. The mechanism of cell death was deduced to be apoptosis by the activation of apoptotic proteins such as caspase-3, and by the inhibition of oncoproteins such as c-myc. Stability of the dispersed liposomal formulation was inadequate for long term storage. To address this concern, lyophilized formulations were developed and optimized to maximize long term storage as displayed by short term stability studies. It was determined that targeted drug delivery with liposomal AD 198 was more potent and specific compared to other untargeted formulations.

Document Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Program

Pharmaceutical Sciences

Research Advisor

George C. Wood, Ph.D.

Keywords

AD 198, B cell malignancies, Liposome, Lyophilization, Nanoparticles, Targeted Nanoparticles

Subject Categories

Medicine and Health Sciences | Pharmaceuticals and Drug Design | Pharmacy and Pharmaceutical Sciences

Comments

One year embargo expired May 2016

**Design, Development, Characterization and Testing of CD22 Targeted Long
Circulating Liposomal Drug Delivery Systems for B Cell Malignancies**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Nivesh Kumar Mittal
May 2015

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DEDICATION

For my parents without whose love and support I would be nothing today, my wife Saini, who stood by me even when she did not agree with me, and my sister Molly, who was an inspiration in her own ways.

ACKNOWLEDGEMENTS

I am thankful to all people who have helped me in the successful completion of this dissertation project. I am immeasurably grateful to my advisor Dr. George C. Wood for his research and academic guidance and unconditional support throughout this work. I thank Dr. Laura A. Thoma for her firm commitment to support my research. I am thankful to my academic committee members Dr. Himanshu Bhattacharjee, Dr. Leonard Lothstein, Dr. Timothy D. Mandrell and Dr. Duane D. Miller for their advice, support and help at various stages of the this work.

I extend my sincere thanks to all members of the Plough Center for Sterile Drug Delivery Systems (past and present). I am thankful to Bivash Mandal and Pavan Balabathula for their help, support and suggestions in this work. I thank Dr. Robert Nolly, Dr. Vivian Loveless, Dr. Vinayagam Kannan, Dr. Hari R. Desu, Frank P. Horton, Gwen Stornes, Jennifer L. Hart, Harriet C. Lang, Howard Nguyen and Dr. Elizabeth D. Barker for their support at various stages. I am also thankful to all the members of Dr. Subhash Chauhan group, Dr. Anjaparavanda P. Naren group, Dr. Ram I. Mahato group, Dr. Lawrence M. Pfeffer group and the Imaging Center at The Neuroscience Institute at UTHSC for their help and support. I would also like to thank Dr. Ellen S. Vitetta at The University of Texas Southwestern Medical Center and her group for generously providing essential materials for the study.

I am grateful to University of Tennessee Health Science Center, College of Pharmacy and the Plough Center for Sterile Drug Delivery Systems for the facilities, equipment and financial support. I would like to thank all the faculty and staff of the Department of Pharmaceutical Sciences for their support.

ABSTRACT

Hematological malignances of the B cells affect almost 130,000 people in the United States every year of which approximately 44,000 lose their lives. Therapies for B cell malignancies such as doxorubicin, have limitations due to dose related adverse effects such as neutropenia and cardiomyopathy. AD 198 is a novel PKC-delta activating agent that has cytotoxic superiority over doxorubicin by being able to circumvent resistance mechanisms developed by the cancer cells towards doxorubicin and being cardioprotective from the damage caused to cardiomyocytes by doxorubicin. Targeted delivery of AD 198 is crucial to moderate the non-specific interactions of the chemotherapeutic agent with healthy cells. The objective of this work was to design, develop and evaluate AD 198 loaded liposomal formulations for targeted delivery to CD22 overexpressing B cell cancers. Liposomes were prepared by the classical Bangham method followed by size reduction by extrusion. Anti-CD22 Fab' were generated and conjugated to the long circulating AD 198 liposomes by thioether bonds. Physico-chemical parameters of the CD22 targeted liposomal formulation such as size, zeta-potential, drug encapsulation, drug release and drug targeting were optimized to maximize efficacy and stability of the nanoparticles. In vitro studies established that uptake of the targeted liposomes was via an endocytotic pathway independent of the conventional proteins involved in clathrin and caveolae dependent endocytosis. Uptake was preferentially higher in CD22 overexpressing malignant B cells (Daudi) compared to cells devoid of CD22 (Jurkat). Intracellular localization of the targeted liposomes was observed to be in endolysosomes which suggests that drug release was via lysosomal enzymatic breakdown of the liposomal structure which would release encapsulated AD 198. Cytotoxicity was also observed to be higher in Daudi cells compared to Jurkat cells whereas free solution AD 198 had comparable cytotoxicity in both cell types. The mechanism of cell death was deduced to be apoptosis by the activation of apoptotic proteins such as caspase-3, and by the inhibition of oncoproteins such as c-myc. Stability of the dispersed liposomal formulation was inadequate for long term storage. To address this concern, lyophilized formulations were developed and optimized to maximize long term storage as displayed by short term stability studies. It was determined that targeted drug delivery with liposomal AD 198 was more potent and specific compared to other untargeted formulations.

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LIST OF ABBREVIATIONS

μL	Microliters
λ	Wavelength
ABC	Accelerated blood clearance
AD 198	<i>N</i> -benzyladriamycin-14-valerate
ADC	Antibody drug conjugate
ALL	Acute lymphoblastic leukemia
BCA	Bicinchoninic acid
BMT	Bone marrow transplantation
^{BPC} NeuAc	9- <i>n</i> -biphenylcarboxyl-NeuAc α 2-6Gal β 1-4GlcNac
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
CHEMS	Cholesteryl hemisuccinate
CHOL	Cholesterol
CHOP	Cyclophosphamide, hydroxydaunorubicin, Oncovin and prednisone
CLIC	Clathrin independent carrier
CLL	Chronic lymphocytic leukemia
CME	Clathrin mediated endocytosis
DAPI	4', 6-diamidino-2-phenylindole
DLS	Dynamic light scattering
DMF	Dimethylformamide
DMPC	Dimyristoylphosphatidylcholine
DMSO	Dimethyl sulfoxide
DOPC	Dioleoylphosphatidylcholine
DOPE	Dioleoylphosphatidylethanolamine
DOX	Doxorubicin
DSC	Differential scanning calorimetry
DSPC	Distearoylphosphatidylcholine
DSPE-PEG ₂₀₀₀ -COOH	Distearoyl- <i>n</i> -(3-carboxypropionoyl)poly(ethyleneglycol)succinyl) phosphatidylethanolamine
DTT	Dithiothreitol
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylene diamine tetra acetic acid
EPC	Egg phosphatidylcholine
EPR	Enhanced permeability and retention
Fab'	Antigen binding fragment
Fc	Constant fragment of antibody/crystallizable fragment
FL	Follicular lymphoma
FUDR-dO	3', 5'-O-dioleoyl-5-fluorouridine deoxyribose
Fv	Variable fragment of antibody

G	Gram
GEEC	GPI-anchored protein-enriched early endosomal compartment
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High pressure liquid chromatography
HSPC	Hydrogenated soy phosphatidylcholine
HZ-PEG ₂₀₀₀ -DSPE	Hydrazide-PEG ₂₀₀₀ -DSPE
IC ₅₀	Concentration at which 50% of cells die
ILS	Increase in life span
IVIVC	<i>In-vitro</i> - <i>in vivo</i> correlation
K	Thousand
L	Liter
LCCTLA	Long circulating CD22 targeted liposomal AD 198
LCLA	Long circulating liposomal AD 198
LDL	Low density lipoprotein
LL1	Anti-CD74 antibody
Lyo-LCCTLA	Lyophilized LCCTLA
mAb	Monoclonal antibody
mal-DSPE-PEG ₂₀₀₀	Maleimide-distearoyl phosphatidylethanolamine – polyethylene glycol 2000
Mal-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[maleimide(polyethylene glycol)-2000]
MALT	Mucosa-associated lymphoid tissue
MDR	Multi-drug resistance
mL	Milliliters
MLCCTLA	Magnetic LCCTLA
MLCLA	Magnetic LCLA
MLV's	Multilamellar vesicles
MM	Multiple myeloma
MPB-PE	N-4-(p-maleimidophenyl) butyryl) phosphatidylethanolamine
mPEG ₂₀₀₀ -DSPE	1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000]
mPEG ₂₀₀₀ -PE	mPEG ₂₀₀₀ -phosphatidylethanolamine
MPS	Mononuclear phagocytic system
mTorr	Millitorr
MTS	Mean time of survival
MWCO	Molecular weight cut-off
M-β-CD	Methyl-beta-cyclodextrin
N	Normal
Na ₂ HPO ₄	Disodium phosphate
NaCl	Sodium chloride

NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NaF	Sodium fluoride
NaVO ₄	Sodium orthovanadate
NBD-PC	12-[n-(nitrobenz-2-oxa-1, 3-diazol-4-yl) amino] dodecanoyl phosphatidylcholine
NBD-PE	12-[n-(nitrobenz-2-oxa-1, 3-diazol-4-yl) amino] dodecanoyl phosphatidylethanolamine
NCTD	Norcanthridin
ng	Nanogram
NHL	Non-Hodgkin's lymphoma
NHS	N-hydroxysuccinimide
NL-DOX	Non-liposomal DOX
nm	Nanometers
No.	Number
pAKT	Phosphorylated protein kinase B
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
P-gp	P-glycoprotein
PKC	Protein kinase C
PLD	Pegylated liposomal doxorubicin
PMPC	Palmitoyl-myristoylphosphatidylcholine
PMSF	Phenylmethylsulfonylfluoride
POPC	Palmitoyl-oleoylphosphatidylcholine
PVDF	Polyvinylidene fluoride
q.s.	Quantity sufficient
RES	Reticulo-endothelial system
ROS	Reactive oxygen species
RT	Room temperature
SATA	N-succinimidyl-s-acetylthioacetate
scFv	Short chain variable fragment of antibody
SCID	Severely combined immune deficiency
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SGML	Sphingomyelin
SIL-DOX	Stealth® doxorubicin immunoliposomes
SL-DOX	Stealth® liposomal DOX
SLL	Small lymphocytic lymphoma
SMPC	Stearoyl-myristoylphosphatidylcholine
SPC	Soy phosphatidylcholine
SPDP	N-hydroxysuccinimidyl-3-(2-pyridyldithio) propionate
SUV's	Small unilamellar vesicles
TBS	Tris buffered saline

TBST	Tris buffered saline and tween® 20
TCEP	Tris(2-carboxyethyl)phosphine
TEM	Transmission electron microscopy
T _g	Glass transition temperature
TO	Triolein
US FDA	United states food and drug administration
V	Volts
VCR	Vincristine
VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor
vs	Verses

CHAPTER 1. INTRODUCTION*

1.1. Statement of the Problem

Cancers are tissue/organ specific and may manifest as solid tumours such as breast cancer or lung cancer or can be circulating as in hematopoietic malignancies. B cell malignancies are characterized as hematopoietic malignancies (Harris et al., 2000, Mittal et al., 2014). Hematopoietic malignancies mostly occur due to uncontrolled proliferation of the leukocytes. These include the leukemias and lymphomas (Swerdlow et al., 2008). One of the main causes for the development of B cell malignancies are genetic abnormalities. Various genes are over expressed such as cell cycle control genes, cell cycle checkpoint genes and DNA synthesis and replication genes. As an example, the gene *Ki67* is commonly used to determine the extent of proliferation of the malignancy (Alizadeh et al., 2000). In the United States, B cell malignancies are the most common cause of cancer in children at birth and up to 19 years of age. Approximately 130,000 people in the United States are expected to be diagnosed with a B cell malignancy in the year 2015, of which about 44,000 will lose the battle against this lethal disease. (The Leukemia and Lymphoma Society, 2014, Delarue et al., 2013)

Depending on the type of cancer, chemotherapy is often considered to be a debilitating aspect of therapeutic intervention. The stage of progression of the tumour determines the treatment options and can range from surgery to chemotherapy and/or radiation (Brannon-Peppas and Blanchette, 2004, Love et al., 1989). Although the type and stage of cancer dictates the treatment options, chemotherapy is the primary treatment option for haematological malignancies. Limitations of solution chemotherapy include lack of cell specificity, development of resistance mechanisms and debilitating adverse effects such as loss of function of healthy tissue, nausea, vomiting and tiredness (Zhang et al., 2007, Sahoo et al., 2007).

To this end, the past decade has seen significant advancements in the field of nanotechnology and has provided researchers with the means to overcome some of these limitations and develop superior drug delivery systems. One type of nanoparticles are liposomes, that are nanosized vesicles with unilamellar or multilamellar phospholipid bilayers that surround aqueous compartments. **Figure 1.1** illustrates the most important components of a liposome. Depending on the physicochemical characteristics of the therapeutic molecules, they can be encapsulated either in the bilayer or in the aqueous core of the liposome. These specialized nanoparticulate carrier systems can be designed to deliver a range of therapeutic entities ranging from small molecule drugs to peptides, proteins and nucleic acids.

They can be made to target specific cancer cells thus increasing drug efficacy and

*Adapted with permission. Mittal, N. K., Bhattacharjee, H., Mandal, B., Balabathula, P., Thoma, L. A. & Wood, G. C. (2014). Targeted liposomal drug delivery systems for the treatment of B cell malignancies. *J Drug Target*, 22, 372-86.

Figure 1.1. Components of unilamellar liposomal drug formulations

Notes. Hydrophilic drugs are encapsulated in the aqueous core, whereas hydrophobic drugs are incorporated into the lipid bilayer (Zamboni, 2005, Molema, 2001). The bilayer is principally composed of a phospholipid such as phosphatidylcholine. Cholesterol molecules impart some degree of rigidity to the bilayer thus increasing bilayer stability, mDSPE-PEG₂₀₀₀ (methoxy-distearoyl phosphatidylethanolamine – polyethylene glycol 2000) molecules make the liposome surface more hydrophilic which aid in escape from removal by the immune cells, mal-DSPE-PEG₂₀₀₀ (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]) serves as an anchor where the targeting ligands can be conjugated, and the targeting ligand can be any molecule that can be specific for a particular cancer tissue. The targeting ligands shown here are half-antibody molecules and Fab' (antigen binding fragment) of the antibody. These systems are modified to make them more compatible with the type and or physiology of the targeted tissue (Davis et al., 2008).

reducing the non-specific actions of the drug, which eventually reduces the adverse effects (Bharali and Mousa, 2010). Liposomes aim at achieving selectivity towards the malignant cells by harnessing the targeting ability of several cell surface markers which are over-expressed exclusively in certain malignant cells. CD19, CD20, CD22 and CD74 are a few of the markers over-expressed on malignant B cells (Sapra and Allen, 2002).

Another significant feature of these systems is their ability to overcome multi-drug resistance (MDR) which is a major problem in chemotherapy (Thierry et al., 1993). One of the mechanisms for MDR is via P-glycoprotein (P-gp), an efflux pump, which drives the therapeutic molecules out of the cancer cells. Nanoparticles such as liposomes are designed to utilize the enhanced permeability and retention (EPR) effect to exit the blood vessels in the tumour and target the surface receptors on tumour cells. **Figure 1.2** shows how the liposomes then enter the cells by endocytosis without being recognized by P-gp producing a longer activity at the cancerous cell (Brigger et al., 2002).

The treatment of blood malignancies employing targeted particulate drug delivery systems still presents unique challenges and considerable research has been focused towards the development of targeted liposomal formulations for B cell leukemias and lymphomas. A number of studies have demonstrated the advantages of targeted liposomal systems encapsulating doxorubicin or vincristine (Mayer et al., 1994, Rodriguez et al., 2009, Sapra et al., 2004, Sarris et al., 2000). In this study, liposomal encapsulation of newer anti-neoplastic agents which are superior to doxorubicin are considered. *N*-benzyladriamycin-14-valerate (AD 198) is a newer lipophilic anthracycline, which is superior to doxorubicin (DOX) in murine cancer models and has novel biochemical pathways and pharmacological properties (He et al., 2005). It has been shown that the cells that had developed (multiple drug resistance) MDR toward DOX, were sensitive to AD 198 (Cai et al., 2010, Edwards et al., 2013, Ganapathi et al., 1989). The cardiotoxicity manifested by DOX is also absent in AD 198 and that was one of the rationales for AD 198 development. While the pharmacological and biochemical superiority of AD 198 over DOX has been proven by Ganapathi et al (Ganapathi et al., 1989), its usefulness in targeted drug delivery was yet to be determined. In the present study an AD 198 loaded targeted liposomal system for B cell malignancies has been developed.

1.1.1. The drug – AD 198

Figure 1.3 shows the structure of *N*-benzyladriamycin-14-valerate or AD 198, a protein kinase C (PKC) activating agent that demonstrates cytotoxic superiority over doxorubicin (Hofmann et al., 2007). It does so by circumventing multiple mechanisms of drug resistance to which doxorubicin is susceptible. The mechanism of action of AD 198 (**Figure 1.4**) induced cell death is entirely different from that of doxorubicin. Doxorubicin functions by preferential binding to chromosomal DNA of intact cells (Mompalmer et al., 1976).

Conversely, AD 198 functionality does not depend on binding to DNA. It

Figure 1.2. Nanoparticles can overcome surface efflux pump mediated drug resistance

Notes. Nanoparticles are designed to utilize the EPR effect to exit blood vessels in the tumor, to target surface receptors on tumor cells, and to enter tumor cells by endocytosis before releasing their drug payloads. This method of delivery allows for high intracellular drug concentrations that can overcome efflux-pump mediated drug resistance (Davis et al., 2008)

Chemical Formula: C₃₉H₄₃NO₁₂
Molecular Weight: 717.76

Figure 1.3. Structure of *N*-benzyladriamycin-14-valerate (AD 198)

Figure 1.4. AD 198 mechanisms of action

Notes: AD 198 actions are not very well defined. Information known is that AD 198 binds to PKC, thus activating the holoenzyme. This activation is sufficient to produce pro-apoptotic effects. Later the catalytic segment (CS) dissociates from the PKC- δ . The CS activates at least three downstream molecules. One is PLS3 (phospholipid scramblase 3) which depolarizes the mitochondria releasing cytochrome c (Cyt C). Cyt C has two effects, one is to release Ca^{+2} ions from the endoplasmic reticulum and second to activate caspases (primarily caspase 3). Caspase 3 eventually results in apoptosis. The second molecule activated by CS is PLS1 (phospholipid scramblase 1) which depolarizes the cell membrane that causes the cell to undergo apoptosis. However, AD 198 is also cytotoxic in cells that do not express PLS1 such as HL60 cells. The CS also phosphorylates Rad9, which in turn depolarizes the nuclear membrane also resulting in apoptosis.

functions by the activation of a PKC- δ induced cascade of apoptotic mechanisms (He et al., 2005, Lothstein et al., 2006). AD 198 does not display the cardiotoxic property of doxorubicin. Moreover, it is cardioprotective to the cardiomyocytes from the damage caused by doxorubicin therapy (Cai et al., 2010). Previously it was known that the cardiotoxicity of doxorubicin is due to the generation of reactive oxygen species (ROS) from the semi-quinone ring. Although the same ring is also present in AD 198 structure, Hofmann et al hypothesized that the cardioprotective nature of AD 198 was due to its ability to activate PKC- ϵ (Hofmann et al., 2007). PKC- ϵ is a key component of protective ischemic preconditioning which prevents cardiac damage (Murry et al., 1986). Recent studies have now proven that the cardiotoxicity of doxorubicin is due to its activity as a poison to topoisomerase II beta (TOP2B). Zhang et al (Zhang et al., 2012) have shown that the deletion of TOP2B in mice prevents doxorubicin mediated cardiotoxicity.

Nevertheless, AD 198 is no wonder drug with all advantages and no limitations. Recently, an unpublished drug evaluation study of solution AD 198 in dogs conducted by Paradox Pharmaceuticals Inc. shows that AD 198 has adverse effects such as neutropenia and thrombocytopenia similar to other chemotherapeutic drugs. **Figures 1.5 – 1.8** are results from this study that indicate that there was some level of liver and kidney injury after solution AD 198 therapy. As specified earlier, these injuries are due to interactions of the drug with non-target or healthy tissues. Development of a targeted long circulating liposomal AD 198 drug delivery system will impart specificity to the drug action thus increasing efficacy and reducing adverse effects.

1.1.2. The disease – B cell malignancies

B cell malignancies are characterized as hematopoietic malignancies (Harris et al., 2000). Hematopoietic malignancies mostly occur due to the uncontrolled proliferation of the leukocytes, which include the leukemias and lymphomas. B cell malignancies can be categorized into two broad classes – precursor B cell neoplasms, which include B cell lymphomas and leukemias, and the mature B cell neoplasms (Swerdlow et al., 2008, Harris et al., 2000). **Table 1.1** summarizes the classification of B cell neoplasms. Both lymphomas and leukemias are often included in the same classification, as both solid and circulating phases as present in lymphoid neoplasms. For example, B cell chronic lymphocytic leukemia (CLL) and B cell small lymphocytic lymphoma (SLL) are different manifestations of neoplasm of the same cell. The distinction between the two being that CLL is primarily circulating whereas SLL presents itself at the lymph nodes. B cell malignancies can either be indolent or rapidly proliferating. CLL and follicular lymphoma (FL) are rather indolent (Alizadeh et al., 2000) compared to the other types of B cell malignancies. This is derived primarily due to the observation that they manifest as clusters in proximity to the resting B cells.

Figure 1.5. Mean change in neutrophil count after solution AD 198 treatment (n=3)

Notes. Mean drop in white blood cells (WBC's), mainly neutrophils was approximately 40% on day 7 after treatment with solution AD 198 (*unpublished drug evaluation study of solution AD 198 in dogs conducted by Paradox Pharmaceuticals Inc*). Error bars represent standard deviation around the mean.

Figure 1.6. Alanine transaminase (ALT) levels after AD 198 treatment (n=3)

Notes. ALT levels measure liver health and change in these levels indicate liver damage/disease. In this figure we can see a drop in ALT levels by almost 50% after solution AD 198 treatment (*unpublished drug evaluation study of solution AD 198 in dogs conducted by Paradox Pharmaceuticals Inc*). Error bars represent standard deviation around the mean.

Figure 1.7. Mean change in alkaline phosphatase levels after solution AD 198 treatment (n=3)

Notes. Alkaline phosphatase levels are also indicative of liver health. Typically, increased levels of alkaline phosphatase denote diseases of the liver/ liver damage. In this study, alkaline phosphatase levels rose almost 4 times the normal range (*unpublished drug evaluation study of solution AD 198 in dogs conducted by Paradox Pharmaceuticals Inc*). Error bars represent standard deviation around the mean.

Figure 1.8. Mean blood urea nitrogen (BUN) after solution AD 198 treatment (n=3)

Notes. BUN is indicative of renal health. If diet is kept approximately the same, increased levels of BUN indicate lower excretion by kidneys indicating kidney damage. Post solution AD 198 treatment BUN levels were higher by 60% of the normal levels (*unpublished drug evaluation study of solution AD 198 in dogs conducted by Paradox Pharmaceuticals Inc*). Error bars represent standard deviation around the mean.

Table 1.1. Types of B cell neoplasms

Precursor B cell neoplasm	Mature (peripheral) B cell neoplasms
Precursor B-lymphoblastic leukemia/lymphoma (precursor B cell acute lymphoblastic leukemia)	B cell chronic lymphocytic /small lymphocytic lymphoma B cell prolymphocytic leukemia Lymphoplasmacytic lymphoma Splenic marginal zone B cell lymphoma (+/- villous lymphocytes) Hairy cell leukemia Plasma cell myeloma/plasmacytoma Extranodal marginal zone B cell lymphoma of MALT type Nodal marginal zone B cell lymphoma (+/- monocytoïd B cells) Follicular lymphoma Mantle-cell lymphoma Diffuse large B cell lymphoma Mediastinal large B cell lymphoma Primary effusion lymphoma Burkitt's lymphoma/Burkitt cell leukemia

1.1.3. Therapies for B cell malignancies

Currently available therapies for haematological malignancies of the B cells can be divided into conventional, advanced and experimental therapies. These therapies are currently in use, those which are used by a few patients today and those in which most are currently under clinical trials. Conventional therapies include treatment options like chemotherapy, bone marrow transplantation (BMT) and radiation therapy (Leonard, 1998).

Chemotherapy is a treatment modality, employing anti-neoplastic drug's administered either parenterally, or orally to eradicate malignant cells. Combinations of two or three chemotherapeutic agents are common treatment regimens, with CHOP being the standard (Leonard, 1998). CHOP therapy consists of cyclophosphamide, hydroxydaunorubicin (doxorubicin), Oncovin® (vincristine) and prednisone. For an overview of the most currently used chemotherapeutic drugs the reader is advised to read a report by the Leukemia and Lymphoma Society (The Leukemia and Lymphoma Society, 2014). Chemotherapeutic agents function by truncating the growth and division of cancer cells that have a much higher rate of development compared to normal tissues. However, the non-specific nature of these treatments, result in loss of healthy tissue. This property is seen to cause considerable adverse effects which are due to the inherent toxicity of the chemotherapeutic agents (Mackey et al., 2012, Soultati et al., 2012, Marchese et al., 2011). In recent years attempts have been focused on eradicating these adverse effects using the application of nanoparticulate drug delivery systems.

Advanced therapies include biological therapies such as cytokines, monoclonal antibodies (Gupta and Kanodia, 2002, Cheson and Leonard, 2008) and antibody drug conjugates (ADC's)(Carter and Senter, 2008, Senter, 2009), whereas experimental therapies include approaches that are based on sophisticated drug delivery platforms (mainly targeted nanoparticles such as liposomes) that directly and indirectly show promise of effectively treating blood malignancies. Nanoparticles such as liposomes can also be made to target specific tissues by immobilizing target specific molecules on their surface (Torchilin, 1994). One such class of molecules are antibodies and antibody conjugated liposomes are often referred to as immunoliposomes.

In the following sections emphasis has been given specifically on liposomal systems developed for B cell malignancies. Types of targeting, barriers to delivery of liposomes to B cell malignancies, specific approaches in active targeting to malignant B cells, the impact of liposomal construct design and selections of targeting agents are extensively discussed.

1.2. Nanoparticle Targeting

Most research activities initiated and studied employing targeted drug delivery systems are associated with solid tumours. A mechanistic understanding of nanoparticle

targeting to malignant tissues can be explained by two well-studied phenomenon; 1) passive targeting and 2) active targeting (Danhier et al., 2010). These processes have been extensively discussed in the literature and the reader is directed to multiple reviews for a comprehensive study of these phenomenon (Hirsjärvi et al., 2011, Byrne et al., 2008). Briefly, passive targeting takes advantage of the EPR effect. Here the nanosized particles tend to accumulate in the tumours due to leaky angiogenic vasculature and poor lymphatic drainage. The EPR effect has been shown to be effective in particles of only a particular size range, the cut-off for which is between 400-600 nm. However, the EPR effect is not seen in sizes below 100 nm (Society, 2013, Matsumura and Maeda, 1986). This is because the smaller particles can diffuse freely in and out of the tumour vasculature. Therefore their concentration in the tumour site falls once their concentration in the plasma decreases. Nanoparticles behave as macromolecules which are big enough to escape renal clearance (Longmire et al., 2008). Even though they cannot pass through normal vasculature, extravasation through tumour vasculature can take place with ease due to the EPR effect. Thus their concentration inside the tumour increases steadily and goes many folds above that of the plasma as the lymphatic drainage in the tumour is poor (Greish, 2007, Iyer et al., 2006). This effect can be exemplified by the study of a polymer-protein conjugate SMANCS (Styrene maleic acid neocarzinostatin). SMANCS is neocarzinostatin modified with a synthetic polymer [(styrene)₁₋₃(maleic acid₄₋₇/anhydride₁)]. Maeda and co-workers have shown that these particles accumulate in the tumours at a higher concentration than the protein alone (Maeda et al., 1979, Maeda, 2001). This phenomenon was attributed to the presence of a high concentration of vascular permeability factors such as bradykinin, nitric oxide, peroxynitrate and VPF (vascular permeability factor, which is identical to VEGF) in the tumour tissue all of which are shown to be retained inside the solid tumours in excess of 100 hours. Excessive accumulation in the lymph nodes is also noticed for these macromolecules in contrast to the small molecule drugs. The efficacy of targeting via the EPR effect has been shown in many types of solid tumours (Maeda et al., 2000). **Figure 1.9** displays how EPR occurs in cancerous tissues.

However, once in the tumour microenvironment, these particles may not be taken up by the cells or release the drug. It is due to this uncertainty; that most bio-recognition molecules are conjugated onto the surface of the nanoparticle to enhance specificity. This is an active targeting approach which not only distinguishes between normal cells and cancer cells but also between different types of cancer (Wang and Thanou, 2010). These moieties decide the final destination of the particles, which are cell surface proteins overexpressed on cancer cells. **Figure 1.10** shows that once the ligand is bound to its receptor on the cancer cell, the particle is either engulfed by the process of receptor mediated endocytosis (Davis et al., 2008) or its interaction with the cell surface induces drug leakage. This leaking drug ultimately enters the cell via diffusion (Düzgüneş and Nir, 1999).

Figure 1.9. Mechanism of the EPR effect

Notes. **(A)** Normal Tissue: Nanoparticles (NPs) generally do not exocytose through normal tissue which has normal development of endothelial and smooth muscle cells. **(B)** Malignant Tissue: NPs in the effective size range extravasate the abnormal vasculature having wide fenestrations.

Figure 1.10. Nanoparticles can enter cells via endocytosis

Notes. This figure shows a transmission electron micrograph of nanoparticles at the surface of a cancer cell, entering the cell and within endocytic vesicles (Davis et al., 2008).

1.3. Barriers for Delivery of Chemotherapeutics to B Cell Malignancies Using Immunoliposomes as Carriers

The key contrast between targeting solid tumors and targeting cancers that have a largely floating population of malignant cells, such as B cell malignancies, is that solid tumors can be targeted via both the targeting procedures; i.e. passive and active targeting. Thus, when chemotherapeutic agents are targeted to a solid tumor, for example breast cancer, using an immunoliposomal delivery system, the malignant cells will experience a drug concentration which is a result of the EPR effect in addition to the binding of the immunoliposome to its specific receptor on the malignant cells. However, passive targeting would not be an option for the floating malignant cells as the EPR would be ineffective due to lack of the anatomy required for EPR. The only method available to target circulating malignant cells would be by active targeting, which is by targeting specific overexpressed receptors on the surface of the B cells primarily. Hence, targeting immunoliposomal chemotherapy to B cell cancers is not the same as targeting the same delivery system to solid tumors. A number of additional barriers to targeting immunoliposomes to cancers have been discussed by Peer et al (Peer et al., 2007). A brief discussion on the most significant factors is given here.

The efficacy of antibody targeted drug delivery systems relies immensely on the specific binding of the targeting monoclonal antibody (mAb)-drug delivery system conjugate with the receptor to which it is targeted (DiJoseph et al., 2006). To actively target immunoliposomes to a particular type of cancer, the choice of surface receptor targeted is critical. To maximize targeting efficiency, the surface receptor must be overexpressed on the malignant cells relative to the normal cells. In the case of B cells, CD19 is one such receptor that is overexpressed on malignant B cells. An optimum receptor density would be 10^4 - 10^5 copies per cell (Peer et al., 2007). However, as one particular receptor is constantly targeted, the immune system potentially down-regulates or even eliminates the antigen being targeted. This is known as 'immunoediting' (Dunn et al., 2002).

Also, CD19 is an internalizing receptor, which means that upon ligand binding, it undergoes receptor mediated endocytosis, thus delivering the entire drug load inside the CD19+ malignant B cell. This results in higher drug concentrations inside the cell compared to the immunoliposomes targeted to non-internalizing receptors such as CD20 (Sapra and Allen, 2002). The binding affinity between the ligand and receptor must also be optimum as shown by Allen (Allen, 2002). A higher affinity between the receptor and ligand promotes binding but eventually prevents internalization.

Another debate exists regarding the use of whole mAbs or their fragments. As discussed by Allen (Allen, 2002), use of whole antibodies for targeting purposes is always advantageous due to the presence of two binding sites on a single antibody. However, the crystallizable fragment (Fc) of a whole mAb can also bind to the Fc receptor of normal cells thus increasing immunogenicity. Another advantage of using whole mAb is the stability it has during long term storage. Although advantages of employing whole antibodies rather than their fragments for targeting purposes exists,

research is slowly shifting towards the use of fragmented antibodies since they are safer and display reduced non-specific binding (Cheng and Allen, 2008, Loomis et al., 2010, Sapra et al., 2004).

Rapid clearance by the reticulo-endothelial system (RES) and MPS (mononuclear phagocytic system) is another challenge faced by lipid based drug delivery systems. This can be overcome by using PEGylated liposomes. However, this is not always the case as there are also non-pegylated liposomes such as Daunosome® and Myocet® in the market today, although their circulation times are not as long as the pegylated liposomes such as Doxil®.

Pegylated liposomes are themselves susceptible to a phenomenon called accelerated blood clearance (ABC) (Ishida et al., 2006). This process causes the rapid clearance of pegylated liposomes from circulation upon repeated injection. PEG specific IgM antibodies are secreted by the B cells (Ichihara et al., 2011), which increase in concentration after the first dose is injected. Thus, the first dose is unaffected by this phenomenon. However the second dose is exposed to the anti-PEG IgM's secreted and is thus rapidly cleared. Ishida et al. reported that this phenomenon is inversely dependent on the concentration of the phospholipids injected. A concentration of 5 μ M total phospholipids failed to induce ABC; however, a concentration of 1 μ M did induce the immune response. Another criterion for the immune reaction as reported by the group, is that DOX loaded pegylated liposomes will mount a much weaker response as compared to placebo liposomes. Therefore it is essential to identify the appropriate concentration of drug and lipid to facilitate maximum circulation time in the blood.

1.4. Targeted Liposomal Systems for B Cell Malignancies

It has been suggested that targeting of B cell lymphomas can occur by leveraging the EPR effect (Xiao et al., 2011). However, this is yet to be demonstrated and lacks literature evidence. The answer may be inherent in the problem itself where it is known that the leukemias generally do not manifest as solid tumour and hence do not exist as a malignant tissue with leaky vasculature. This physiological fact precludes the use of the passive targeting approach via the EPR effect. This being said, different types of lymphomas have different manifestations and in some instances the lymph nodes are affected. In such manifestations of the disease the EPR effect may be used effectively (Xiao et al., 2011), but for malignant cells in circulation, active targeting becomes vital.

In one approach for targeted delivery the drug molecule is conjugated to the antibody directly. These molecules are known as ADC's. This has limitations in terms of loss of antibody specificity, drug activity, long purification times, and stability and also in terms of the economic viability, as coupling each drug molecule with a molecule of antibody, proves to be an expensive therapeutic option. These shortcomings have encouraged the development of other systems that are capable of carrying a higher drug load with the usage of comparatively fewer antibodies, carbohydrate molecules or any other targeting ligands, compared to the number of drug molecules that they carry. There

are a number of ADC products used in the clinic today such as Gemtuzumab ozogamicin and Inotuzumab ozogamicin. Gemtuzumab is specific for CD33 and Inotuzumab is specific for CD22. Both these receptors are overexpressed in the respective B cell malignancies (Alley et al., 2010). A major advantage of a liposomal drug delivery system is the potential for surface modification lending it as a versatile system. A number of targeting molecules or ligands conjugated to nanoparticulate drug delivery systems have been explored for the treatment of haematological malignancies (Lopes de Menezes et al., 1998, de Menezes et al., 1999, Ishida et al., 2001, Sapra and Allen, 2004, Sapra et al., 2004, Allen et al., 2005, Cheng and Allen, 2008). Additionally, the use of specific lipids such as, lipid-PEG conjugates when inserted into the liposomal structure, afford superior targeting and pharmacokinetic behaviours (Newman et al., 1999).

1.5. Epitopes Targeted by Liposomal Systems for B Cell Malignancies

One of the advantages of using liposomes for drug delivery is their ability to afford a higher therapeutic index from drugs with a low therapeutic index (Bhattacharjee et al., 2010). Another advantage is their biodegradable nature that has made them an attractive option to deliver the cytotoxic drugs for the treatment of cancer. Currently there is at least one liposomal product that is approved by the FDA for use in patients with acute lymphoblastic leukemia (ALL) (Rodriguez et al., 2009). **Table 1.2** gives comprehensive information about liposomal drugs that are currently approved for marketing or in different stages of clinical and pre-clinical trials. The formulation characteristics of this liposomal product, called Marqibo®, are discussed later in this section (Sarris et al., 2000). A liposomal annamycin product which shows promise for treatment of paediatric relapsed ALL is currently in phase 1 clinical trials (Booser et al., 2002). Another liposomal product called LEM-ETU, encapsulating Mitoxantrone, is in Phase 1 clinical trials (Chang and Yeh, 2012). A review of liposomal drug products currently approved by the FDA and those in various stages of clinical trials can be found in Allen and Cullis (Allen and Cullis, 2013) and Chang and Yeh (Chang and Yeh, 2012). Maqibo® is a passively targeted liposome and so are the ones in clinical trials mentioned above. This review of literature attempts to summarize the efforts of various groups with a focus on actively targeted liposomal systems for the therapy of lymphoproliferative disorders.

Targeting may be achieved actively or in a passive way. In case of active targeting, for B cells, the targets can be either internalizing such as CD19, CD22 and CD74 or non-internalizing such as CD20 (Sapra and Allen, 2002). The mechanism by which the drug reaches its site of action (the nucleus for most drugs) is different for both types of targets. For non-internalizing epitopes, the drug gets released slowly at the cell surface and subsequently enters the cell by passive diffusion or other transport mechanisms. It is speculated that the rate at which the drug diffuses away from the site exceeds the rate at which the drug enters the cell and thus the complete drug load is not utilized even though it is delivered near the site of action.

However, in case of internalizing epitopes, the drug load in the liposome is

Table 1.2. Liposomal drugs currently approved for marketing or in pre-clinical/clinical trials

Drug	Receptor targeted	Model	Results	Reference
<i>Marketed</i>				
Vincristine (Marqibo®)	Untargeted	Approved by FDA for use in humans	--	(Sarris et al., 2004)
<i>Clinical Trials</i>				
Annamycin (L-annamycin, Phase II)	Untargeted	Human refractory ALL patients	62% of patients had an efficacy signal with complete clearing of circulating peripheral blasts.	(Wetzler et al., 2013)
<i>Pre-clinical Studies</i>				
Dexamethasone	CD74	CB-17 SCID Mice	CD74-IL-DEX vs. CD74-ILs Difference in days of survival: 13.56	(Mao et al., 2013)
Doxorubicin	CD19	CB-17 SCID Mice	Mean survival time: Free DOX: 29 days Anti-CD19-HSPC [DOX]: 47 days	(Allen et al., 2005)
Doxorubicin	CD22	Human Burkitt lymphoma cell line, Raji	Compared to PLD, IL-PLD are 3.1 to 5.4 times more cytotoxic in CD22+ Raji cells	(O'Donnell et al., 2010)
Doxorubicin	CD22	CB-17 SCID Mice	Both PLD and IL-PLD were more efficacious and showed reduced cytotoxicity compared to free DOX.	(Tuscano et al., 2010)

delivered inside the cells so the possibility of the drug diffusing away from the site of action is eliminated or reduced. After the liposome binds to its internalizing target, such as CD22, the complete immunoliposomal complex is endocytosed by a process described as receptor mediated endocytosis (Sapra and Allen, 2002). Receptor mediated endocytosis is the process by which cells internalize molecules or particles upon receptor interaction. Once the complex is inside the cell, the drug is released due to action of lysosomal and endosomal enzymes. This should result in higher concentrations of the drug inside the cell compared to non-internalizing epitope targeted systems.

Four targets for treatment of B cell malignancies that have been studied extensively are CD19, CD20, CD22 and CD74. Of these CD19, CD22 and CD74 are internalizing whereas CD20 is non-internalizing. In this literature review an attempt has been made to summarize the findings of several research groups that have studied these targets. **Table 1.3** provides a concise format of the most important information in the literature. Also given are the ratios of the formulation excipients used in the studies.

1.5.1. CD19

CD19, an internalizing epitope, is a differentiation antigen from the B cell lineage and is expressed early in its cycle (Rickert et al., 1995). Initially, it was shown that DOX loaded CD19 targeted liposomes were superior to the free drug, untargeted liposomes, free anti-CD19 antibody and combinations thereof (Lopes de Menezes et al., 1998). The liposomes were prepared using the same method as described by Cheng et al (Cheng and Allen, 2008). Briefly, the dried lipid films were hydrated using 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 140 mM NaCl buffer (pH 7.4). The hydrated liposomes (multilamellar vesicles, MLV's) were sequentially extruded at 65°C through a series of polycarbonate filters of pore sizes from 0.4 µm - 0.08 µm to give SUV's (small unilamellar vesicles). For preparing hydrazide derivatized liposomes the mPEG₂₀₀₀-DSPE (1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]) was substituted with hydrazide-PEG₂₀₀₀-DSPE (HZ-PEG₂₀₀₀-DSPE). Doxorubicin was loaded using the ammonium sulfate gradient method as described by Haran et al. The hydrazide coupling method was utilized to couple the anti-CD19 mAb to the long circulating liposomes (Benita, 2006). These liposomes were tested in malignant B cells expressing CD19 surface antigens (Namalwa Cells). Compared to untargeted liposomes, these gave three times higher binding to B cells. Studies in SCID (severely combined immune deficiency) mice showed that CD19 targeted SIL-DOX (DOX loaded stealth® immunoliposomes) compared to untargeted or free DOX gave a much higher therapeutic effectiveness and also long term survivors (2/9 in one study and 4/9 in another).

Although CD19 targeted DOX loaded liposomes accumulate less rapidly compared to free DOX, the cellular levels of DOX were several folds higher in cells treated with the targeted liposomes compared to cells treated with non-targeted liposomal DOX (de Menezes et al., 1999). It was also seen that the targeted liposomes appeared mainly at the cell surface after 1 hour, with some DOX sequestered in vesicular structures

Table 1.3. Summary of literature on liposomal systems for B cell malignancies

Drug	Receptor targeted	Lipid ratio	Ligand	Ligand binding procedure	Ligand amount	Size (nm)	Drug loading	Reference
Doxorubicin	CD19	HSPC:CHOL:mPEG ₂₀₀₀ -DSPE, 2:1:0.1	HD37 mAb/ Fab'/ScFv	Post Insertion	Ligand: Mal-PEG ₂₀₀₀ -DSPE, 1:10	100 ± 10	0.26 µmol DOX/µmol phospholipid	(Cheng and Allen, 2008)
Doxorubicin	CD19	HSPC:CHOL:mPEG ₂₀₀₀ -DSPE/ Hz-PEG ₂₀₀₀ -DSPE, 2:1:0.10	Anti-CD19 mAb	Hydrazone	Ligand: PL, 1:1000	110 ± 10	140-160 µg DOX/µmol phospholipid	(Lopes de Menezes et al., 1998)
Doxorubicin	CD19	HSPC:CHOL:Hz-PEG ₂₀₀₀ -DSPE, 2:1:0.10 HSPC:CHOL:mPEG ₂₀₀₀ -DSPE:Mal-PEG ₂₀₀₀ -DSPE, 2:1:0.08:0.02	Anti-CD19 mAb	Hydrazone Thioether	Ligand: PL, 1:2000	95-110	0.26 mmol DOX/mmol phospholipid	(de Menezes et al., 1999)
Doxorubicin	CD19	Major Lipid:CHOL:mPEG ₂₀₀₀ -DSPE, 2:1:0.10 Major lipid = DMPC, DOPC, DSPC, PMPC, HSPC, POPC, SMPC)	Anti-CD19 mAb	Thioether	-	100 ± 20	NA	(Charrois and Allen, 2004)

Table 1.3. Continued

Drug	Receptor targeted	Lipid ratio	Ligand	Ligand binding procedure	Ligand amount	Size (nm)	Drug loading	Reference
Doxorubicin	CD19	HSPC:CHOL:mPEG ₂₀₀₀ -DSPE/Hz-PEG ₂₀₀₀ -DSPE, 2:1:0.1	Anti-CD19 mAb	Hydrazone	50 µg ligand/µmol PL	95-110	150 µg DOX/µmol	(Lopes de Menezes et al., 2000)
Doxorubicin	CD19	DOPE/CHEMS	Anti-CD19 mAb	Thioether	Ligand: PL, 1:2000	120 ± 10	NA	(Ishida et al., 2001)
Doxorubicin	CD19	Non-pH Sensitive: HSPC:CHOL:mPEG ₂₀₀₀ -DSPE, 2:1: 0.01 pH-sensitive: DOPE:mPEG ₂₀₀₀ – DSPE:Mal- PEG ₂₀₀₀ -DSPE, 10:0.4: 0.1 DOPE:CHEMS:mPEG ₂₀₀₀ -DSPE:Mal-PEG ₂₀₀₀ -DSPE, 6:4:0.24: 0.6	Anti-CD19 mAb	Thioether	Ligand: PL, 1:2000	95-110	150µg DOX/µmol phospholipid	(Kirchmeier et al., 2001)

Table 1.3. Continued

Drug	Receptor targeted	Lipid ratio	Ligand	Ligand binding procedure	Ligand amount	Size (nm)	Drug loading	Reference
Doxorubicin	CD19	HSPC:CHOL:Hz-PEG ₂₀₀₀ -DSPE, 2:1:0.10 HSPC:CHOL:mPEG ₂₀₀₀ -DSPE:Mal-PEG ₂₀₀₀ -DSPE, 2:1:0.08:0.02 HSPC:CHOL:mPEG ₂₀₀₀ -DSPE/Hz-PEG ₂₀₀₀ -DSPE, 2:1:0.10	Anti-CD19 mAb	Thioether Or Hydrazone Or Post Insertion	Ligand: PL, 1:2000	NA	NA	(Allen et al., 2002d)
Doxorubicin	CD22	DSPC:CHOL:PEG ₂₀₀₀ -DSPE/ ^{BPC} NeuAc-PEG-DSPE, 60:35:5	^{BPC} NeuAc	Ethyl amine	Ligand:PL, 1:19	100 ± 10	Loading efficiency 90%	(Chen et al., 2010)
Doxorubicin	CD22	HSPC:CHOL: PEG ₂₀₀₀ -DSPE:dl- α -tocopherol, 56.1:38.2:5.5:0.2	HB22.7	Post Insertion	NA	PLD: 118 ± 10 IL-PLD: 165 ± 10	NA	(O'Donnell et al., 2010)
Doxorubicin	CD22	NA	Anti-CD22-ScFv	NA	200 μ g ligand for 10-20 mg PL	80-100	NA	(Loomis et al., 2010)
Doxorubicin	CD19 or CD20	HSPC:CHOL:mPEG ₂₀₀₀ -DSPE, 2:1:0.1 HSPC:CHOL:mPEG ₂₀₀₀ -DSPE- Mal-PEG ₂₀₀₀ -DSPE, 2:1:0.08:0.02	α CD19 α CD20	Thioether	Ligand: PL, 1:2000	100 ± 10	NA	(Sapra and Allen, 2002)

Table 1.3. Continued

Drug	Receptor targeted	Lipid ratio	Ligand	Ligand binding procedure	Ligand amount	Size (nm)	Drug loading	Reference
Vincristine or Doxorubicin	CD19	SGML:CHOL:mPEG ₂₀₀₀ -DSPE, 55:40:5 SGML: CHOL: mPEG ₂₀₀₀ -DSPE: Mal-PEG ₂₀₀₀ -DSPE, 55:40:4:1 HSPC:CHOL:mPEG ₂₀₀₀ -DSPE, 2:1:0.1. HSPC:CHOL:mPEG ₂₀₀₀ -DSPE:Mal-PEG ₂₀₀₀ -DSPE, 2:1:0.08:0.02	Anti-CD19 mAb or anti-CD19 mAb Fab' fragment	Thioether	Ligand: PL, 1:2000 Or Ligand: PL, 1:1000	120-130 or 100 ± 10	NA	(Sapra et al., 2004)
	CD19 or CD20	SGML:CHOL:mPEG ₂₀₀₀ -DSPE, 55:40:5. SGML: CHOL: mPEG ₂₀₀₀ -DSPE: Mal-PEG ₂₀₀₀ -DSPE, 55:40:4:1 HSPC:CHOL:mPEG ₂₀₀₀ -DSPE, 2:1:0.1. HSPC:CHOL:mPEG ₂₀₀₀ -DSPE:Mal-PEG ₂₀₀₀ -DSPE, 2:1:0.08:0.02	αCD19 IgG2a) or αCD20 (IgG1)	Thioether	Ligand: PL, 1:2000	120 ± 10 or 100 ± 10	NA	(Sapra and Allen, 2004)
Vincristine	Untargeted	EPC: CHOL or DSPC: CHOL, 55:45	--	--	--	120-1000	Loading efficiency 95%	(Mayer et al., 1994)

Table 1.3. Continued

Drug	Receptor targeted	Lipid ratio	Ligand	Ligand binding procedure	Ligand amount	Size (nm)	Drug loading	Reference
Norcanthridin	CD19	SPC:CHOL:mPEG ₂₀₀₀ -PE, 2:1:0.1 SPC:CHOL:mPEG ₂₀₀₀ -PE: Mal-PEG ₂₀₀₀ -DSPE, 2:1:0.08:0.02	Anti-CD19 mAb	Post Insertion	Ligand: Mal-PEG ₂₀₀₀ -DSPE, 1:250/50/25	Untargeted 104.12 Targeted 118.32	Loading efficiency 46.51%	(Zhang et al., 2010)
FUdR-dO	CD74	EPC:DPPE-PEG ₂₀₀₀ -Mal: FUdR-dO, 1:0.2:0.1	Anti-CD74 (LL1)	Thioether	NA	~110	NA	(Lundberg et al., 2004)
Milatuzumab	CD74	HSPC:CHOL:mPEG ₂₀₀₀ -DSPE, 2:1:0.1 HSPC:CHOL:mPEG ₂₀₀₀ -DSPE, 2:1:0.08:0.02	Anti-CD74 (Milatuzumab)	Thioether	Ligand: PL, 1:2000	NA	NA	(Hertlein et al., 2010)
Dexamethasone	CD74	EPC:CHOL:mPEG ₂₀₀₀ -DSPE, 65:33.5:1.5 HSPC:CHOL:mPEG ₂₀₀₀ -DSPE:Mal-PEG ₂₀₀₀ -DSPE	Anti-CD74	Thioether	Ligand: PL, 1:1000	~103	NA	(Mao et al., 2013)

in the cells, compared to the untargeted formulation, which appeared only at the surface of the cells. After two hours almost all of the targeted formulation was sequestered into the vesicular structures inside the cells with the untargeted formulation still only present at the surface. After 48 hours incubation it was shown that the targeted formulation started accumulating DOX inside the nucleus, which is its site of action. Menezes et al have experimentally acquired a 5 fold higher cytotoxicity compared to the untargeted formulation, and this too, selectively in CD19+ cells only (de Menezes et al., 1999). In this study two different types of conjugations were analysed, hydrazide and thioether. The HSPC (hydrogenated soy phosphatidylcholine):CHOL (cholesterol):HZ-PEG₂₀₀₀-DSPE liposomes were prepared using the same ratio of lipids and the method as described above in Cheng et al (Cheng and Allen, 2008). The HSPC:CHOL:mPEG₂₀₀₀-DSPE:mal -PEG₂₀₀₀-DSPE liposomes were prepared in the molar ratio 2:1:0.08:0.02 and using the same method as the other set of targeted liposomes. It was seen that the hydrazide coupling method had a coupling efficiency of 32% and the thioether method had a coupling efficiency of 80%.

Other than the rate of uptake, another important parameter that must be studied for targeted formulations is the effect of variable release rates of the drug from the liposome on cytotoxicity. This study has been performed by Charrois et al (Charrois and Allen, 2004). They have shown that the drug release rates of DOX from untargeted liposomes are responsible for toxicities in an orthotropic 4T1 murine mammary carcinoma model. Liposomes with three release rates were studied – fast, intermediate and slow. Liposomes with varying release rates were prepared by altering the liposome fluidity by changing the fatty acyl chain length and/or degree of saturation of the phosphatidylcholine in the liposomes. The lipids used in the formulations were, primary lipid lipid:CHOL:mPEG₂₀₀₀-DSPE and the molar ratios in which they were used were 2:1:0.1. The primary lipids that they tested were DMPC (dimyristoylphosphatidylcholine), DOPC (dioleoylphosphatidylcholine), DSPC (distearoylphosphatidylcholine), HSPC, PMPC (palmitoyl-myristoylphosphatidylcholine), POPC (palmitoyl-oleoylphosphatidylcholine) and SMPC (stearoyl-myristoylphosphatidylcholine). The method of liposome preparation and drug loading was the similar to the one described in Cheng et al (Cheng and Allen, 2008). Of the seven lipid formulations tested, the liposomes prepared using DOPC, POPC, and DSPC were selected for studies in tumor bearing mice. Liposomes prepared from DOPC gave fastest release rates, POPC gave intermediate and DSPC gave the slowest drug release rates. The researchers tested the concentration of DOX in the tumour and in cutaneous tissues. It was seen that the formulation with the slowest release rates gave the highest DOX concentrations in both the tumour and the cutaneous tissues and also best therapeutic activity, whereas the fastest releasing formulations gave the lowest concentrations in both. However, unexpected toxicities were noticed in case of the intermediate formulations and these toxicities had no particular pattern with respect to the composition of the liposomes. It was speculated then that this toxicity may be controlled by targeting the formulations with anti-CD19 antibody.

In a related study (Allen et al., 2005) conducted in Namalwa cells by conjugating CD19 specific antibodies to the liposomes via the thioether bond by using mal-PEG-

DSPE (Benita, 2006). Five different formulations comprising of HSPC, DPPC, PMPC, DOPC and POPC were tested. Their drug release rates varied from 11.2 -85.5 hours in the order POPC<DOPC<PMPC<DPPC<HSPC. The molar ratio of the lipids, major lipid:CHOL:mPEG₂₀₀₀-DSPE was 2:1:0.1. For preparing targeted liposomes, 0.02 mole% of the mPEG₂₀₀₀-DSPE was substituted with mal- PEG₂₀₀₀-DSPE. Liposomes were prepared in the same method as described by Cheng et al. (Cheng and Allen, 2008). The results revealed that there was a significant increase in the life span of all test groups. The test group for CD19 targeted slow release formulation gave an increase in life span (ILS) of 88% with one long term survivor compared to the non-targeted one that gave only 12%. The CD19 targeted fast release formulation gave an ILS of 46% compared to only 12% of the non-targeted. The intermediate release results for ILS were all below the fast and slow release formulations but the CD19 targeted formulations gave comparatively better results compared to the non-targeted formulations. This pair of studies showed that the toxicity and the therapeutic effect of a formulation is the result of liposome composition, mechanism of liposome uptake and the drug release rates from the liposome.

A similar formulation targeted to CD19+ B cells has shown to be efficacious in patients with multiple myeloma (MM) (Lopes de Menezes et al., 2000). They coupled anti-CD19 antibody to the long circulating liposomes using the hydrazide method (Benita, 2006). To promote visualization, a fluorescent lipid, 12-[N-(nitrobenz-2-oxa-1,3-diazol-4-yl) amino] dodecanoyl phosphatidylethanolamine (NBD-PE), 0.1%, was incorporated into the liposomes when preparing them. Liposomes were composed of HSPC:CHOL:mPEG₂₀₀₀-DSPE/HZ-PEG₂₀₀₀-DSPE in the ratio 2:1:0.1. The method of preparation was the same as described in Cheng et al. (Cheng and Allen, 2008). Targeted liposomes carrying DOX showed higher binding to and cytotoxicity against the CD19+ ARH77 MM cell line, than did the non-targeted liposomal DOX or liposomal DOX that was targeted using isotype matched antibodies. The internalizing nature of CD19 was noted by the observation of fluorescence in the early endosomes that were formed. *Ex vivo* studies were also done using the peripheral blood mononuclear cells (PBMC) from patients of MM. These cells were treated in three separate groups with free DOX, untargeted liposomal DOX (DOX-SL) and targeted DOX immunoliposomes (DOX-SIL-anti-CD19). It was seen that the free DOX was equally cytotoxic to both B and T cells, the DOX-SL were minimally toxic to both B and T cells whereas the SIL-DOX-anti-CD19 were selectively cytotoxic for B cells in PBMC. This study showed that targeted liposomes had an IC₅₀ at a 0.98 µM concentration of DOX whereas untargeted liposomes had an IC₅₀ of 10.4 µM DOX, which suggests that antibody targeted liposomes are more effective than untargeted therapies.

DOX appears to be the molecule of choice so far for researchers working on B cell malignancies. Few studies have been undertaken to compare the efficacy of liposomal encapsulation of DOX with other cytotoxic drugs. Liposomal encapsulation of vincristine (VCR) is also known to reduce the neurotoxic effects that are elicited by its free drug form (Sarris et al., 2000). VCR gets concentrated more specifically in lymph nodes and in tumours than in nerves. The method of preparation of VCR liposomes is briefly described. Liposomes comprising of sphingomyelin (SGML) and CHOL were

prepared at a lipid concentration of 100 mg/ml. 1 ml of VCR sulfate at a concentration of 100 mg/ml was added to the preformed liposomes followed by addition of 5 ml sodium phosphate for injection (14.2 mg/ml). This mixture was heated for 10 minutes at 60-65°C. These liposomes are currently approved for use in patients with relapsed non-Hodgkin's lymphomas (NHLs) and ALL. The study concluded that liposomal VCR can be used in patients with relapsed NHL with only a fraction of patients showing neurotoxicity. Thus, it is suggested that liposomal VCR can also be used in previously untreated patients.

Sapra et al have compared targeting liposomal VCR and liposomal DOX to CD19 via the whole antibody or just it's the Fab' fragment (Sapra et al., 2004). The untargeted VCR liposomes were prepared using SGML: CHOL: mPEG₂₀₀₀-DSPE at a molar ratio of 55:40:5. The CD19 targeted VCR liposomes were composed of SGML: CHOL: mPEG₂₀₀₀-DSPE: Mal-PEG₂₀₀₀-DSPE at a molar ratio of 55:40:4:1. Briefly, the dried lipid film was hydrated in a 300 mM citrate buffer to give MLV's. The liposomes were then extruded through a series of polycarbonate membranes. VCR was loaded using a transmembrane pH dependant procedure. Untargeted DOX liposomes were composed of HSPC:CHOL:mPEG₂₀₀₀-DSPE at a molar ratio of 2:1:0.1. CD19 targeted DOX loaded liposomes were composed of HSPC:CHOL:mPEG₂₀₀₀-DSPE:Mal-PEG₂₀₀₀-DSPE at a molar ratio of 2:1:0.08:0.02. The antibody or its Fab' fragment was attached to the liposomes via the Mal-PEG-DSPE using the thiol reaction (Benita, 2006) and all the above described formulations were tested in Namalwa cells. It was seen that the targeted liposomes using the whole antibody (SIL [α CD19]) or the Fab' fragment (SIL [Fab']) had a higher binding compared to the non-targeted liposomes. The (SIL [Fab']) had a longer circulation time than (SIL [α CD19]). VCR gave faster release rate from the liposomes than DOX and also VCR formulations gave a better therapeutic effect compared to DOX formulations. For DOX formulations the (SIL [Fab']) was more efficacious than the (SIL [α CD19]). However, for VCR, both the formulations were equally effective.

Liposomal VCR emerges more efficacious compared to liposomal DOX. The reason for this is that the efficacy of liposomal VCR is largely dependent upon two parameters. First, it is dependent on the composition of the vesicles. Using egg phosphatidylcholine (EPC)/cholesterol for preparing vesicles, the ILS does is not significant compared to free VCR. However, when distearoylphosphatidylcholine (DSPC)/cholesterol vesicles are prepared, ILS increased to values as high as 133%. Secondly, it was seen that liposomal VCR activity is also largely dependent on particle size. If the size of the vesicles is increased from 100 nm to 1 μ m, the ILS falls from 133.3% to 55.6% (Mayer et al., 1994). In this study, the vesicles were prepared in a method similar to the one described by Sarris et al. (Sarris et al., 2000). The only difference here is that five freeze thaw cycles were used followed by ten cycles of extrusion via polycarbonate membranes. EPC: CHOL vesicles were prepared in a molar ratio 55:45, and the DSPC: CHOL vesicles were also prepared using the same molar ratios.

Norcanthridin (NCTD) is another molecule for which similar studies have been performed. For the study testing the efficacy of CD19 targeted liposomal NCTD,

untargeted liposomes were prepared using SPC (soy phosphatidylcholine):CHOL:mPEG₂₀₀₀-PE at a molar ratio of 2:1:0.1. The CD19 targeted liposomes were prepared using SPC:CHOL:mPEG₂₀₀₀-PE (mPEG₂₀₀₀-phosphatidylethanolamine):Mal-PEG₂₀₀₀-DSPE at a molar ratio of 2:1:0.08:0.02. The antibody used was the human anti-CD19 monoclonal antibody 2E8. The liposomes were prepared using the film dispersion method followed by extrusion via stacked polycarbonate membranes, and the antibody incorporated using the post insertion method (Allen et al., 2002a) to give 2E8-NCTD-liposomes. The formulation was tested in the human leukemic B cell line NALM-6 and an MTT assay showed specific cytotoxicity toward NALM-6 cells in a dose and time dependent manner and the viability of these treated cells was much lower than the same cells treated with the untargeted formulation or free NCTD (Zhang et al., 2010). However, the efficacy of this formulation was not tested *in vivo*, thus it is difficult to arrive at a conclusion as to how effective targeting or liposomal encapsulation of this molecule may be to ILS or number of long term survivors.

One of the novel ways for targeting CD19 was recently studied by Ishida and co-workers. They have explored the possibility of making stimuli sensitive targeted liposomes sensitive to change in pH (Ishida et al., 2001). This study tested CD19 targeted and untargeted variants of two types of liposomes, one being pH sensitive and the other not sensitive to pH. The pH sensitive liposomes were prepared by means of a disulphide bond introduced in mPEG₂₀₀₀-DSPE to give mPEG-S-S-DSPE. This link was known to break down at pH 5.5. The major lipid used for preparing the pH sensitive liposomes was DOPE (dioleoylphosphatidylethanolamine) or DOPE and CHEMS (cholesteryl hemisuccinate). CHEMS is a stabilizing molecule which assists DOPE in forming a bilayer structure at neutral pH. For the non-pH sensitive liposomes the major lipid used was HSPC. Detailed molar ratios of the ten formulations tested and their procedure for preparation can be referred to in Ishida et al. (Ishida et al., 2001). Briefly, the dried lipid films were hydrated using an appropriate buffer and extrude via a series of polycarbonate membranes. These CD19 targeted liposomes were prepared using the anti-CD19 antibody, which was coupled to the liposomes using the maleimide derivatized PEG (mal-PEG) to give a thioether bond (Benita, 2006). *In vitro* leakage, nuclear accumulation, cytotoxicity and *in vivo* elimination in BALB/c inbred mice was studied. It was observed that the pH sensitive liposomes showed enhanced delivery of DOX to the nuclei of cells and thus higher cytotoxicity compared to non-pH sensitive liposomes. This result was seen in human plasma but not in culture media. The *in vitro* study concluded that pH sensitive formulations delivered drug more efficiently and had a higher cytotoxicity than the other formulations. The *in vivo* studies also suggested that CD19 targeted pH sensitive formulations were the most effective in ILS compared to other formulations. The non-pH sensitive CD19 targeted liposomes gave a mean survival time of 42 days and a percent ILS of 101%, whereas the pH sensitive CD19 targeted liposomes gave a mean survival time of 54 days and an ILS of 156%. These results were statistically significant and exhibited that using pH sensitive delivery can increase the therapeutic efficacy of targeted liposomes.

Another method employed to form pH sensitive liposomes is by using PE's like DOPE (Kirchmeier et al., 2001). As already mentioned, these lipids are known to form a

bilayer entrapping the drugs at physiological pH or at pH 8.0. However, when the pH is dropped to below physiological the lipid assumes a non-bilayer state and instantaneously releases its contents. The pH at which it assumes this non-bilayer structure can be dropped even lower by including some stabilizing molecules such as CHEMS. Thus, when liposomes that include DOPE/CHEMS in their formulation are internalized via one of the internalizing molecules (CD19/CD22), they would be in the cytoplasm in vesicular forms until lysosomes fuse with these endosomes lowering the pH and thus releasing the liposomal contents into the cytoplasm in a pH dependent manner. It was possible for Kirchmeier and co-workers to study this mechanism by monitoring the rate of drug accumulation in the nuclei. The five types of formulations of DOX tested were free DOX, pH sensitive targeted, pH sensitive untargeted, non-pH sensitive targeted and non-pH sensitive untargeted liposomes. Non-pH sensitive liposomes were composed of HSPC:CHOL:mPEG₂₀₀₀-DSPE at a molar ratio of 2:1: 0.01 or, for mAb coupling experiments, of HSPC:CHOL:mPEG₂₀₀₀-DSPE:Mal- PEG₂₀₀₀-DSPE at a molar ratio of 2:1:0.08:0.02. pH-sensitive liposomes were composed of either DOPE:mPEG₂₀₀₀-DSPE/Mal-PEG₂₀₀₀-DSPE at a molar ratio of 10:0.4:0.1 or DOPE:CHEMS:mPEG₂₀₀₀-DSPE/Mal-PEG₂₀₀₀-DSPE at a molar ratio of 6:4:0.24: 0.6. These studies suggest the pH sensitive targeted liposomal formulation gave the most rapid release from lysosomes and the highest cytotoxicity. For an in depth review on stimuli sensitive nanocarriers, the reader is advised to read (Torchilin, 2009).

1.5.2. CD22

Another internalizing epitope is CD22. It is a B cell specific member and belongs to an immunoglobulin like lectin family which in addition to recognizing the ligands also internalizes them thus delivering the entire drug load inside the cell (Powell and Varki, 1994). Since it belongs to the lectin family, it retains the potential of binding to polysaccharides or oligosaccharides. It has been shown that CD22 positive B cells can be targeted using glycan ligands which are attached onto the liposomal surface by using N-hydroxysuccinimide (NHS) activated pegylated lipids (Chen et al., 2010). They have used DSPC:CHOL:PEG₂₀₀₀-DSPE in a 60:35:5 molar ratio to prepare the untargeted naked liposomes. To prepare the CD22 targeted liposomes, they first conjugated the high affinity glycan ligand 9-*N*-biphenylcarboxyl-NeuAc α 2-6Gal β 1-4GlcNAc (^{BPC}NeuAc) with an N-hydroxy succinimide (NHS) activated pegylated lipid, via an ethyl amine linkage. This lipid-ligand conjugate, ^{BPC}NeuAc-PEG-DSPE, is then substituted for PEG₂₀₀₀-DSPE and CD22 targeted liposomes are prepared. Liposomes were prepared by dissolving the lipids in a mixture of chloroform and DMSO (dimethyl sulfoxide). This solution was lyophilized for 16 hours following which the lipids flakes were hydrated with water to give a final phospholipid concentration of 10 mM. This lipid dispersion is then extruded through stacked polycarbonate filters. DOX was loaded remotely using gradients of ammonium sulfate. This liposomal formulation was tested for efficacy in a standard Daudi lymphoma model in SCID mice and the results showed that the ^{BPC}NeuAc liposomes were far more efficacious compared to the naked, non-targeted ones. The control group that was injected with just PBS (phosphate buffered saline)

showed a mean time of survival (MTS) of 50 days compared to 73 days and 100 days for 2% and 5% ^{BPC}NeuAc coupled liposomes.

Another research group has targeted CD22 using the antibody HB22.7 which is specific for CD22 only (O'Donnell et al., 2010). The method they used to prepare pegylated liposomal doxorubicin (PLD) has been described by Sakakibara et al. (Sakakibara et al., 1996). Briefly, an ethanol solution of HSPC:CHOL:PEG₂₀₀₀-DSPE:dl- α -tocopherol in a molar ratio of 56.1:38.2:5.5:0.2 was injected into ammonium sulfate desferal and shaken at 60°C for 1 hour. This liposomal suspension was then extruded through polycarbonate filters of predetermined sizes. DOX was loaded by the process of remote loading as previously described (Haran et al., 1993). The CD22 specific HB22.7 mAb was successfully coupled to the surface of pegylated liposomal DOX to give immunoliposomal PLD (IL-PLD) using the post insertion method (Allen et al., 2002a). Immunofluorescence staining exhibited binding of these IL-PLD's to CD22 expressing cells and the cytotoxicity observed specifically in CD22+ cells. For IL-PLD, the group have reported an IC₅₀ which is 3.1 to 5.4 times lower than that of PLD in CD22+ cell lines. Tuscano et al later studied the efficacy, biodistribution and the pharmacokinetics of these CD22 targeted IL-PLD's in xenograft mouse models and the results further strengthened their previous findings (Tuscano et al., 2010). Both targeted and untargeted liposomal formulations increased efficacy and reduced toxicity compared to non-liposomal DOX (NL-DOX). PLD also had a higher half-life compared to NL-DOX. PLD and IL-PLD were less myelotoxic compared to the NL-DOX.

Internalization of ligands by CD22 is a temperature dependant process. This was shown by Loomis et al. (Loomis et al., 2010) in a DOX loaded liposomal system, targeted via an anti-CD22 immunotoxin, HA22. Liposomes were prepared using the probe sonication method as described by Puri et al. (Puri et al., 2008). Briefly, a lipid film was formed which was reconstituted in HBSE buffer (10 mM HEPES, 150 mM NaCl, 9.1 mM EDTA, pH 7.5). These MLV's were sonicated for 10 minutes using a probe sonicator, which gave liposomes in the size range 100-150 nm diameter. Temperature dependant internalization was shown by comparing internalization at two temperatures and results show successful internalization at 37°C and not at 4°C. Two to three fold higher accumulation and 2 to 4 fold higher killing rates were noted for targeted liposomes in CD22+ Raji cells compared to CD22- SUP-T1 cells. However, it must be noted that at 4°C, which is an unfavourable temperature for the growth of the above cells, other processes may also cease which may in turn affect the internalization. Therefore, further studies are necessary to show that for an *in vitro* study it is only the internalization that is affected at 4°C and no other cell processes.

All internalizing epitopes do not have equivalent efficacy of internalization. A study has compared the potential of internalization of CD22 to CD19. The study used two immunotoxins, FMC63 (Fv)-PE38 targeting CD19 and RFB4 (Fv)-PE38 (BL22) targeting CD22. The lymphoma cell lines that they have used had 4-9 times more surface expressed CD19 compared to CD22. Still the amount of RFB4 (Fv)-PE38 (BL22) internalized by CD22 (2-3 times more immunotoxin internalized than the number of CD22 molecules) over one hour was far more than the amount of FMC63 (Fv)-PE38

internalized by CD19 (5.2% to 16.6% of the surface bound immunotoxin). It was also seen that the intracellular reservoir of CD22 greatly decreased after internalization which proved that CD22 was actually involved in the internalization process (Du et al., 2008). Therefore, CD22 appears to be a better target for use in targeted therapy for B cell related cancers.

1.5.3. CD74

CD74 is a part of the HLA class II major histocompatibility complex. It is overexpressed on malignant B cells and displays the property of receptor mediated internalization. A few research groups have utilized this feature of the CD74 antigen to target drug loaded liposomes to malignant B cells. Lundberg et al. (Lundberg et al., 2004) have compared the efficacy of targeting CD74+ malignant B cells with targeted and untargeted emulsions and liposomes. The emulsion was composed of Triolein (TO), EPC, Polysorbate 80, DPPE-PEG₂₀₀₀-MAL, and the anti-neoplastic agent 3', 5'-O-dioleoyl-5-fluorouridine deoxyribose (FUdR-dO) in the ratio 2:2:0.8:0.6:0.3. The liposomes were composed of EPC, DPPE-PEG₂₀₀₀-MAL, and FUdR-dO in the ratio 1:0.2:0.1. The liposomes were prepared by hydrating the dried lipid films in HEPES buffer followed by five freeze thaw cycles and subsequent sonication for two minutes. The anti-CD74 antibody (LL1) was covalently attached to the liposome using the thioether bond (Benita, 2006). These formulations along with solution FUdR were tested for cytotoxicity in CD74+ Raji cells and the IC₅₀ values were 2.5 ± 0.2 , 5.3 ± 0.3 and 7.0 ± 0.3 μ M for the LL1-emulsion, LL1-liposomes and the solution FUdR. The researchers have concluded that the study demonstrated the potential of utilizing LL1 conjugated drug-carriers for site specific drug delivery of anti-neoplastic agents.

CD74 signalling is associated with inhibition of apoptosis and cell proliferation (Binsky et al., 2007, Gore et al., 2008). Disruption of this signalling pathway presents a potential therapeutic option for the treatment of CLL and other CD74+ malignancies. This function of the pathway has been utilized by Hertlein et al. (Hertlein et al., 2010) to target liposomes coupled to Milatuzumab, an anti-CD74 mAb, to malignant B cells. They have demonstrated that Milatuzumab by itself mediates direct cytotoxicity by a mechanism involving the aggregation of CD74 on the cell surface. Furthermore, the targeting of milatuzumab incorporated into liposomes potentiates the cytotoxic activity of the mAb. The immunoliposomes for this study were prepared using the components and ratio as described by Sapra and Allen (Sapra and Allen, 2002).

Dexamethasone was used as a model drug to develop liposomes specific for CD74+ B cell malignancies by Mao et al. (Mao et al., 2013). They have prepared liposomes as described by Sapra and Allen (Sapra and Allen, 2002) and compared the in vitro and in vivo effects of solution dexamethasone with CD74 targeted dexamethasone loaded immunoliposomes. The results show that CD74 targeted liposomes were preferentially taken up by the CD74-positive B cells compared to the CD74-negative T cells. The immunoliposomal dexamethasone was also more cytotoxic in the CD74-positive B cells and primary CLL cells. Furthermore, the immunoliposomal

dexamethasone displayed an enhanced therapeutic efficacy against a CD74 positive B cell model in SCID mice engrafted with Raji cells (Mao et al., 2013).

1.5.4. CD20

CD20 is a non-internalizing type of antigen that has been targeted for B cell malignancies. It is a phosphoprotein specifically expressed on the surface of all B cells progressively increasing in concentration until maturity (Golay et al., 1985, Bofill et al., 1985). Sapra and co-workers (Sapra and Allen, 2002) have compared the efficacy of targeting DOX loaded liposomes to internalizing (CD19) and non-internalizing (CD20) epitopes. Untargeted liposomes were prepared using HSPC:CHOL:mPEG₂₀₀₀-DSPE at a molar ratio of 2:1:0.1 and for targeted liposomes 0.02 mole percent of mPEG₂₀₀₀-DSPE was substituted with Mal-PEG₂₀₀₀-DSPE. Liposomes were prepared by hydration of thin lipid films as described previously followed by extrusion through stacked polycarbonate membranes (de Menezes et al., 1999). The thiol reaction (Kirpotin et al., 1997) was used to attach the anti-CD19 and the anti-CD20 antibodies to the liposomes and studied their efficacy in SCID mice that were injected with Namalwa cells at the tail vein. Using a confocal fluorescence microscopy, they showed that both anti-CD19 tagged and anti-CD20 tagged liposomes bound the B cells with equal affinity. However, fluorescence in the cytoplasm was not seen in the anti-CD20 tagged liposomes, compared to the anti-CD19 tagged liposomes, which showed bright red fluorescence in the cytoplasm. This result can be attributed to the non- internalizing nature of the CD20 epitope. *In vivo* studies in SCID mice injected with Namalwa cells suggested that targeting internalizing epitopes like CD19 increased the life spans relative to treatment with liposomes targeted to non-internalizing epitopes such as CD20. DOX loaded immunoliposomes targeted to CD20 showed an ILS of 24.3% where those targeted to CD19 had an ILS of 65.2%.

However, it is not that targeting only the internalizing epitopes would give a higher cytotoxicity when compared to non-internalizing ones. A study (Sapra and Allen, 2004) compared the effects of using DOX loaded liposomes targeted to CD19, CD20 or combination of both, to VCR loaded liposomes targeted similarly, in SCID mice injected with Namalwa cells. For the doxorubicin loaded liposomes, the ratio of lipids used, the formulation process and the process for mAb attachment was the same as described previously (de Menezes et al., 1999), whereas for the VCR loaded liposomes, the parameters and process were the same as described previously by Sapra et al. (Sapra et al., 2004). Results showed that the VCR-loaded liposomes targeted to CD20 gave far better increase in life spans and long term survivors (93% and 2/7) compared to similarly targeted liposomes loaded with DOX (24% and 0/7). Even better results were reported using a combination of CD19 and CD20 targeted liposomes. With VCR an increase of 258% in life span was reported with 5/7 long-term survivors and 76% and 0/7 in case of DOX.

Menezes et al have compared cytotoxicity profile, half-lives, cellular internalization and *in vivo* stability of targeted liposomes prepared by various coupling methods. The liposome formulation process and the molar ratios of the lipids used were

the same as described previously (de Menezes et al., 1999). Their results show that liposomes prepared by the Mal-PEG-DSPE method have a short plasma half-life whereas the methods which eliminate or mask the Fc region of the ligand have half-lives similar to untargeted liposomes. Liposomes prepared by the post insertion method showed half-lives comparable to ones prepared by conventional coupling procedures. These systems were tested in different animal models of either haematological malignancies, pseudo metastatic disease of solid tumours, and their results have shown that successful *in vivo* targeting was achieved when the target was either small or at a position easily accessible by the vasculature and/or had an internalizing epitope (like CD19) that was being targeted (Allen et al., 2002d).

1.6. Hypothesis and Specific Aims

130,000 Americans are diagnosed with a blood malignancy every year and approximately 44,000 lose their battle against this deadly disease. Leukemias are the most common cause of cancer in children and young adults less than 20 years of age and almost 3/4th of these are ALL. Chemotherapy, the primary therapy for blood malignancies has debilitating adverse effects such as loss of function of healthy tissue, thrombocytopenia, neutropenia, nausea, vomiting and tiredness (Zhang et al., 2007, Sahoo et al., 2007). These adverse effects can be attributed largely to the non-specific interactions of the drug with healthy tissues of the body and drug specific toxicities such as cardiomyopathy with DOX therapy and neuropathy with VCR therapy. The non-specific interactions can be moderated by using drug delivery systems that can deliver drugs specifically to the malignant cells/tissues and the drug specific toxicities can be decreased or even eliminated by using superior molecules such as AD 198.

Entrapment of AD 198 in liposomes should alter bio-distribution of the molecule, thereby minimizing dose related toxicities and improving therapeutic efficacy. However, conventional liposomes are prone to rapid clearance by the RES and premature drug leakage. Therefore the physicochemical parameters of the liposomal formulation such as size, charge, drug encapsulation and drug release will be optimized to maximize safety and efficacy of the drug delivery system. Also, to ensure targeted delivery to malignant cells primarily, an active targeting approach will increase drug concentration in the target malignant cells particularly. For this purpose, the AD 198 loaded liposomal system will be targeted to CD22 using Fab' fragments of an anti-CD22 monoclonal antibody. CD22 is a B cell specific receptor of the lectin binding family and is overexpressed on malignant B cells which undergoes receptor mediated endocytosis. This should improve uptake of AD 198 in the malignant B cells preferentially.

The efficacy and specificity of the formulation can be verified using *in vitro* models of the malignant B cells. Cellular uptake and cytotoxicity studies along with tracing levels of key cell cycle regulatory molecules should give a well-defined understanding of the potential of the system compared to solution formulations of AD 198.

Long term stability of the liposomal formulation would be a challenge given that the liposomal components are prone to oxidation in aqueous media. Removal of the water from the final formulation by lyophilization should increase stability of the liposomal formulations. However, water being an integral component of liposomal formulations, removal of water would prove detrimental to the final product. Therefore, the lyophilized formulations and the lyophilization process needs to be optimized to warrant an active and stable final drug product. This lyophilized product should be more stable than the liquid liposomal dispersions but equally effective.

The main objective of this project is the design and development of a stable and effective liposomal formulation for AD 198 that would be specific for CD22 overexpressing malignant B cells. In keeping with this objective, the following hypotheses were proposed.

1. AD 198 encapsulated CD22 targeted liposomal formulations can be developed to deliver drug preferentially to CD22 overexpressing malignant B cells.
2. The liposomal formulation will have approximately the same cytotoxicity as solution AD 198 over prolonged periods.
3. The formulation will be endocytosed via the clathrin- and caveolin-independent endocytotic pathway and localized in lysosomes within the malignant cells where it will eventually release AD 198. The targeted liposomal drug will activate classical apoptotic pathways and suppress oncoproteins.
4. The dispersed liquid formulation will not be adequately stable for long term storage and thus a lyophilized formulation will be required to enhance stability.

To test these hypotheses, my specific aims were to:

1. **Develop and characterize prototype formulations of long circulating liposomal AD 198.** Here I tested the various excipients in different ratios for developing the basic liposomal formulations of AD 198. Formulation and process parameters were identified and optimized to achieve an acceptable prototype formulation.
2. **Conjugate a targeting ligand to the liposomes.** Basic liposomes prepared were conjugated with a fragment of the anti-CD22 antibody RFB4 to give CD22 targeted liposomes. Conjugation efficacy was tested and the average number of antibody molecules per liposome were calculated.
3. **Evaluate *in vitro* cellular uptake and cytotoxicity of long circulating CD22 targeted liposomal AD 198.** *In vitro* studies were performed in target and non-target cells to substantiate preferential uptake and cytotoxicity in the

target population of CD22 overexpressing malignant B cells.

4. **Evaluate *in vitro* intracellular trafficking and effect on induction of apoptosis.** To ascertain the route of uptake and intracellular localization of the CD22 targeted liposomal AD 198 in the target cells studies were performed over short period and various analytical techniques such as flow cytometry and confocal and electron microscopy were employed.
5. **Enhancement and evaluation of stability of long circulating CD22 targeted liposomal AD 198.** Since the stability of liposomes in dispersed conditions during storage was a major concern, the optimized liposomal dispersion was lyophilized. The lyophilization cycle parameters and formulation was optimized. The formulation that displayed optimum physicochemical characteristics was tested for stability for 30 days and compared to the dispersed formulation.

CHAPTER 2. DEVELOPMENT AND CHARACTERIZATION OF PROTOTYPE FORMULATIONS OF LONG CIRCULATING LIPOSOMAL AD 198 (LCLA)

Alec Bangham and R.W. Horne first saw liposomes under the electron microscope in 1964 when they dispersed dry lecithin in water. They noted that these tiny, closed, bilayered structures formed spontaneously and resembled the bilayers of living cells (Bangham and Horne, 1964). They were initially referred to as 'Bangasomes' and it was only later that a Greek word was coined for them. '*Lipos*' meaning fat and '*somas*' meaning body gave rise to liposomes. It was not until 1971, that Gregoriadis et al successfully encapsulated enzymes into liposomes to be used as a vehicle for drug delivery (Gregoriadis et al., 1971, Gregoriadis and Ryman, 1971). However, they were quick to notice some of the limitations to the system, such as accumulation of the particles in the liver and were able to conclude that these systems could be used successfully only for drug delivery to the spleen and the liver (Gregoriadis and Ryman, 1972a, Gregoriadis and Ryman, 1972b). Therefore they started development work on more complex systems for delivery to other diseases or affected organs (Gregoriadis, 1973). Soon after Gregoriadis discovered that the liposomal concentration was slightly higher in the tumor than other tissues in the body and also concluded that this was due to higher vascularization of the tumor tissue (Gregoriadis et al., 1974).

Ever since the liposomes were discovered as a vehicle for drug delivery, various aspects have surfaced that govern the effectiveness of the final delivery system. Composition of liposomes is one such aspect which determines physico-chemical parameters such as liposome size, surface charge or bilayer fluidity (Kersten and Crommelin, 1995, Lian and Ho, 2001, Kaasgaard and Andresen, 2010, Torchilin, 2005, Drummond et al., 1999, Maurer et al., 2001, De Gier et al., 1968). These factors primarily affect the behavior of the liposomal dispersion *in vivo*. For example, the rate of uptake by the RES increases with the increase in size of the liposome (Lian and Ho, 2001). With the inclusion of PEG to make long circulating liposomes, the effective upper size for liposomes is between 150-200 nm (Lian and Ho, 2001). As for surface charge the RES works differently. Neutral liposomes have a lower tendency to be taken up by the RES, but then they are unstable due to their tendency to aggregate. Thus, liposomes are formulated as anionic. This negative charge increases stability but also increases the rate of uptake by the RES. This limitation is overcome by making the surface more hydrophilic which avoids recognition by the RES. Surface hydrophilicity is increased by modifying the surface with hydrophilic polymers such as PEG (Lian and Ho, 2001).

Bilayer fluidity is another parameter that governs stability of the final formulation. Fluidity of the bilayer depends on the lipids it is composed of. Every lipid has a glass transition temperature (T_g), which is the temperature at which 50% of the lipid changes from gel to liquid phase. Below the T_g the lipids are in a well-ordered gel phase, whereas above the T_g the lipids become more fluid and lack order. It is the T_g at which maximum drug leakage is observed (Lian and Ho, 2001). Lipids with high melting points such as cholesterol can also be incorporated into the membrane to ensure low fluidity

(Drummond et al., 1999). It is essential that when developing liposomes, these parameters be optimized to ensure the safety, stability and effectiveness of the final drug product. In the following sections the studies that were performed for optimization of the formulation of LCLA have been described and the results discussed.

2.1. Materials and Methods

2.1.1 Materials

HSPC, EPC and mPEG₂₀₀₀-DSPE were purchased from Avanti Polar Lipids, Alabaster, AL, USA, cholesterol was purchased from Sigma-Aldrich Co. LLC, St. Louis, MI, USA, chloroform, methanol, Whatman® Nucleopore track etched polycarbonate membranes, 200 proof ethanol, 10x PBS (phosphate buffered saline) and HPLC (high pressure liquid chromatography) grade water, Slide-A-Lyzer® MINI Dialysis Devices, 3.5K MWCO (molecular weight cut off), 0.5 mL capacity, ammonium hydroxide and 80% formic acid and immobilized pepsin were purchased from Thermo Fisher Scientific, Waltham, MA, USA, ultrapure nitrogen was purchased from Nexair, Memphis, TN, USA, Sephadex G50 pre-filled macro SpinColumns® were purchased from Harvard Apparatus, Holliston, MA, USA, Total Recovery HPLC vials were purchased from Waters, Milford, MA, USA.

2.1.2. Thermal characterization (T_g) of LCLA ingredients and formulation

The glass transition (T_g) temperature is critical for liposomal formulations since it determines the temperature at which the formulation is supposed to be extruded. For this, the ingredients and the LCLA ingredient mixtures were thermally analyzed using a DSC (TA Instruments Q2000 differential scanning calorimeter equipped with refrigerated cooling system). Five to ten mg of the excipient, drug or the excipient-drug mixture were loaded onto T-Zero aluminum pans and hermetically sealed. An empty pan prepared in a similar manner was used as reference. The samples were equilibrated at 25°C and then cooled at 5°C/min to -50°C. After equilibration, the samples were heated at the rate of 10°C/min to 200°C.

2.1.3. Preparation and formulation optimization of long circulating liposomal AD 198 (LCLA)

The liposomes were prepared by the Bangham method followed by extrusion via polycarbonate membranes. Briefly, the lipids and drug were weighed accurately and dissolved in 3 mL's 9:1 solvent mixture of chloroform:methanol in a round bottom flask. A thin lipid film was formed at the bottom of the flask by evaporating the solvent using a BUCHI Rotavapor®. Rotations were maintained at rotation speed no 3 and temperature was maintained at 40°C using a BUCHI heating bath. This step was carried out for 1 hour

following which the vacuum was released and the water bath heated to 65°C. Simultaneously, 1X PBS (phosphate buffered saline) was prepared from the 10X PBS and added to the thin lipid film for hydration. Rotations were maintained at the number 3 setting. Hydration was carried out for 1 hour which gave MLV's. The MLV's were extruded through polycarbonate filters in two steps to give SUV's. Extrusion was carried out using LIPEX® Extruders purchased from Northern Lipids, Burnaby, BC, Canada, connected to a high pressure ultrapure nitrogen tank. In the first step of extrusion, polycarbonate membranes of two sizes, 100 nm (nanometers) and 200 nm, were stacked and the drug loaded liposomes extruded only once using 450 psi pressure. In the second step, the resulting liposomes were extruded three times via 80 nm and 100 nm stacked membranes again using 450 psi pressure. Since there was some loss of volume during the rehydration and extrusion process, the final volume is made up to 3 mL's with 1x PBS.

2.1.4. Removal of un-encapsulated AD 198

The un-encapsulated drug was removed using Sephadex - G50 prefilled macro-column. Briefly, the dried G50 gel was rehydrated using 1X PBS for 15 minutes and centrifuged using a Thermo Scientific IEC CL31R centrifuge at 4°C for 4 minutes at 1500 rpm. The resulting gel was washed three times using 150 µL's of blank liposomes containing no AD 198, under the same centrifugation conditions as mentioned above. This was to block any non-specific retention of drug loaded liposomes in the column. Then 150 µL's of the AD 198 loaded liposomes were passed through the treated column. The final eluate was reconstituted to 150 µL's.

2.1.5. Analysis of liposomal encapsulated drug content

Encapsulated AD 198 content in the liposomes was calculated using a Waters Alliance e2695 HPLC (High Pressure Liquid Chromatography) coupled to a Waters 2998 UV Photodiode Array Detector. Samples were prepared at a dilution factor of 20. Briefly, 50 µL of the purified liposomes were dissolved in 950 µL of 1:1 methanol: ethanol. Samples were briefly vortexed to give a clear solution and 300 µL's transferred to Waters total recovery HPLC vials. These vials were loaded into the auto sampler of the HPLC separations module. The column used for separation was Waters Nova-Pak® C18 4µm, 3.9x150 mm and was maintained at 30°C throughout the separation process. The mobile phase was a 70:30 Acetonitrile: pH 4.0 Ammonium Formate buffer. The ammonium formate buffer was prepared by adding 3.85 mL's of ammonium hydroxide to 950 mL's of HPLC grade water. The pH was adjusted to 4.0 using 80% formic acid and the volume was made up to 1 L. The flow rate for the mobile phase was maintained at 1.2 mL/minute, the injection volume was 20 µL's and the run time for each injection was 7 minutes. AD 198 eluted between 3 and 4 minutes and was detected at a wavelength (λ) of 254 nm.

2.1.6. Analysis of liposome size and zeta (ζ) potential

The final liposomal drug was diluted 40 times using HPLC water and the liposome size and ζ -potential was measured using a Malvern Zetasizer Nano ZS.

2.1.7. Determination of phospholipid concentration

To calculate the amount of HSPC retained in the final formulation of LCLA, total phospholipids were estimated using the following procedure. 2.703 G (grams) ferric chloride hexahydrate and 3.04 G ammonium thiocyanate was dissolved in 100 mL distilled water and mixed to give ferrithiocyanate reagent. To determine the concentration of HSPC in the LCLA dispersion, an HSPC standard curve was made ranging from 10 to 60 $\mu\text{g/mL}$. Analysis of these standards was done as follows. A mixture of 2 mL chloroform, 2 mL ferrithiocyanate reagent and 100 μL of the standard solution was made for each standard and vortexed vigorously for exactly one minute each. The mixture was allowed to settle and the lower layer containing chloroform was aspirated carefully and transferred to a 1 mL quartz cuvette. The absorbance for each standard was measured at λ 488 against a chloroform blank. Samples of LCLA were prepared in the same method and the absorbance measured. Absorbance of the standard vs. HSPC concentration was plotted for each standard concentration and the unknown amount of HSPC in the LCLA sample was determined.

2.1.8. LCLA dissolution

Drug release in 1x PBS at pH 7.4 and 37°C was tested for the LCCTLA's as described by Zhang et al (Zhang et al., 2008). Briefly, 100 μL 's of the LCLA's were placed in Slide-A-Lyzer® MINI Dialysis Devices, 3.5K MWCO, 0.5 mL capacity. 5 time points were tested; 6, 12, 24, 48 and 72 hours. Each sample was tested in triplicates. The sample loaded dialysis devices were loaded into floats introduced into a 3000 mL beaker containing 3000 mL of 1x PBS preheated to 37°C and dissolution started. Samples were taken out at the pre-determined time points and the drug content measured by HPLC as stated earlier.

2.1.9. LCLA characterization by TEM (transmission electron microscopy)

Liposome size and shape were studied by TEM. LCLA was prepared as stated above. Samples for TEM were prepared by loading approximately 10-15 μL of the LCLA dispersion onto a copper grid. The sample was air dried for a short period (5-10 minutes) and stained with Uranyl Acetate to increase contrast and electron density since Uranyl Acetate stains lipids. The sample loaded copper grids were then loaded into the TEM and images taken at various magnifications.

2.1.10. LCLA numerical characterization

LCLA was characterized for its drug encapsulation per liposome. This was done by using the following assumptions, the average size of the liposomes was 120 nm (diameter), the lipid bilayer thickness was 5 nm (Gramse et al., 2013) and the cross sectional area of a phosphatidylcholine head-group was 0.71 nm² (Torre et al., 2007).

2.2. Results and Discussion

EPC and HSPC are two lipids that were studied for their effect on the encapsulation of AD 198. Both lipids are derived from natural sources and both are biodegradable. However, HSPC and EPC differ in their composition such that EPC is a more crude mixture between saturated and unsaturated lipids of varying chain lengths, whereas HSPC is primarily composed of saturated lipids of a fixed chain length. The effect that this has on the lipid bilayer is that the longer chain length lipids in EPC increase the thickness of the bilayer (not the size of the liposome) (Lian and Ho, 2001). The more saturated a lipid is the higher is its transition temperature, thus imparting lesser fluidity and possibly increased stability (Lian and Ho, 2001). A variable length between the two tail group chains also reduces the order of packing in the liposomal bilayer. Saturated lipids such as HSPC also have higher circulation half-lives compared to lipids with un-saturations such as EPC (Gregoriadis and Senior, 1980, Senior, 1987).

2.2.1. Thermal characterization of the excipients, drug and excipient-drug mixture

Figure 2.1 shows results for the thermal characterization. The thermograms for AD 198, CHOL, mPEG₂₀₀₀-DSPE, mal-PEG₂₀₀₀-DSPE, HSPC and the optimized ratio of drug and excipients for the LCLA formulation have been shown. The highest T_g that is observed is that for the drug, AD 198 at 64.81°C. The T_g for the drug-excipient mixture is at approximately 51°C. The values for the other excipients are melting points (PEG and CHOL). Cholesterol melts at approximately 148°C but the peak at 42°C is possible from a different isomer. Since the highest T_g was 64.81°C, the rehydration of the lipid bilayer and the extrusion was carried out at 65°C.

2.2.2. Effect of lipid composition in the bilayer

Studies were performed using different lipids at various ratios to optimize the physico-chemical properties of the liposomal bilayer. **Figure 2.2** shows EPC and HSPC formulations studies in various ratios for their effect on AD 198 encapsulation and liposomal size. It explained that EPC and HSPC did not necessarily control the degree of AD 198 encapsulated in the liposome or the liposomal size. However, the dependence of ζ -potential on the HSPC:EPC ratio was evident as portrayed in **Figure 2.3**. As the ratio of HSPC:EPC was increased the ζ -potential on the liposome became more negative.

Figure 2.1. DSC thermograms of excipients, drug and excipient-drug mixture

Notes. The thermograms for AD 198, CHOL, mPEG₂₀₀₀-DSPE, mal-PEG₂₀₀₀-DSPEm HSPC and the optimized ratio of drug and excipients for the LCLA formulation have been shown. The highest T_g that is observed is that for the drug, AD 198 at 64.81°C. The T_g from the drug-excipient mixture is at approximately 51°C. The values for the other excipients are melting points (PEG and CHOL). Cholesterol melts at approximately 148°C but the peak at 42°C is possible from a different isomer. Since the highest T_g was 64.81°C, the rehydration of the lipid bilayer and the extrusion was carried out at 65°C.

Figure 2.2. Effect of lipid composition on AD 198 encapsulation and liposomal size (n=3)

Notes. At the end of the extrusion cycles the product volume was made up to batch size before AD 198 encapsulation and size were measured. Theoretical concentration of AD 198 used was 500 µg/mL. The liposome size and AD 198 encapsulation was determined after the free AD 198 was removed using G50 Sephadex prefilled macro columns. EPC and HSPC did not necessarily control the degree of AD 198 encapsulated in the liposome or the liposomal size. Error bars represent standard deviation around the mean.

Figure 2.3. Effect of lipid composition on ζ -potential (n=3)

Notes. ζ -potential is dependent on the HSPC:EPC ratio. As the ratio of HSPC:EPC was increased the ζ -potential on the liposome became more negative. Error bars represent standard deviation around the mean.

This may possibly be attributed to the chloride ion on the EPC molecules interacting with the cationic nature of AD 198. The cationic AD 198 molecules may adsorb onto the anionic EPC and form a positive coat thus giving near zero or positive ζ -potential. However, when HSPC concentration was higher than the EPC concentration, the electronegativity from the O^- ion on the HSPC was not as high compared to the chloride ion on the EPC, which gave lesser AD 198 adsorption onto the liposomal surface resulting in more negative ζ -potential.

2.2.3. Effect of total phospholipids on AD 198 encapsulation, liposomal size and ζ -potential

Liposomes were prepared using the Bangham method in increasing concentrations of total HSPC. Four concentration of HSPC tested were 25, 50, 75 100 and 125 mM. The drug concentration was maintained at 2000 $\mu\text{g/mL}$. The batch with 25mM HSPC failed to extrude. The possible reason for that may be attributed to the excess amount of drug in the system that the little amount of carrier (HSPC) would need to encapsulate. Thus, the system could not be extruded. The results for 50 – 125 mM HSPC are shown in **Figures 2.3, 2.4 and 2.5**. It would be logical to believe that with increase in the amount of HSPC in the system, which is the major encapsulating material, the amount of encapsulated drug would tend to increase. However, **Figure 2.4** suggests that the amount of drug encapsulated may not be related to the amount of lipid in the system. This result can be explained with **Figure 2.5** which explains that during processing, a considerable amount of phospholipid was being lost as the total lipid content was increased beyond 75 mM. In **Figure 2.5** we can see that the amount of HSPC loss during processing at 75 mM HSPC is approximately 25% (almost the same as 50 mM). However at 100 and 125 mM the amount of phospholipid loss was more than 40 %, whereas the benefits in terms of AD 198 encapsulation and liposomal size were negligible (**Figure 2.4**). Although there was a pronounced increase in the net negative charge on the liposomal surface, as depicted in **Figure 2.6**, the size of the liposome increased considerably which warranted the conclusion of total phospholipid concentration at 75 mM to be optimum. An additional justification for the conclusion was that the extrusion time (result not shown here) with total phospholipid concentration above 75 mM increased considerably (more than 10 times).

The increase in the negative charge of the liposomes when the phospholipid concentration is increased from 50 mM to 75 mM and then not such a defined increase from 75 mM to 100 mM and 125 mM can possibly be explained with the excess amount of AD 198 that remains un-encapsulated. Since AD 198 has a cationic nature and HSPC an anionic nature, the excess AD 198 in the dispersion could be adsorbed onto the liposomal surface thus shielding the anionic nature of HSPC.

Figure 2.4. Effect of total phospholipid concentration on liposome size and AD 198 encapsulation (n=3)

Notes. Theoretical concentration of AD 198 used was increased to 2000 µg/mL. Liposomes were prepared using only HSPC as the major lipid. EPC was not used from this point forward. The amount of AD 198 encapsulated is not related to the amount of lipid in the system. This result can be explained with **Figure 2.5**. Error bars represent standard deviation around the mean.

Figure 2.5. Optimization of HSPC concentration (n=3)

Notes. A considerable amount of phospholipid was lost in processing as the total lipid content was increased beyond 75 mM. The amount of HSPC loss during processing at 75 mM HSPC is approximately 25% (almost the same as 50 mM). However at 100 and 125 mM the amount of phospholipid loss was more than 40 %, whereas the benefits in terms of AD 198 encapsulation and liposomal size were negligible (**Figure 2.4**). Error bars represent standard deviation around the mean.

Figure 2.6. Effect of total phospholipid concentration on ζ -potential (n=3)

Notes. Although there was a pronounced increase in the net negative charge on the liposomal surface, the size of the liposome increased considerably which warranted the total phospholipid concentration at 75 mM to be optimum. Error bars represent standard deviation around the mean.

2.2.4. Effect of AD 198 concentration on AD 198 encapsulation and ζ -potential

Five concentrations of AD 198 ranging from 500 $\mu\text{g/mL}$ to 2500 $\mu\text{g/mL}$ were tested to see their effect on AD 198 encapsulation and ζ -potential. **Figure 2.7** shows that as the AD 198 concentration was increased from 500 -1500 $\mu\text{g/mL}$ the AD 198 encapsulation increased steadily. However when the AD 198 concentration was increased from 1500 to 2000 $\mu\text{g/mL}$, the AD 198 encapsulation almost doubles. However, if the AD 198 concentration was increased further, there was not a considerable increase in AD 198 encapsulation. The ζ -potential was relatively constant up to 2000 $\mu\text{g/mL}$. However, once the AD 198 concentration reached 2500 $\mu\text{g/mL}$, the ζ -potential became lesser negative. Again, this can be attributed to the excess un-encapsulated cationic AD 198 adsorbing onto the predominantly anionic HSPC liposomes, which will shield the negative charge due to HSPC. Hence, 2000 $\mu\text{g/mL}$ was selected as the optimum AD 198 concentration.

2.2.5. Effect of cholesterol concentration on AD 198 encapsulation and liposomal size

Cholesterol is incorporated into the membrane to obtain an optimum bilayer fluidity (Drummond et al., 1999, Lian and Ho, 2001). However, with encapsulation of hydrophobic drugs in the bilayer it is important to consider effects of cholesterol concentrations on the encapsulation of the drug even before bilayer fluidity is considered. This is shown in **Figure 2.8** which depicts the results from a study on the effects of increasing cholesterol concentrations. Four concentrations of cholesterol ranging from 5 mM to 30 mM were studied. Liposomal size was relatively similar for 5 and 10 mM cholesterol concentrations. However, the size increased considerably at 15 and 30 mM cholesterol. This may be attributable to the liposomal bilayer trying to accommodate both the AD 198 and the increasing cholesterol molecules which could possibly increase the liposomal size. AD 198 concentration falls suddenly when cholesterol concentration was increased from 15 mM to 30 mM. Since part of the cholesterol molecular structure is similar to that of AD 198, the excess cholesterol molecules trying to occupy volume within the bilayer may be pushing out the AD 198. This was also in agreement with the liposomal size results. Once excess AD 198 was pushed out, the liposomal size reduced by approximately 50 nm. These results suggest that in view of AD 198 encapsulation and liposomal size approximately 10 mM cholesterol was optimum for LCLA.

2.2.6. Optimization of mPEG₂₀₀₀-DSPE concentration

To determine the optimum amount of mPEG₂₀₀₀-DSPE, four concentrations of mPEG₂₀₀₀-DSPE ranging from 1 to 5 mole% were studied. **Figure 2.9** represents the effect of mPEG₂₀₀₀-DSPE concentration on liposomal size and ζ -potential. Liposomal size was observed to increase with increasing concentrations of mPEG₂₀₀₀-DSPE.

Conversely, ζ -potential was observed to become more negative with increase in

Figure 2.7. Effect of AD 198 concentration on AD 198 encapsulation and ζ-potential (n=3)

Notes. As the AD 198 concentration was increased from 500 -1500 µg/mL the AD 198 encapsulation increased steadily. However when the AD 198 concentration was increased from 1500 to 2000 µg/mL, the AD 198 encapsulation almost doubles. However, if the AD 198 concentration was increased further, there was not a considerable increase in AD 198 encapsulation. The ζ-potential was relatively constant up to 2000 µg/mL. However, once the AD 198 concentration reached 2500 µg/mL, the ζ-potential became lesser negative ($p < 0.05$). Again, this can be attributed to the excess un-encapsulated cationic AD 198 adsorbing onto the predominantly anionic HSPC liposomes, which will shield the negative charge due to HSPC. Hence, 2000 µg/mL was selected as the optimum AD 198 concentration. Error bars represent standard deviation around the mean.

Figure 2.8. Effect of cholesterol concentration on AD 198 encapsulation and liposome size (n=3)

Notes. Liposomal size was relatively similar for 5 and 10 mM cholesterol concentrations ($p>0.05$). However, the size increased considerably at 15 and 30 mM cholesterol ($p<0.05$). This may be attributable to the liposomal bilayer trying to accommodate both the AD 198 and the increasing cholesterol molecules which could possibly increase the liposomal size. AD 198 concentration falls suddenly when cholesterol concentration was increased from 15 mM to 30 mM. This may be due to the excess cholesterol molecules trying to occupy volume within the bilayer. This was also in agreement with the liposomal size results. Once excess AD 198 was pushed out, the liposomal size reduced by approximately 50 nm. These results suggest that in view of AD 198 encapsulation and liposomal size approximately 10 mM cholesterol was optimum for LCLA. Error bars represent standard deviation around the mean.

Figure 2.9. Effect of mPEG₂₀₀₀-DSPE concentration on ζ -potential and liposome size (n=3)

Notes. Liposomal size was observed to increase with increasing concentrations of mPEG₂₀₀₀-DSPE. Error bars represent standard deviation around the mean. Conversely, ζ -potential was observed to become more negative with increase in mPEG₂₀₀₀-DSPE concentration which may be attributable to the anionic nature of mPEG₂₀₀₀-DSPE. mPEG₂₀₀₀-DSPE is a large molecule (MW = 2805.497). The increase in the number of mPEG₂₀₀₀-DSPE molecules may cause the net size of the liposome to increase as justified by Woodle and co-workers (Woodle et al., 1992). However, a balance needs to be maintained between optimum size and optimum ζ -potential. Hence, 2 mole% mPEG₂₀₀₀-DSPE was selected as the optimum concentration.

mPEG₂₀₀₀-DSPE concentration which may be attributable to the anionic nature of mPEG₂₀₀₀-DSPE. mPEG₂₀₀₀-DSPE is a large molecule (MW = 2805.497). The increase in the number of mPEG₂₀₀₀-DSPE molecules may cause the net size of the liposome to increase as justified by Woodle and co-workers (Woodle et al., 1992). However, a balance needs to be maintained between optimum size and optimum ζ -potential. Hence, 2 mole% mPEG₂₀₀₀-DSPE was selected as the optimum concentration.

2.2.7. LCLA dissolution

Long circulating liposomal AD 198 was tested for its release characteristics in 1x PBS at 37°C. **Figure 2.10** shows that approximately 30% AD 198 release was observed over the first 12 hours following which only about 10% more AD 198 was released over the next 48 hours. This may be due a biphasic release in which the drug is released in two separate phases. Possibly there may be micelles which are less stable compared to liposomes which may be releasing the drug faster than liposomes. Once micelle drug release is over the liposomal AD 198 release predominantly manifests the kinetics.

The release is also in conjunction with the proposed theory of active targeting in which the liposome requires a certain period of time before it encounters a CD22+ malignant B cell, binds to it and gets internalized. The drug must largely be released once the liposome is inside the malignant cell. Therefore the delayed release of AD 198 over >72 hours is beneficial for the LCLA drug delivery system altogether.

2.2.8. TEM analysis of LCLA

LCLA was analyzed by TEM. **Figure 2.11** was taken at 30,000x and **Figure 2.12** was taken at 150,000x magnification respectively. In **Figure 2.11** we can see the vast population of LCLA's all over the copper grid. Some parts of the image are in focus and other are out of focus due to the multiple layers of the sample. If we look at **Figure 2.12** which is at a higher magnification, we are able to better identify the liposome size and the shape. The size as shown by the two scale bars are at 88.7 and 85.3 nm. This size is in agreement with the process used to produce them. The smallest diameter of the membrane used for extrusion was 80 nm. Since liposomes are waxy spherical structures a little larger vesicle may be able to squeeze out of an 80 nm pore. However, compared to the size range from the DLS (dynamic light scattering) instrument from Malvern, which is in the range of 120 – 140 nm, the size given by the TEM is more accurate. This is because the dynamic light scattering instrument gives the hydro-dynamic size of a nanoparticle. The Malvern Zetasizer® Nano ZS calculates particle size by converting the velocity of a particle in a certain media into its size by using the Stokes-Einstein equation. There are many variables in this equation which may not be accurately estimated at each measurement.

Also, when the nanoparticles move through the suspending medium, a thin layer of charged ions move along with it, which alter its velocity in the medium, which in turn

Figure 2.10. LCLA dissolution at 37°C (n=3)

Notes. Approximately 30% AD 198 release was observed over the first 12 hours following which only about 10% more AD 198 was released over the next 48 hours. This may be due a biphasic release in which the drug is released in two separate phases. Possibly there may be micelles which are less stable compared to liposomes which may be releasing the drug faster than liposomes. Once micelle drug release is over the liposomal AD 198 release predominantly manifests the kinetics. The release is also in conjunction with the proposed theory of active targeting in which the liposome requires a certain period of time before it encounters a CD22+ malignant B cell, binds to it and gets internalized. The drug must largely be released once the liposome is inside the malignant cell. Therefore the delayed release of AD 198 over >72 hours is beneficial for the LCLA drug delivery system altogether. Error bars represent standard deviation around the mean.

No.5.007.tif

Print Mag: 30000x @ 7.0 in

14:30 10/20/11

500 nm

HV=60.0kV

Direct Mag: 20000x

AMT Camera System

Figure 2.11. TEM of LCLA at magnification 30,000x (magnified on next page)

No.5.006.tif

Print Mag: 150000x @ 7.0 in

14:27 10/20/11

100 nm

HV=60.0kV

Direct Mag: 100000x

AMT Camera System

Figure 2.12. TEM of LCLA at magnification 150,000x

would have an impact on the size. Therefore, size displayed on an electron microscopic image of the nanoparticles would be relatively more accurate.

The shape of the particles in the image looks roughly spherical but not uniformly spherical for all particles. This may be attributable to the hollow nature of liposomes which would collapse when water was removed. However, the polydispersity of the particles in the image can be noted to be relatively low. Calculated by DLS, the polydispersity of this sample was 0.071.

2.2.9. Number of AD 198 molecules/liposome

The number of HSPC molecules per mL of the LCLA were calculated by using the Avogadro's number, 6.023×10^{23} molecules/mole. This gave 3.25×10^{22} HSPC molecules/mL of LCLA as per the analyzed concentration of HSPC, 54 mM from the HSPC assay results. The number of AD 198 molecules/mL of LCLA were calculated similarly from the analyzed concentration of AD 198 to be 1250 $\mu\text{g/mL}$. This gave 1.05×10^{21} AD 198 molecules/mL of LCLA. The outer surface area of the liposomal bilayer was calculated from **Equation 2.1**.

$$\text{Outer Surface Area} = 4\pi r^2 \quad (\text{Eq. 2.1})$$

Here 'r' was the outer radius of the liposome. The thickness of the bilayer was denoted 'h'. Then the inner surface area was calculated from **Equation 2.2**.

$$\text{Inner Surface Area} = 4\pi(r - h)^2 \quad (\text{Eq. 2.2})$$

The cross sectional area of a phosphatidylcholine head-group was denoted as 'a' and the number of HSPC molecules per liposome calculated from **Equation 2.3**.

$$\text{Number of HSPC molecules/liposome} = \frac{4\pi[r^2 + (r - h)^2]}{a} \quad (\text{Eq. 2.3})$$

$$\text{Number of HSPC molecules/liposome} = \frac{17.69[60^2 + (60 - 5)^2]}{0.71} = 117,196$$

This gave us the number of liposomes/mL from **Equation 2.4**.

$$\text{Number liposomes/mL} = \frac{\text{Number of HSPC molecules/mL}}{\text{Number of HSPC molecules/liposome}} \quad (\text{Eq. 2.4})$$

$$\text{Number liposomes/mL} = \frac{3.25 \times 10^{22}}{117,196} = 2.77 \times 10^{17}$$

Number of AD 198 molecules per liposome were calculated from **Equation 2.5**.

$$\text{Number of AD 198 molecules/liposome} = \frac{\text{Number of AD 198 molecules/mL}}{\text{Number of liposomes/mL}} \quad (\text{Eq. 2.5})$$

$$\text{Number of AD 198 molecules/liposome} = \frac{1.05 \times 10^{21}}{2.77 \times 10^{17}} = 3790$$

2.3. Summary and Conclusions

The formulation and process parameters were identified and optimized in the preparation of LCLA. Lipid film formation parameters were optimum at 40°C and rotation speed at number 3. With the same rotations, rehydration was performed optimally at 65°C. For extrusions of the LCLA, extrusion was performed in two steps. In the first step the stacked membranes used had a cut off pore diameter of 100 nm and 200 nm, a pressure of 450 psi at 65°C. This cycle was performed only once. In the second step extrusion was performed using the same parameters only using stacked 80 nm and 100 nm membranes. The second step was performed thrice. Using these parameters the following composition of LCLA formulation ingredients were found to be optimum; HSPC was the lipid of choice and was used at 75 mole %, mPEG₂₀₀₀-DSPE 2 mole %, Cholesterol 10 mole % and AD 198 2 mg/mL. The physicochemical parameters of the optimized formulation were as follows; size 115-145 nm, ζ-potential -8 to -15 mV, AD 198 encapsulation 1000-1500 µg/mL and dissolution of not more than 30% AD 198 occurred for 72 hours. The size of the LCLA as per TEM was found be in the range of 80-90 nm. This variability in size measurement could be attributed to the way the Zetasizer calculated nanoparticle size using DLS which calculated hydrodynamic size. We inferred that LCLA size as shown by the TEM was more accurate since it did not take into consideration the ionic cloud that was present on the surface of the LCLA as done by DLS. TEM micrographs indicated spherical morphology of the liposomes. Given the assumptions of size and lipid bilayer thickness, the number of HSPC molecules per liposome were calculated to be approximately 117,196 and the number of AD 198 molecules were calculated to be 3790 per liposome. These liposomes serve as a platform for the delivery of AD 198.

CHAPTER 3. DEVELOPMENT OF PROTOTYPE FORMULATION OF LONG CIRCULATING CD22 TARGETED LIPOSOMAL AD 198 (LCCTLA)

To make actively targeted liposomes, there are two principal methods for coupling ligands onto the liposomal surface, covalent and non-covalent methods (Benita, 2006). Generally, covalent linkage affords a more stable bonding between the ligand and liposomes whereas non-covalent linkage is dependent on weaker electrostatic and hydrophobic interactions (Benita, 2006, Nobs et al., 2004). Weaker interactions can be susceptible to breakdown due to minor changes in environmental conditions such as temperature or pH, leading to loss of targeting efficiency. An exception to this is the biotin-avidin conjugation method. This is a non-covalent conjugation method which has bonding energies comparable to covalent bonds (Miyamoto and Kollman, 1993).

3.1. Covalent Coupling

For the covalent attachment of a targeting ligand, a hydrophobic anchor with the desired functional groups for conjugation is necessary. Generally the anchor is a phospholipid, such as phosphatidylethanolamine or phosphatidylinositol, which is embedded in the liposomal bilayer. There are different ways in which the anchor can be included onto the liposomal bilayer. The most frequently used method embeds the anchor into the bilayer during liposome formation followed by the reaction for attachment of the ligand. A variation to this approach is to first carry out the reaction for attachment of the ligand to the anchor followed by inclusion of this complex while preparing liposomes (Li et al., 2010). Another alternative is to prepare the liposome and the anchor attached to the ligand discretely, followed by incubation of the anchor-ligand with the preformed long circulating liposomes (Cheng and Allen, 2008).

Although a number of anchor ligands have been explored, palmitic acid was the first reported long chain fatty acid employed for covalent coupling of targeting ligands. Huang and co-workers have reported the use of a succinimidyl ester of the fatty acid as a viable substrate for covalent conjugation, wherein the ester can react with primary amines lending a stable amide bond (Huang et al., 1980). It was only later that phospholipids were employed for conjugation purposes. Phosphatidylethanolamine is the most widely used because it is relatively simpler to modify for the required functional groups. Chen et al have successfully conjugated glycan ligands of certain receptors to the liposome using phosphatidylethanolamine as the anchor (Chen et al., 2010).

Ligands on the other hand usually carry the necessary functional groups required for conjugation, but if they are absent, methods have been devised to introduce them by attachment of ligands. **Figure 3.1** depicts the covalent reactions that are mostly used for coupling ligands to the anchors. These reactions utilize the thioether, disulfide, and hydrazone linkages, because the functional groups required for these linkages are most often already present on the ligand. Cross-linking reactions between the primary amines or the carboxylic acid functions on the surface of the liposomes with the primary amine

Figure 3.1. Schematic diagram of the different coupling methods

Notes. Reaction between maleimide and thiol functions **(A)**, formation of a disulfide bond **(B)**, reaction between carboxylic acid and primary amine group **(C)**, reaction between hydrazide and aldehyde function **(D)**, and cross-linking between two primary amine **(E)**.

of the ligands have also been explored (Benita, 2006).

The thioether bond is a stable bond. Covalent linkages based on this linkage generally require a multistep reaction for coupling ligands. However, since the ligand or the protein may be devoid, inaccessible or may have insufficient number of native thiol groups, chemical modifications are required.

Such modifications can be accomplished by using cross-linking agents or by reducing the existing disulphide linkages within the ligand (Sapra and Allen, 2002, Kirpotin et al., 1997, Sapra and Allen, 2004, Cheng and Allen, 2008, Ishida et al., 2001, Sapra et al., 2004, Loomis et al., 2010, Kirchmeier et al., 2001). Commonly used cross linkers are N-hydroxysuccinimidyl-3-(2-pyridyldithio) propionate (SPDP) and N-succinimidyl-S-acetylthioacetate (SATA). The most frequently used anchors are N-(4-(p-maleimidophenyl) butyryl) phosphatidylethanolamine (MPB-PE) and maleimide-derivatized PEG₂₀₀₀ (Mal-PEG) (Benita, 2006).

Another reliable method involves coupling two thiol functions to give a disulfide bond (Benita, 2006). This too is a multi-step reaction as thiolated ligands need to be made available by reduction of the disulfide bonds or by using cross linking agents like SATA or SPDP. Using these agents conjugated protein bound dithiopyridine is obtained, which protects the disulfide bond but can easily be reduced using dithiothreitol (DTT). These thiolated ligands can then be attached to the anchor using maleimide groups as shown previously or to pyridyldithio groups on the anchor to form disulfide bonds (Martin et al., 1981).

Although the SATA coupling reactions can be utilized for small molecules as targeting ligands, such reactions may be detrimental to targeting ligands of biological origin. The reaction conditions can always pose a potential risk of the protein fragment losing its conformation while the modification is being made. In such scenarios, the carboxylic acid functional groups on the surface of liposomes can also be utilized to cross link with the primary amines of the ligands. For carrying out this bonding, distearoyl-N-(3-carboxypropionylpoly(ethyleneglycol)succinyl)phosphatidylethanolamine (carboxyl-PEG₂₀₀₀-DSPE) has often been used (Magalhaes et al., 1995). The C-terminal end of the polyethylene glycol (PEG) provides the carboxylic acid function. A stable amide bond is yielded when a cross linking agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) is added which forms an acyl amino ester which reacts with the primary amine of the ligand. This method of coupling is advantageous over the other two above mentioned methods because no prior modification of the ligand is required which reduces the risk of denaturation and activity loss. Therefore careful and extensive studies must be conducted to verify the conjugation of the ligand with the anchor and also to validate the activity of the ligand. Similar to the carboxylic acid functional group, the primary amine on anchor have also been used to cross link with the primary amine of the ligands and reaches a coupling efficiency of 60% (Torchilin et al., 1978).

Hydrazide groups can be introduced onto the liposomal surface that can covalently bind antibodies using their carbohydrate moieties. The aldehyde groups are

obtained by mild oxidation reactions of the carbohydrates on the constant region of the heavy chain of the antibody. These aldehyde groups in turn react with the hydrazide groups on the surface of the liposomes to yield a hydrazone linkage (Lopes de Menezes et al., 1998, Lopes de Menezes et al., 2000, Benita, 2006, de Menezes et al., 1999).

The expanding concept of "click chemistry" has also been exploited by Hassane and co-workers wherein ligands on the surface of preformed SUV's have been conjugated in an efficient method. In such conjugation reactions mild conditions are employed in aqueous buffer systems and in the presence of metal ion chelators such as copper. This approach is exemplified by the conjugation of liposomes comprising of a synthetic lipid carrying a terminal alkyne function to an unprotected α -D-mannosyl derivative carrying a spacer arm functionalized with an azide group (Hassane et al., 2006). Although this method is mild and efficient, it suffers from the limitation of use of copper catalysts. These reagents have been associated with degradative side-reactions that are oxidative in nature and render the bilayer leaky (Agard et al., 2004). The reaction scheme for conjugation utilizing click chemistry has been depicted in **Figure 3.2**.

3.2. Non-covalent Coupling

Non-covalent interactions are an alternative way to couple ligands to liposomes. These methods have the advantage of incorporating the ligand more easily compared to the covalent methods and doing this without using aggressive reagents. However, the unreliability of the conjugation and the loss of activity of the targeting ligand have led to the wider acceptance of covalent attachment procedures when compared to non-covalent linkages. The methods that have been investigated previously include innovative ways of synthesizing lipid conjugated antibodies, which are essentially lipids conjugated to single chains of antibodies. These lipo-proteins are then incorporated into the liposomal membranes or adsorbed onto preformed liposomes (Laukkanen et al., 1995, Laukkanen et al., 1993). However, it may be debated that this method does not involve chemical reactions thus the ligand may detach and diffuse away from the surface of the liposome.

Another technique includes the use of haptens that are displayed on the surface of the vesicles (Benita, 2006). These liposomes tend to bind to their targets in the presence of their specific antibodies. In a related study, hapten derivatized phosphatidylethanolamine (PE) were prepared and incorporated into the surface of the liposomes. However, a major drawback of using this method is that these liposomes deliver very little drug to the tumour cells. The reason is that the soluble hapten inhibits the liposomes from binding to the target cells.

One of the initial approaches to tag liposomes with antibodies was to heat aggregate the antibodies onto the surface of the liposomes (Benita, 2006). Here the interaction between the liposome and the aggregated immunoglobulin's is based on electrostatic and hydrophobic interactions. These interactions are preferentially with the Fc (constant fragment) region of the immunoglobulins. Bonding via these sites is usually not very stable and displays poor efficiency of coupling of the antibody on the liposomal

Figure 3.2. Reaction scheme for click chemistry

Notes. **1** = 2-Carboxyethyl 2,3,4,6-tetra-O-acetyl-1-thio-β-D-galactopyranoside, **2** = *O*-(2-Aminoethyl)-*O'*-(2-azidoethyl)undecaethylene glycol, **3** = *N*-[(2-(2-Azidoethoxy)ethoxy)₁₀ethyl-(1-deoxy-2,3,4,6-tetra-*O*-acetyl-thio-α-D-mannopyranosyl)]propionamide, **4** = *N*-[2-(2-Azidoethoxy)ethoxy₁₀)-ethyl(1-deoxy-1-thio-α-D-mannopyranosyl)]propionamide, **5** = *N*-[2-(2-(2-(2-(2,3-Bis(hexadecyloxy)propoxy)ethoxy)ethoxy)ethyl)]hex-5-ynamide.

To a solution of **1** (ligand) in dichloromethane (CH₂Cl₂), *N,N'*-Dicyclohexylcarbodiimide (DCC) and *N*-Hydroxysuccinimide (NHS) are added and stirred for 45 minutes at room temperature (RT) under argon. A solution of **2** in CH₂Cl₂ containing *N,N*-Diisopropylethylamine (DIEA) is added and stirred for 18 hours. Then, DIEA and 0.2 equivalents of **2** are added and stirred for 48 hours. This gives a precipitate of **3** which is removed and purified. **3** is dissolved in methanol (MeOH) and potassium carbonate (K₂CO₃) is added with a few drops of water. This deprotection reaction is performed under argon at RT for 4 hours. The reaction product gives **4** which is a yellow coloured oil and is separated and purified. DCC and NHS are added to a solution of 5-hexynoic acid in CH₂Cl₂. Functionalized vesicles are prepared by adding the anchor **5**, which carries the alkyne moiety, to the lipid solution of the lipids in chloroform/methanol (9/1, v/v). The functionalized liposome and the azidomannose (**4**) are then incubated together with bathophenanthrolinedisulphonate (L), CuSO₄, Na-Ascorbate and NaCl in HEPES buffer at a pH of 6.5 and stirred for 6 hours at RT. This gives liposomes conjugated to mannose residues on the surface.

surface, thus the amount of antibody getting coupled onto the surface cannot be controlled. Another major drawback of this method is that the heat aggregated antibody acts as an antigen and eventually the host can develop inhibitory antibodies against it (Purohit et al., 2006).

A non-covalent bond that has a binding constant comparable to covalent bonds is the avidin – biotin interaction. This is also a multi-step approach in which one can use a biotinylated lipid molecule such as DSPE-PEG-Biotin, which is embedded in the liposomal surface while the liposome is processed followed by avidin attachment to the biotin on the surface of the liposome. The biotinylated antibody is then attached to the avidin thus giving antibody coupled liposomes (Loughrey et al., 1987). **Figure 3.3** shows the conjugation of ligands to nanoparticles using the avidin-biotin reaction.

3.3. Post Insertion Method

The post insertion method is different from all the other methods previously described. It can be used to conjugate whole antibodies, antibody fragments, peptides or other ligands. In this method, the ligand of choice is coupled to micelles composed of Mal- PEG- DSPE using the thiol reaction (Benita, 2006), which are then incubated with the preformed drug loaded liposomes under the conditions that allow the transfer of the ligand-PEG-DSPE onto the liposomes (Allen et al., 2002a, Zhang et al., 2010). For example, the authors have described the transfer of ligand coupled mPEG-DSPE to DOX or vincristine (VCR) loaded vesicles at the transition temperature of the lipid mix, 60 – 65 °C for 0.5 to 1 hour. Time and temperature are the only two variables on which the conjugation is dependent. The advantage of this method is that the condition for conjugation is decoupled from the conditions for the preparation of liposomes. The features of targeted liposomes prepared by this method are not different from those prepared by covalent or non-covalent methods.

Other than the type of conjugation utilized, the choice of ligand for targeting is also important. If the ligands are antibodies they can be used as the whole antibody molecule, the Fab' or as a fragment of the antibody chain for conjugation. All three ligands have been successfully utilized in targeting cancer cell specific epitopes. However, their efficacies differ to some extent due to their altered half lives in the blood. Cheng et al (Cheng and Allen, 2008) have compared SIL-DOX targeted to CD19 using either whole monoclonal antibody (HD37mAb), the Fab' fragment (HD37 Fab') or HD37–c-myc–Cys–His5 single chain Fv (variable fragment), HD37-CCH, which is only a part of the Fv fragment of the antibody HD37. The HD37-CCH was produced by recombinant DNA technology in *E. coli*. For antibody conjugation, a modification of the post-insertion method was utilized (Iden and Allen, 2001). Briefly, the HD37-CCH and the HD37 F(ab')₂ were reduced with DTT in HBS. After removal of unreacted DTT, the HD37-CCH or HD37 Fab' were immediately coupled to the Mal-PEG₂₀₀₀-DSPE micelles at a molar ratio of 1:4 [short chain variable fragment (scFv) or Fab':Mal-PEG₂₀₀₀-DSPE]. The whole mAb HD37 was thiolated using Traut's reagent. The thiolated mAb was coupled to Mal-PEG₂₀₀₀-DSPE/mPEG₂₀₀₀-DSPE (1:4 molar ratio) micelles at a molar

Figure 3.3. Ligand attachment to nanoparticles using the biotin-avidin reaction

Notes. A biotinylated lipid molecule such as DSPE-PEG-Biotin, is embedded in the liposomal surface while the liposome is processed followed by avidin attachment to the biotin on the surface of the liposome. The biotinylated antibody is then attached to the avidin giving antibody coupled liposomes.

ratio of 1:10 (mAb:Mal-PEG₂₀₀₀-DSPE). The micelles conjugated with the three distinct forms of the HD37mAb were then incubated with preformed liposomes. Once studied in mice, it was observed that the immunoliposomes tagged with HD37 Fab' had the longest circulation half-lives and also were most effective in prolonging survival times compared to SIL-DOX targeted via either HD37-CCH or HD37mAb. This can be attributed to the rapid clearing of the HD37mAb by the liver and spleen by Fc mediated uptake and the clearance of HD37-CCH by the liver due to the poly-His and the c-myc tags in the scFv construct.

Since the Fab' has a longer circulation half-life compared to the whole antibody as shown by a number of groups (Cheng and Allen, 2008, Sapra et al., 2004), in the present study, anti-CD22 Fab' was used for targeting LCLA to CD22+ B cell malignancies. Since the thioether bond displayed to be most stable of the conjugation techniques, it was used for the conjugating the anti-CD22 Fab' to the mal-DSPE-PEG₂₀₀₀ derivatized liposomes. The method used for the conjugation and the studies performed to validate it are briefly outlined below.

3.4. Materials and Methods

3.4.1. Materials

Mal-DSPE-PEG₂₀₀₀ was purchased from Avanti Polar Lipids, Alabaster, AL, USA, Thermo Scientific™ CL-XPosure™ Film (X-Ray Film), Sepharose CL4B gel filtration gel, anhydrous citric acid and empty PD-10 columns were purchased from Thermo Fisher Scientific, Waltham, MA, USA, anti-CD22 monoclonal antibody (RFB4) was a generous gift from the lab of Dr. Ellen Vitetta, University of Texas, Southwestern Medical Center, Dallas, TX, USA, empty macro SpinColumns® were purchased from Harvard Apparatus, Holliston, MA, USA, Amicon® Ultra – 0.5 mL centrifugal filters, Ultracel® - 100K and Ultracel® - 30K were purchased from Millipore, Bellericka, MA, Laemmli buffer, polyacrylamide gels were purchased from Bio-Rad, Hercules, CA, USA, and materials for SEM (scanning electron microscopy) were generously provided by Bioscience Research Center, College of Dentistry, UTHSC.

3.4.2. Fab' generation from whole anti-CD22 antibody

The antibody was first purified by passing through a G50 prefilled macro column. The Fc (constant fragment) region was first digested using immobilized pepsin. Briefly, the immobilized pepsin was first separated from the vehicle by loading the immobilized pepsin suspension into an empty macro column and centrifuging it for 2 minutes at 5000g and 4°C. The purified anti-CD22 monoclonal antibody was then incubated with the immobilized pepsin at pH 3.0, 37°C for 6 hours. pH 3.0 was adjusted using 1M citric acid solution. After the given time, the antibody was collected by centrifuging the immobilized pepsin and antibody digest in an empty macro spin column at 5000g for 2

minutes at 4°C. The collected antibody digest was then incubated with 10 µl of 5 mM TCEP (*tris*(2-carboxyethyl)phosphine) at room temperature for 1 hour. This gave 2Fab' fragments from each molecule of antibody. The resulting digest mix was purified by filtration using two filters 100K and 30K MWCO and the appropriate fraction containing the 50 kD Fab' was collected and used for conjugation. A schematic of this reaction is given in **Figure 3.4**.

3.4.3. Conjugation of Fab' to liposomes to give long circulating CD22 targeted liposomal AD 198 (LCCTLA)

For conjugation with antibody, liposomes were prepared in the same method as mentioned above, only 50% of m-DSPE-PEG₂₀₀₀ was replaced with mal-DSPE-PEG₂₀₀₀ to serve as an anchor for the antibody. 100 µL of the Fab' was incubated with an equal volume of the maleimide derivatized liposomes at 4°C for 12-15 hours. Following incubation, unconjugated antibody fragments were removed by gel filtration chromatography using Sepharose CL4B gel. Briefly, the 70% gel slurry in ethanol was filled in an empty PD-10 column and centrifuged at 1000g for 150 seconds at 4°C to remove the ethanol. Three 1x PBS washes followed the removal of ethanol. The column was then saturated with placebo liposomes in three separate runs and then the 200 µL of targeted liposomes were passed through the column. The final reaction for the conjugation between the maleimide derivatized liposomes is depicted in **Figure 3.5**. The resulting solution is analyzed for proof of conjugation by western blotting.

3.4.4. Verification of conjugation

Conjugation of the 50 kD Fab' fragment to the liposomes was verified by western blotting as done by Oliveira et al (Oliveira et al., 2010). Briefly, 4 samples were studied: the targeted liposomes, the fraction higher than 100 kD, the fraction below 50 kD and the whole antibody were quantified for total protein by the BCA assay and 20 µL (10 µL sample and 10 µL Laemmli buffer) of an equal concentration sample of protein (250 ng) were loaded into a 4-15% polyacrylamide gel. Samples were run at 100V for approximately one hour (until the Laemmli dye reached the end of the gel). The protein bands were then transferred from the gel onto a PVDF (polyvinylidene fluoride) membrane. The membrane was probed with a mouse secondary antibody and the blot was developed on an x-ray film.

3.4.5. Calculation of number of antibody molecules per liposome

The number of anchors (maleimide groups) and the number of antibody molecules per liposome were calculated by first calculating the number of liposomes as in the previous chapter. Then number of antibody molecules and maleimide were calculated in one mL of the LCCTLA by using Avogadro's number and substituting the values in **Equation 3.1**.

Figure 3.4. Generation of anti-CD22 Fab' fragments

Notes. Purified anti-CD22 monoclonal antibody was incubated with immobilized pepsin at pH 3.0, 37°C for 6 hours. The partially digested antibody was collected by centrifuging the immobilized pepsin and antibody digest in an empty macro spin column at 5000g for 2 minutes at 4°C. The collected antibody digest was then incubated with TCEP for 1 hour. This gave 2Fab' fragments from each molecule of antibody.

$$\text{Number of } x \text{ molecules/liposome} = \frac{\text{Number of } x \text{ molecules/mL}}{\text{Number of liposomes/mL}} \quad (\text{Eq 3.1})$$

Where 'x' may be the maleimide or anti-CD22 Fab'.

3.4.6. SEM evaluation of morphology of LCCTLA nanoparticles

Morphology of LCCTLA particles were analyzed by SEM. Briefly, 100 µL of the LCCTLA particles were loaded onto standard SEM pin stub mounts. They were dried overnight and coated with gold dust for 20 seconds. The samples were then viewed under the Carl Zeiss EVO LS15 scanning electron microscope.

3.5. Results and Discussion

3.5.1. Verification of conjugation

A western blot of the conjugated liposomes was run with the other fractions and the whole antibody. The results are shown in **Figure 3.6B**. Results for whole antibody and the fraction below 50 kD are not shown here. The whole antibody gave a very intense band at the 150 kD region whereas the concentration of protein for the fraction below 50 kD was very low and bands were too faint to be seen. However, the fraction larger than 100 kD has been shown here. **Figure 3.6A** shows the possible combinations of the antibody digests that may be produced and may show up on the blot. Fab' would be 50 kD each, F(ab')₂ would be 100 kD, the Fc region has been digested by pepsin thus would be broken into very small peptides possible smaller than 10 kD, and undigested antibody would be 150 kD.

In **Figure 3.6B** in the targeted liposomes lane, we see a clear band at 50 kD. Since we have removed other fragments such as the Fc pepsin digests and whole antibody from the 50-100 kD fraction, this is the possibly band for the anti-CD22 Fab'. In the lane with the fraction >100 kD, we see two bands, one at 150 kD and one at 100 kD. The 150 kD band most possibly is the whole anti-CD22 whole antibody whereas the 100 kD band could be the F(ab')₂. The appearance of a strong band at 50 kD in the LCCTLA sample proves that conjugation between the liposomes and the Fab' was successful.

3.5.2. Number of anti-CD22 Fab' and maleimide per liposome

2.2 mg/mL of mal-DSPE-PEG₂₀₀₀ was added to make LCCTLA particles. This equals 748 µM of mal-DSPE-PEG₂₀₀₀. Using Avogadro's number we get 4.5×10^{20} mal-DSPE-PEG₂₀₀₀ molecules/mL. As specified in the method, the number of maleimide's per liposome can be calculated by the **Equation 3.2**.

A.

B.

Figure 3.6. Verification of anti-CD22 Fab' conjugation

Notes. **(A) Anti-CD22 possible digest variants.** Fab' would be 50 kD each, F(ab')₂ would be 100 kD, the Fc region has been digested by pepsin thus would be broken into very small peptides possible smaller than 10 kD, and undigested antibody would be 150 kD. **(B) Verification of conjugation of anti-CD22 Fab' by western blotting.** A clear band at 50 kD was observed. Since I removed other fragments such as the Fc pepsin digests and whole antibody from the 50-100 kD fraction, this is the possibly band for the anti-CD22 Fab'. In the lane with the fraction >100 kD, two bands were observed, one at 150 kD and one at 100 kD. The 150 kD band most possibly is the whole anti-CD22 whole antibody whereas the 100 kD band could be the F(ab')₂.

$$\text{No. of maleimide molecules/liposome} = \frac{\text{No. of maleimide molecules/mL}}{\text{No. of liposomes/mL}} \quad (\text{Eq. 3.2})$$

$$\text{Number of maleimide molecules/liposome} = \frac{4.5 \times 10^{20}}{2.77 \times 10^{17}} \approx 1626$$

The number of anti-CD22 Fab' molecules were calculated in a similar method. The analyzed concentration of Fab' fragments in LCCTLA was 313 µg/mL which equals 6.26 µM. Using the Avogadro's number the number of anti-CD22 Fab' molecules/mL were calculated to be 3.77×10^{18} molecules/mL. Substituting these numbers in **Equation 3.3**:

$$\text{Number of Fab'/liposome} = \frac{\text{Number of Fab'/mL}}{\text{Number of liposomes/mL}} \quad (\text{Eq. 3.3})$$

$$\text{Number of Fab'/liposome} = \frac{3.77 \times 10^{18}}{2.77 \times 10^{17}} \approx 13 \text{ anti-CD22 Fab'/liposome}$$

3.5.3. Morphology of LCCTLA particles by SEM

Figure 3.7 is a scanning electron micrograph of LCCTLA particles. It was observed that the particles were spherical in shape and were approximately 130 – 150 nm in size. Some of the nanoparticles appear to be aggregated which may be due to drying of the sample in an uncontrolled environment. Also, only a single layer of the LCCTLA particles is visible in the image, and therefore 20 seconds of gold dust coating can be reduced to 15 seconds or less if deeper layers of the dried LCCTLA are to be viewed.

3.6. Summary and Conclusions

The 50 kD anti-CD22 Fab' was successfully produced by the digestion and reduction of the whole RFB4 anti-CD22 antibody. It was effectively isolated using two filters of 100 kD and 30 kD MWCO filters. The results from the western blot analysis suggest that the thioether bonding was effectively utilized to conjugate the anti-CD22 Fab' to the maleimide derivatized liposomes to give LCCTLA. 1% mal-DSPE-PEG₂₀₀₀ was optimum for the conjugation as suggested by approximately 1626 maleimide molecules per liposome as compared to only 13 anti-CD22 Fab' molecules per liposome. There was an excess of maleimide for every antibody molecule and that is how it is suggested it should be so that maximum number of antibody molecules can be conjugated to the liposomes.

Figure 3.7. SEM image of LCCTLA (Magnification - 8000x)

Notes. It was observed that the particles were spherical in shape and were approximately 130 – 150 nm in size. Some of the nanoparticles appear to be aggregated which may be due to drying of the sample in an uncontrolled environment. Also, only a single layer of the LCCTLA particles is visible in the image, and therefore 20 seconds of gold dust coating can be reduced to 15 seconds or less if deeper layers of the dried LCCTLA are to be viewed.

CHAPTER 4. *IN VITRO* EVALUATION OF CELLULAR UPTAKE AND CYTOTOXICITY OF LCCTLA

Confocal and fluorescence microscopy is routinely utilized to study the uptake of molecules or particles into cells (Ducat et al., 2011). Laser scanning confocal microscopy (CSLM) is an established technique to obtain high resolution images of a variety of biological specimens (Suh et al., 1998). Daudi cells are used by researchers for their high expression of CD22 and CD19 which can be exploited as potential targets for malignant B cell nano-therapies (Chen et al., 2010, Carnahan et al., 2007, DiJoseph et al., 2006, Du et al., 2008, Loomis et al., 2010, Tuscano et al., 2010). These cells were derived from a 16 year old Black male with Burkitt's lymphoma. Daudi cells are tumorigenic in nude mice and can be used to implant tumors or disease in animals for study. These cells are also very sensitive to interferon- α and their growth is inhibited by very low concentration of this interferon. Jurkat cells are malignant T cells that can be used to compare results with Daudi cells since they are devoid of the CD22 and CD19 receptors. These cells were originally derived from the peripheral blood of a 14 year old boy with a cancer of the T-cells. Jurkat cells are characteristically used in the studies of cytokines since they produce interleukin-2. Treatment of these cells with fluorescent nanoparticles or with nanoparticles loaded with fluorescent molecules enables intracellular nanoparticle tracking.

Flow cytometry is another technique that is extensively used for a variety of study applications including cellular uptake of drug loaded nanoparticles such as liposomes (Huth et al., 2006, Wender et al., 2000, Koller-Lucae et al., 1999, Ducat et al., 2011). Using a fluorescent drug molecule or incorporating a fluorescent lipid such as NBD-PC into the liposome bilayer, enables quantitative analysis of cellular nanoparticle uptake by flow cytometry. The number of nanoparticles internalized will be proportional to the fluorescence intensity (read by flow cytometry). Higher fluorescence intensity corresponds with a higher nanoparticle uptake. Flow cytometry is also used to measure cellular association of the nanoparticles to the cells. Receptor mediated endocytosis is an ATP dependent process (Schmid and Carter, 1990) and its activity is greatly reduced at 4°C (Dautry-Varsat et al., 1983). To confirm the mechanism of uptake of LCCTLA into CD22 expressing Daudi cells the cells can be incubated at 4°C and their uptake compared to cells incubated at 37°C.

One way of determining the therapeutic efficacy of drug loaded liposomes is by determining the viability of the cancer cells under equivalent doses over the same period of treatment as free drug formulations. The MTT assay (Mosmann, 1983) is an assay that has been used for decades now to measure the number of viable cells in culture. In the MTT assay, live cells are detected by conversion of the MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into insoluble formazan in the presence of NAD(P)H dependent enzymes. The purple colored insoluble formazan dye can be solubilized by using solubilizing agents such as DMSO or an acidic solution of a strong detergent such as SDS (sodium dodecyl sulfate). The absorbance of this reactant

mixture is measured at λ 570 nm. The absorbance of the mixture increases with cell viability. Therefore the more potent a drug, the lesser the absorbance will be.

In the following segment, studies were conducted to ascertain AD 198 uptake and cytotoxicity in CD22 overexpressing Daudi cells and CD22 deficient Jurkat cells. To determine uptake, fluorescent LCCTLA and LCLA were prepared by substituting 0.125 mole% of HSPC with NBD-PC (a fluorescent lipid). For cellular cytotoxicity, the non-fluorescent liposomes were used.

4.1. Materials and Methods

4.1.1. Materials

Daudi and Jurkat cells were purchased from ATCC, Manassas, VA, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich Co. LLC, St. Louis, MI, USA, NBD-PC was purchased from Avanti Polar Lipids, Alabaster, AL, USA, SDS, DMF (dimethylformamide), DMSO, 80% acetic acid and 1N HCl (hydrochloric acid) were purchased from Thermo Fisher Scientific, Waltham, MA, USA, and Vectashield® cell mounting medium with DAPI (4', 6-diamidino-2-phenylindole) was purchased from Vector Labs, Burlingame, CA, USA.

4.1.2. Cellular uptake of LCLA and LCCTLA by flow cytometry

To determine and compare cellular uptake in CD22+ Daudi and CD22- Jurkat cells CD22 targeted liposomes were prepared in the same method as specified previously only 0.125 mole % of the HSPC was substituted with an equal mole % of NBD-PC for fluorescence imaging. Six time points were tested ranging from 5 minutes to 4 hours (Huth et al., 2006). 10 ml each of Daudi and Jurkat cells at a cell density of 7×10^5 /ml were grown in T25 flasks for each time point. At each time point, both cell types were treated with two types of 1 μ M AD 198 formulations, LCLA and LCCTLA separately. At the end of the each time point the cells were centrifuged at 4°C and 100g for 4 minutes. The pellet obtained was washed with 1x PBS thrice and the final cell pellet was re-suspended in 1 mL of 1x PBS and tested for fluorescence intensity for NBD-PC per 10,000 cells using the BD Accuri™ C6 Flow cytometer.

4.1.3. Cellular uptake of LCLA and LCCTLA by confocal scanning laser microscopy

Jurkat and Daudi cells were prepared and treatments were given in the same method as in the method for the determination of cellular uptake by flow cytometry (Huth et al., 2006). In the present study one time point was tested at 30 minutes. The cell pellet obtained after three PBS washes was re-suspended in a single drop of Vectashield® cell

mounting medium with DAPI. These cells were mounted onto a microscope slide and covered by a Fisherbrand number 1 coverslip. The slide was allowed to dry in a fume hood for approximately 30 minutes and the edges are sealed using a transparent nail polish. The nail polish was also allowed to dry in a fume hood for another 30 minutes after which the slide was viewed under a Nikon Eclipse E800 confocal scanning laser microscope. The lasers were set at the wavelength for NBD-PC, AD 198 and DAPI and images were taken.

4.1.4. Evaluation of LCCTLA cytotoxicity

To determine cellular cytotoxicity, the MTT assay was used (Mosmann, 1983). Briefly, Daudi and Jurkat cells were grown to the required cell density. The assay was set up in 96 well plates. Three different formulations of AD 198 were tested; LCCTLA, LCLA and free AD 198. Free AD 198 was prepared in DMSO such that the final concentration of DMSO was less than 3% in any treatment well. Two time points were prepared for each treatment, 24 hours and 48 hours. For the 24 hour treatment 17,500 cells per well were plated and for the 48 hour treatment 8750 cells per well were plated. Treatment was done for 10 concentrations of each of the three AD 198 formulations ranging from 0.01 μ M to 3 μ M and the control was cells with no drug treatment. These plates were incubated at 37°C and 5% CO₂ for the study time period. At the end of the study time (24 or 48 hours) 15 μ L of 5 mg/mL concentration of MTT dye was added to each well of study and incubated under the same conditions mentioned above for 4 more hours. Following the 4 hour incubation the insoluble formazan dye formed as a result of the reaction was dissolved in 100 μ L of solubilization buffer (20% SDS in 50% DMF, 0.5% of 80% acetic acid and 0.4% 1N HCl) and incubated for 3 hours. After incubation absorbance is read at λ 570 nm using the Molecular Devices, SpectraMax M2e® microplate reader.

4.2. Results and Discussion

4.2.1. Cellular uptake of LCLA and LCCTLA

Flow cytometry and confocal laser scanning microscopy were utilized to determine the uptake of LCLA and LCCTLA in Daudi and Jurkat cells. **Figure 4.1** summarizes the results for cellular uptake of both formulations in both Daudi and Jurkat cells. It was observed that the maximum uptake at each time point was for LCCTLA in Daudi cells. The least uptake was seen with LCLA treated Jurkat cells at every time point. Daudi cells treated with LCLA and Jurkat cells treated with LCCTLA had intermediate uptake. Maximum uptake in Daudi cells treated with LCCTLA was understandable due to the CD22 receptor being overexpressed on the Daudi cells and the LCCTLA having the antibody for this overexpressed receptor. However, the Jurkat cells having higher uptake with LCCTLA than LCLA is more complicated to explain. One theory we suggest is that the Jurkat cells may have some receptor on their surface with

Figure 4.1. Time dependent cell uptake of LCLA and LCCTLA in Daudi and Jurkat cells (n=3)

Notes. It was observed that the maximum uptake at each time point was for LCCTLA in Daudi cells compared to other cell types and formulations ($p < 0.05$ for all groups). The least uptake was seen with LCLA treated Jurkat cells at every time point. Daudi cells treated with LCLA and Jurkat cells treated with LCCTLA had intermediate uptake. Maximum uptake in Daudi cells treated with LCCTLA was understandable due to the CD22 receptor being overexpressed on the Daudi cells and the LCCTLA having the antibody for this overexpressed receptor. However, the Jurkat cells having higher uptake with LCCTLA than LCLA is more complicated to explain. One theory we suggest is that the Jurkat cells may have some receptor on their surface with which the anti-CD22 Fab' non-specifically interacts, thus causing higher uptake. Also, it was seen for Daudi cells treated with LCCTLA, the uptake plateaued at approximately one hour. Therefore, maximum uptake had already taken place by one hour. Error bars represent standard deviation around the mean.

which the anti-CD22 Fab' non-specifically interacts, thus causing higher uptake. Also, it was seen for Daudi cells treated with LCCTLA, the uptake plateaued at approximately one hour. Therefore, maximum uptake had already taken place by one hour. These results were consistent with CSLM results (**Figure 4.2**) which indicated a fluorescence pattern in the cells consistent with the flow cytometry data.

4.2.2. Analysis of cytotoxicity of LCCTLA

Once that the cellular uptake studies and the mechanism of liposome uptake was known, cellular cytotoxicity studies were performed. As mentioned above three different formulations were tested in two cell types, CD22 expressing Daudi and non-CD22 expressing Jurkat cells. The study was performed to see the cytotoxic effect of the formulations at two lengths of exposure, 24 and 48 hours. **Figures 4.3** to **4.6** depict the results from this study. **Figure 4.3** exhibits the cytotoxic effects of the three formulations, free drug, LCLA and LCCTLA over 24 hours in Daudi cells. The difference in cytotoxicity between different formulations is clearly pronounced. All through from 0.01 μM to 3 μM AD 198 concentration, free AD 198 turns out to be the most cytotoxic, with an IC_{50} of approximately 0.25 μM . LCLA has an IC_{50} at about 0.5 μM , but LCCTLA have an IC_{50} of 1.5 μM . Up to 1 μM LCLA was more cytotoxic than LCCTLA and almost equally cytotoxic at 1.5 μM . At 2 μM and 3 μM , LCCTLA displayed higher cytotoxicity. This may be attributable to the process of receptor mediated endocytosis which may possibly take some additional time to endocytose the targeted liposomes. The Jurkat cells are devoid of these receptors and thus we see the results as displayed in **Figure 4.4**, which are the results for the 24 hour study in Jurkat cells. Since the CD22 receptor is absent in Jurkat cells, LCCTLA is not as cytotoxic as LCLA or free AD 198.

Moving on to the 48 hour cytotoxicity study, a steadily increasing cell death pattern was noticed in both Jurkat (**Figure 4.5**) and Daudi cells (**Figure 4.6**) from 0.01 μM to 0.5 μM for all formulations. Then from 0.75 μM up to 3 μM , there is not much of a pronounced difference between concentrations and also between the formulations. This result may be attributed to the overburdening of the cells with drug in all types of formulations. Since the drug is not being cleared as in *in vivo* systems, all the drug from the formulation eventually enters the cells and kills them. This may be controlled by developing a system that would mimic blood circulation as in whole animals. One such model was developed by Budha et al at the University of Tennessee Health Science Center (Budha et al., 2009). Nevertheless, *in vivo* models would always give more accurate representations of how the drug would behave in clinical settings.

4.3. Summary and Conclusions

In vitro studies for determination of cellular uptake and the MTT assay for cell cytotoxicity were successfully conducted. The cellular uptake of AD 198 formulations result suggested that uptake of LCCTLA was highest in Daudi cells and reached a plateau between 30 minutes and 1 hour. Next highest uptake was seen for LCCTLA in Jurkat

Figure 4.2. CSLM images of cellular uptake studies in Daudi and Jurkat cells (n=3)

Notes. Maximum uptake of the CD22 targeted liposomes in Daudi cells was confirmed by confocal microscopy. These results corroborated the results from the flow cytometry studies.

Figure 4.3. 24 hours Daudi AD 198 cytotoxicity (n=3)

Notes. Refer to section 4.2.2 for a detailed discussion. Error bars represent standard deviation around the mean.

Figure 4.4. 24 hours Jurkat AD 198 cytotoxicity (n=3)

Notes. Refer to section 4.2.2 for a detailed discussion. Error bars represent standard deviation around the mean.

Figure 4.5. 48 hours Jurkat cytotoxicity (n=3)

Notes. Refer to section 4.2.2 for a detailed discussion. Error bars represent standard deviation around the mean.

Figure 4.6. 48 hours Daudi cytotoxicity (n=3)

Notes. Refer to section 4.2.2 for a detailed discussion. Error bars represent standard deviation around the mean.

cells, which could be due to non-specific binding of the anti-CD22 antibody on other receptors on the Jurkat cell surface. These results were confirmed with CSLM images of liposome uptake, where again LCCTLA fluorescence in Daudi cells was highest compared to other cells and formulations. 24 hour cell cytotoxicity results suggested a delay in the LCCTLA cytotoxicity in Daudi cells, but ultimately, this cytotoxicity was the highest as portrayed in **Figure 4.3**. The delay could be attributed to the process of receptor mediated endocytosis which could be the rate limiting step for drug action. The 48 hour results possibly were lacking drug clearance which was not built into the model, due to which the cytotoxicity of free drug, LCLA and LCCTLA was relatively similar. Thus, it was concluded that further studies were required in whole animal models which would better mimic clinical settings.

CHAPTER 5. *IN VITRO* ANALYSIS OF INTRACELLULAR TRAFFICKING AND EFFECT ON INDUCTION OF APOPTOSIS

The process of endocytosis has evolved as a method to bring nutrients and other molecules into the cell (Sigismund et al., 2012). However, endocytosis happens to be more than just for transport purposes. It is a method of communication with the extracellular environment and is deeply engrained and intertwined with cell signaling and intracellular communications (Sigismund et al., 2012). Receptor mediated endocytosis is one of the many types of endocytotic mechanisms. It can be either constitutive or ligand induced. Both processes are very similar apart from the fact that ligand induced requires to be triggered by receptor-ligand interaction. The internalized receptors may or may not be returned to the cell surface, but if they do, they do in a location that maximizes their utility. The invagination and the membrane ripping to form endosomes requires a great deal of energy and thus these processes require energy in one form or the other (Doherty and McMahon, 2009).

Specific pathways for endocytosis utilize adaptor proteins to employ additional molecules such as clathrins or caveolins. Depending on the molecule required for the endocytosis to be successfully completed the pathways are better known as clathrin-mediated endocytosis or caveolae-mediated endocytosis (CME). Other endocytotic pathways such as macropinocytosis also exist which function independent of receptors. LCCTLA could employ either one of these pathways. Sanders et al first observed nanoparticle endocytosis in rat intestinal epithelia cells when they administered 220 nm polystyrene particles intra-gastrically (Sanders and Ashworth, 1961). To determine if the pathway is energy dependent, uptake studies can be performed at 4°C at which ATP dependent processes cease or slow down to a rate at which they become negligible. Comparing uptake results from this with uptake results at 37°C will conclusively suggest the uptake mechanism.

Once confirmed that active endocytotic mechanisms are involved, the next step would be to determine the specific endocytotic pathway. Two types of endocytotic pathways exist, phagocytosis and pinocytosis. Phagocytosis is the primary mechanism for engulfment of opsonized particles by macrophages whereas pinocytosis is the mechanism for cells to take in fluid, solutes or suspensions containing small particles such as nanoparticles. Pinocytosis can be classified into macropinocytosis, clathrin mediated endocytosis, caveolae mediated endocytosis and caveolae and clathrin independent endocytosis (Wang et al., 2011). Clathrin mediated endocytosis takes place in all mammalian cells. It is dependent on the GTPase activity of dynamin, which helps form the clathrin coated vesicles (Pucadyil and Schmid, 2009). This is the main mechanism by which CD22 internalizes its ligands (O'Reilly et al., 2011). The caveolae dependent endocytotic pathway is one that can bypass fusion with lysosomes and is a preferred pathway of entry for pathogens to avoid acid labile degradation. This is mainly a caveolin dependent process. Caveolins are proteins which exist in most cells. There are three isoforms, Caveolin-1, 2 and 3. However, lymphocytes such as B cells are devoid of caveolins (Benmerah and Lamaze, 2007). Macropinocytosis is a caveolae and clathrin

independent process. It is induced by growth factor, is driven by energy supplied by actin and is largely responsible for internalizing surrounding fluid into vacuoles (Benmerah and Lamaze, 2007, Mercer and Helenius, 2009). However, another pathway exists that is still not very well understood. This is known as the clathrin and caveolin independent pathway. It is so named because of its inherent nature to function even after being devoid of both the classical endocytotic proteins. Information that is known about this pathway is that it functions using either Arf6, Cdc42 or Rhoa (Mayor and Pagano, 2007). Dynamin is also known to play a significant role in this pathway although its function is not yet deeply understood. The endocytotic apparatus may contain clathrin independent carrier (CLIC) or a GPI-anchored protein-enriched early endosomal compartment (GEEC) (Benmerah and Lamaze, 2007).

Of the methods used to study the mechanisms of the endocytotic pathways, two main methods can be utilized, markers and inhibitors. Markers such as low density lipoprotein (LDL) (Duit et al., 2010) or transferrin (Liu et al., 2010), which are known to enter cells via specific pathways can be used. Another method is to use inhibitors specific to a particular pathway is to block the pathway. Inhibitors such as genistein, methyl- β -cyclodextrin (M- β -CD) or filipin can be used to block caveolae dependent endocytosis, chlorpromazine can be used to block clathrin mediated pathway and amiloride can be used to block macropinocytosis. Also, mutant cell lines that lack a particular protein required by a particular pathway can also be used (Kou et al., 2013).

Apart from the receptor-ligand interaction or the type of cell, the physicochemical characteristics of the nanoparticle also plays a role in which pathway is used for endocytosis. The size of nanoparticles has always been believed to play a considerable role in the endocytosis of nanoparticles (Zhang et al., 2009). For endocytosis to take place, the size range has been 10 nm – 500 nm (Benmerah and Lamaze, 2007). Larger particles are engulfed via macropinocytosis. Clathrin mediated pathways are efficient with approximately 100 nm sized particles whereas caveolae mediated pathway is more efficient with 60-80 nm particles (Benmerah and Lamaze, 2007). Other investigators suggest that the size may not be important for the endocytotic process (Huang et al., 2002). Surface charge plays an important role. Given that the cell membrane is negatively charged interaction of cationic particles with the cell surface is higher whereas anionic particles may be endocytosed via interaction with the positive charge on the proteins in the membrane (Yeung et al., 2008). Hydrophobic particles have a higher interaction with the cell but then are picked up by the RES relatively quickly, and require a balance between hydrophobic and hydrophilic groups on the surface (Kou et al., 2013).

Another parameter of particle trafficking was the final fate of the nanoparticle in the cell post endocytosis. The theory of endocytosis states that endosomes are usually formed post-endocytosis (Kou et al., 2013). However, since the liposomes need a slightly acidic environment to aid in immediate drug release, the endosomes containing the LCCTLA need to fuse with the lysosomes to bring the pH down and aid in drug release (Belchetz et al., 1977, Chu et al., 2005). The localization of liposomes post endocytosis can be viewed in a couple of different ways. The best way is to tag lysosomes with LysoTracker®, a dye that is available from Life Technologies, which binds to lysosomes

or endolysosomes. Another method is to view the cells under a TEM. If the nanoparticles can be made electron dense, they can be visualized inside the endosomes using a TEM.

The effect of AD 198 to induce apoptosis was studied by monitoring the protein expressions of specific proteins that are the hallmarks of apoptosis or cancer such as caspase-3 or phosphorylated protein kinase B (pAKT) (Edwards et al., 2013). The expressions of these proteins can be quantitatively measured by running a western blot and probing for the specific proteins of interest.

In the following section, studies that were conducted to determine the route and mechanism of uptake of LCCTA, the intracellular localization of LCCTLA and the effect of LCCTLA on expression of hallmark apoptotic and oncogenic proteins are reported.

5.1. Materials and Methods

5.1.1. Materials

Amiloride, genistein, M- β -CD and chlorpromazine were purchased from Sigma-Aldrich Co. LLC, St. Louis, MI, USA, LysoTracker® Deep Red was purchased from Thermo Fisher Scientific, Waltham, MA, USA, Iron oxide nanoparticles were purchased from Ocean NanoTech, San Diego CA, USA, antibodies for c-myc #5605, pAKT #4058, caspase-3 #9662 and anti-mouse secondary #7076 were purchased from Cell Signaling Technologies, Danvers, MA, USA and antibodies for pJNK (sc-571), β -actin (sc-130065) and anti-rabbit secondary (sc-2357) were purchased from Santa Cruz Biotechnology, Dallas, TX, USA. TEM sample preparation materials were generously provided by the Imaging Center at the Neuroscience Institute at UTHSC, Memphis, TN, USA.

5.1.2. Energy dependent or independent pathway for LCCTLA internalization

To confirm that the mechanism of uptake of LCCTLA particles into Daudi cells was by receptor mediated endocytosis cellular association studies were performed (Oliveira et al., 2010). Briefly, 10 ml of Daudi cells at a cell density of 7×10^5 /ml were grown in two separate T25 flasks. One was pre-cooled to 4°C and treated with 1 μ M fluorescent LCCTLA for one hour and the other was treated with 1 μ M fluorescent LCCTLA at 37°C for one hour. . At the end of the time point the cells were centrifuged at 4°C and 100g for 4 minutes. The pellet obtained was washed with 1x PBS thrice and the final cell pellet was re-suspended in 1 mL of 1x PBS and tested for fluorescence intensity for NBD-PC per 10,000 cells using the BD Accuri™ C6 Flow cytometer.

5.1.3. Route of uptake of LCCTLA into Daudi cells

To determine the mechanism of entry of LCCTLA into the Daudi cells, various inhibitors were used to block specific pathways and then the uptake analyzed as done in the determination of CD22 targeted liposomal drug uptake in cells by flow cytometry. As per Douglas et al (Douglas et al., 2008) three specific inhibitors were used; amiloride for micropinocytosis, genistein for caveolae mediated endocytosis and chlorpromazine for clathrin mediated endocytosis. 10 mL Daudi cells at a concentration of 7×10^5 cells/mL were incubated with the inhibitors for one hour at the following specific concentrations; amiloride 10 μ M, genistein 0.2 μ M and chlorpromazine 10 μ g/mL. Following this the inhibitor treated cells were treated with 1 μ M fluorescent CD22 targeted liposomal AD 198 (as prepared in section 4.1.2) for 1 hour. At the end of treatment the cells were processed in the same method as for the determination of LCCTLA uptake in Daudi cells by flow cytometry.

5.1.4. Intracellular trafficking of LCCTLA by TEM

5.1.4.1. MLV preparation and treatment of Daudi cells. To view the intracellular localization of LCCTLA in the Daudi cells (Gao et al., 2013, Suresh et al., 2014), 15 nm sized magnetic iron oxide nanoparticles in water (with carboxylic acid functional group) were processed into the liposomes (Päuser et al., 1997). Briefly, 1% w/w iron oxide nanoparticles were added to the 1x PBS that was used to rehydrate the dried lipid film. The MLV's thus obtained were sonicated for 5 seconds with 5 minute intervals. During the sonication and the interval the liposomal suspension was kept in ice. The sonication is repeated three times to give magnetic LCLA (MLCLA). The anti-CD22 Fab' was conjugated as specified in section 3.2.3 to give magnetic LCCTLA (MLCCTLA). 10 mL Daudi cells at a density of 7×10^5 cells/mL were treated with 1 μ M of the MLCCTLA for 4 hours. After treatment, the cells were centrifuged at 100g for 5 minutes at 4°C. The pellet was washed with ice cold 1x PBS thrice and suspended in 1mL 2.5% glutaraldehyde in 1x PBS.

5.1.4.2. TEM sample preparation. Once the cells were fixed in 2.5% glutaraldehyde overnight they were spun down at 100g for 5 minutes and washed with 10x PBS thrice for 20 minutes each. Then the cells were stained with a 4% osmium tetroxide in PBS solution for 1 hour at room temperature and excess stain washed off using 10x PBS thrice for 20 minutes each. The cell sample was then dehydrated in an ethanol (EtOH) series gradually increasing from 50% EtOH to 100% EtOH in four steps. Each dehydration cycle was done once for 10 minutes and the 100% EtOH thrice. The cells were then infiltrated with 50% Spurr's resin in EtOH under rotation overnight. Infiltration was continued with 100% Spurr's resin the next day thrice for 2 hour periods. With the 100% Spurr's resin, the centrifugation was done at 800g for 20 minutes. The cells were then embedded in fresh Spurr's resin in a mold and cured at 65°C for 48 hours. Once the resin hardened the sample block was placed in an ultramicrotome and

approximately 80 nm sections cut using a diamond knife. Chloroform was used to smoothen the sections. The sections were loaded onto copper grids and stained with Uranyl Acetate and Lead Citrate to increase contrast and electron density. The grids were then inserted into the TEM for viewing.

5.1.5. Intracellular trafficking of LCCTLA by CLSM

To confirm that the intracellular vesicles the magnetic liposomes were observed in were endolysosomes, cells were stained with endolysosome specific dye (Wu et al., 2014, Jiang et al., 2012). Briefly, 10 mL Daudi cells were cultured to a cell density of 7×10^5 cells/mL and treated with 10 μ L of placebo fluorescent CD22 targeted liposomes (as prepared in section 4.1.2. without any AD 198) for 1 hour. During the final 5 minutes of treatment, 50 nM LysoTracker® Deep Red was added to the treated cell culture. The media was then removed by centrifugation and the cell pellet thus obtained was washed 3 times using 1x PBS. The final cell pellet was suspended in a single drop of Vectashield® cell mounting medium with DAPI. This final cell suspension was mounted onto a microscope slide and covered by a Fisherbrand number 1 coverslip. The slide was allowed to dry in a fume hood for approximately 30 minutes and the edges were sealed using a transparent nail polish. The nail polish was also allowed to dry in a fume hood for another 30 minutes after which the slide was viewed under a Nikon Eclipse E800 confocal scanning laser microscope. The lasers were set at the wavelength for NBD-PC, LysoTracker® Deep Red and DAPI and images were obtained.

5.1.6. Effect of LCCTLA on cell cycle regulatory molecules by western blot

To study the efficacy of the drug delivery system on induction of apoptosis in the cancer cells, the cells were treated with the LCCTLA and the expression of 4 proteins was monitored, Caspase 3, c-myc, p-JNK, pAKT. β -actin was used as a control to signify equal loading. The process has been briefly outlined below.

5.1.6.1. Sample preparation. 10 mL of Daudi cells at a density of 7×10^5 cells/ml were treated with 1 μ M of LCCTLA at three separate time points, 2, 4 and 6 hours. The control was the same density and volume of cells but without any drug treatment. At the end of the treatment, cells were centrifuged in a Thermo Scientific Sorvall Legend X1 centrifuge at 100g for 4 minutes at 4°C. The pellet obtained was washed with 1x PBS thrice and then suspended in 70 μ L whole cell lysis buffer [Tris HCl 50mM, NaCl (sodium chloride) 150 mM, Triton X-100 1%, SDS 0.1%, EDTA (ethylenediamine tetraacetic acid) 5mM, Na₂HPO₄ (disodium phosphate) 30 mM, NaF (sodium fluoride) 50 mM, NaVO₄ (sodium orthovanadate) 0.5 mM, PMSF (phenylmethylsulfonylfluoride) 2mM and protease inhibitor at 1 μ L/106 cells]. This cell suspension was sonicated using a Virtis Virsonic® Ultrasonic Cell Disrupter. The samples were sonicated thrice for 5 seconds each with 5 minute intervals. During the intervals, the cell debris suspension was

placed on ice. The cell debris suspension was then centrifuged at 1000 rpm at 4°C for 10 minutes and the supernatant collected. The pellet obtained was discarded.

5.1.6.2. Polyacrylamide gel electrophoresis (PAGE). The protein content in the supernatant collected was quantified using the microplate BCA (bicinchoninic acid) protein assay. A volume of protein was calculated such that all samples had an equal concentration of protein. These whole cell protein samples were then mixed with an equal volume of 2x Laemmli sample buffer and boiled for 5 minutes. Samples were then cooled to room temperature (RT) for 3 minutes and loaded onto a 4-15% polyacrylamide gel of 50 μ L well capacity. The buffer reservoir was filled up with electrode running buffer (composition in Appendix) to the given mark. The electrodes were connected to a Bio-Rad power pack and samples were electrophoresed at 100V for approximately one hour (until the Laemmli sample dye reached near the bottom of the gel).

5.1.6.3. Blotting. The gel was then loaded onto a transfer cassette to transfer the protein bands onto a PVDF membrane. The sized membrane was first soaked in 100% methanol followed by transfer buffer (composition in Appendix). It was then placed over the gel in the cassette and the transfer apparatus set up. A stir bar was placed at the bottom of the buffer reservoir along with a freezer pack. Transfer buffer was then poured into the reservoir to a level such that the freezer pack was fully submerged and also covered the cassette completely. The electrodes were connected to a Bio-Rad power pack and transfer was done at 100V for one hour.

5.1.6.4. Primary and secondary antibody probing. Once the bands were transferred to the membrane, the membrane is blocked using either 5% powdered milk or 5% BSA (bovine serum albumin) in TBST (Tris buffered saline and Tween® 20) (composition in Appendix). The blocking was done at RT for 1 hour on a shaker. After the blocking was complete, excess milk or BSA was washed off the membrane with TBST (a quick wash) and the primary antibody for a specific protein was added at a dilution of 1:1000 (only β -actin 1:40,000) in TBST. The blot was incubated with the primary antibody for 12-15 hours at 4°C on a rocker. At the end of the incubation period, the excess primary antibody was washed off the membrane using TBST. The wash was done thrice for 5 minutes each. Then, the membrane was incubated with secondary antibody specific for the particular antibody used (anti-rabbit for c-myc, pJNK, pAKT and caspase-3 and anti-mouse for β -actin) at a dilution of 1:25000 for 1 hour at RT. The excess secondary antibody was then washed off the membrane using TBST thrice for 15 minutes each.

5.1.6.5. Analysis. Once the secondary antibody was probed onto the membrane, the substrate (hydrogen peroxide+ luminol) was added and briefly incubated for 1-2 minutes. The membrane was then loaded onto an x-ray film cassette and covered with a fresh x-ray film. This film was then developed and the results recorded.

5.1.6.6. Membrane stripping. The membrane was reused for probing another protein with another antibody. For this, the previous antibody was removed by a process called stripping and was done by a stripping buffer (composition in Appendix). The membrane already bound with the primary and secondary antibody was incubated with the stripping buffer for 5 minutes at 45°C following which it was washed with TBST and was reused for probing the next protein starting at step number 5.1.6.4.

5.2. Results and Discussion

5.2.1. Cellular association

Figure 5.1 shows the results for the cellular association studies. It was observed that the LCCTLA uptake in Daudi cells was significantly reduced when the uptake study was being performed at 4°C compared to when it was done at 37°C. As we already know receptor mediated endocytosis is a specific process that requires ATP for appropriate functioning and is also temperature dependent (with optimum temperature being 37°C). It may be possible that the reduced uptake in the 4°C study group was due to the uptake mechanism for the LCCTLA being receptor mediated endocytosis. Since the mechanism is greatly reduced and even possible halted, the uptake seen may be a result of the receptor associated LCCTLA being internalized before the washes were done. This result suggests that the mechanism of uptake of LCCTLA into CD22 expressing Daudi cells is receptor mediated endocytosis.

5.2.2. LCCTLA particles are endocytosed into cells by a clathrin- and caveolae-independent pathway

Figure 5.2 shows the results for LCCTLA uptake under the effect of inhibitors for certain specific pathways. The control data is for uptake results under no inhibitor use. Compared to the control, the other pathways that were inhibited, macropinocytosis, caveolae mediated internalization or clathrin mediated internalization, LCCTLA uptake seems to have somewhat increased if not significantly. These results suggest that none of these pathways is the mechanism for uptake of LCCTLA in CD22 expressing Daudi cells, which in turn suggests that LCCTLA uptake could be via the fourth pathway which is independent of clathrin or caveolin proteins and that the uptake of LCCTLA was not affected in the presence of these inhibitors because its uptake was not dependent on those pathways (Kou et al., 2013). This complete mechanism for this pathway is not yet currently understood especially the late stage of vesicle formation.

Figure 5.1. Cell association of LCCTLA in Daudi cells (n=3)

Notes. It was observed that the LCCTLA uptake in Daudi cells was significantly reduced when the uptake study was being performed at 4°C compared to when it was done at 37°C ($p < 0.05$). As we already know receptor mediated endocytosis is a specific process that requires ATP for appropriate functioning and is also temperature dependent (with optimum temperature being 37°C). It may be possible that the reduced uptake in the 4°C study group was due to the uptake mechanism for the LCCTLA being receptor mediated endocytosis. Since the mechanism is greatly reduced and even possible halted, the uptake seen may be a result of the receptor associated LCCTLA being internalized before the washes were done. This result suggests that the mechanism of uptake of LCCTLA into CD22 expressing Daudi cells is receptor mediated endocytosis. Error bars represent standard deviation around the mean.

Figure 5.2. LCCTLA uptake in Daudi cells under different inhibitors (n=3)

Notes. The control data is for uptake results under no inhibitor use. Compared to the control, the other pathways that were inhibited, macropinocytosis, caveolae mediated internalization or clathrin mediated internalization, LCCTLA uptake seems to have somewhat increased if not significantly. These results suggest that none of these pathways is the mechanism for uptake of LCCTLA in CD22 expressing Daudi cells, which in turn suggests that LCCTLA uptake could be via the fourth pathway which is independent of clathrin or caveolin proteins and that the uptake of LCCTLA was not affected in the presence of these inhibitors because its uptake was not dependent on those pathways (Kou et al., 2013). This complete mechanism for this pathway is not yet currently understood especially the late stage of vesicle formation. Error bars represent standard deviation around the mean.

5.2.3. LCCTLA nanoparticles were localized intracellularly in endosomes

Figure 5.3 shows a whole Daudi cell image acquired by TEM post-MLCCTLA treatment. Two parts of the image have been enlarged in **Figures 5.4** and **5.5**. As in **Figure 5.4** an MLV inside an endocytotic vesicle can be observed. The size of each of the three dark structures inside the vesicle are approximately 70 nm in diameter, indicating that these are the SUV's inside the outer, larger MLV. In **Figure 5.5** another endocytotic vesicle carrying SUV's was observed. These SUV's are approximately 50 nm in diameter. The size of these vesicles correspond with the size of the MLCCTLA (data not shown here). This data confirms that the intracellular localization of the MLCCTLA is in endosomes. Later, these endosomes must fuse with lysosomes to form endolysosomes to release AD 198.

5.2.4. The endosomes fuse with lysosomes to give endolysosomes

Figure 5.6 shows the images for the study to determine if the endosome in which the LCCTLA were localized were endolysosomes. The cells shown here are Daudi cells treated with 10 μ L of placebo LCCTLA and LysoTracker® Deep Red (a dye that binds to late endolysosomes). After washing off the excess LCCTLA, the images showed red circular structures inside the Daudi cells (white arrows). Since the dye for tagging the endolysosomes was red, it was deduced that these circular red structures were the endolysosomes. The liposomes were processed with NBD-PC, a fluorescent green lipid, which also show up in the images as bright green (green arrows). When these two images were overlaid, it was observed that the endolysosomes and the LCCTLA were co-localized inside the cells (yellow arrows). This establishes that the LCCTLA were intracellularly localized in endosomes (as from **Figures 5.4** and **5.5**) and these endosomes were later fusing with the lysosomes to give endolysosomes (**Figures 5.6**)

5.2.5. LCCTLA activates classical apoptotic pathways

With LCCTLA treatment in Daudi cells, it was observed that expression of oncogenic markers decreased and apoptotic markers increased. **Figure 5.7A** shows the proteins that were affected along with their functions and **Figure 5.7B** gives a comparison of their expressions with the control. C-myc is a marker for Burkitt's lymphoma and is a regulator gene that codes for a transcription factor (Finver et al., 1988). The protein is multi-functional playing roles in apoptotic inhibition and cell cycle progression. Treatment with LCCTLA reduced the expression of c-myc in a time dependent manner as shown in **Figure 5.7B**.

LCCTLA, once internalized is believed to release drug due to pH reduction in the endolysosomes. The intracellularly released AD 198 binds to PKC holoenzyme. Later the catalytic segment (CS) dissociates. The CS activates PLS3 which depolarizes the mitochondria releasing Cyt C. The Cyt C activates caspase 3. Caspase 3 being an apoptotic protein eventually results in apoptosis (He et al., 2005). Thus, we see a time

IONP DAUDI_017
IONP in Daudi
Print Mag: 15900x @ 7.0 in
2: 29: 27 PM 1/29/2015

2 μ m
Direct Mag: 10000x
AMT Camera System

Figure 5.3. TEM image of LCCTLA intracellular localization in Daudi cells

Notes. Refer to section 5.2.4 for a detailed description.

IONP DAUDI_014

IONP in Daudi

Print Mag: 79800x @ 7.0 in

2: 22: 42 PM 1/29/2015

500 nm

Direct Mag: 50000x

AMT Camera System

Figure 5.4. Enlarged from Figure 5.3 showing an MLV of MLCCTLA inside an endosomal structure

Notes. Refer to section 5.2.4 for a detailed description.

IONP DAUDI_015

IONP in Daudi

Print Mag: 63800x @ 7.0 in

2: 27: 45 PM 1/29/2015

500 nm

Direct Mag: 40000x

AMT Camera System

Figure 5.5. Enlarged from Figure 5.3 showing SUV's of MLCCTLA inside an endosomal structure

Notes. Refer to section 5.2.4 for a detailed description.

Figure 5.6. CLSM image of intracellular localization of LCCTLA in endolysosomes

Notes. Refer to section 5.2.5 for a detailed description.

Figure 5.7. Effect of LCCTLA particles on expression of hallmark apoptotic and oncogenic proteins

Notes. **(A)** Effect of LCCTLA on expression of caspase-3, pAKT, c-myc and pJNK, and **(B)** Western blot results for the expression patterns post LCCTLA treatment for 3 time points, 2, 4 and 6 hours. Refer to section 5.2.6 for a detailed description of the result.

dependent increase in the concentration of caspase 3 as depicted in **Figure 5.7B**, which is a hallmark of cell death via apoptosis. There is a possibility for AD 198 to activate caspase-3 by other pathways but these are not well understood yet.

Protein kinase B also known as Akt is a serine threonine specific protein kinase which plays a key role in multiple process in the cell such as cell proliferation, transcription and apoptosis. It is capable of initiating cell survival via growth factor dependent and independent pathways (Nicholson and Anderson, 2002). Phosphorylated Akt is the activated form of Akt which is necessary for it to activate or deactivate its substrates (Sarbasov et al., 2005, Edwards et al., 2013). In **Figure 5.7B**, we can see the expression of pAKT going down in a time dependent manner compared to the control which means that LCCTLA also inhibited cell proliferation via suppression of pAKT.

JNK's or c-Jun N-terminal kinases are master protein kinases that regulate many processes in the cell such as inflammation, cell proliferation and differentiation and apoptosis. They belong to the mitogen-activated protein kinase family. Their active role in cancer development is now well-established (Bubici and Papa, 2014). JNK is activated by phosphorylation (pJNK). pJNK in turn phosphorylates multiple protein depending on its isoform. **Figure 5.7B** shows a time dependent suppression of pJNK by treatment of Daudi cells with LCCTLA, thus proving that cell proliferation is also inhibited by suppression of pJNK.

5.3. Summary and Conclusions

Studies to verify the *in vitro* pathways undertaken and mechanisms employed by the LCCTLA to enter, release drug and eradicate the malignant cells were performed. It was observed that the uptake pathway of LCCTLA was primarily energy dependent. However, the known mechanisms of uptake such as the clathrin or caveolin dependent pathways were possibly not the pathways for uptake of the LCCTLA. Results suggest a relatively newer method for uptake called the clathrin- and caveolae-independent pathway. This pathway functioned independently of the proteins required for the conventional endocytotic pathways. Little is currently known about this pathway. What is known is that this pathway works by recruiting proteins such as Arf6, Cdc42, Rhoa and dynamin.

The intracellular localization of LCCTLA was determined by CLSM and TEM. By TEM we established that the LCCTLA were located in endosomes. However, using LysoTracker® deep red under the CLSM, it was understood that the endosomes were more specifically endolysosomes. Once the liposomes were intracellularly located in the endolysosomes, the low pH would break down the bilayer formation of the LCCTLA, releasing the AD 198 load.

The expression of specific proteins in the cells are markers for specific processes and pathways proceeding in the cell. Expression profiles of proteins engaged in processes such as apoptosis and cell proliferation were successfully monitored. Post-LCCTLA

treatment in Daudi cells, caspase-3 expression, an apoptotic protein, increased in a time dependent manner. Conversely, the expression of certain proteins which aid in cell proliferation, such as c-myc, pJNK and pAKT was significantly lowered.

These results suggest that CD22 targeted drug delivery system is successfully delivering the AD 198 cargo inside the malignant cells. The uptake mechanism works differently from the well documented pathways but efficiently delivers LCCTLA inside the cells. The drug is released due to the fusion of endosomes with the lysosomes and works well in comparison with the free AD 198 solution drug (Edwards et al., 2013).

CHAPTER 6. LYOPHILIZATION OF LCCTLA TO ENHANCE LONG TERM STABILITY

Results from studies conducted earlier in this dissertation have shown the potential efficacy and specificity of LCCTLA in the treatment of B cell malignancies. However, one of the limitations in using dispersed liposomal systems for therapeutic purposes is their physical instability due to aggregation, particle fusion and drug leakage from the bilayer (van Winden, 2003) when they are in the dispersed liquid form. This instability is because of non-elastic collisions and the fluid behavior of the lipid bilayer resulting in drug leakage from the particles.

One of the ways to address this limitation is to remove the water from the nanoparticulate dispersion which limits both the Brownian motion nanoparticles in the dispersion and the fluidity of the lipid bilayer. However, water constitutes an essential component of the liposome superstructure. If removed it could result in collapse of the liposome structure. Therefore certain additives need to be added to the liposomal dispersion to ensure that the removal of water does not negatively impact the stability of the dispersion (Abdelwahed et al., 2006b).

Removal of water to prolong the stability of the dispersion can be done by a process known as lyophilization. Removal of water is achieved by freezing the dispersion and subliming away the ice by exposing the frozen dispersion to a relatively lower partial pressure of water vapor. This process exerts stresses on the nanoparticulate dispersion especially the stress of freezing and dehydration. Stress from freezing is due to the crystallization of ice which causes separation of phases into ice and the cryo-concentrated phase (composed of the nanoparticles and other components such as buffering salts). This greatly increases the tonicity of the cryo-concentrated phase which may potentially impact the formulation negatively (Abdelwahed et al., 2006b, Abdelwahed et al., 2006a). Stress from drying is due to the fact that water interacts with the hydrophilic phosphate head groups which plays a key role in the formation of liposomes. Removal of this water will result in a complete collapse of the system (van Winden, 2003). Although rehydration with water may result in the reformation of the vesicles, yet the complete process could increase drug leakage. To address this issue, lyo and cryoprotectants are utilized to reduce or eliminate the stresses due to freezing (cryoprotectants) and drying (lyoprotectant).

As water freezes, the cryo-concentrated phase gets pushed into smaller and smaller pockets as the ice crystals get larger. Cryoprotectants function by dissolving and lowering the melting point of water. This process is known as vitrification. By allowing salts and other molecules to remain undisturbed in their natural locations, vitrification avoids the damage caused by freezing. Cryoprotectants increase the size of the pockets of the cryo-concentrated phase to an extent that damage due to phase separation becomes negligible.

Lyoprotectants on the other hand work by the theory of replacement. As specified, the hydrophilic phosphate head groups require water to maintain the liposomal structure and removal of water could cause them to collapse. Non-reactive disaccharides such as sucrose function as lyoprotectants. It is hypothesized that the sugar molecules form hydrogen bonds with the polar head groups during the drying process (Abdelwahed et al., 2006b). Therefore it is assumed that the sugar molecules serve as substitutes for water. Inclusion of lyoprotectant can form an amorphous matrix around the vesicles and protect the vesicles from fusion and prevent rupture of the vesicles due to crystallization of ice (Koster et al., 1994).

Trehalose is another disaccharide that is widely used as a lyoprotectant for the stabilization of liposomes (Crowe et al., 1984, Crowe et al., 1985). In preliminary studies performed, trehalose did not show a significant difference from sucrose for AD 198 retention or particle size and charge maintenance (data not presented in the present text). Sucrose in effect gave marginally better results compared to trehalose. This may be attributed to the fact that trehalose demonstrates crystallization during the dehydration process which may contribute to some degree of vesicle rupture (Sundaramurthi and Suryanarayanan, 2009). In the following sections, studies performed to determine the appropriate concentration of sucrose required to efficiently stabilize lyophilized liposomes is discussed. Also presented are results for a stability comparison of lyophilized LCCTLA and dispersed LCCTLA.

6.1. Materials and Methods

6.1.1. Materials

Ultrapure sucrose was purchased from Thermo Fisher Scientific, Waltham, MA, USA and T-Zero aluminum pans were purchased from TA Instruments, New Castle, DE, USA.

6.1.2. Determination of Tg

The Tg of maximally freeze concentrated amorphous phase of mixture of liposomes and sucrose was determined by DSC. About 10-20 μ L of the liposome mixture prior to lyophilization was placed in T-Zero aluminum pans and hermetically sealed. An empty pan prepared in a similar manner was used as reference. The samples were equilibrated at 25°C and then cooled at 5°C/min to -70°C. After equilibration, the samples were heated at the rate of 10°C/min to 25°C.

6.1.3. Preparation of lyophilized LCCTLA (Lyo-LCCTLA)

The liposomes were prepared in the same method as previously specified in Chapter 2 section 2.1.2. However, for including lyoprotectants in the hydrophilic core of the liposome, sucrose was dissolved in the 1x PBS that was used for hydrating the dry lipid film. The lyoprotectant for the external phase was added after conjugation of the Fab' and removal of unconjugated fragments. Volume was made up to the batch size (if necessary) after all the sucrose was dissolved.

6.1.4. Lyophilization formulation optimization

500 μ L's of the final LCCTLA along with the dissolved sucrose was loaded into 2 mL pharmaceutical amber glass vials. The vials were appropriately labelled and covered with lyophilization rubber closures. The rubber closures had a small vent that remained open during lyophilization for water vapor to be extracted. The vials were then loaded into the lyophilizer (Virtis Advantage 2.0) and the cycle was started. The cycle was as follows: Thermal treatment included ramping the temperature down to -45°C over a period of 180 minutes. The samples were then held at -45°C for 240 minutes to make sure the sample was frozen evenly throughout. Following this the drying phase was started. The drying was divided into two phases; the primary drying phase and the secondary drying phase. For the primary drying phase the temperature was ramped up from -45°C to -35°C in 30 minutes. The vacuum was pulled down to 100 mTorr (millitorr). This condition was maintained for 720 minutes to aid maximum drying. Following this the secondary drying was started where the temperature was ramped up to 10°C over a period of two hours. Vacuum was maintained at 100 mTorr. This condition was maintained for 240 minutes. The end of secondary drying completed the lyophilization cycle and samples were overlaid with ultrapure nitrogen and crimp sealed with flip-off caps. The formulations containing the various lipid:sucrose ratios were then examined for their moisture content, drug retention, particle size and ζ -potential.

To determine AD 198 retention, lyophilized LCCTLA were reconstituted with 500 μ L of HPLC grade water and free AD 198 removed by gel chromatography using G25 sephadex gel. Samples were prepared and the AD 198 content in the eluate was determined by HPLC as specified in Chapter 2, determination of encapsulated AD 198. The gel chromatography eluate was also used to determine the size and ζ -potential. Moisture content of the lyophilized LCCTLA cake was determined by Karl Fischer titration (Scholz, 1984).

6.1.5. Retention of Fab' activity post-lyophilization

Lyophilization is a process that exerts stress on the weaker molecular bonds of certain molecules such as hydrogen bonding in proteins. Thus it is important to confirm the activity of an antibody post-lyophilization. Cellular uptake studies in Daudi cells were performed to confirm the activity of the Fab' in the LCCTLA and compared to the uptake

of non-lyophilized dispersed LCCTLA. The protocol followed was the same as in 4.1.2, cellular uptake of LCLA and LCCTLA by flow cytometry.

6.1.6. Stability study

A four week stability study was conducted on the lyophilized samples and compared with liquid liposomal dispersion samples. Lyo-LCCTLA samples were prepared in the procedure described above. Liquid liposomal dispersion stability samples were prepared in the same method as used for preparing LCCTLA. The liquid samples were also loaded into the same vials and sealed using the same container closure system used for preparing Lyo-LCCTLA samples for stability. Both formulations were tested for stability at two temperatures, $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$, and $25^{\circ}\text{C} \pm 3^{\circ}\text{C}$. Each formulation was tested at 5 time points; 0, 7, 14, 21 and 31 days (1 month). Parameters tested at each time point were drug retention, liposome size and ζ -potential. Moisture content was tested twice, once at 0 time and once at 31 days.

6.2. Results and Discussion

6.2.1. The Tg values for sucrose-LCCTLA formulation increased with an increase in sucrose concentration

The lyophilization cycle was designed based on the Tg of the liposome sucrose mixtures as determined by DSC. The heating profiles for mixtures of liposomes containing increasing concentrations of sucrose is shown in **Figure 6.1**. The Tg of these mixtures containing 2x to 8x sucrose of the total lipid content was between -34 and -39°C . Consequently, for the lyophilization cycle, the liposome sucrose mixtures were frozen to -45°C and held for 240 minutes and the rest of the drying cycle begun as stated earlier.

6.2.2. 8x sucrose proved to be optimum for lyo-LCCTLA

At the selected parameters for the lyophilization cycle (**Figure 6.2**), the results for the optimization of sucrose concentration are presented in **Figure 6.3**. The sucrose concentration for the internal core of the liposome was observed to be optimum at approximately 84 mM or 67 mg/mL (results not presented in the study). Keeping the internal sucrose concentration constant at 84 mM, the external sucrose concentration was increased such that the total sucrose concentration was between 2x and 8x of the total lipid concentration. The control for the study was a formulation with no sucrose (internal and external). It was observed that as the sucrose concentration was increased from 0x to 8x, the AD 198 retention post lyophilization increased from 23% to 98%. There was almost a direct correlation between the sucrose concentration and drug retention. Size and ζ -potential conversely were not significantly affected beyond 2x sucrose concentration.

Figure 6.1. Effect of sucrose concentration on T_g

Notes. The T_g of these mixtures containing 2x to 8x sucrose of the total lipid content was between -34 and -39°C. Consequently, for the lyophilization cycle, the liposome sucrose mixtures were frozen to -45°C and held for 240 minutes and the rest of the drying cycle begun as stated earlier.

Figure 6.2. Phases of a lyophilization cycle for LCCTLA

Notes. The temperature was reduced to -45°C over a period of 180 minutes. The samples were then held at -45°C for 240 minutes to make sure the sample was frozen evenly throughout (freezing phase in blue). Following this the drying phase was started. The drying was divided into two phases; the primary drying phase (green phase) and the secondary drying phase (red phase). For the primary drying phase the temperature was ramped up from -45°C to -35°C in 30 minutes. This temperature was maintained for 720 minutes to aid maximum drying. Following this the secondary drying was started where the temperature was ramped up to 10°C over a period of two hours. This temperature was maintained for 240 minutes.

Figure 6.3. Effect of increasing sucrose concentration on post-lyophilization size and drug retention (n=3)

Notes. The sucrose concentration for the internal core of the liposome was observed to be optimum at approximately 84 mM or 67 mg/mL (results not presented in the study). Keeping the internal sucrose concentration constant at 84 mM, the external sucrose concentration was increased such that the total sucrose concentration was between 2x and 8x of the total lipid concentration. The control for the study was a formulation with no sucrose (internal and external). It was observed that as the sucrose concentration was increased from 0x to 8x, the AD 198 retention post lyophilization increased from 23% to 98%. There was almost a direct correlation between the sucrose concentration and drug retention. Size and ζ -potential conversely were not significantly affected beyond 2x sucrose concentration. The size also decreased at the 6x and 8x concentration of sucrose. Error bars represent standard deviation around the mean. This study gives the impression that 8x sucrose concentration was optimum for lyophilization of LCCTLA to retain AD 198 in the lipid bilayer and also preserve the size and charge of the liposomes.

The size also decreased at the 6x and 8x concentration of sucrose. This study gives the impression that 8x sucrose concentration was optimum for lyophilization of LCCTLA to retain AD 198 in the lipid bilayer and also preserve the size and charge of the liposomes.

Moisture content is a critical parameter to maintain the stability of lyophilized drug products. As per the US FDA (United States Food and Drug Administration), the residual moisture content in a lyophilized product with protein must be between less than 1% to 5% (FDA, 1990). The moisture content for the lyo-LCCTLA was approximately 1.48% immediately after lyophilization.

6.2.3. Activity of Fab' was retained post-lyophilization

Activity of the anti-CD22 Fab' was confirmed by cellular uptake studies in Daudi cells to ensure that the targeting ligand was behaving in the same manner after lyophilization compared to the un-lyophilized product. The result is represented in **Figure 6.4**. By cell cytometry, it was observed that the cellular uptake of the lyo-LCCTLA was not significantly different compared to uptake by un-lyophilized LCCTLA.

6.2.4. Lyo-LCCTLA are more stable than liquid LCCTLA for one month

A stability comparison was done between the Lyo-LCCTLA and liquid LCCTLA at two temperatures, 5°C and 25°C. The results in **Figure 6.5** represent the % AD 198 retention on every stability time point. It was observed that the Lyo-LCCTLA were most stable at 5°C compared to other temperatures and other formulations. Lyophilized formulation performed far better at both temperatures (5°C and 25°C) compared to the liquid LCCTLA formulation. On day 28 of the study, Lyo-LCCTLA had a 102% AD 198 retention at 5°C and 96% at 25°C, whereas the liquid LCCTLA formulation had a 69% AD 198 retention at 5°C and 55% at 25°C. Size and ζ -potential were not affected for both formulations stored at either temperature. The liquid formulations were additionally stored in an inverted position, to maximize the container closure interaction, for the 14 day and 28 day time point. These inverted samples also did not show a significantly different AD 198 retention or liposomal size and ζ -potential compared to the upright samples of the same formulation at the same temperature. Moisture content on day 0 was 1.48% whereas at 5°C and 25°C on day 28 it was 1.08% and 1.34%.

These results suggest that the lyophilized liposomal formulations are more stable compared to liquid liposomal formulations. One possible justification for this may be that the lipids tend to get oxidized in the presence of water as explained by Winden et al (van Winden, 2003). Once the water was removed (almost completely) by lyophilization, the potential for degradation by oxidation was reduced.

Figure 6.4. Fab' activity before and after lyophilization (n=3)

Notes. The cellular uptake of the lyo-LCCTLA was not significantly different compared to uptake by un-lyophilized LCCTLA ($p < 0.05$). Error bars represent standard deviation around the mean.

Figure 6.5. LCCTLA stability comparison for liquid and lyophilized formulations at 5°C and 25°C (n=3)

Notes. The Lyo-LCCTLA were most stable at 5°C compared to other temperatures and other formulations. Lyophilized formulation performed far better at both temperatures (5°C and 25°C) compared to the liquid LCCTLA formulation. On day 28 of the study, Lyo-LCCTLA had a 102% AD 198 retention at 5°C and 96% at 25°C, whereas the liquid LCCTLA formulation had a 69% AD 198 retention at 5°C and 55% at 25°C. Size and ζ -potential were not affected for both formulations stored at either temperature. The liquid formulations were additionally stored in an inverted position, to maximize the container closure interaction, for the 14 day and 28 day time point. These inverted samples also did not show a significantly different AD 198 retention or liposomal size and ζ -potential compared to the upright samples of the same formulation at the same temperature. Moisture content on day 0 was 1.48% whereas at 5°C and 25°C on day 28 it was 1.08% and 1.34%. These results suggest that the lyophilized liposomal formulations are more stable compared to liquid liposomal formulations. One possible justification for this may be that the lipids tend to get oxidized in the presence of water as explained by Winden et al (van Winden, 2003). Once the water was removed (almost completely) by lyophilization, the potential for degradation by oxidation was reduced. Error bars represent standard deviation around the mean.

6.3. Summary and Conclusions

Liposomes for lyophilization were successfully processed by including the appropriate amount of sucrose in the aqueous core of the LCCTLA and in the external phase. Based on the results for the thermal analysis of the sucrose liposome mixtures, the optimum freezing temperature was determined to be -45°C . Thus the liposomes were frozen at -45°C . The lyophilization cycle is depicted in **Figure 6.2**. A sucrose concentration of 8 times the lipid concentration was determined to be optimum. Of the 8x sugar concentration 84 mM or approximately 67 mg/mL sucrose was optimum for the internal aqueous core. Post-lyophilization, the moisture content was determined to be approximately 1.48%, which was within the acceptable limits as specified by the FDA (FDA, 1990). Activity of the anti-CD22 Fab' was retained post lyophilization which was determined by a comparable cellular uptake of the LCCTLA in Daudi cells before and after lyophilization.

Stability studies of the liquid and lyophilized formulation concluded that lyophilized liposomes were evidently more stable compared to the liquid dispersed formulations. This lead to the conclusion that while water is an integral part of the liposomal structure, its presence was detrimental to their long term stability. Thus, it is necessary to store these formulations in the dehydrated form. Also they must be stored at 5°C to avoid degradation of the drug substance in the formulations.

CHAPTER 7. SUMMARY AND CONCLUSIONS

B cell malignancies are a serious health concern killing almost 44,000 people in the United States every year. CHOP is the standard of therapy. DOX is a part of CHOP therapy that displays dose related cardiotoxicity which is one of the reasons many patients need to opt out of CHOP. AD 198 is an analogue of DOX which is superior to DOX in murine cancer models and has novel biochemical pathways and pharmacological properties. One of the common drawbacks of chemotherapeutic agents is depletion of the healthy population of neutrophils and thrombocytes. This is also displayed by AD 198. Cytopenias are mainly due to non-specific interactions of the drug with healthy cells of the body. Thus, a targeted long circulating AD 198 loaded drug delivery system that would impart specificity to the drug action and reduce adverse effects was sought. Nanotechnology has been actively pursued as a means of providing alternative drug delivery systems for disease targeted therapy. Liposomes, a specific type of nanoparticle comprising of lipid bilayers turned out to be an attractive option due to its biocompatible nature of excipients and its ability to accommodate drugs of a wide range of physico-chemical characteristics. In addition, an active targeting approach could be applied to liposomes which offer a wide repertoire of surface modification options to choose from. A targeting ligand such as an antibody could be conjugated to a specific functional group built into the liposomal structure. The main objective of this dissertation work was to design, develop, characterize and test long circulating AD 198 loaded targeted liposomes that would be cytotoxic specifically to malignant B cells.

A prototype formulation of AD 198 loaded liposomes (LCLA) was developed that would be able to encapsulate maximum AD 198 and have optimum parameters for effective delivery of the encapsulated drug. The optimized composition of LCLA was as follows. HSPC was the lipid of choice and was used at 75 mole %, mPEG₂₀₀₀-DSPE 2 mole %, cholesterol 10 mole % and AD 198 2 mg/mL. The physicochemical parameters of the optimized formulation were as follows; size 115-145 nm, ζ -potential -8 to -15 mV, AD 198 encapsulation 1000-1500 μ g/mL and dissolution of not more than 30% AD 198 occurred for 72 hours. The size of the LCLA as per TEM was found to be in the range of 80-90 nm. TEM micrographs indicated a roughly spherical morphology of the liposomes. It was calculated that the number of HSPC molecules per liposome were approximately 117,196 and the number of AD 198 molecules were approximately 3790 per liposome.

To achieve an active targeting approach it was necessary to conjugate a ligand for a specific receptor on the surface of the malignant B cells. For this purpose, CD22 was selected as the receptor to be targeted on the malignant B cells. CD22 was selected due to its property of receptor mediated endocytosis upon interaction with the ligand and also because it was overexpressed on malignant B cells. The anti-CD22 monoclonal antibody, RFB4, was selected as the targeting ligand. Since earlier studies by other research groups had proven that the circulating half-life of targeted liposomal systems was higher if targeted using just the Fab' conjugated to the liposomes rather than the whole antibody, only the anti-CD22 Fab' conjugation was optimized. Numerous methods for conjugating a ligand to the liposome exist. The one selected for conjugating the anti-CD22 Fab' to the

LCLA was with the thioether bond. The reason for selecting this strategy was its minimum use of harsh reagents and provision of a strong covalent chemical bond ensuring stability of the targeted liposomal system. Proof of conjugation by the thioether bonding was provided by a western blot of the targeted liposomes which evidently portrayed a band at the 50 kD region which is be the molecular weight of the anti-CD22 Fab'. It was calculated that every liposome displayed an average of approximately 13 anti-CD22 Fab' molecules.

Whether or not the 13 anti-CD22 Fab' molecules were sufficient to effectively target and deliver the liposomal AD 198 to the malignant B cells was determined by testing the long-circulating CD22 targeted liposomal AD 198 (LCCTLA), *in vitro* in CD22 overexpressing Daudi cells and comparing this result with non-CD22 expressing Jurkat cells. It was seen that Daudi cells had a significantly higher uptake of the LCCTLA compared to Jurkat cells, which confirmed specificity of the delivery system. The MTT assay results for cell cytotoxicity suggested a delay in cell kill for Daudi cells treated with LCCTLA, but this could be explained by the method of endocytosis that they underwent which would take more time compared to the diffusion mechanism for LCLA and solution AD 198. Nevertheless, cytotoxicity by the LCCTLA in Daudi cells was highest for a 24 hour study. However, the results from the 48 hour study warranted studies in animals in which unbound drug would be cleared such that clinical settings could better be mimicked.

The functioning of the LCCTLA after cellular was explained by the several studies performed *in vitro*. Cellular association studies determined that the endocytotic mechanism was an energy dependent mechanism and it was further ascertained that the mechanism of endocytosis was a clathrin- and caveolin-independent pathway. This pathway has not been fully understood yet particularly the later stages. However, it was successfully determined that after endocytosis, the liposomes were localized in endolysosomes. This result deduced that the drug release would take place due to liposomal breakdown by the low lysosomal pH. Once the drug was released into the cytosol, it functioned via the activation of apoptotic proteins such as caspase-3 and the suppression of oncoproteins such as c-myc. These verifications were deduced by protein expression studies performed in Daudi cells post-LCCTLA treatment.

Since the long term storage of liposomes was an issue due to lipid degradation by oxidation, water was removed by the process of lyophilization. The lyophilization cycle parameters were successfully optimized and the formulations of the liposomes were also optimized so that they would not collapse during dehydration. It was determined that a lipid:sucrose ratio of 1:8 was required to retain maximum AD 198 post lyophilization. Via a short term stability study it was also ascertained that the lyophilized formulation was most stable at 5°C and was more robust than the liquid liposomal dispersions.

In conclusion, targeted drug delivery with AD 198 was more potent and specific compared to other untargeted formulations and the main hypothesis of the dissertation was accepted. Further studies in small animal models are necessary to ascertain the

efficacy of the system in more clinically relevant models. The stability of LCCTLA was improved by dehydrating them in a temperature and pressure controlled environment.

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APPENDIX. BUFFERS FOR WESTERN BLOTTING

1x Electrode running buffer (2L)

Glycine	28.8 G
Tris base	6.04 G
SDS	2.0 G
Water	q.s.

Transfer buffer (1L)

Tris Base	3.03 G
Glycine	14.4 G
Methanol	200 mL
Water	q.s.

10x TBS (Tris buffered saline)

Tris HCl	500 mM
NaCl	1500 mM

1x TBST

10x TBS	100 mL
Tween 20 (10%)	10 mL
Water	890 mL

Stripping buffer (100 mL)

SDS 10%	20 mL
Tris HCl	12.5 mL
β -mercaptoethanol	0.8 mL
Water	q.s.

VITA

Nivesh K. Mittal was born in Kolkata, India in 1984. He received his Bachelor of Biotechnology degree from YCMO University, Nashik, India and his Master of Science in Biotechnology from West Bengal University of Technology, India. He worked at Sanmour Pharma, a contract research organization in Thane, India as Junior Manager, Formulations for about a year. Nivesh joined the Ph.D program in Pharmaceuticals and Drug Design at the University of Tennessee Health Science Center in 2010 with Dr. George C. Wood as his major advisor.

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