Monoclonal Antibody and Liposomal Nanoparticle-based Targeting Therapies for
Chronic Lymphocytic Leukemia

DISSERTATION

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By

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Abstract

Chronic Lymphocytic Leukemia (CLL), representing 30% of leukemia, remains the most common hematologic malignancy in the western world. It is diagnosed by peripheral blood immunophenotyping with co-expression of biomarkers, such as CD5, CD19, CD20 and CD23, on B cell surface and with more than 5,000 B lymphocytes in one μl. The first-line treatment for CLL includes monotherapy or combined chemotherapy of alkylating agents (e.g. chlorambucil and cyclophosphamide), purine analogues (e.g. fludarabine and cladribine) and monoclonal antibody based immunotherapy (e.g. rituximab and alemtuzumab) CD20 directed therapeutic antibodies such as rituximab and ofatumumab are widely used in CLL therapy. However, there are still limitations of currently existing first-line treatments for CLL due to nonspecific cytotoxicity induced by drug or relapse caused by immunotherapy. Therefore, it is crucial to develop therapies that are efficiently and specifically targeting to the malignant B-CLL cells. Herein, we explore monoclonal antibody (mAb) and liposomal nanoparticles-based therapeutic strategies for CLL with the goal to achieve efficient and directed drug/gene delivery with minimal side effects.

Firstly, a relatively new antibody, anti-CD37, was combined with one of the two classical antibodies, anti-CD19 or anti-CD20 to form dual-ligand immunoliposome
(dILPs) delivery systems to show higher targeted delivery efficiency and more cytotoxicity to CLL cells compared to single-ligand immunoliposomes (sILPs). Among all combined dILPs, CD19/CD37 dILPs demonstrated the highest delivery efficiency to primary B cells from CLL patients. The improvement in specificity and efficiency made by dILPs revealed the advantages of combining mAbs with complementary features such as high expression level, unique specificity and fast internalization.

In Chapter 3, a novel anti-ROR1 mAb, 2A2-IgG based immunoliposome (ILPs) delivery system specifically targeting to B-CLL cells was developed and tested. Besides investigating dILPs and 2A2-IgG based ILPs without any drug, two small molecule drugs, FTY720 and OSU2S, with potent therapeutic efficacy but significant side effect due to off-target selection were formulated into liposomes to realize targeted delivery and used as model drugs to further evaluate the delivery systems. Drug loaded in liposomes with or without antibody targeting provided different pharmacokinetics profiles compared with free drug in vivo and showed improved targeted delivery, enhanced killing efficiency and reduced the off-target killing of T lymphocytes in vitro. Additionally, characterization of liposomal FTY720 and OSU-2S were also presented in Chapter 4 with physical-chemical characterization studies and pharmacokinetics evaluation.

In addition to drug delivery, mAb targeted liposomal nanoparticle systems can also deliver oligodeoxynucleotides to CLL cells, which was demonstrated with an in vivo study of anti-CD20 targeted lipoplex nanoparticle containing ODN G3139 in SCID mice engrafted with Raji cells.
Overall, this study explored liposomal nanoparticles-based therapies using inserted new mAb and dual mAb for both targeting and therapeutic functions. Our results proved their advantages of delivering drug or gene, providing evidence of potential new strategies for CLL treatment.
Dedication

This dissertation is dedicated to my loving family.
Acknowledgments

Eventually, it comes the moment to end my long journey as a Ph.D. student. Not along with the great helps from numerous people could I reach such achievement. So, first of all, I want to say a sincere “Thank You!” to all who has supported.

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the first person with a Ph.D. degree in my family, and such accomplishment should attribute to all my family members.

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1.1 Introduction

Cancer has long been an enigma to medical practitioners and researchers. It was not until the nineteenth century that advances in surgical technique and anesthesia offered surgery as a possible treatment for cancer. Since that time, researchers have pressed on in exploring novel frontiers for treatment. In the 1960s, the advent of radiation therapy allowed for localized tumor treatment. Another technique, bone marrow transplantation in leukemia, was also utilized for cancer therapy. However, neither surgery nor radiation or the combination of the two was efficient in controlling the metastatic cancers; thus, chemotherapy was applied to eliminate all malignant cells. Chemotherapy can be very effective, but its broad spectrum of activity ends up damaging normal cells as a consequence. Over the past few decades, characteristics and pathways of different cancer types have been fully identified and validated; however, the generation of new therapeutic strategies that are effective, yet benign towards normal cells remains a critical challenge. This type of treatment may be realized by targeting therapy that directly induces toxicity or specifically deliver drugs to the malignancies. One of the currently
available targeting therapy approaches involves identification and development of compounds that are specific to their molecular-level targets. For example, Gleevec (imitanib mesylate; Novartis) employs 2-phenylaminopyrimidine to competitively inhibit ATP binding to the Abl kinase and was the first selective tyrosine kinase inhibitor approved for cancer treatment in 2001. Similarly, Iressa (gefitinib) performs as a selective oral epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor for advanced non-small-cell lung cancer. Generation of therapeutic monoclonal antibodies also provides an alternative for targeted therapy. Within the past two decades, a number of therapeutic monoclonal antibodies have been approved for cancer treatment, including trastuzumab (Herceptin) targeted to HER-2 overexpression in breast cancer, rituximab (Rituxan) targeted to CD20 for low-grade B-cell Non-Hodgkin’s lymphoma treatment, and alemtuzumab (Campath) targeted to CD52 for chronic lymphocytic leukemia. In addition, selective targeting can be achieved by linking ligands with cytotoxic reagents and directing them towards cancer cells. These ligand-targeted therapies have been expected to improve the therapeutic efficacy over conventional anti-cancer drugs. Relative to conjugating or coupling a ligand to anticancer drug directly, targeted liposomal anticancer drugs are advantageous due to the high drug-to-ligand ratio as well as increased binding avidity. Therefore, ligand-targeted liposomal nanomedicine is rapidly emerging as leading modality for cancer therapy.

Liposomes are biocompatible drug carriers for both hydrophilic agents and hydrophobic compounds. Coating liposomes with polyethylene-glycol (PEG) helps prevent and inhibit uptake of liposomes by the reticuloendothelial system (RES), thus
significantly prolonging circulation time of liposomes in the blood stream\textsuperscript{11}. Increased tumor accumulation can also be achieved owing to the enhanced permeability and retention effect\textsuperscript{11-13}. The development of coupling procedures that allow the attachment of ligands, including antibodies or antibody fragments\textsuperscript{14,15}, growth factors, transferrin\textsuperscript{16}, and vitamin folate\textsuperscript{17,18} to the terminal end of PEG provides a practical method for targeted liposomal delivery. Such features make liposomes ideal and promising drug delivery systems for targeted anti-cancer drug delivery.

Unlike non-targeted liposomal therapy or antibodies immunotherapy, there are still no FDA approved targeted liposomal drugs. However, ligand-targeted liposomal anti-cancer drugs have showed improved survival rates in a variety of disease models\textsuperscript{19}. As the first immuno targeted liposomal drug formulation, the anticancer drug, doxorubicin, encapsulated in sterically stabilized liposomes (Doxil®) has been applied to various animal models of human diseases, such as anti-CD19 or anti-CD20 liposomal doxorubicin in murine models of human B lymphoma\textsuperscript{14,20}, anti-HER-2 targeted in murine models of human metastatic breast cancer\textsuperscript{15}, and anti-GD2-targeted\textsuperscript{21} or NGR-targeted in murine models of human neuroblastoma\textsuperscript{22}.

1.2 Liposome delivery systems

Liposomes are nano-sized particles (10-400 nm) composed of phospholipid bilayers self-assembling to form a vesicle in aqueous solution with hydrophilic heads pointing outward towards aqueous phases and hydrophobic tails pointing away from aqueous phases (Figure 1.1). Different therapeutic purposes can be achieved by incorporating
different functional lipids such as PEGylated (stealth), cationic, pH-sensitive, or derivatized targeting agents \(^{23-27}\).

1.2.1 Liposomes as drug delivery vectors

1.2.1.1 Liposome composition

Characteristics of liposomes, including the mean diameter, polydispersity index, zeta potential, loading capacity, drug release, antitumor activity, and intracellular uptake of encapsulated drug are influenced by liposome lipid composition\(^{28}\). In Figure 1.2, several commonly used lipids and components are listed. DOTAP and DC-Chol are widely used because of their cationic nature, while Egg-PC and Cholesterol are often used as helper lipids. PEG-DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]) is the PEGylated component that helps prevent aggregation of liposomal particles and protect liposomes from recognition by the immune system\(^{29}\).

Phospholipids are the most conventional groups of amphiphilic surfactant molecules and are the major component of the biological membrane. The most common natural phospholipid is the phosphatidylcholine (PC) also known as lecithin.

Sterols are another important group of amphiphilic components which aid incorporation of lipid molecules to liposome structures, thus enhancing vesicular stability of liposomal nanoparticles. One of the most applied molecules used to increase liposome stability is cholesterol. Cholesterol, a modulator of lipid fluidity, has been used in many formulations to stabilize the systems against the formation of aggregates by repulsive steric or electrostatic effects\(^{30}\).
1.2.1.2 Preparation methods

Features of liposomes are also influenced by preparation methods\textsuperscript{28}. Various liposome preparation techniques have been developed on laboratory or industrial scales and have been reported in many scientific papers and review articles\textsuperscript{31,32}. Generally, the preparation methodology can be divided into three categories: (1) mechanical methods, (2) organic solvent replacement methods, and (3) size transformation or fusion methods.

The sonication method is considered the most widely used mechanical method for the preparation of liposomal vesicles. They include the probe sonication for small volumes at high energy and bath sonications at lower energy for larger volumes\textsuperscript{33}. Another mechanical method, microfluidization or homogenization method is a reproducible process for large scale manufacture of liposomes with good aqueous phase encapsulation\textsuperscript{31}. Unilamellar liposomes can also be obtained through extrusion under moderate or high pressures (250-1000 psi) at temperatures higher than the lipid transition temperature (\(T_m\))\textsuperscript{34}.

Historically, the reverse-phase evaporation method is a breakthrough in liposome technology. After removing the solvent from the lipid mixture under pressure by a rotary evaporator, lipids are re-dissolved in the organic phase in which the reverse phase vesicles will form. Then the emulsion is obtained and the solvent is removed by evaporation to a semisolid gel resulting in reverse phase evaporation vesicles. This method is commonly used to encapsulate large macromolecules with high efficiency\textsuperscript{32}. The other widely used organic solvent removal method is the ethanol injection method. The lipid is injected rapidly through a fine needle into an excess of saline or other
aqueous medium, and since ethanol can easily pass through the lipid bilayers, it can be easily diluted and removed by dialysis or diafiltration. This method does not apply harsh mechanical forces to create liposomes but instead relies on lipids to self-assemble, making it ideal for encapsulating water-soluble biomolecules, such as genes or proteins\textsuperscript{35}.

The freeze-dried rehydration method is also pretty commonly used in the synthesis of liposomes. It is based on the organic removal thin film method with the resulting suspension of reagents and the liposomal film subjected to several cycles of freezing and thawing. By using such freeze-dried rehydration method, a high encapsulation ratio can be achieved due to the close contact between lipid bilayers and reagents to be encapsulated. But such fusion is strongly restrained by the ionic strength of medium selection and the phospholipid concentration.

1.2.1.3 Liposomes as nano-particulate drug carriers

As therapeutic nanoparticles, liposomes can prolong the half-life of drugs in systemic circulation by reducing immunogenicity (\textbf{Figure 1.3B}) and deliver drugs in an accumulated and targeted manner with reduced systemic side effects (\textbf{Figure 1.3C and D}). Moreover, they can also enhance the solubility of poorly-soluble drugs in water (\textbf{Figure 1.3A}), release drug at a protracted rate so as to reduce the administration frequency, and realize simultaneous multi-drug delivery for combination therapy with synergistic effects (\textbf{Figure 1.3E}). Additionally, liposomal nanoparticles lack substrates for ATP-binding cassette proteins, resulting in minimized drug-resistance\textsuperscript{36}.

Due to the specific characteristics of liposomes, therapeutic nanoparticles have shown greater anticancer efficacy and lower toxicity than their corresponding free drug
countercparts. Up to date, more than twenty active reagents have been encapsulated into liposome formulations and have entered clinical applications or trials (Table 1.1). Among these liposomal drugs, Marqibo®, which consists of vincristine sulfate, has been approved by the FDA in 2012 to treat rare leukemia such as chromosome (Ph) negative acute lymphoblastic leukemia (ALL) and Non-Hodgkin’s lymphoma, and is under a phase III study in Philadelphia. The potent vinca alkaloid anti-mitotic is encapsulated in the aqueous core of sphingomyelin-based liposomes and has been developed to facilitate high-concentration, targeted drug delivery and continuous drug release kinetics \textit{in vivo}^{37}. Another example is the liposomal mitoxantrone, LEM-ETU, which is synthesized in multi-lamellar liposomes after charge interactions with cardiolipin. It has been under investigation of a phase I clinical trial for leukemia treatment\cite{38}. In 2005, PEGylated liposomal doxorubicin was combined with Rituximab, cyclophosphamide, vincristine and prednisone (DR-COP) for aggressive B-cell Non-Hodgkin’s lymphoma in a phase II trial\cite{39}. However, elucidation of the mechanism behind liposomal drug delivery has not been done because of several major challenges such as \textit{in vivo} drug release and intracellular trafficking. Further studies are necessary to better understand the mechanism of anticancer drug delivery to targeted tumor tissues and cells. It is also important to comprehensively analyze the process of drug release from liposomes. The relationship between the biodistribution of liposomal drugs and their toxicity in organs such as liver is also worthy of deeper study\cite{36}. 

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1.2.1.4 Liposomal drug loading

Theoretically, hydrophobic drugs can partition into the lipid hydrocarbon region and hydrophilic drugs can be trapped in the interior aqueous compartment. There are mainly two different ways to encapsulate bioactive compounds into liposomes: (1) passive encapsulation and (2) active loading. Many factors, such as encapsulation efficiency, lipid-to-drug ratio, drug retention, liposome and drug stability, as well as ease of preparation and scale-up, are greatly influenced by the encapsulation protocol\textsuperscript{35}.

Passive entrapment processes include all techniques discussed previously in Section 1.2.1.2. Although passive entrapment is a viable strategy for encapsulating hydrophilic drugs, trapping efficiencies are usually less than 50% if organic solvents or detergents are not used. Hydrophobic drugs can usually be co-solubilized in the organic solvent with lipids and subsequently dispersed in aqueous buffer\textsuperscript{40}.

Active loading includes techniques that involve drug loading in preformed liposomes. Such processes exhibit a membrane potential or transmembrane pH gradient. For instance, the pH-gradient loading method includes two steps, the generation of the pH gradient with low intra-liposomal pH and the subsequent loading of the drug. Many anticancer or antibiotic drugs are weak bases so they can be accumulated into liposomes by transmembrane pH gradient\textsuperscript{41}. Liposomal uptake in response to potential or pH difference has been demonstrated for several lipophilic amino-containing drugs in addition to doxorubicin\textsuperscript{42}, including vinblastine\textsuperscript{43}, dibucaïne\textsuperscript{44,45}, and dopamine\textsuperscript{46}. Active loading is independent of lipid composition and is considered to be the optimal choice for
formulations with high drug-to-lipid ratios, encapsulation efficiency, and improved retention property.

### 1.2.2 Liposomes as gene delivery vectors

Recombinant DNA technologies and studies of gene function and gene therapy all depend on the successful delivery of nucleic acid into cells \textit{in vitro} and \textit{in vivo}\textsuperscript{32}. Several barriers including limited chemical stability in serum, rapid blood clearance and poor cellular uptake of free gene reagents \textit{in vivo} have restricted the extensive application of gene based therapy\textsuperscript{25,47,48}. Cationic liposomes are the most accessible and appropriate transfection vector among various synthetic carriers currently available for gene therapy\textsuperscript{23,49}. Usually, cationic lipids have a positively charged head-group that interacts with negatively charged genes so that cationic liposomes can protect genes from enzymatic degradation and provide reduced renal clearance.

Additionally, most liposomal formulations used for gene delivery consist of neutral helper lipids to improve transfection efficiencies\textsuperscript{50-52}. By incorporating DC-Chol and other cholesterol derivatives into the lipoplex assembly, increased \textit{in vivo} transfection efficiency as well as reduced cytotoxicity has been realized\textsuperscript{53,54}. In general, gene delivery by anionic lipids is not very efficient compared to cationic lipids, however, anionic liposomes have been explored as potential gene delivery vehicles as they are more stable upon storage and exhibit less cytotoxicity\textsuperscript{55,56}. Various divalent cations, such as Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, Mn\textsuperscript{2+} and Ba\textsuperscript{2+}, have been incorporated into systems to condense nucleic acids...
before envelopment by anionic lipids. Ca\(^{2+}\) has yielded the highest transfection efficiency due to its enhanced DNA binding affinity\(^{57,58}\).

### 1.2.3 Rational design of targeting liposomes

The main purpose of using a targeted liposome delivery system is to help the active drug or gene within liposomes arrive at the selective location in the disease site. In contrast to the method of directly conjugating a targeting ligand with the therapeutic agent, the use of liposomes for targeted delivery has several advantages. Firstly, there might be limited functional groups on a drug molecule available for direct ligand conjugation. Secondly, the biological activity of the drug molecule can be compromised by direct conjugation of the ligand so that the additional construction of a cleavable linker to enable drug release following endocytosis is required. Moreover, multiple drug molecules can be delivered upon internalization of a single liposome, whereas only single type of drug molecules is delivered by the uptake of directly conjugated agents\(^{35}\). Generally speaking, targeting liposomes to tumor tissues can be broadly categorized into two major mechanisms: passive and active.

#### 1.2.3.1 Passive targeting

Non-targeted nanoparticle formulations demonstrate selective tumor targeting as a consequence of passive targeting; they do not interact with cancer cells directly and have limited tumor penetration. Such site-specific delivery is realized based on the physicochemical characteristics of liposomal nanoparticles and the clearance kinetics or biodistribution depend on factors like particle size, surface charge and other
physicochemical properties\textsuperscript{35,59,60}. In cases of solid tumors, unique hyper permeable vasculature and impaired lymphatic drainage make tumor tissues comparably permeable to macromolecules and nanoparticles, enabling the selective extravasation of long-circulating nanoparticles via the permeability and retention (EPR) effect even without any active targeting strategy\textsuperscript{61-63}. Usually, the sizes of long-circulating liposomes are less than 200 nm with most nanoparticles accumulating at tumor site while particles larger than 400 nm are easily uptaken by the reticuloendothelial system (RES) trapping effect\textsuperscript{35,64}. Alternatively, passive targeting utilizing macrophages by the RES effect or macrophage activating factors to regulate and increase the cytokine level, thus, inducing the activation of monocytes; such a mechanism is particularly promising for metastasized cancer treatment\textsuperscript{65,66}.

1.2.3.2 Active targeting

Active targeting refers to the directed movement of the lipid vesicles to the targeted organ, tissue or cells before the release of encapsulated bioactive agents. It can be achieved via modifications to the liposomal structure, such as using thermo-labile, pH-sensitive, peptide, or antibody coated lipids\textsuperscript{67}. The major strategy for modification of liposomes is by linking with targeting ligands on the liposome surface, such as with antibody or antibody fragment, folic acid and transferrin. Antibodies have been among the most commonly used ligands in such formulations\textsuperscript{68}. The linking or targeting of ligands to the surface of long-circulating nanoparticles can help liposomal nanoparticles specifically bind to cancer cells that express corresponding receptors or target molecules. Such formulations have been designed and widely produced to enhance the therapeutic
activity of the encapsulated anticancer drugs\textsuperscript{7,69,70}. The rationale for the specific binding of ligand-modified nanoparticles with receptors on cancer cells is based on: (1) the over-expressed or unique antigenic receptors on the surface of cancer cells relative to normal cells, (2) the specificity and high binding affinity of targeting ligands to receptors, and (3) the possibility of intracellular delivery by cell-mediated endocytosis via interactions between the ligand and the receptor. Receptor-mediated endocytosis provides a means for nanocarriers entering into the cells with the internalized nanoparticles ending up in small vesicles of endosomes which then undergo a rapid maturation to late endosomes, fusing with each other or lysosomes.\textsuperscript{71,72} Notably, many studies related to solid tumors revealed no significant improvement generated by receptor targeted liposomal nanoparticles compared to non-targeted ones, mainly due to the reticuloendothelial system (RES) effects and reaching tumor site via EPR effect. In such cases, the targeting ligands may not have much effect given the limited access to the receptors\textsuperscript{73,74}. Targets on the tumor endothelial cells, vascular compartment, or vasculature are the most applicable candidates for active targeting liposomes with relatively quicker binding of nanoparticles to the target sites.

\subsection*{1.3 Antibody-based therapy in cancer}

\subsubsection*{1.3.1 Antibody for cancer therapy}

Although monoclonal antibodies (mAbs) have been developed and widely used as a valuable therapeutic approach, the application of mAb in cancer treatment has not been
curative to cancer as a single reagent\textsuperscript{75}. mAbs approved by the FDA for oncologic use and their mechanisms of action are listed in Table 1.2 \textsuperscript{76}.

Chimeric or humanized antibodies can efficiently activate immune effectors by inducing antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity to destruct malignant cells. In solid tumors, mAbs also must overcome obstacles of the vascular endothelium to reach the target site and take effect\textsuperscript{77}. ADCC occurs when the Fab’ fragment of mAbs recognizes and binds to the targeted cells while the Fc fragment of the mAbs is engaged with Fc receptors on monocytes, macrophages, and natural killer cells, resulting in phagocytosis and/or antibody dependent cytotoxicity of the targeted tumor cell \textsuperscript{78,79}. CDC refers to the initiation of the complement system with the binding of mAbs, thus resulting in the lysis of targeted cells with holes formed by the membrane attack complex\textsuperscript{80}.

In addition to direct targeting to antigens on cancer cell, mAbs also target to secreted mediators of biological pathways. For example, the marketed bevacizumab (Avastin®) targets to vascular endothelial growth factor (VEGF) that serves as crucial tumor neovascularization factor\textsuperscript{81}. mAbs targeting to inhibitory antigens on T lymphocytes, killer cell Ig-like receptors (KIRs), or programmed cell death protein 1 receptor (PD-1) block the immunosuppressive functions, thus overcoming immune evasion by T cells or NK cells. \textsuperscript{82-84}

In cancer therapy, most mAbs available on the market are canonical, which are usually monospecific and full-length IgG molecules. But noncanonical mAbs, including antibody-drug conjugates (ADCs), bispecific antibodies, engineered antibodies and
antibody fragments and/or domains, are rapidly growing in the pipeline with alternative mechanisms of action to provide innovative medicines and clinical outcomes in the near future\(^8\). The well-validated antigens, such as epidermal growth factor receptor (EGFR), CD20 and HER2, are targets for a total of 18 mAbs in clinical study and 8 mAbs on the market. Other frequent targets for anticancer mAbs include CD19, CD22, CD38, CD70, GD2, PD-1, fibronectin, angiopoietin 2, etc.\(^8\). These anticancer mAbs have spanned the clinical application of a majority of cancers, including but not limited to breast cancer, colorectal cancer, prostate cancer, lymphoma and leukemia.

1.3.2 CLL and antibodies for CLL

CLL is the most common leukemia that is characterized by progressive accumulation of malignant B lymphocytes in the blood, bone marrow, and lymphatic tissues\(^8\). Although predominantly seen in adults over 70 years old, younger patients under the age of 50 years old also can be found\(^8\). Most CLL patients are asymptomatic at diagnosis with gradual proliferation and accumulation of small CD5+/CD19+/CD23+ lymphocytes\(^8\). These abnormal leukocytes are resistant to programmed cell death in the microenvironment. Due to the indolent nature of CLL, patients at earlier stage (Rai 0 or Binet A) are not treated, but monitored since the current treatment of CLL only shows improved outcome with patients at advanced stages\(^9\). The life expectancy of CLL patients varies due to the heterogeneous nature of the disease\(^9\).

The first-line treatment for CLL includes monotherapy or combined chemotherapy of alkylating agents (e.g. chlorambucil and cyclophosphamide), purine analogues (e.g.
fludarabine and cladribine), and monoclonal antibody based immunotherapy (e.g. rituximab and alemtuzumab). Combination therapies including FR (fludarabine, rituximab), FCR (fludarabine, cyclophosphamide, rituximab) and rarely R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone) combination immunochemotherapy are among the frontline therapy in many B cell malignancies. However, the majority of patients will relapse after treatment and some will even become refractory to the initial therapy. Additionally, though several novel drugs have shown promising therapeutic effects in vitro, the numerous undesired side effects in vivo caused by nonspecific cytotoxicity has limited their application in the clinics. Therefore, it is crucial to develop therapies that efficiently and specifically target to malignant B-CLL cells. Some new therapies include protein kinase C inhibitor UCN-01, cyclin-dependent kinase inhibitor flavopiridol, Bruton tyrosine kinase (BTK) inhibitor ibrutinib (formerly PCI-32765), and phosphatidylinositol 3-kinase δ (PI3K) inhibitor GS-1101 (formerly CAL101), all of which are currently under investigation to treat CLL with potential breakthrough.

In the past decade, monoclonal antibodies have been performing a major role in CLL therapy. Immunotherapy with rituximab has been the major breakthrough in the treatment of B cell malignancies. Rituximab is a chimeric anti-CD20 mAb that is most commonly used in treating CLL, but has shown limited activity as a monotherapy. While patients have variable response to therapy, most of them will eventually develop resistance and relapse. Ofatumumab is a fully humanized mAb designed to target the novel epitope on the CD20 molecule on CLL cells and it has shown superior antitumor effects with
enhanced CDC effects in rituximab resistant cells\textsuperscript{104-106}. Alemtuzumab is a humanized mAb targeting to CD52 antigen with antiproliferative effects mainly via ADCC and CDC\textsuperscript{107}. It is also approved for the treatment of CLL. Alemtuzumab is effective in CLL patients, but the widespread expression of CD52 on many immune cells, including T lymphocytes, NK cells, neutrophils, macrophages and monocytes, makes therapy with alemtuzumab highly immunosuppressive\textsuperscript{107-109}.

In addition to the currently available mAbs in clinic therapy of CLL, several novel mAbs have also been under investigation in clinic with promising applications. XmAb5574, as a novel engineered anti-CD19 mAb, has demonstrated potent ADCC in CLL and is currently being evaluated in a phase I clinical trial\textsuperscript{110}. TRU-016 is a novel small compound that targets tetraspanin family CD37 and has been investigated in relapsed CLL patients with improved therapy by augmenting NK cells and inducing Fc-mediated cellular cytotoxicity\textsuperscript{111}. Dacetuzumab, a humanized mAb against CD40 was also reported in a phase I study in relapsed CLL with 41% of patients showing stable disease\textsuperscript{112}. The anti-leukemic activity of a primatized mAb, lumiliximab, which targets CD23 and mediates ADCC and CDC in relapsed CLL has also been reported\textsuperscript{113}. New monoclonal antibodies in CLL clinical development are listed in Table 1.3\textsuperscript{114}.

1.3.3 Combination of monoclonal antibodies for cancer therapy

Although antitumor activity of mAbs has been investigated and shown in clinic, it is unlikely that those mAbs can cure cancer as a single drug. For instance, the anti-EGFR mAb, cetuximab, failed to inhibit EGFR phosphorylation in EGFR-mutant cells, but
mutations in EGFR are an important factor in cancer. Similarly, the mutation of the complement factor C1q resulted in reduced or abolished efficacy of rituximab in activating CDC. Thus, the use of additional mAbs and the combination of multiple mAbs targeting to antigens involved in different signaling pathways could be important. The alternative immunostimulatory activities may allow for synergic effects. Antibodies are able to target distinct, non-overlapping mechanisms which are helpful in avoiding the deficiency of treatment caused by mutation against one antibody. Combination of mAbs has demonstrated equal or greater effect compared to single cross-linked mAb treatment in all cell lines tested and cross-linking with secondary antibodies is believed to function by generating stronger and/or more sustained signals compared to simple ligation of surface antigens with primary mAbs to the antigens alone.

Galiximab (an anti-CD80 mAb) can be safely combined with rituximab for relapse or refractory of follicular lymphoma according to a phase I/II study, and has generated momentum as a potential approach to avoid or delay chemotherapy and its associated toxic effects, with the possibility of integrating with other lymphoma therapies. In the case of breast cancer, pertuzumab blocks the HER2 receptor while trastuzumab prevents HER2 dimerization or interaction, and the combination of these two mAbs has demonstrated significantly higher percentage of elimination of tumor with chemotherapy prior to surgery. The combination of anti-CD20 and anti-CD47 mAbs boosting the efficacy in non-Hodgkin’s lymphoma (NHL) indicates a promising treatment to elicit fewer side effects than rituximab plus chemotherapy for blood cancers. A novel class of bispecific antibody in phase II trials, blinatumomab, has been developed by
incorporating two scFvs that bind CD3 and CD19. The anti-CD3 fragment triggers T cells while the other anti-CD19 fragment binds to CD19+ cells in close proximity, resulting in T cell activation and subsequent depletion of the target cell over the simultaneous binding\textsuperscript{75}.

### 1.4 Development of immunoliposomes (ILPs) for cancer therapy

#### 1.4.1 Overview

ILPs are targeting liposomes which feature antibodies on the liposome surface. This provides a modality for high affinity interactions with target antigens on the cell surface (Figure 1.4)\textsuperscript{126-129}. The application of ILPs as drug vehicles combines advantages of both liposome delivery systems and antibody specificity, with potentially additive or synergistic effects between the signaling antibodies and the encapsulated drug\textsuperscript{130,131}. In some cases, empty antibodies incorporated into ILPs have been shown to mediate potent cytotoxicity \textit{in vitro} compared to that caused by mAb cross-linking\textsuperscript{132}.

Chronic cancer therapy usually requires large or multiple doses of antibodies for extended periods of time. The production of antibody fragments, such as Fab’, Fv, and scFv, that can be readily expressed in microbial systems is a large-scale and cost-efficient solution. ILPs coupling the antibody fragments or whole mAb with various techniques further improved the \textit{in vivo} circulation and biodistribution for the liposome or antibody delivery system\textsuperscript{133}.  

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The advantages of ILPs have been demonstrated in a number of experimental cancer studies. The treatment of the PEG-modified immunoliposomal doxorubicin conjugated with cancer-reactive human mAbs, GAH, with a positive ratio over 90% against stomach cancer, has strongly increased efficacy to gastrointestinal cancers\textsuperscript{130}. The Fab’ fragments of anti-disialoganglioside (GD\textsubscript{2}) immunoliposomal doxorubicin presented increased selectivity and efficacy in inhibiting neuroblastoma cells’ proliferation compared to free drug and non-targeted liposomal drug\textsuperscript{21}. Anticancer drugs formulated into liposomes, with either anti-CD19 IgG2a or Fab’ fragments, or anti-CD52 mAb conjugated for targeting, all have exhibited not only higher association with B-cell malignancies, but also induced additional cytotoxicity\textsuperscript{19,134,135}. Anti-HER2 ILPs also enhanced targeted delivery of doxorubicin to HER2 breast cancer and displayed a prolonged circulating distribution of doxorubicin\textsuperscript{15,136}. Currently there is a scFv-targeted HER-ILP doxorubicin under phase I study (MM302)\textsuperscript{85}. A summary of the concurrent investigated list of mAb-based targeted delivery for cancer therapy is listed in Table 1.4.

ILP-based targeted delivery of antitumor drugs has been considered as a promising system for rational improvement of cancer therapy. Ideally, the ILP-anticancer drug delivery system should be applicable to all encapsulated drug molecules, so that successful delivery can become therapeutically active upon binding of ILPs to their target epitopes \textit{in vivo}\textsuperscript{137}. However, the application of ILPs in the clinic is still limited due to manufacturing and quality control issues\textsuperscript{7}. Major pharmaceutical achievements in the development of ILP formulations, such as the stability of liposomes, the uniformity of
lipid nanoparticles, and the elimination of ligand conjugation variance, are still a necessity to make ILP-formulated chemotherapies pharmaceutically viable.

1.4.2 Choice of ligands and coupling methods for ILPs

To achieve optimized delivery efficacy of ILPs, several strategies should be considered. For antibodies, the targets must be reactive with the target cell and not cross-react greatly with healthy tissue so as to ensure specificity\textsuperscript{131}. On the other hand, the internalizing epitope is necessary for ILPs to increase the cellular uptake of the drug competing with diffusion of drug at the cell surface\textsuperscript{20,138}.

Various types of coupling methods have been developed to attach antibodies to phospholipids while maintaining their biological activity, including covalent coupling and thiolated antibody linkages to maleimided phosphatidylethanolamines by disulfide bonds\textsuperscript{139}. The major drawback of such direct coupling of antibodies to the liposome surface is the possibility of the strong shielding effect of PEG chains that prevent the interaction between the antibody and the target antigen, depending on the PEG chain length (Figure 1.4B)\textsuperscript{139}. Other commonly used coupling methods to immobilize antibody onto the terminus of the PEGylated liposome (Figure 1.4C) include biotin-avidin-biotin bridge, functionalized PEG derivative linkers, employment of liposomal carboxyl group, etc.\textsuperscript{140-142}. The biotinylated lipids presenting in the lipid vesicle membrane can absorb avidin under diffusion controlled conditions, and biotinylated molecules can specifically bind to absorbed avidin to realize the conjugation\textsuperscript{143}. Commercially available PEG derivatives with functional groups such as aldehyde and sulfhydryl groups linked to PEG
have minimized steps for conjugation. Similarly, the lipid moiety bearing an active carboxyl group can bind directly to amino groups on proteins and other molecules\textsuperscript{144}. Yet these methods lead to a random orientation of the antibody, and thus will reduce its efficiency as the active antigen binding site might be blocked. Therefore, the efficacy of conjugated antibodies will not be comparable with free antibodies at a similar concentration\textsuperscript{19,140-142,145}. One of the solutions is to use IgG Fc-binding proteins (protein A or protein G) conjugated liposomes for directly controlled mAb coupling with the Fab’ facing the targeted cells and tissue\textsuperscript{141,142,146}. Despite the stability of whole mAbs during the prolonged storage, the existence of the Fc domain may initiate non-specific binding to normal tissues through Fc receptors, particularly on macrophages \textit{in vivo}\textsuperscript{7,139}. To overcome immunogenicity and also to maximize the binding efficiency, F(ab’)\textsubscript{2}, Fab’ and scFv fragments lacking the Fc domain but with complementary activating regions are useful for ILP immobilization. F(ab’)\textsubscript{2} fragments can be still quite stable with two binding regions joined by disulphide bonds; and the disulphide bonds can be laminated under certain reducing conditions, yielding two Fab’ fragments each with a thiol (-SH) group. This is practical for coupling the fragments to ILPs\textsuperscript{133,139,140}.

In this study, immunoliposomes were prepared by a post-insertion method. mAbs were first thiolated in the lysine group by Traut’s reagent (2-iminothiolane) followed by reaction with micelles prepared from maleimide PEG-DSPE to form Ab-coated micelles. A post-insertion method was adopted to incorporate the Ab-coated micelles to the previously prepared non-targeted liposomes forming Ab-targeted liposomes for targeted delivery (\textbf{Figure 1.5}).
As shown in Figure 1.6, the insertion of mAb does not alter the morphology of liposome nanoparticles, and the mAb creates a layer less than 5 nm on the surface of liposomes that cannot be distinguished by cryo-TEM at the current resolution. Alternative means of liposomal ligand conjugation methods include non-covalent methods without chemical reactions, such as avidin-biotin interaction, and lipid-tagged antibodies for direct incorporation\textsuperscript{68}.

Some other naturally occurring ligands to cell-surface receptors such as vitamins, hormones, and growth factors may also be used as immuno-tolerant homing devices and they are potentially advantageous over antibodies with less immunogenicity and lower preparation cost\textsuperscript{137}.

1.4.3 Immunoliposomes for B cell malignancies

Hematological malignancies (e.g. lymphomas, multiple myeloma, or leukemia) are attractive candidates for immunoliposome-based therapy, as circulating tumor cells are easily accessible targets for i.v. administered ILPs\textsuperscript{137}. Internalization is necessary to increase the efficiency of drug delivery to malignant cells within the vasculature.

1.4.3.1 CD19 based ILPs

CD19 is known as B-lymphocyte antigen and is present on human B lymphocytes at all stages of maturation, but is lost on terminally differentiated plasma cells. Recently, it has been identified as a major BCR-independent regulator of MYC-driven B cell neoplasm\textsuperscript{147}. CD19-ILPs with various drugs are the most representative, widely and frequently targeted drug delivery systems investigated in several studies, especially those
involving in B malignancies\textsuperscript{148-152}. CD19 is emerging as a promising target for B cell lymphoma and leukemia as it is expressed more commonly and in greater amounts than CD20 with efficient internalization on all B cells, but not found in other healthy tissues\textsuperscript{75}. Though conventional antibodies targeting CD19 exhibited limited activity in preclinical studies, several novel CD19 antibodies generated in commercial clinical programs have shown promising results in clinical trials. Among the candidates, blinatumomab under phase 2 clinical trials in relapsed/refractory acute lymphocytic leukemia (ALL) and non-Hodgkin lymphoma (NHL) is expected to be the first CD19 agent on the market in 2014. Other mAbs targeted to CD19 include the Fc-engineered humanized Ab MEDI-551, XmAb-5574 and MDX-1342, and all are in either phase I or II clinical trials for relapsed/refractory CLL\textsuperscript{75}. CD19-ILP doxorubicin (DXR) showed improved selectivity in B lymphoma (Namalwa cells) and prolonged survival time of Raji-bearing mice, relative to untargeted DXR or free DXR, by attaching different fragments of anti-CD19 mAb (HD37) onto B cells\textsuperscript{150}. Other CD19-ILP DXR studies, mainly based on B-cell lymphoma, were also reported and arrived at the same conclusions both \textit{in vitro} and \textit{in vivo}\textsuperscript{149,152}. The inhibitory effect of CD19-ILP sodium butyrate revealed that the mechanism was altered by CD19-ILPs (mAb J3-119) by blocking the transcriptional and translational machinery in cancer cells\textsuperscript{151}. It has also been demonstrated that CD19-ILP imatinib induced specific and efficient death of Philadelphia chromosome-positive (Ph\textsuperscript{+}) ALL cells with fewer side effects than free imatinib\textsuperscript{148}. 

\textit{\textsuperscript{75}CD19 is emerging as a promising target for B cell lymphoma and leukemia as it is expressed more commonly and in greater amounts than CD20 with efficient internalization on all B cells, but not found in other healthy tissues. Though conventional antibodies targeting CD19 exhibited limited activity in preclinical studies, several novel CD19 antibodies generated in commercial clinical programs have shown promising results in clinical trials. Among the candidates, blinatumomab under phase 2 clinical trials in relapsed/refractory acute lymphocytic leukemia (ALL) and non-Hodgkin lymphoma (NHL) is expected to be the first CD19 agent on the market in 2014. Other mAbs targeted to CD19 include the Fc-engineered humanized Ab MEDI-551, XmAb-5574 and MDX-1342, and all are in either phase I or II clinical trials for relapsed/refractory CLL. CD19-ILP doxorubicin (DXR) showed improved selectivity in B lymphoma (Namalwa cells) and prolonged survival time of Raji-bearing mice, relative to untargeted DXR or free DXR, by attaching different fragments of anti-CD19 mAb (HD37) onto B cells. Other CD19-ILP DXR studies, mainly based on B-cell lymphoma, were also reported and arrived at the same conclusions both \textit{in vitro} and \textit{in vivo}. The inhibitory effect of CD19-ILP sodium butyrate revealed that the mechanism was altered by CD19-ILPs (mAb J3-119) by blocking the transcriptional and translational machinery in cancer cells. It has also been demonstrated that CD19-ILP imatinib induced specific and efficient death of Philadelphia chromosome-positive (Ph\textsuperscript{+}) ALL cells with fewer side effects than free imatinib.}
1.4.3.2 CD20 based ILPs

CD20 is a 297-amino acid transmembrane phosphoprotein of the calcium channel with expression on the surface of all mature B cells\textsuperscript{153-155}. mAbs targeting to CD20 including rituximab, veltuzumab, and ofatumumab are all approved or under clinical trials for the treatment of CLL, with rituximab the most commonly used for immunotherapy\textsuperscript{156}. Rituximab works by binding to CD20 resulting in cytotoxicity with a not entirely known mechanism. However, several proposed mechanisms reveal that rituximab may act via (1) induction of antibody-dependent cellular cytotoxicity (ADCC), (2) elicitation of lymphoma cell death through complement-dependent cytolysis (CDC), and (3) direct induction of apoptosis\textsuperscript{95,96}. In concurrent first-line treatment of CLL, rituximab has been used for chemo-immunotherapy combined with fludarabine by sensitizing resistant tumor cells to the agents\textsuperscript{90,92,155,157,158}.

CD20-ILPs (rituximab) were synthesized in our group with the formulation of DC-Chol:Egg-PC:PEG-DSPE at a molar ratio of 28/70/2 by the ethanol dialysis method. The ILPs are 50–60 nm in mean diameter and slightly positively charged. These nanoparticles showed preferential uptake by B-CLL cells in PBMC cells, as well as by Raji cells from the mixture of Raji and Jurkat cells, with the capacity of B-cell specific targeting correlated well with the CD20 expression level.

1.4.3.3 CD37 based ILPs

CD37 antigen is a glycoprotein with a molecular weight of 40-52 kDa and belongs to the tetraspanin superfamily with four transmembrane domains\textsuperscript{159}. It is strongly expressed on mature B lymphocytes but not on plasma cells\textsuperscript{160}. The engineered protein,
CD37-small modular immunopharmaceutical (CD37-SMIP), has a single chain variable region with specificity for human CD37 and effector domains from human IgG1 to provide relative selectivity for B cells, but not for resting T cells. This mediates potent caspase independent apoptosis and ADCC, which is directly proportional to the CD37 expression level\textsuperscript{161}. Based on the apoptosis achieved by cross-linking of CD37 molecules, CD37-SMIP-coated liposomes (CD37-ILPs) were synthesized in our lab and proven to activate apoptosis-related signaling \textit{in vitro} by inducing CD37 clustering on malignant B cells. This provides the evidence that CD37-targeted ILPs can potentially be used for targeted drug delivery for chemotherapeutic agents and RNA-based therapies.

1.4.3.4 CD74 based ILPs

CD74 is the protein known as HLA class II histocompatibility antigen gamma chain encoded by the CD74 gene\textsuperscript{162}. It is expressed on the surface of melanoma, dendritic cells, T-cell, and B-cell lymphoma at a restricted level, but at a higher degree on B malignancies\textsuperscript{163,164}. It has been clarified that CD74 regulates B-cell differentiation by inducing a signaling cascade leading to activation of NF-\textit{\textkappa}B and augmented expression of BCL-X\textsubscript{L}, thus promoting survival and growth of the cells\textsuperscript{165,166}. Unlike rituximab, anti-CD74 mAbs is rapidly internalized, presumably due to the function of CD74 in antigen trafficking, and this process is also preserved with the CD74-lipid drug-carrier complexes\textsuperscript{118,132,167}. As a monotherapy reagent, cross-linked anti-CD74 mAb, such as milatuzumab, showed greatly improved antiproliferative effects in cells expressing CD74\textsuperscript{132}. Moreover, it has been demonstrated to mediate direct cytotoxicity in CLL cells by aggregating CD74 on the cell surface\textsuperscript{120,132,167}. With milatuzumab, the anti-CD74 mAb
is currently under clinical evaluation for CLL treatment, CD74-ILPs were created in our lab with the formulation of Egg-PC:Chol:PEG-DSPE at a molar ratio of 64/35/1. For specificity evaluation, CD74-ILPs labeled with calcein showed specific binding to CD74 positive Raji cells, but no binding to CD74 negative Jurkat cells, with IgG-ILPs as the isotype control. In the Raji xenograft model, CD74-ILPs, with or without dexamethasone payload, significantly prolonged the survival of mice (Figure 1.7). Although the mechanism is unknown, these results showed that CD74-ILPs are a viable candidate for treating CLL.

1.4.3.5 CD52-based ILPs

CD52 is a 21-kDa to 28-kDa glycosyl-phosphatidylinositol anchored membrane glycoprotein highly expressed on all normal B and T lymphocytes, monocytes, macrophages, and natural killer cells. It has been found to be strongly expressed in all leukemia cells including CLL. The anti-CD52 mAb, alemtuzumab (Campath 1-H) has been approved by the FDA for CLL treatment after the demonstration of its efficacy in patients with fludarabine-refractory CLL. It remains the only available agent approved by FDA in patients with del (17p) lacking function of the p53 gene. As to ILPs, alemtuzumab has been conjugated to liposomal oligodeoxyribonucleotide (ODN) forming CD52-ILP-ODN, showing targeted delivery to CD52\textsuperscript{high} Raji cells and primary B cells.
1.5 **ROR1 as a target for CLL treatment**

The receptor tyrosine kinases (RTKs) and their ligands are attractive molecular targets for cancer therapy as they are critical in the development and progression of many cancers and play a key role in regulating normal cellular processes, including signaling, cell-cell interaction, differentiation, proliferation, and survival, etc.\(^\text{169}\). The receptor-tyrosine-kinase-like orphan receptor 1 (ROR1) belongs to the ROR family of RTKs and has currently been identified as a restricted marker for CLL, as over 95\% of CLL cells express ROR1 on the surface while other normal mature healthy B lymphocytes do not\(^\text{170-172}\).

The ROR1 gene has a coding region of 2814 bp, with a predicted 937-amino-acid sequence. The molecular weight of the protein varies between 100 and 130 kDa depending on the glycosylation pattern. ROR1 is composed of an extracellular part including an Ig-like domain, a cysteine-rich domain (CRD) and a kringle (KNG) domain, as well as an intracellular tyrosine kinase and a proline-rich domain\(^\text{173}\).

ROR1 acts as the Wnt receptor and undergoes complex post-translational modifications by glycosylation and mono-ubiquitination\(^\text{173}\). Silencing of ROR1 with specific siRNA induces apoptosis of CLL cells\(^\text{174}\). The ROR1 protein level also increases following IL-6-induced phosphorylation of signal transducer and activator of transcription 3 (STAT-3)\(^\text{175}\). Several monoclonal antibodies targeting to ROR1 have been developed and investigated, owning to the promising specificity to treat CLL\(^\text{176,177}\). Compared to non-progressive patients, the ROR1 expression is significantly more frequently observed in progressive patients both at the gene expression and protein levels\(^\text{176}\). Though several
monoclonal antibodies against ROR1 have been shown to induce apoptosis of CLL cells, ROR1 may serve as an ideal target for armed rather than naked monoclonal antibodies due to its relatively low cell surface density\textsuperscript{177}.

Daneshmanesh et al.\textsuperscript{176} identified five anti-ROR1 mAbs against RTKs and all mAbs induced apoptosis in the absence of complement or added effector cells in CLL cells but not in normal B cells. The most effective mAbs were against CRD and KNG, with superior killing efficiency compared to rituximab. Another group\textsuperscript{177} produced mAbs as the first generation of chimeric rabbit/human Fab and IgG1 that bind to ROR1. Among the panel of mAbs they tested, all mAbs demonstrated high affinity and specificity for epitopes of ROR1 but with no induction of apoptosis or mediated CDC and a low ADCC activity; thus it was suggested that those mAbs would be more suitable as armed mAbs for conjugates rather than naked mAbs. Baskar et al.\textsuperscript{(Baskar, Wiestner et al. 2012)} also demonstrated that a ROR1-immunotoxin can induce apoptosis selectively to ROR1 positive cells in MCL cell lines\textit{in vitro}, thus providing evidence of targeted delivery via anti-ROR1 antibodies for promising specific treatment of malignancies including CLL and MCL with minimized immunogenicity and reduced side effects caused by off-targeting.
1.6 FTY720 and its derivative OSU-2S

1.6.1 FTY720

FTY720 (Fingolimod) is a synthetic compound based on modification of the natural immunosuppressant, myriocin (ISP-1)\textsuperscript{178, 179}. It was approved by FDA in 2010 as the first orally bioavailable drug for patients with multiple sclerosis (MS) to reduce relapse and delay disease progression to disability\textsuperscript{180}.

In addition to MS, FTY720 showed promising \textit{in vitro} and \textit{in vivo} preclinical activity in leukemia and lymphoma disease models, including chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), NK-cell leukemia, and mantle cell lymphoma (MCL)\textsuperscript{181, 182, 183, 184, 185}. FTY720 has also been tested in phase III clinical trials as an immunosuppressant for kidney transplantation\textsuperscript{186, 187} and is a candidate drug for therapy of heart failure and arrhythmias due to its cardio-protective effects\textsuperscript{188,189}. Mechanistic studies have shown that FTY720 functions as an immunomodulator by affecting lymphocyte production, trafficking, infiltration, and apoptosis\textsuperscript{190}. Several studies have provided evidence that FTY720 induces T cell apoptosis both \textit{in vitro} and \textit{in vivo}\textsuperscript{185,191-194}, which is unfavorable for treatment of B-cell malignancies. Previous \textit{in vitro} studies indicated that FTY720 induces down-modulation of Mcl-1, but not Bcl-2 in CLL cells, and its toxicity in CLL cells is dependent on activation of PP2a, but not S1P receptor. This alternative mechanism of FTY720-induced apoptosis in CLL differs from the mechanism of FTY720 in MS\textsuperscript{195,196}. 

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FTY720 is sparingly soluble in aqueous buffer. Maximum solubility of 0.2 mg/ml in 1:1 ethanol/PBS (pH 7.2) is achieved by dissolving the drug first in ethanol and then diluting with aqueous buffer. Notably, free FTY720 is not stable in aqueous buffer/solution and requires daily fresh preparation\textsuperscript{193}. The current available capsule formulation has high oral bioavailability of ~90\% \textsuperscript{197} and enables daily administration with dose proportional pharmacokinetics to achieve active steady-state levels in MS patients at 0.5 mg daily. However, to be clinically effective in B-cell malignancies, higher steady-state levels will need to be achieved as lymphopenia in humans is treated with doses of FTY720 at or above 1 mg/day, while more effective lymphopenia is achieved at 2.5 mg/day \textsuperscript{198,199}. Furthermore, alternative formulations that enable targeting of tumor cells without impacting T lymphocytes and other non-target tissues will be necessary.

1.6.2 OSU-2S

OSU-2S was synthesized by Chen and colleagues as a novel derivative of FTY720, an immunosuppressive agent developed from fungal sphingosine analog ISP-1\textsuperscript{194,200,201}. As previously described, FTY720 exhibited potent preclinical and clinical activity against diseases including multiple sclerosis\textsuperscript{178,202}, various forms of leukemia\textsuperscript{182,184,195}, and organ rejection in kidney transplantation\textsuperscript{186}. FTY720 functions mainly through phosphorylation by sphingosine kinase 2 (SphK2)\textsuperscript{194}, interaction with the sphingosine-1-phosphate (S1P) receptors\textsuperscript{203,204}, and apoptosis induced by dependent activation of protein phosphatase 2A (PP2A)\textsuperscript{205}. However, the immunosuppressive properties and related side effects of
FTY720 have limited its further development for cancer therapy. To overcome this limitation, OSU-2S was designed to avoid interactions with the S1P receptor, and it therefore lacks immunosuppressive activity\textsuperscript{183,206}.

In preclinical assay, OSU-2S induced cytotoxicity in cells representing chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), acute lymphoblastic leukemia (ALL), and T cells\textsuperscript{206}. It also showed high efficiency in suppressing hepatocellular carcinoma tumor growth \textit{in vivo}\textsuperscript{194}. Unlike FTY720, OSU-2S has been shown not to be phosphorylated by Spk2 and is devoid of S1P1 activity. Instead, OSU-2S uniquely targets protein kinase C, involving caspase 3, 8 and 9 signaling, increased LC3-II levels, and induced phosphorylation of Ser 591 of the SHP1 phosphatase\textsuperscript{183,194,206}. Though the detailed mechanism of OSU-2S still remains to be clarified, existing data on OSU-2S have confirmed that it is a potent anti-tumor and nonimmunosuppressive analogue of FTY720.

1.7 Liposomal targeted delivery overcomes immunostimulatory effects of G3139

1.7.1 Free G3139 induces immunostimulation in CLL B cells

Gene therapy approaches, using therapeutic oligonucleotides (ONs), including antisense oligodeoxynucleotides (AS-ODNs), small interfering RNA (siRNA), and more recently discovered microRNA (miRNA), is an emerging interest in hematological malignancies\textsuperscript{48,207,208}. However, clinical trials using ONs in hematologic malignancies have shown limited success due to their instability during delivery as well as their cell
stimulatory properties and associated cytokine release syndrome\textsuperscript{209}. G3139 (Genasense or oblimersen sodium) has been the most studied AS-ODN. G3139 has the 18-base sequence of 5'-3': TCT CCC AGC GTG CGC CAT, which is complementary to Bcl-2 mRNA in the region encoding the first six amino acids of Bcl-2\textsuperscript{210,211}. Bcl-2 is a well characterized member of the Bcl family with multiple anti-apoptotic functions that prevent cell death from multiple mechanisms\textsuperscript{212}. Thus, over-expression of Bcl-2 can dramatically increase the resistance to therapeutics that promote mitochondrial and endoplasmic reticulum mediated death in a variety of cancer types. It has been shown in CLL that Bcl-2 protein is dramatically over-expressed as compared to normal B-cells and contributes in promoting resistance to fludarabine\textsuperscript{213,214}. Though with promising inhibition effects in Bcl-2 protein in preclinical study, G3139 exhibited a lower tolerated dosage in phase I study in CLL compared to other diseases, mainly due to cytokine release and immune activating symptoms unique to CLL\textsuperscript{215,216}. The two CpG dinucleotide motifs contained in the G3139 sequence induce a potent cytokine response due to immune activation via toll-like receptor 9 (TLR9) in murine models\textsuperscript{217}.

Bcl-2 is down-regulated in some CLL patients, but is also up-regulated in a more significant fraction of others, particularly at low or suboptimal concentrations. Consistent with this, the observed limitations in the clinical activity with G3139 may be attributed to the confounding effects of antisense mechanism and immune activation\textsuperscript{215,216,218}. Subsequently, the modest improvement and severe side effects observed in clinical activity failed the approval of G3139 in phase III study.
1.7.2 CD20-ILPs containing G3139 showed selectivity binding and induced Bcl-2
down-regulation in B CLL cells in vitro

In our previous study, a novel formulation of G3139 encapsulated into rituximab
congjugated immune nanoparticles (RIT-ILPs) was synthesized and characterized\textsuperscript{219}. It
was hypothesized that the application of liposomal technology would alter endocytosis
mechanism of G3139 by prolonging its retention in early endosome, instead of in late
endosome/lysosome where TLR is primarily found. Such mechanism has been
subsequently demonstrated by an in vitro study with free G3139 or RIT-ILP G3139
incubated with CLL cells and co-stained with TLR9 and markers for early endosome and
lysosome (Figure 1.8). RIT-ILPs mediate early endosomal compartmentalization of
G3139, resulting in the inhibition of TLR9-driven immunostimulatory effects and
enhanced Bcl-2 down-regulation in contrast to free G3139. Additionally, rituximab was
chosen as a targeting reagent for its specificity to B cells and for its minimal adverse
effects in CLL patients. RIT-ILPs themselves showed preferential binding and uptake by
B CLL cells in peripheral blood mononuclear cells (PBMCs) and RIT-ILP G3139 with
specific selectivity to B CLL cells led to significantly enhanced down-regulation of Bcl-2
and increased sensitivity to fludarabine with CLL cells. Consistently, significant
reduction in cell survival was also observed in CLL B cells treated with RIT-ILP G3139,
compared to those treated with HER-ILP G3139 as non-targeting negative control or free
G3139. In both separated B cells and whole blood from CLL patients, RIT-ILP G3139
demonstrated diminished immunostimulatory effects with decreased cytokine release as
well as the induction of co-stimulatory molecules. The development of RIT-ILP G3139
provides a highly promising application of oligonucleotide delivery and therapy with potent clinical efficacy.

1.8 Summary and dissertation overview

The main purpose of the current work is to develop and investigate a liposomal nanoparticle system with monoclonal antibodies aimed at targeted delivery in chronic lymphocytic leukemia treatment. Monoclonal antibodies involved in this study are all comparatively emerging and unique to CLL. Four major chapters with different emphasis are included.

In Chapter 2, a dual-ligand immunoliposome (dILPs) delivery system based on anti-CD37 antibody was developed and characterized. Targeting selectivity and killing efficiency of dILPs were evaluated in biological in vitro tests in both cell lines and CLL patient samples.

In Chapter 3, a novel anti-ROR1 mAb, 2A2-IgG, was introduced to liposomal nanoparticles, forming an emerging single mAb-based ILP delivery system for potential specific CLL targeting therapy. The specificity and efficiency studies for 2A2-ILP were carried out in cell lines, CLL patients cells, normal donors, and in ROR1 transgenic mice.

To prove the concept of targeted delivery therapy for CLL with the dILPs and 2A2-ILPs, two small molecule drugs with potent therapeutic efficacy but significant side effects were chosen as model drugs. In Chapter 4, both FTY720 and OSU-2S were encapsulated into liposomes as a new formulation to realize targeted delivery. A liquid chromatography-tandem mass spectrometry (LC/MS-MS) method that can
simultaneously quantify FTY720 and OSU-2S was developed and validated. Pharmacokinetic study of both drugs in free and liposomal form was evaluated in ICR mice. FTY720 and OSU-2S have also been applied to dILPs and 2A2-ILPs, respectively, to demonstrate the efficiency of the targeted delivery systems in CLL.

In Chapter 5, an *in vivo* study of anti-CD20 targeted lipoplex nanoparticles with ODNs was reported. The therapeutic efficiency of the rituximab conjugated ILP G3139 was evaluated in a Raji engrafted model in SCID mice. To be consistent to our previous *in vitro* experiments, the alternative effect of G3139 in RIT-ILP was also investigated through immune-stimulation assays.
Table 1.1. Approved and emerging liposome formulations\textsuperscript{23}

<table>
<thead>
<tr>
<th>Active agent (product name)</th>
<th>Composition</th>
<th>Stealth</th>
<th>Company, year of product marketing</th>
<th>Application</th>
<th>Trial phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>DaunoXome\textsuperscript{9} (daunorubicin)</td>
<td>DSPC/CHOL</td>
<td>no</td>
<td>Nexstar Pharmaceuticals, 1995</td>
<td>Kaposi's sarcoma</td>
<td>Approved</td>
</tr>
<tr>
<td>DOXIL\textsuperscript{9} (doxorubicin)</td>
<td>SoyPC/CHOL/DSPE-PEG</td>
<td>yes</td>
<td>Sequus Pharmaceuticals, 1997</td>
<td>Kaposi's sarcoma</td>
<td>Approved</td>
</tr>
<tr>
<td>Myocet\textsuperscript{7} (doxorubicin)</td>
<td>EPC/CHOL</td>
<td>no</td>
<td>Elan Pharma, 2000</td>
<td>Metastatic breast cancer</td>
<td>Approved</td>
</tr>
<tr>
<td>SPI-077 (cisplatin)</td>
<td>SoyPC/CHOL/DSPE-PEG</td>
<td>yes</td>
<td>Sequus Pharmaceuticals</td>
<td>Head and neck cancer, Lung cancer</td>
<td>Phase II/III</td>
</tr>
<tr>
<td>Lipoplatin\textsuperscript{TM} (cisplatin)</td>
<td>SoyPC/DPPG/CHOL</td>
<td>yes</td>
<td>Regulon Inc.</td>
<td>Several cancer type</td>
<td>Phase II/III</td>
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<tr>
<td>S-CKD602 (camptothecin analog)</td>
<td>—</td>
<td>yes</td>
<td>Alza Co.</td>
<td>Several cancer type</td>
<td>Phase I</td>
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<tr>
<td>Aroplatin (oxaliplatin analog)</td>
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<td>no</td>
<td>Antigenics Inc</td>
<td>Colorectal cancer</td>
<td>Phase II</td>
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<td>DepoCyt (paclitaxel)</td>
<td>DOPE/DPPG/CHOL/triolein</td>
<td>no</td>
<td>SkyPharma 1999</td>
<td>Lymphomatous meningitis</td>
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<td>LEP-ETU (paclitaxel)</td>
<td>DOPE/CHOL/cardiolipin</td>
<td>no</td>
<td>NeoPharm Inc</td>
<td>ovarian, breast, and lung cancer</td>
<td>Phase I</td>
</tr>
<tr>
<td>LEM-ETU (mitoxantrone)</td>
<td>DOPE/CHOL/cardiolipin</td>
<td>no</td>
<td>NeoPharm Inc</td>
<td>leukemia, breast, stomach, liver, ovarian cancers</td>
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<td>LE-SN38 (irinotecan)</td>
<td>DOPE/CHOL/cardiolipin</td>
<td>no</td>
<td>NeoPharm Inc</td>
<td>advanced cancer</td>
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</tr>
<tr>
<td>MBT-206 (paclitaxel)</td>
<td>DOPE/DOPC/triethylammoniumpropionate</td>
<td>no</td>
<td>MediGene AG</td>
<td>Anti-angiogenic properties Breast cancer</td>
<td>Phase I</td>
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<td>OSI-211 (lactotetacan)</td>
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<td>Enzon Co.</td>
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<td>Non-Hodgkin's lymphoma</td>
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<td>Atragen\textsuperscript{®} (t retinoic acid)</td>
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<td>INX-0125 (vinorelbine)</td>
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<td>advanced cancer</td>
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<td>breast cancer</td>
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<td>no</td>
<td>Fujtsuka USA Inc and Nexstar Pharm 1997</td>
<td>Fungal infections in immuno-compromised patients</td>
<td>Approved</td>
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<tr>
<td>Nycostran\textsuperscript{®} (nisticin)</td>
<td>DMPC/DMPG/CHOL</td>
<td>no</td>
<td>Aronex Pharm</td>
<td>Fungal infections in immuno-compromised patients</td>
<td>Phase II/III</td>
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Table 1.2. Monoclonal antibodies currently FDA approved in oncology and their mechanisms of action

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<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>FDA-approved indication</th>
<th>Approval in Europe*</th>
<th>Mechanisms of action</th>
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<tbody>
<tr>
<td><strong>Naked antibodies: solid malignancies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trastuzumab (Herceptin; Genentech):</td>
<td>ERBB2</td>
<td>ERBB2-positive breast cancer, as a single agent or in combination with chemotherapy for adjuvant or palliative treatment</td>
<td>Similar</td>
<td>Inhibition of ERBB2 signalling and ADCC</td>
</tr>
<tr>
<td>humanized IgG1</td>
<td></td>
<td>ERBB2-positive gastric or gastro-oesophageal junction carcinoma as first-line treatment in combination with cisplatin and capecitabine or 5-fluorouracil</td>
<td></td>
<td></td>
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<tr>
<td>Bevacizumab (Avastin; Genentech/Roche):</td>
<td>VEGF</td>
<td>For first-line and second-line treatment of metastatic colon cancer, in conjunction with 5-fluorouracil-based chemotherapy; for first-line treatment of advanced NSCLC, in combination with carboplatin and paclitaxel, in patients who have not yet received chemotherapy; as a single agent in adult patients with glioblastoma whose tumour has progressed after initial treatment; and in conjunction with IFNα to treat metastatic kidney cancer</td>
<td>Similar</td>
<td>Inhibition of VEGF signalling</td>
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<td>humanized IgG1</td>
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<td></td>
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<tr>
<td>Cetuximab (Erbitux; Bristol-Myers Squibb):</td>
<td>EGFR</td>
<td>In combination with radiation therapy for the initial treatment of locally or regionally advanced SCCHN; as a single agent for patients with SCCHN for whom prior platinum-based therapy has failed; and palliative treatment of pretreated metastatic EGFR-positive colorectal cancer</td>
<td>Similar</td>
<td>Inhibition of EGFR signalling and ADCC</td>
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<td>chimeric human-murine IgG1</td>
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<td>Panitumumab (Vectibix; Amgen):</td>
<td>EGFR</td>
<td>As a single agent for the treatment of pretreated EGFR expressing, metastatic colorectal carcinoma</td>
<td>Similar</td>
<td>Inhibition of EGFR signalling</td>
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<tr>
<td>human IgG2</td>
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<td>Iplimumab (Yervoy; Bristol-Myers Squibb):</td>
<td>CTLA4</td>
<td>For the treatment of unresectable or metastatic melanoma</td>
<td>Similar</td>
<td>Inhibition of CTLA4 signalling</td>
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<td>human IgG1</td>
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<td><strong>Naked antibodies: haematological malignancies</strong></td>
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<td>Rituximab (Mabthera; Roche):</td>
<td>CD20</td>
<td>For the treatment of CD20-positive B cell NHL and CLL, and for maintenance therapy for untreated follicular CD20-positive NHL</td>
<td>Similar</td>
<td>ADCC, direct induction of apoptosis and CDC</td>
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<td>Alemtuzumab (Campath; Genzyme):</td>
<td>CD52</td>
<td>As a single agent for the treatment of B cell chronic lymphocytic leukemia</td>
<td>Similar</td>
<td>Direct induction of apoptosis and CDC</td>
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<td>humanized IgG1</td>
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<td>Ofatumumab (Arzerra; Genmab):</td>
<td>CD20</td>
<td>Treatment of patients with CLL refractory to fludarabine and alemtuzumab</td>
<td>Similar</td>
<td>ADCC and CDC</td>
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<tr>
<td>human IgG1</td>
<td></td>
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<td><strong>Conjugated antibodies: haematological malignancies</strong></td>
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<td>Gemtuzumab ozogamicin (Mylotarg; Wyeth):</td>
<td>CD33</td>
<td>For the treatment of patients with CD33-positive acute myeloid leukaemia in first relapse who are 60 years of age or older and who are not considered candidates for other cytotoxic chemotherapy; withdrawn from use in June 2010</td>
<td>Not approved in the European Union</td>
<td>Delivery of toxic payload, calicheamicin toxin</td>
</tr>
<tr>
<td>(Mylotarg; Wyeth):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>humanized IgG4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brentuximab vedotin (Adcetris; Seattle Genetics):</td>
<td>CD30</td>
<td>For the treatment of relapsed or refractory Hodgkin’s lymphoma and systemic anaplastic lymphoma</td>
<td>Not approved in the European Union</td>
<td>Delivery of taxol payload, auristatin toxin</td>
</tr>
<tr>
<td>chimeric IgG1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>131I-labelled ibritumomab tiuxetan (Zevalin; IDEC Pharmaceuticals): murine IgG1</strong></td>
<td>CD20</td>
<td>Treatment of relapsed or refractory, low-grade or follicular B cell NHL previously untreated follicular NHL in patients who achieve a partial or complete response to first-line chemotherapy</td>
<td>Similar</td>
<td>Delivery of the radiolabeled anti-CD20 monoclonal antibody</td>
</tr>
</tbody>
</table>
Table 1.3. New monoclonal antibody in CLL clinical development\textsuperscript{114}

<table>
<thead>
<tr>
<th>Agent</th>
<th>Target</th>
<th>Humanized/chimeric</th>
<th>Direct cell death</th>
<th>ADCC</th>
<th>CDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDX-1342</td>
<td>CD19</td>
<td>Humanized</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>XmAb6574</td>
<td>CD19</td>
<td>Humanized</td>
<td>Modest</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Ofatumumab</td>
<td>CD20</td>
<td>Humanized</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GA-101</td>
<td>CD20</td>
<td>Humanized</td>
<td>Yes</td>
<td>Yes</td>
<td>Modest</td>
</tr>
<tr>
<td>PRO131921</td>
<td>CD20</td>
<td>Humanized</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Veftuzumab</td>
<td>CD20</td>
<td>Humanized</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>LFB-R803</td>
<td>CD20</td>
<td>Humanized</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Lumilumab</td>
<td>CD20</td>
<td>Humanized</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>TRU-016</td>
<td>CD37</td>
<td>Humanized</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>SGN40</td>
<td>CD40</td>
<td>Humanized</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>HCD122</td>
<td>CD40</td>
<td>Humanized</td>
<td>No</td>
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<td>No</td>
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<tr>
<td>MDX-1411</td>
<td>CD70</td>
<td>Humanized</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>Milatuzumab</td>
<td>CD74</td>
<td>Humanized</td>
<td>Yes</td>
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<td>No</td>
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<tr>
<td>Ipilimumab</td>
<td>CTLA-4</td>
<td>Humanized</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>VEGF</td>
<td>Humanized</td>
<td>No</td>
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Table 1.4. Targeted liposomes in advanced phase of trial

<table>
<thead>
<tr>
<th>Targeted with</th>
<th>Encapsulated drug</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HER2 (trastuzumab)</td>
<td>DOXIL®</td>
<td>breast, ovarian cancer</td>
</tr>
<tr>
<td>Anti-EGF</td>
<td>doxorubicin, vinorelbine,</td>
<td>solid tumors</td>
</tr>
<tr>
<td></td>
<td>methotrexate, DNA</td>
<td></td>
</tr>
<tr>
<td>Anti CD19</td>
<td>vincristine</td>
<td>lymphoma</td>
</tr>
<tr>
<td>Anti CD22</td>
<td>doxorubicin</td>
<td>anti-B-cell lymphoma</td>
</tr>
<tr>
<td>Anti CD19</td>
<td>imatinib</td>
<td>ALL</td>
</tr>
<tr>
<td>Anti-betal integrin</td>
<td>doxorubicin</td>
<td>several cancers</td>
</tr>
<tr>
<td>Anti GD2</td>
<td>doxorubicin</td>
<td>neuroblastoma</td>
</tr>
<tr>
<td>GAH MAb</td>
<td>doxorubicin</td>
<td>gastric, colon and breast cancer</td>
</tr>
<tr>
<td>Anti-EGF receptor</td>
<td>RNA</td>
<td>brain cancer</td>
</tr>
</tbody>
</table>
Figure 1.1. Schematic diagram of a bilaminar liposome

Shown is a lipid nanoparticle structure composed of lipid molecules. The assembly formed lipid bilayer (brown area) is suitable for lipid-soluble drugs and the hydrophilic core is appropriate for water-soluble reagents. PEGylated protective layer shields liposomes from clearance by macrophages and allows immobilization of ligands for active targeting.
Figure 1.2. Chemical structure of commonly used lipids

Shown are the representatives of commonly used lipids. Cationic lipids such as DOTAP and DC-Chol have better affinity with oligonucleotides. Lipids like Egg-PC and cholesterol function as helper lipids to stabilize the structure of liposomes. PEG (2000)-DSPE modified liposomes and prevent them from being recognized by immune system and opsonizing proteins.
Figure 1.3. Advantages of using liposomal nanoparticles for anti-cancer delivery

(A) Liposomal nanoparticles improve the solubility of drug; (B) liposomal nanoparticles prolong the circulation time of drug in blood vessels; (C) liposomal nanoparticles enhance the accumulation of drug in targeted tumor tissues; (D) the targeting ligands immobilized allow drug uptake via endocytosis, thus increasing intracellular drug; (E) controlled and stable drug release can be achieved via liposomal drug delivery; (F) liposomal nanoparticles lack substrates for ATP-binding cassette proteins, resulting in minimized drug-resistance.
Figure 1.4. PEGylated liposomes with antibody immobilized

(A) Schematic of PEGylated liposome (B) PEGylated ILPs where the antibody is bound directly to the liposome surface or (C) to the distal tip of the PEG chain.
Ab-targeted liposomes are prepared by a post insertion method. MAbs are first thiolated at the lysine group by Traut’s reagent (2-iminothiolane) followed by reaction with micelles prepared from maleimide PEG-DSPE to form Ab-coated micelles. A post insertion method is adopted to incorporate the Ab-coated micelles to the previously prepared non-targeted liposomes forming the Ab-targeted liposomes for targeted delivery.
Figure 1.6. Morphological analysis of liposomes and immunoliposomes

Cryo-TEM images of (A) liposome nanoparticles without antibody and (B) rituximab-conjugated anti-CD20 immunoliposomes. The insertion of mAb does not alter the morphology of liposome nanoparticles, and the mAb created a layer less than 5 nm on the surface of liposomes that cannot be distinguished by cryo-TEM at the current resolution.
CB-17 SCID female mice were injected with $2 \times 10^6$ Raji Burkitt lymphoma cells through the tail vein using a mouse tail illuminator. DEX was given through i.p. injection at 5mg/kg in free or liposomal form 3 times a week for 5 weeks. Hind-limb paralysis was considered as the end point. All control groups showed symptoms within day 14-15 days post engraftment. CD74-ILPs increased the mean survival by 6.35 days compared with CD74 treatment alone. CD74-ILP-DEX survived 13.56 days and 29.4 days longer than CD74-ILPs group and controls (n=14, $P<0.0001$), respectively. Kaplan-Meier estimates of survival for treatments and engraftments were plotted, and the survival for each treatment was calculated with 95% confidence intervals. (DEX: Dexamethasone; HER: Herceptin)
Figure 1.8. Differential compartmentalization by free G3139 and RIT-ILP-G3139 in CLL B cells

Purified CLL B cells were incubated with free FAM-G3139 (2 μM) or RIT-ILP-FAM-G3139 (1 μM) for 0.5 h. Cells were washed, fixed and stained with anti-EEA-1 or anti-LAMP-1 and TLR9 antibodies for confocal observation. The white arrows indicate the co-localization (purple dots). Scale bar=10 μm (EEA-1: Early endosome antigen 1 protein; LAMP-1: Lysosomal-associated membrane protein 1; TLR: Toll-like receptor).
CHAPTER 2

IMPROVED TARGETED DELIVERY TO B CHRONIC LYMPHOCYTIC LEUKEMIA CELLS BY ANTI-CD37 BASED DUAL-LIGAND IMMUNOLIPOSOMES

2.1 Introduction

Despite therapeutic advances in chemo and immunotherapeutic agents for chronic lymphocytic leukemia, the undesirable adverse effects due to the non-specific effect of chemotherapeutic agents on unintended target cells remains to be addressed. The introduction of the anti-CD20 monoclonal antibody rituximab (RIT)\textsuperscript{220-222} has substantially impacted CLL therapy\textsuperscript{95,220,223}. RIT, when given in combination with fludarabine and cyclophosphamide, has been shown to extend survival in symptomatic CLL\textsuperscript{220,223,224}. In addition to rituximab, alemtuzumab that targets CD52, an antigen expressed on normal lymphocytes as well as many T- and B-cell neoplasms has been used for first-line treatment for CLL\textsuperscript{221,222}. The immunosuppressive effects of alemtuzumab caused by T and NK cell depletion, however, impose limit to the use of this agent in aged patients. New antibodies targeted against CD19, CD40, CD23 and CD74 are being tried clinically for the treatment of CLL\textsuperscript{121,161,225,226}. Recently, CD37 antigen
has been identified as a potential target for therapy in B-cell malignancies\textsuperscript{161,227,228}. CD37, a 40–52kDa glycoprotein, is highly expressed on B cells and has limited or no expression on other hematopoietic cells such as T cells and NK cells\textsuperscript{229,230}. In particular, the CD37 expression level on B-CLL cells is uniformly present and relatively over-expressed\textsuperscript{161,228}.

B-cell lymphomas and leukemia often involve multiple, different pathological factors and pathways. Therapeutic efficacy of most of the marketed monospecific antibodies is attributed to their interaction with a single target. Therefore, simultaneous engagement of multiple targets either via the combination of two Abs or by a bispecific antibody (BsAb) is likely to provide better clinical efficacy and/or reach a broader patient population\textsuperscript{231-233}. The improved therapeutic efficacy of combining milatuzumab and RIT mAbs has already been demonstrated in the preclinical model of mantle cell lymphoma (MCL)\textsuperscript{234}. In addition, the bispecific, anti-CD20/CD22 antibodies and anti-CD20/CD74 antibodies also demonstrated the enhanced immunotherapy for B-cell lymphomas and leukemia\textsuperscript{231,235}.

Specific and efficient \textit{in vivo} delivery of therapeutic agents to target B-CLL cells still remains a major challenge in the clinical use. To address these issues, monoclonal antibody conjugated nanocarriers such as immunoliposomes (ILPs) have been increasingly recognized as a promising strategy for selective delivery of anti-cancer drugs to B-CLL cells\textsuperscript{135,225,236}. In addition, recent efforts on dual-ligand mediated delivery approaches offers the potential to improve selectivity and efficiency over single-ligand approaches\textsuperscript{237}. The dual-ligand immunoliposomes (dILPs) have shown the improved therapeutic effects of anti-cancer drugs in B-cell malignancies\textsuperscript{14,238}. A schematic
illustration of dILPs concept is shown in Figure 2.1. Despite the obvious promise, dILPs against antigens co-expressed on the same cells have not been investigated in CLL.

In this chapter, a novel approach using anti-CD37 monoclonal antibody conjugated ILP for efficient targeted delivery to B chronic lymphocytic leukemia cells was identified. To achieve maximal benefits for all patients, the new treatment strategy through dILPs with anti-CD37 Ab combined with either anti-CD19 Ab or anti-CD20 Ab was proposed and characterized by utilizing the fast internalization feature of anti-CD37 Ab and the comparatively higher expression of anti-CD19 or anti-CD20 Ab.

2.2 Materials and methods

2.2.1 Reagents

Egg phosphatidylcholine (Egg PC) and methoxy-polyethylene glycol (MW ~ 2,000 Da)-distearoyl phosphatidylethanolamine (PEG-DSPE) were obtained from Lipoid (Newark, NJ). DSPE–PEG–maleimide (DSPE–PEG–Mal) was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Cholesterol (Chol), calcein hydrophilic dye, 2-Iminothiolane (Traut's reagent) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Rituxan (rituximab) was obtained from Genentech, Inc (South San Francisco, CA). Purified anti-human CD37 and anti-human CD19 monoclonal murine antibodies were purchased from BD Biosciences (Catalogue #555456 and #555410, San Diego, CA). Alexa 488 labeled anti–mouse secondary antibody was from Invitrogen (Catalogue #A-11001, Carlsbad, CA).
2.2.2 Cell culture

Raji Burkitt’s lymphoma, Daudi, Romas, RS11846 and Jurkat cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and penicillin (100 U/mL)/streptomycin (100 µg/ml; Sigma-Aldrich, St. Louis) at 37°C in an atmosphere of 5% CO2. Blood was obtained from patients with informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the institutional review board (IRB) of The Ohio State University (Columbus, OH). All patients examined in this series had immunophenotypically defined CLL as outlined by the modified 96 National Cancer Institute criteria. CLL B cells were isolated from freshly donated blood using ficoll density gradient centrifugation (Ficoll-Paque Plus, Amersham Biosciences, Piscataway, NJ). Enriched CLL fractions were prepared by using the “Rosette-Sep” kit from Stem Cell Technologies (Vancouver, British Columbia, Canada) according to the manufacturer’s instructions.

2.2.3 Immuno-surface staining and flow cytometry

Cell lines (0.5×10^5/ml) or B-CLL cells (1.0×10^5/ml) were incubated with PE-labeled anti-CD20, anti-CD19, and anti-CD37 as well as mouse IgG1 isotype control antibody (BD Biosciences, Catalogue #555623, #555413, #555749, San Diego, CA; Beckman Coulter, Catalogue #IM0458, Brea, CA), at 4°C for 30 min. The cells were then spun down at 300 g for 10 min and rinsed twice with cold phosphate-buffered saline (PBS,
pH=7.4) and then analyzed by flow cytometry on a Beckman Coulter EPICS XL (Beckman Coulter) to determine antigen expression levels. A minimum of 10,000 events were collected under the LIST mode for each assay. The data was analyzed using WinMDI software.

### 2.2.4 Selectivity study of CD37 antibody in PBMC

For surface staining, PBMC cells were incubated with PE labeled anti-CD19 (as a B cell marker) or PE labeled anti-CD3 (as a T cell marker) on ice for 30 min. The cells were washed twice with cold PBS (pH=7.4) and analyzed by flow cytometry. To study B-cell selectivity, PBMC cells were incubated with FITC labeled CD37 (Beckman Coulter, Catalogue #IM0457, Brea, CA) on ice for 30 min. The cells were then spun down and rinsed twice with cold PBS (pH=7.4). The treated cells were further stained with PE labeled anti-CD19 or anti-CD3 (BD Biosciences, Catalogue #555413 and #555333, San Diego, CA) antibodies to identify B and T cell populations respectively. After another washing and spin-down, the cells were analyzed by flow cytometry.

### 2.2.5 Antibody internalization assay

Cells were incubated with fluorochrome labeled antibodies (PE-CD20, PE-CD19 and PE-CD37) at 37°C for 30, 60, 120 and 240 min. The antibody control was added at 0 min on ice to ensure that internalization reaction did not occur till temperature was raised to 37°C. After incubation, extracellular bound antibodies were removed with stripping
buffer (100 mM glycine, 100 mM NaCl (pH 2.5) thus allowing detection of only internalized fluorochrome labeled mAb by flow cytometry. Appropriate IgG isotypes were used as negative controls. Internalization is defined as time-dependent increase in the Mean Fluorescent Intensity (MFI) after acidic washing by stripping buffer, which removed any surface bound antibody.

2.2.6 Combinatorial antibody microarray

Three pure (anti-CD19, anti-CD20, and anti-CD37) and combined dual antibodies were printed onto Nexterion® slide H using a non-contact piezoelectric arrayer (Perkin Elmer, Waltham MA), at the same total antibody concentration (0.5 mg/mL). Each sample was arrayed in triplicate with a spot center-to-center distance of 400 μm into a subarray of 6.8 mm x 6.8 mm. Cells, from B-CLL patients, were labeled with CFSE (carboxy-fluorescein diacetate, succinimidyl ester; Invitrogen, Carlsbad CA) according to manufacturer’s protocol. After incubation of B-CLL cells (1.5x10^6/ml) with the Ab microarray for 1 hr at room temperature, the microarray slide was carefully dip-washed in PBS solution to remove unbound cells and then was imaged. Microarray images were acquired by ProScanArray fluorescence scanner (Perkin Elmer, Waltham MA). Slides were scanned at 5μm resolution. Image quantification and analysis were performed by using ScanArray Express 3.0 (Perkin Elmer, Waltham MA).
2.2.7 Preparation of immunoliposomes

A lipid mixture of EggPC/Chol/PEG-DSPE=65/34/1.0 (molar ratio) was dissolved in ethanol. The lipids formed a homogeneous thin lipid layer in rotor vapor. Calcein hydrophilic dye (50 µM, pH=8.0) was incorporated in the lipid mixture by re-hydration as a green fluorescent marker. Particle size was reduced to ~ 100 nm range by high pressure extrusion with nuclear polycarbonate membranes (pore sizes: 0.2 and 0.1 mm, Northern Lipids).

A post-insertion method was adopted to incorporate antibody ligands into preformed liposomes carrying calcein. Antibody was reacted with 10× Traut’s reagent (2 hr, room temperature) to yield sulphydryl modified antibodies, which were then reacted to micelles of Mal-PEG-DSPE at a molar ratio of 1:10, and then incubated with calcein loaded liposomes for 1 hr at 37°C. For the binding study, the post-inserted immunoliposomes carrying calcein were further separated to remove free PEG conjugated antibodies by CL-4B column. The particle sizes of ILPs were analyzed using a NICOMP Particle Sizer Model 370 (Particle Sizing Systems, Santa Barbara, CA). The volume-weighted Gaussian distribution analysis was used. The zeta potential (ξ) was determined on a ZetaPALS (Brookhaven Instruments Corp., Worcestershire, NY).

2.2.8 Uptake of ILPs and dILPs by fluorescence microscopy

Binding and internalization of the ILPs and dILPs in Raji cells were examined by fluorescence microscopy. Cells were incubated with anti-CD20 ILPs, anti-CD37 ILPs and anti-CD20/CD37 dILPs for 4 hrs at 37°C and washed twice with PBS, followed by
fixation with 2% paraformaldehyde for 30 min. The cells were mounted on a poly-D-lysine coated cover glass slide (Sigma-Aldrich, St. Louis, MO). Green fluorescence of FAM-ODN was analyzed, and images were produced by using a Zeiss 510 META Laser Scanning Confocal Imaging Systems at 600X of magnificence (Carl Zeiss MicroImaging, Inc., NY, USA).

2.2.9 Apoptosis assay

Apoptosis activity was determined in primary CLL cells after 24-hour incubation with ILPs or dILPs by annexin V / PI staining. Samples from same patients were treated with mAbs in the presence or absence of an anti-IgG Fc crosslinker as controls. The degree of apoptosis induction is displayed as the total percentage of annexin V and PI double negative cells normalized to untreated condition.

2.2.10 Statistical analysis of data

Statistical analyses were performed by Center for Biostatistics at The Ohio State University. Linear mixed effect models were used to estimate unrestricted covariance structures and produce robust hypothesis tests. $P$-values were adjusted by Holm’s method. A significance level of $\alpha = 0.05$ was used for all tests.
2.3 Results

2.3.1 CD37 is an attractive target for efficient delivery to B-CLL cells

CD37 has been recently identified as a novel target for the treatment of B cell malignancies. It is predominantly expressed on B cells, and has minimal or no expression on other types of blood cells, including T and NK cells. To further demonstrate its specific delivery to B but not T cells, the PBMCs isolated from patients with CLL were treated by anti-CD37-FITC, followed by anti-CD19-PE or anti-CD3-PE. As shown in Figure 2.2A, two-color flow cytometry confirmed that anti-CD37-FITC was selectively bound to B cells that were CD19 (+) but not by other cell populations including T cells identified by anti-CD3-PE. Thus, CD37 can serve as a good targeting ligand for the drug delivery of B cell malignancies.

We further evaluated the levels of expression of CD37, CD19 and CD20 antigens in B cell lines and primary CLL B cells using fluorochrome-labeled antibodies by FACS (Figure 2.2B and C). CD37 levels are comparable with CD19 levels and their variations of expression were small in both B cell lines and B-CLL cells. In addition, the average expression of CD19 or CD37 on Raji cells is comparable to that observed on B-CLL cells.

2.3.2 Internalization study of antibodies

The internalization rate of antibody bound to surface antigens is an important consideration factor for choosing appropriate candidates for immunotoxins and antibody mediated drug delivery. In this content, antibodies directed against CD37, CD19 and
CD20 were examined for internalization in Raji cell line (Figure 2.3). Compared to anti-CD20 antibodies, anti-CD19 and anti-CD37 consistently exhibited increased internalization at a faster rate in Raji cells (n=3). Within only 15 min, over 7.5% of anti-CD37 antibody already has been internalized into cells while only 3.5% of the well-known internalizing antibody, anti-CD19, has been uptaken by the cells. The maximum internalization of anti-CD37 was observed at 2 hr with 13.3% of antibody internalized. This supports our selection of anti-CD37 as the primary antibody to mediate B-cell specific targeting delivery of drug loaded immuno-nanoparticles.

2.3.3 Screening of optimal Ab combination for targeted delivery to B-CLL cells through combinatorial antibody microarray

Antibody microarray assays can be used for quantitative immunophenotyping of leukemia cells in a high throughput manner. In contrast to its usual application for detection of certain CD antigens on cells, we intended to borrow this technology for evaluation of binding efficiency of the probable single or combined antibodies suitable for ILPs based targeting delivery. In our pilot microarray screening, we have chosen four candidate antibodies targeting to CD19, CD20, CD52 and CD37, respectively. Anti-CD19 antibodies have been used for CLL diagnosis, and recently CD19 has thrived to be targeted for CLL therapy. CD20 has been the most well-validated target for CLL therapy over the past decade with the antibody rituximab, ofatumumab and GA101 available already. Alemtuzumab as the anti-CD52 antibody has already been used as a secondary line for CLL therapy. And CD37 is a relative new but promising target with the anti-
CD37 antibody TRU-016 and other for clinical trials undergoing. As the pilot microarray showed the non-specificity of anti-CD52 Ab, so our Ab microarrays were constructed by printing the CD19, CD20, CD37 antibodies and their dual combinations. The highest binding fluorescence intensity in the Ab microarray was set as 1 (Fig. 2.4A) and quantitative binding profiles of cells from nine B-CLL patients on the combinatorial antibody microarrays were shown (Fig. 2.4B and C). The levels of cell binding across the spots printed with single antibody solutions (anti-CD19, anti-CD20, anti-CD37) clearly varied from patient to patient. For all nine patients the highest binding occurred on spots printed with mixtures of two antibodies. The greater binding efficiencies in all 9 tested primary CLL cells were validated when dual combinations of antibodies such as CD20/CD37 and CD19/CD37 were used (n=9, \( P < 0.05 \) between all single antibody groups compared to CD20/CD37 or CD19/CD37 group).

### 2.3.4 Synthesis and optimization of anti-CD37 based dILPs for enhanced targeting delivery

Based on results from the antibody microarray, we designed and synthesized a series of a mAb conjugated ILPs. The “post-insertion” method was used to construct ILPs with different compositions as shown in Figure 1.5\(^{225,238}\). The internalization rate of antibody is an important consideration factor for choosing appropriate candidates for immunotoxins and antibody mediated drug delivery\(^7,20\).

To determine the optimal mAb ratio for dILPs, we evaluated various combinations of CD37 antibody and CD20 antibody on ILPs through the flow cytometry analysis in both
Daudi and primary B-CLL cells isolated from CLL patients. The binding efficiencies of anti-CD20/CD37 dILPs reached the highest value at the 50/50 composition compared to 100/0 or 0/100 (single Ab ILPs) for both Daudi and B-CLL cells (n=3, \( P < 0.0001 \)) (Figure 2.5A). Furthermore, the dual-mAb ILPs worked better than single-mAb ILPs. Keeping the 50/50 composition as a constant, we examined the delivery efficiency of two dILPs (CD19/CD37 and CD20/CD37) in other B cell lines (Figure 2.5B and C). The MFI of CD20/CD37 dILPs reached 14.8 while the corresponding single ILPs, CD20-ILP and CD37-ILP had the MFI of 6.9 and 8.5, respectively. Consistent with Daudi cells, dual CD20/CD37 dILPs showed the strongest delivery efficiency compared to individual mAb ILPs and CD19/CD37 dILPs with over 25% to 330% increased MFI in all cell lines investigated.

Fluorescence microscopy was used to compare the uptake and cellular localization of anti-CD20 ILPs, anti-CD37 ILPs and anti-CD20/CD37 (50/50) dILPs encapsulated calcein in Raji cells (Figure 2.5D). Cells were incubated with anti-CD20 ILPs, anti-CD37 ILPs and anti-CD20/CD37 dILPs for 4 hrs at 37°C and washed twice with PBS, followed by fixation with 2% paraformaldehyde for 30 min. Fluorescence microscopy revealed a high level of uptake of anti-CD37 ILPs by Raji cells, while anti-CD20 ILPs had only limited uptake by Raji cells and most of green fluorescence was just bound on the cell surface. Anti-CD20/CD37 dILPs demonstrated the highest cellular uptake.

2.3.5 Validation of synergistic effect of anti-CD37 based dILPs on B-CLL cells
To further confirm the synergistic effect, the anti-CD37 based dILPs were also examined on B-CLL cells. Among all tested B-CLL cells, the CD19/CD37 dILPs demonstrated the highest delivery efficiency compared to all individual mAb ILPs and the CD20/CD37 dILPs. The synergistic effect of CD19/CD37 combination was significant compared to CD20/CD37 dILPs (n=11, \( P = 0.002 \)) with 43.3% increase in binding to B-CLL cells. And CD20/CD37 dILPs enhanced the delivery and binding efficiency compared to CD20 ILPs with over 2.36 fold change in MFI in B-CLL cells. The CD37 ILPs have much better delivery efficiency than CD19 and CD20 ILPs, further confirming the benefits of using CD37 as a targeting ligand (Figure 2.6A and B). It should be noticed that the delivery efficiency of CD20 ILPs varied greatly on patient cells because of the variable CD20 antigen expression on B-CLL cells\(^{213,226,244}\). This observation is consistent with the greater binding efficiency found in the antibody microarray experiment.

2.3.6 Single-antibody immunoliposomes showed similar cytotoxicity to crosslinking effect in CLL cells

Previous studies has revealed that ILPs may cause cross-linking effect similar to that from anti-Fc receptor\(^{132,245}\), the same phenomenon was observed with CD37 in our study after 24 hr treatment (Figure 2.7). B-CLL cells isolated from six patients showed equivalent cytotoxicity responding to 0.25μg/ml of CD37 in the ILPs form as that of 0.5μg/ml of CD37 with the existence of anti-Fc linker, and over 40% cytotoxicity were induced. By increasing the concentration of CD37-ILPs to 0.5μg/ml of CD37, there were
more cells being directed to apoptosis though it is not significant compared to the anti-Fc cross-linking effect with 20% further apoptosis induced in cells (n=6, $P=0.0536$, 0.5μg/ml ILP vs. CD37+anti-Fc).

2.3.7 Daul-antibody ILPs significantly improved killing efficiency in CLL patients compared with single ILPs

To prove our hypothesis that dILPs not only increase the binding efficiency of nanoparticles to the targets, B-CLL cells, but also induce more cytotoxicity, combinations of dILPs were tested with B-CLL cells with investigation of cross-linking effect of CD19-ILPs and CD20-ILPs. All three mAb ILPs more or less represented the cross-linking effect caused by ILPs (Figure 2.8). Furthermore, compared to their single ILPs form, both CD19 and CD20 exhibited greater effect with up till 44% more on inducing apoptosis of B-CLL cells irrespective of combining with each other or with CD37. While CD37 dILPs did not show enhanced cytotoxicity compared to its single ILPs form, an enhancing effect of CD37 dILPs were observed with CD19 or CD20 without payload.

2.4 Discussion

In this chapter, the combinatorial antibody microarray technology was adapted to quickly identify the optimal antibody combinations for individual patient cells. For proof of concept, two other B-cell specific antibodies, anti-CD19 and anti-CD20, were combined with anti-CD37 antibody to enhance selectivity and efficacy. Consistent with antibody microarray, anti-CD37 dILPs is shown to provide highly specific targeting
efficacy to both leukemia cell lines and chronic lymphocytic leukemia patient B cells. Compared with the single antibody targeted ILPs, the anti-CD19/CD37 dILPs clearly demonstrated synergistic effect and provided the best delivery efficiency and cytotoxicity to B chronic lymphocytic leukemia cells, whereas the anti-CD20/CD37 dILPS were most efficient for delivery to leukemia cell lines. Our findings suggest that the anti-CD37 based dILPs may provide a personalized nanomedicine strategy for the treatment of B-cell malignancies.

Monoclonal antibodies such as RIT are widely used for the treatment of B-CLL and other B-cell malignancies. However, B-CLL is often multifactorial in nature, and involves redundant or synergistic action of disease mediators. Consequently, blockade of multiple, different pathological factors and pathways may result in improved therapeutic efficacy\textsuperscript{232,233}. This result can be achieved by combining multiple drugs, antibodies and bispecific antibodies (BsAb). The dual targeting strategy including the combination of two Abs and a BsAb has already been demonstrated in preclinical models, and such an approach is also being investigated in the clinic, such as the anti-CD20/CD74 bispecific antibodies in mantle cell and lymphoma reported by Gupta et al., as well as the engineered two-in-one antibody targeting to VEGF and HER2 for breast cancer, etc\textsuperscript{231,234,235}.

One of the major challenges in targeted drug delivery for leukemia treatment is associated with limitations in the choice of diverse potential targeting molecules for individualized therapy. Antibody targeted nanoparticles such as ILPs are a promising strategy for the treatment of B-malignancies including CLL\textsuperscript{127,225}. In this study, we
demonstrate that CD37 antibody is a good target ligand for efficient B cell specific delivery. Anti-CD37 based dILPs showed a synergistic effect on targeted delivery to both B cell lines and B-CLL cells. Therefore, the anti-CD37 based dILPs may provide better therapeutic efficacy for the treatment of B-CLL.

The appropriate selection of mAb or mAb fragments are important to achieve effectively targeted delivery of ILPs. The receptor expression and internalization rate of mAbs are two key factors for determining the mAb selection. Generally, the higher the antigen density, the greater the therapeutic effects of ILPs. In addition, an antibody with a higher internalizing rate can often provide better delivery efficiency of ILPs. For B-cell malignancies, CD19, CD20 and CD37 have been recognized as good targets for immunotherapy\textsuperscript{121,227,246}. It was found that doxorubicin loaded anti-CD19 ILPs caused higher cytotoxicity than anti-CD20 ILPs in B-lymphoma cells (Namalwa) and its xenograft mouse model. Anti-CD37 is a highly B-cell specific antibody (\textbf{Figure 2.1}). CD37 is highly expressed on mature B cells but not on other hematopoietic cells\textsuperscript{161,229,230}. Furthermore, anti-CD37 provided a much higher internalizing rate when compared to CD19 and CD20. Hence, Anti-CD37 is an excellent candidate for the targeting delivery to B-CLL cells.

The targeting efficiency of ILPs is also well correlated with the antigen expression level (or antigen density) on the target cells\textsuperscript{7,225}. ILPs with antibody against a high antigen density can significantly improve the therapeutic responses. Given the difficulties and unpredictability associated with increasing the desired antigen density on the target cells for \textit{in vivo} applications, we resorted to dual targeting approach using two antibodies
against antigens expressed on the same cell. This has been demonstrated by the combination of immunotoxins showing significantly improved therapeutic responses compared with single immunotoxin therapy\(^7\). And dual-targeting can potentially overcome the mutation in one of the antigens with another one is still functioning. The upregulated cell surface antigen in tumor cells can serve as the second target that will be helpful to discriminate tumor cells from normal cells. In B-CLL cells, the single antigen expression on the cell surface generally varies from patient to patient. Thus, it is useful to combine several antibodies (e.g. CD37, CD19 and CD20) together to achieve higher binding and delivery efficiency of ILPs for individual patients. Additionally, the CD37 antibody based dual targeting may also increase the B-cell selectivity of ILPs because CD19, CD20 and CD37 antibodies are very B-cell type specific. To enhance the delivery efficiency of anti-CD37 mediated ILPs, two anti-CD37 based dILPs, anti-CD20/CD37 dILPs and anti-CD19/CD37 dILPs, were designed and constructed. We found that the composition of 50/50 on dILPs could provide the best results. The enhanced binding efficiencies of anti-CD19/CD37 and anti-CD20/CD37 dILPs over the single-mAb ILPs are clearly demonstrated. The combination of CD20/CD37 on dILPs showed the greatest delivery efficiency for leukemia lines, whereas the combination of CD19/CD37 was found to be superior for delivery of ILPs to B-CLL cells.

To identify candidate mAbs or mAb combinations that could efficiently bind to leukemia cells, we have used antibody microarrays to quantitatively characterize the binding efficiencies of various antibodies and their combinations. The combination of anti-CD19 and anti-CD37 showed the highest binding efficiency (Figure 2.2), which
agrees well with the delivery efficiency results of ILPs determined by flow cytometry. The combinatorial antibody microarray technology is simpler, faster and less expensive than the synthesis of many ILPs and dILPs, thus, it can be utilized as a tool to screen and select the optimal combination of antigen targets for specific B-CLL patients before preparing ILPs. The apparent antigen expression level measured by the antibody microarray should be useful for the design of “personalized” nanomedicine for B-CLL therapy and other B-malignancy.

Our recent work indicates that ILPs can provide a novel therapeutic strategy even without anti-cancer drug payload. Owing to the strong cross-linking effect caused by mAb on ILPs, the anti-CD74 ILPs showed the potent cell killing on B-CLL cells, although the underlying mechanisms remain unknown. Combination of monoclonal antibody therapy has shown synergistic effect in B cells leukemia, therefore, the dual mAb ligands on the same ILPs may potentially show the synergistic cell killing on B-CLL cells, which is partly confirmed in our study with enhanced cytotoxicity by adding CD37 to the original single CD19 or CD20 ILPs forming the CD37-based dILPs. The improved cytotoxicity may be attributed to the recently described SHP-1 dependent death of CLL cells caused by CD37 which remains to be investigated. Synergistic targeting of CD37 and CD19 by dILPs over the single-targeted ILPs suggested that the CD37 dual targeting strategy is likely to improve the clinical efficacy of dILPs and reach a broader patient population for B-CLL. Since liposomes are capable of carrying a variety of drugs, it is possible to encapsulate chemotherapy drugs for CLL such as fludarabine, flavopiridol and dexamethasone in ILPs and dILPs. Together, our results presented in this
study demonstrate the utility of such a dual ligand delivery approach. The application of such an approach may ultimately provide the ability to tailor ligand-targeted nanocarriers to fit the profile of individual CLL patient, allowing for patient-specific treatments.
Figure 2.1. Schematic of dual-antibody immunoliposomes (ILPs)

By simultaneously bearing both two B-cell specific antibodies, dual-Ab ILPs can more efficiently target to B cells expressing multiple antigens than single-Ab ILPs.
Figure 2.2. CD37 is an optimal candidate for targeted delivery to B-CLL cells
Figure 2.2 continued

C

(A) Selective binding of anti-CD37 to CD19+ B cells but not CD3+ T cells in human PBMC. For surface staining, the PBMC cells were incubated with or without CD37-FITC on ice for 30 min and washed twice with cold PBS. Then, the treated cells were further stained with PE labeled anti-CD19 (B-Cell marker) or PE labeled anti-CD3 (T-Cell marker) on ice for another 30 min and rinsed twice with cold PBS. (B) Mean fluorescent intensity of antigen expression levels on cell lines and (C) mean fluorescent intensity of antigen expression levels on CLL patients (n=6). (MFI: Mean Fluorescent Intensity)
Figure 2.3. Determination on internalization rates of various mAbs

Determination on internalization rates of various mAbs. Cells were incubated with fluorochrome labeled antibodies (CD20-PE, CD19-PE and CD37-PE) at 37°C for 15, 30, 60, 120 and 240 min and extracellular bound antibodies were removed with stripping buffer thus allowing detection of only internalized fluorochrome labeled antibody by flow cytometry. Appropriate IgG isotypes were used as negative controls. Internalization is defined as time-dependent increase in the Mean Fluorescent Intensity (MFI) after acidic washing by stripping buffer, which removed any surface bound antibody (n=3, mean ± SD).
Figure 2.4. Binding efficiency of B-CLL cells on combinatorial antibody microarrays
A library of antibody mixtures containing three antibodies (anti-CD19, anti-CD20 and anti-CD37) at equal total concentrations with all possible combinations was used for microarray assay. Total antibody concentration was maintained at 0.5mg/ml. The CFSE (carboxy-fluorescein diacetate, succinimidyl ester; Invitrogen, Carlsbad CA) fluorescence labeled B-CLL patient cells (1.5X10⁶/ml) were incubated with the Ab microarray. After removal of unbound cells, the quantified mean spot intensities were acquired and data (n=3, mean ± SD) was presented as relative binding efficiency. (A) Representative of Ab microarrays constructed by printing the CD19, CD20, CD37 antibodies and their dual combinations at different concentrations. The highest binding fluorescence intensity in the Ab microarray was set as 1. (B and C) Quantitative binding profiles of cells from nine B-CLL patients on the combinatorial antibody microarrays (n=9). Linear mixed effect models were used to estimate unrestricted covariance structures and produce robust hypothesis tests. Holm’s method was used to adjust for multiplicity.
Figure 2.5. Optimization of mAb ratio in dILPs for efficient delivery to leukemia cell lines and B-CLL cells
Figure 2.5 continued

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Figure 2.5 continued

(A) Effect of mAb ratio in dILPs on Daudi and B-CLL cells. Indicated ratios of anti-CD20 and anti-CD37 antibodies were immobilized onto liposomes with the post insertion method previously described. The binding efficiency was determined by MFI via flow cytometry detection (n=3, $P < 0.0001$ for all 100/0 vs. 50/50 or 50/50 vs. 0/100 in Daudi or B-CLL cells). Linear mixed effect models were used to estimate unrestricted covariance structures and produce robust hypothesis tests. Holm’s method was used to adjust for multiplicity. (B) Comparison of delivery efficiency of ILPs on B cell lines. Fluorescent labeled ILPs were incubated with different cell lines for 30 min followed by twice wash, and MFI were determined by flow cytometry. (C) Histogram comparison of anti-CD20 ILPs, anti-CD37 ILPs and CD20/CD37 (50/50) dILPs. MFI for CD20-ILPs, CD37-ILPs and CD20/CD37-ILPs were 12.3, 13.5 and 18.9, respectively. (D) Confocal microscopy analysis on the enhanced cellular uptake by CD20/CD37 (50/50) dILPs compared with the two single-Ab ILPs. Cells were incubated with anti-CD20 ILPs, anti-CD37 ILPs and anti-CD20/CD37 dILPs for 4 hrs at 37°C and washed twice with PBS, followed by fixation with 2% paraformaldehyde for 30 min. Anti-CD20/CD37 dILPs demonstrated the highest cellular uptake.
Figure 2.6. Delivery efficiency of various ILPs on B-CLL cells
Figure 2.6 continued

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(A and B) 1×10^6 B-CLL cells were incubated with indicated calcein encapsulated ILPs and dILPs encapsulating calcein for 1.0 hr at 37°C. Following washing away free ILPs, the fluorescence intensity was determined by flow cytometry (n=11). Linear mixed effect models were used to estimate unrestricted covariance structures and produce robust hypothesis tests. Holm’s method was used to adjust for multiplicity.
Figure 2.7. Cytotoxic effect on B-CLL cells with single anti-human CD37 ILPs in comparison with cross-linking effect

B-CLL cells (1×10^6) were co-cultured with indicated reagents for 24 hr and cytotoxicity was determined via annexin V FITC/ PI analysis by flow cytometry (n=6, mean ± SD). Data was analyzed by mixed effect model and p-values were adjusted by Holm’s method.
Figure 2.8. Enhanced cytotoxicity by anti-CD37 based dILPs in B-CLL cells

B-CLL cells (1×10^6) were co-cultured with indicated reagents with total of 0.5 μg/ml of mAbs for 24 hr and cytotoxicity was determined via annexin V FITC/ PI analysis by flow cytometry (n=9, mean ± SEM). Data was analyzed by mixed effect model and p-values were adjusted by Holm’s method.
CHAPTER 3

2A2 IgG-BASED IMMUNOLIPOSOMES AS A SPECIFIC TARGETED DELIVERY SYSTEM TO CHRONIC LYMPHOCYTIC LEUKEMIA

3.1 Introduction

As previously introduced, novel therapy options that efficiently and specifically target to malignant cells are still warranted for chronic lymphocytic leukemia (CLL) treatment. In the previous chapter, the anti-CD37 based dual-antibody immunoliposomes (dILPs) have been introduced for potential application of targeting delivery of drugs to treat B-CLL. However, though strongly expressed on the surface of malignant B lymphocytes, CD19, CD20 and CD37 are also expressed on normal peripheral B lymphocytes; Alemtuzumab, which targets to CD52 also is widely used for treating CLL patient as single agent or in combination with combined with chemotherapy. Expression of CD52 in B and T cells results in depletion of both leukemic B cells and immune-competent T lymphocytes. The off-target effects of the antibodies on unintended immune cells results in an immune compromised state leading to adverse effects such as hepatitis B virus reactivation and cytomegalovirus reactivation during their application in CLL treatment. These limitations have led to a search for novel
cell surface antigens with restricted expression on CLL cells.

The receptor-tyrosine-kinase-like orphan receptor 1 (ROR1) has been recently identified as a restricted B-CLL marker, as over 95% of CLL B cells express ROR1 on the surface while other normal mature healthy B lymphocytes do not\textsuperscript{170-172}. ROR1 acts as the Wnt receptor and undergoes complex post-translational modifications by glycosylation and mono-ubiquitination\textsuperscript{173} and silencing of ROR1 with specific siRNA induces apoptosis of CLL cells\textsuperscript{174}. Several monoclonal antibodies targeting to ROR1 have been developed and investigated, owing to its promising specificity to treat CLL\textsuperscript{176,177}. Compared to non-progressive patients, ROR1 is expressed significantly in progressive patients both at the gene expression and protein levels\textsuperscript{176}. Several monoclonal antibodies against ROR1 have been shown to induce apoptosis of CLL cells. The relatively low cell surface density indicates ROR1 as a potential target for armed rather than naked monoclonal antibodies\textsuperscript{177}.

2A2-IgG is a mouse monoclonal antibody, directed against N-terminal epitopes in the extracellular region of human ROR1\textsuperscript{169}. Immunoliposomes (ILPs) for cancer therapy are designed nanoparticles with incorporated onto the liposome surface, thus they can serve as efficient drug delivery vehicles that utilize the antibody specificity and liposomal delivery\textsuperscript{126,127}.

In this chapter, we describe synthesis and characterization of a novel ILP delivery system which incorporates 2A2-IgG onto the surface of liposome nanoparticles specifically targeting to CLL. By using a newly developed human ROR1 transgenic mouse that expressed ROR1 in B cells, the specificity of the 2A2-IgG-ILPs was further
investigated.

3.2 Materials and methods

3.2.1 Reagents

2A2-IgG was kindly provided by Christoph Rader, National Cancer Institute and this has been previously described\textsuperscript{169}. Egg phosphatidylcholine (Egg PC), methoxy-polyethylene glycol (MW≈2,000Da)-distearoyl phosphatidyl-ethanolamine (PEG-DSPE) were purchased from Lipoid (Newark, NJ). 2-Iminothiolane (Traut’s reagent), cholesterol (Chol) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). DSPE-PEG-maleimide (DSPE-PEG-mal) was obtained from Avanti Polar Lipids (Alabaster, AL).

3.2.2 Preparation of 2A2-ILPs

Liposomes were firstly synthesized with composition of Chol: Egg-PC: PEG-DSPE (molar ratio = 33.5: 65: 1.5) by rapidly injecting lipids dissolved in absolute ethanol into 10 times of phosphate buffered saline (PBS, pH7.4). 2A2-IgG was reacted with 10× Traut’s reagent at room temperature for 1 hr at pH 8.0 to yield 2A2-IgG-SH, thus it further reacted with DSPE-PEG-mal at a molar ratio of 1:10 to form micelles at pH 6.8 for 1 hr and followed by further incubation with liposomes at the lipid to 2A2-IgG ratio of 2000:1 at 37\textdegree{}C for 1 hr. Fluorescent-labeled liposomes (LP-calcein or LP-FAM-G3139) were prepared as previously described\textsuperscript{251}.
3.2.3 Cell separation and culture

CLL patient samples were obtained following informed consent under a protocol approved by the Institutional Review Board of The Ohio State University. Healthy donor blood in leukopacks was obtained from the Red Cross in Columbus. All B cells from CLL patients or healthy donors were isolated from freshly donated blood by ficoll (Ficoll-Paque Plus, Amersham Biosciences, Piscataway, NJ) density gradient centrifugation after negative selection by Rosette-Sep kit (Stem Cell Technologies, Vancouver, British Columbia, Canada) as per the manufacturer’s instruction. PBMCs from patients and healthy donors were obtained following ficoll separation of peripheral blood. All cells were incubated and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine and 1% penicillin-streptomycin at 37°C with 5% CO₂.

hROR1 transgenic mouse splenocytes were used to test the specificity of 2A2-ILPs. Spleens from either transgenic mouse or non-transgenic mouse were collected in sterile hood. Briefly, a cell strainer (BD Biosciences, San Jose, CA) was used to filter splenocytes from the mashed spleen tissues in PBS. After incubating the separated cells in ACK lysis buffer (154.95 mM NH₄Cl, 10mM KHCO₃, 0.01mM EDTA) on ice for 10 min, purified splenocytes were harvested at 1000 rpm for 10 min centrifugation.

3.2.4 Selectivity study

Cell lines or PBMC cells were co-incubated with 2A2-IgG followed by secondary PE-conjugated anti-mouse IgG antibody (Abcam, Cambridge, MA), as well as either anti-
CD19 FITC or anti-CD3 FITC antibodies (BD Biosciences, Catalogue #555412 and #555332, San Diego, CA) on ice for 30 min. Cells incubated with 2A2-ILP FAM ODN at 37°C for 30 min were then spun down and rinsed with cold PBS twice and stained with PE conjugated anti-CD19 or anti-CD3 (BD biosciences, Catalogue #555413 and #555333, San Jose, CA) on ice to identify B and T cell populations, respectively. Cells were then washed with PBS and analyzed by flow cytometry and analysis program Kaluza (Beckman Coulter, Inc., Brea, CA). For selectivity study with hROR1 transgenic mouse, mouse splenocytes were incubated with 2A2-ILP FAM ODN for 30 min at 37°C with PBS wash for twice and then were stained by PE conjugated anti-mouse B220 and anti-mouse CD8 (BD Biociences, San Jose, CA) to identify B and T cell populations, respectively. Biotinlyated anti-human ROR1 (anti-hROR1) antibody (R&D systems, Catalogue #BAF2000, Minneapolis, MN) and corresponding streptavidin Phycoerythrin (PE) secondary antibody (R&D systems, Catalogue #F0040, Minneapolis, MN) were used as controls. Non-transgenic littermates or C57BL/6 mice were used as controls for transgenic animals as needed.

3.2.5 Preparation of Alexa fluor-488 labeled antibody

2A2-IgG was fluorescently labeled by conjugating to an amine-reactive compound, Alexa fluor 488 5-SDP ester (Invitrogen, Carlsbad, CA). 2A2-IgG solution was exchanged to sodium bicarbonate buffer by dialysis against 0.1M sodium bicarbonate solution within Slide-A-Lyzer Dialysis Unit (Thermo scientific, Rockford, IL). Alexa fluor 488 5-SDP ester was added into 2A2-IgG solution and incubated at room
temperature for 1hr followed by dialysis against PBS overnight. The resultant 2A2-IgG-Alexa 488 was collected and sterilized via 220 nm polymer membrane filter and stored at 4°C. In parallel, polyclonal mouse IgG (mIgG) was used to obtain Alexa fluor-488 mIgG.

### 3.2.6 Internalization study

The internalization rate of antibodies was determined by measuring and comparing the mean fluorescent intensity of fluorescently-labeled antibodies, FITC-CD19 (BD Biosciences, Catalogue #555412, San Jose, CA), FITC-CD37 (Beckman Coulter, Catalogue #IM0457, Brea, CA) and biotinylated 2A2-IgG (kindly provided by Dr. Christoph Rader, NCI) followed by streptavidin-conjugated FITC (R&D systems, Catalogue #F0030, Minneapolis, MN). 2×10⁶ cells were stained with approximately 1μg/mL antibodies on ice for 30 min in PBS. Cells were washed twice with ice-cold PBS followed by centrifugation. Cells were then incubated at 37°C for different time points from 15 min to 4 hr. At each time point, cells were placed on ice and surface-bound antibodies were removed by 500 µl of stripping buffer (100 mM glycine, 100 mM NaCl (pH 2.5)) at room temperature for 2 min before washing twice with ice-cold PBS. Appropriate IgG isotypes were used as negative controls. Internalization is defined as time-dependent increase in the mean fluorescent intensity (MFI) detected by flow cytometry after removal of any surface bound antibodies and normalized to MFI at 30 min on ice as the 100% antibody expression on the cell surface.
3.2.7 Laser-scanning confocal microscopy

Laser scanning confocal microscopy was used to further examine binding and internalization of 2A2-IgG. Cells were treated with Alexa fluor-488 2A2-IgG or anti-CD19 FITC antibody for 20 min on ice followed by incubation at 37°C for 15 min. Unbound antibodies were washed away by stripping buffer and fixed with 2% paraformaldehyde for 30 min. Cells incubated with 2A2-IgG on ice without stripping buffer wash was used as a negative control. Nuclei were stained with 20 μM DRAQ5™ for 5 min at room temperature. Cells mounted on glass slide were monitored and analyzed by Zeiss 510 META Laser Scanning Confocal Imaging Systems and LSM Image software (Carl Zeiss Microimaging, Inc., NY) under green and blue fluorescence for Alexa fluor-488 and DRAQ5, respectively, with 600X magnification.

3.2.8 Evaluation of apoptosis and cell viability

Cell apoptosis was determined by annexin V-FITC / propidium iodide (PI) staining (BD Bioscience, San jose, CA) followed by flow cytometry analysis. Cells (1×10⁶) were treated with the corresponding agent for 24 hr and stained with annexin V-FITC / PI according to manufactures’ protocol. Results were presented as % live cells, defining by % annexin V⁻ and PI⁻ cells of treated groups.

3.2.9 Statistical analysis of data

Data were analyzed by mixed-effect models, accounting for observational dependencies among various treatments. Holm’s method was used to adjust multiplicity
for primary end points. A significance level of $\alpha = 0.05$ was used for all tests.

3.3 Results

3.3.1 2A2 IgG targeting to hROR1 showed specificity to CLL cells but not normal B cells

It has been reported that ROR1 is selectively expressed on B-CLL cells but not other normal B cells, normal blood cells or normal tissues$^{171}$. To confirm the specificity of 2A2-IgG, B cells or PBMC from CLL patients or healthy donors, cell lines available in our lab, including Jurkat, Raji, RV4;11, Mino, 697, Mec-1, Mec-2, Ramos and Jeko, were treated with primary antibody (mouse IgG / goat anti-hROR1 IgG / mouse anti-human 2A2 IgG) and secondary antibody (anti-mouse IgG PE / anti- goat IgG PE) on ice for 30min. After each binding step with a indicated antibodies the cells were washed with ice-cold PBS twice. The secondary IgG PE were used as negative controls and the commercial available hROR1 IgG was used as a positive control. Cell lines tested included Mec-1, Mec-2, Jeko, Ramos, Raji, Mino, RS4;11, Jurkat, and 697. Among all cell lines tested (Figure 3.1A), 697, Jeko, Mino and RS4;11 cells showed hROR1 expression on the cell surface while Jurkat, Mec-1, Mec-2 and Ramos did not show hROR1 expressed on cell surface with either 2A2-IgG or anti-hROR1 IgG. Raji cells that expressed low levels of hROR1 was only stained with commercial anti-hROR1 antibody but not 2A2-IgG

Similarly, B cells from healthy donors and CLL patients were stained by 2A2-IgG
and anti-hROR1 IgG followed by corresponding secondary antibody and it confirmed that normal B cells did not express ROR1 but only B cells from CLL patients had surface expression of ROR1 (Figure 3.1B). Furthermore, the investigation of ROR1 expression on PBMC cells from CLL patients revealed that there is a population of cells expressing ROR1 which are stained by 2A2-IgG and anti-hROR1 antibody (Figure 3.1C), which presumably includes B cells as reported previously. Based on all current results, the binding efficiency, selectivity and affinity of 2A2-IgG was similar to the positive control anti-hROR1 antibody.

3.3.2 2A2-IgG is an internalization antibody

An requirement for an ideal targeting antibody used in generating a immunoliposomal formulation is dictated by its ability to be internalized by the targeted cells. To evaluate the suitability of its use in ILP formulation, the internalization rate of 2A2-IgG was evaluated with B-CLL cells. Two other relevant internalization antibodies, anti-CD19 and anti-CD37 antibodies, were used as controls. As shown in Figure 3.2A, 15% to 20% of anti-CD19 and anti-CD37 antibodies were internalized into cells at 15 min, but till 4 hr, the maximum internalization rate was only around 30%. Meanwhile, 40% 2A2-IgG has already been significantly internalized by B-CLL cells at 60 min compared to anti-CD19 or anti-CD37 antibodies (n=7, P<0.001). 2A2-IgG was continuously internalized by cells reaching >60% of antibody internalized compared to the original total amount of antibody binding on cell surface on ice as the basis. The high efficiency of internalization validated the suitability of 2A2-IgG as an excellent targeting antibody. The confocal
microscopy image in Figure 3.2B also showed that 2A2-IgG was internalized by cells after 15 min incubation at 37°C, just like the well-known internalizing anti-CD19 antibody did.

3.3.3 2A2-ILPs selectively bind to B-CLL cells

In section 3.1, 2A2-IgG, anti-hROR1 antibody, was shown to selectively bind to B-CLL cells. In an attempt to develop and evaluate 2A2-ILPs in CLL, we synthesized 2A2-ILP-calcein fluorescent labeled liposomal particles for further investigation on the selectivity of 2A2-ILPs. PBMCs from CLL patients or normal B cells from healthy donors were co-incubated with 2A2-ILP calcein, anti-CD19 PE for identifying B cells and anti-CD3 PE for identifying T cells. As seen in Figure 3.3A, in PBMCs from CLL patients, B cells and T cells can be clearly distinguished by anti-CD19 and anti-CD3 antibodies, respectively. 2A2-ILP calcein was co-stained with anti-CD19 PE+ B cells but not with anti-CD3 PE+ T cells, resembling that 2A2-ILPs selectively bind to hROR1+ B-CLL cells only. In Figure 3.3B, normal B cells did not uptake any 2A2-ILP calcein consistent with the lack of expression of hROR1 in normal B cells. Comparing the mean fluorescent intensity of 2A2-ILP calcein among B-CLL cells, normal B cells and T cells, B-CLL cells were dominantly selected by 2A2-ILP calcein while normal B cells or T cells did not uptake 2A2-ILPs with low mean fluorescent intensity (n=8, B-CLL cells vs. normal B cells or T cells, P<0.0001 ).
3.3.4 2A2-IgG does not induce cytotoxicity

It has been previously reported that 2A2-IgG alone was not able to induce significant direct cytotoxicity, CDC or ADCC in primary CLL cells or hROR1-positive cell lines. Since the cross-linking effect of anti-CD19 and anti-CD37 antibodies was observed in Chapter 2, we evaluated the direct cytotoxic effects of 2A2-ILP. \(10^6\) cells of cell lines, hROR1+ B-CLL cells and normal B cells were treated with 2A2-IgG, 2A2-IgG cross-linked with anti-Fc antibody or 2A2-ILPs at 1.5 \(\mu\)g/mL for 24 hr and cytotoxicity was evaluated by flow cytometry via Annexin V/PI assay. As shown in Figure 3.4A to B, 2A2-IgG with or without cross linker did not induce cytotoxicity in cell lines, including Jurkat, Jeko, 697 and Mino cells, or human cells.

3.3.5 2A2-ILPs can be specifically delivered to B220+ cells in hROR1 transgenic mice

In order to further characterize human ROR1 targeted nanoparticle we employed our recently generated human ROR1 transgenic mouse model to evaluate the efficiency and specificity of 2A2-ILPs to hROR1 positive cells \textit{in vivo}. Mouse splenocytes were separated as described above and incubated with 2A2-ILP FAM ODN for 30 min at 37°C to allow the binding and cellular uptake by the targeted cells. Anti-mouse B220 conjugated PE was used to stain B cells while anti-mouse CD8 antibodies were used to stain T cells. Cellular binding studies using fluorescence-labeled FAM ODN showed selective binding activity of 2A2-ILPs to splenocytes from hROR1 transgenic mouse but not that from non-transgenic mouse (Figure 3.5A). The specific delivery of 2A2-ILPs
was demonstrated as well in the two-color flow cytometry. The majority cells from hROR1 transgenic mouse that were bound by 2A2-ILP FAM ODN were B220+ cells, but only little 2A2-ILP FAM ODN were delivered to CD8+ cells. As shown in Figure 3.5B, 2A2-ILP FAM ODN exhibited higher delivery in B220+ cells from hROR1 transgenic mouse compared to non-transgenic mouse with more than ten-fold changes. In contrast to the B220+ cells, CD8+ cells from either hROR1 transgenic or non-transgenic mouse showed minimal difference with 2A2-ILP FAM ODN.

3.3 Discussion

We have described here a novel strategy to enhance the efficiency and specificity of potential drug delivery application in CLL based on hROR1 targeting 2A2-IgG conjugated ILPs. Our results showed that as an anti-human ROR1 monoclonal antibody, 2A2-IgG selectively binds to CLL cells. Also, several leukemic cell lines were demonstrated expressing hROR1 on the surface. However, Raji, performs as the most commonly used for xenograft model for CLL study did not show high expression level of hROR1. Thus, B-CLL cells were chosen for further characterization of 2A2-ILPs as well as related drug delivery study. The internalization study revealed that 2A2-IgG is a fast internalizing antibody with 60% of bound antibody internalized after 4 hr incubation, which is higher than that of anti-CD19 and anti-CD37 antibodies, thus validating the use of 2A2-IgG for ILP drug delivery for enhanced selectivity and efficiency.

Consistent with the previous reported research, 2A2-IgG does not induce cytotoxicity as a single reagent, and no cross-linking effect was observed in cell lines or B-CLL cells,
irrespective of the presence of cross-linker or liposomes\textsuperscript{169}. Such feature indicated that 2A2-IgG would be more suitable as conjugates, because 2A2-IgG can perform as a purer targeting reagent rather than dual-functional antibodies as anti-CD37 does with both therapeutic and targeting effects. On the other hand, due to the limited cell surface density, hROR1 is considered to be preferably as the target for armed rather than naked monoclonal antibodies\textsuperscript{177}, though researchers have also described several mAbs that induced killing activity by ADCC or CDC\textsuperscript{176}.

The specificity of 2A2-ILPs as a drug delivery system was also evaluated using human cells. Similar to free 2A2-IgG, 2A2-ILPs labeled with fluorescent FAM ODN selectively bind to B-CLL cells but not to either normal B cells or human T cells. In a novel B cell specific promoter/enhancer driven human ROR1 transgenic mouse model, 2A2-ILPs also showed selectivity in binding to splenic B cells from human ROR1 transgenic mouse but not to that from non-transgenic mouse.

Following detailed characterization of the 2A2-ILPs we have developed, a model drug can be chosen to further evaluate the efficiency of 2A2-ILPs as an emerging drug delivery system for CLL treatment. The hROR1 transgenic mouse can be used for future \textit{in vivo} therapeutic study with prolonged investigation on the depletion efficacy hROR1+ cells with 2A2-ILP delivery system. Furthermore, it can also be mated with other leukemia model, such as TCL1 transgenic mouse, to create a double transgenic mouse model which is more appropriate for preclinical study.

Compared to the current antibodies available in clinical use, anti-ROR1 antibodies meet its unique specificity as well as the improved efficiency requirements. The anti-
ROR1 mAbs against cysteine-rich (CRD) or kringle (KNG) domains or ROR1 were reported to be significantly more effective than rituximab\textsuperscript{176}. And the targeting specificity is also obviously shown in other studies as well as our 2A2-ILPs related characterization.

The delivery system described here can be extended to other therapeutic reagents and small molecules that can be incorporated to suitable liposomal formulation. Also the newly developed 2A2-ILPs can be applied to not only CLL treatment but also to other non-Hodgkin lymphomas and B cell malignancies which express ROR1 surface antigen.
Figure 3.1. Surface expression of hROR1
Cells (1×10^6) were co-incubated with 2A2-IgG followed by secondary PE-conjugated anti-mouse IgG antibody on ice for 30 min. Cells were then washed with PBS and analyzed by flow cytometry and the analysis program Kaluza with excess numbers processed. (A) Surface expression of 2A2 IgG was tested with different cell lines including Jurkat, Raji, RV4;11, Mino, 697 and Jeko. (B) Normal B cells from healthy donors (left panel) were not stained by anti-hROR1 antibody or 2A2-IgG while CLL B cells (right panel) were all positive to both 2A2-IgG and anti-hROR1 IgG. (C) Representative of PBMC cells from two CLL patients showing hROR1 expression in PBMC.
A higher internalization percentage was reached by 2A2-IgG on B-CLL cells (n=7, 2A2-IgG vs. CD19 or CD37, mean ± SD, P<0.001). Data were analyzed by mixed-effect models, accounting for observational dependencies among various treatments. Holm’s method was used to adjust multiplicity for primary end points. Cells were treated with Alexa fluor-488 2A2-IgG for 20 min on ice followed by incubation at 37°C for 15 min. Unbound antibodies were washed away by stripping buffer and fixed with 2% paraformaldehyde for 30 min. Cells incubated with 2A2-IgG on ice without stripping buffer wash was used as a negative control. Nuclei were stained with 20 μM DRAQ5™ for 5 min at room temperature. Cells mounted on glass slide were monitored and analyzed by Zeiss 510 META Laser Scanning Confocal Imaging Systems and LSM Image software (Carl Zeiss Microimaging, Inc., NY) under green and blue fluorescence for Alexa fluor-488 and DRAQ5, respectively, with 600X magnification.
Figure 3.3. Selectivity binding of 2A2-ILPs to B-CLL cells
Figure 3.3 continued

B

C

\[ P < 0.0001 \]

\[ P < 0.0001 \]

Continued
Cells (1×10^6) were co-incubated with 2A2-ILP calcein and/or antibodies at 4°C for 30 min. After washing away free ILPs or antibodies, MFI of 2A2-ILP and antibodies were determined by flow cytometry. (A) PBMC cells from CLL patients (n=8) and (B) normal B cells from healthy donors (n=8) with anti-CD19 PE and anti-CD3 PE antibodies to distinguish B cells and T cells, respectively. (C) Mean fluorescent intensity showing of binding affinity of 2A2-ILP calcein to CLL B cells, normal B cells and T cells (n=8, *P*<0.0001). Data were analyzed by mixed-effect models, accounting for observational dependencies among various treatments. Holm’s method was used to adjust multiplicity for primary end points.
Figure 3.4. Failure of 2A2-IgG to induce cytotoxicity

Cells (1×10^6) were treated with the corresponding agent at 1.5 µg/mL of 2A2-IgG for 24 hr and stained with annexin V-FITC/PI followed by flow cytometry. Results were presented as % live cells, defining by % annexin V and PI cells of treated groups. (A) cell lines (n=3, mean ± SD, P>0.05) or (B) human cells (n=6, mean ± SD, P>0.05). Data were analyzed by mixed-effect models, accounting for observational dependencies among various treatments. Holm’s method was used to adjust multiplicity for primary end points.
Figure 3.5. Selective binding of 2A2-ILPs to splenocytes from hROR1 transgenic mouse but not non-transgenic mouse.
hROR1 transgenic mouse splenocytes were separated and used to testify the specificity of 2A2-ILPs. Splenocytes (1×10^6) from transgenic or non-transgenic mice were incubated with 2A2-ILP FAM ODN for 30 min at 37°C. Anti-mouse B220 and CD8 PE were used to distinguish B cells and T cells, respectively.
CHAPTER 4

DESIGN AND CHARACTERIZATION OF NOVEL LIPID-BASED OF OSU-2S AND FTY720 FOR POTENTIAL APPLICATION OF TARGETING DELIVERY THERAPY

4.1 Introduction

In the previous two chapters, a novel CD37-based dual-antibody immunoliposomal and a CLL specific 2A2-IgG immunoliposomal delivery systems were introduced, respectively. The characterizations were all based on delivery systems themselves without drug payloads. However, as a successful drug delivery system, it is also necessary to encapsulate drugs to realize multi-functions including targeting delivery of the loaded drug to reduce off-target side effects and increase the drug efficiency.

FTY720 (Fingolimod) is a synthetic compound based on modification of the natural immunosuppressant, myriocin (ISP-1)\textsuperscript{178,179} was the first orally bioavailable drug for patients with multiple sclerosis (MS) to reduce relapse and delay disease progression to disability\textsuperscript{180}. In our related preclinical studies, FTY720 showed promising \textit{in vitro} and \textit{in vivo} activity in leukemia and lymphoma disease models, including chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), NK-cell leukemia, and mantle cell lymphoma (MCL)\textsuperscript{181, 182, 183, 184, 185}. Several studies have provided evidence that FTY720
induces T cell apoptosis both in vitro and in vivo\textsuperscript{185,191-194}, which is unfavorable for treatment of B-cell malignancies. Previous in vitro studies indicated that FTY720 induces down-modulation of Mcl-1 but not Bcl-2 in CLL cells, and its toxicity in CLL cells is dependent on activation of PP2a but not S1P receptor.

OSU-2S was synthesized by Chen and colleagues as a novel derivative of FTY720\textsuperscript{194,200,201}. To overcome the limitation of FTY720, such as its immunosuppressive properties and related side effects for cancer therapy, OSU-2S was designed to avoid interaction with S1P receptor, and it therefore lacks immunosuppressive activities\textsuperscript{252,253}.

In preclinical assay, OSU-2S induced cytotoxicity in cells representing CLL, MCL, ALL and T cells\textsuperscript{253}. Differently from FTY720, OSU-2S has demonstrated not phosphorylated by Spk2, and devoid of S1P1 activity. Instead, OSU-2S uniquely targeted protein kinase C, involving caspase 3, 8 and 9 signaling, increased LC3-II levels, and induced phosphorylation of Ser 591 of the SHP1 phosphatase\textsuperscript{183,194,253}. Though the detailed mechanism of action for OSU-2S remains to be clarified, existing data on OSU-2S confirm that it is a potent anti-tumor and nonimmunosuppressive analogue of FTY720.

FTY720 and OSU-2S are sparingly soluble in aqueous buffer. Notably, free FTY720 and OSU-2S are not stable in aqueous buffer/solution and requires daily fresh preparation\textsuperscript{193}. Furthermore, alternative formulations that enable targeting of tumor cells without impacting T lymphocytes and other non-target tissues will be necessary.

In this chapter, liposomal carriers for FTY720 (LP-FTY720) and OSU-2S (LP-OSU-2S) were synthesized and characterized, including their physicochemical, morphological and pharmacokinetic properties. The development and validation of a sensitive and
selective liquid chromatography-tandem mass spectroscopy (LC-MS/MS) method for simultaneously quantify FTY720 and OSU-2S in mouse plasma will be described. After applying this method to characterize pharmacokinetics in mice dosed with FTY720 and OSU-2S within different formulations, either through intravenous and intraperitoneal routes of administration, a comprehensive PK profile of FTY720 and OSU-2S will be presented. The pharmacokinetic data for this novel agent indicates OSU-2S has favorable disposition with relatively low clearance and long exposures of active drug concentrations. This and additional pharmacokinetic data will support optimization of dose regimens for future preclinical efficacy studies. Finally, the application of LP-FTY720 and LP-OSU-2S to the ILPs delivery system showed the promising efficiency of the targeting delivery systems with these two model drugs.

4.2 Materials and methods

4.2.1 Materials

FTY720 and OSU-2S were synthesized at The Ohio State University Comprehensive Cancer Center Medicinal Chemistry Core Facility as previously described\textsuperscript{254,194}. Sphingosine C17 (Sph-17) was purchased from Avanti Polar Lipids (Alabaster, AL). LC-grade ethyl acetate, water and methanol were purchased from Fisher Scientific (Waltham, MA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO). Mouse plasma was obtained from Lampire Biological Laboratories, Inc. (Pipersville, PA). Egg phosphatidylcholine (Egg PC) and methoxy-polyethylene glycol (MW ~ 2,000Da)-
distearoylphosphatidylethanolamine (PEG-DSPE) were obtained from Lipoid (Newark, NJ). Cholesterol (Chol), Mal-PEG-DSPE and Sphingosine C17 (Sph-17) was purchased from Avanti Polar Lipids (Alabaster, AL). 2-Iminothiolane (Traut’s reagent) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Chemical structure of FTY720, OSU-2S and Sph-17 were shown in Figure 4.1.

4.2.2 Experimental animals

ICR mice with weight ranging from 25 to 30 g (age 6 weeks) were purchased from Charles River Lab (Wilmington, MA). ROR1 transgenic mice were developed in the lab based on Black C57BL/6 wild type mice. All work performed on animals was approved by the IACUC at The Ohio State University.

4.2.3 Liposomal drug preparation

LP-FTY720 and LP-OSU-2S were prepared by ethanol injection method. FTY720 or OSU-2S was dissolved in absolute ethanol at 10 µM. Lipids with composition of Chol: Egg-PC: PEG-DSPE (molar ratio = 33.5: 65: 1.5) was dissolved in ethanol at 40mg/ml. Fully mixed lipids and FTY720 or OSU-2S at a weight ratio of 20:1 were rapidly injected into phosphate buffered saline (PBS, pH7.4) with 9 times more volume to form LP-FTY720 or LP-OSU-2S. Empty liposomes were prepared with equal volume of ethanol as substitute for FTY720 or OSU-2S. The liposomes were then dialyzed against HEPES-buffered saline (HBS, 145mM NaCl, 20mM HEPES pH7.4) overnight using a DispoDialyzer (Spectrum Labs, Rancho Dominguez, CA) with a molecular weight cut-
off of 10,000 Da to remove non-encapsulated drug. For fluorescent liposomes, fluorescein modified FAM-G3139 (Alpha DNA, Inc., Montreal, Canada) dissolved in PBS were mixed with lipids and FTY720 or OSU-2S in ethanol at G3139: lipids (weight ratio) = 1: 12.5 followed by dialysis against PBS to remove free G3139 and drug. Immunoliposomes (ILPs) with monoclonal antibodies (anti-CD19, anti-CD20 or anti-CD37) (anti-CD19 and anti-CD37 from BD Biosciences Pharmingen Catalogue #555456 and #555410, San Jose, CA; anti-CD20 used was rituximab) and IgG (negative control) immobilized onto the surface of LP-FTY720 were prepared as described previously with a post-insertion method\textsuperscript{132}. 2A2-IgG provided by our collaborators were immobilized onto the surface of LP-OSU-2S\textsuperscript{169}. Briefly, antibody (Ab) was reacted with 10×Traut’s reagent (2 h, room temperature) to yield Ab-SH at pH 8.0. Ab-SH was then reacted to form micelles of Mal-PEG-DSPE at a molar ratio of 1:10 at pH 6.8, and then incubated with LP-drug for 1 h at 37°C. The ILPs were further purified on a Sepharose CL-4B size exclusion column to remove free antibody.

4.2.4 Characterization of LP-FTY720 and LP-OSU-2S

The size distribution of LP-FTY720 and LP-OSU-2S was determined by dynamic light scattering (DLS) (Brookhaven Instruments Corporation BI200SM, Holtsville, NY) using the laser wavelength of 632.8 nm at 90° detection angle. Three batches of LP-FTY720 and LP-OSU-2S were measured independently and the mean diameter by volume ± standard deviation was reported. The zeta-potential of LP-FTY720 and LP-OSU-2S were evaluated by a ZetaPALS zeta-potential analyzer (Brookhaven Instruments
Corporation, Holtsville, NY). Liposomes were diluted in 1 × PBS buffer and measured independently with 10 runs each at room temperature. The Smoluchowski model was used to calculate the zeta-potential and the mean ± standard deviation was reported.

4.2.5 Morphology study

The morphology of nanoparticles were observed with a cryogenic transmission electron microscope (Cryo-TEM). Briefly, the sample containing LP-drug was applied to a glow-discharged holey carbon/formvar film coated grid in a controlled environment vitrification system at constant humidity and temperature of 25°C. Samples were immediately plunged into a bath of liquid ethane for vitrification after gently blotting. Maintained at -172°C, the prepared grid was transferred to Gatancryo-holder and imaged in low-dose mode with FEI Tecnai G2 Spirit TEM equipped with bioTWIN optics operating at 120 kV. Images were recorded using a Gatan CCD camera.

4.2.6 Stock, standard and quality control sample preparation

Stock solutions (1mg/mL) of FTY720, OSU-2S or Sph-17 were prepared in methanol and stored at -80°C. Standard solutions of FTY720 and OSU-2S were prepared by dilution of stock solutions in 50% methanol with concentrations ranging from 10 to 30,000 ng/mL. Standard and quality control (QC) plasma samples were prepared by spiking 10μL standard solution into 100μL mouse plasma containing 10 ng/mL Sph-17. QC samples were prepared at 10, 100 and 1000 ng/mL for validation.
4.2.7 Sample processing

Every 100 μL of mouse plasma samples, either for validation or pharmacokinetic studies, were spiked with IS (final concentration of 10ng/mL). Samples were mixed well via vortex and were extracted with 1 mL of ethyl acetate by shaking for 60 min. After centrifugation at 11,000×g for 10 min, samples were placed on dry ice for 1 min to solidify the lower plasma layer, and the upper ethyl acetate layer was transferred and collected in a glass tube for evaporation under a gentle stream of nitrogen. Residues in each tube were reconstituted with 120 μL of 50% methanol and were transferred into microcentrifuge tubes for another 10 min centrifugation at 11,000×g. Supernatants were transferred to autosampler vials for analysis.

4.2.8 Liquid chromatography-tandem mass spectrometry analysis

A rapid and robust liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the quantification of FTY720 and OSU-2S in mouse plasma on a Shimadzu LC system (comprising two LC-20 AD pumps, a SIL-20AC autosampler, DGU-20A5 degasser and a CBM-20A system controller) coupled to a TSQ Quantum Ultra mass spectrometer with electrospray ionization source. LC separation was performed on a ZORBAX Extend C18 column (50×2.1mm I.D, 3.5 μm particle size) at room temperature with a total run time of 4 min for each sample. A linear gradient elution was used with water/0.1% formic acid as mobile phase A and methanol/0.1%formic acid as mobile phase B at a flow rate of 200 μL/min. The gradient was as follows: 0.0 min (30% A and 70% B), 1.0 min (30% A and 70% B), 3.0 min (100% B), 4.5 min (100% B), 4.6
min (30% A and 70% B) and 6.0 min (30% A and 70% B). MS monitoring was carried out in positive ion mode with selected reaction monitoring. The monitored ion transition channels were m/z 308.00→255.00 for FTY720, 322.00→268.00 for OSU-2S and 286.00→238.00 for Sph-17 with 30 ms scan times. MS parameters were optimized by a direct infusion of 10 μg/mL FTY720, OSU-2S and internal standard Sph-17 at a flow rate of 10 μL/min via a syringe pump. The ion spray voltage, skimmer offset and tube lens offset were set to 5000, 10 and 100 V, respectively. Argon collision gas pressure was 1.5 mTorr, and nitrogen sheath and auxiliary gas were set to 30 and 15 (arbitrary unit), respectively. Collision energy was 15% for FTY720, 13% for OSU-2S and 16% for Sph-17. The ion transfer tube was maintained at 325ºC.

4.2.9 Plasma assay validation

The intra-day and inter-day precision of FTY720 and OSU-2S were determined in six replicates of QC samples at concentrations of 10, 100 and 1,000 ng/mL in the same day or on the second day after sample preparation, respectively. Mean concentrations and coefficients of variation (relative standard deviation; %CV) were calculated from the six replicates. The accuracy of the assay was determined by comparing the corresponding calculated mean concentrations with nominal concentrations. The lower limit of quantification (LLOQ) was defined as the lowest concentration that could be detected with accuracy between 80% and 120% and precision less than or equal to 20%.

Matrix effects and recovery of FTY720 and OSU-2S from mouse plasma were determined in three separate batches of QC samples. Batches (a) prepared directly in 50%
methanol with IS, (b) sample and IS spiked with 50% methanol during reconstitution, post extraction and drying of mouse plasma, and (c) extract of OSU-2S and IS in mouse plasma followed by reconstitution in 50% methanol. The matrix effects and recovery of FTY720, OSU-2S and IS were calculated by the ratio of peak areas from batches (c)/(b) and (b)/(a), respectively.

For short-term stability, QC samples were prepared in triplicate and evaluated after 6h. For long-term stability, QC samples were prepared in triplicate and stored at -80°C for 21 days until analysis. For freeze-thaw stability, triplicate QC samples were frozen at -80°C and thawed at room temperature followed by immediate return to -80°C with three freeze-thaw cycles.

4.2.10 *In vitro* release of FTY720 and OSU-2S from the liposomes

Rates of drug release from liposomes were studied by measuring retention of FTY720 or OSU-2S in the liposome fraction. LP-FTY720 or LP-OSU-2S was dialyzed against PBS (pH 7.4) or 10% fetal bovine serum (FBS) in RPMI 1640 media at 37°C. Samples were collected at different time points (0, 30min, 1, 2, 4, 8, 12 and 24 h), and liposomes were lysed with methanol. Concentration of FTY720 or OSU-2S was measured by LC-MS/MS as described above. The percentage of drug remaining in the liposome fractions was calculated by comparing to the original total drug amount.

4.2.11 Apoptosis assay

Primary B-cells from CLL patients were separated and incubated as previously
described\textsuperscript{182}. Apoptosis activity was determined in primary CLL cells after 24-hr incubation with indicated agents by annexin V-FITC/PI staining. The degree of apoptosis induction is displayed as the total percentage of annexin V-FITC and PI double negative cells normalized to untreated condition.

4.2.12 Immunoblot analysis

Whole cell lysates were prepared using lysis buffer (10mM Tris pH 7.4, 150 mM NaCl, 1\% Triton X-100, 1\% deoxycholic acid, 0.1\% SDS, 5mM EDTA) containing protease and phosphatase inhibitors. Proteins were analyzed by immunoblot following standard procedures\textsuperscript{195} using anti-Mcl-1 antibody (S-19) from Santa Cruz Biotech (Catalogue #sc-819, Santa Cruz, CA). Digital quantification was completed using a ChemiDocstation (Bio-rad, Hercules, CA).

4.2.13 Mouse pharmacokinetic studies

All work performed on animals was approved by the Institutional Animal Care and Use Committee at The Ohio State University. No signs of toxicity in the mice were observed throughout all studies. Pharmacokinetic analyses were performed using WinNonlin Professional software (v. 5.2, Pharsight, Mountain View, CA).

For FTY720, plasma pharmacokinetics of free FTY720 and LP-FTY720 were evaluated in ICR mice (Charles River Lab, Wilmington, MA). In groups of five, mice were intravenously injected with FTY720 at a dosage of 5 mg/kg body weight through tail vein. Blood samples were collected in EDTA-containing tubes at various time points,
and plasma was immediately isolated by centrifugation and stored at -80°C. FTY720 in 50μL plasma was mixed with 50 μL of blank mouse plasma and internal standard (final concentration 1μM), extracted with ethyl acetate, dried and reconstituted in 120 μL of 50% methanol. Samples were analyzed using LC-MS/MS as described above.

For OSU-2S, sterile dosing solutions of OSU-2S were prepared as follows: OSU-2S was first dissolved in DMSO with vortex followed by adding Cremophor EL and phosphate buffered saline (PBS, pH 7.4) to produce 1.0 mg/mL OSU-2S in 10% DMSO, 18% Cremophor EL and 72% PBS. Female ICR mice 6 weeks of age (Harlan Laboratories, Madison, WI), were dosed with OSU-2S at 5 mg/kg body weight (ranging from 25-33g) via intravenous tail vein or intraperitoneal injection. At 15 different time points of 5, 10, 20, 30, 45 min, 1, 1.5, 2, 4, 8, 12 and 24h post dosing, 5 mice were sacrificed by CO2 asphyxiation and blood was collected via cardiac puncture followed by transferring into heparinized tubes. After centrifugation, plasma was separated and collected for storage at -80°C till processing and analysis.

For 2A2-ILP-OSU-2S pharmacokinetic study, non-targeted LP-OSU-2S and 2A2-ILP-OSU-2S were intravenously injected into C57BL/6 mice and ROR1 transgenic mice, respectively, at the dosing level of 5 mg/kg of OSU-2S. At different time points within 8 hr, one mouse from each group was sacrificed and blood was collected via cardiac puncture and plasma was separated after centrifugation in a heparinized tube for LC/MS-MS analysis.
4.2.14 Statistical analysis

In pharmacokinetic study, data were analyzed by Student’s t-tests using MiniTAB software (Minitab Inc., State College, PA). For cell line assays, linear mixed effect models were used to estimate unrestricted covariance structures and produce robust hypothesis tests. Holm’s method was used to adjust for multiplicity. A significance level of $\alpha = 0.05$ was used for all tests.

4.3 Results

4.3.1 Design and preparation of liposomal OSU-2S and FTY720

The lipids chosen for our study (Chol: Egg-PC: PEG-DSPE; molar ratio = 33.5: 65: 1.5) are widely used to encapsulate small molecule drugs. Compared to this cholesterol based formulation, another formulation composed of cholesterylhemisuccinate: Egg-PC: PEG-DSPE; molar ratio = 33.5: 65: 1.5 induced more cytotoxicity and had less favorable properties. Characteristics of all empty liposomes, LP-FTY720 and LP-OSU-2S are shown in Table 4.1. The mean diameter by volume of nanoparticles did not change following addition of FTY720 or OSU-2S into the composition. FTY720 and OSU-2S altered the zeta potential of nanoparticles from - 4.10±0.34 to 3.99±1.67 mV and 5.41±0.12 mV. By measuring and calculating the amount of FTY720 and OSU-2S before and after dialysis against PBS with LC-MS/MS, the drug entrapment efficiency was 85.2±5.72% and 83.4 for FTY720 and OSU-2S, respectively. Morphological analysis of LP-FTY720 was carried out by using Cryo-TEM (Figure 4.2). The empty liposome
nanoparticles showed uniform structures with one lipid-bilayer, while the LP-FTY720 exhibited a bilamellar structure. FTY720 was presumably incorporated into the lipid-bilayer, which is typical for hydrophobic small molecules.

4.3.2 Development and validation of LC/MS to accurately measure FTY720 and OSU-2S

4.3.2.1 LC-MS/MS method conditions

The full-scan mass spectrum of FTY720 and OSU-2S displayed the expected parent peak at m/z 308 and 322, correspondingly, and the product ion spectrum displayed a prominent peak at m/z 255 and 286, respectively, as shown in Figure 4.3A and B. For the internal standard compound Sph-17, the full mass and product ion spectrum were obtained at m/z 268 and 238, respectively (Figure 4.3C). LC conditions evaluated included a C18 column with gradient elution using water and methanol with 0.1% formic acid as described above. Retention times for FTY720, OSU-2S and Sph-17 were approximately 2.7, 2.6 and 3.7 min, respectively, with Gaussian peaks and clear baseline separation. A set of typical chromatograms of blank mouse plasma with and without FTY720 or OSU-2S at 1ng/mL is presented in Figure 4.4A to C, and total ion chromatograms of separated 100 ng/mL FTY720, OSU-2S and Sph-17 are displayed in Figure 4.4D to F, respectively.

4.3.2.2 Method validation

The standard curve for FTY720 or OSU-2S included eight calibration points and ranged from the LLOQ at 1 ng/mL to 3000 ng/mL. Intra-day and inter-day validation
experiments were carried out with six replicates each at 10, 100, 1000 ng/mL. For FTY720, as shown in Table 4.2, the intra-day and inter-day accuracy of these samples ranged narrowly from 99-104% and 105-111%, respectively while the relative standard deviations of both intra-day and inter-day were below 7.36%, ranging from 1.60% to 7.36%. For OSU-2S, as shown in Table 4.3, the intra-day and inter-day accuracy of these samples ranged narrowly from 103-106% and 108-111%, respectively while the relative standard deviations of both intra-day and inter-day were below 10.5%, ranging from 2.24% to 10.5%.

Matrix effect was evaluated in triplicate QC samples at 10, 100, 1000 ng/mL FTY720 or OSU-2S with 100ng/mL Sph-17 as described above. The matrix effects at three QC levels of FTY720 ranged from 43.8% to 55.9%. Recovery of FTY720 ranged from 58.4% to 69.0%. The matrix effects at three QC levels ranged from 58.4% to 69.0%. Recovery of OSU-2S in this method ranged from 47.6% to 61.1%. Relative standard deviation of both matrix effect and recovery of FTY720 and OSU-2S were below 15%. Sph-17, as the internal standard, had an average matrix effect of 41.05% and recovery of 56.67% in this developed method. The detailed recovery and matrix effect values of FTY720 and OSU-2S are shown in Table 4.4 and Table 4.5, respectively.

Short-term stability of FTY720 and OSU-2S in mouse plasma was studied with standard samples kept for 6 hr at 4°C. Results indicated both signals of FTY720 and OSU-2S were stable under these conditions. Long-term freezer and freeze-thaw stability for OSU-2S in mouse plasma was evaluated with QC samples at 10, 100 and 1000 ng/mL as described above. Results indicated good freezer stability of both FTY720 and OSU-2S.
through 21 days with accuracy between 85% and 115%.

4.3.3 In vitro release study of LP-FTY720 and LP-OSU-2S

The in vitro release kinetics of FTY720 or OSU-2S from liposomes was determined. As shown in Figure 4.5A and B, the LP-drug was stable in PBS and in serum at 37°C. For LP-FTY720, the release over a time period of 12 hr was <40%, while at 24 hr the liposomes still retained over 50% FTY720 in both media. For LP-OSU-2S, after 24 hr dialysis, above 60% OSU-2S was still retained in liposome in both PBS and serum. The results indicated LP-FTY720 and LP-OSU-2S were adequately stable in PBS and in serum.

4.3.4 Cytotoxicity of LP-FTY720

The cytotoxicity of LP-FTY720 and LP-OSU-2S were evaluated using \(1 \times 10^6\) B-CLL patient cells. In addition, equivalent empty liposomes and free FTY720 or OSU-2S diluted in PBS were incorporated as controls. After incubating with different concentrations (0.3, 0.6, 1.25, 2.5, 5, 7.5, 10, 15, 20 μM) of FTY720, OSU-2S or empty liposomes, cells were stained with annexin V / PI for flow cytometry analysis. The sigmoidal dose-response curves (Figure 4.6A and B) indicates that cells treated with increasing concentrations of both FTY720 or LP-FTY720, OSU-2S or LP-OSU-2S showed a dose-dependent decrease in viability corresponding to a concomitant decreased amount of annexin V-/PI- cells. The EC\(_{50}\) values of free FTY720 and LP-FTY720 were 9.3 μM and 5.7 μM, respectively, while EC\(_{50}\) values of free OSU-2S and LP-OSU-2S...
were 3.1 μM and 4.9 μM. The obtained EC<sub>50</sub> values of LP-FTY720 and LP-OSU-2S provided an optimal dose level that we can use for further evaluation of LP-FTY720 and LP-OSU-2S with targeting reagents.

Previous study showed that FTY720 induces down-modulation of Mcl-1 in CLL B cells<sup>195</sup>, therefore the expression of Mcl-1 protein was evaluated in CLL patient B cells after the LP-FTY720 treatment at FTY720 concentration of 5μM and compared with free FTY720. The Mcl-1 protein expression was successfully reduced by 40% and 43% by free FTY720 in DMSO and LP-FTY720, respectively, compared to that of untreated samples, respectively (Figure 4.7).

4.3.5 Pharmacokinetic study of FTY720 and LP-FTY720

Pharmacokinetics of LP-FTY720 was determined in ICR mice with free FTY720 as comparison. As shown in the plasma FTY720 concentration vs. time plot (Figure 4.8), the FTY720 in the liposomes was cleared at a slower rate compared to free FTY720. Plasma concentration data were evaluated with non-compartment methods, and resulting pharmacokinetic parameters are displayed in Table 4.6. The highest observed concentration (C<sub>max</sub>) of LP-FTY720 was twice that of free drug, suggesting LP-FTY720 was retained in plasma and did not distribute as extensively to other compartments. LP-FTY720 clearance was approximately one third that of free drug, and terminal phase half-life was approximately 50% greater with LP-FTY720. These data are consistent with expectations that LP-FTY720 has increased plasma exposure compared to free drug.
4.3.6 Pharmacokinetics of OSU-2S

The validated LC-MS/MS assay was applied in a pharmacokinetic study of OSU-2S after IV and IP administration in ICR mice. Mice injected at 5mg/kg OSU-2S were euthanized at time points between 5 min and 24 hrs, and blood was collected through cardiac puncture. Separated plasma was further extracted and processed for analysis, and the OSU-2S concentration versus time curves after IV and IP injection were plotted in Figure 4.9. Non-compartmental pharmacokinetic parameter estimates are presented in Table 4.7. Mean maximum observed concentration for IV and IP administration routes were 2803 ± 285 ng/mL and 305 ± 55 ng/mL, respectively. Areas under the concentration vs. time curves indicated that the IP administration route systemic availability was approximately 46%.

4.3.7 ILPs loaded with FTY720 can improve targeted delivery and enhance killing efficiency

Antibody-targeted ILPs have shown improved drug delivery outcomes in therapy across multiple cancers\textsuperscript{15,71,132,255}. ILPs utilize antibodies for specific targeting of therapeutic reagents, efficiently and specifically delivering the encapsulated drug to its targeted cell or tissue. We chose three monoclonal antibodies, anti-CD19, anti-CD20 and anti-CD37, which are commonly used in CLL characterization or treatment \textsuperscript{110,159,161}. The amount of mAbs was optimized to a suboptimal concentration, taking advantage of the targeting but not killing effect of mAbs. To further investigate the efficacy of targeted delivery of ILP-FTY720, CLL cells were treated with 3 μM FTY720 at a concentration of
antibodies of 0.2 μg/mL for 24 hr. These antibody concentrations are known to be only mildly cytotoxic when the antibodies are administered alone (data not shown). As shown in Figure 4.10, both CD19-ILP-FTY720 and CD37-ILP-FTY720 mediated significantly increased cytotoxicity to CLL B cells compared to non-targeted LP-FTY720 (n=5, P<0.05). Our findings indicated that encapsulating FTY720 into liposomes and targeting it with mAbs can induce target cell apoptosis and affect normal cellular functions resulting in significant apoptosis of target cells compared to the corresponding ILP groups without FTY720 (n= 5, P<0.001).

4.3.8 2A2-ILP-OSU-2S enhanced killing efficiency and reduced off-target effect in CLL therapy

To confirm the selectivity of 2A2-ILPs as a targeted delivery system specific to B-CLL cells, B-CLL cells separated from CLL patients and normal B cells obtained from healthy donors were used to compare the targeting delivery efficiency of 2A2-ILP-OSU-2S. 1E⁶ cells were incubated with free OSU-2S, LP-OSU-2S, IgG-ILP-OSU-2S or 2A2-ILP-OSU-2S at 5μM of OSU-2S and 0.1μg/ml mAbs in total for 24 hr before annexin V / PI analysis by flow cytometry. As shown in Figure 4.11A and B, after normalized the untreated group, B-CLL cells and normal B cells did not alter responses to the treatment of free OSU-2S, LP-OSU-2S or IgG-ILP-OSU-2S. However, the percentage of annexin V-/PI- cells in two populations significantly varied. B-CLL cells were greatly more killed by 2A2-ILP-OSU-2S due to the targeting effect of 2A2-IgG, while normal B cells exhibited similar killing efficiency with 2A2-ILP-OSU-2S, IgG-ILP-OSU-2S or LP-
OSU-2S, resembling the non-targeted delivery of OSU-2S in the 2A2-ILPs form to the ROR1 negative normal B cells.

4.3.9 2A2-ILP-OSU-2S differs pharmacokinetics profiles in ROR1 transgenic mouse

It has been reported in several papers that targeting ILPs may alter the PK parameters of the payload drug in xenograft models with targeted cells or tissues in body due to the antibody immobilized on the surface of liposomes. Thus, a pilot PK study was carried out with the 2A2-ILP-OSU-2S in ROR1 transgenic mouse. According to the limitation of mice available, mice age and gender were randomly mixed and the liposomal OSU-2S without targeting mAb was dosed at 5 mg/kg to C57BL/6 wild type mice while 2A2-ILP-OSU-2S was dosed at 5 mg/kg of OSU-2S to ROR1 transgenic mice. Time points for plasma collection covered from 5 min to 4 hr after dosing. At different time point, one mouse from each group was sacrificed and plasma was collected from blood via cardiac puncture. After quantify the concentration of OSU-2S via the developed LC/MS-MS method, the concentration versus time curve was shown in Figure 4.12. The PK curves clarified that PK profiles of 2A2-ILP-OSU-2S is significantly different from that of non-targeting LP-OSU-2S. However, the real factor results in the difference still needs to be further explored.

4.4 Discussion

Compared to free drugs, liposomal drugs may provide an advantage by decreasing the toxicity and increasing the circulation time\textsuperscript{256-258}. In this study, we loaded FTY720 and OSU-2S, respectively, into a liposomal carrier and characterized their
physicochemical, biological and pharmacokinetic properties. Additionally, immune-targeted LP-FTY720 and LP-OSU-2S for CLL was investigated.

The structure of FTY720 and OSU-2S both share the amphiphilic feature of phospholipids and hence the classic ethanol injection method was employed to synthesize LP-FTY720 and LP-OSU-2S. Studies related to size measurement, encapsulation efficiency evaluation and in vitro drug release kinetics revealed that the composition of lipids and loading method resulted in nanoparticles suitable for delivery.

The development of LP-FTY720 or LP-OSU-2S provides a more favorable efficacy/toxicity profile for these promising agents compared to their free form. Cytotoxicity studies showed that LP-FTY720 was slightly more potent than free FTY720 in CLL patient cells. Though reduced cytotoxicity was observed with LP-OSU-2S relative to its free form, the cytotoxicity efficiency was reasonable for a liposomal formulation. Moreover, empty liposomes were not toxic to cells indicating that the observed cytotoxicity with LP-FTY720 or LP-OSU-2S was due to the encapsulated FTY720 or OSU-2S but not the liposome formulation components. The modulation of McI-1 protein expression was evaluated as a representative readout for the LP-FTY720 pharmacodynamic read out. As expected, LP-FTY720 successfully decreased McI-1 protein expression to the same level as free FTY720. Further studies will be useful to ensure free drug and LP-FTY720 operate through the same mechanisms\textsuperscript{259}, and the accordance of mechanism of LP-OSU-2S can be also investigated as long as the detailed mechanism of OSU-2S has been clarified.

The development and validation of a sensitive LC-MS/MS method to accurately
quantify FTY720 and OSU-2S in mouse plasma is presented. The assay has an LLOQ of 1 ng/mL and is accurate and precise within the standard curve range up to 3000 ng/mL. The method is adequately sensitive for characterizing pharmacokinetics of FTY720 and OSU-2S throughout 24 h after 5 mg/kg IV or IP dosing. Validation data for the assay indicate it is a reliable and stable method with minimal degradation of FTY720 and OSU-2S in both long-term and short-term storage.

Pharmacokinetic data demonstrated LP-FTY720 had higher plasma $C_{max}$ and overall exposure of FTY720 compared to free drug. This will be clearly beneficial for targeting hematologic diseases where the target is located in the blood compartment. Although we did not evaluate in vivo pharmacokinetics of ILPs, we anticipate exposures would be similar or potentially even greater since antibodies will target ILPs to circulating blood cells.

It has been the first time to report pharmacokinetic data for OSU-2S as a new small molecule drug. As per our current results, OSU-2S is adequately absorbed from the peritoneal cavity with bioavailability of 46%. The mean observed $C_{max}$ of 0.305 μg/mL (~1μM) after 5 mg/kg IP injection occurs at 10 min post dosing. Further pharmacokinetic studies will be required to confirm higher doses that will achieve plasma levels equal to or greater than the previously reported 1.5-2.5 μM EC$_{50}$ of OSU-2S in hepatocellular carcinoma $^{194}$. The pharmacokinetic data for this novel agent indicates OSU-2S has favorable disposition with relatively low clearance and long exposures of active drug concentrations. This and additional pharmacokinetic data will support optimization of dose regimens for future preclinical efficacy studies.
In addition, we used FTY720 as the model drug to validate the efficiency of anti-CD19, anti-CD20 and anti-CD37 ILPs in targeted delivery as well as cell cytotoxicity. Though the chosen antibodies were also used as single killing drugs in pre-clinical or clinical studies\textsuperscript{110,159,260}, sub-toxic concentrations of antibodies were chosen as targeting reagents in these proof of principal studies. Compared to non-targeted LP-FTY720, ILPs with each of the antibody conjugated formulation improved potency and delivery of FTY720 to CLL patient B cells. Further optimization of FTY720 and antibody concentration and antibody type will be necessary to optimize activity and \textit{in vivo} efficacy. Moreover, rationale choice of targets and antibodies will enable expansion of the LP-FTY720 formulation into other diseases where FTY720 has shown a therapeutic benefit.

LP-OSU-2S has been conjugated with 2A2-IgG for more specific targeting delivery to CLL. The results indicated that 2A2-ILP-OSU-2S specifically killed more efficient to B-CLL cells but not to normal B cells or T cells with no ROR1 expression. The pilot pharmacokinetic comparison of non-targeting and targeting LP-OSU-2S in ROR1 transgenic mouse revealed that the slower clearance of 2A2-ILP-OSU-2S prolonged the circulation time of OSU-2S in blood and reduced the percentage of drugs being distributed into liver. Further pharmacokinetic study including the tissue distribution of targeted ILP-OSU-2S would be more helpful to fully understand the mechanism of targeting delivery of drug \textit{in vivo}. And therapeutic study in ROR1 transgenic mice can better indicate the efficacy of 2A2-ILP-OSU-2S for any potential clinical application.

In conclusion, efficient liposomal FTY720 or OSU-2S formulations have been
designed and evaluated. The formulations exhibited high drug loading ratio, prolonged in vitro release rate and beneficial pharmacokinetic properties in vivo. They also provide alternative degradable vehicles for administration of FTY720 or OSU-2S with increased efficacy and decreased potential for off-target effects. And these two liposomal model drugs combined with ILPs provided basic characterized information on targeted delivery of small molecule drugs to treat CLL. Further preclinical studies are warranted to define the safety, mechanism and therapeutic efficacy of these novel formulations with the targeting delivery systems.
Table 4.1. Characterization of liposomal nanoparticles

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle size (nm)</th>
<th>Polydispersity index (PI)</th>
<th>Entrapment efficiency (EE) (%)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty liposome</td>
<td>159.1 ± 5.40</td>
<td>0.186 ± 0.038</td>
<td>N/A</td>
<td>−4.10 ± 0.34</td>
</tr>
<tr>
<td>LP-FTY720</td>
<td>157.5 ± 2.91</td>
<td>0.193 ± 0.074</td>
<td>85.2 ± 2.72</td>
<td>3.99 ± 1.67</td>
</tr>
<tr>
<td>LP-OSU-2S</td>
<td>157.2 ± 3.98</td>
<td>0.168 ± 0.056</td>
<td>90.09 ± 2.69</td>
<td>5.41 ± 1.12</td>
</tr>
<tr>
<td>2A2-ILP-OSU-2S</td>
<td>163.3 ± 4.31</td>
<td>0.184 ± 0.043</td>
<td>88.25 ± 4.81</td>
<td>0.32 ± 1.75</td>
</tr>
</tbody>
</table>

Data represent the mean ± S.D. of three separate experiments (n=3).
Table 4.2. FTY720 intra-day and inter-day accuracy and precision

<table>
<thead>
<tr>
<th>QC (ng/mL)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/mL (% accuracy)</td>
<td>%CV</td>
</tr>
<tr>
<td>10</td>
<td>9.93 (99)</td>
<td>5.91</td>
</tr>
<tr>
<td>100</td>
<td>103.71 (104)</td>
<td>1.60</td>
</tr>
<tr>
<td>1000</td>
<td>998.26 (100)</td>
<td>4.02</td>
</tr>
</tbody>
</table>

Values indicate mean concentrations and % accuracy. Relative standard deviation (% CV) is from six replicates (n=6).
### Table 4.3. OSU-2S intra-day and inter-day accuracy and precision

<table>
<thead>
<tr>
<th>QC (ng/mL)</th>
<th>Mean calculated concentrations</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Intra-day</strong></td>
<td><strong>Inter-day</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ng/mL (% accuracy)</td>
<td>%CV</td>
<td>ng/mL (% accuracy)</td>
</tr>
<tr>
<td>10</td>
<td>10.27 (103)</td>
<td>10.50</td>
<td>11.11 (111)</td>
</tr>
<tr>
<td>100</td>
<td>103.55 (103)</td>
<td>6.49</td>
<td>108.65 (109)</td>
</tr>
<tr>
<td>1000</td>
<td>1105.96 (106)</td>
<td>5.73</td>
<td>1108.37(108)</td>
</tr>
</tbody>
</table>

Values indicate mean concentrations and % accuracy. Relative standard deviation (% CV) is from six replicates (n=6).
Table 4.4. Recovery and matrix effect of FTY720

<table>
<thead>
<tr>
<th>QC (ng/mL)</th>
<th>Recovery (%)</th>
<th>Matrix Effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>55.91</td>
<td>64.50</td>
</tr>
<tr>
<td>100</td>
<td>55.92</td>
<td>58.43</td>
</tr>
<tr>
<td>1000</td>
<td>43.79</td>
<td>69.04</td>
</tr>
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Indicated values represent means (n=3) of plasma QC samples.
Table 4.5. Recovery and matrix effect of OSU-2S

<table>
<thead>
<tr>
<th>QC (ng/mL)</th>
<th>Recovery (%)</th>
<th>Matrix Effect (%)</th>
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<tbody>
<tr>
<td>10</td>
<td>61.08</td>
<td>49.67</td>
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<tr>
<td>100</td>
<td>59.83</td>
<td>51.43</td>
</tr>
<tr>
<td>1000</td>
<td>47.57</td>
<td>51.73</td>
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</table>

Indicated values represent means (n=3) of plasma QC samples.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Free FTY720</th>
<th>LP-FTY720</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>mg/kg</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>$\lambda_z$</td>
<td>1/hr</td>
<td>0.0357</td>
<td>0.0244</td>
</tr>
<tr>
<td>$T_{1/2\lambda_z}$</td>
<td>hr</td>
<td>19.44</td>
<td>28.36</td>
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<tr>
<td>$C_{max}$</td>
<td>mg/L</td>
<td>5.572</td>
<td>12.505</td>
</tr>
<tr>
<td>$V_d$</td>
<td>L/kg</td>
<td>21.81</td>
<td>9.60</td>
</tr>
<tr>
<td>AUC0-$\infty$ _obs</td>
<td>hr*mg/mL</td>
<td>6.24</td>
<td>20.78</td>
</tr>
<tr>
<td>CL</td>
<td>mL/hr/kg</td>
<td>777.81</td>
<td>234.61</td>
</tr>
</tbody>
</table>

Indicated values are non-compartmental model parameter estimates (n=5). $\lambda_z$ Elimination rate constant; $T_{1/2\lambda_z}$ Elimination half life; $C_{max}$ observed maximum concentration; $V_d$ apparent volume of distribution; AUC0-$\infty$ _obs area under the observed concentration-time curve estimated to infinity.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>IV</th>
<th>IP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>mg/kg</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>( \lambda_z )</td>
<td>1/hr</td>
<td>0.0445</td>
<td>0.0443</td>
</tr>
<tr>
<td>( T_{1/2\lambda_z} )</td>
<td>hr</td>
<td>15.59</td>
<td>15.64</td>
</tr>
<tr>
<td>( T_{\text{max}} )</td>
<td>min</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>( C_{\text{max}} )</td>
<td>( \mu g/mL )</td>
<td>2.803</td>
<td>0.305</td>
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<tr>
<td>( V_z/F_{\text{obs}} )</td>
<td>L/kg</td>
<td>68.89</td>
<td>144.05</td>
</tr>
<tr>
<td>( \text{AUC}<em>{0-\infty</em>{\text{obs}}} )</td>
<td>hr*( \mu g/L )</td>
<td>1522.13</td>
<td>698.25</td>
</tr>
<tr>
<td>( \text{CL} / F_{\text{obs}} )</td>
<td>L/hr/kg</td>
<td>3.06</td>
<td>6.38</td>
</tr>
<tr>
<td>( F )</td>
<td>%</td>
<td>100</td>
<td>45.87</td>
</tr>
</tbody>
</table>

Indicated values are non-compartmental parameter estimates (n=5). \( \lambda_z \), elimination rate constant; \( T_{1/2\lambda_z} \), elimination half life; \( T_{\text{max}} \), time of \( C_{\text{max}} \); \( C_{\text{max}} \), observed maximum concentration; \( \text{AUC}_{0-\infty_{\text{obs}}} \), area under the observed concentration-time curve extrapolated to infinity; \( V_z/F_{\text{obs}} \), estimated volume of distribution divided by \( F \); \( \text{CL} / F_{\text{obs}} \), clearance divided by \( F \); \( F \), bioavailability.
Figure 4.1. Chemical structure of (A) FTY720, (B) OSU-2S and the internal standard (C) Sph-17
Figure 4.2. Morphological analysis of LP-FTY720 by Cryo-TEM

CryoTEM images of (A) empty liposomes were unilamellar nanoparticles while (B) LP-FTY720 had a unique bilamellar structure.
Figure 4.3. Mass spectra of FTY720, OSU-2S and Sph-17
Figure 4.3 continued

C

Spectra were obtained from direct infusion of 10μg/mL of each compound in positive electrospray ionization mode. The full-scan mass spectrum of (A) FTY720 displayed the expected parent peak and product peak at m/z 308 and 255, respectively. (B) OSU-2S displayed the expected parent peak and product ion spectrum displayed a prominent peak at m/z 322 and 286, respectively. (C) For the internal standard compound Sph-17, the full mass and product ion spectrum were obtained at m/z 268 and 238, respectively.
Figure 4.4. Total ion chromatograms in mouse plasma.
Chromatograms from (A) blank plasma and (B) plasma spiked with 1ng/mL FTY720 (retention time = 2.68 min) and (C) 1 ng/mL OSU-2S (retention time = 2.63 min). Total ion chromatogram showing relative separation of (E) 100 ng/mL FTY720 (2.66 min), (F) 100 ng/mL OSU-2S (2.59 min) and (G) 100 ng/mL Sph-17 (3.68 min).
Figure 4.5. *In vitro* release of FTY720 or OSU-2S from liposome nanoparticles

(A) FTY720 or (B) OSU-2S entrapped liposome in PBS was incubated at 37°C and dialyzed against PBS or 10% serum. At different time points, the liposomes were collected and disintegrated by methanol. The amount of drug retained and released from liposomes were measured by LC-MS/MS (n=3, mean ± SD).
Figure 4.6. Comparison of EC$_{50}$ of free drug and liposomal drug in B-CLL patient cells

Purified CD19$^+$ lymphocytes from CLL patients were incubated with indicated concentrations of empty liposomes, free drug or liposomal drug for 24 hr and were evaluated by flow cytometry with annexin V/PI staining. The data shown represent percentages of annexin V-PI- viable cells with SD that are normalized to untreated media control. (A) The EC$_{50}$ values of free FTY720 and LP-FTY720 were 9.3 μM and 5.7 μM (n=6, mean ± SEM), respectively, while (B) EC$_{50}$ values of free OSU-2S and LP-OSU-2S were 3.1 μM and 4.9 μM (n=5, mean ± SEM).
Figure 4.7. LP-FTY720 down-regulates Mcl-1 protein in CLL patient cells via western blot

(A) Representative western blot of Mcl-1 protein level after treating CLL cells at $1 \times 10^6$ cell/mL with 5 μM FTY720 for 20 h. (B) Summary of western blots results of Mcl-1 down-regulation levels of CLL patient cells, LP-FTY720 vs. Liposome treated group (n=4, mean ± SD, **P<0.01). Shown fold change has been normalized to untreated group for each patient accordingly. Linear mixed effect models were used to estimate unrestricted covariance structures and produce robust hypothesis tests. Holm’s method was used to adjust for multiplicity.
Figure 4.8. Plasma concentration vs. time curve of FTY720 and LP-FTY720 after i.v. bolus injection in mice

FTY720 was injected through the tail vein (5mg/kg) in groups of 5 mice each. At indicated time points mouse plasma was collected and FTY720 was extracted for analysis by LC-MS/MS. Error bars stand for standard deviations (n=5, mean ± SD, P<0.03). Data were analyzed by Student’s t-tests with P<0.05 considered as significantly different.
Figure 4.9. Plasma concentration vs. time curve of OSU-2S

OSU-2S was injected through i.v. or i.p. (5mg/kg) in groups of 5 mice each. At indicated time points mouse plasma was collected and OSU-2S was extracted for analysis by LC-MS/MS. Data points are mean ± SD of OSU-2S in mouse plasma after intravenous and intraperitoneal administration (n=5).
Figure 4.10. Improved delivery efficiency and increased cytotoxicity of ILP-FTY720 on B-CLL cells

B-CLL cells (1×10^6) were treated with 3 μM FTY720 at a concentration of antibodies of 0.2 μg/mL for 24 hr before annexin V/PI analysis by flow cytometry (n= 5, mean ± SD, **P<0.001). Linear mixed effect models were used to estimate unrestricted covariance structures and produce robust hypothesis tests. Holm’s method was used to adjust for multiplicity.
Figure 4.11. Targeted delivery of 2A2-ILP-OSU-2S with specific cytotoxicity on B-CLL cells

B-CLL cells or B cells (1×10^6) from healthy people were incubated with different formulation of FTY720 at 5μM of OSU-2S and 0.1μg/ml mAbs in total for 24 hr before annexin V / PI analysis by flow cytometry (mean ± SEM). (A) 2A2-ILP-OSU-2S significantly induced improved cytotoxicity in B-CLL cells compared to non-targeting formulations (n=8, P<0.01), while (B) in normal B cells, no difference in cytotoxicity induction with 2A2-ILP-OSU-2S or negative controls (n=4). Linear mixed effect models were used to estimate unrestricted covariance structures and produce robust hypothesis tests. Holm’s method was used to adjust for multiplicity.
Figure 4.12. Pilot pharmacokinetic study with plasma concentration vs. time curve of OSU-2S from non-targeted or targeted liposomal form.

At different time point, one mouse from each group was sacrificed and plasma was collected from blood via cardiac puncture. (A) Liposomal OSU-2S without targeting mAb was dosed at 5 mg/kg to C57BL/6 wild type mice while (B) 2A2-ILP-OSU-2S was dosed at 5 mg/kg of OSU-2S to ROR1 transgenic mice. Note: mice age and gender were mixed due to the limited amount of mice available.
CHAPTER 5

LIPOSOMAL TARGETED DELIVERY OVERCOMES IMMUNOSTIMULATORY EFFECTS OF OLIGONUCLEOTIDE BASED THERAPY IN VIVO

5.1 Introduction

The poor intracellular delivery, suboptimal target down modulation and CpG motif mediated immune stimulation of CLL B cells have all attribute to the limited application of G3139 in recent clinical trials. To overcome such effects, a novel strategy of using liposomal nanoparticles with B cell specific antibody targeted delivery to minimize the immunomodulatory effect and maximize the B cell specific antisense effect of G3139 has been explored in our group previously. In principle, rituximab was selected as the targeting antibody which has been performing as a clinically safe and efficacious CD20 directed therapeutic antibody in CLL treatment. In our study, rituximab (RIT) was conjugated onto G3139 lipopolyplexes to construct RIT-ILPs for improving efficiency and specificity of G3139 delivery to CLL B cells. Compared to free G3139, G3139 encapsulated in RIT-ILPs demonstrated significantly reduced CLL B cells activation which was attributed to weaker NF-kB activation, reduced expression of co-stimulatory molecules and pro-inflammatory cytokine release. Our results also showed that RIT-ILP
mediated delivery of G3139 resulted in efficient BCL-2 down-regulation that directly led to the spontaneous apoptosis and the increased sensitivity to currently approved fludarabine treatment in CLL B patient cells. Based on CD20 targeting RIT-ILPs, G3139 can be selectively delivered to CD20+ CLL B cells in PBMCs, which may minimize its non-specific uptake by other immune cells in vivo.

Our investigation also indicated that differential cellular trafficking was realized by RIT-ILP G3139 by triggering alternative endocytosis in CLL B cells. The RIT-ILP mediated delivery of G3139 prolonged its retention in early endosomal compartments while free G3139 was predominantly localized in late endosome or lysosome.

To further evaluate and demonstrate the potential application of RIT-ILP-G3139 in direct targeting to CD20+ cells and conquering the limitation of free G3139, in this chapter, an in vivo study by using a Raji xenograft model followed by treatment of RIT-ILP-G3139 is presented. In addition to therapeutic study, biomarkers that have been studied in in vitro study were also performed to compare the consistency of our hypothesis by using RIT-ILP-G3139 for improvement of treating CLL.

5.2 Materials and methods

5.2.1 Reagents and oligodeoxynucleotides (ODNs)

Phosphorothioate oligos G3139 (5’- TCT CCC AGC GTG CGC CAT-3’) were custom synthesized by Alpha DNA, Inc (Montreal, Canada). Egg phosphatidylcholine (Egg PC) and methoxy-polyethylene glycol (MW ~ 2,000 Da)-disteroyl
phosphatidylethanolamine (PEG-DSPE) were obtained from Lipoid (Newark, NJ). 3β-[N-(N’, N’- Dimethylaminoethane)-carbamoyl] Cholesterol (DC-Chol) and DSPE-PEG-maleimide (DSPE-PEG-Mal) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Rituxan (rituximab) and trastuzumab (herceptin) were obtained from Genentech, Inc (South San Francisco, CA). Traut’s reagent (2-Iminothiolane) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

5.2.2 Cell culture

Raji Burkitt’s B-cell lymphoma cell line obtained from American Type Culture Collection (Manassas, VA) were cultured and incubated in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and penicillin (100 U/mL)/streptomycin (100 μg/mL; Sigma-Aldrich, St. Louis) at 37 °C with 5% CO₂.

5.2.3 Preparation of rituximab conjugated liposomal ODN nanoparticles

An ethanol dilution method was used to prepare the ODN nanoparticles as previously reported. Briefly, lipid mixture (DC-Chol: Egg-PC: PEG-DSPE at molar ratios of 28.0: 70.0 : 2.0) dissolved in ethanol were mixed with protamine sulfate in citrate buffer (20mM, pH = 4) at a lipid : protamine mass ratio of 12.5: 0.3. G3139 in citric acid (20mM, pH =4) added into lipid-protamine mixture at an lipid : ODN : protamine mass ratio of 12.5: 1: 0.3. The formed nanoparticles solution was dialyzed against citrate buffer to remove free G3139 and further dialyzed against HEPES-buffered saline (HBS)
(145mM NaCl, 20mM HEPES, pH=7.4) overnight in a DispoDialyzer (Spectrum Labs, Rancho Dominguez, CA) at a molecular weight cut-off of 10,000 Da. Rituximab (RIT) was incorporated into nanoparticles carrying ODNs by a post-insertion method. RIT was reacted with 10× Traut’s reagent (2 h, room temperature) to yield RIT-SH at pH 8.0. RIT-SH was then reacted to form micelles of Mal-PEG-DSPE at a molar ratio of 1:10 at pH 6.8, and then incubated with ODN nanoparticles for 1 h at 37 °C. Thus, immunonanoparticles (ILPs) with a RIT to a total lipid ratio of 1: 4000 (0.025 mol %) were prepared. Similarly, herceptin (HER) was also synthesized using the same method and performed as control. The ILPs were further purified on a Sepharose CL-4B size exclusion column to remove free antibody.

5.2.4 In vivo study in Raji xenograft model

Raji (2×10^6 cells/mouse) cells were injected intravenously (i.v) into female NOD/SCID mice at age of 6 weeks and were housed in accordance to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the Ohio State University. Free G3139, Her-ILP-G3139 or RIT-ILP-G3139 prepared at 0.2 mg/ml G3139 in saline and were injected via intra-peritoneal (i.p.) injections at a dose of 5mg/kg by alternative days for two weeks (7 injections each mouse) 3 days post inoculation. Animals were monitored daily for signs of illness and sacrificed immediately if meeting the early removal criteria such as hind limb paralysis, respiratory distress or 30% body weight loss while body weight was measured every day before day 30. After day 30, the mice were weighed twice a week and observed for signs of illness. Hind-limb paralysis was
considered as the primary endpoint and survival time was used primarily for evaluation. Cured mice in the treated group were kept for 6 months post-engraftment. For the in vivo Bcl-2 down-regulation study, free G3139 or RIT-ILP-G3139 was injected at a dose of 5 mg/kg for three doses (days 10, 12 and 14) and were sacrificed on day 15. The cells isolated from bone marrow were assessed for the level of Bcl-2 protein expression and percentage of human CD20 positive cells by flow cytometry whereas mouse plasma was collected to determine the IFN-γ and IL-6 levels by ELISA using commercial kits (R&D Systems, Inc., MN).

5.2.5 Histopathological and immunohistochemical (IHC) analyses

Histopathological and immunohistochemical staining were conducted by Comparative Pathology and Mouse Phenotyping Shared Resource at The Ohio State University. For pathological analysis, at the endpoint, tumor samples were fixed in 10% phosphate-buffered formalin solution. As well, the tissue sections were collected and fixed in 10% phosphate-buffered formalin solution for 24 hr and then tissue fractions were stained with hematoxylin and eosin (H&E). For IHC analysis, tumor samples were stained with mAbs against human CD45 (Catalogue #AHP1726, AbD Serotec, Raleigh, NC), anti-human CD20 (Dako, Catalogue #M0755, Carpinteria, CA) or anti-Bcl-2 antibody (Catalogue #ab-692, Abcam, Cambridge, MA) followed by staining with horseradish-peroxidase-conjugated IgG (provided by Shared Resource from OSU).
5.2.6 Flow cytometry

At the endpoint of each mouse, the expression level of cell surface markers on bone marrow cells were analyzed by staining with fluorescent antibodies against CD20 and CD86, or isotype control Ab (BD Biosciences, Catalogue #555623, #555658 and #555749, San Diego, CA) at 4°C for 30 min. The cells were then spun down at 1200 rpm for 10 min and washed twice with cold phosphate-buffered saline (PBS, pH=7.4) and then analyzed by flow cytometry on a Beckman Coulter EC 500 (Beckman Coulter) to determine the corresponding antigen expression levels. A minimum of 10,000 events were collected for each assay. Mean fluorescence intensity (MFI) and positive cell percentages were determined. The data were analyzed using WinMDI software.

5.2.7 Cytokine assays

In Raji xenograft model, plasma from mice treated with PBS, free G3139, HER-ILP-G3139 or RIT-ILP-G3139 were collect for cytokine evaluation by the endpoint of the study. Human IL-6 and IFN-γ level was measured by ELISA following the protocol provided by the vendor (R&D Systems, Inc., MN).

5.2.8 Statistical analysis of data

Statistical analysis was performed by statisticians in the Center for Biostatistics, the Ohio State University. Linear mixed-effects models were used for analyses of cell samples. Kaplan-Meier estimates of survival for treatments and engraftments were plotted, and the survival for each treatment was calculated with 95% confidence intervals.
A significance level of $\alpha = 0.05$ was used for all tests. SAS software (SAS Institute, Inc., Cary, NC) was used for all statistical analyses.

5.3 Results

5.3.1 SCID mice engrafted with Raji cells

An aggressive Raji cell-inoculated disseminated xenograft mouse model was chosen to evaluate the *in vivo* therapeutic efficacy of RIT-ILP-G3139, as primary CLL B cells were not applicable to engraft in immunodeficient mice with recapitulation of the disease. Histological analysis of tissue sections from SCID mice engrafted with Raji cells revealed extensive infiltration of donor human B cells in lymph nodes, thymus and bone marrow 14-18 days post inoculation (Figure 5.1). Multifocal neoplastic cell infiltration was also observed in the central nerve system and brain\textsuperscript{161}.

5.3.2 RIT-ILP loaded with G3139 showed significant therapeutic effect in a SCID mouse xenograft Raji model

A significant *in vivo* therapeutic effect of RIT-ILP loaded with G3139 was demonstrated in a SCID mouse xenograft Raji model (Figure 5.2). The median survival time for placebo controls was 15 to 18 days without liposomal G3139 treatment. CD20-targeting RIT-ILP-G3139 showed very significant therapeutic efficacy with >80% survival rate (n=14) on day 120 of the study as a single agent. Compared to the mice treated with free G3139, RIT-ILP-G3139 treated mice significantly prolonged survival
with less than six dosing through i.p. injection ($P<0.0001$), while the control HER-ILP-G3139 that showed hind-limb paralysis before day 40 in all mice was also not competitive to CD20-targeting RIT-ILP-G3139 ($P = 0.0009$). Thus, the \textit{in vivo} therapeutic effect of RIT-ILP-G3139 was consistent with our previous \textit{in vitro} observations.

### 5.3.3 RIT-ILP-G3139 can more efficiently down-regulate the Bcl-2 protein expression than G3139 did in xenograft Raji model

To further illustrate the underlying mechanism of the potent therapeutic effect by RIT-ILP-G3139, we carried out another \textit{in vivo} study in Raji xenograft model by starting treatment on day 10 after inoculation and total three treatments were given for each group. On day 15, mice were sacrificed and bone marrow was collected for further analysis. As shown in Figure 5.3, intracellular staining of anti-Bcl-2 antibody by using flow cytometry and immunohistochemical staining of bone marrow both demonstrated down-regulation of the Bcl-2 protein expression in the RIT-ILP-G3139 treated group compared to free G3139 treated group. Bcl-2 level in bone marrow from mice treated with free G3139 was slightly up-regulated (104 ± 59.34%, n=4, mean ± SEM), whereas, Bcl-2 down-regulation was reached in average with mice treated with RIT-ILP-G3139 (67.8 ± 24.8%, n=4, mean ± SEM). This result was also consistent with the previous \textit{in vitro} western blot and indicates that RIT-ILP-G3139 can more efficiently down-regulate the Bcl-2 protein expression in Raji engrafted model as well.
5.3.4 RIT-ILP-G3139 can more efficiently deplete CD20+ cells derived from bone marrow of Raji engrafted mice

As described above, after the corresponding treatment, mice bone marrow cells were separated and analyzed by flow cytometry using antibodies directed against human CD20 antigen to evaluate the depletion of transferred tumor cells. As shown in Figure 5.4, n the separated PBMCs from bone marrow, around 55% cells were stained by PE anti-human CD20 antibody without treatment, resembling the aggregation of inoculated Raji cells in bone marrow of SCID mice in the Raji engraft model. After triplicate treatments, free G3139 and RIT-ILP-G3139 depleted 10% and 20% CD20+ cells, respectively, in each 10000 cells counted in flow cytometry.

5.3.5 Cytokine levels were consistently reduced by RIT-ILP-G3139 in Raji engrafted mice

The CpG mediated activation effect is one of the key limitations of G3139 in clinical application with severe side effects. Previous co-localization study in Figure 1.7 has shown that RIT-ILP-G3139 differs endosomal compartmentalization of G3139 via prolonged early endosome rather than late endosome, reduced the potent cytokine response due to immune activation via TLR9. Our in vitro study has demonstrated that the efficiency of G3139 encapsulated in RIT-ILP is contributed by both cross-linking effect of RIT-ILP as well as early endosomal compartmentalization. Hence, we examined cytokine production in plasma from Raji engrafted SCID mice to further prove that RIT-ILP-G3139 inhibits cytokine release. The ELISA assay results
showed that, though only modest levels of plasma IL-6 and IFN-γ were detected in the mice treated with free G3139, the levels of IL-6 and IFN-γ were found to be consistently reduced in the RIT-ILP-G3139 treated group compared to mice treated with free G3139 (Figure 5.5). For IL-6, all groups did not show significant difference with around 30 to 48 pg/ml expression in all groups. But for IFN-γ level, though all treated groups induced increasing expression, but free G3139 and HER-ILP-G3139 induced more IFN-γ with 107 pg/ml and 75 pg/ml, respectively; however, IFN-γ level in RIT-ILP-G3139 group was only 38 pg/ml, which was significantly lower than that induced by free G3139 (n=4, P=0.03). Likely to what we observed in vitro, the cytokines levels were least induced in RIT-ILP-G3139 treated group. The HER-ILP-G3139 as a negative control of rituximab also inhibited cytokine release compared to free G3139, but not as efficient as RIT-ILP-G3139.

5.3.6 Co-stimulation molecules were not up-regulated by RIT-ILP-G3139

In related studies, free G3139 can significantly induce activation markers such as CD40, CD86 and HLA-DR, thus, with the expression of such molecules, efficient T cell interactions can be recruited and activated to protect cells from death via co-stimulation effect. And it is another limitation of clinical use for CpG containing ODNs as well. The in vitro study revealed that RIT-ILP-G3139 reduced induction of these co-stimulatory molecules with CLL B cells, since RIT-ILPs has altered the cellular uptake and trafficking pathways of G3139. However, such phenomena were not observed with Raji cells. As shown in Figure 5.6, bone marrow cells from Raji xenograft model SCID mice
also have been stained with anti-CD86 antibodies for detection of CD86 induction levels. Free G3139 induced 1.5 fold changes of CD86 expression level in mice compared to unstimulated PBS treatment, which is not significant. RIT-ILP-G3139 reduced CD86 level. The up-regulation of CD86 molecules were not observed in cells from bone marrow which is in agreement with our in vitro data in Raji cells.

### 5.3.7 Altered spleen sizes due to RIT-ILP-G3139

In addition to elevated production of cytokine and co-stimulatory molecules, free G3139, HER-ILP-G3139 or RIT-ILP-G3139 treatment also resulted in the enlargement of spleens as compared to the saline-treated mice on Day 15 after treatment for three times. After engraftment of Raji cells and triplicate treatments, the average size of mice with engraftment in PBS group was 76.2 mg while all the treated mice had enlarged spleens from 70.4 mg to 154.5 mg. Among these three groups, free G3139 induced the most size increase in spleen at 120.3 mg in average. However, in the therapeutic study, after seven times dosing, the spleen sizes of mice all dropped and among which, RIT-ILP-G3139 mice at the end points all had spleens at 35.2 mg in average, which is even smaller than the mean weight from the PBS control groups at 56.3 mg. The enlargement of spleen may due to engraftment of Raji cells as mice in PBS also had a larger spleen, however, the injection of G3139 also contributed to the increased spleen size with 87.6 mg averagely. The spleen sizes exhibited more than 60% reduction in RIT-ILP-G3139 group while the decrease was only 25 % in free G3139 gourp. In the therapeutic study, mice treated with free G3139 all died around the similar days after engraftment as the
PBS group, thus resembling free G3139 induced splenomegaly immunostimulation in 
*NOD-SCID* mice. The decrease of spleen sizes in the RIT-ILP-G3139 group could be the 
result of recovery after treatment, but compared to HER-ILP-G3139 group and free 
G3139, it has already shown improved efficiency in clearing out the malignant cells with 
induced side effects.

### 5.4 Discussion

Herein we have used a Raji xenograft model with *NOD-SCID* to evaluate targeting 
efficiency of RIT-ILP-G3139 *in vivo*. Mice treated with RIT-ILP-G3139 had high 
survival ratio after two-week treatment, which was with significantly improved efficiency 
compared with other control groups as all other mice died due to hide limb paralysis 
related to the engrafted cells. Furthermore, other biomarkers including stimulatory 
molecules, cytokine release and Bcl-2 expression level were also measured as what was 
done in the previous *in vitro* study by using purified CLL B cells and whole blood from 
CLL patients. Our *in vivo* therapeutic efficacy and the corresponding *ex vivo* results were 
all consistent to that obtained *in vitro* conclusion, indicating that RIT-ILP encapsulated 
G3139 provided profound evidence in antitumor effect with Bcl-2 antisense effect as well 
as independent of CpG immune stimulation. According to our results, the antisense ODN 
delivery by ILPs could be of great benefit since this approach eliminates adverse 
immunological effects.

Compared to mice treated with free G3139, mice treated with RIT-ILP-G3139 
exhibited more depletion of CD20+ cells in bone marrow, induced less cytokines in
plasma and reduced expression of stimulatory molecules. The splenomegaly and elevated plasma levels of IL-6 and IFN-γ suggest that G3139 can be immunostimulatory even in NOD-SCID mice. As NOD-SCID mice lack functional B and T cells and deficit NK cells, the observed pro-inflammatory cytokine response is less likely attributed to NK cells. But by using RIT-ILP-G3139, such stimulated effects can be suppressed.

Based on these exciting preclinical data, further clinical development of RIT-ILP-G3139 is warranted to resurrect the use of G3139 in CLL and other B cell malignancies in which BCl-2 is implicated in pathogenesis and survival. A similar approach utilizing a different antibody to tumor specific antigens might also make this approach relevant to other malignancies. Indeed application of this approach in selective targeting and to circumvent immunostimulatory properties of novel oligonucleotide based therapeutics including antisense, siRNA and microRNA has broader implications in targeted therapy in lymphoid and other malignancies.
Figure 5.1. Analysis of Raji xenograft model

Representative H&E staining and IHC staining of human CD45 and human CD20 of tissue sections from Raji cell–inoculated SCID mouse.
Figure 5.2. Survival study for Raji-SCID mouse treated with various formulated G3139

SCID mice engrafted with $2 \times 10^6$ Raji cells on Day 0. The 10-day treatment was initiated at 5mg/kg via i.p. injection on Day 4 till Day 14 as the first mouse in placebo group showed symptom of hind-limb paralysis (n=6 for PBS and free G3139 groups, n=14 for HER-ILP-G3139 and RIT-ILP-G3139). Kaplan-Meier estimates of survival for treatments and engraftments were plotted, and the survival for each treatment was calculated with 95% confidence intervals.
Mice were engrafted by Raji cells and started to be treated by free G3139 (5 mg/kg), RIT-ILP-G3139 (5 mg/kg) or HER-ILP-G3139 (5 mg/kg) on Day 10 after inoculation and total three treatments were given for each group. On Day 15, mice were sacrificed and bone marrow was collected (A) Relative Bcl-2 expression determined by flow cytometry with intracellularly Bcl-2 staining (mean fluorescent intensity normalized to PBS group) (n=3, mean ± SEM, P=0.1296). Linear mixed-effects models were used for analyses with Holm’s method to adjust multiplicity. (B) IHC staining of Bcl-2 on bone marrow. The arrows indicate intracellular human Bcl-2 staining.

Figure 5.3. Analysis of Bcl-2 expression level in mouse bone marrow from Raji xenograft mice
Figure 5.4. Reduction in hCD20+ cells from bone marrow of Raji xenograft mice

Mice were engrafted by Raji cells and started to be treated by free G3139 (5 mg/kg), RIT-ILP-G3139 (5 mg/kg) or HER-ILP-G3139 (5 mg/kg) on Day 10 after inoculation and total three treatments were given for each group. On Day 15, mice were sacrificed and bone marrow was collected for CD20 staining and detected by flow cytometry (n=4, mean ± SEM). Linear mixed-effects models were used for analyses and Holm’s method was used to adjust multiplicity for primary end points.
Figure 5.5. Cytokine level in plasma of mice from Raji xenograft mice

Cytokine productions of IL-6 (n=4, mean ± SEM) and IFN-γ (n=4, mean ± SEM) in plasma from Raji xenografted mice received triplicate treatment of PBS, free G3139 (5 mg/kg) and RIT-ILP-G3139 (5 mg/kg) or HER-ILP-G3139 (5 mg/kg). Cytokine level of plasma was determined by ELISA. Linear mixed effect models were used for analyses and Holm’s method was used to adjust multiplicity.
Figure 5.6. Changes in induced co-stimulatory molecule CD86 in human CD19+ cells from bone marrow cells from Raji xenograft model

Mice were engrafted by Raji cells and started to be treated by free G3139 (5 mg/kg), RIT-ILP-G3139 (5 mg/kg) or HER-ILP-G3139 (5 mg/kg) on Day 10 after inoculation and total three treatments were given for each group. On Day 15, mice were sacrificed. Fold changes of co-stimulatory molecules CD86 in cells collected from bone marrow was determined by staining with anti-CD86 antibodies and evaluated by flow cytometry. Data was normalized to the PBS treated group (n=4, mean ± SEM). Linear mixed effect model were used to estimate unrestricted covariance structures and produce robust hypothesis and Holm’s method was used to adjust for multiplicity.
CHAPTER 6

SUMMARY AND PERSPECTIVES

This dissertation focused on the development and preclinical characterization of several immunoliposomal delivery systems that can potentially be applied to improve the therapeutic efficiency for CLL treatment. The novel dILPs based on anti-CD37 mAb combined with either anti-CD19 mAb or anti-CD20 mAb demonstrated improved binding efficiency to CLL B cells and enhanced killing effect in CLL B cells. By conjugating 2A2-IgG on the surface of liposomes, the new 2A2-IgG-ILPs showed specific selectivity and cytotoxicity to CLL B cells and ROR1+ cells. The liposomal formulations of FTY720 and its derivative, OSU-2S, have been developed and characterized, and the prospective application for future targeting delivery has been investigated in vitro with CLL as a model. The in vivo study of rituximab-conjugated ILP G3139 (RIT-ILP-G3139) was investigated in a Raji xenograftment model, which confirmed our previous in vitro study and demonstrated the therapeutic benefits of RIT-ILP-G3139. These findings and innovations have great potential to promote and explore new nanocarrier strategies for CLL treatment in the near future. Figure 6.1 summarizes the major contributions of this work.
6.1 Anti-CD37 based dual-antibody immunoliposomes

To our best knowledge, this is the first time that dual-mAb ILPs are investigated in CLL. To screen for the proper mAb combination for CLL patient cells, we have utilized a combinatorial antibody microarray technology to quantitatively characterize binding efficiencies of single and dual antibodies in a systematic and high throughput manner. Combination of multiple monoclonal antibodies has shown synergistic therapeutic effects in B cells leukemia\textsuperscript{247,248}, therefore, the dual mAbs on the same ILPs may potentially enhance the killing of CLL B cells. This has been demonstrated in our study even without drug payload. Compared to single ILPs or mixed single ILPs, dILPs should have several following advantages, including increased binding, uptake and cytotoxicity to the targets, synergistic signaling killing, complementary effects between antibodies and reduced steric hindrance.

The anti-CD19 and anti-CD37 antibodies used in this study are commercial reagents which are not designed for clinical use in humans. They can be replaced by antibodies approved for use in human clinical trials such as Xmab-5574 for CD19 and TRU-016 for CD37. Such replacement will be helpful to promote the future application of dILPs in clinical use as either drug delivery systems or therapeutic reagents. Another candidate of mAb that can be used in a dILP system is blinatumomab, which combines a CD3 site for T cells and a CD19 site for the target B cells\textsuperscript{264}. The conjugation of such a bispecific antibody on liposomes theoretically will reduce the steric hindrance between T cells and
B cells with nanoparticle as a more variable hinge linker, and also increase T cell to B cell ratio to enhance the killing efficiency of B malignancies.

The cytotoxicity induced by either single or dual ligand ILPS are exciting, but further details need to be investigated to explore the mechanism of ILPs-induced cytotoxicity in CLL. It will be important to find out whether ILPs alter the mechanism of free antibodies, such as ADCC and CDC, and how antibodies based cross-linkers on ILPs induce cytotoxicity.

Since liposomes are capable of carrying multiple drugs, it is possible to encapsulate chemotherapy drugs such as fludarabine, flavopiridol and dexamethasone in dILPs to enhance the drug efficacy for CLL treatment, but further related preclinical and clinical investigation will be necessary. Recently, it has been reported that upon ligation of CD37, two signaling pathways are stimulated through either phosphorylation of the N-terminal ITIM-like motif of CD37 by LYN kinase, leading to SHP1-dependent BIM up-regulation and cell death, or through tyrosine phosphorylation of the C-terminal ITAM-like motif of CD37 with the activation of PI3Kδ and AKT, phosphorylation of GSK3β and promotion of cell survival\textsuperscript{159}. Future strategies could encapsulate a PI3Kδ isoform-specific inhibitor, such as CAL-101 and LY 294002, or OSU-2S targeting to SHP1 to increase the synergy effect on killing CLL cells and provide clinical rationale for combination therapy.

6.2 2A2-IgG-based immunoliposomes

Our study is the first to use the anti-human ROR1 antibody for immunoliposomal delivery. The development of 2A2-IgG-ILPs specifically targeting to the unique marker
of malignant cells in CLL may become a new landmark for the targeting therapy. We have shown the advantages and specificity of the targeting effect of 2A2-IgG-ILPs in the ROR1 transgenic mouse.

Further therapeutic evaluation of 2A2-IgG-ILP based drug delivery, such as specific ROR1+ cell clearance in peripheral blood in ROR1 transgenic mice with small molecule drugs such as FTY720 and OSU-2S will be necessary to promote the future application of anti-human ROR1 ILPs into clinical use as a targeting strategy for CLL and other non-Hodgkin lymphoma expressing ROR1. Additionally, liposomal drug delivery remains poorly understood for mechanisms of in vivo drug release and intracellular trafficking. Future pharmacokinetic or co-localization tissue distribution studies in transgenic mice with drug encapsulated in the ILPs are important for translational research and for better understanding the mechanism of anticancer drug delivery to targeted tumor tissues and cells. ILPs should be able to deliver drugs or reagents more efficiently to the target cells or tissues yet reduce drug exposure to other organs such as liver and kidney compared to free drugs. Such studies will provide useful information about tissue distribution and can further characterize the feasibility of ILP-based targeting delivery.

6.3 FTY720, OSU-2S and the corresponding liposomal formulated drugs

Other major contributions this work has made include the novel liposomal formulation of FTY720 and OSU-2S, and the LC/MS-MS method that can simultaneously quantify these two drugs. The liposomal formulations not only improved the solubility of FTY720 and OSU-2S, but also provided a platform for targeting delivery.
of FTY720 or OSU-2S to diseases that might respond to these drugs. Even without targeting, the liposomal design may reduce the dosage or dosing intervals with a prolonged circulation time of drugs in plasma. The LC/MS-MS method is useful for further preclinical studies related to OSU-2S and FTY720 because it can be further developed to simultaneously quantify OSU-2S and its metabolites in mouse and human plasma and thus provide more comprehensive pharmacokinetic and pharmacodynamic information. In the current work we have presented the PK profiles of OSU-2S or LP-FTY720 with single dose at one dose level. Further characterization of PK with multiple doses and dose levels, as well as characterizing in vivo PD response of proper biomarkers for the drugs, will be critical for a full understanding of the PK-PD relationships and for optimizing dosing regimens.

In some cases, liposomal formulations may alter the drug mechanism compared to the free drug. For instance, liposomal G3139 altered the compartmentalization resulting in increased efficacy with reduced immunostimulatory effects. Similarly, it is unknown whether LP-FTY720 or LP-OSU-2S work through different mechanisms relative to the corresponding free drug. In our work, we have demonstrated that both liposomal FTY720 and free FTY720 down-regulate Mcl-1 protein levels. However, further investigations in both in vitro and in vivo experiments to explore currently known pathways involved in FTY720 and OSU-2S, such as protein phosphatase 2A (PP2A), will be necessary to confirm the mechanism of LP-FTY720 and LP-OSU-2S. It may also be useful to evaluate LP-FTY720 in other diseases such as multiple sclerosis and MCL. Since in those diseases free FTY720 functions through alternative mechanisms with S1PR binding in multiple
sclerosis but downregulation of Cyclin D1 in MCL. Knowing the mechanism of LP-FTY720 in multiple diseases will further facilitate its clinic use, especially since all lipids and FTY720 have already been approved by the U.S. FDA.

As a novel molecule, there is still much to learn about the PK and PD properties of OSU-2S. Further PK/PD studies of OSU-2S will be required for optimizing dosage level and frequency for preclinical or clinical therapeutic studies. Several biomarkers may be selected to monitor OSU-2S activity at the target site or in surrogate tissues. Such markers include Ser591 SHP1 and PP2A. The signaling pathway of OSU-2S in CLL still requires further characterization, and thus additional useful biomarkers may be identified.

6.4 Rituximab-conjugated ILP-G3139

The in vivo study of rituximab-conjugated G3139 in the Raji xenograft model described in Chapter 5 is significant as it confirmed our hypothesis in the previous in vitro studies that RIT-ILP-G3139 can reduce immunostimulatory effects of free G3139 and improve its gene silencing efficacy in CLL. Our study revealed that the immunoliposomal formulation can alter the intracellular pathway of the oligonucleotide to enhance its therapeutic efficacy. In addition to G3139 ODN, oligonucleotide based therapies including other antisense ODNs, siRNAs and miRNAs can be evaluated using the similar immunoliposomal formulation. A similar strategy utilizing relevant antibodies to tumor specific antigens might also extend this approach to other malignancies in addition to CLL. As in our current therapeutic study, the dosage and frequency of treatment was selected based on reference from other groups, a thorough in vivo PK/PD
study will be essential to further optimize the conditions for dosing, which would facilitate application of CD20-ILP-G3139 in future clinical use.

6.5 Stability and quality control of ILPs

The stability and quality control of dual-ILPs and single-ILPs are critical for ILP based immunotherapy. Although fast and simple, the current bulk mixing methods for large scale manufacturing usually result in non-uniform nanoparticle sizes and compositions which could greatly affect the transfection and cytotoxicity, particularly for multi-component nanoparticles. Figure 6.2 shows the cryogenic-transmission electron microscopy (cryo-TEM) images of structures of lipoplex nanoparticles prepared by the bulk mixing method composed of Quantum dots and ODN. The majority of nanoparticles encapsulated ODN only, and there were large concentrations of free Qdots. One possible approach to minimize such variation is to combine the sucrose gradient ultracentrifugation method and cryo-TEM. After centrifugation, the concentrated target fractions can be selected and confirmed by cryo-TEM to improve the efficacy of multi-functional nanoparticles. Figure 6.3 shows the structures of multifunctional liposomal nanoparticles in several fractions after centrifugation. Nanoparticles with different densities were separated into several fractions, and liposomal ODN without Qdots were collected from the upper fraction while the ideal multi-component nanoparticles encapsulating both Qdots and ODN were collected and concentrated from the lower fraction. This technique can be applied to ILPs with encapsulated drug to improve the quality of ILP-drug.
In this study, we used the post-insertion method to covalently couple the thiolated antibody on PEG-DSPE chains followed by insertion into the liposomal nanoparticles. The major drawback of this method is the uncontrolled random orientation of mAbs, which may cause the active antigen binding site to be blocked. Also, despite the stability of whole mAbs during prolonged storage, the existence of the Fc domain may initiate non-specific binding to normal tissues through Fc receptors, in addition to non-specific uptake by macrophages in vivo\textsuperscript{7,139}. To overcome the immunogenicity and also to maximize the binding efficiency, F(ab’)\textsubscript{2}, Fab’ and scFv fragments lacking the Fc domain but with complementary activating region can be used to replace the whole mABs in the ILP formulation. F(ab’)\textsubscript{2} fragments are still quite stable with two binding regions joined by disulphide bonds; and the disulphide bonds can be cleaved under reducing conditions to yield two Fab’ fragments each with a thiol (\(-\text{SH}\)) group for coupling the fragments to ILPs\textsuperscript{133,139,140}. Such methods will enhance the quality control of targeting without losing stability of ligand conjugation, and will be practical for the manufacturing of ILPs for potential application in future clinical use.
Figure 6.1. Summary of main contributions in this work
Figure 6.2. Cryogenic-transmission electron microscopy (cryo-TEM) images of structures of lipoplex nanoparticles prepared by the bulk mixing method.

Lipids were mixed with ODN and Quantum dots to form the multi-component lipoplex and morphology of obtained nanoparticles was observed via cryo-TEM.
Figure 6.3. Structures of multifunctional liposomal nanoparticles in several fractions after centrifugation

After ultracentrifugation, nanoparticles with different density were separated into several fractions. (A) Cryo-TEM image of nanoparticles from upper fraction with liposomal ODN and (B) cryo-TEM image of nanoparticles from lower fraction with the multi-component nanoparticles encapsulating both Qdots and ODN.


