TARGETING CD37 AND FOLATE RECEPTOR FOR CANCER THERAPY: STRATEGIES BASED ON ENGINEERED PROTEINS AND LIPOSOMES

DISSERTATION

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ABSTRACT

One of the lingering challenges in cancer therapy is to selectively destroy malignant cells and minimize the toxicity to normal tissues. The field of therapeutic targeting has thus been attractive with the ultimate goal of developing anti-cancer agents that work like “magic bullets”. Herein, we explore therapeutic approaches through targeting to CD37 and folate receptor, two promising cellular surface markers that can be utilized for antibody and liposome-based targeted therapies.

In the first part, a novel recombinant CD37-targeted small modular immunopharmaceutical (CD37-SMIP) and nanoscale liposomal particles were used to target CD37 molecule. CD37 represents an attractive target for immunotherapy in B cell malignancies, but has been neglected in the past. We first demonstrated specific expression of CD37 surface antigen on B but not T cells in peripheral blood mononuclear cells (PBMC) from chronic lymphocytic leukemia (CLL) patients. Crosslinking the CD37 resulted in dose and time dependent apoptosis by CD37-SMIP in CLL B cells. In addition, CD37-SMIP induced antibody dependent cellular cytotoxicity (ADCC) but not complement dependent cytotoxicity (CDC) in CLL cells. In vivo therapeutic efficacy of CD37-SMIP was demonstrated in a Raji cell xenograft mouse model. These findings provide strong justification for CD37 as a therapeutic target and introduce SMIP as a novel class of targeted therapies for B cell malignancies. Furthermore, immunoliposomes specific to CD37 were constructed using post insertion method to validate the use of
liposomes as a carrier to potentiate the effect of CD37-SMIP. CD37-immunoliposomes specifically bound to CD37\(^+\) cells and induced apoptosis without the need for a crosslinking secondary antibody, a mechanism different from that mediated by CD37-SMIP molecule. These data suggest that CD37-immunoliposomes can act as a novel formulation design for CD37-SMIP, and may also be utilized for delivery of therapeutic agents, such as chemotherapies and nucleic acid-based therapies.

In the second part, folate receptor (FR) was investigated as a cellular target for liposomal delivery of chemotherapeutic and radiation therapeutic agents. In the first study, cholesterol derivatives, PEG-cholesterol and folate-PEG-cholesterol, were synthesized for PEGylation and FR targeting. These two compounds were incorporated into the lipid bilayer of FR-targeted liposomal doxorubicin. The physicochemical properties, binding affinity as well as \textit{in vivo} circulation of these particles were investigated. These studies suggested that cholesterol is a viable bilayer anchor for synthesis of FR-targeted liposomes. In addition, liposomes have been a main focus of tumor selective boron delivery strategies in boron neutron capture therapy (BNCT). Three novel carboranyl cholesterol derivatives were incorporated into the lipid bilayer components for the construction of FR-targeted boronated liposomes for BNCT. These liposomes were taken up extensively in FR overexpressing KB cells \textit{in vitro} and the uptake was effectively blocked in the presence of free folate. These data collectively suggest that FR-targeted liposomes can be used as a delivery strategy for this class of novel carboranyl cholesterol derivatives.
Dedicated to my wife
ACKNOWLEDGMENTS

*It is good to have an end to journey toward,*

*but it is the journey that matters in the end.*

*-Ursula K. LeGuin*

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CHAPTER 1

INTRODUCTION

1.1. Overview of current research

One of the lingering challenges in cancer therapy is to selectively destroy malignant cells and minimize the toxicity to normal tissues. The field of targeting has thus been attractive with the ultimate goal of developing anti-cancer agents that work like “magic bullets”. This can be realized by several approaches, including antibodies, engineered antibody-like protein therapeutics, chemotherapy-antibody conjugates, radioimmunotherapy, immunotoxins, antibody-enzyme conjugates, photodynamic therapy, magnetic drugs, ligand-coated targeted drug delivery systems, antibody-coated targeted drug delivery systems.

The concept of using antibodies to selectively act on cancer cells has been around for many years, but has only been until recent decades that there was a wave of development of engineered monoclonal antibody-based protein therapeutics. Another significant explosion of activity is to use targeted drug delivery system, such as liposomes coated with ligands or antibodies, for specific delivery of therapeutic agents that by themselves do not distinguish normal vs. malignant tissues. Numerous targets have been in the past exploited for cancer treatment, including but not limited
to folate receptor, transferrin receptor, HER2, VEGF, integrin, IL-2 receptor, CD19, CD33, CD20, CD52, CD22, CD40, and CD74.

One longstanding interest of mine has been the development of targeted treatment for cancer therapy. My overall goal of research is thus to utilize our current knowledge in understanding tumor specific cellular markers to design targeted therapy with the potential to be translated into future clinical application. During the last 5 years with Dr. Lee and Dr. Byrd, I have had the opportunity to investigate this through two different approaches: engineered protein as novel therapeutic reagents and targeted liposomes as drug delivery vesicles. In particular, I have utilized two cellular surface targets that are specifically expressed on cancer cells. One of such targets is FR, which is over-expressed on acute myelogenous leukemia (AML) cells and other types of cancers. The other target is CD37, a B cell-specific marker for immunotherapy in chronic lymphocytic leukemia (CLL) and non Hodgkin’s lymphoma (NHL). Therapeutic approaches aimed at targeting these 2 molecules for antibody or liposome-based therapies are presented in this dissertation.

In the first part of my research, CD37 was investigated as a target for immunotherapy in B cell malignancies. CD37 is B cell surface antigen that represents an attractive target for immunotherapy in B cell malignancies, but has been neglected in the past. In collaboration with Trubion Pharmaceutical Inc., we have investigated CD37-specific small modular immunopharmaceutical (CD37-SMIP) for treatment against CLL and NHL. These studies are described in Chapter 2-4.
In Chapter 2, we first demonstrate specific expression of CD37 surface antigen on CLL B cells. Significant induction of apoptosis by CD37-SMIP against primary CLL cells was observed. The apoptosis induced by CD37-SMIP was correlated with levels of CD37 surface expression. The apoptosis occurred independent of caspase activation. The effector function of CD37-SMIP is shown to involve antibody dependent cellular cytotoxicity (ADCC) but not complement mediated cytotoxicity (CDC) in CLL cells. The ADCC function was mediated by natural killer (NK) cells but not naïve or activated monocytes. In addition, combination of fludarabine and CD37-SMIP further synergized the direct cytotoxicity in vitro.

In Chapter 3, we demonstrated that CD37-SMIP has significant cytotoxicity against B-cell lymphoma/leukemia cell lines in vitro and in vivo. Several leukemia and lymphoma cell lines were tested for CD37 expression. Results showed high levels of expression of CD37 on NHL cell lines. Cytotoxicity was observed with CD37+ cell lines through induction of apoptosis and ADCC. Moreover, significant in vivo therapeutic efficacy was demonstrated in a Raji cell xenograft mouse model. We also illustrated that the in vivo effect of this engineered protein appeared to be dependent on NK cells. These findings provide strong justification for CD37 as a therapeutic target and introduce small novel small modular immunopharmaceuticals as a novel class of targeted therapies for B cell malignancies.

Chapter 4 deals with a unique CD37-targeted immunoliposomes formulation using CD37-SMIP. This is based on the observation that CD37-SMIP induced apoptosis upon secondary crosslinking. Therefore, we hypothesized that liposomes
can be utilized as a carrier to potentiate the effect of CD37-SMIP. Immunoliposomes specific to CD37 were constructed by coupling CD37-SMIP to liposomes. CD37-immunoliposomes specifically bound to CD37$^+$ cells, including primary CLL cells and CD37$^+$ B cell lines. These liposomes induce apoptosis directly upon binding to the target, CD37. CD37-immunoliposomes showed significant level of apoptosis upon direct binding to the target, without a requirement for crosslinking, a mechanism different from CD37-SMIP molecule. These data suggest immunoliposomes as a novel formulation design for engineered antibodies that require crosslinking to kill CLL cells. In addition, CD37-immunoliposomes are also potential valuable targeted delivery systems that can be utilized for delivery of other therapeutic agents, such as chemotherapies and nucleic acid-based therapies, to CLL cells.

The second part of this dissertation is concerned with the folate receptor as a target for treatment in cancer. The work covers Chapter 4 and 5. In Chapter 4, cholesterol, a biocompatible material and an important component in biomembranes, was investigated as an alternative anchor for polyethulene glycerol (PEG) and folate. In this study, cholesterol derivatives were synthesized for PEGylation and folate receptor targeting, and incorporated into the bilayer of folate receptor-targeted liposomal doxorubicin. The colloidal stability of these cholesterol derivative-containing liposomes was superior to non-PEGylated liposomes, indicating that steric barrier provided by mPEG-cholesterol can efficiently inhibit aggregation of liposomes. Folate receptor-targeting activity of these liposomes was demonstrated by \textit{in vitro} cell binding studies on folate receptor-overexpressing KB cells. In addition, \textit{in vivo} circulation of cholesterol-anchored liposomes was prolonged compared to non-
PEGylated liposomes. These studies suggest that cholesterol is a viable bilayer anchor for synthesis of PEGylated and FR-targeted liposomes.

In chapter 6, we further extended the FR-targeted delivery strategy for tumor selective delivery in boron neutron capture therapy (BNCT), a binary radiotherapeutic method for the treatment of cancer. Three novel carboranyl cholesterol derivatives were incorporated for the construction of FR-targeted boronated liposomes for BNCT. *In vitro* studies were carried out to exam the uptake level of these liposomes in FR overexpressing KB cells. These data collectively suggest that FR-targeted liposomes can be used as a delivery vehicle for this class of novel carboranyl cholesterol derivatives.

Listed in the Appendices are some of my other efforts during graduate study that have contributed to publications in collaboration with other colleagues. Most of them are closely related to liposomal drug delivery and targeted therapy. In Appendix A, we designed liposomal delivery system for co-encapsulation of fludarabine and mitoxantrone. Our results showed that the fludarabine and mitoxantrone in fixed ratio can induce synergistic effect by co-encapsulation. This strategy can be potentially utilized in combination with CD37-targeted immunoliposomes to treat hematologic malignancies such as CLL. In Appendix B, my contribution to develop a CD52 positive *in vivo* animal model for preclinical evaluation of CD52-specific therapies is listed. Appendix C is a project aimed at process development of FR-targeted liposomal doxorubicin. We used solvent exchange system, tangential filtration system
and high pressure homogenization for pilot scale production of these targeted liposomes.

1.2. Antibody-based therapy in cancer treatment

The concept of using monoclonal antibodies (MAbs) as “magic bullet” for treatment of disease was first introduced soon after the discovery of MAbs by Kohler and Milstein 30 years ago.\(^1\) Since then, a vast range of antibodies have been evaluated as pharmaceuticals, based on different cell killing mechanisms. The development of MAbs as successful targeted therapy is best evidenced by FDA’s approval of multiple MAbs and others currently in phase III clinical trials. Hematological malignancies are particularly attractive for engineered protein development, evidenced by the large number of clinical trials aimed at using this type of therapy in diseases such as non Hodgkin’s lymphoma (NHL), follicular lymphoma and chronic lymphocytic leukemia (CLL).

This is particularly true for B-cell lymphoproliferative disorders where two therapeutic antibodies have been approved. Rituximab (Rituxan\(^{®}\)), a recombinant chimeric anti-CD20 MAb, was the first MAb approved by FDA for treatment of cancer in 1997.\(^2,4\) Another humanized rat antibody to CD52, alemtuzumab (Campath-1H), was also approved for treatment of refractory CLL 4 years later.\(^5\) The list of approved antibody therapeutics will continue to grow with more than 30 antibodies currently in late-phase clinical trials.\(^6\) Variety of hematological malignancy-related antigens have been extensively evaluated, including CD20,\(^3,7-9\) CD52,\(^5\) CD33,\(^10,11\) CD40,\(^12\) CD23,\(^13\) CD74,\(^14\) CD47,\(^15\) HLA-DR,\(^16\) CD80,\(^17\) CD22,\(^18\) and CD19.\(^19,20\)
There are several key features to consider when a new therapeutic antibody is designed for clinical development: (1) the specificity of target antigens for tumor, (2) the selectivity of therapeutic antibodies for the target antigen, (3) the cancer cell killing effect and mechanism, (4) the safety of antibodies in humans, and (5) the ability to produce sufficient antibody quantity with a stable formulation for implementation of clinical trials.

Among these features, the specificity of antigen (Ag) target and the selectivity of antibody are the overriding factors. This makes the basis for MAb to be fundamentally different from traditional chemotherapeutic modalities. Advances in protein engineering have greatly facilitated the selection of high-specificity and high affinity therapeutic MAbs. However, the selection of specific tumor markers, particularly against solid tumors such as breast, lung and colon cancer, has proven to be extremely difficult in part due to inaccessibility of suitable target antigens. Therefore, clinical benefit is more favorable for hematological malignancy, especially such as chronic lymphocytic leukemia where the cancer cells are fully accessible to MAbs and have more selective agents.

The cancer cell-killing effect is another critical factor to be considered to achieve optimal therapeutic effect. This is directly related to the specificity of the Ab-Ag binding, but also highly relies on the selection of constant region, as it contains domains important for the effector function and other killing mechanisms. Various mechanisms have been proposed with substantial in vitro and in vivo studies to support some of the proposed mechanisms of action of MAbs in cancer therapy. These include ligand binding blockage, modulation of intracellular signaling, agonist
and antagonist activity,\textsuperscript{12,21} induction of apoptosis,\textsuperscript{3-5,14-16,22} complement dependent cytotoxicity (CDC),\textsuperscript{3-5} antibody dependent cell-mediated cytotoxicity (ADCC)\textsuperscript{3-5,14} and targeted delivery of therapeutic agents, such as toxin,\textsuperscript{23} radioisotope,\textsuperscript{7,8} and cytokine.\textsuperscript{24} For nonconjugated MAb, direct apoptosis and effector function (CDC and ADCC) are by far the most significant functions relevant to the effective cell-killing. However, the exact understanding of the \textit{in vivo} mechanisms involved in antibody mediated killing is still not fully defined. Even for some clinically approved MAbs with established efficacy, such as rituximab and alemtuzumab, the precise molecular and cellular mechanisms for B cell depletion remain uncertain.

Another challenge toward successful development of MAb-based immunotherapy is the safety issue. Although MAbs are considered very well tolerated as compared to chemotherapeutic agents, side effects are still considerable obstacles in early development. Undesired human anti-mouse antibody response, for example, has prevented murine MAbs from multiple administrations in early clinical trials. A partial solution to the antiglobulin response is the production of recombinant chimeric antibodies in which the rodent constant regions are replaced with human constant regions to diminish antigenicity and improve immune effector cell and complement recruitment. Furthermore, the rodent-specific residues in variable regions could still be exchanged by human sequences and humanized MAbs could be produced by grafting rodent complementarity determination sequence into human framework sequences in the variable region. Finally, the full humanization can be realized by direct selection of human-like V region sequences from combinatorial or synthetic phage libraries, and then coexpressed with human constant regions.
Another challenge is the reliable production of MAbs. For production of antibodies that are difficult to be generated using conventional hybridoma method, either for practical or ethical reasons, phage display library method has been a more recent approach. Genes encoding Fv or Fab structures of MAbs were cloned and expressed to bacteriophage vectors for antigen-specific immunoglobulin fragments production. However, Fv fragments are not potent enough to be used directly for therapy, and they are usually constructed with suitable human constant regions into expression vectors.

The last challenge before moving the therapeutically effective MAb to clinical trial is production of stable formulation. This is particularly true when the protein concentration is as high as 10 mg/mL, which is necessary for systemic or subcutaneous administration. Protein aggregation, immunogenecity, poor long-term storage stability profiles, poor manufacturability or scalability are frequently faced in protein therapy development process. In fact, this formulation problem sometimes may not arise until late in the product development cycle and can cause expensive delays in reaching clinical trials or the market. Therefore, addressing the question by formulation development in early phase development such as in preclinical study or phase I study is vital to mitigate these problems.

1.3. Liposomes as nanoparticulate drug carriers

Liposomes are spherical self-closed structures composed of lipophilic bilayers with entrapped hydrophilic core. The development of small size, stable, long-circulating, targeted liposomes has led to an exciting era in the pharmaceutical
application of nanotechnology. Giving the unique structure of liposomes compared to other drug delivery systems, both lipophilic and lipophobic therapeutic agents can be delivered by liposomes. The biocompatibility of liposomes if used with proper lipid composition also makes liposome attractive as safe and effective drug carriers. A variety of therapeutic agents have been incorporated into liposomes. Several have reached clinical use. These include liposomal doxorubicin (Doxil™)\textsuperscript{25}, daunorubicin (Daunoxome\textsuperscript{TM})\textsuperscript{26}, amphotericin B (Amphotec\textsuperscript{TM}, Ambisome\textsuperscript{TM}, Abelcet\textsuperscript{TM})\textsuperscript{27}, cytarabine (Depocyte\textsuperscript{TM}), and verteporfin (Visudyne\textsuperscript{TM}). Numerous liposomal formulations are in clinical trial, including vincristine, all-trans retinoid acid, topotecan, and cationic liposome-based therapeutic gene transfer vectors. Many more are in preclinical evaluation. Besides potential use in systemic gene delivery, cationic liposomes are routinely used as transfection reagents for plasmid DNA and oligonucleotides in the laboratory. The use of liposomes can be deliberately engineered to possess unique properties, such as long systemic circulation time, pH sensitivity, temperature sensitivity and target cell specificity. These are achieved by selecting the appropriate lipid composition and surface modification of the liposomes, thus expanding the application of liposomes for drug delivery.

Liposomal delivery of anticancer drugs has been shown to greatly extend their systemic circulation time, reduce toxicity by lowering plasma free drug concentration, and facilitate preferential localization of drugs in solid tumors based on increased endothelial permeability and reduced lymphatic drainage, or enhanced permeability and retention (EPR) effect\textsuperscript{28}. Clearance of drugs in a liposomal formulation is
mediated by phagocytic cells of the reticuloendothelial system (RES), primarily located in the liver and spleen. Drugs can be loaded during liposome preparation through methods such as passive entrapment. Alternatively, they can also be loaded after liposome preparation, through a process called “remote loading” utilizing the ion gradient across the membrane as in the case of doxorubicin. Compared to free drugs, liposomal drugs are not subjected to rapid renal clearance. Instead they are cleared by RES, thus exhibiting prolonged systemic circulation time \(^{29}\). RES clearance of liposomes can be reduced by surface coating with polyethyleneglycol (PEG). Passive targeting effect has also been shown in liposomal drug delivery to solid tumors, where liposomes preferentially localize in these tumor tissues due to the enhanced permeation and retention (EPR) effect\(^{30}\).

1.4. Development of targeted liposomes for cancer therapy

Liposomes can be targeted to specific cell populations via incorporation of a desired targeting moiety, such as a chemical conjugated ligand, antibody, or antibody fragment directed against the surface molecules. Targeted delivery of these liposomes can greatly improve their selectivity to cancer cells and facilitate their cellular uptake and intracellular drug release. Examples of such targeted liposomes are folate-conjugated liposomes targeting to acute mylogenous leukemia, CD19-targeted immunoliposomes for non-Hodgkin’s lymphoma (NHL) therapy\(^ {31}\), and anti-HER2 immunoliposomal doxorubicin targeting to HER2-overexpressing breast cancer cells\(^ {32}\).
A variety of techniques have been described to incorporate targeting moieties to liposomes. First, ligands such as folate can be conjugated to a lipid composition such as PEG-DSPE or PEG-Chol. This amphophilic lipid composition can be constructed into the lipid bilayer during the formation of membrane and leave the ligand outside of the particles.\textsuperscript{33-41} The second method is to first include a reactive lipid crosslinker exposed outside of the liposomes after the formation of bilayer membrane.\textsuperscript{32,42-49} Thus the exposed crosslinker can react with activated targeting moieties such as thiolated proteins (antibody, scFv, or transferrin etc.). The most useful thiolation reagent is 2-iminothiolane, also known as Traut’s reagent. There are 2 types of immunoliposomes based on the antibody coupling strategy. Type one involves direct attachment of the antibodies to the lipids,\textsuperscript{43,48} and the type two involves linking the antibodies to the terminal ends of reactive PEG derivates.\textsuperscript{32,42,46,48,50} Most of these coupling techniques lead to a random antibody orientation at the liposome surface. A significant contribution to the development of the type two immunoliposome, recently being adopted for formulation of HER2-targeted immunoliposomes, is an antibody coupling method called “post-insertion” method\textsuperscript{32}. This method involves formation of micelles of lipid-derivatized antibodies first, which is then followed by transferring the antibody-coupled PEG-lipid micelles to the preformed liposomes, thus converting the conventional liposomes to immunoliposome through a simple one-step incubation method. Maleimide-terminated PEG-DSPE is a coupling lipid that has been validated to successfully convert commercially available liposomal doxorubicin (Doxil/Caelyx) to targeted sterically stabilized liposomal doxorubicin. The last post insertion method seems highly promising for future clinical development.
Immunoliposomes are targeted liposomes with specific antibody or antibody fragments on their surface to enhance the specific delivery to targets, usually to cancer cells as a major research interest. It is emerging as the third generation of the nanocapsule drug delivery system, representing a novel strategy for cancer-targeted drug delivery.

The feasibility of using immunoliposomes as drug delivery vesicles is a result of parallel advances both in the monoclonal antibody (MAb) and liposome technology. This tumor-specific high payload delivery vesicle, as we call “magic missile”, combines the tumor-targeting properties of MAbs and drug delivery advantages of liposomes, thus representing one of the most advanced form of targeted drug delivery system. Several studies have been carried out, reporting successful targeting and enhanced therapeutic efficacy. For example, CD19-targeted immunoliposome has been developed by Allen et al for delivery of doxorubicin and antisense oligodeoxynucleotides. Harata et al designed imatinib-CD19-liposome and demonstrated specific and efficient death of Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL) cells. In another study, Huwyler et al. used OX26 MAb-mediated liposomal targeting of transferrin receptor to overcome blood brain barrier. Park et al. moved anti-HER2 immuoliposomal doxorubicin to Phase II clinical trial for breast cancer therapy, and recently Matsumura et al. also conducted clinical trials using MCC-465, a GAH-targeted immuoliposomal doxorubicin formulation, in human stomach cancer.

The advantages of liposome-based targeted delivery system reside in: (1) selective delivery of high payload of cytotoxic agents towards malignant cells, not to normal
cells; (2) avoidance of compound structure modification, because drugs are encapsulated into particles instead of through covalent bond conjugation; (3) pharmacokinetic advantages, such as prolonged systemic circulation, optimized tissue distribution and reduced toxicity.

1.5. **CD37 as a target for immunotherapy in B cell malignancies**

1.5.1. **CD37 is a B cell-specific surface marker**

CD37 antigen is a glycoprotein of molecular weight 40-52 kDa, usually with two N-linked carbohydrate chains attached to a 26kDa core protein. It belongs to tetraspan superfamily, proteins with 4 transmembrane domains, which also includes proteins such as CD9, CD53, CD63, CD81, and CD82. It is strongly expressed on mature B lymphocytes, including normal and neoplastic cells. Its expression is observed in post pre-B cell stage, maintained on peripheral B cell, but not on plasma cells. Characterization of CD37 in mice revealed that the expression level of CD37 on resting and activated T cells, monocytes, granulocytes and neutrophil granulocytes is low. There is no CD37 expression on NK cells, platelets or erythrocytes. The immunohistochemistry on frozen sections using monoclonal antibody (MAb) indicated CD37 has a B cell-restricted staining pattern.

The function of CD37 is yet unclear, but is believed to be involved in signal transduction that plays a role in the regulation of cellular development, activation, growth and motility. Studies using CD37 knockout mice showed that CD37 is not essential for B-cell development, but, instead, acts as a nonclassical costimulatory molecule or by directly influencing antigen presentation via complex formation with
MHC class II molecules. Association of CD37 with other tetraspan superfamily antigens (CD53, TAPA-1, and R2/C33) as well as CD19, CD21 has been reported to coprecipitate with DR antigens by immunoprecipitation, suggesting that CD37 may be involved in antiproliferative and signaling effects. In addition, observation of CD37 in multivesicular endosomes and exosomes secreted by human B lymphocytes reflected possible involvement of CD37 in intracellular trafficking.

Due to its constant expression in nearly all mature B-cells, CD37 serves as a very attractive target for B cell malignancies involving mature B cells, including chronic lymphocytic leukemia (CLL), non Hodgkin’s lymphoma (NHL) and hairy cell leukemia (HCL).

1.5.2. CD37-targeted therapeutics in hematological malignancies

The CD37 antigen is one potential target that has not been adequately evaluated. In preclinical studies, radiolabelled murine CD37 MAb, MB-1, was examined in a xenograft athymic nude mouse model, and tumor size was found to be significantly decreased. In another study, bispecific MAbs with reactivity to high-affinity Fc receptor for IgG (Fc gamma RI/CD64) and CD37 have been investigated with the aim to treat NHL by macrophage-mediated phagocytosis. Significant phagocytosis of B lymphocytes by macrophages was found in this study. However, efforts to target CD37 clinically have been limited. MB-1, a $^{131}$I labelled murine MAb, was evaluated in patients with non-Hodgkin’s B-cell lymphoma, where both CD37 and CD20 antibodies were evaluated. Despite clinical responses observed
in these studies, CD20 was chosen as the target antigen by many for therapeutic antibody therapy and no subsequent efforts have been made to target CD37.

Given the relative selectivity of CD37 as a B-cell antigen and potential therapeutic target, a CD37-small modular immunopharmaceutical (SMIP) was developed by Trubion Pharmaceutical, using variable regions (VL and VH) from G28-1 hybridoma and engineered constant regions encoding human IgG1 domains (hinge, CH2 and CH3). (Figure 1.1) Therefore, CD37-SMIP was improved from mouse CD37 MAb, with most of the mouse sequence replaced with human portion, and has greatly reduced immunogenicity. Initial expressions were performed by transfection of COS 7 monkey kidney cells and screened for specific binding to human B cell lines. The selected recombinant expression plasmid was used to transfect Chinese hamster ovary (CHO) cells, and further selected under methotrexate pressure. The final stably expressing cell line was used in production of the fusion protein, which was purified from CHO culture supernatant by chromatography. To enhance the production of sufficient high quality material, acceptable pharmacokinetics, and therapeutic efficacy, several technical considerations were made. These modifications provided a production efficiency that will allow sufficient production of CD37-SMIP for clinical investigation and were further screened for their ability to recruit effector cells to mediate cellular cytotoxicity. In addition, CD37-SMIP was engineered to have a molecular weight above that filtered by the glomerulus to avoid rapid elimination. This size feature of the CD37 SMIP offers the potential advantage of an extended half life in vivo compatible with other biologic therapies such as monoclonal antibodies.
1.5.3. *Therapeutic challenges in chronic lymphocytic leukemia*

Chronic lymphocytic leukemia (CLL) currently constitutes a substantial portion of hematopoietic malignancies. The annual incidence of CLL represents the most common form of leukemia in United States. However, despite extensive efforts in developing novel therapeutics, CLL is still presently regarded as incurable.\(^{77-79}\) For many years, only marginally effective therapies that rarely yield complete responses are available for CLL therapy.

The traditional therapeutic approach to CLL has been using chemotherapeutic regimens, such as chlorambucil, cyclophosphamide-vincristine-prednisone\[COP\]; cyclophosphamide-doxorubicin-prednisone \[CAP\]; cyclophosphamide-doxorubicin-vincristine-prednisone\[CHOP\], which can produce higher response rates than alkylating agents but not longer survival rates.\(^{80}\) The real breakthrough was from fludarabine, a purine analog, which produced not only higher response rates but also a longer disease-free progression than chlorambucil.\(^{81}\) Another breakthrough is the introduction of MAbs, including rituximab and alemtuzumab into the CLL therapy.\(^{4,79}\) Because MAbs act on a different mechanism from chemotherapeutic agents, they alone or in combination, lead to effective therapy for patients with CLL. Recently, fludarabine has been built as the “golden standard” into new treatment regimens, with or without other chemotherapeutic agents and MAbs that enhance the effect of the purine analogs. Retrospective comparison of fludarabine alone and fludarabine plus rituximab combination indicated the addition of rituximab may prolong progression-free survival and overall survival in previously untreated CLL patients.\(^{82}\) Other combination therapy approaches have also been investigated, such as
fludarabine plus mitoxantrone, combination of fludarabine, cyclophosphamide and rituximab (FCR), fludarabine plus alemtuzumab, etc. Another encouraging observation is to use fludarabine along with cyclophosphamide and mitoxantrone (FCM), with or without rituximab or alemtuzumab, where increased response rates and molecular response were observed, indicating an important message that eradication of minimal residue disease could be achieved in patients with previously treated CLL. Some other new therapies currently under investigations also include, transcriptional repression–inducer depsipeptide, protein kinase C inhibitor UCN-01, kinase inhibitor flavopiridol, phosphodiesterase inhibitor theophylline.

Regardless of all the active research conducted directed against multiple therapeutic targets, so far, no therapeutic regimen can truly cure patients with CLL, and the eradication of minimal residue disease continues to be the biggest challenge, which could possibly be addressed by research herein.

1.6. Folate receptor targeted liposomes for cancer therapy

Targeted drug delivery is a promising strategy to improve both the efficacy and safety of treatment. This is especially attractive for cancer therapy, as most anticancer drugs have only marginal therapeutic index. Various cellular surface receptors and antigens have been targeted through different strategies. Among these, folate receptor (FR) has been one of the targets extensively investigated. FR is selectively amplified on human malignant cells, and can take up folate and its analogs through a receptor-mediated endocytosis process. FR-targeted liposomes can be utilized to deliver
therapeutic agents to FR positive cells\textsuperscript{35}. These liposomes have been evaluated for the targeted delivery of a wide variety of agents.

1.6.1. \textit{Folate receptor as a cancer-specific cellular marker}

FR, also known as folate-binding proteins (FBP), is an N-glycosylated protein with high binding affinity to folate. FR has three isoforms, \( \alpha \), \( \beta \) and \( \gamma/\gamma' \). FR-\( \alpha \) and FR-\( \beta \) are glycosyl-phosphatidylinositol (GPI)-anchored membrane bound proteins\textsuperscript{97,98}, whereas FR-\( \gamma/\gamma' \) are constitutively secreted\textsuperscript{95,99,100}. The \( \gamma' \) isoform is a truncated form of FR-\( \gamma \)\textsuperscript{101}. These isoforms display different patterns of tissue specificity, and they also present differential ligand stereospecificities\textsuperscript{95}. The two membrane receptor subtypes, \( \alpha \) and \( \beta \), share high amino acid sequence identity (70%), but are distinguishable by differential affinities for folic acid and stereoisomers of reduced folates (affinities for folic acid: FR-\( \alpha \) \( K_d \approx 0.1 \text{nM} \)\textsuperscript{102}, FR-\( \beta \) \( K_d \approx 1 \text{nM} \)\textsuperscript{103}, and FR-\( \gamma \) \( K_d \approx 0.4 \text{nM} \)\textsuperscript{99}).

Expression of FR is both tissue-specific and differentiation dependent. The expression of FR has been identified by immunohistochemical staining, reverse transcription-polymerase chain reaction (RT-PCR), western blotting, and 3H-folic acid binding both in normal and malignant tissues\textsuperscript{104,105}. Functional FR expression is absent in most normal tissues, with the exception of the luminal surface of certain epithelial cells\textsuperscript{96}, where it has limited accessibility to the blood stream. On the other hand, FR-\( \alpha \) is consistently expressed in several carcinomas\textsuperscript{105}, especially in non-mucinous ovarian carcinomas, uterine carcinomas, testicular choriocarcinomas, ependymomas, and pleural mesotheliomas, and less frequently in breast, colon, and
renal cell carcinomas.\textsuperscript{95,96,106} Correlation of FR-\( \alpha \) expression level and histologic grade has also been shown in ovarian and breast cancers, suggesting FR-\( \alpha \) as a possible malignant cellular marker for cancers\textsuperscript{107}. Methods for upregulating FR-\( \alpha \) expression using anti-estrogens and glucocorticoid agonists have been recently published.\textsuperscript{108,109} The effect of these agents is further enhanced by histone deacetylase (HDAC) inhibitors\textsuperscript{109}. FR-\( \beta \) is a differentiation marker in the myelomonocytic lineage during neutrophil maturation\textsuperscript{110} and is amplified in activated monocytes and macrophages\textsuperscript{111}. However, FR-\( \beta \) in neutrophils is unable to bind folate due to aberrant post-translational modifications\textsuperscript{112}. FR-\( \beta \) is also expressed in a functional form in chronic myelogenous leukemia (CML), in 70\% of acute myelogenous leukemias (AML)\textsuperscript{100,110,113,114}, and in activated macrophages associated with rheumatoid arthritis\textsuperscript{115}. FR-\( \beta \) expression is regulated by retinoid receptors and can be upregulated by all-trans retinoic acid (ATRA)\textsuperscript{116}. The effect of ATRA is further enhanced by HDAC inhibitors. The selective amplification of FR-\( \alpha \) and FR-\( \beta \) expression in human cancers suggests possible roles for FRs as cancer specific cellular markers and their potential utility as targets for drug and gene delivery to these diseases. FR-\( \gamma/\gamma' \) are reported to be specific to hematopoietic tissues, but are expressed at very low levels.\textsuperscript{100} FR-\( \gamma/\gamma' \) are in secreted form, thus have limited application for targeted drug delivery, but may serve as a serum marker to monitor certain hematological malignancies.\textsuperscript{100} The additional possibility of selective FR upregulation in the target cells might further enhance the targeting strategy.
1.6.2. Targeting FR for cancer therapy

The selective amplification of FR expression in both human solid tumors and leukemia suggests its utility as a potentially valuable target for drug and gene delivery. Both monoclonal antibodies against FR and folate itself have been evaluated as targeting moieties for targeting to the FR.

Two monoclonal antibodies (MAbs), MOv18 and MOv19, have been produced against FR-alpha on ovarian cancers. Both are murine MAbs of the IgG1 class raised against a poorly differentiated ovarian carcinoma, recognizing two distinct epitopes. Clinical studies on radioimmunoscintigraphy using $^{131}$I-MOv18 have been carried out in ovarian cancer patients and demonstrated improved targeting of ovarian cancer. In addition, alpha-emitter At-211 conjugated to MOv18 has been found to prolong survival in a murine peritoneal tumor model developed by i.p.-inoculation of OVCAR-3 cells.

Folate itself has in fact been the focus of recent development of FR targeting ligand. This is possibly because: (1) folate is a low molecular weight (MW = 441) ligand, and has high affinity to FR; (2) the conjugation chemistry of folate is defined and relatively easy to carry out; (3) folate is a compound of unlimited availability; and (4) folate lacks immunogenicity in contrast to murine MAb. The conjugation of folate via its $\gamma$-carxocyl has been shown to preserve its binding affinity to the FRs. Recent studies also found that the glutamate residue of folic acid is not critical for FR recognition, and only pteroic acid is essential for FR binding. Similar to the uptake process of the vitamin folate, FRs mediate the cellular internalization of folate conjugates via receptor-mediated endocytosis, therefore this highly specific event can
be utilized to promote uptake of folate-therapeutic agent conjugates in FR positive cancer cells.

Early proof-of-concept *in vivo* studies were conducted using either radiolabeled or fluorochrome-labeled folic acid. The selectivity has also been documented in tumor-bearing mice using radiolabeled folate conjugates, such as 67Ga-deferoximaine-folate, 99mTc-hydrazinonicotinic acid (HYNIC)-folate and 111In-diethylenetriamine pentaacetic acid (DTPA)-folate as potential folate-based radiopharmaceuticals\(^\text{119,120}\). Among these, 111In-DTPA-folate has been further evaluated in human clinical trials. Concentration of radioactivity in abdominal masses was found in women suspected of ovarian cancer, and only the kidneys and livers in some patients displayed significant retention of 111In-DTPA-folate. This reagent was developed by Endocyte Inc., an Indiana-based biopharmaceutical company, to be a folate-targeted radiopharmaceutical imaging agent (FolateScan\(^\text{TM}\)), and is currently in Phase II clinical trial designed to evaluate whether FolateScan\(^\text{TM}\) can be used to detect and make a disease or pathological assessment of ovarian and endometrial cancer.

A wide range of therapeutic agents have also been evaluated for enhanced cancer cell selective delivery. (Figure 1.2) These include chemotherapeutic agents\(^\text{121}\), radiopharmaceuticals\(^\text{122}\), prodrug-converting enzymes\(^\text{123}\), protein toxins\(^\text{124}\), T-cell specific antibodies\(^\text{125}\), haptens\(^\text{123}\), gene transfer vectors\(^\text{126,127}\), antisense oligodeoxyribonucleotides (ODNs)\(^\text{128}\), polymeric drug carriers\(^\text{129}\), and liposomes\(^\text{33-40,112,130-133}\) carrying a variety of therapeutic agents. These conjugates potentially have broad applications in numerous imaging and therapeutic modalities.
Based on the action site of therapeutic agents, application of FR-targeted delivery can be categorized into two types. For hydrophilic compounds with limited permeability through cell membrane, FR offers an efficient drug uptake pathway that can both specify and facilitate the accessibility of therapeutic active agents to cancer cells. Folate-conjugates enter FR positive cells through receptor-mediated endocytosis, which is different from passive permeation process. Delivery to cancer cells through a process different from traditional drug transportation has potential to make the drug more accessible to cancer cells. On the other hand, for drugs that are ready to take effect on the surface of cells, such as immunomodulator and prodrug-activating enzymes, FR may also acts as a concentrator for these types of drugs. FRs are generally over expressed on the cellular surface of various cancer types, and upon binding of FR drug conjugates they may enrich these therapeutic agents on the cell surface. Therefore, application of FR-targeted delivery can be exploited for variety of compounds.

Taken together, FR-targeting has been investigated as a valuable approach to enhance delivery of variety of agents. Thus, the interest in exploiting this receptor for targeting application is still increasing as demonstrated by the active researches conducted in both academia and industry. Among these, FR-targeted liposome, first designed and investigated by Lee\(^{38,39}\), has been attractive because of the advantages it possesses as a useful delivery system both for hydrophilic and hydrophobic agents.
FR-targeted liposomes as drug delivery carriers

FR-targeted liposomes can be prepared by including a small fraction (e.g., 0.1 mole%) of a lipophilic folate derivative into the lipid composition. Both folate-polyethyleneglycol (M.W. 3350)-distearoyl phosphatidylethanolamine (folate-PEG-DSPE) and folate-PEG-cholesterol (folate-PEG-chol) have been synthesized and shown to be effective in targeting liposomes to the FR. The synthesis of folate-PEG-DSPE and folate-PEG-Chol can be realized through two stages. First, an intermediate folate-PEG-amine is synthesized by reacting NHS-activated folate and reacted with PEG-bis amine and further purified using a Sepharose column. This folate-PEG-NH$_2$ is then ready to react with N-succinyl-DSPE to form folate-PEG-DSPE, or with cholesteryl chloroformate to form folate-PEG-chol. Another synthetic method has also been described by Gabizon, which is to couple FA to readily attainable H$_2$N-PEG-DSPE with the mediation of carbodiimide. These two synthesized lipophilic folate-derivatives have both been shown to be good components to be incorporated to liposomes. Compared to the synthesis of folate-PEG-DSPE, the synthesis of folate-PEG-chol is relatively simpler, and is less costly. With regard to stability, cholesterol also provides better chemical stability, and it is also known to be able to increase the structural integrity of lipid membranes through tight packing. The molecule of cholesterol is neutrally charged, different from the negatively charged DSPE molecule, thus can possibly provide better targeting effect when used as a lipophilic anchor to incorporate the folate molecule.
The preparation of FR-targeted liposomes is relatively straightforward, as the targeting lipid component is ready to be included into the lipid composition during the formation of membrane. A small percentage (0.1% to 0.5%, molar ratio) of folate-PEG-DSPE or folate-PEG-chol in the lipid can yield targeting effect to FR. Relatively long linker between folate and the lipid anchor (e.g., PEG 3350) was found to be necessary for FR binding of the liposomes\textsuperscript{38}. FR targeting was compatible with incorporation of 3 mole% of PEG-DSPE, which is required for prolonging the \textit{in vivo} circulation time of the liposomes. Lab scale production of these targeted liposomes can be performed by a thin-layer hydration followed by a polycarbonate membrane extrusion method. For scaling up, alternative methods such as liquid phase emulsification, high pressure homogenization and tangential flow filtration can be used. Recently our lab has successfully validated a processing method combining homogenization, ultrafiltration and remote loading for FR-targeted liposomal doxorubicin at clinical relevant scale quantity. Using this method, three consecutive batches of liposomes with mean particle size around 100nm was produced. Another alternative method for folate-PEG-Chol incorporation has not yet been investigated, which is to use “post insertion” method similar to HER2-targeted immunoliposome production, by incubating micelles of folate-PEG-cholesterol/PEG-cholesterol to pre-formed liposomal drugs. The latter method avoided the ligand stability concern during the liposome production, and thus has the potential to be used for future cGMP grade production of FR-targeted liposomes. Both hydrophilic drugs and hydrophobic drugs can potentially be loaded to the targeted particles through remote loading (doxorubicin, daunorubicin, vincristine, and topotecan) or passive entrapment
(paclitaxel, and ATRA), and therefore this FR-targeted liposomal drug delivery represents a valuable approach for FR-positive cancer therapy. (Figure 1.3)

Cellular uptake of FR-targeted liposomes has been characterized using KB cells, a FR-α (+) human oral carcinoma cell line. Binding of the liposomes has a relatively slow kinetics, occurring over several hours, presumably due to their size. The specific binding of these liposomes to KB cells was saturable and could be blocked by excess free folate in the binding media\textsuperscript{38}. Interestingly, the apparent affinity of the folate-conjugated liposomes to KB cells was much higher than that of free folate. This is due to multivalent binding of the folate ligand on the liposomes with the FRs on the cellular surface\textsuperscript{38}. The folate-liposomes are internalized into a low pH compartment via receptor-mediated endocytosis and the encapsulated drug molecules are released into the cytoplasm to induce cytotoxicity\textsuperscript{37}. (Figure 1.4.)

Drug delivery properties of FR-targeted liposomes have been studied in vitro using liposomes loaded with chemotherapeutic agents, such as doxorubicin\textsuperscript{39}, daunorubicin\textsuperscript{40}, and cisplatin. Lee et al. first reported the in vitro effect of doxorubicin and showed these targeted liposomes showed ~ 86-fold greater cytotoxicity in KB cells compared to non-targeted control liposomes\textsuperscript{39}. The enhancement in cytotoxicity was correlated with the increase in doxorubicin uptake and could be blocked by excess free folate\textsuperscript{39}. Furthermore, a study by Goren et al. showed that the delivery of doxorubicin via FR-targeted liposomes bypassed Pgp-dependent drug efflux in drug resistant FR-α(+) M109 murine lung carcinoma cells in vitro and in an adoptive assay in mice\textsuperscript{36}. These data suggest that this delivery modality might overcome drug
resistance in these tumor cells\textsuperscript{36}. More recently, folate-liposomal doxorubicin was studied in FR-β(+) KG1 human AML cells and similarly showed enhanced cytotoxicity relative to non-targeted control liposomes\textsuperscript{112}, and the effect was enhanced by FR-β upregulation using ATRA\textsuperscript{112}. Therefore, both FR-α and FR-β are potential targets for folate-conjugated liposomes.

In addition to chemotherapeutics, a variety of agents have been loaded into FR-targeted liposomes and evaluated \textit{in vitro}. These include fluorescent\textsuperscript{38} probes, BNCT agents\textsuperscript{134}, photosensitizers\textsuperscript{135}, antisense oligodeoxyribonucleotides against the EGF receptor\textsuperscript{136}, and plasmid DNA carrying luciferase reporter gene\textsuperscript{37,41}. Similar formulations of FR-targeted lipid nanoparticles containing paclitaxel have also been reported recently\textsuperscript{137}. In each of these studies, the FR-targeted formulation was shown to be much more efficient in cellular uptake and biological activity compared to the non-targeted control formulation.

The in-vivo clearance rate for folate-conjugated liposomal doxorubicin was faster than non-targeted control liposomes in mice engrafted with FR-overexpressing M109 or human KB carcinoma or mouse J6453 lymphoma\textsuperscript{34}. This result also suggested a possible role of folate receptor in the differential rates of clearance\textsuperscript{34}. Plasma clearance kinetics of FR-targeted liposomes radiolabled with 67Ga was studied in rats. These liposomes showed enhanced plasma clearance rate and higher uptake in the liver and the spleen compared to non-targeted control liposomes\textsuperscript{34}. This effect was even more pronounced in rats that were placed on a folate free diet, suggesting that FR expression in the RES organs might be responsible for the increase in the rate of
liposome clearance\textsuperscript{34}. One possible explanation is that FR-targeted liposomes, by virtue of its multivalency, can interact with the FR-β that is present at a relatively low level in the RES cells.

In biodistribution studies in murine tumor models, FR-targeted liposomes did not show enhanced distribution in tumors relative to non-targeted liposomes. This might be due to the fact that passive accumulation of the liposomes via the EPR effect was the predominant factor in tumor localization of both targeted and non-targeted liposomes, since endothelial extravasation was the rate-limiting step in tumor localization. In addition, FR-mediated enhancement in uptake of the targeted liposomes might be offset by the more rapid plasma clearance of these liposomes as discussed above [60]. Moreover, in solid tumors, FRs on tumor cell surface are not directly exposed to the circulation, thus not fully accessible by circulating FR-targeted liposomes. Therefore, extravasation, instead of specific binding, is the rate-limiting factor of liposome distribution in solid tumors.

An important observation \textit{in vivo} is that macrophages also have high uptake of FR-targeted liposomes. In a study done in an ovarian carcinoma ascites mouse model, results showed that macrophages take up most FR-targeted liposomes efficiently in the ascites fluid even in the presence of FR positive ascitic tumor cells\textsuperscript{138}, although other reports did show increased FR-targeted liposome uptake in FR(+) ascetic tumor cells\textsuperscript{34}. The relative uptake of folate-liposomes by these two cell populations was likely determined by the relative FR expression levels.\textsuperscript{34}
The therapeutic efficacy of folate-liposomal doxorubicin has been evaluated in several murine tumor models. In a KB cell xenograft model of athymic mice on a folate-free diet, folate-liposomal doxorubicin given i.p. at 10 mg/kg x 6 injections showed significant greater tumor inhibition compared to non-targeted liposomes. However, in another study using a M109R-HiFR syngraft model in BALB/c mice on a regular folate-containing diet, folate-liposomal doxorubicin given i.v. at 8 mg/kg x 4 injections showed a similar therapeutic efficacy to non-targeted control liposomes. The differences in these studies might be partly due to the different routes of administration, animal diet, and dosages applied. A challenge in demonstrating a therapeutic benefit of folate-liposomal doxorubicin is the excellent antitumor activity of PEGylated liposomal doxorubicin used as a control, which has optimal pharmacokinetic properties and EPR effect and are much more effective than the free drug. Further studies in additional models are likely needed to confirm possible therapeutic benefits of FR-targeted liposomal doxorubicin.

A possible mechanism for increased therapeutic efficacy for the folate-liposomal doxorubicin in solid tumor is altered intratumoral drug distribution. These liposomes might be more efficiently internalized by FR(+) tumor cells and or tumor-infiltrating macrophages that are FR(+). This in turn results in an increase in drug release with the tumor. In contrast, non-targeted liposomal doxorubicin may remain extracellular and be distributed in the interstitial space and releases drug more slowly. This may result in a bioavailable (free) drug concentration that falls below the minimum effective concentration (MEC) for inducing tumor cell apoptosis. Therefore, cellular
internalization and associated drug release may be a critical mechanism of liposomal
drug action in solid tumors and a rate limiting factor in overall drug delivery pathway.
The relative importance of FR expression on tumor cells and tumor infiltrating
macrophages and their role in uptake of folate-coated liposomes warrant further
investigation.

Leukemia is a better disease target as compared to solid tumors for this targeted
delivery strategy. This is because malignant cells are circulating in leukemia patients,
therefore are fully accessible by targeted liposomes. However, these liposomes need
to overcome several barriers in solid tumors before reaching cancer cells. Several
studies have already reported the antitumor efficacy of FR-targeted liposomal
anthracyclines in leukemia. In both L1210JF murine lymphocytic leukemia ascites
model and a KG-1 human acute myelogenous leukemia xenograft models, FR-
targeted liposomal doxorubicin and daunorubicin when administered i.p \textsuperscript{112}, showed
improvement in survival of treated mice compared to non-targeted control liposomes
or the free drug in the L1210JF model \textsuperscript{112}. In both mice models, folate-liposomal
doxorubicin given i.p. was also more effective in prolonging animal survival. \textsuperscript{112} The
relatively greater accessibility of leukemia cells in ascites tumors might have
contributed to the superior efficacy of the targeted liposomes.

Interestingly, pretreatment of mice with ATRA, an agent known to upregulate
FR-β expression in KG-1 cells \textit{in vitro}\textsuperscript{112}, before the injection of liposomes was also
shown to enhance the efficacy. An increase in long-term survival in these mice as a
result of FR-targeted liposomal doxorubicin was shown from 12.5\% to 60\%. 
Therefore, administrating ATRA to upregulate FR-β expression seems to be a potentially promising therapeutic strategy to be combined with the FR-targeted therapy.

1.6.4. FR-targeting in gene/antisense ODN delivery

Gene therapy is a promising approach for the treatment of cancer. The main obstacle for the clinical application of cancer gene therapy is the lack of gene transfer vectors that are safe, efficacious, and tumor selective. In recent years, targeted gene delivery through cellular receptors, using either viral or nonviral vectors, is emerging as a novel approach to enhance the efficacy of tumor-selective gene delivery.¹²⁸ The folate receptor (FR), which is absent in most normal tissues and elevated in over 90% of ovarian carcinomas and at a high frequency in other human malignancies, is an attractive tumor selective target. FR-targeted vectors include folate-derivatized adenoviruses, cationic polymers, cationic liposomes, and pH-sensitive liposomes. In addition, FR-targeted liposomes have been evaluated for the targeted delivery of antisense oligodeoxyribonucleotides.¹³⁹,¹⁴⁰ These vectors have invariably shown impressive FR-selectivity in cell culture assays and, in addition, shown promising tumor-specific gene transfer activity in several in vivo models. There are important theoretical advantages for FR-targeted vectors over traditional non-targeted vectors in therapeutic gene and oligodeoxyribonucleotide delivery in vivo to cancer cells. Further preclinical characterization of these vectors is, therefore, warranted to determine their potential utility in cancer gene therapy.
**FR-targeted nonviral vectors.** Cationic polymers and cationic liposomes have the ability to form electrostatic complexes with plasmid DNA and facilitate its cellular uptake via charge-mediated interactions. Such a non-specific mechanism of delivery is unlikely to be effective in gene transfer to tumor cells via systemic administration, given the high concentration of plasma proteins and blood cells in the circulation. In contrast, receptor-based targeting is a promising mechanism to facilitate tumor-selective gene delivery *in vivo*. Several FR-targeted vector formulations have been designed to combine the DNA binding properties of the cationic polymers and/or cationic liposomes and the FR affinity of an attached folate ligand. In these vectors, folate was either directly linked to a polymer or a lipid component, or indirectly linked via a PEG spacer. The types of formulations included polyplexes, lipoplexes, and lipopolyplexes. Charge ratios and vector composition were optimized in order to achieve the best possible balance between gene transfer activity and FR selectivity in transfection studies.

**FR-targeted polyplexes.** Several cationic polymer-folate conjugates have been synthesized for FR-targeted gene delivery. These include poly-L-lysine (PLL)-folate\(^{126,127,141}\), PLL-PEG-folate\(^{141}\), polyethylenimine (PEI)-folate\(^{142,143}\), PEI-PEG-folate\(^ {143}\), and pDMAEMA-PEG-folate\(^ {144,145}\).

First, Gottschalk et al. showed that folate-PLL conjugate was able to mediate FR-dependent gene delivery to receptor positive cells, albeit with relatively low efficiency.\(^{127}\) This was expected given the lack of endosomal lytic activity by PLL. Highly efficient delivery was, however, obtained when a replication-defective
adenovirus was added to facilitate endosomal disruption. Another report by Mislick et al.\textsuperscript{126} showed similar results with PLL-folate using chloroquine as a lysosomotropic agent. These studies suggested that a long spacer between folate and the polyplexes was not particularly essential and that endosome disruptive agent plays a critical role in PLL polyplex mediated gene delivery. However, Leamon et al. synthesized a PLL-PEG-folate conjugate and showed that it could facilitate efficient FR-dependent gene delivery without additional vector components.\textsuperscript{141} Furthermore, Ward et al. showed that PLL/DNA complexes, modified with folate via a PEG linker, exhibited both long systemic circulation time and efficient uptake by FR+ HeLa cells. In this study, the length of the PEG spacer was found to be an important factor in determining FR targeting efficiency. This formulation strategy, i.e., post polyplex formation conjugation with folate and PEG, was similarly applied to another cationic polymer, pDMAEMA, in a study by van Steenis et al., which produced similar results in FR+ OVCAR-3 ovarian carcinoma cells.

Guo et al.\textsuperscript{143,146} synthesized PEI-PEG-folate and showed that it can mediate FR-dependent delivery of reporter genes in FR+ cells. Unlike PLL, PEI has inherent endosomal lytic activity and can transfect cells efficiently without the help of another helper component. FR selectivity of the transfection complexes was overshadowed by non-specific transfection at high positive/negative charge (N/P) ratios but was shown to be maximized at the neutral charge ratios.

In summary, it appears that incorporation of a long PEG spacer between folate and a cationic polymer and a component with endosomal or lysosomal lytic property
are important, but might not be essential, for efficient FR-selective gene delivery. High FR selective gene transfer can only be obtained at neutral charge ratios, where the in vitro transfection activity might not be at a maximum. PEGylation appeared to be a useful method to prolong the systemic circulation time of the FR-targeted polyplexes. Folate conjugation and PEGylation following polyplex formation might be the preferred method since this does not interfere with DNA condensation by the cationic polymer, which is more efficiently accomplished with non-pegylated cationic polymers.

**FR-targeted lipoplexes.** Several cationic liposome formulations that incorporate a lipophilic folate derivative as a targeting entity have been studied. Xu et al.\textsuperscript{147} showed that delivery of p53 gene therapy using cationic liposomes conjugated to folate enhanced the antitumor efficacy of conventional chemo- and radiotherapy. The specific structure of the targeting component used was, however, not described. Folate-PEG-DSPE and folate-PEG-cholesterol, when combined with a cationic lipid RPR209120 and DOPE, formed lipoplexes with greatly reduced normal tissue gene transfer and efficient in vivo tumor gene transfer, although no increase in tumor accumulation was observed compared with non-targeted lipoplexes.\textsuperscript{148} In another study, Dauty et al. prepared an FR-targeted cationic liposomal vector incorporating 2 mole\% DPPE-PEG3340-folate, and a cationic dithiol-detergent (dimerized tetradecyl-ornithinyl-cysteine, (C14Corn)\textsubscript{2}) as the lipid component and showed efficient FR-dependent cellular uptake and transfection. The disulfide bond in this novel lipid can
be reduced, in response to the intracellular environment, into surfactant-like molecules, which might facilitate endosomal escape.

**FR-targeted lipopolyplexes.** LPDI type lipopolyplexes consist of a ternary complex of cationic liposomes, DNA-condensing polycation, and plasmid DNA. In a report by Reddy et al., polyplexes prepared from protamine were mixed with cationic liposomes containing folate-cys-PEG-PE as a targeting ligand and DOPE as a helper lipid. The resulting vector showed superior transfection activity in FR+ M109 murine lung carcinoma cells as well as in ascitic cells derived from L1210A murine lymphocytic leukemia cells.

LPDII type lipopolyplexes consist of a ternary complex of anionic liposomes, polycation, and plasmid DNA. Lee et al. reported the formulation of the first LPDII type vector, in which DNA was first complexed to PLL and then mixed with pH-sensitive anionic liposomes composed of DOPE/CHEMS/folate-PEG-DOPE (6:4:0.01 mole/mole). At low lipid-to-DNA (L/D) ratios, the vectors carried a net positive charge and transfected the cells independent of the FR. In contrast, at high L/D ratios, the vectors carried a net negative charge and mediated FR-dependent transfection in FR+ KB cells but not in FR- CHO cells. These first generation LPDII vectors were inactivated by the presence of serum. A variation of this vector formulation was, therefore, developed by Gosselin et al. as covalently cross-linked by dithiobis (succinimidylpropionate) (DSP) or dimethyl 3,3’-dithio-bis-propionimidate (DTBP), as a DNA condensing agent and a lipid composition of diolein/CHEMS/folate-PEG-Chol (6:4:0.05 m/m). The disulfide crosslinked PEI can
theoretically be reduced in the intracellular environment, which facilitates the cytosolic release of the associated plasmid DNA following cellular entry. Diolein was selected as a helper lipid due to its ability to promote cubic phase formation. The resulting FR-targeted vectors exhibited improved gene transfer activity in the presence of serum compared to the DOPE based FR-targeted LPDII vectors. Furthermore, Shi et al. reported efficient gene delivery using an LPDII vector that incorporated PEI as a DNA condensing agent and a cationic/anionic lipid pair, composed of DDAB/CHEMS/Tween 80/folate-PEG-PE, as a novel pH-sensitive endosome disruptive component and achieved excellent *in vitro* transfection results in serum containing media. Another FR-targeted LPDII-type formulation was reported by Reddy et al. In their formulation, PLL/DNA polyplexes with a net positive charge were combined with pH-sensitive liposomes composed of DOPE/cholesterol/ N-citraconyl-DOPE/folate-PEG-DOPE. The component N-citraconyl-DOPE is hydrolyzed at endosomal pH thereby, unmasking the fusogenic properties of DOPE. The resulting vector mediated highly efficient FR-dependent gene transfer.

**FR-targeted delivery of antisense oligodeoxyribonucleotides (ODNs).** ODNs, like gene constructs, are negatively charged molecules. They are, however, of a much smaller size and, therefore, present a different set of challenges for their effective delivery. To address the issue of serum stability, ODNs containing modified backbones, such as phosphorothioates, were developed and showed promise in clinical trials. Nevertheless, ODNs are currently administered via continuous
infusion due to their limited systemic circulation time. Developing a better delivery system that has prolonged circulation time and selectivity for tumor cells would undoubtedly improve the clinical potential of ODN drugs.

First, FR-targeted liposomes were evaluated as a potential vehicle for ODN delivery. In a study by Wang et al., a 15-mer antisense to the EGF receptor gene (AEGFR2) was entrapped in FR-targeted liposomes and evaluated in cultured KB cells. The FR-targeted liposomal ODNs exhibited greater efficacy in cellular growth and EGFR expression inhibition compared to free ODNs and non-targeted liposomal ODNs. A similar system based on liposomal entrapment was evaluated by Leamon et al. for the FR-targeted deliver of 35S or FITC-labeled ODNs. The study found that a PEG linker length of 1,000 daltons between folate and the lipid anchor was sufficient for full FR targeting efficiency. Approximately 105 liposomes per cell were taken up at 1 hr in cultured KB cells in that study. Despite showing the anticipated FR selectivity in cell culture, a further biodistribution study in nude mice carrying KB tumor xenografts showed only enhanced uptake in the liver but not in the tumor. This observation was consistent with earlier results from Guo et al., which indicated that 111In-labeled FR-targeted liposomes showed similar tumor localization compared to non-targeted liposomes. A possible explanation for the lack of an in vivo targeting effect of folate-derivatized liposomes is that their tumor localization was limited by the permeability of the tumor vasculature rather than the capacity of tumor cell binding.
Formulations based on cationic lipids have also been evaluated for ODN delivery. A recent study by Rait et al. showed that FR-targeted cationic liposomes were more efficient than the non-selective lipofectinTM transfection agent in delivering anti-HER-2 ODNs into breast cancer cells both in vitro and in vivo.\textsuperscript{155,156} The optimal cationic liposome composition was found to be DDAB/DOPE (1:1). Compared to free ODNs, folate-coated cationic liposomal ODNs exhibited prolonged systemic circulation time and increased tumor localization. Interestingly, treatment with these ODNs resulted in the chemosensitization of the tumor cells, both in culture and in a murine xenograft, to docetaxel, regardless of HER-2 expression status of these cells. A similar study was reported by Zhou et al.,\textsuperscript{157} in which an ionizable aminolipid 1, 2-dioleoyl-3-(dimethylammonio) propane (DODAP) and an ethanol-containing buffer system were used to achieve 60-80\% ODN entrapment efficiency. The formulation was found to be superior to non-targeted liposomes in ODN delivery in FR+ cells, which was unaffected by the presence of serum in the culture media.

In addition to liposomal carriers, ODNs have also been directly linked to folate. Li et al. showed that folate linked to the 3’ terminus of an anti-c-fos phosphorothioate/phosphodiester chimeric ODN showed FR-dependent (8-fold higher) uptake in the FR+ FD2008 ovarian cancer cells but not in receptor negative Chinese Hamster Ovary (CHO) cells and at the same time enhanced cellular growth inhibition in the FR+ cells by these ODNs.

**Factors affecting FR-targeted delivery in vivo.** Several factors have the potential to adversely affect FR-targeted gene transfer in vivo. The first is the presence of
endogenous folate in systemic circulation, which potentially can block FR binding. We believe that competition from the endogenous form of folate, i.e., 6S-N-5-methyltetrahydrofolate (at 1-50 nM in human plasma), which has ~30x low affinity for the FR compared to folic acid, should not significantly impede FR binding of multivalent folate conjugates, including folate-derivatized gene transfer vectors, which typically exhibit a much higher apparent affinity.

A second concern is that FR is expressed in the apical membrane of the kidney proximal tubules. Since the size of folate-coated gene transfer vectors precludes glomerular filtration, these FRs are inaccessible from blood circulation to these vectors.

A third obstacle is that, given the size of gene transfer vectors, escaping the vasculature and intratumoral diffusion could be limiting to targeted delivery. To address this issue, formulation parameters can potentially be optimized to improve the pharmacokinetic properties of the vectors. For example, the vectors can be PEGylated to reduce plasma protein binding and RES uptake, which results in extended systemic circulation time. In addition, the size of the vector can be kept under 300 nm since this is the approximate limit for efficient tumor extravasation.

Intratumoral diffusion rate of vectors will remain a limiting obstacle for tumor-directed gene delivery. Gene therapy strategies that do not rely on transgene delivery to a high percentage of tumor cells are, therefore, much more likely to lead to clinical success using any of the existing vectors, with the exception of, perhaps,
replication-competent infective viral vectors. Antisense ODNs, much smaller in size, should encounter a lesser obstacle associated with intratumoral distribution. For example, ODNs released from an FR-targeted vector can diffuse deeply into a solid tumor within a reasonable timeframe. The design strategy for ODN delivery vectors might, therefore, be focused on controlling the rate of release of ODNs from the vector and optimizing the pharmacokinetic properties of ODNs.

Finally, insufficient levels of FR expression on tumor cells in patients might be another obstacle for the development of FR-targeted vectors. A possible strategy to overcome this barrier is to induce FR expression upregulation in tumor cells by co-administration of anti-estrogen (for FR-α) or retinoid receptor ligands (for FR-β), which have recently been shown to greatly increase FR expression in tumor cells. Upregulation of FR-β using all-trans-retinoic acid (ATRA) in KG-1 human AML cells has been shown to enhance the FR-dependent cytotoxicity of FR-targeted liposomal doxorubicin as well as its therapeutic efficacy in a murine ascitic tumor model derived from KG-1 acute myelogenous leukemia cells.158
Fig. 1.1. Schematic diagram of CD37-specific Small Modular ImmunoPharmaceutical (SMIP). The molecule is constructed by a single chain variable region (scFv) as a binding domain, a hinge domain, and effector domains consisted of CH2 and CH3 domains from human IgG1.
Fig. 1.2. Folate-conjugates for FR-targeted delivery. A wide range of therapeutic agents have also been evaluated for enhanced cancer cell selective delivery. These include chemotherapeutic agents, radiopharmaceuticals, DNA and antisense oligodeoxynucleotide, prodrug converting enzymes, antibodies, boron neutron capture therapy agents, polymeric drug carriers, and liposomes carrying a variety of therapeutic agents.
**Fig. 1.3. FR-targeted liposome.** The ligand, folate, is conjugated to a lipophilic anchor (PEG3350-DSPE or PEG3350-chol) and incorporated to the lipid bilayer. PEG2000-DSPE is also included in the construction of the liposome membrane, thus prolonging the systemic circulation of these particles through avoiding the RES uptake. Hydrophilic anticancer reagents can be loaded to the aqueous core through active loading or passive entrapment, and hydrophobic reagents can be loaded to the lipid bilayer through formation of electrostatic complex or passive entrapment. The structures of two synthetic targeting lipids (F-PEG-DSPE and F-PEG-Chol) are also shown.
Fig. 1.4. **FR mediated endocytosis of folate-liposomes.** FR-targeted liposomes are internalized upon specific binding with FRs on cell surface through a receptor-mediated endocytosis process. These endosomes are transported and converted to late stage endosomes. The fusion of these late endosomes with lysomes facilitate the release of cytotoxic compounds inside the cells, thus kills cell specifically.
LIST OF REFERENCES


13. Rosenwasser LJ, Busse WW, Lizambri RG, Olejnik TA, Totoritis MC. Allergic asthma and an anti-CD23 mAb (IDEC-152): results of a phase I, single-dose, dose-escalating clinical trial. The Journal of Allergy and Clinical Immunology. 2003;112:563-570


43. Sunamoto J, Sato T, Hirota M, Fukushima K, Hiritani K, Hara K. A newly developed immunoliposome—an egg phosphatidylcholine liposome coated with pullulan bearing both a cholesterol moiety and an IgMs fragment. Biochimica Et Biophysica Acta. 1987;898:323-330


61. Moore K, Cooper SA, Jones DB. Use of the monoclonal antibody WR17, identifying the CD37 gp40-45 Kd antigen complex, in the diagnosis of B-lymphoid malignancy. J Pathol. 1987;152:13-21


82. Byrd JC, Rai K, Peterson BL, Appelbaum FR, Morrison VA, Kolitz JE, Shepherd L, Hines JD, Schiffer CA, Larson RA. Addition of rituximab to fludarabine may


97. Lacey SW, Sanders JM, Rothberg KG, Anderson RG, Kamen BA. Complementary DNA for the folate binding protein correctly predicts anchoring to the membrane by glycosyl-phosphatidylinositol. The Journal of Clinical Investigation. 1989;84:715-720


CHAPTER 2

CD37-SMIP INDUCES CELL DEATH IN CLL CELLS

2.1. Introduction

B-cell chronic lymphocytic leukemia (CLL), with an annual incidence of 8100 new cases, is the most common form of leukemia in United States.\(^1\) However, despite extensive efforts in developing novel therapeutics, CLL is still presently regarded as incurable.\(^2,^3\)

CD37 is a member of the tetraspans transmembrane family (TSTF)\(^4-9\). It is highly expressed on normal and neoplastic mature B cells, but not on pre-B cells or plasma cells.\(^10,11\) The expression level of CD37 on resting and activated T cells, monocytes, granulocytes is low. There is no CD37 expression on NK cells, platelets or erythrocytes\(^10,11\). The function of CD37 is yet unclear, but is believed to be involved in signal transduction that plays a role in the regulation of cellular development, activation, growth and motility.\(^12,13\)

Immunotherapy using monoclonal antibody (MAb) is emerging as a safe, selective method for the treatment of cancer. Several antibodies, including rituximab\(^14,15\) and alemtuzumab\(^16-20\) have been already introduced as novel therapies for the
treatment of CLL and NHL. The effectiveness and side effect profile of these antibodies resides in the antigen selectivity for tumor cells relative to normal cells. Antibodies such as rituximab are more selective for B-cells and its therapeutic application is not associated with significant toxicity or immunosuppression. In contrast, alemtuzumab targets the more broadly expressed CD52 antigen and is associated with more immunosuppression. 

Given the modest activity observed with rituximab monotherapy in CLL and often life threatening immunosuppression and infections associated with alemtuzumab, pursuit of antibodies targeting alternative antigens is a high priority for this disease. The CD37 antigen is highly expressed on B cells in a relatively selective manner. However, there have been relatively few studies evaluating it as a therapeutic immune based target. Adriamycin linked to G28, an anti-CD37 antibody, has been evaluated in mouse and showed effects. MB-1, a murine MAb, was labeled with 131[I] and evaluated in a lymphoma xenograft nude mouse model, and latter on in patients with non-Hodgkin’s B-cell lymphoma. It was found that although tumor response at nonmarrow ablative doses can be observed, the myelosuppressssion associated with this approach limited its application in clinic.

The aim of this study is to evaluate the effect and mechanism of CD37-SMIP, a recently developed engineered protein, in primary CLL cells. This molecule consists of a single chain variable region with specificity for human CD37 and effecor domains from human IgG1. We hypothesized that CD37-SMIP can bind specifically
to CD37 antigen expressed on CLL surface, and induce cell death through induction of direct apoptosis and mediation of effector functions.

2.2 Results

2.2.1. CD37 is a specific marker on B cells from CLL patients

CD37 antigen expression level was first examined by immunostaining using mouse anti-human CD37 antibody. The percentage of CD37 positive cells and mean fluorescence intensity (MFI) of purified B cells from CLL patients, as well as results from normal T cell samples from healthy donors, were summarized in Table 2.1. Results from this set of experiment showed greater than 90% (96% ± 3%) of the CLL cells expressed CD37 compared to 3% ± 2% in resting T cells from 5 normal healthy donors. Double staining in human peripheral blood mononuclear cells (PBMCs) using PE-CD37 and FITC-CD3 (T cell marker), FITC-CD19 (B cell marker) and FITC-16 (NK cell marker) also revealed that CD37 is specifically expressed on B cells, not on T cells or NK cells. (Figure 2.1) These results indicated that CD37 is highly expressed on B cells from CLL patients, and can be potentially utilized as a “non-CD20” target for immunotherapy. CLL as a common type of B cell malignancy is a suitable disease target for development of CD37-targeted treatment.

2.2.2. CD37-SMIP binds to CLL cells specifically

The specificity of CD37-SMIP binding to CD37 antigen on CLL surface was demonstrated by Figure 2.2. CD37-SMIP binds to CLL cells with relatively high affinity, illustrated by the high percentage of positive cells (96%) and the mean
fluorescence intensity at approximately the same level as mouse anti-human CD37 antibody. The addition of CD37-SMIP (5μg/mL), not trastuzumab, rituximab or alemtuzumab, could effectively block the binding of FITC-CD37 on CLL cells. This also proved that the binding of FITC-CD37-SMIP is specific to CD37 antigen on CLL cells. We also conducted double staining experiments on freshly separated human peripheral blood mononuclear cells from 3 CLL patient samples. A T-cell specific marker FITC-conjugated CD3 or a pan-B cell surface marker FITC-conjugated CD19 was used in combination to PE-labeled CD37-SMIP to define the specificity of CD37-SMIP binding. Results showed only CD19+ B cells were bound with CD37-SMIP, while < 1%CD3+ T cells were bound. (Figure 2.3.) These 2 sets of data supported the specificity of CD37-SMIP to CD37 antigen on CLL cells.

2.2.3. CD37-SMIP induces B-cell specific cell death through induction of apoptosis

Having confirmed the specific binding of CD37-SMIP to CD37 molecules in primary B-CLL cells, we next sought to determine whether the binding could also lead to apoptosis in the absence of effector cells as noted previously with other effective B-CLL therapeutic antibodies such as alemtuzumab and rituximab. Purified CLL cells were treated with CD37-SMIP in the presence or absence of a secondary cross-linking goat anti-human IgG antibody (Fc specific). While no apoptosis was observed with either crosslinking antibody or only with CD37-SMIP, addition of secondary anti-human Fc cross-linking antibody promoted maximal apoptosis (Fig. 2.4). The CD37-SMIP mediated apoptosis is shown to be dose dependent with
maximal effect seen at 5μg/mL, the CD37 saturating dose of CD37-SMIP in B-CLL cells (Fig 2.5.). Analysis of 14 additional B-CLL patient samples demonstrated significantly increased cytotoxicity mediated by CD37-SMIP compared to rituximab or alemtuzumab. Figure 2.6. demonstrate the cumulative results of these experiments with CD37-SMIP promoting more apoptosis (44.9±14.7%) than that observed with the rituximab (21±17.6%, P<0.001) and alemtuzumab (35.6±6.41%, P = 0.04). Figure 2.7. demonstrate that apoptosis induced by CD37-SMIP in B-CLL cells correlates with the expression of the target CD37 antigen (p = 0.002). Consistent with this, CD37-SMIP fails to mediate apoptosis in T-cells lacking any significant CD37 expression (Fig 2.8.). These data provide definitive evidence that CD37-SMIP induced apoptosis in B-CLL cells is correlated to the level of expression of CD37 antigen and is not cytotoxic to normal T-cells.

2.2.4. CD37-SMIP Induced Apoptosis in CLL Cells is Caspase-Independent

Apoptosis in CLL cells after antibody treatment can occur through various mechanisms involving activation of caspase dependent and independent pathways. In this context, we examined the mechanism by which CD37-SMIP induced apoptosis in B-CLL cells. Fludarabine is widely used as a therapeutic agent in B-CLL and mediates death through a caspase dependent pathway in B-CLL cells. Treatment of B-CLL cells with fludarabine (6.6μM) promotes modest apoptosis by 24 hours that is inhibited by a pan caspase inhibitor z-VAD-fmk (Figure 2.9) The caspase dependence of fludarabine is further supported by demonstrable processing of the inactive pro-
form of caspase 3 and cleavage of downstream target poly (ADP-ribose) polymerase (PARP) following fludarabine treatment as shown in Figure 2.10. Contrasting with fludarabine, CD37-SMIP promotes rapid apoptosis at 24 hours that is not prevented by z-VAD-fmk treatment as shown in Figure 2.10. Furthermore, caspase 3 and downstream PARP are not processed following treatment with CD37-SMIP. These data collectively suggest that CD37-SMIP induces apoptosis through a novel pathway different from fludarabine in primary B-CLL cells.

2.2.5. **CD37-SMIP efficiently mediates ADCC but not CDC**

We next investigated whether the modified Fc region on CD37-SMIP is efficient in mediating ADCC and CDC against primary CD19⁺ CLL cells. Peripheral blood mononuclear cells (PBMCs) from normal healthy volunteers mediated ADCC in the presence of CD37-SMIP (39.3% ± 18%, E:T = 50:1) but not in the presence of the negative control trastuzumab (5.3% ± 5%, E:T = 50:1). (Figure 2.11.). Further, for all effector/target ratios tested, the ADCC with CD37-SMIP was found to be significantly higher than the ADCC with either alemtuzumab (14.5% higher; 95% CI: 9.0%, 19.9%; p < 0.0001) or rituximab (15% higher; 95% CI: 9.7%, 20.6%; p < 0.0001) that are directed against CD52 and CD20 molecules, respectively. In contrast to their ability to mediate ADCC function, CD37-SMIP or rituximab failed to mediate CDC against CLL cells, while alemtuzumab effectively mediated CDC *in vitro* (Figure 2.12.).
2.2.6. **CD37-SMIP synergizes with fludarabine in inducing apoptosis in CLL cells**

Fludarabine is currently a standard therapy for CLL and has been combined with variety of MAbs in treatment. Due to different apoptosis mechanism associated with CD37-SMIP (caspase independent apoptosis), which is different from fludarabine-induced apoptosis in CLL cells, we further hypothesized that addition of CD37-SMIP to fludarabine can potentiate the cytotoxicity. The interaction between CD37-SMIP and fludarabine was thus tested using MTT method, where purified CLL B cells were treated with these 2 drugs in different concentration combinations (1:5 serial dilution starting from 7.3 μg/mL for fludarabine, and 1:5 serial dilution starting from 20 μg/mL for CD37-SMIP). The cytotoxicities were expressed as relative reduction in absorbance compared to media control at 24 hr after continuous incubation. The IC$_{50}$ of each combination was calculated from the viability curve and plotted on an isobologram analysis graph, where the line was drawn from the IC$_{50}$ of CD37-SMIP or fludarabine when used alone, and the dots were plotted using the concentrations of each reagent when applied in combination to reach 50% cell death. Dots above, on or below the line indicate antagonistic, additive, and synergistic effect, respectively. Results from one representative patient from a total of 8 patients were presented in Figure 2.13. The synergism between the fludarabine and CD37-SMIP was found at several different dose combinations, indicated by the dots below the iso-effect line.
2.3. Conclusion and discussion

Herein, we describe a new targeted therapeutic approach using the SMIP directed at the lineage specific CD37 antigen in CLL. CLL is currently incurable and available antibody therapies targeting CD20 (rituximab) or CD52 (alemtuzumab) are either less effective or highly immunosuppressive. CD37 therefore represents a potentially superior target for engineered protein based therapy, based upon either expression features relative to CD20 or selectivity relative to CD52.

Our data herein against primary CLL cells demonstrate CD37-SMIP is superior to both rituximab and alemtuzumab relative to direct cytotoxicity and ADCC efficacy. Additionally, CD 37 targeted SMIP therapy has B-cell selectivity that contrasts with alemtuzumab\textsuperscript{35}. The studies by our group herein demonstrate that CD37-SMIP mediates apoptosis through a novel caspase independent pathway not utilized by rituximab or fludarabine in primary CLL B cells. This finding of alternative mechanisms as compared to rituximab provides a potential explanation of why synergy between these two antibodies is observed.

Our data also demonstrates that apoptosis by CD37-SMIP is directly proportional to CD37 surface antigen expression. We also demonstrate that CD37-SMIP has relative selectivity for B-cells and does not bind to resting T-cells, indicating that it will not be immunosuppressive. More importantly, our studies demonstrate that CD37-SMIP mediates potent caspase independent apoptosis and ADCC that is directly proportional to the amount of CD37 antigen on the primary tumor cells and dependent in part upon signal transduction.
Both apoptosis and ADCC observed with CD37-SMIP in CLL appear to be greater than that observed with rituximab or alemtuzumab, two alternative therapeutic antibodies currently utilized in the clinic. Furthermore, evaluation of aggregation of CD37 in lipid rafts and down-stream transmembrane signaling is important to understand the mechanism of apoptosis.

Finally, the novel engineered structure of CD37-SMIP makes it a smaller molecule, thus allowing for potential improved tumor penetration that may contribute to improved efficacy. Detailed pharmacokinetics and assessment of both tumor and protected site (e.g. central nervous system) penetration warrants further investigation. These observations were subsequently confirmed in vivo, where the therapeutic efficacy of CD37-SMIP is similar to improved than that observed with rituximab in studies demonstrated in the next chapter. Based upon these exciting preclinical data, further clinical development of CD37-SMIP in CLL is warranted.

2.4. Material and methods

Reagents and antibodies

CD37-SMIP (G28-1 scFv-Ig) and fluorescein isothiocyanate (FITC)-labeled CD37-SMIP were provided by Trubion Pharmaceuticals, Seattle, WA. Phycoerythrin (PE)-labeled mouse anti-human CD37 antibody (clone BL14), PE-labeled isotype control mouse IgG1, FITC-labeled annexin V, and propidium iodide (PI) were purchased from BD Pharmingen, San Diego, CA. Benzylxy-carbonyl-val-ala-asp [Ome] fluoromethylketone (Z-VAD-fmk) was purchased from EMD Biosciences (San Diego, CA). Alemtuzumab was produced by Ilex Pharmaceuticals (San Antonio,
TX) and purchased commercially. Rituximab and trastuzumab were produced by Genentech (San Francisco, CA) and purchased commercially. Goat anti-human IgG antibody (Fc gamma fragment specific) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-Caspase-3 (mAb AR-14) was a gift from Dr. John Reed, Burnham Institute, La Jolla, CA. Anti-poly(ADP-ribose) polymerase (PARP, MAb C-2-10) was from Oncogene Research. Anti-phosphotyrosine antibody clone 4G10 was from Upstate Biotechnology (Lake Placid, NY). Anti-GAPDH antibody was from Chemicon International Inc. (Temecula, CA). Herbimycin was purchased from Sigma (St. Louis, MO).

**Patient sample processing and cell culture**

All the patients enrolled in this study had immunophenotypically defined B-CLL as outlined by the modified 96 National Institute criteria. Blood was obtained from patients with informed consent under a protocol approved by the hospital internal review board. All of the B-CLL patients had been without prior therapy for a minimum of two months. B-CLL cells were isolated immediately following donation using ficoll density gradient centrifugation (Ficoll-Paque Plus, Amershan Biosciences, Piscataway, NJ). Isolated mononuclear cells were incubated 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. Freshly isolated B-CLL cells were used for all the experiments described herein except for the surface staining. Samples used were more
than 90% B cells as determined by CD19 surface staining and fluorescence-activated cell sorting (FACS) analysis. For those samples with less than 90% B cells, negative selection was applied to deplete non-B cells using B cell Isolation Kit II (Miltenyi Biotec, Auburn, CA) or by “Rosette-Sep” kit from Stem Cell Technologies (Vancouver, British Columbia, Canada) according to the manufacturer’s suggested protocol. Normal T-lymphocytes were separated from human peripheral blood mononuclear cells (PBMC) using negative selection using Pan T Cell Isolation Kit II (Miltenyi Biotec Inc, CA, USA).

**In vitro treatment of cells with antibodies**

Cells were suspended in media at a density of $1 \times 10^6$ cells/mL immediately after isolation. CD37-SMIP treatment was applied at a concentration of 5 μg/mL to the cell suspension, except for the dose kinetic studies. All other antibodies (trastuzumab, rituximab, and alemtuzumab), were used at 10 μg/mL. The cross-linker, goat anti-human IgG (Fc specific) was added to the cell suspension 5 minutes after adding the primary antibodies, at a concentration 5 times that of the primary antibodies (i.e. 25 μg/mL for 5 μg/mL). For all CD37-SMIP treatment, a group of samples with the same concentration of trastuzumab treatment was applied as isotype control. In addition, a group of samples with no treatment was collected as media control, and a group treated with fludarabine (6.6 μM) was also set up as a positive control. For examination of caspase inhibition, 150 μM Z-VAD-fmk was added 10 minutes prior to the addition of the indicated reagents.
Assessment of apoptosis by flow cytometry

The apoptosis of cells was measured using annexin V-FITC/propidium iodide (PI) staining followed by FACS analysis according to manufacture’s protocol (BD Pharmingen). Unstained cell sample, and cells stained with annexin V-FITC or PI only were also processed for compensation. Results were presented as % cytotoxicity, which was defined as (% annexin V+ and/or PI+ cells of treatment group) – (% annexin V+ and/or PI+ cells of cells of media control). FACS analysis was performed using a Beckman-Coulter EPICS XL cytometer (Beckman-Coulter, Miami, FL). Ten thousand events were collected for each sample and data was acquired in list mode. System II software package (Beckman-Coulter) was used to analyze the data.

Cell surface immunostaining and flow cytometry analysis

For CD37 surface expression analysis, B-CLL cells were isolated as described above, and used fresh or preserved in cryovials in 10% dimethyl sulfoxide (DMSO), 40% FBS, and 50% RPMI media in -180°C for less than 4 months. The expression level of CD37 was not altered due to the cryopreservation process. Cryopreserved samples were quickly thawed and washed twice in ice cold PBS before use. Cells were incubated with PE-labelled anti-CD37, CD37-SMIP or mouse IgG1 isotype control antibodies at 4°C for 30 minutes. The cells were then spun down at 300 g for 10 minutes and rinsed twice with cold PBS and analyzed by FACS. For the binding study, cells were pre-incubated with CD37-SMIP or trastuzumab (5 μg/mL) for 5 minutes followed by PE-labeled anti-CD37 mouse IgG. The incubation and wash
procedure was identical to the surface staining protocol. For cell lines, cells were split the night before and fresh cells were used for immunostaining as described for B-CLL cells.

**MTT assay**

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Chemical C. St. Louis, MO] assay was performed to measure the cell viability with method previously described with minor revision. Briefly, $10^6$ cells in 100 μl RPMI 1640 media with or without antibodies were seeded in triplicates to 96wells plates. Cells were then incubated at 37°C in an atmosphere of 5% CO$_2$ for 24 hours and 96 hours. Then at set time points, 50 μL of MTT working solution (2 mg/mL, prepared from 5 mg/mL MTT reagent mixed with RPMI 1640, 2:3, v/v) was added to each well, and the plates were incubated at 37°C in an atmosphere of 5% CO$_2$ for 8 hours. The plates were then centrifuged and the supernatant was removed. The cells were then lysed using 100 μL lysis solution and the absorbance 540 nm was measured with a plate reader. Cell viability was expressed as the percentage of absorbance compared with media control.

**Western blotting**

Immunoblot assays were performed with the multiple antigen detection (MAD) immunoblotting method. Whole cell lysates were prepared and stored in -80°C. Protein concentration in the lysates was quantified by the bicin choninic acid (BCA) method (Pierce, Rockford, IL). Protein samples (50 μg) were separated by
SDS-PAGE (10% for PARP and 12% for Caspase-3 blots), and transferred to 0.2 μm nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Following incubation with indicated primary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA) were used as secondary antibodies. The immune complexes were detected using a chemiluminescent substrate kit as described by the manufacturers. (SuperSignal, Pierce Inc. Rockford, IL).

**Antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) assay**

ADCC activity was determined by standard 4-hour $^{51}$Cr-release assay. $^{51}$Cr-labeled target cells ($1 \times 10^4$ B-CLL cells, Raji cells, or 697 cells) were placed in 96-well plates and various concentrations of antibodies were added to wells. Effector cells (PBMC, NK cells or monocytes from healthy donors) were then added to the plates at indicated effector to target (E:T) ratios. After 4-hour incubation, supernatants were removed and counted in a gamma counter. The percentage of specific cell lysis was determined by: $\%$ lysis = $100 \times (ER-SR)/(MR-SR)$, where ER, SR, and MR represent experimental, spontaneous, and maximum release respectively. ER was measured from each treatment well where antibodies were added, SR was measured from the minimum release wells where no drug or lysis buffer was added, and MR was measured from the maximum release wells where lysis buffer was added to release 100% of the cells.
For CDC assay, B-CLL cells at $10^6$/mL were suspended in RPMI media, media with 30% autologous plasma from the patient blood samples, and media with 30% heat-inactivated (56°C, 30 minutes) plasma. Cells were then treated with antibodies for 10 minutes. After incubation at 37°C for 1 hour, cells were pelleted and resuspended with 100μL 1x binding buffer (BD Pharmingen), then stained with 5 μL of PI solution (BD Pharmingen). The extent of CDC was measured by FACS analysis of PI⁺ cells in duplicate samples.

**Statistical analysis of data**

Analysis was performed by statisticians in the Center for Biostatistics, the Ohio State University, using SAS software (SAS Institute Inc. Cary, NC, USA). Comparisons were made using a two-sided $\alpha = 0.05$ level of significance. Mixed effects models were used to account for the dependencies in the cell donor experiments, and analysis of variance (ANOVA) was used for the cell line experiments. Synergy hypotheses were tested using interaction contrasts.
Table 2.1. Expression of CD37 on CLL cells and T cells.
CLL1-CLL6 are primary CLL B cell samples from 6 B-CLL patients. T1-T5 are normal T cell samples from 5 healthy donors. Cells were immunoseparated and stained with PE-labelled mouse monoclonal antibody to human CD37 or an isotype control, PE-mouse IgG1, and analyzed by flow cytometry. Cells were gated based on viability on forward and side scatter. % positive stands for percentage of positive cells from 10,000 cells analyzed. MFI is the mean fluorescent intensity of the total cell population.
Figure 2.1. Specific expression of CD37 on B cells from CLL patients.
PBMCs (1x10^6 cells each) from CLL patients were double stained with FITC- or PE-labeled antibodies at 4°C, and analyzed by flow cytometry. CD3, CD19 and CD16 antibodies were used as a T cell, B cell, and NK cell markers, respectively. Number in each panel indicates the percentage of double positive cells in each staining. Results are representative of 4 patient samples.
Figure 2. Specific binding of CD37-SMIP to CD37 on CLL cells. Purified CD19⁺ B cells from CLL patients were used for the staining. Cells (1x10⁶) were stained with FITC-labeled antibodies at 4°C, and analyzed for percentage of positive cells by flow cytometry. Trastuzumab, alemtuzumab, rituximab or CD37-SMIP were used at 5 μg/mL 5 mins before the addition of FITC-CD37 to test the blockage effect. Numbers in each panel stand for the percentages of positive stained cells.
Figure 2.3. Specific binding of CD37-SMIP on B cells, not on T cells in PBMCs from CLL patients.

PBMCs from CLL patients (1x10⁶) were used for surface staining at 4°C using method described in “Material and Methods”. FITC-labeled CD37-SMIP was used to define the binding, PE-CD3 was used as a T cell marker, and PE-CD19 was used as a B cell marker. Percentages in each panel stand for double positive cell population. Results are representative of at least 3 independent experiments.
Figure 2.4. Apoptosis induced by CD37-SMIP treatment at 24 hr.
Purified CLL cells were treated with trastuzumab (Her), rituximab (Rit) or alemtuzumab (Cam) at 10 μg/mL, and Tru 16.4 or CD37-SMIP at 5 μg/mL for 24 hr. Numbers are percentages of dead cells defined by the percentage of Annexin V positive and/or PI positive cells over the media control measure. Representative staining results from 14 patient samples are shown.
Figure 2.5. Dose and time dependent induction of cytotoxicity by CD37-SMIP.
Purified CLL B cells (1x10^6) were treated with indicated concentrations of CD37-SMIP with cross-linker α-Fc. The direct cell death at indicated time points was assessed by FITC-Annexin V/PI staining. Percentage cytotoxicity shown represents Annexin V^+ and/or PI^+ cells normalized with the media control. Error bars represent standard deviation between CLL patient cell samples (n=4).
Figure 2.6. Summary of CD37-SMIP induced direct cytotoxicity.

CLL B cells isolated from 14 patients were subjected to indicated treatment for 24 hrs with or without crosslinker (α-Fc). Cell death was examined with FITC-Annexin V and PI. Percentage cytotoxicity shown represents Annexin V+ and/or PI+ cells normalized with the media control. Error bars represent standard deviation between 14 B-CLL patient samples. Statistical analysis of difference between antibody treatments is shown by P values: * P < 0.001, ** P < 0.001, *** P = 0.04.
Figure 2. 7. Correlation of CD37 expression level and direct cytotoxicity by CD37-SMIP.
MFI (CD37): Mean fluorescence intensity of different CLL cell samples by CD37 antibody cell surface staining. % cytotoxicity shown represents Annexin V⁺ and/or PI⁺ cells normalized with the media control.
Figure 2. CD37-SMIP induced cytotoxicity in B but not T cells.
Results shown is summary of FITC-Annexin V+ and/or PI+ cells normalized with media control in normal T cells (white bar; n = 5) or B-CLL cells (filled bar; n = 15) 24 hours post- treatment with CD37-SMIP or trastuzumab (both 5 μg/mL with crosslinker). Error bars represent standard deviation among the samples.
Figure 2. 9. *z*-VAD-fmk failed to inhibit CD37-SMIP induced cell death.*
Freshly isolated B cells from 5 CLL patients were treated with crosslinker alone (α-Fc), trastuzumab + α-Fc, CD37-SMIP + α-Fc, or fludarabine in the presence or absence of the pan caspase inhibitor *z*-VAD-fmk (150 μM). Percentage cytotoxicity shown represents Annexin V⁺ and/or PI⁺ cells normalized with the media control. Error bars represent standard deviation between 5 B-CLL patient cell samples.
Figure 2. 10. CD37-SMIP failed to induce activation of caspases in CLL cells. Cell lysates from primary CLL B cells treated with indicated conditions for 24 hours were assessed by Western blotting to detect PARP and caspase-3 cleavage. UV-irradiated Jurkat cell lysate was used as a positive control for both PARP and caspase 3 cleavage. GAPDH was used as a loading control.
Figure 2. 11. CD37-SMIP induced ADCC against CLL B cells. Trastuzumab, CD37-SMIP, alemtuzumab or rituximab mediated ADCC was evaluated using fresh human PBMC as effector cells and CD19+ primary CLL B cells as target cells at the indicated effector: target (E:T) ratios. Data shown here are summary of 6 patient samples and error bars represent standard deviation between patients. For 25:1 E/T ratio, P < 0.0001 (CD37-SMIP vs. alemtuzumab) and P < 0.0001 (CD37-SMIP vs. rituximab).
Figure 2. CD37 SMIP does not mediate CDC.
Primary CLL B cells were treated with media, trastuzumab, CD37-SMIP or alemtuzumab in the presence of media, human plasma or heat inactivated human plasma for 1 hour. The CDC function was evaluated by propidium iodide staining and flow cytometry analysis. Summary of the results from 4 CLL patient samples are shown. Standard deviations between patients are shown by error bars.
Figure 2.13. Synergistic effect between CD37-SMIP and fludarabine.
Freshly selected CLL cells were treated with fludarabine and CD37-SMIP at indicated concentration combinations. Cell viabilities were analyzed using MTT assays at 24 hr. % viability stands for the percentage of absorbance by MTT analysis normalized by media control. (A) CLL cell viability after treatment with fludarabine at a fixed concentration and CD37-SMIP at variable concentrations by 1:5 serial dilutions. (B) CLL cell viability after treatment with CD37-SMIP at a fixed concentration and fludarabine at variable concentrations by 1:5 serial dilutions. (C) Isobologram analysis of interaction between the two compounds in treating CLL cells, using IC$_{50}$ data from the A and B panels. An isoeffect line of additivity was drawn between the IC$_{50}$ of fludarabine and CD37-SMIP used as a monotherapy. Synergistic effect was observed, especially at suboptimal concentrations of each agent, demonstrated by the fact that all the dose pairs fall below the line. Results are representative from 4 CLL patient samples.
LIST OF REFERENCES


CHAPTER 3

CD37-SMIP DEMONSTRATES *IN VIVO* THERAPEUTIC EFFICACY AGAINST B CELL MALIGNANCIES

3.1. **Introduction**

In chapter 2, we have identified CD37 as a specific cellular surface antigen that can be utilized for targeted therapy against CLL. Our results have also revealed that CD37-SMIP binds specifically and induces apoptosis to B cells from CLL patients, not to other cell types, such as T cells and NK cells that are important for innate immunity functions. Therefore, CD37-targeted immunotherapy, as compared to CD52 is more selective and is not associated with significant toxicity or immunosuppression.\(^1\)-\(^4\)

In addition to CLL, NHL is another B cell lymphoproliferative disorder that represents a number of challenges. Treatment of NHL can be categorized into chemotherapy, immunotherapy, radiotherapy and stem cell transplantation. Traditionally, alkylating agents and purine nucleoside analogs are used in combinations, but none of these have been proven curative. Monoclonal antibodies, such as rituximab, have been shown to have significant clinical efficacy towards this
Especially when given in combination with chemotherapies, MAbs can increase the effectiveness of other treatments and increase the length of the remission produced by the treatment. In some types of lymphomas, addition of rituximab has been shown to increase patients’ survival compared to standard chemotherapy alone.\textsuperscript{5, 7, 9} However, resistance to rituximab treatment is frequently observed in treatment against lymphoma. In addition, the expression level of CD20 in patients are variable, and there are patients that do not respond to the rituximab treatment due to the diminished antigen expression level on the cancer cell surface.\textsuperscript{5, 10} Therefore, exploring “non-CD20” antigens overexpressed on NHL cells for immunotherapy represents another promising direction to overcome problems associated with resistance to rituximab treatment.

Herein, we validate CD37 as an exciting therapeutic target for NHL therapy. We also provide strong \textit{in vitro} and \textit{in vivo} evidence to support clinical development of novel CD37-SMIP in NHL and related B cell malignancies.

3.2. Results

3.2.1. Expression of CD37 in B cell lines

We initially tested a panel of lymphoblastic B-cell lines, representing a variety of lymphoma/leukemia types for expression levels of CD37. (Table 3.1.) Results revealed significant levels of CD37 expression in Raji (62%, MFI = 11), Daudi (100%, MFI = 189), Ramos (100% MFI = 122), and RS11846 (99%, MFI = 70) lymphoblastic cell lines while MEC-1 B-CLL cells (23%, MFI = 12) had medium
expression. Both 697, an acute lymphoblastic lymphoma cell line (0.1% MFI = 0.93) and WAC B-CLL cell line (0.3%, MFI = 1) were negative for this antigen. We utilized the Raji and Ramos cells as two representative CD37+ cell lines and 697 cells as a CD37- cell line for our initial in vitro studies.

3.2.2. CD37-SMIP kills CD37+ B cells through apoptosis and ADCC

CD37-SMIP promoted apoptosis in Ramos (CD37+) or Raji cells (CD37+) with a cross-linking antibody, whereas no cytotoxicity was noted in the 697 (CD37-) cell line (Figure 3.1). Trastuzumab, a humanized monoclonal antibody towards HER2, did not induce any cytotoxic effect to these cells. The existence of secondary crosslinker, goat anti-human IgG (α-Fc), was also found to be critical to potentiate the apoptosis induced by CD37-SMIP (data not shown). This result indicated the specificity of CD37-SMIP induced apoptosis towards CD37+ cells.

Comparison of the effectiveness of CD37-SMIP induced apoptosis to other MAbs that are currently used in NHL treatment, such as rituximab and alemtuzumab, in parallel with isotype control trastuzumab and media control, were processed in 2 Raji cell lines for determining the level of direct cytotoxicity by CD37-SMIP. (Figure 3.2.) In Raji cells, the level of cell death induced by CD37-SMIP exposure was similar to that achieved by rituximab. Raji cells lack significant CD52 expression and were not sensitive to alemtuzumab treatment, unless presented as a CD52 high expression clone.

We also examined ADCC effect following treatment of target cells with CD37-SMIP. Healthy donor derived PBMCs were used as effector cells at various
effector to target (E:T) ratio from 50 to 12.5. Correlation between cell lines (Raji vs. 697) and treatment was suggested (p < 0.0001), indicating no effect of CD37-SMIP in 697 (CD37⁻) cells, but a strong effect in Raji (CD37⁺) cells (Figure 3.3). A group of controls lacking effector cells were also set up in this experiment, and no significant CD37-SMIP induced cell death was observed, indicating the involvement of effector cells in the ADCC in the 4 hour incubation time, eliminating the potential interference from direct cytotoxicity in this assay.

Similar to the result in primary CLL cells, no CD37-SMIP-mediated CDC was observed in Raji cells (Figure 3.4.) An interesting point to note is that Raji cells are not susceptible to alemtuzumab by CDC, because of lack of CD52 expression in the parenteral cell line. However, rituximab has been shown to be very efficient in activating CDC in this Raji cell line.

3.2.3. Establishement of CD37⁺ lymphoma mouse models

Advances in the clinical management of B cell malignancies have been largely empirical without established experimental in vivo model systems. Preclinical in vivo testing in a relevant mouse model for target validation and therapeutic efficacy evaluation is a critical step to evaluate a targeted therapy. This is especially important for preclinical evaluation of CD37-targeted therapies. Attempts to establish CD37⁺ lymphoma mouse models have not so far been reported. It is thus one of our aims to establish a relevant mouse model for testing the therapeutic effect of CD37-targeted therapeutics.
We initially used non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse for engraftment of CD37+ Raji cells. In parallel, CD37 697 cells were also inoculated at the same condition, to serve as a negative control with comparable background for the *in vivo* testing. Purified cells at exponential phase suspended in 100 μL saline were inoculated through mouse tail vein to 4-6 week old NOD/SCID mice at two different amounts: 5 x10⁶ or 1x10⁷ cells. The survival time and the body weight of each mouse were monitored after the inoculation. Results from this study (Figure 3.5) showed, both Raji cells and 697 cells can be engrafted in NOD/SCID mice, with a mean survival time of 16 days (5x10⁶ cells) and 17 days (1x10⁷ cells) in Raji cell inoculated mouse, and 32 days (5x10⁶ cells) and 39 days (1x10⁷ cells) in 697 cell inoculated mouse. Body weight of each mouse after inoculation was monitored twice every other week, and presented as percentage of that on the day of inoculation (Figure 3.5). Our finding indicated that there was correlation between body weight loss and survival time, and suggested that body weight loss might be able to be used as another indicator to monitor disease progress.

In addition to this, we obtained a luciferase-transfected Raji (Luc-Raji) cell line with an intention to establish a CD37+ mouse model that can be used to provide real-time *in vivo* validation of targets and therapeutic efficacy for CD37-specific reagents. This was first validated using an *in vitro* study to find out if there is a correlation with the number of cells and luminescence intensity using IVIS imaging system. Luc-Raji cells were plated to 24-well plate at various numbers and luciferase activity was detected by IVIS 10 min after addition of luciferin to the media. Interestingly, there was a linear positive correlation between the number of cells and
luminescence intensity within the range of 10,000 – 1,000,000 that we have tested. (Figure 3.6) A pilot in vivo inoculation in SCID mice was carried out. However, the result was inconclusive, potentially due to the mice used were at old age (over 10 weeks) and the bioluminescence was too deep to be penetrate the animal body to be detected by the CCD camera in the IVIS (data not shown). Further bioluminescence imaging studies in animals with modified inoculation protocol may provide more useful information on the feasibility of using this in vivo bioluminescence imaging of disseminated lymphoma for preclinical evaluation.

CD37+ transgenic mouse represents a better disease model for human CLL and NHL disease, which is more relevant for preclinical evaluation of CD37-targeted therapies. Human CD37 cDNA expression plasmid were constructed and transfected to several human and mouse cell lines in our preliminary experiments for validation of expression. First, a CD37+ 697 cell line was used for the transfection. Electrophoration in 697 cells allowed 25% transfection efficiency at 24 hr post transfection, with a cell viability of 55% (Figure 3.7). The expression level dropped over time as an indication of transient expression. Another 2 mouse B cell lines were tested further to validate the CD37 expression. Results in this experiment using VWEHI and 70Z3 indicated that the same CD37 plasmid can also be expressed in the mouse cell lines, and be recognized by both mouse anti-human CD37 MAb and CD37-SMIP. (Figure 3.8)

Finally, we established a Raji cell–inoculated disseminated leukemia/lymphoma xenograft SCID mouse model. Sixteen days post-inoculation, SCID mice revealed neoplastic cell infiltrates throughout the body, as revealed by
tissue sections (Figure 3.9). Extensive replacement by morphologically and histologically distinguishable tumor cells was observed in the lymph nodes, thymus, bone marrow, liver, spleen, soft tissue, and middle ear. In central nerve system (CNS), marked multifocal neoplastic cell infiltration was observed in meninges, and mild multifocal mineralization was also observed in brain. This was accompanied by neuropathy, degenerative spinal nerves and marked paresis of the hind legs at 17-20 days that preceded death by 1-2 days. In vitro, Raji cells in culture are also human CD19 and human CD20 positive (data now shown). Tumor cells derived from the bone marrow of engrafted mice maintained expression of human CD37 and human CD20 (Figure 3.10), making this a valid model for in vivo investigation of CD37-SMIP and the relevant control antibody rituximab.

3.2.4. Therapeutic efficacy by CD37-SMIP in a Raji xenograft mouse model

We next determined the therapeutic efficacy of CD37-SMIP relative to both trastuzumab and rituximab in this Raji cell-engrafted xenograft model. The depletion of transferred human B cells in the bone marrow was confirmed by flowcytomtery using antibodies directed against human CD19, CD20 or CD37 molecules (Figure 3.10). Consistent with this, hematoxilin-eosin staining and immunohistochemical analysis of tissue sections of thymus and lymph node with anti-human CD45 antibody revealed loss of human B cells in the CD37-SMIP treated but not untreated control groups (Figure 3.11). Compared with placebo-treated (17 days; 95% CI: 16, 17; P<0.001) or isotype-treated mice (17 days; 95% CI: 16, 17; P<0.001), the median survival time for CD37-SMIP-treated mice (54 days, 95% CI: 45, 85) was
significantly prolonged (Figure 3.12). The hazard ratio and 95% confidence interval for rituximab vs. CD37-SMIP (1.57; 95% CI: 0.87, 2.8; p = 0.137) suggest that CD37-SMIP is not inferior and could in fact be superior to rituximab. These data are in agreement with our in vitro data suggesting that CD37-SMIP may be comparable to or exceed rituximab efficacy.

3.2.5. NK cells contribute to CD37-SMIP induced ADCC in vitro and in vivo

The efficient ADCC function prompted us to evaluate the potential effector cell populations that may be involved in CD37-SMIP mediated ADCC. We compared the ability of PBMC, NK or monocyte effector cells to mediate ADCC in vitro. Figure 3.13 shows that NK cells enriched from PBMC, mediated an increase in CD37-SMIP dependent ADCC compared to normal mononuclear effector cells. In contrast, enriched monocytes failed to cause CD37-SMIP dependent ADCC (NK cells: 49.2% ± 2.6%, PBMC: 29.0% ± 2.1%, monocytes: 5.4% ± 1.8%). Activation of monocytes by IFNγ also failed to result in CD37-SMIP mediated ADCC in CLL cells (data not shown). The importance of NK cells in CD37-SMIP mediated ADCC was not unique to this target as demonstrated by similar findings of NK cell importance over monocytes in alemtuzumab mediated ADCC (Figure 3.13). These results indicate that NK cells, but not monocytes are the major effector cell population in mediating the CLL cell death induced by CD37-SMIP.

Our in vitro studies suggested that NK cells but not naïve or activated monocytes are critical mediators of CD37-SMIP dependent ADCC. To determine the in-vivo importance of NK cells, we evaluated the efficacy of CD37-SMIP or
rituximab treatment in the above described mouse model depleted of NK cells using anti-asialo GM1 antibody. Depletion of NK cells following anti-asialo GM1 antibody treatment, resulted in reduction of YAC-1 cell targeted cytotoxicity by the mouse splenocytes (Figure 3.14). Consistent with this finding, immunostaining of peripheral blood mononuclear cells using mouse NK cell specific CD122 and DX5 antibodies also showed decreased NK cells (data not shown). Consistent with these in-vitro results, the in-vivo therapeutic efficacy of CD37-SMIP or rituximab was significantly compromised by depletion of NK cells (Figure 3.15). The median survival time of CD37-SMIP treated mice decreased from 51 days (95% CI: 38, 78) to 27 days (95% CI: 25, 37) (p = 0.017) with the depletion of NK cells. Similar result was also observed with the rituximab-treated group (49 days vs. 25 days), which is consistent with an earlier report34. The depletion of NK cells failed to exhibit significant effect to the trastuzumab control groups (16 days vs. 17 days, p = 0.16). These data provide further support to the importance of NK cells in mediating CD37-SMIP efficacy in vivo.

3.3. Conclusion and discussion

The development of targeted protein therapies that depend upon signaling and ADCC has been unsuccessful to date due to limited production capability and poor pharmacokinetic features relative to therapeutic antibodies. Pre-clinical development of the first CD20 directed SMIP Tru15 has demonstrated absence of immunogenicity in cynomologus monkeys, and superior B-cell depletion as compared to rituximab.11 Preliminary results of the first phase I study with Tru15 demonstrated it to be
clinically well tolerated, and successful at depleting B-cells in a dose dependent manner with an extended serum half-life (12-19 days).\textsuperscript{12} Thus, development of CD37-SMIP for clinical investigation in B cell malignancies based upon our data herein represents an obvious extension of this exciting targeted therapeutic class of agents directed at a B-cell selective antigen not yet pursued.

The unique engineered features of SMIP-based therapies may offer an advantage over classic antibody based-therapies including improved effector function, target affinity, and apoptotic signaling. The novelty of this work lies in the unique design of SMIP molecule and the use of CD37 as a target for unconjugated engineered protein immunotherapy. In our study, we demonstrate the effectiveness of the CD37 antigen as a therapeutic target in lymphoma cell lines representing NHL and other B cell malignancies. The effectiveness and side effect profile of antibody-based cancer therapies reside in the selectivity for tumor cells relative to normal cells. Additionally, we provide \textit{in vitro} and \textit{in vivo} evidence of the importance of NK cell function to the effectiveness of this novel SMIP therapy. Our data provide strong justification for further pre-clinical studies of CD37-SMIP to facilitate its effective transition into the clinic for the treatment of B cell non-Hodgkin’s lymphoma.

In summary, to our knowledge these are the first reports documenting both the relevance of CD37 as an important target for B-cell malignancies and the potential therapeutic advantage of CD37-SMIP for the treatment of NHL. Based upon these exciting preclinical data, further clinical development of CD37-SMIP is warranted.
3.4. Material and methods

Development of disseminated leukemia xenograft SCID model. Female 4-6 week-old C.B.-17 SCID mice (Taconic Farm, Germantown, NY) were housed in pathogen-free, isolated cages. To ensure the consistency of engraftment, Raji cells were frozen in cryovials (1x10^7 cells each) and stored in a vapor-phase liquid nitrogen environment (-180°C). Before in vivo inoculation, cryopreserved cells were thawed, cultured for 10 days, and examined for the viability, CD37, CD20 and CD19 positivity, and sensitivity to CD37-SMIP or CD20 antibody treatment in vitro. Only cells with > 90% viability and > 50% CD37 positivity were used for injection. Cells were resuspended in PBS at room temperature at a density of 10^7 cells/mL, in 200μL (2x10^6 cells) and inoculated i.v through tail vein using a mouse tail illuminator (Braintree Scientific, Braintree, MA). Untreated SCID mice developed symptomatic central nervous system, liver and pulmonary metastasis that resulted in death from massive tumor burden 17-20 days after inoculation. Tissues obtained from sacrificed tumor-bearing SCID mice were subjected to histopathological examination to confirm the presence of disease. Gross pathology, histology, histopathology and immunohistochemical analysis of the tissues from treated and untreated mice were performed at the Ohio State University Comprehensive Cancer Center mouse phenotyping core facility. The residual human Raji cells were evaluated in formalin-fixed paraffin embedded tissues section of 4-5 microns by immunohistochemistry using monoclonal mouse anti-human CD45 (BD Pharmingen) followed by biotinylated goat anti-mouse secondary antibody. Counterstaining was done with Richard-Allan Hematoxylin 2. Slides were then dehydrated and coverslipped. All
washes were carried out in DakoCytomation Wash Buffer (Tris-Buffered Saline/Tween 20). Bone marrow cell suspension was prepared from flushing fresh marrow-containing femurs with PBS, and stained with PE-labeled antibodies to check the existence of human leukemia cells.

Transfection of human CD37 expression vectors in human and mouse B cell lines. For nucleofection of 697, VWEHI and 70Z3 cells, basic Nucleofector Kit (Amaxa) was used. Cells were passaged one day before transfection to insure that cells are in their logarithmic growth phase. On the day of transfection, Nucleofector solution V was prepared by adding 0.5 mL supplement to 2.25 mL nucleofector solution and mixing gently. The supplemented nucleofector solution was prewarmed to room temperature. Culture medium containing serum and supplement was also prewarmed at 37°C in a 15mL tube. 12-well plates was prepared by filling the appropriate number of wells with 1.5mL culture medium and pre-incubate plates in humidified 37°C/5% CO₂ incubator. Cells were prepared and washed with RPMI 1640 without serum, and resuspended in 1mL Nucleofector solution V to a final concentration of 1x 10⁶ cells/100uL. DNA (2 μg) was added to the cell suspension (1x 10⁶). 100uL cells suspension with DNA was transferred into a cuvette and inserted into the holder to apply program M13 (VWEHI and 70Z3 cells) and T-16 (697 cells). The cuvette was removed immediately after the program has finished, and the cells were transferred from the cuvette by adding 500uL prewarmed culture medium into 12-well plate. Cells were incubated in incubator. Gene expression can be
checked beginning from 24 hr. For GFP, the expression can be observed as early as 4 hours.

**In vitro and in vivo bioluminescent imaging.** Cell suspensions were made from fresh luciferase-transfected cell culture at the density of $1 \times 10^6$ cell/mL and seeded to 24 well plate. Serial dilution using RPMI 1640 (containing 10% FBS) was used to prepare cell suspension at reduced density. D-luciferin (Xenogen) was dissolved at 15 mg/mL in sterile PBS and 10uL was added to the media 5 min before imaging. For *in vivo* imaging, mice were injected with luciferin. Light emission was measured by IVIS and LivingImage software (Xenogen). Signal intensity was quantified as the total counts measured over the region of interest.

**In vivo therapeutic efficacy evaluation in xenograft model.** Antibody treatment was started 3 days after inoculation of the Raji cells. CD37-SMIP or rituximab dissolved in 1mg/mL saline were injected via tail vein, and maintained every other day *i.v.* schedule for 2 weeks (5 mg/kg/injection, 7 injections each mouse). Placebo (saline) and isotype control (trastuzumab) were administered at the same schedule and dose. Animals were monitored daily for signs of illness and sacrificed immediately if hind limb paralysis, respiratory distress or 30% loss in body weight was noted. Body weight was measured once every week. Survival time (paralysis time) was used as primary endpoint for evaluation. For NK cell depletion, anti-asialo GM1 antibody (30 μg/injection in 200 μL saline) was injected to mouse through tail vein at -6 d, -1 d, 4 d, 9 d, and 14 d after Raji cell inoculation. At day 10, 50 μL peripheral blood
obtained by retroorbital bleeding was analysed for NK cells by flowcytometry to confirm the depletion effect. In a separate group, 4 control SCID mice were treated with or without the same dose of anti-asialo GM1 antibody and the splenocytes were prepared 2 days after the injection for cytotoxicity experiment using YAC-1 cells as target cells.

**Assessment of apoptosis by flow cytometry.** The apoptosis of cells was measured using annexin V-FITC/propidium iodide (PI) staining followed by FACS analysis according to manufacture’s protocol (BD Pharmingen). Unstained cell sample, and cells stained with annexin V-FITC or PI only were also processed for compensation. Results were presented as % cytotoxicity, which was defined as (% annexin V\(^+\) and/or PI\(^+\) cells of treatment group) – (% annexin V\(^+\) and/or PI\(^+\) cells of cells of media control). FACS analysis was performed using a Beckman-Coulter EPICS XL cytometer (Beckman-Coulter, Miami, FL). Ten thousand events were collected for each sample and data was acquired in list mode. System II software package (Beckman-Coulter) was used to analyze the data.

**Cell surface immunostaining and flow cytometry analysis.** Cell lines were split the night before and fresh cells were used for immunostaining. Fresh cells were incubated with PE-labelled anti-CD37 (BD Pharmingen, Clone BL14), CD37-SMIP or mouse IgG\(_1\) isotype control antibodies at 4\(^{\circ}\)C for 30 minutes. The cells were then spun down at 300 g for 10 minutes and rinsed twice with cold PBS and analyzed by FACS.
Antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) assay. ADCC activity was determined by standard 4-hour $^{51}$Cr-release assay. $^{51}$Cr-labeled target cells (1 × $10^4$ B-CLL cells, Raji cells, or 697 cells) were placed in 96-well plates and various concentrations of antibodies were added to wells. Effector cells (PBMC, NK cells or monocytes from healthy donors) were then added to the plates at indicated effector to target (E:T) ratios. After 4-hour incubation, supernatants were removed and counted in a gamma counter. The percentage of specific cell lysis was determined by: % lysis = 100 x (ER-SR)/(MR-SR), where ER, SR, and MR represent experimental, spontaneous, and maximum release respectively.

For CDC assay, B-CLL cells at $10^6$/mL were suspended in RPMI media, media with 30% autologous plasma from the patient blood samples, and media with 30% heat-inactivated (56°C, 30minutes) plasma. Cells were then treated with antibodies for 10 minutes. After incubation at 37°C for 1 hour, cells were pelleted and resuspended with 100μL 1x binding buffer (BD Pharmingen), then stained with 5 μL of PI solution (BD Pharmingen). The extent of CDC was measured by FACS analysis of PI$^+$ cells in duplicate samples.

Statistical analysis of data. Analysis was performed by statisticians in the Center for Biostatistics, the Ohio State University, using SAS software (SAS Institute Inc. Cary, NC, USA). Comparisons were made using a two-sided $\alpha = 0.05$ level of significance. Mixed effects models were used to account for the dependencies in the cell donor experiments, and analysis of variance (ANOVA) was used for the cell line
experiments. Synergy hypotheses were tested using interaction contrasts. Kaplan-Meier survival functions and log-rank tests were used to compare animal survival between different treatment groups.
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Table 3.1. Analysis of surface expression of CD37, CD19 and CD20 staining on B cell lines.

Fresh cells (1x10^6) were stained using mouse anti-human CD19, CD20 and CD37 antibodies at 4°C and analyzed by flow cytometry. % + stands for the percentage of positive cells from 10,000 cells analyzed. MFI is the mean fluorescent intensity of the total cell population.
Table 3.2. Surface expression of CD19, CD20, CD37 and CD52 on Raji and luciferase-transfected Raji cells.

Fresh cells (1x10^6) were stained using mouse anti-human CD19, CD20 and CD37 antibodies (isotype control: mouse IgG1), or rat anti-human CD52 antibody (isotype control: rat IgG1) at 4°C and analyzed by flow cytometry. %+: percentage of positive cells from 10,000 cells analyzed. MFI: mean fluorescent intensity of the total cell population. Luc-Raji: luciferase transfected Raji cells.
Figure 3.1. CD37-SMIP induced direct cytotoxicity in CD37+ (Raji and Ramos), but not in CD37- (697) human B cell lines. Cytotoxicity was measured using FITC-annexin V/PI staining at 24 hours post treatment as described in “Material and Methods”. Representative results from 3 independent experiments are shown. Error bars are SD between triplicate samples within the same experiment.
Figure 3.2. CD37-SMIP induced direct cytoxicity in Raji B cell lines comparable to that seen with alemtuzumab or rituximab.

In vitro direct cytotoxic effects of CD37-SMIP (5μg/mL), alemtuzumab (10 μg/mL) or rituximab (10 μg/mL) in the presence or absence of cross-linker (α-Fc) was evaluated by FITC-Annexin V/PI staining at 24 hours post treatment as described in “Material and Methods”. Raji parent clone (CD52−) and variant CD52+ clones were used in these studies.
Figure 3.3. CD37-SMIP induced ADCC in CD37+ Raji Cells but not in CD37-697 B cell line.

Transtuzumab (10 μg/mL), CD37-SMIP (5 μg/mL), alemtuzumab (10 μg/mL) or rituximab (10 μg/mL) were treated against Cr-51 labeled cells (5,000 each well) in flat bottom 96-well plates. Fresh isolated PBMCs from healthy donors at indicated effector: target (E:T) ratios were incubated with the target cells for 4 hr. % of relative cytotoxicity was calculated by the relative release of Cr-51 activity after the 4hr incubation as described in “Material and Methods”.
Figure 3.4. Rituximab but not CD37-SMIP mediated CDC function against Raji B cell line.

Raji cells (1x10^6) were treated with media, trastuzumab (10 μg/mL), CD37-SMIP (5 μg/mL), rituximab (10 μg/mL), or alemtuzumab (10 μg/mL) in the presence of media, 30% human plasma or 30% heat inactivated human plasma for 1 hour. The CDC function was evaluated by propidium iodide staining and presented as % PI positive cells in response to the various treatments. Results shown are representative of 3 independent experiments.
Figure 3. 5. Survival time (upper panel) and body weight change (lower panel) of NOD/SCID mice after inoculation of Raji or 697 cells. Cells were inoculated in 6-8 well NOD/SCID mice through tail vein at indicated amount in 100 μL PBS suspension. % body weight was calculated by normalizing using the body weight at the time of inoculation. 3 mice in each group were used.
Figure 3.6. Measurement of luminescence intensity for detection of luciferase-transfected Raji cells (Luc-Raji) using IVIS. Upper panel: image of luminescent cell suspension in a 24 well plate. Lower panel: correlation of cell amount and luminescence intensity. Luc-Raji cells were cultured in RPMI 1640 media with 10% FBS and resuspended in media. Cells were diluted beginning from $1 \times 10^6$ using media by 1:2 serial dilution method, and 10 $\mu$L D-Luciferin (15mg/mL) was added to each well 5 min before imaging using IVIS system. Signal intensity was quantified as the total counts measured over the area of each well.
Figure 3.7. Transfection of CD37 plasmid in 697 cells.
Upper panel: transfection efficiency and viability analysis by flow cytometry. 1x10^6 cells were transfected with 2 μg DNA using electrophoration as described in “Material and Methods”. Program T-16 was used. Transfection efficiency was calculated using % green fluorescent protein positive cells. Viability was calculated using PI staining. Lower panel: Expression of CD37 in 697 cells. 1x10^6 cells were transfected with human CD37 cDNA expression plasmid (2 μg) using electroporation. The CD37 expression was detected by immunostaining using FITC-labeled mouse anti-human CD37 antibody at 48 hr after electroporation. The percentage stands for the CD37+ cells by immunostaining. Non-transfected 697 cells were used as a control.
Figure 3.8. Transfection of CD37 plasmid into mouse B cell lines. Fresh cells (1x10⁶) were transfected with 2 μg DNA using Nucleofector (Amaxa) as described in “Material and Methods”. Program M-13 was used. Cells were collected for staining after incubation for 24 hr. GFP expression was detected by flow cytometry to determine the transfection efficiency. Cells were also stained for CD37 expression using both FITC-labeled mouse anti-human CD37 antibody and FIT-labeled CD37-SMIP. Number on each panel stands for the percentage of positive cells after transfection. Shaded area is the non-transfected control.
Figure 3.9. H&E staining of tissue sections from Raji cell inoculated SCID mouse (placebo-treated).
i, Thymus infiltrated with neoplastic cells. ii, Lymphoid atrophy/hypoplasia in spleen. iii, Extramedullary hematopoiesis in liver (arrows). iv, Atrophic lymph node partially replaced by tumor cells. v, Markedly atrophic cervical lymph node. vi, Spinal nerve showing rare swollen axons (arrow). vii, Neoplastic cells in meninges over cerebellum. viii, Neoplastic cells in vertebral bone marrow and within spinal canal. ix, Neoplastic cells in bone marrow.
Figure 3. 10. Flowcytometric analysis of bone marrow suspension cells.
Bone marrow cells (1x10^6) prepared from SCID mice 17 days post inoculation were stained with human antibodies. Mice were either treated with placebo (untreated) or CD37-SMIP (treated), and the percentage of human CD19^+, human CD20^+, or human CD37^+ were analyzed by cell surface staining with PE-labelled antibodies. Numbers on each panel stand for percentage of positive cells. Shaded area is the staining result of isotype control in the same experiment.
Figure 3.11. Depletion of infiltrated human CD45+ cells in CD37-SMIP treated but not control mice.
Histological (H&E Staining) analysis of thymus (upper panels) and lymph node (lower panels) from control untreated (left panels) or CD37-SMIP treated (right panels) mice. Insert shows presence of human CD45+ cells in the untreated but not in CD37-SMIP treated mice as detected by immunohistochemistry of the tissue sections.
Figure 3. Evaluation of therapeutic efficacy of CD37-SMIP in Raji cell-inoculated SCID mice.
Raji cell inoculated SCID mice were treated with saline (n=22), negative control trastuzumab (n=22), rituximab (n=32) or CD37-SMIP (n=32). Treatment started 3 days after inoculation, and followed i.v. dose of 5mg/kg every other day for 2 weeks. Survival was monitored by hind leg paralysis. Comparison among different groups was made by log-rank test: P < 0.001 between CD37-SMIP and trastuzumab, and P = 0.124 between CD37-SMIP and rituximab.
Figure 3. 13. *In vitro* evaluation of CD37-SMIP induced ADCC function by NK cells, monocytes, or PBMCs effector cells.

Effect of purified NK cells, monocytes or PBMC effector cells to mediate CD37-SMIP, trastuzumab or Alemtuzumab dependent ADCC function against CD19+ primary CLL B cells as target cells was evaluated at the indicated effector:target (E:T) ratios by standard chromium release assay as described in the materials and methods section. Data shown here is summary of results from 6 patient samples and error bars represent standard deviation between patients.
Figure 3. 14. Decreased ex-vivo NK cell activity in splenocytes from mice treated with asialo GM1 antibody.
Splenocytes from control or mice treated with NK cell depleting asialo GM1 antibody were tested for their ability to mediate cytotoxicity against NK cell target cell. Solid and broken lines represent % lysis mediated by cells from NK undepleted and depleted mice respectively.
In vivo therapeutic efficacy of Trastuzumab, CD37-SMIP or Rituximab were compared in control (NK+) or anti-asialo GM1 antibody treated (NK-) Raji xenograft model described in Fig 5. Survival is determined based on the paralysis time after inoculation. Log-rank test was applied for statistical analysis.
LIST OF REFERENCES


CHAPTER 4

CD37-IMMUNOLIPOSOMES INDUCE APOPTOSIS THROUGH CROSSLINKING IN CLL CELLS

4.1. INTRODUCTION

B cell malignancies represent various types of lymphoma and leukemia developed as a result of abnormal changes that B cells undergo during their life cycle. Although the cause of these diseases are not fully understood, the pathogenesis is attributed to deregulation of cell growth and apoptotic pathways. Treatment strategies that can promote apoptosis in these diseases are thus attractive.

For decades, chemotherapies, either as monotherapy or in combination, have been used for treatment against B cell malignancies. However, development of biotechnology, especially the introduction of monoclonal antibody (MAb) therapeutics, has dramatically changed the treatment paradigm in this area. In fact, due to the relatively well characterized cell surface antigens on B cells, immunotherapy approach using engineered MAbs has made B cell malignancies ideal candidates for targeted therapy. Rituximab (Rituxin) is the first engineered chimeric monoclonal antibody for treatment against non Hodgkin’s lymphoma (NHL), and has already been established in wide ranges of clinical practice since its approval by FDA in 1997. Another humanized monoclonal antibody, alemtuzumab (Campath) was approved for treatment of refractory chronic lymphocytic leukemia (CLL) several
years later. In addition, antibodies directed against molecules such as CD40, CD22, CD74, CD23, and CD80 are currently under development, some of which already showed very promising results in late phase clinical trials.

CD37 is a tetraspan superfamily member and represents a heavily glycosylated glycoprotein with a molecular weight of 40-52kDa. Despite the fact that little is known about the function of this cell surface antigen, CD37 is an extremely attractive target for treatment against CLL and other types of B cell malignancies. This is because, CD37 is a lineage specific antigen, similar to CD20, specifically expressed on B cells, and has minimal or no expression on other types of blood cells, including T cells, neutrophils, monocytes, and NK cells, etc. The expression level of CD37 on malignant B cells has made it appropriate for development of CD37-targeted immunotherapy, since CLL, NHL and hairy cell leukemia all have CD37 positivity. This is particularly attractive for CLL, because the CD37 expression level on CLL cells are relatively high, in contrast to the dim and variable expression of CD20 on CLL cells.

Our previous studies described in Chapter 2 and 3 showed that an engineered protein specific to human CD37 antigen, CD37-small modular immunopharmaceutical (CD37-SMIP), containing variable regions from anti-CD37 Ab and engineered constant regions encoding human IgG1 domains (hinge, CH2 and CH3), binds to and kills CLL cells through induction of caspase-independent apoptosis and antibody-dependent cellular cytotoxicity, both in vitro and in vivo. We also found that signaling-induced apoptosis failed to occur by direct binding of CD37-SMIP to CD37 antigen. Instead, the direct cytotoxicity caused by CD37-SMIP
is dependent on crosslinking of CD37 molecules using a goat anti-human IgG. Our finding also indicated that upon hypercrosslinking using the secondary antibody, CD37-SMIP induced tyrosine phosphorylation of several proteins, which eventually lead to caspase-independent apoptosis in CLL cells. The precise identification and the role of these tyrosine phosphorylated proteins are not known, nevertheless, these data indicated that hyper-crosslinking plays a critical role on initiating and amplifying CD37-SMIP induced cell death. Similar crosslinking dependent cytotoxicity has been reported for other therapeutically relevant molecules, such as CD20. Despite the fact that the exact mechanisms of rituximab in mediating B cell depletion is not fully understood, several studies have showed evidence for rituximab-induced apoptosis is dependent on crosslinking of CD20 molecules using goat anti-human IgG, or Fc receptor-bearing cells, or by immunobilization on plates in vitro\textsuperscript{23,24}. The apoptosis induced by rituximab hypercrosslinking is associated with detergent-insoluble lipid rafts that are enriched with cholesterol, and membrane CD20 clustering has been shown to attenuate the apoptosis through calcium mobilization and activation of kinases\textsuperscript{25}. The in vivo importance of Fc receptors on effector cells in mediating cell death has also been demonstrated\textsuperscript{26,27}, where depletion of B cells was not observed in mice deficient of Fc receptor, further supporting the involvement of Fc:Fc receptor interaction in the antibody-induced cell death. These studies suggest Fc receptor-bearing cells as possible mediators of hyper-crosslinking to cause apoptosis in treatment against B cell malignancies using CD37-SMIP. However, lack of effector cells is common in CLL patients, especially after chemotherapy, and this may account for the failure of some patients’ response to rituximab treatment.
It is therefore our hypothesis that crosslinking of CD37 molecules can be achieved by CD37-SMIP-coated liposomes (CD37-Lip), which potentially could be a practical approach to activate apoptosis-related signaling *in vivo* without participation of Fc receptor bearing effector cells. (Figure 4.1) Immunoliposomes directed CD37 antigen expressed on B cells were constructed with CD37-SMIP molecules linked to distal PEG chains exposed on liposome surface. Addition of PEG allows the long circulation time of liposomes through escaping from the uptake by reticular endothelial system. Coating of monovalent CD37-SMIP molecules to liposome surface can potentially lead to a novel therapeutic approach using multivalent long circulating nanoscale particles that can mediate cytotoxicity in malignant B cells. The augmentation from multivalent binding will also compensate the delinquent expression level of CD37 in some malignant B cells, especially in a subset population of CLL patients, and potentially increase the response rate or reverse the resistance towards CD37-SMIP therapy due to shedding or internalization of the surface antigen. Therefore, the current immunoliposome approach represents another strategy that can potentially be utilized as a platform technology for *in vivo* effect augmentation of engineered therapeutic proteins, which are dependent on involvement of Fc receptors and sufficient antigen density for apoptosis induction.

4.2. RESULTS

4.2.1. Preparation and characterization of CD37-immunoliposomes

CD37-specific immunoliposomes (CD37-Lip) were prepared by a “post insertion” method as previous described\textsuperscript{28-30}. This method involves the preparation of
CD37-SMIP conjugated PEG-DSPE micelles and nontargeted liposomes separately, and subsequently fusing these 2 components at 45°C to allow the insertion of lipophilic DSPE into the lipid bilayer. (Figure 4.2). CD37-SMIP was first thiolated on the terminal NH₂ group on lysine, which exists outside of complementarity determination region. The thiolated CD37-SMIP has a reactive -SH group that can further link to maleimide groups at the distal end of PEG chain on micelles. Dipalmitoyl phosphatidylcholine (DPPC) was used as a major lipid composition, due to its low phase transition temperature (Tₘ) at 37°C that will allow post insertion to occur at relative low temperature (45°C). This has advantages compared to the previous reported 60°C procedure²⁹ because of less deactivation of protein at this temperature. mPEG-DSPE was added to mal-PEG-DSPE to facilitate the formation of micells and it also provides PEG coating to liposome surface that can potentially prolong the in vivo circulation time. The mean diameter of liposomes following transfer of micelles into liposomes were determined by both laser photon dynamic correlation and transmission electron microscopy analysis. Sizes of all the 3 formulations used in our experiments were around 150 nm (Figure 4.3), which will exhibit delayed removal from the circulation, and will be able to extravasate into tissues in the case of lymphoma therapy. Coupling efficiencies of CD37-SMIP to liposomes were determined by size exclusion chromatography after post insertion. Electrophoretic analysis of the elutions (Figure 4.4) confirmed that CD37-SMIP exists mainly in the liposomal portions (eluted between 3-5 ml), and the percentage of uncoupled CD37-SMIP is less than 10 % based on BCA assay analysis of protein concentrations. Approximately 35-40 CD37-SMIP or herceptin molecules were
coated to each liposomal particle, based on protein and lipid concentration analysis. The calculation is based on surface area estimation assuming the particle size of 150 nm.

4.2.2. Specific binding of CD37-immunoliposomes to CD37+ B cells

The binding profiles of CD37-Lip were determined using fluorescence labeled liposomes. Calcein, a hydrophilic membrane impermeable green fluorescent dye was encapsulated into the hydrophilic core, and R-18, a lipophilic red fluorescent dye was integrated to lipid bilayer during the liposome preparation. Nontargeted liposomes (NT-Lip) and an isotype conjugated liposomes (Her-Lip) were used as controls. Her-Lip was prepared using herceptin, a humanized protein targeting to HER2. This is a receptor that is known to be absent on human B cells. Results from fluorescence microscopy (Figure 4.5) showed that CD37-Lip bound to CLL cells, as demonstrated by the high fluorescence intensity on both FITC and rhodamine channels; in contrast, neither NT-Lip nor Her-Lip showed significant fluorescence under microscope. The cells were also analyzed by flowcytometry and the fluorescence intensities on FITC channel were illustrated on Figure 4.6, where CD37-Lip bound with high level towards CLL cells, while Her-Lip had only minimal background binding similar to NT-Lip. In addition, a panel of B cell lines with different expression level of CD37 were tested for specific binding of CD37-Lip. Figure 4.7 showed that Ramos, Raji, Daudi, and RS11846 had high specific binding towards CD37-Lip. In contrast, 697, WAC, MEC-1 and Jurkat cell lines were negative for CD37-Lip binding. These results are consistent to our earlier CD37 surface antigen staining using FITC-labeled mouse
anti-CD37 monoclonal antibody. The relative CD37 expression level of these cell lines as quantified by mean fluorescence intensity (MFI) after CD37 MAb binding was plotted with MFI of each cell line after liposome binding (Figure 4.8). The data indicated positive correlation trend between these 2 parameters, as demonstrated by the $R^2 = 0.793$. This result further supported that CD37-Lip has specific binding to CD37 antigens on malignant B cells.

4.2.3. Apoptosis induced by CD37-Lip in CD37+ B cell lines

The cell apoptosis caused by CD37-Lip treatment was assessed by Annexin V/propidium iodide (PI) staining and quantified by flow cytometry. Raji (Figure 4.9) and Ramos (Figure 4.10), which are known to have CD37 expression were used as two representative B cell lines. In each cell line, the media control had viability over 90%. CD37-Lip induced 23% cell death in Raji cells at 24 hr (Figure 4.9), which is equivalent to the levels observed by crosslinked CD37-SMIP by secondary antibody (25%), and is superior to non-crosslinked CD37-SMIP (1%), NT-Lip (1%) or Her-Lip (0%). Similar results were observed in another CD37+ B cell line, Ramos (Figure 4.10), where CD37-Lip caused 34% cell death at 24 hr, similar to the level observed with crosslinked CD37-SMIP (32%). No significant cell death was observed with other controls. These data, consistent with the specific binding results, supported that CD37-Lip selectively produced a cytotoxic effect markedly greater than the same amount of non-crosslinked CD37-SMIP.
4.2.4. Direct induction of cell death by CD37-Lip in CLL cells

To assess whether CD37-Lip can induce cell death through direct binding to CLL cells, both annexin V/PI staining and MTT methods were used to detect the cell viability. Annexin V/PI staining results obtained from CLL cells showed, while no significant cell death (5%) was observed with treatment of non-crosslinked CD37-SMIP (5 μg/mL) in CLL cells at 24 hr, CD37-Lip was able to bring down the percentage of viable CLL cells (annexin V⁻/PI⁻) to 60%, although addition of 5x secondary antibodies (25 μg/mL) to CD37-SMIP was also able to reduce the viability to 39%. NT-Lip and Her-Lip did not cause apoptosis in CLL cells, indeed, slight increase of cell viability was observed in both cases. In a separate assay, MTT was used to analyze the relative viable cell numbers after 48 hr treatment. CLL cells were subjected to 1:4 serial diluted concentrations of liposomes or CD37-SMIP starting from 50 μg/mL. Results in Figure 4.12 illustrated that the cell death induced by CD37-Lip was equivalent to the level of goat anti-human IgG (α-Fc) crosslinked CD37-SMIP at most concentrations. In contrast, no significant cell death was observed with the addition of NT-Lip or Her-Lip, except at high concentrations (50 μg/mL and 12.5 μg/mL), where 5 – 20% cell death relative to the media control was observed.
4.2.5. *No induction of ADCC or CDC by CD37-Lip in CLL cells*

Antibody dependent cellular cytotoxicity (ADCC) (Fig. 4.13) and complement dependent cytotoxicity (CDC) (Fig.4.14) were also conducted to exam the effector function by CD37-Lip. While our initial hypothesis is that lipid may be able to act as a mediator for attachment of effector cells and complements, our data failed to demonstrat significant level of ADCC or CDC. ADCC assay by standard chromium-51 release assay using Raji cells as target cells and human PBMCs as effector cells only revealed minimal level of cytotoxicity with these CD37-Lip compared to CD37-SMIP. In another assay using PI staining for detection of CDC effect, no significant PI positive cell population was noted with the addition of 30% human plasma. These data indicate that direct cytotoxicity is the major cell killing mechanism associated with these liposomes.

4.3. **DISCUSSION**

Herein we have described an approach of using immunoliposomes as a crosslinking particle to activate the apoptosis mediated by CD37 clustering on malignant B cells. This is to some extent similar to the approach of using high valent homodimners, which has been reported to be able to induce higher apoptosis rate in treatment against cancer cells\(^{32,33}\). Homodimer of rituximab, another chimeric antibody targeting to CD20 on B cell surface, has been shown to have induce apoptosis and necrosis independent of Fc receptors.\(^{33}\) However, the current
immunoliposome approach is superior to the homodimer strategy, because it combines the specificity of CD37-SMIP, along with the long circulation associated with delayed clearance of liposomes in vivo. The advantages of this as compared to homodimers also exist in: first, it provides feasibility to reach high valency over 40 that cannot be achieved by homodimers; second, liposomes after PEGylation can prolong the circulation of engineered proteins and reduce the immunogenicity; and finally it avoids costly and challenging protein engineering procedures, thus is more viable for pharmaceutical development.

Consistent with the multivalent CD37-Lip binding to target cells, we observed clumping of cells within 1 hour post treatment in B cell lines and CLL B cells. The formation of cell clumps that consist of 10-20 cells were also found with the treatment of crosslinked CD37-SMIP, but not with CD37-SMIP alone, NT-Lip or Her-Lip. The role of cell clustering remains to be evaluated. The direct role of CD37-Lip induced clustering of CD37 molecules on cell surface and subsequent activation of death signals, the capping on cell membrane, and other related mechanisms to the cell death remain to be elucidated.

Furthermore, the current design of CD37-targeted immunoliposomes can also be used for targeted drug delivery, such as for chemotherapeutic agents and RNA-based therapies. This MAb-based targeted delivery strategy, especially when used for treatment in CLL, has such advantages: (1) Liposomes encapsulated with high payload of cytotoxic agents have unrestricted access to malignant cells compared to solid tumors, where extravasation is necessary; (2) Selective eradication of malignant cell population can be achieved, thus preserving immunological responses in patients;
(3) Potential synergistic effect with encapsulated therapeutics can be achieved, based on different killing mechanisms. Potential challenges with this immunoliposome approach are (1) stability of drug encapsulation and antibody conjugation, and (2) immunogenicities against immunoliposomes. These have to be ruled out before promoting this promising strategy for future clinical application.

Taken together, we believed that liposomes as nanoscale vesicles can be used for delivery of engineered proteins, such as CD37-SMIP. This is a unique approach different from previous reported application of immunoliposomes only as delivery vesicles for encapsulated chemotherapeutics, ignoring the fact that Abs themselves on liposomal surface are active and can have biological effect to be utilized for treatment. Therefore, immunoliposomes, in addition to being a high payload encapsulation particles, can potentially been utilized as a platform technology for delivery of MAb-based therapies. Further in vivo therapeutic efficacy studies and in vitro mechanistic studies are warranted to provide more promise for promoting this technology for future clinical application.
4.4. MATERIAL AND METHODS

Materials

CD37-SMIP was produced by Trubion Pharmaceuticals (Seattle, WA). Dipalmitoyl phosphatidylcholine (DPPC) and polyethylene glycol$_{2000}$-distearoylphosphatidylethanolamine (PEG-DSPE) were purchased from Genzyme (Cambridge, MA). Maleimide-derivatized polyethylene glycol$_{2000}$-distearoylphosphatidylethanolamine (Mal-PEG-DSPE) was purchased from Shearwater Polymers (Huntsville, AL). Octadecyl rhodamine B chloride (R-18) was purchased from Molecular Probes (Eugene, OR). Cholesterol, 2-iminothiolane (Traut’s reagent), calcein, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 2-(N-morpholine)-ethane sulphonic acid (MES), and Sepharose CL-4B were purchased from Sigma (St. Louis, MO). Nuclear polycarbonate membranes (pore sizes: 0.2 and 0.1 μm) were purchased from Northern Lipids (Vancouver, BC). Disposable preloaded Sephadex G-25 (PD-10) column was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). PE-labeled CD37 antibody, isotype control (mouse IgG1), and FITC-annexin V/propidium iodide staining kit were purchased from BD Bioscience (San Jose, CA). RPMI 1640 medium, penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). All other chemicals were of analytical grade.
Preparation of liposomes

Non-targeted liposomes (NT-Lip) were composed of DPPC/Chol at a 55:45 ratio. CD37-targeted liposomes (CD37-Lip) and HER2-targeted liposomes (Her-Lip) were composed of DPPC/cholesterol/PEG-DSPE/mal-PEG-DSPE (50/45/4/1, molar ratio). Antibody to lipid ratio is 1:1000 (molar). 2-iminothiolane to antibody ratio is 10:1 (molar). pH 8.0 HEPES buffer was prepared from 25mM HEPES, 140mM NaCl, and 5mM EDTA. pH 6.5 buffer was prepared from 25mM HEPES, 5mM EDTA, and 25mM MES. 2-iminothiolane was prepared at 0.1 mg/mL in pH 8.0 HEPES buffer before the antibody thiolation, and purged with N₂. Liposomes were prepared using thin layer film method as we described previously. Particle size was reduced by high pressure extrusion (Northern Lipid, Vancouver, BC) at 60°C using polycarbonate membranes. For fluorescent labeling, 0.001% (molar) R-18 was incorporated into the lipid mixture during the thin layer film preparation to give red fluorescence. 10 mM calcein was used as hydration solution for thin layer film to give green fluorescence.

For protein coupling, a post insertion method was used with modifications. CD37-SMIP or herceptin was thiolated using 2-iminothiolane in pH 8.0 HEPES buffer at room temperature for 45 min. Separation of activated antibodies from unreacted 2-iminothiolane was realized using Sephadex PD-10 desalting column eluted with pH 6.5 buffer purged with N₂. Lipid mixtures, composed of Mal-PEG-DSPE and PEG-DSPE at 1:4 molar ratio, were first hydrated using pH 6.5 HEPES buffer at 0.333 mM in 65°C water bath with occasional vortexing to form micelles.
Immediately after the PD-10 column elution, thiolated antibodies were added into micelles at a ratio of 10:1 (Mal-PEG-DSPE: Ab), and waited for the coupling reaction to happen overnight at room temperature. On the second day, micelles were added to the preformed liposomes at a ratio of 1:20 (molar ratio) and incubated at 45°C for 1 hr. Non-coupled Ab was separated on a Sepharose CL-4B column using pH 7.4 PBS as eluent. All liposomal suspensions were filtered through 0.22 μM PES syring filters to ensure sterility before being preserved in 4°C for further use. Liposomes were stable for at least 1 month under these conditions.

**Size exclusion chromatography and SDS-PAGE analysis**

Sepharose CL-4B (10mL) was loaded to a 1.5x15cm column, and equilibrated with 20mL pH 7.4 PBS. Liposomal samples were loaded to the top of the column and eluted with PBS. Fractions were collected at each mL for determination of protein concentration using BCA assay (Bio-Rad). 4-12% NuPAGE Bis-Tris Gels (Invitrogen) were used for SDD-PAGE, and 15 μL samples were loaded with reducing sample buffer after boiling for 2 min. Gels were run in NuPAGE MES SDS running buffer (Invitrogen) at 200 vol, and visualized by SimplyBlue™ SafeStain (Invitrogen) according to manufacture’s procedure.

**Particle size determination of liposomes**

Transmission electron microscopy (TEM) and laser photon correlation spectroscopy analysis were used to characterize the particle size. TEM was performed after negative staining of liposomes with uranyl acetate (UA). Briefly, the liposomal
samples were placed onto 100 mesh grids. After 3 minutes, excess solution was wiped off with filter paper and the grid was immersed with 2% UA. The UA solution was removed after 1 min, and the grids were washed twice with 200 μL distilled water. The grids were then imaged on a Philips CM 12 Transmission Electron Microscope (Campus Microscopy and Imaging Facility, OSU, Columbus, OH). 55,000 fold magnification photos were taken from the suspended liposomal particles. The mean particle diameter and distribution were measured by photon correlation spectroscopy on a NICOMP Particle Sizer Model 370 (Santa Barbara, CA). Briefly, 50μl liposomal samples were suspended in 500 μl normal saline, and the normalized Gaussian-distributed volume-weighted particle size was collected after 3 runs.

**Flowcytometry analysis for in vitro cell binding assays**

Binding of immunoliposomes to cells were performed by washing cells twice with cold PBS. Cells (1x10⁶) from each sample were resuspended with 0.5mL cold PBS in culture tubes. Cells were then incubated with fluorescence labeled liposomes or PE-labeled anti-CD37 at 4°C for 30 minutes. PE-labeled mouse IgG₁ was used as an isotype negative control for anti-CD37 MAb according to manufacture’s instruction. At the end of the 30 minutes incubation, cells were spun down at 300g for 10 minutes and rinsed twice with cold PBS, and the fluorescence was analyzed by FACS.
**Fluorescence microscopy analysis**

For analysis of liposome binding by fluorescence microscopy, cells were stained with identical procedure as for flow cytometry and washed in pH 7.4 PBS. Cells were then spun down and transferred to glass slide using glycerol mounting solution (50% glycerol, 50% phosphate-buffered saline) right before microscopic analysis. Slides were observed under a Nikon phase-contrast fluorescence microscope immediately after preparation. Green fluorescence of calcein and red fluorescence of R-18 were analyzed using FITC and rhodamine channels on the microscope, respectively.

**Determination of viability and apoptosis**

MTT assay was performed to measure the CLL cell viability. Briefly, serial dilution of drugs were conducted in 96-well plates to make a range of protein concentration in 100μl media. 10⁶ cells in 100μl RPMI media were subsequently seeded in triplicates to the 96-well plates. Cells were incubated for 48 hr. 50μl of MTT working solution (2mg/ml, prepared from 5mg/m MTT reagent mixed with RPMI 1640 2:3 v/v) was added to each well, and the plates were incubated for 8 hours before centrifugation, removing the supernatant, dissolution in 100 μl lysis solution (DMSO). The dissolved purple solution were measurement at O.D.540 with a plate reader. Cell viability was expressed as the relative percentage of absorbance compared with the media control.
The apoptosis of CLL cells and cell lines after incubation with liposomes or antibodies was measured using annexin V-FITC/PI staining with FACS analysis according to manufacture suggested protocol. Cells at a density of 1x10^6 cells/mL were cultured either in 12-well plates or culture tubes with indicated treatments. At 24 hr, 5x 10^5 cells in 200μl 1x binding buffer were stained with 5μL FITC-annexin V and 5μL PI, and kept in dark at room temperature for 15 minutes before suspension with the binding buffer (300 μl) and analyzed by flow cytometry. Cells without staining, cells staining with only annexin V, and cells stained with only PI were also processed for compensation. For all flow cytometry experiments, FACS analysis was performed using a Beckman-Coulter EPICS XL cytometer (Beckman-Coulter, Miami, FL). Fluorophores were excited at 488nm. 10,000 cells were counted for each sample. Viable cells were defined as annexin V−/PI− cell population.
Figure 4.1. Proposed crosslinking mechanism by CD37-immunoliposomes on CLL B cells.
Demonstrated in the left panel is the proposed apoptosis mechanism through signaling events mediated by crosslinking of CD37 antigens on CLL B cells. The specific binding of CD37 and CD37-SMIP is potentiated by the binding of a secondary Ab crosslinker (goat anti-human Fc), and activates several unknown proteins through phosphorylation of tyrosines, which eventually leads to caspase-independent apoptosis in CLL B cells. In our proposed research herein, the crosslinking mechanism can be achieved by using a multivalent immunoliposome, which has ~40 CD37-SMIP molecules attached to the surface of each particle with a diameter in the 150 nm range. The crosslinking by CD37-Lip can potentially initiate sigaling events that induce death in CLL B cells.
Figure 4.2. Schematic demonstration of CD37-immunoliposomes preparation. CD37-targeted liposomes were prepared by a post insertion method. CD37-SMIP was first thiolated at the lysine group using Traut’s reagent (2-iminothiolane). The thiolated CD37-SMIP was further reacted with micelles prepared from maleimide-PEG-DSPE and mPEG-DSPE to form CD37-SMIP coated micells. Non-targeted liposomes were prepared separately. A post insertion method was adopted to incorporate the CD37-SMIP coated micelles to the non-targeted liposomes for the formation of CD37-targeted liposomes.
Fig. 4.3. **Transmission electron microscopy analysis of CD37-Liposomes.**
Particle size and distribution of nontargeted liposomes (NT-Lip), herceptin-coated liposomes (Her-Lip), and CD37-targeted liposomes (CD37-Lip) were characterized by transmission electron microscopy after negative staining of liposomes using uranyl acetate. Photos were taken at 55,000 x ole magnification, and each bar in the figure stands for a size of 500 nm.
Fig. 4.4. Electrophoretic analysis of CD37-immunoliposomes chromatography elution portions. CD37-liposomal suspension (0.5 mL) was subjected to pass through a Sepharose CL-4B size exclusive column (10 mL beads in 1.0x10cm column), eluted by pH 7.4 PBS. Fractions were collected at each mL and 15 μL from each of the fractions was loaded to a SDS-PAGE gel. Numbers on the top of the gel stand for the elution volume, thus 3-5mL is the liposomal fraction, and 10-12 mL is the non-incorporated CD37-SMIP fraction. Molecular marker and CD37-SMIP were loaded separately on the left.
Figure 4.5. Specific binding of CD37-immunoliposomes to CLL B cells. CLL B cells were incubated with liposomes labeled with calcein (green) and R-18 (red) at 4°C for 45 min. Cells were observed under fluorescence microscope after 2 washes of cold PBS. Phase contrast (upper), rhodamine channel image (middle), and FITC channel image (lower) were captured.
Figure 4.6. Specific binding of CD37-immunoliposomes to CD37⁺ B cells (flow cytometry analysis).

CLL B cells (1x10⁶) were incubated with liposomes labeled with calcein and R-18 at 4°C for 45 min and washed twice with cold PBS. Cells were analyzed by flow cytometry using FITC filter. 10,000 events were collected. NT-Lip: nontargeted liposomes (black); Her-Lip: herceptin-coated liposomes (red); CD37-Lip: CD37-targeted liposomes (green).
Figure 4.7. Binding of liposomes to cell lines.
Cells (1x10^6) after binding with NT-lip, Her-Lip or CD37-Lip at 4°C were analyzed by flow cytometry. Mean fluorescence intensity (MFI) of each treatment is compared between different cell lines.
Figure 4.8. Correlation of CD37-immunoliposome binding and CD37 antigen expression on cell lines.
Mean fluorescence intensity (MFI) of each cell line after binding with CD37-Lip was obtained from flow cytometry analysis, same as in Fig. 4.7. CD37 antigen expression was analyzed after surface staining using PE-labeled CD37 mAb, and MFI of each cell line was expressed as CD37 expression on x axis. The correlation between the CD37-Lip binding and CD37 expression was analyzed by linear correlation, demonstrated by $R^2 = 0.793$. 
Figure 4.9. Direct induction of cell death in Raji cells by CD37-immunoliposomes.

Fresh Raji cells (1x10^6) were treated in media with liposomes at concentration equivalent to 5 μg/mL protein, or empty liposomes with equivalent lipid concentration to targeted liposomes. Herceptin (10 μg/mL) and CD37-SMIP (5 μg/mL) were added to culture, with or without crosslinker (goat anti-human Fc) at a concentration 5 fold of the primary antibody. Cell viability was analyzed by FITC-annexin/PI staining after 24 hr treatment, and the % viability was expressed as % of annexin/PI cells in 10,000 cells analyzed. Error bars stand for standard deviations between 3 independent experiments.
Figure 4.10. Direct induction of cell death in Ramos cells by CD37-immunoliposomes.

Fresh Ramos cells (1x10⁶) were treated in media with liposomes at concentration equivalent to 5 μg/mL protein, or empty liposomes with lipid concentration equivalent to targeted liposomes. Herceptin (10 μg/mL) and CD37-SMIP (5 μg/mL) were added to culture, with or without crosslinker (goat anti-human Fc) at a concentration 5 fold of the primary antibody. Cell viability was analyzed by FITC-annexin/PI staining after 24 hr treatment, and the % viability was expressed as % of annexin/PI- cells in 10,000 cells analyzed. Error bars stand for standard deviations between 3 independent experiments.
Figure 4.11. Direct induction of cell death in CLL cells by CD37-immunoliposomes.

Primary CLL B cells (1x10⁶) separated from patients were treated in media with liposomes at concentration equivalent to 5 μg/mL protein, or empty liposomes with lipid concentration equivalent to the targeted liposomes. Herceptin (10 μg/mL) and CD37-SMIP (5 μg/mL) were added to culture, with or without crosslinker (goat anti-human Fc) at a concentration 5 fold of the primary antibody. Cell viability was analyzed by FITC-annexin/PI staining after 24 hr treatment, and the % viability was expressed as % of annexin⁻/PI⁻ cells in 10,000 cells analyzed as compared to each of the media controls. Data are summarized from 3 patients after normalization using media controls.
Figure 4.12. MTT cytotoxicity analysis in CLL cells.

CLL B cells ($5 \times 10^5$) in 100 μL media were seeded to each well of 96-well plates, after 1:5 serial dilutions of drugs in 100 μL media. For NT-Lip, the amount was added equivalent to have same lipid as CD37-Lip to act as a vehicle control. Crosslinker (a-Fc) was 5x concentration of CD37-SMIP. Cells were incubated for 48 hr before addition of 50 μL MTT solution for additional 8 hr incubation. Cell viability was normalized using the absorbance of media controls. Error bars stand for standard deviations between triplicated wells. Data are representative from 3 patients.
Figure 4.13. Lack of ADCC by CD37-immunoliposomes.
ADCC was analyzed using a 4-hr chromium-51 release assay. Target cells used are Raji cells, and effector cells are PBMCs from normal healthy donors. E:T ratios ranging from 50:1 to 0 were differentiated by different colors. Relative cell death was calculated using the equation in the “Material and Methods”. Error bars stand for standard deviations between triplicated wells.
Figure 4.14. Lack of CDC by CD37-immunoliposomes in Raji cells.
CDC was analyzed using PI staining and flowcytometry after treating Raji cells (1x10^6) with liposomes or proteins for 1hr. 30% human plasma or heated inactivated plasma was added to provide complements in the media. % PI positive was obtained from the flow data analyzed from 10,000 events without normalization.
LIST OF REFERENCES


CHAPTER 5

CHOLESTEROL AS AN ANCHOR FOR FR-TARGETED LIPOSOMES

5.1. Introduction

Liposomes are vesicles consisting of one or more self-assembled lipid bilayers enclosing aqueous compartment(s). They have potential applications as drug carriers due to properties such as sustained release, altered pharmacokinetics, increased drug stability, ability to overcome drug resistance and target specific tissues. Liposomal drugs have increasingly been evaluated in clinical trials, which have led to several approved drugs on the market, including sterically stabilized liposomal doxorubicin (Doxil®) for Kaposi’s sarcoma and ovarian cancer, and liposomal amphotericin B (Ambisome®) for fungal infection. Liposome technology has broad potential applications, such as in delivery of cancer chemotherapy, anti-inflammatory therapy, cancer imaging agents, cosmetics and gene therapy.

Folate receptor (FR)-targeted liposomes have been developed for treatment of malignancies overexpressing folate receptors, such as ovarian cancer and acute myelogenous leukemia. Folate receptors α and β are glycosylphosphatidylinositol-anchored membrane proteins with high affinities for folate that are frequently overexpressed in solid tumor and myeloid leukemias, respectively. In contrast, most normal tissues lack expression of either FR. The
selective amplification of FR expression among human malignancies suggests a potential utility as a selective marker that can be exploited in targeted drug delivery. Targeting FRs through folate-conjugation is an attractive strategy, because of the relatively small size of folate compared to monoclonal antibody and its lack of immunogenicity. Compared to non-targeted formulations, FR-targeted liposomes retained many inherent advantages of liposomal formulations. In addition, it may improve therapeutic index of the drug by providing folate receptor-based cellular uptake of the anticancer agent.

An important factor in the design of liposomal formulations is formulation stability, including both in vitro and in vivo. Colloidal stability of liposomes is primarily determined by their surface properties. The in vivo fate of liposomal particles and the rate of drug release in vivo are mainly determined by particle size, surface charge, hydrophilicity of the drug, fluidity of the lipid bilayer, and the presence of targeting agents. Liposomes are cleared from systemic circulation by the reticuloendothelial system (RES), mainly in liver and spleen. Development of long-circulating liposomes that are able to escape RES uptake has been regarded as a milestone in the advancement of liposome technology. Poly (ethylene glycol) (PEG) coating has been used to produce “stealth liposomes”, which have been shown to prolong the circulation of these particles, and to enhance accumulation in solid tumors based on the enhanced permeation and retention (EPR) effect. Moreover, PEG has also been used as a linker for lipid conjugation of targeting molecules, such as folate, transferin, peptide, antibody, and antibody fragments. Compared to direct conjugation to lipids in the bilayer, PEG linker-
based conjugation can serve to overcome steric hindrance on the liposomal surface and enable cellular receptor recognition even in the presence of surface PEGylation required for long circulation. Several lipids have been evaluated as hydrophobic anchors for PEG, including distearoylphosphatidylethanolamine (DSPE)\(^3,15,26,45,53-55\), dioleoylphosphatidylethanolamine (DOPE)\(^56\), cramides\(^54\), phosphatidic acid\(^57\), and cholesterol\(^7,58\).

FR-targeted liposomes have been investigated for tumor specific delivery of reagents for chemotherapy\(^22-27,59\), boron neutron capture therapy\(^60,61\), and immunotherapy\(^36,62\). These liposomes contain folate-PEG-DSPE from 0.1 to 1\%(mol) in the lipid composition that enables the liposomes to target FR-overexpressing cells through binding to FRs on the cell surface\(^14,15,25-27\). However, cholesterol-anchored long circulating liposomes have not been investigated for folate receptor-targeted delivery.

In the current study, we investigated cholesterol as an anchor both for PEGylation and for folate. Two derivatives, methoxy poly (ethylene glycol)-cholesterol (mPEG-Chol) and folate-PEG-cholesterol (F-PEG-Chol) were synthesized and incorporated into liposomes (Figure 5.1). The liposomes were characterized for their stability and \textit{in vivo} circulation time. The significance of cholesterol-based bilayer anchor in liposome development is discussed.
5.2. Results

5.2.1. Synthesis of liposomes containing cholesterol derivatives

Samples were prepared by high pressure homogenization and stored in 4°C for less than 1 week. The particle sizes of these samples in PBS were determined by photon correlation spectroscopy. Processing by homogenization was able to produce the mean diameter of the liposomes in the range of 90nm -110nm, by controlling the number of cycles, temperature and maximum pressure. Addition of PEG derivatives facilitated the formation of homogenous population of small particles, indicated by the reduced number of cycles and processing pressure needed to reach the 100nm size range, compared to no-PEG composition (I).

Negative staining TEM was used to visualize the liposomes. Structural features of liposomes prepared by compositions either without PEG derivatives (I), with DSPE-based derivatives (II, IV), or cholesterol-based derivatives (III, V) were visualized by TEM (Figure 5.2), which enabled determination of size distribution and degree of aggregation. EM pictures showed that spherical vesicles with features of small unilamellar vesicles (SUV). The addition of PEG derivatives seemed to promote narrower size distribution. We thus concluded that supplementing lipid bilayers with mPEG-Chol facilitated the formation of homogenous particles.
5.2.2. Effect of PEG-cholesterol on particle stability in vitro

Surface coating using mPEG-DSPE has been known to stabilize the liposomes via steric hindrance\(^6,8,63\). We analyzed the effect of PEGylated cholesterol derivatives on FR-targeted liposomes in a long-term stability test. The samples used had similar initial mean particle sizes (90-110nm) after preparation. The encapsulation efficiency (EE) of doxorubicin was >95% in all five formulations. Tangential flow diafiltration did not change the particle size of liposomes (<10%, mean particle size change). Liposome size and drug retention while in storage at 4ºC were monitored over a period of 12 month (Figure 5.3). Our results (Figure 5.3a) indicated that mPEG-Chol was able to stabilize the liposomes, for both the FR-targeted formulation (V) and the non-targeted formulation (III). The change in mean particle diameter (V: 106 nm to 125 nm) followed similar pattern as that of DSPE-anchored PEGylated liposomes (IV: 101 nm to 129 nm). In contrast, non-PEGylated liposomes (I) had a significant particle size increase (97 nm to 281 nm), either because of liposome aggregation or fusion. A similar stabilization effect was also observed in non-targeted formulation (II and III). We also examined drug release rate of liposomes over the storage period, as another indicator of particle stability. Figure 3b illustrated the percentage of drug entrapment efficiency (EE), as an indicator for drug release (release % = 1 – EE %). The data (Figure 5.3b) showed that mPEG-Chol-incorporated formulations had <5% drug release over a 12-month period (III: 1.1%, V: 4.6), which was comparable to mPEG-DSPE based formulations (II: 2.0%, IV: 4.8) and significantly less than non-
PEGylated formulation (I: 19.3%). In conclusion, PEG coating to the FR-targeted liposomal surface through a cholesterol anchor in the bilayer was effective in promoting particle stability \textit{in vitro}, possibly by reducing interactions among liposomal particles via steric hindrance.

5.2.3. \textit{Effect of cholesterol derivatives on the rate of drug release from FR-targeted liposomes}

The time course of drug release at physiological temperature (37\(^\circ\)C) was determined for various liposomal formulations. As shown in Figure 5.4, incorporation of mPEG-Chol as PEGylation moiety and F-PEG-Chol as a targeting moiety did not change the \textit{in vitro} release profile of liposomal doxorubicin compared to formulation IV, which incorporated DSPE derivatives at the same molar ratio. Interestingly, the release of PEGylated formulations under this condition seemed faster than non-PEGylated liposomes at 96 hr. Nevertheless, the kinetics of doxorubicin release presented similar pattern between liposome compositions containing cholesterol and DSPE-anchored PEG, in both the non-targeted formulations and FR-targeted liposomes.

5.2.4. \textit{Binding of cholesterol-anchored FR-targeted liposomes to KB cells}

The targeting efficacy of cholesterol-anchored FR-targeted liposomes to FR-overexpressing cells was assessed both by flow cytometry and fluorescence microscopy. Calcein, a membrane impermeable green fluorescent dye was encapsulated into the hydrophilic core. First, we confirmed FR expression level in KB cells by flow cytometry. As shown in Figure 5.5a, FRs on KB cells were shown by
the binding of polyclonal rabbit anti-FR antibody. Cellular binding of liposomes containing folate-PEG-Chol (composition V) was significantly higher than that of the non-targeted liposomal calcein (composition I), and this increase could be blocked by free folate (V+Folate) (Figure 5.5b). We also demonstrated specific binding of these FR-targeted liposomes to KB cells by fluorescence microscopy (Figure 5.5c). The binding of cholesterol-anchored FR-targeted liposomal calcein was similar to that of DSPE-anchored FR-targeted liposomes (iv). Only background nonspecific binding was shown in the nontargeted formulation (i).

5.2.5. **Effect of PEG-Chol on systemic circulation time of the liposomes**

The clearance of liposomes was assessed by determining the blood circulation times of the various formulations. The clearance rate of vesicles was related to factors such as the surface charge, hydrophilicity, presence of targeting ligand, and particle size. In this study, we prepared all 5 formulations with similar particle sizes, so that differences in circulation time mainly reflected the effect of PEG coating through different bilayer anchors. Figure 5.6 showed that PEGylated liposomes via the cholesterol anchor (III and V) had increased circulation time over the non-PEGylated liposomes (I), as shown by the sustained doxorubicin concentration in the circulation over a 72 hr period. Plasma concentration-time profiles were analyzed using an iv bolus 2-compartment model, and major pharmacokinetic parameters, $t_{1/2\beta}$, AUC, and CL, are summarized in Table 1. PEGylation via either DSPE or cholesterol anchor greatly reduced the clearance of drugs and increased AUC. Clearance of targeted liposomes (IV and V) was faster than that of the non-targeted liposomes (II and III),
which may suggest increased uptake by macrophages. (Table 5.1) Compared with DSPE-anchored PEGylated liposomes, cholesterol-anchored PEGylated liposomes were cleared faster, possibly because DSPE provided greater *in vivo* anchoring stability for PEG than cholesterol.

5.3. Discussion

FR-targeted liposomal doxorubicin (FLD) is a promising formulation that has been shown to target FR expressing tumor cells. Previous results obtained in our lab have demonstrated *in vivo* antitumor efficacy of FLD both in a KB cell murine xenograft 23 and AML ascites models22. These efforts can potentially lead to the development of FLD as a clinical agent for the treatment of FR (+) tumors such as ovarian cancer and AML. The DSPE derivatives (mPEG-DSPE, F-PEG-DSPE) have previously been used in synthesis of FR-targeted liposomes14,15,21-24 25-27. However, this might not have been the optimal design from a developmental point of view.

Cholesterol as an anchor may have advantages over DSPE. First, incorporation of DSPE as a lipid anchor introduces a negative charge to the liposome surface, which may lead to additional plasma protein binding 64. In contrast, cholesterol, a neutral molecule and component of most liposome formulations, is electrically neutral. Secondly, DSPE molecule is not as chemically stable as cholesterol, thus is susceptible to degradation during the storage57. Thirdly, the cost of DSPE is more than 100 times that of cholesterol, which is less desirable for product development. Thus, the replacement of DSPE with cholesterol is potentially more
cost-effective. The *in vivo* circulation time of cholesterol-anchored liposomes, however, is not as long as the DSPE-anchored liposomes. This may be due to the breakdown of carbonate linker *in vivo* or the loss of PEG-Chol by lipid transfer. PEG-conjugates have been reported previously to hydrolyze *in vivo*\textsuperscript{57}. In addition, lipid monomers have been shown to transfer from one lipid phase to another. In fact this has been the basis for the “post insertion” method for preparation of immunoliposomes\textsuperscript{13,53}. Therefore, this formulation might be suitable for applications where a “long lasting” PEG coat is not desired. It is also possible that folate-PEG-Chol targeted liposomes were more easily captured by the folate receptor on activated macrophage, which can be utilized for treatment of chronic inflammatory diseases such as rheumatoid arthritis.\textsuperscript{65-68}

In summary, we have prepared and evaluated FR-targeted liposomes incorporating cholesterol derivatives mPEG-Chol and F-PEG-Chol. The particle properties, such as the morphology, stability, *in vitro* release, targeting efficacy, *in vivo* circulation, were evaluated in parallel with liposomes containing DSPE-anchored derivatives. Our data indicated that cholesterol was a viable alternative anchor to DSPE for anchoring PEG and folate on liposomal doxorubicin. Results reported herein provide information that PEGylated cholesterol derivatives can be used to stabilize liposomes and provide FR-targeting capacity. Cholesterol anchor is also a valuable tool for PEG conjugation and can be used for anchoring other types of targeting ligand, such as monoclonal antibody and transferrin.
5.4. Material and Methods

Materials

Distearoylphosphatidylethanolamine (DSPE) was purchased from Genzyme (Boston, MA). Hydrogenated soybean phosphatidylcholine (HSPC), monomethoxy-polyethyleneglycol(2000)-distearoylphosphatidylethanolamine (mPEG-DSPE) was purchased from Lipoid (Newark, NJ). Cholesterol, cholesteryl chloroformate, doxorobucin, PEG, PEG-bis-amine, folic acid and Sepharose CL-4B chromatography resin were purchased from Sigma (St. Louis, MO). Spectra/Por DispoDialyzer dialysis tubes (1 mL, MWCO 10,000) were purchased from Spectrum Lab (Rancho Dominguez, CA). Tissue culture media RPMI 1640 without folic acid, fetal bovine serum (FBS), and antibiotics were purchased from Life Technologies (Rockville, MD). The KB human oral carcinoma cell line (ATCC # CCL-17) was a gift from Dr. Philip Low, Purdue University (West Lafayette, IN). Anti-FR rabbit antiserum, control rabbit serum and secondary fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG were obtained from Dr. Manohar Ratnam, Medical University of Ohio (Toledo, OH). Folate-polyethyleneglycol (MW~3,350)-distearoylphophatidylethanolamine (F-PEG-DSPE) was synthesized as previously described \textsuperscript{14,15}.

Synthesis of mPEG-Cholesterol and Folate-PEG-Cholesterol

mPEG-cholesterol was synthesized as a cholesteryl carbonate in one step reaction of monomethoxypoly(ethylene glycol) (M-PEG) and cholesteryl carbonate.
chloroformate (CholCOCl) with some modifications from previously described method. Briefly, a mixture containing 1mmol CholCOCl, 0.05 mmol tetrabutylammonium chloride, and 1.5mmol NaOH (in 6M solution) in 10mL CH₂Cl₂ was magnetically stirred in a round bottom flask. 1mmol m-PEG dissolved in 5mL CH₂Cl₂ was added dropwise to the mixture. The reaction was carried out under nitrogen and continuous stirring at room temperature for 7 days. The mixture was filtered to remove NaCl, and CH₂Cl₂ was evaporated. The solid residue was suspended in ethyl acetate and purified on a silica gel column and eluted by ethanol/CH₂Cl₂ (9:91, v/v). The recovered fraction was dried under vacuum and then stored at -20°C before use. mPEG-Chol was analyzed by silica gel thin layer chromatograph (TLC) using ethanol/CH₂Cl₂ (88:12) as the mobile phase. The product mPEG-Chol had an Rf of 0.09, different from CholCOCl (Rf = 0.93) and cholesterol (Rf = 0.7). F-PEG-Chol was synthesized by formation of carbamate. Briefly, folate-PEG-amine was synthesized by reacting PEG-bis-amine with 1.1 molar excess of NHS-folate in DMSO. A 1.1 molar excess of cholesteryl chloroformate was added to folate-PEG-amine in chloroform, and the reaction was carried out overnight at room temperature. The product was dried under vacuum, and washed twice with diethyl ether to remove residual CholCOCl. The purity of F-PEG-Chol was determined by silica gel TLC using a solvent system of CH₂Cl₂/methanol (70:30, v/v) plus trace amount of acetic acid.
Liposome Preparation

Liposomes were prepared using an emulsification-homogenization-diafiltration method. First, lipid mixtures were dissolved in t-butanol in different compositions: I. HSPC/Chol (60/40, mol ratio), II. HSPC/Chol/mPEG-DSPE (57/38/5, mol ratio), III. HSPC/Chol/mPEG-Chol (57/38/5, mol ratio), IV. HSPC/Chol/mPEG-DSPE/F-PEG-DSEP (57/38/4.5/0.5, mol ratio), V. HSPC/Chol/mPEG-Chol/F-PEG-Chol (57/38/4.5/0.5, mol ratio). The lipid solution was then injected to magnetically stirred 300mM ammonium sulfate buffer at 60°C. The emulsified liposomal suspensions were further subject to high pressure homogenization using a EmulsiFlex C5 unit (Avestin, Ottawa, Ontario) for 5 passes. The ammonium sulfate solution in the homogenized liposomes suspension was replaced with neutral 14% sucrose solution by a diafiltration method using Labscale TFF system (Millipore, Billerica, MA). Doxorubicin in 20mg/mL stock solution was added to the empty liposome suspension, and incubated in a 60°C water bath. Histidine buffer (pH 7.5) was then added to initiate loading of the drug. The loading efficiency was greater than 95% after incubation for 30 min. For calcein-containing liposomes, lipid solution in t-butanol was injected into 50mM calcein solution instead of ammonium sulfate solution at a 1:9 volume ratio, and the suspension was passed through the homogenizer. Free calcein and organic solvent were removed from the suspension by passing through a disposable desalting PD-10 column packed with Sephadex G-25 medium (Amersham Pharmacia, Piscataway, NJ) eluted with PBS. Final products were filtered through a 0.22μm PES membrane and stored at 4°C.
before use. Drug entrapment efficiency (EE) was measured by running samples through a Sepharose CL-4B column, and calculated by the following equation:

\[
EE\% = \frac{(\text{Drug conc. after column}) \times (\text{Drug volume after column})}{(\text{Drug conc. before column}) \times (\text{Drug volume before column})} \times 100\%
\]

Transmission Electron Microscopy (TEM) and Photon Correlation Spectroscopy Analysis

TEM analysis was performed after negative staining of liposomes with uranyl acetate, as described previously. Briefly, the liposomal samples were placed onto 100 mesh grids. After 3 minutes, excess solution was wiped off with filter paper and the grid was immersed with 2% UA. The uranyl acetate solution was removed after 1 min, and the grids were washed twice with 200 μL distilled water. The grids were then imaged on a Philips CM 12 Transmission Electron Microscope (Campus Microscopy and Imaging Facility, OSU, Columbus, OH). Photos at 55,000 fold magnification were taken. The mean particle diameter and distribution were measured by photon correlation spectroscopy on a NICOMP Particle Sizer Model 370 (Santa Barbara, CA). Briefly, 50μl liposomal samples were suspended in 500 μl normal saline, and the normalized Gaussian-distributed volume-weighted particle size was obtained after 3 runs.

Kinetics of Doxorubicin Release

The in vitro release profile of liposomal doxorubicin was measured by monitoring changes in doxorubicin concentration using a dialysis method. This
method was based on creation of a sink condition maintained by a large volume of PBS that would not result in drug precipitation given its solubility of > 10μg/mL in PBS. Briefly, 1 mL liposome samples were added to Spectra/Por DispoDialyzer (MWCO 10,000) and incubated in 400 mL PBS at 37°C. Constant magnetic stirring (100 rpm) was applied to the dialysis beaker. At set time points, 1 mL samples were taken from the PBS and the released free doxorubicin was measured using a Perkin-Elmer spectrofluorometer (Wellesley, MA) with excitation and emission at 470 nm and 580 nm respectively. The percentage of retention was calculated based on normalization using the original concentration before dialysis.

**Cell Binding Study**

KB cells were cultured as a monolayer in folate-free RPMI 1640 media supplemented with penicillin, streptomycin and 10% fetal bovine serum. Culture was maintained in a humidified atmosphere containing 5% CO₂ at 37°C, and split one day before the binding study. One million KB cells resuspended in cold phosphate buffered saline (PBS) were stained with anti-FR rabbit antiserum and secondary fluorescein isothiocyanate (FITC)-goat anti-rabbit IgG to determine FR expression level. Cell binding with non-targeted liposomal calcein (LC) and FR-targeted liposomal calcein (FLC) were performed by incubation at 4°C for 45 min. One mM folate was used to compete with the FLC for FR binding. FACS analysis was performed using a Beckman-Coulter EPICS XL cytometer (Beckman-Coulter, Miami, FL). Fluorescence measurement was performed using filter set for detection of emission at 488nm. Cells were gated on live population, and ten thousand events
were collected for each sample. Data was acquired in list mode and System II software package (Beckman-Coulter) was used to analyze the data.

**Plasma Clearance Study**

Male BALB/c mice (20g, 6-8 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA). Mice (6 per group) were injected with different formulations at a drug dose of 6mg/kg via the tail vein. Blood samples (50 μL) were collected at 15min, 1h, 5h, 8h, 24h, 48hr and 72hr after injection. The volume collected was less than 20% of the total blood volume over a 3 day period. Blood samples were then mixed with 0.5mM EDTA-PBS (250 μL) followed by centrifugation to obtain plasma. Plasma protein was precipitated during extraction of doxorubicin with 2.5mL of acidic isopropanol. A standard curve for doxorubicin in acidic isopropanol was prepared using fluorometry, and the doxorubicin concentration in the plasma was determined based on the standard curve. Plasma concentration data were analyzed by WinNonlin 5.0 (Pharsight Corp., Mountain View, CA).
Table 5.1. Pharmacokinetic parameters of liposomal doxorubicin administered to BALB/c mice.
The mice received 6 mg/kg of doxorubicin via i.v. bolus injection. $t_{1/2\beta}$, $\beta$-phase half life. AUC: Area under the curve. CL: total plasma body clearance.
Figure 5.1. Structure of phospholipid and cholesterol derivatives
Figure 5.2. Transmission electron microscopic graph of various liposome preparations.
Photos were taken at 55,000 x magnification from the suspended liposomal particles after negative staining. I, Non-PEGylated, non-targeted liposomes. II, DSPE-PEGylated, non-targeted liposomes. III, Cholesterol-PEGylated, non-targeted liposomes. IV, DSPE-anchored PEGylated targeted liposomes, V, Cholesterol-anchored PEGylated targeted liposomes. Bar =100nm.
Figure 5.3. Long term stability of folate liposomal doxorubicin at 4°C, characterized by particle size and encapsulation. a. Particle size analysis of liposomes stored in 4°C. Mean particle size was measured using photo correlation method (volume weighted) by taking samples at each of the indicated time point. b. Drug encapsulation ratio analysis of liposomes stored in 4°C. Percentage of doxorubicin encapsulated in the liposomes was measured by Sepharose CL-4B chromatography.
Figure 5.4. Time-course of the *in vitro* drug release from liposomes in PBS at 37°C. Liposomal doxorubicin in different formulations were contained in dialysis tubes, and 400mL PBS was used as a sink condition. The buffer was magnetically stirred and incubated in 37°C. The release of doxorubicin was determined using fluorometer as described in the methods. Data is presented as the % retention, standing for the percentage of drug amount remained inside the dialysis tube at the time of measurement.
Figure 5.5. *In vitro* cell binding properties of targeted liposomes incorporated with cholesterol-based derivatives.

**a.** Folate receptor expression of KB cells. Cells were stained with normal rabbit serum (Control), or anti-FR rabbit serum (Anti-FR) and secondary FITC-goat anti-rabbit IgG and measured by flow cytometry. **b.** Specific binding of FR-targeted cholesterol-anchored liposome encapsulated calcein (V) to KB cells. 5mM free folate was used to compete with the specific binding (V+Folate). Non-targeted liposomal calcein (I) was used as a control to show the non-specific binding. **c.** Fluorescence microscopy graphs of trypsinized KB cells incubated with calcein-containing liposomes formulated with non-targeted formulation (I), DSPE-anchored targeted formulation (IV) and cholesterol-anchored targeted formulation (V). 5mM folate was also used to compete with the specific binding (W/ folate). The same slide was visualized in phase-contrast mode (Contrast) or fluorescence mode (Fluorescent) to demonstrate the level and percentage of binding.
Figure 5.6. Plasma doxorubicin concentration-vs.-time curve in mice. Liposomal doxorubicin (6mg/kg) in 5 indicated formulations was injected through tail vein. Data points represent the doxorubicin concentration in the murine plasma at the given time after the administration. Each value was an average of 6 mice, and the error bars represent the standard deviation.
LIST OF REFERENCES


43. Klibanov, A.L., Maruyama, K., Beckerleg, A.M., Torchilin, V.P. & Huang, L. Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target. Biochimica Et Biophysica Acta 1062, 142-8 (1991).


CHAPTER 6

FOLATE RECEPTOR TARGETED DELIVERY OF CHOLESTEROL DERIVATIVES FOR BORON NEUTRON CAPTURE THERAPY

6.1. INTRODUCTION

Boron neutron capture therapy (BNCT) is a binary chemotherapeutic method for the treatment of cancer, which is based on the nuclear reaction between boron atoms and low energy thermal neutrons. Naturally occurring elemental boron has two stable isotopes namely boron-10 ($^{10}$B) and boron-11 ($^{11}$B). The abundant isotope is $^{11}$B (around 80%), however the most distinguishing property of $^{10}$B is its high neutron capture cross section for thermal (slow) neutrons. Hence the reaction of a neutron with $^{10}$B yields two charged particles, a $^4$He nucleus and a $^7$Li nucleus, each of which are able to kill tumor cells due to their high linear energy transfer. For successful BNCT, a minimum of 20-30 $\mu$g of non-radioactive $^{10}$B per gram of tumor tissue is required. Another key requirement for the success of BNCT is the selective delivery of high amounts of boronated compounds to tumor cells, while at the same time the boron concentration in the cells of surrounding normal tissue should be kept low to minimize the damage to normal tissue.

Liposomes have been a main focus of tumor selective boron delivery strategies in BNCT since research activities in this area were initiated by Hawthorne and Yanagie in the early 90’s. Design strategies for boron containing liposomes for BNCT centered both on non-targeted and tumor-targeted formulations. The latter
included liposomes conjugated to transferrin\textsuperscript{6}, the epidermal growth factor (EGF)\textsuperscript{7}, antibodies\textsuperscript{8}, alpha (v)-integrin specific RGD peptides\textsuperscript{9}, and folic acid\textsuperscript{10,11}. Most liposomes designed for BNCT contained hydrophilic low molecular weight boron agents, which presumably located in the aqueous core of the liposomes during preparation. These boron agents were e.g. BSH\textsuperscript{12,13} and BPA\textsuperscript{14}, both of which have been used in clinical BNCT\textsuperscript{1}, negatively charged boron clusters with or without simple substitution patterns\textsuperscript{2,15}, as well as carborane cage substituted polyamines\textsuperscript{11}, acridines\textsuperscript{7,8}, porphyrins\textsuperscript{16}, carbohydrates\textsuperscript{17}, and nucleosides\textsuperscript{18}.

Phospholipids are common lipid bilayer components of liposomes and have proven to be effective anchors for boron entities in form of dual-\textsuperscript{19,20} or single chain \textit{nido}-carboranyl phospholipid mimics. Cholesterol is another major component of the mammalian cell membrane and most liposomal formulations, which is transported in the blood stream by low density lipoprotein (LDL). Owing to its natural carrier capability and receptor-targeting specificity, LDL has been extensively studied as a drug carrier\textsuperscript{22}. Therefore, the development of boron-containing compounds that mimic physiological cholesterol and cholesterol esters is potentially an effective approach for boron delivery to cancer cells both via liposomes and LDL, and thus, extensive efforts in BNCT drug development have focused on such structures\textsuperscript{23-30}.

To the best of our knowledge, all synthetic approaches direct towards boron-containing cholesterol derivatives have focused on the attachment of carboranyl moieties to the hydroxyl group of cholesterol via ether or ester linkages. In the present manuscript, we report a novel strategy for the design and synthesis of
carboranyl cholesterol mimics. This design strategy is based on a groundbreaking concept developed by Endo et al. for the synthesis of a carboranyl analogues of estradiol\textsuperscript{31,32}. In analogy to Endo’s carboranyl estradiols, both the B and C ring of cholesterol were replaced with a carborane cluster in the boronated cholesterol mimics described in this paper. The design and synthesis of these structures, their incorporation into liposomes, and the evaluation of the capacity of these liposomes for efficient boron delivery are the subjects of this paper. Both the folate receptor (FR)\textsuperscript{33,34} and the vascular endothelial growth factor receptor-2 (VEGFR-2)\textsuperscript{35,36} have been identified as important molecular targets for cancer therapy in recent years. The capacity of the designed boronated liposomes for incorporation of receptor-targeting ligands such as folate, and the results of \textit{in vitro} uptake and cytotoxicity studies with the resulting constructs are discussed.

6.2. RESULTS

6.2.1. Incorporation of Carboranyl Cholesterol Mimics into Liposomes.

Compounds I-III (Figure 6.1) were formulated into non-targeted and FR-targeted liposomes using the thin-layer evaporation method\textsuperscript{10}, and the particle sizes and lamellarities were further defined using ultrasonication followed by extrusion. A fixed drug to lipid ratio (1:20) was applied for all the 3 compounds. First, we characterized the incorporation efficiency using size-exclusion chromatography. Liposomal and free compound fractions were separated at different elution volumes, and the boron concentrations were measured by ICP-AES. The results, shown in Figure 6.2, indicated that all three compounds had > 90% incorporation efficiency (I
= 99.7%, II = 94.3%, and III = 96.8%). For all liposomal formulations prepared subsequently from compound I for in vitro release and cell uptake studies, > 90% incorporation efficiency was confirmed with the same method (data not shown). Samples for in vitro release and cell uptake studies were all purified using the Sepharose CL-4B column to eliminate free compound. Compound I was selected for all further studies because of its good structural overlap with cholesterol and its high incorporation efficiency.

6.2.2. Size and Lamellarity Characterization of Liposomal Formulations.

For liposomal delivery of BNCT agents, as well as other anticancer therapeutics, the size distribution and lamellarity of liposomes are of great importance for parameters such as stability, drug release profile, in vivo half-life, and tissue distribution. Small, homogeneous, and unilamellar particles are preferred for optimal delivery to malignant tissues, as they are able to escape reticular endothelial system (RES) uptake. We applied transmission electron microscopy (TEM) and photon correlation spectroscopy for the analysis of the physical properties of conventional, non-targeted and FR-targeted liposomes. After negative staining, both FR-targeted and non-targeted liposomal formulation of compound I were inspected with TEM (Figure 6.3AI and 6.3CI) for particle size distribution and lamellarity observation. As a control, conventional liposomes (DPPC/Cholesterol [55%;45%]) were also constructed under identical conditions and analyzed accordingly (Figure 4BI). Since the TEM method does not allow the particle size quantification, the same samples were also analyzed for mean particle size and distribution using a laser particle sizer.
in parallel (Figure 6.3AII-6.3CII). These data demonstrate that homogenous small unilamellar vesicles (SUVs) were formed by using compound I both for non-targeted and FR-targeted liposomal formulations. The mean particle sizes for all three formulations ranged from 82.4 to 95.5 nm with size distributions around 30-40 nm. No major differences were found in appearance, size distribution, and lamellarity between conventional DPPC/cholesterol liposomes, non-targeted and FR-targeted liposomal formulations of compound I.

6.2.3. **In Vitro Release of Liposomal Formulations of Compound I.**

The performance of boron-containing liposomes *in vivo* is highly affected by the stability of incorporation of the boron entities. Therefore, a standard *in vitro* release experiment \(^5\) was conducted using PBS as the media at 37 °C, and the stability of incorporation of compound I both in FR-targeted and non-targeted liposomal formulation was evaluated. Control liposomes were constructed with cholesterol instead of compound I, and thus, calcein, a green fluorescent dye, was encapsulated into the liposomal hydrophilic compartment of all liposomal formulation to analyze stability via fluorescence measurements of calcein release. Samples were taken at different time points beginning at 0.5 h after the initiation of incubation, and calcein release was measured by absorbance (Figure 6.4A). Boron concentrations of samples obtained at 0 h and 96 h were determined by ICP-AES (Figure 6.4B). Overall, both data sets indicate that a 45% molar ratio of compound I was stably incorporated into the vesicles, and that the release of calcein followed a pattern similar to that of conventional DPPC/Cholesterol liposomes. For both the non-
targeted and FR-targeted formulation, the calcein release was less than 30% at 24 hours, which was not significantly different from the control liposome. At later time points, however, the targeted formulation appeared to release calcein, and presumably also boron, at an increased rate. This could be due to degradation of the receptor-targeting ligand, folate-PEG-cholesterol, or insufficient anchoring to the lipid bilayer, which has been observed previously by us in similar studies using doxorubicin as the incorporated compound. Nevertheless, non-targeted liposomal formulations showed a release profile comparable to the control liposomes, with more than 50% calcein and boron remaining unreleased at 96 h. The release rate of FR-targeted liposomes at least until 48 h should be sufficient for selective receptor-mediated boron delivery to tumor cells for BNCT.

6.2.4. In Vitro Uptake of FR-targeted Liposomal Formulations of Compound I.

We determined the in vitro delivery of compound I to KB cells, an established FR-expressing oral carcinoma cell line, in form of non-targeted liposomal formulations as well as in FR-targeted liposomal formulations in the presence and absence of free 5mM free folate. No significant cytotoxicity was noted during the 4 hour incubation time based on a microscopic inspection of the cells. Cellular uptake of the liposomes was determined by measuring cell-associated boron concentrations with DCP-AES. The obtained data are summarized in Figure 6.5. The non-targeted liposomal formulation of compound I appeared to bind in a non-specific way to KB cells (30 μg boron/g cells), which have a very low back ground level of boron (<1 μg boron/g cells, data not shown) in cell culture environment. Specific targeting to FR
was demonstrated by increased boron levels for FR-targeted liposomal formulation of compound I (267 μg/g cells). FR specificity was further demonstrated by the reduction of the boron concentration of FR-targeted boronated liposomes that were incubated with KB cells in the presence of 5mM free folate (9 μg/g cells). All three liposomal formulations were co-labeled with calcein and specific binding was also confirmed by the increased fluorescence intensity in KB cells treated with FR-targeted boronated liposomes, as demonstrated by fluorescence microscopy (data not shown).

6.3. Materials and methods

Materials. Di-palmitoylphosphatidyl choline (DPPC) was purchased from Genzyme (Boston, MA). Monomethoxy-polyethylene glycol(2000)-distearoylphosphatidylethanolamine (PEG-DSPE) was purchased from Lipoid (Newark, NJ). Cholesterol, calcein, and Sepharose CL-4B were purchased from Sigma (St. Louis, MO). Folate-polyethylene glycol_{2000}-cholesterol (folate-PEG-Chol) was synthesized as described previously. Spectra/Por DispoDialyzer dialysis tubes (1 mL, MWCO 10,000) were purchased from Spectrum Lab (Rancho Dominguez, CA). Tissue culture media RPMI 1640 without folic acid, fetal bovine serum (FBS), and antibiotics were purchased from Life Technologies (Rockville, MD). The KB human oral carcinoma cell line (ATCC # CCL-17) was gift from Dr. Philip Low, Purdue University (West Lafayette, IN).
**Preparation of Liposomes.** Non-targeted liposomes, control liposomes, and FR-targeted liposomes were prepared using the thin-layer evaporation method\textsuperscript{45}. The particle size was reduced by ultrasonication and extrusion. The compositions for each liposomal formulations were as follows: non-targeted liposome (DPPC: Compound I: PEG-DSPE, 55:40:5, mol%), control liposome (DPPC: cholesterol, 55:45, mol%), and FR-targeted liposome (DPPC: Compound I: PEG-DSPE: folate-PEG-cholesterol, 55:40:4.5:0.5, mol%). Lipids were first dissolved in chloroform, dried to a thin film in a round-bottom flask under nitrogen, and then further dried using vacuum for 4 hours. The dried lipid was then resuspended using 10 mM calcein solution and pulsed-sonicated for 5 minutes. The resulting liposomal suspension was passed through a 100 nm polycarbonate membrane for 5 circles using a high-pressure extruder (Northern Lipid, Vancouver, British Columbia, Cannada). A 10 mL Sepharose CL-4B column equilibrated with pH 7.4 phosphate buffered saline (PBS) was applied to separate the liposomal portions from low molecular weight components. Elutions were collected in 1 mL-quantities and subjected to the determination of boron concentration by ICP-AES as described in a following section on boron measurements.

**In Vitro Release Analysis of Liposomes.** The *in vitro* release profile of non-targeted liposomes, control liposomes, and FR-targeted liposomes was measured both by calcein release and determination of boron concentrations via ICP-AES. Briefly, 1 mL of Sepharose CL-4B column-purified liposome samples were added to 1 mL
dialysis tubes (MWCO 10,000) and incubated in 500 mL PBS at 37°C. Constant magnetic stirring (100 rpm) was applied to the dialysis beaker. At set time points, 1 mL samples were taken from the beaker and the released calcein was measured using a Perkin-Elmer spectrofluorometer (Wellesley, MA) with excitation and emission at 485 and 520 nm respectively. Excitation and emission slits were set to 5 nm. The boron concentrations of liposomal suspensions before and after the dialysis were also measured by ICP-AES. The percentage of retention was calculated based on normalization using the original concentration before dialysis.

**Boron Uptake Study of Liposomes in FR-Positive KB Cells.** KB cells were cultured as a monolayer in folate-free RPMI 1640 media supplemented with penicillin, streptomycin, and 10% FBS in a humidified atmosphere containing 5% CO₂ at 37°C. After reaching confluence, KB cells were collected with trypsin/EDTA, washed with PBS, and suspended in folate-free RPMI media at a density of 2 x 10⁷ cells/mL. Boronated FR-targeted or non-targeted liposomes (40 μg/mL boron) were added to the media and cells were incubated under CO₂ atmosphere at 37°C for 2 h. Subsequently, cells were centrifuged at 400 g for 10 minutes and the media removed. Cells were washed twice with cold PBS by resuspension and centrifugation at 400 g. Cell pellets were then collected, and boron concentrations were determined by direct current plasma-atomic absorption emission spectroscopy (DCP-AES)⁴⁸. All the treatments and measurements were triplicated.
Transmission Electron Microscope (TEM) and Photon Correlation Spectroscopy Analysis. The consistencies of non-targeted liposomes, control liposomes, and FR-targeted liposomes constructed with compound I were investigated with TEM after negative staining with uranyl acetate (UA) as described previously 50. Briefly, the liposomal samples were placed onto 100 mesh grids. After 3 minutes, excess solution was wiped off with filter paper and the grid was immersed with 2% UA. The UA solution was removed after 1 min, and the grids were washed twice with 200 μL distilled water. The grids were then imaged on a Philips CM 12 Transmission Electron Microscope (Campus Microscopy and Imaging Facility, OSU, Columbus, OH). 55,000 fold magnification photos were taken from the suspended liposomal particles. The mean particle diameter and distribution were measured by photon correlation spectroscopy on a NICOMP Particle Sizer Model 370 (Santa Barbara, CA), and the normalized Gaussian-distributed volume-weighted particle size was collected after 3 runs.

Boron Determinations. Boron concentrations of samples obtained from boron uptake studies with KB cells were obtained by DCP-AES as described previously 48. Boron concentration for all other samples were obtained by inductively coupled plasma atomic emission spectroscopy (ICP-AES) with a Varian VISTA AX™ ICP spectrometer system. The Varian™ argon-purged echelle spectrometer, which has a bandpass ≤10 pm at wavelengths below 260 nm, enabled intensity measurements free of spectral interferences for the four neutral-atom boron lines used in this study:
208.889 nm, 208.956 nm, 249.678 nm and 249.772 nm. All relative intensities for boron spectral lines were based on background-corrected peak heights. A CCD detector, maintained at –34 °C by a Peltier cooler, generated all spectrum-based electronic signals processed by the spectrometer system. The central axial region of a 1.3 kw, 40 MHz ICP was aligned optically with the axis of the acceptance angle of the echelle spectrometer. Sample solutions were injected into the high-temperature plasma as aerosols produced by a PFA nebulizer. The aerosols first passed through a 50 mL Polycon cyclone spray chamber from which fine aerosol particles were swept by an argon stream through a PTFE tube and alumina injector tube into the plasma. In the plasma, free atoms and ions were produced and excited to emit quantized radiation. Calibration was achieved with one blank and four accurately diluted aliquots of a single-element standard solution. The boron concentration in the original standard was certified traceable to the National Institute of Standards and Technology (NIST) by the supplier, Inorganic Ventures (Lakewood, NJ). All dilutions, including those for samples, were accomplished in polyethylene tubes on a mass basis enabled by an analytical balance capable of measurements to 0.1 mg. The diluent was 0.5 M nitric acid in 18.2 MΩ-cm water. Samples (1 mL) were diluted by factors that ranged from 3 to 15 to provide the 3 mL solution volume needed for nebulization into the ICP. Calibration functions were second-degree polynomial least-square regression fits with correlation coefficients greater than 0.995. Concentration values obtained by this approach generally are within 10% of actual concentrations. Certified reference materials were not available for further corroboration of accuracy.
6.4. Discussion

Three carboranyl cholesterol derivatives were synthesized and analyzed applying novel molecular design concepts. All three compounds have structural features and physicochemical properties that are very similar to those of cholesterol. One of the synthesized boronated cholesterol mimics was stably incorporated into non- and FR-targeted liposomes. No major differences were found in appearance, size distribution, and lamellarity between conventional DPPC/cholesterol liposomes, non-targeted, and FR-targeted liposomal formulations of this carboranyl cholesterol mimic. The calcein release properties of conventional DPPC/cholesterol liposomes and non-targeted boronated liposomes were comparable and were reflected by the boron release rate of the latter. The calcein and boron release rates of FR-targeted boronated liposomes were higher than those of non-targeted boronated liposomes but the overall stability of the latter was acceptable for the purpose of tumor-selective boron delivery for BNCT. There was no apparent in vitro cytotoxicity in FR overexpressing KB cells when these were incubated with FR-targeted liposomal formulations of this carboranyl cholesterol derivative. The obtained results convincingly demonstrate that the novel carboranyl cholesterol mimics are excellent lipid bilayer components for the construction of non-targeted and receptor targeted boronated liposomes for BNCT of cancer.
Fig. 6.1. Structures of cholesterol and boronated cholesterol mimics I-III
Fig. 6.2. Incorporation of compound I-III into liposomes.
Encapsulation of boron compounds was determined by eluting 0.5 mL liposomal samples through a 10 mL Sepharose CL-4B column with pH 7.4 PBS. Fractions were collected for determination of boron concentration by ICP-AES. The encapsulation efficiencies were 99.7% for compound I, 94.3% for compound II, and 96.8% for compound III.
Fig. 6.3. Characterization of size distribution and lamellarity of liposomal formulations of compound I.
(A), non-targeted boronated liposome; (B), non-boronated control liposome (DPPC/cholesterol); (C), FR-targeted boronated liposome. (I) Morphology of the liposomes visualized by Transmission Electronic Microscopy (TEM). Negative staining was applied to observe the particle size and lamellarity of liposomes. Formation of small unilamellar vesicle (SUV) was confirmed for all the 3 formulations of compound I. (II) Particle sizes measured by photon correlation method. Numbers shown are the mean particle size ± standard deviation (volume-weighted) of the individual samples.
Fig. 6.4. *In vitro* release profile of liposomal formulations of compound I in pH 7.4 PBS at 37°C. 

(A) Compound I was formulated into liposomes with different compositions at a 1:20 drug/lipid ratio. In parallel, DPPC/cholesterol control liposomes without boron components were prepared using the same protocol. All liposomal formulations contain a 10 mM solution of calcein. The percentage of calcein release was determined by fluorescence and absorbance measurements. (B) Boron concentrations of the liposomal suspensions were measured at 0 h and 96 h by ICP-AES. NT: Boronated liposomes (non-targeted), Control: control liposomes (no boron), FR: FR-targeted boronated liposomes. Error bars shown in Figure 5B are based on standard deviations calculated from three measurements.
**Fig. 6.5. Cellular uptake of liposomal formulations of compound I.**

NT: Non-targeted boronated liposomes without targeting ligands. FR: FR-targeted boronated liposomes. FR + folate: FR-targeted boron-containing liposomes were co-incubated with 5mM free folate. 2x10^7 cells in 1 mL media were incubated with liposomes containing 40 μg/mL boron. Each data point represents the mean of boron amount in the cell lysates normalized to 1 x 10^9 cells (equivalent to 1 gram tissue). Error bars stand for standard deviations of the normalized boron amount in triplicates.
LIST OF REFERENCES


CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

7.1. Conclusions

This dissertation project has been focused on the development of targeted cancer therapies directly against two promising cellular targets: CD37 and FR, both of which are highly attractive due to their overexpression and high specificity in several types of diseases. More specifically, the research presented herein has explored targeting strategies through two innovative yet developmental viable approaches: engineered proteins and liposomes. Based on these findings, we believe that CLL (for CD37) and AML (for FR) are likely to be the most promising disease targets for translation of these novel therapies into clinical development.

To the best of our knowledge, this is the first report on utilizing a CD37-specific engineered MAb-like molecule for treatment in CLL. CD37 has been in the past largely neglected for development, despite two inconclusive clinical trials using radiolabeled mouse MAb\textsuperscript{1,2}. Our study, however, revoked the promise of CD37 as a very exciting target for treating hematologic malignancies, especially for CLL. In fact,
research described in this dissertation has not only evaluated CLL as a promising disease target for CD37-based therapy, but also provided comprehensive information on the therapeutic efficacy and mechanisms of CD37-SMIP \textit{in vitro} and \textit{in vivo}. These studies paved the way for clinical development of CD37-SMIP in CLL and other types of CD37$^+$ hematologic malignancies. In addition, our unique approach, combining drug delivery and MAb technology, also allowed us to explore the possibility of designing liposome-based CD37-SMIP formulation for treatment in CLL. This nanoscale formulation design, liposomal CD37-SMIP, is likely to have several potential advantages, such as independence on crosslinking molecules, optimized tissue distribution, longer half life, and reduced immunogenicity. We believe that this liposomal approach, in addition to be an alternative formulation design for CD37-SMIP, may have the potential to be explored as a platform technology for delivery of other therapeutic engineered proteins in treating cancers.

On the other aspect, FR-targeted therapeutic strategies have in fact being around for more than 2 decades, and are still in very active exploratory phase by multiple groups. Although the idea of using FR-targeted liposomes itself has been presented in the past, the studies detailed in the current dissertation still contributed significantly to the FR-targeting field. Our work has provided important information about using cholesterol, a low cost biocompatible lipid composition, for anchoring PEG and folate to the liposome bilayer. This approach is valuable, especially from pharmaceutical development point of view, because it provides an alternative design that is more relevant for \textit{in vivo} application and commercial development. Similarly,
although the concept of using FR-targeted liposomes for delivery of BNCT agents itself is not new to the field, our research on the delivery of novel boron-containing cholesterol mimics is more or less an approach of “Old Wine in a New Bottle”. This study provided useful information about the feasibility of delivering novel BNCT agents that represent unique structural features and therapeutic advantages. This certainly contributed to the BNCT field, where the delivery issue has always being vital important due to the nature of the radiotherapy in brain tumor.

In summary, these studies paved the way for investigation of CD37-targeted immunotherapies and provided further basis for clinical development of FR-targeted liposomes. We believe that future development attempting to translate our increasing knowledge of cellular surface molecule-targeted therapy will eventually turn these “magic bullets” into full display and benefit patients with leukemia and other types of cancers.

7.2. Future directions

7.2.1 Development of CD37-SMIP for hematologic malignancies

One of our ongoing efforts is to translate CD37-SMIP into clinic for treatment of CD37+ hematologic malignancies, represented by CLL. CD37-SMIP differentiates itself from several other therapies used in CLL by mediating apoptosis through a novel caspase independent pathway not utilized by other therapies in this disease. In fact, synergistic effect between CD37-SMIP and fludarabine, a know caspases
activator and so far the most active agent for CLL therapy, has been demonstrated in our study. The difference in CD37-SMIP mechanism of action as compared to rituximab is further supported by a preliminary report by Trubion Pharmaceutical, demonstrating synergy between rituximab and CD37-SMIP in lymphoma xenograft models. Based on these findings, further studies investigating combinational therapies are likely to lead to fruitful results, and will eventually benefit the translation of CD37-SMIP based therapy in clinic.

The success of therapeutic antibodies to eliminate malignant B cells appears to be greatly influenced by ability to mediate ADCC, as demonstrated by both pre-clinical in vivo studies and clinical trials in NHL, where high affinity FcγRIIIa polymorphisms associated with enhanced ADCC are linked to superior response to rituximab. The critical role of FcγR in in vivo effects of therapeutic antibodies has also been supported by studies in mice deficient of FcγR. In CLL cells, however, rituximab mediates relatively poor ADCC, possibly explaining diminished efficacy of this antibody. Our data provide evidence that CD37-SMIP mediates significantly greater selective ADCC against CLL cells and related B-cell lymphoma cell lines as compared to rituximab and alemtuzumab. ADCC mediated by CD37-SMIP in CLL cells occurs predominately through human NK cells. Despite macrophage/monocytes-mediated ADCC activity being observed with several antibodies, we did not find that CD37-SMIP is associated with phagocytic efficacy in our studies. How the absence of CD37-SMIP induced phagocytosis will differentiate this engineered protein from other Ab-based therapeutics will be interesting to further define the in vivo function of this molecule. Further studies on the importance of
FcγRIIIa polymorphisms towards SMIP-based therapeutics will also be important to elucidate the clinical activity for this class of SMIP molecules as compared to existing Ab therapeutics. The ultimate test of CD37 SMIP efficacy will require pursuit of clinical studies in patients with CLL and related diseases.

The development of targeted engineered protein therapies that depend upon signaling and ADCC has been unsuccessful to date due to limited production capability and poor pharmacokinetic features relative to therapeutic antibodies. Preclinical development of the first CD20 directed SMIP Tru15 has demonstrated absence of immunogenicity in cynomologus monkeys\(^9\), and superior B-cell depletion as compared to rituximab\(^9,10\). Preliminary results of the first phase I study with Tru15 demonstrated it to be clinically well tolerated, and successful at depleting B-cells in a dose dependent manner with an extended serum half-life (12-19 days)\(^9\). Thus, development of CD37-SMIP for clinical investigation in CLL based upon our data herein represents an obvious extension of this exciting targeted therapeutic class of agents directed at a B-cell selective antigen not yet pursued. The unique engineered features of SMIP-based therapies may offer an advantage over classic antibody based-therapies including improved effector function, target affinity, and apoptotic signaling. Detection of immunogenicity-related neutralizing antibody response against CD37-SMIP after multiple injections at varying frequencies in an immunocompetent model will be valuable to further investigate the immune response towards this class of engineered proteins with modified Fc region. In addition, pharmacokinetic studies to define the plasma half life, and biodistribution studies to define the targeting effect will also be important to determine the clinical application
of this novel targeted therapy in CD37⁺ lymphoid malignancies. Based upon these exciting preclinical data, further clinical development of CD37-SMIP is warranted.

7.2.2 Development of FR-targeted liposomes

Targeted drug delivery is a promising approach to deliver neoplastic agents selectively to cancer cells and improve the therapeutic index of chemotherapy. Folate receptor outstands among various tumor markers because of the specificity and relatively high level of endocytosis, therefore represents a very attractive target for developing targeted delivery for future cancer therapy. FR-targeted liposomal delivery is a promising strategy for treatment of FR⁺ tumors and leukemias. Although a variety of agents have been delivered to FR⁺ tumor cell *in vitro* using these liposomes, relatively few reports address the *in vivo* properties of these liposomes. However, at least some studies indicated a therapeutic advantage of folate-liposomal doxorubicin over the non-targeted control⁹⁻¹³. Leukemia, which has greater accessibility from the systemic circulation, may present an even better therapeutic target for these liposomes. The concept of sensitizing AML cells with FR-β-upregulating agents such as ATRA may further bring the approachability to this FR-targeted liposomal drug delivery to clinical trials.

Several critical factors to be considered for designing *in vivo* application of FR-targeted liposomes for cancer therapy include: FR expression level on tumor cells relative to normal cells, the accessibility of liposomes to these receptors, the intratumor diffusion rate of vectors and the presence of endogenous folate in systemic
circulation. The first obstacle of insufficient levels of FR expression on tumor cells in patient could be possibly overcome by inducing FR expression upregulation by co-administration of anti-estrogen or retinoid receptor ligands. To address the second concern of accessibility, optimization of formulation parameter is necessary to reduce the particle size, reduce the plasma protein binding, and avoid the RES and other normal tissue uptake. For solid tumor delivery, priming the vasculature in tumor to increase the distribution of targeted particles is a rational approach. The concern that the presence of endogenous folate in systemic circulation can block FR binding is not a critical one. We believe this should not constitute a barrier, as the endogenous form of folate has lower affinity for the FR compared to folic acid, thus should not significantly impede the binding of multivalent folate-coated liposomes.

Clinical success in targeted drug delivery area has so far been limited by the lack of a suitable cellular target and/or a high degree of difficulty in the production of clinical quantities of targeted drug carriers. FR-targeted liposomes represent an outstanding candidate for clinical development, with potential application in the treatment of AML and other FR-overexpressing solid tumors. Interesting questions for future studies include, first the possible role of receptor upregulation in promoting targeted therapy, both for FR-α and FR-β positive tumors, and secondly the cellular population responsible for enhancement in therapeutic response in solid tumors, i.e., relative roles of tumor infiltrating macrophages and the tumor cells themselves. Since activated macrophages have increased FR-β expression, it is possible that folate-conjugates can effectively target tumor infiltrating macrophages in non-FR
expressing tumors, thus extending the FR-targeting strategy to receptor negative tumors.

The area of folate receptor targeting remains an area of great excitement, suggested by large number of recent publication and reports from industrial development. So far, no clinical study targeting FR for therapeutic purpose has been carried out. Given the advantages of the FR-targeting strategy, further preclinical studies are urgently needed to define mechanisms of \textit{in vivo} targeting and the potential for clinical efficacy. FR-targeted liposomal doxorubicin represents an outstanding candidate for clinical development, with enormous potential application in the treatment of AML. Furthermore, future application to other tumors with a high frequencies of FR overexpression, such as ovarian and lung carcinomas also is likely to happen.

For gene and antisense molecule delivery, there have now been many reports showing unequivocally the ability of FR-targeted vectors to efficiently deliver genes and antisense ODNs into tumor cells. Early \textit{in vivo} studies examining the circulation time, gene transfer tissue selectivity, and therapeutic efficacy showed equally promising results. Given the diverse types of gene transfer vectors that have been evaluated for FR-targeted delivery, it clearly would be helpful to compare their relative performances. There is, however, great difficulty in making such a comparison since these vectors are not generally available. Further complicating matters are the multitude of factors affecting gene transfer efficiency, including the net charge of vectors, component ratios, FR expression level in the cell, and specific
cell line’s susceptibility to a specific vector formulation. Furthermore, there is generally a lack of correlation between *in vitro* and *in vivo* gene transfer efficacies of gene transfer vector formulations, due to the very different parameters that determine these two processes. Nevertheless, before initiation of clinical studies, additional improvements in the gene transfer efficiency of any of the reported FR-targeted vectors are likely necessary. Further efforts aimed at optimizing FR-targeted vector formulation for systemic administration is warranted and should lead to the clinical evaluation of these vectors for cancer gene therapy delivery in the foreseeable future.

7.2.3 *Targeting CD37 and FR for siRNA delivery*

Another exciting area that warrants extensive investigation is to utilize CD37 or FR as a target for delivery of nucleic acid-based therapeutic molecules, to offer the potential for treatment of particular cancers without significant side effects. Small interfering RNA (siRNA), modulating gene expression at the transcriptional level, has been growingly recognized as a desired approach for treatment of cancers, especially hematological malignancies.

The utility of these CD37 and FR as targets for delivery is based on the following favorable facts. First, both CD37 and FR are highly expressed on certain type of diseases, with high specificity. Secondly, both targets are cellular surface molecules that undergo fast internalization after binding, which can be utilized for targeted drug delivery through a receptor-mediated endocytosis pathway. Thirdly,
high affinity ligands or antibodies are available for the design of targeting vesicles that carry high payload of therapeutic agents. Finally, with regard to the diseases targets, CLL (CD37) and AML (FR), RNA-based therapy has been extremely exciting, due to the fact that multiple genes (Bcl-2, Bcl-XL, Mcl-1 and etc.) have been identified that directly related to the impaired apoptosis. Therefore, efficient and specific delivery of siRNA to reduce or silent the expression of certain gene targets is likely to lead to promising outcome, and is a therapeutic strategy that worth pursuing.
LIST OF REFERENCES


Byrd, J. C., B. L. Peterson, et al. (2005). "Treatment of relapsed chronic lymphocytic leukemia by 72-hour continuous infusion or 1-hour bolus infusion of
flavopiridol: results from Cancer and Leukemia Group B study 19805."


Polyak, M. J. and J. P. Deans (2002). "Alanine-170 and proline-172 are critical determinants for extracellular CD20 epitopes; heterogeneity in the fine specificity of CD20 monoclonal antibodies is defined by additional requirements imposed by both amino acid sequence and quaternary structure." Blood 99(9): 3256-62.


Abstract

Fludarabine-based combination therapies are commonly used to treat low-grade lymphoma and chronic lymphocytic leukemia (CLL) patients. In vitro and clinical studies have indicated advantages when fludarabine (FLU) and mitoxantrone (MTO) are applied in combination. To further enhance this effect, these two agents were co-encapsulated in liposomes. FLU was passively encapsulated during liposome formation, and MTO was loaded with a transmembrane pH gradient. Entrapment efficiency, particle size, stability, and drug release kinetics were characterized. In vitro cytotoxicity study was carried out in two representative B-cell lines: Wac3CD5 and Raji. Synergism as measured by combination index (CI) was observed in cells treated with liposomes co-encapsulating FLU and MTO. Annexin V/propidium iodide analysis further confirmed that co-encapsulated FLU and MTO improved the percentage of apoptosis among primary CLL cells. These data suggest that adopting liposomes containing co-encapsulated drug combinations constitutes a potential strategy to promote drug synergism and may have utility in the treatment of leukemia and lymphoma.
Introduction

Lymphoproliferative disorders, such as low grade non-Hodgkin’s lymphoma (NHL) and chronic lymphocytic leukemia (CLL), represent a number of challenging diseases. Treatment of NHL and CLL can be categorized into chemotherapy, immunotherapy, radiotherapy and stem cell transplantation.\(^1\) Traditionally, alkylating agents and purine nucleoside analogs are used in combinations, but none of these have been proven curative. Monoclonal antibodies, such as rituximab (Rituxan, anti-CD20), and alemtuzumab (Campath, anti-CD52), have also recently shown significant clinical efficacy.\(^1\) Despite much effort, most of these diseases are still regarded as incurable. Drug resistance and recurrences are frequently observed. Development of novel and more effective therapy is urgently needed. Fludarabine (2-F-arA-AMP, FLU), an adenine nucleoside analogue, is so far the most effective in treating these type of diseases with a relatively high initial response rate and extended progression-free survival as compared to alkylator based therapy.\(^2\text{-}^5\) However, the clinical outcome of FLU monotherapy is unsatisfactory, because it only provides a complete response in a subset of patients, and all patients eventually experience relapse.\(^6\) A number of fludarabine-based combination therapy have been explored, including FLU/doxorubicin\(^7,8\) FLU/cyclophosphamide\(^7,9\text{-}12\), FLU/cytarabine\(^13,14\), FLU/chlorambucil\(^15,16\), and FLU/mitoxantrone regimens\(^17\text{-}29\). The FLU/MTO combination appears to be particular promising and has been further extended to other combinations as well\(^19,20,22,23\). In vitro studies have demonstrated synergism of FLU with MTO in inducing cytotoxicity in B-cell CLL\(^30,31\). Phase I/II studies have also
demonstrated that this combination is effective and well tolerated in relapsed or refractory indolent NHL and also CLL. 26-29

Drug interaction is concentration-dependent and maintenance of appropriate drug ratio is vital for achieving synergism. In fact, a combination can result in synergistic, antagonistic or additive outcomes at different concentration ratios. Therefore, in order to achieve optimized therapeutic efficacy in vivo, dosing schedule is critically important to expose the tumor cells with defined concentrations of reagents. This is challenging for most combination therapies. Unlike in vitro studies where drug concentration is relatively constant, in vivo treatment represents a dynamic system, where drug molecules undergo absorption, distribution, metabolism and elimination, thus the plasma drug concentration is subjected to change over time. For example, FLU32 performs different pharmacokinetic profiles from MTO33-35, and the concentration ratios of these two drugs in plasma will continuously change during the treatment. Optimized dosing schedule is therefore required to achieve optimal therapeutic activity. Alternatively, defined drug delivery system, such as liposomes or nanoparticles, which can synchronize the drug concentrations in vivo, is desired.

Liposomes are well established drug carriers for anticancer agents.36-37 Formulations using liposomal encapsulation have been shown to sustain drug release, alter pharmacokinetic and biodistribution characteristics of chemotherapeutic agent, and lead to increased therapeutic efficacy and/or reduced toxicities36-38. However, there is limited data on co-encapsulation of two different drugs in one liposomal formulation39. Gentamicin and ceftazidime have been co-encapsulated into liposomes and the in vivo synergistic effect has been demonstrated by a rat model of
pneumonia.\textsuperscript{40-42} In another study, isoniazid and rifampicin were successfully co-encapsulated into liposomes, but no study of beneficial effect was presented.\textsuperscript{43-45} To date, no studies have co-encapsulated FLU/MTO nor demonstrated therapeutic benefit of such a combination. Therefore, it is the aim of present study to investigate the application of liposomes as drug delivery system for simultaneous delivery of FLU and MTO as an effective combination therapy strategy for NHL and CLL. Taking into consideration the established results of combined therapy using FLU and MTO, we hypothesized that stealth liposomes can be utilized to co-encapsulate FLU and MTO, and synchronize the release of both drugs from the delivery vehicle to achieve synergistic killing of NHL and CLL cells.

Materials and methods

Reagents

HSPC was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). mPEG\textsubscript{2000}–DSPE was obtained from Genzyme Inc. (Cambridge, MA). Cholesterol (Chol), thiazolyl blue tetrazolium bromide (MTT) and Sepharose CL-4B were purchased from Sigma Inc. (St. Louis, MO). Fludarabine phosphate and mitoxantrone hydrochloride were purchased from Polymed Therapeutics Inc. (Houston, TX). Float-A-Lyzer was purchased from Spectrum Laboratories Inc. (Rancho Dominguez, CA).

Determining fludarabine and mitoxantrone concentration

The HPLC system used for FLU concentration analysis consisted of Beckman 126P Solvent Modules, 166P detector, 507e autosampler and System Gold\textsuperscript{®} software.
A UV variable detector at a wavelength of 254nm and Alltech Versapack C18 column (4.1mm×300mm, 10μm) were utilized. The mobile phase consisted of 5:95 (v/v) mixture of methanol and 0.1M KH₂PO₄ solution. During the assay, an aliquot of 20μl of sample was injected into the HPLC system at a flow rate of 1.5 ml/min. FLU concentration was quantitatively determined by dissolving liposomes in methanol and using external standards. MTO content in the formulation was determined by dissolving the liposomes in methanol and detected using spectrometry at 608 nm.

**Liposome preparation**

Liposomes composed of HSPC, Chol and mPEG₂₀₀₀-DSPE in a molar ratio of 57:40:3 were prepared by a modified thin-film hydration method. Briefly, lipids were dissolved in chloroform at indicated molar ratios. Solvent was evaporated to dryness under vacuum. The dry lipid film was then hydrated in 0.1M ammonium sulphate solution (pH 5.0) containing 10 mg/ml FLU. The resulting multilamellar vesicle suspension was frozen and thawed for five cycles and then subjected to extrusion under high pressure (500 psi) through two stacks of 0.2 and 0.1 μm polycarbonate filters (Whatman, Inc., Clifton, NJ) using a high-pressure extruder (Northern Lipids Inc., Vancouver, Canada). Free FLU was removed by dialysis against 300 fold phosphate buffer solution (pH 7.4) three times in 24 h. An aliquot of 100μl liposome sample was taken from inside the dialysis membrane and applied to Sepharose CL-4B column for measurement of free and encapsulated FLU. Fractions were collected and FLU concentration was analyzed by HPLC after dissolution of the liposomes using
100% methanol to determine the encapsulation efficiency. MTO solution was added into the dialysis-purified pre-made Lip-FLU at a ratio of 3:1 (FIT to MTO) and loaded at 65°C for 15min. The loading efficiency of MTO was higher than 95%, thus no further purification was applied after the loading. During hydration and extrusion, the temperature was kept above the phase transition temperature ($T_m$) of the lipids. Liposomes containing either FLU or MTO alone were also prepared as controls.

**Drug encapsulation efficiency analysis**

Drug encapsulation efficiency was determined by using size-exclusion chromatography on mini-columns made of Sepharose CL-4B (10ml). Aliquots of the sample (100μl) were loaded onto the column and fractions were collected. Drug concentrations of FLU and MTO in liposomal and free fractions were determined. Encapsulation efficiency of liposomes was calculated as follows:

$$\text{Encapsulation Efficiency} = \frac{\text{Drug}_{\text{liposome}}}{\text{Drug}_{\text{liposome}} + \text{Drug}_{\text{free}}} \times 100\%$$

**Transmission electron microscopy (TEM) and photon correlation spectroscopy analysis**

Liposomes were visualized by TEM after negative staining with uranyl acetate (UA). Briefly, liposomal samples were placed onto 100 mesh grids. Excess solution was wiped off with filter paper after 2 min and the grid was immersed in 2% UA. The UA solution was removed after 1 min, and the grids were washed twice with 200 μL distilled water. The grids were then imaged at 1000 kv on a Philips CM 12
Transmission Electron Microscope (Campus Microscopy and Imaging Facility, OSU, Columbus, OH). Photos were taken at 68,000 x magnification. The mean diameter and particle size distribution were determined using dynamic light scattering (DLS) technique with a Nicomp 370 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA). The laser in this equipment was operated at 632.8 nm at a 90° angle. Data were analyzed automatically by Gaussian Analysis from the Nicomp CW380 version software. Data were reported as volume weighted distribution and represented as mean of at least two measurements.

**In vitro drug release**

In vitro release of drugs from liposomal formulation was analyzed by membrane dialysis against phosphate buffer solution (PBS, pH 7.4) at 37°C. Briefly, aliquots (100μl each) of liposomal sample was first diluted with 400 μl phosphate buffer solution and then transferred into Float-A-Lyzer dialysis cassettes (molecular weight cutoff: 10K, Spectrum Laboratories, Inc.). The cassettes were suspended in temperature-controlled (37°C) flasks containing 300ml of PBS. At set time points, samples were taken from the liposomal samples and analyzed for drug concentrations. For each time point, one cassette was used.

**In vitro cytotoxicity study of liposomes**

B cell chronic lymphocytic leukemia (CLL) cell line, Wac3CD5, and B cell lymphoma cell line, Raji, were cultured in RPMI 1640 culture medium with glutamine supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a
humidified atmosphere containing 5% carbon dioxide. The modified MTT assay was used to assay the IC<sub>50</sub> of liposomes in these 2 cell lines. Briefly, cells harvested from exponential phase cultures were counted by trypan blue exclusion (only cell preparations demonstrating viability > 90% were used) and dispensed within 96-well flat-bottomed culture plates (3×10<sup>4</sup> cells/ 50μl per well for Raji and 6×10<sup>4</sup> cells/ 50μl per well for Wac3CD5). These cells were exposed to serial concentrations of the indicated drugs during culture. After 72 hours of incubation, 15μl of MTT reagent (5mg/ml in PBS) was added to each well for additional 4 h incubation. The blue MTT formazon precipitation was dissolved in 50μl of solution consisted of 10% SDS, 5% isopropanol, and 0.012N hydrochloride acid. The absorbance values at 550nm were determined on a multi-well plate reader. IC<sub>50</sub> was determined from cell viability versus log drug concentration plot using data from studies completed in triplicate.

**Analysis of drug interactions**

The drug interaction between fludarabine and mitoxantrone was analyzed by the “Combination Index” (CI) method\textsuperscript{46,47}. CI is defined as: 

\[
CI = \frac{C_{12} + C_{21}}{C_1 + C_2}
\]

where 

\(C_{12}\) and \(C_{21}\) are concentrations of drug 1 or drug 2 respectively in the combination to induce a defined effect (i.e. 50% cell death), and \(C_1\) and \(C_2\) are drug concentrations required to have the same effect when administered as single agent. In essence, the combination index CI is the ratio of the combination dose to the single-agent doses (isoeffective); consequently, CI<1 indicates synergy, CI=1 reflects additive activity, and CI>1 signifies antagonism.
Patient sample processing and primary cell culture

All the patients enrolled in this study had immunophenotypically defined B-CLL as outlined by the modified 1996 National Cancer Institute criteria. Blood was obtained from patients with informed consent under a protocol approved by the institutional review board. All of the B-CLL patients had been without prior therapy for a minimum of two months. B-CLL cells were isolated immediately following collection using Ficoll density gradient centrifugation (Ficoll-Paque Plus, Amershan Biosciences, Piscataway, NJ). Freshly isolated B-CLL cells were used for all experiments described herein. For those samples with less than 90% B cells, negative selection was applied to deplete non-B cells by “Rosette-Sep” kit from Stem Cell Technologies (Vancouver, British Columbia, Canada) according to manufacture suggested protocol. Isolated mononuclear cells were incubated 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.

Assessment of apoptosis by flow cytometry

Apoptosis of cells was measured by annexin V-FITC/propidium iodide (PI) staining with FACS analysis according to manufacture’s protocol (BD Pharmingen). Results are presented as % death = (% annexin V⁺ and/or PI⁺ cells of treatment group) – (% annexin V⁺ and/or PI⁺ cells of media control). FACS analysis was performed
using a Beckman-Coulter EPICS XL cytometer (Beckman-Coulter, Miami, FL). Ten thousand events were collected for each sample and data were acquired in list mode. System II software package (Beckman-Coulter) was used to analyze the data.

Results and discussion

Formulation development

Several formulations were investigated containing different phospholipids, lipid to drug ratios, charge components, and drug concentrations to optimize the passive loading procedure for FLU. Egg PC was initially used in our study but FLU was released significantly (>15%) from the liposomes when stored at 4°C for three months. In contrast, formulation containing HSPC was highly stable (<1% drug release) under the same condition. A drug-to-lipid ratio of 1:37 was used, which was able to retain ~20% FLU after the dialysis. Didodecyl-dimethyl-ammonium bromide (DDAB), a cationic lipid, was initially included in the formulation to improve the entrapment efficiency. However, there was no increase in the entrapment efficiency, and in fact precipitation was observed in the samples after 3 months, thus it was excluded from the final formulation. mPEG-DSPE was used to introduce steric hindrance, in order to increases liposome stability against aggregation, which was often a problem in working with uncharged lipid bilayers.

After the preparation of liposomal fludarabine (Lip-FLU), MTO was loaded to these pre-made liposomes with FLU encapsulated in the hydrophilic core. The ratio between FLU and MTO was controlled to be 1:3 (MTO:FLU) by adjusting the amount of MTO added to the pre-made Lip-FLU, which have defined FLU
concentration and encapsulation efficiency after the dialysis. The encapsulation of MTO was carried out by a transmembrane pH gradient-driven remote loading procedure. This resulted in very high efficiency (> 95%) as determined by Sepharose CL-4B gel filtration. Interestingly, probably due to its high hydrophilicity, no leakage of FLU was observed during the pH gradient loading procedure. Therefore, we did not conduct additional purification process to remove free MTO or FLU after the pH gradient loading procedure. Fig. A.1 illustrated that FLU was encapsulated in the inner aqueous cavity by passive loading. MTO was loaded into the liposomes by pH gradient generated across the lipid bilayer. We hypothesized that acidic compound FLU and basic compound MTO form a complex in the hydrophilic core that synchronizes the release of the two compounds. Electron microscopy was further applied to define the particle property. Small unilamellar vesicles with size around 150 nm were observed. (Fig. A.2) Interestingly, some crystalline structures (arrows) with altered electron-density were also noted inside some particles, which indicated possible formation of precipitation from FLU and MTO.

Upon optimization, a lead liposomal co-encapsulated FLU/MTO formulation was developed. This consisted of 2 mg/ml of FLU and 0.7 mg/ml of MTO, which was based on the clinical dose ratio of FLU and MTO in combination therapy. The lipid composition was HSPC: cholesterol: mPEG-DSPE (57:40:3, mol %). The final drug encapsulation ratios were 99% (FLU) and 98% (MTO) respectively, where free FLU was removed by dialysis and MTO was loaded with high efficient by a transmembrane pH gradient.
In vitro drug release

The in vitro release assay used was based on dialysis against a large volume of buffer. As shown in Fig.A.3, release of FLU and MTO from liposomes either loaded alone or in co-encapsulated form gave a sustained release profile. Difference was observed from the release kinetics of MTO between liposome formulations containing MTO alone and in co-encapsulated form. For liposomal formulation containing MTO alone, 12.7% and 24.6% drug were released from liposome at 2h and 72h, respectively. Meanwhile, for liposomes containing co-encapsulated FLU/MTO, MTO release was 27.0% and 39.5% at 2h and 72h, respectively, indicating that co-encapsulation accelerated MTO release from liposomes. A slower release profile of liposomes containing FLU in combination with MTO was also observed when compared to liposomes encapsulating FLU alone. Interestingly, it was found that after co-encapsulation of the 2 drugs in the same liposome, there were 32.1% FLU released at 2h and 38.8% FLU released at 72h, suggesting that the release rates of FLU from liposomes were similar to those of MTO. This result suggested that relatively constant ratio between FLU and MTO could be achieved for at least 72h.

The current design of loading procedure also restrains FLU in the inner water phase of liposome, due to its high hydrophilicity. Therefore, the release of FLU was largely dependent on lipids bilayer characteristics. For MTO, it was encapsulated through transmembrane pH gradient, and the compound could form insoluble precipitates within the liposome. The permeability characteristics of MTO in a precipitated form might be less dependent on membrane characteristics or the
presence of a residual transmembrane pH gradient, and thus a slower release profile could be achieved. The co-encapsulation could also allow complex formation between the 2 compounds within the inner water phase of liposomes, and alter the release profile of both compounds. This was demonstrated by the similar drug release rates of FLU and MTO observed in the combination liposome with an increased release of MTO and reduced release of FLU.

**Stability**

The long term stability of liposomes co-encapsulating FLU and MTO as a combination was monitored at 4°C during a period of 3 months. Mean particle size, pH and drug entrapment was used to examine the stability. Results obtained from our study indicated that, there were no significant changes in these parameters during the course of stability study. Both FLU and MTO release were less than 3% at 0.5, 1, 2, and 3 months. No significant change of pH was observed during the 3 month. Mean particle sizes of the FLU-MTO-Lip remained relatively constant (started at 142nm, after 3 month 155nm). This suggested that FLU-MTO-Lip could be potentially a stable formulation for further development.

**In vitro cytotoxicity study**

Liposomes encapsulating FLU and MTO in a combination or alone were exposed to B cell lines for 72h with a series of concentrations. In both Raji and Wac3CD5 cells, FLU-MTO-Lip was more cytotoxic than either drug in liposome alone. (Fig. A. 4) Of perhaps even more interest was the fact that considerably greater
cytotoxicity was observed than equivalent concentrations of either drug in liposome alone. In Raji cell lines (Fig. A. 4a), the IC$_{50}$ values of FLU-lip and MTO-Lip were 2.3 μM and 0.35 μM, respectively, while for FLU-MTO-Lip, the IC$_{50}$ was 0.59 μM (FLU) plus 0.16 μM (MTO). The same concentration of MTO in MTO-Lip could only reduce the viability by 23.8%, while FLU-Lip could barely inhibit the viability of cells. The similar results could be seen as well in the Wac3CD5 cell line (Fig. A. 4b). The IC$_{50}$ values were 102.4 μM for FLU-Lip, 0.092 μM for MTO-Lip and 0.159 μM (FLU) plus 0.043 μM (MTO) for the combination liposome. In contrast, 0.043 μM MTO-Lip could only inhibit about 35% viability of Wac3CD5 cells and 0.159 μM FLU-lip promoted only minimal cytotoxicity to this cell line.

We also evaluated in vitro cytotoxicity of drug combinations utilizing the median-effect analysis method, defined by the combination index (CI) value. CI of FLU-MTO-Lip was obtained to be 0.71 in Raji and 0.47 in Wac3CD5, respectively. Both values are less than 1, suggesting synergism between FLU and MTO in the combination liposomes. (Table A. 1)

**Apoptosis analysis**

The synergistic effect of FLU and MTO when encapsulated in liposomes was further demonstrated in apoptosis analysis using annexin V/propidium iodine staining in primary patient samples. Fresh separated CLL cells were treated with equivalent concentration of the two compounds either in monotherapy formulation or combination therapy formulation. Percentage of apoptotic cells was detected by annexin V/PI staining at 24 hr. Interestingly, FLU-MTO-Lip showed higher
percentage of cell death compared to Flu-Lip or MTO-Lip. Representative results from one of four patients (Fig.A.5) revealed that, FLU-MTO-Lip (56.1 %) induced higher level of death in CLL cells as compared to FLU-Lip (12.4%) or MTO-Lip (40.6 %).

Discussion

Synergism of FLU and MTO in free drug formulations has already been demonstrated by in vitro studies in CLL cells\textsuperscript{31,49}. The combination has also yielded a high response rate in phase I, II and III studies in patients with CLL or NHL.\textsuperscript{26-29} Indeed, this combination with cyclophosphamide\textsuperscript{20,22,25} and rituximab\textsuperscript{26,51} was also demonstrated to be beneficial in follicular lymphoma and mantle cell lymphoma, making it the combination therapy of choice in these diseases. Herein, we have utilized drug delivery technology to further extend this promising therapy by controlling the release profiles of FLU and MTO and allowing for potentially longer co-exposure times to both agents. The optimized liposomal formulation for co-encapsulation was developed and characterized. The fixed ratio between the two drugs was maintained for a sufficient long period (72 hr), so that synergism could be exerted. The 1:3 ratio was adopted based on previous clinical experience of the combined use of these two drugs. This might not have been the optimal ratio and concentration, considering the fact that liposomal drugs and free drugs may have different pharmacokinetics. Therefore, further optimization of the formulation by adjusting the drug ratio might be warranted in future in vivo therapeutic efficacy studies. Nevertheless, the current liposomal formulation co-encapsulating FLU and
MTO represent a design for chemotherapeutics combination, which has the unique characteristics of synchronizing the release kinetics for both drugs, but also may increase the tissue distribution of drugs in solid tumors through enhanced permeability and retention effect.

Combination therapy, especially in cancer treatment, is a rational strategy, to increase the response and tolerability. Anticancer drugs, by different cell killing mechanisms, are promising to ensure treatment effectiveness and safety if applied as appropriate combinations. However, current administration methods for combination chemotherapy regimens are typically developed by escalating individual agents to a maximum tolerated dose, which does not consider each drug has independent pharmacokinetic characteristics after administration \textit{in vivo}. The concept that pharmacological activities are highly dependent on concentration ratio has not yet been fully recognized by physicians in developing combination-based chemotherapy. Drug delivery system could potentially open an avenue different from that of conventional dose escalation studies, in order to improve the therapeutic effect. It is also interesting to note that the administration method of this sustained release formulation is also potentially more advantageous compared with free drug formulations, to avoid the prolonged infusion time in order to maintain the drug concentration above the minimal effective concentration for sufficient time. On the basis of these results, we propose that slow release drug carrier such as liposomes could be utilized as potential approach to address problems that exist in current combination chemotherapy regimen development in clinic. Further \textit{in vivo} studies in tumor bearing mouse model to investigate the pharmacokinetic properties, toxicity
profile, and therapeutic efficacy will be promising to translate this to eventual clinical investigation.

**Conclusion**

In this study, liposomes co-encapsulating FLU and MTO were prepared. Synchronized release of FLU and MTO from combination liposomes was observed. *In vitro* cytotoxicity showed FLU and MTO encapsulated in the same liposome could produce synergism in B cell lines and primary CLL cells. An interesting outcome of the present study was the possibility of co-encapsulating two different chemotherapeutic agents in the same liposomal formulation for lymphoproliferative disorder treatment. This could be a potential novel combined therapy for lymphoproliferative disorder including CLL and NHL.
LIST OF REFERENCES


Table A.1. *In vitro* cytotoxicity of liposomes

<table>
<thead>
<tr>
<th>IC₅₀(μM)</th>
<th>FLU-lip</th>
<th>MTO-lip</th>
<th>FLU-MTO-lip</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Raji</strong></td>
<td>2.3</td>
<td>0.35</td>
<td>0.16 (MTO) plus 0.59 (FLU)</td>
<td>0.71</td>
</tr>
<tr>
<td><strong>Wac3CD5</strong></td>
<td>102</td>
<td>0.092</td>
<td>0.043 (MTO) plus 0.16 (FLU)</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Raji or Wac3CD5 cells at indicated cell density as described in the methods section were incubated for 72h with various concentrations of FLU and MTO. Cell viability was measured by MTT assay, and was calculated based on the absorbance of media control. CI was calculated based on the equation: $CI = \frac{C_{12} \cdot C_{11}}{C_1 \cdot C_2}$
Figure A.1. Schematic illustration of FLU and MTO co-encapsulation in liposomes. FLU is first encapsulated into liposome through passive entrapment. In step 1, NH$_3$ diffuses outside of membrane during dialysis process. In step 2, MTO in deionized form diffuses into the liposomes and get entrapped by the H$^+$. Precipitation of FLU and MTO forms inside liposome upon loading which synchronizes the release of the two compounds.
Figure A. 2. EM analysis of FLU and MTO co-encapsulated in liposomes. Spherical particles with mean diameter less than 150nm were observed. Drug precipitation (arrows) was also observed inside the liposomes.
Figure A. 3. FLU and MTO release profile from liposomes. Release was determined at a 1:600 sink condition using dialysis membrane as described in the Methods. Percentage of release was calculated using the equation: 
\[
\%\text{Release} = \left( \frac{\text{Conc}_{\text{zero}} - \text{Conc}_t}{\text{Conc}_{\text{zero}}} \right) \times 100\%
\]
where Conc\(_{\text{zero}}\) is the drug concentration at initial time and Conc\(_t\) is the drug concentration at indicated incubation time.
A.

Figure A. 4. *In vitro* cytotoxicity study of liposomes coencapsulating FLU and MTO. Raji or Wac3CD5 cells at indicated cell density as described in the methods section were incubated for 72h with serial diluted concentrations of FLU and MTO in different formulations. Cell viability was measured by MTT assay, and calculated based on normalization using absorbance of media control. Results are summary from 3 experiments. A. Cytotoxicity in Raji cells at varying FLU conc. B. Cytotoxicity in Raji cells at varying MTO conc. C. Cytotoxicity in Wac3CD5 cells at varying FLU conc. D. Cytotoxicity in Wac3CD5 cells at varying MTO conc.
Figure A. 5. Apoptosis analysis of liposomal FLU/MTO in primary CLL cells. Purified B cells from patients were treated with FLU-Lip (2.1 μM), MTO-Lip (0.55 μM), or FLU-MTO-Lip (FLU: 2.1 μM, MTO: 0.55 μM) for 24 hr. % cell death indicates dead cell percentage over the media control, defined by Annexin V and/or PI positivity. Data is representative from 4 patients.
ESTABLISHMENT OF A CD52+ RAJI-BURKITT'S LYMPHOMA MOUSE MODEL FOR PRECLINICAL EVALUATION OF CD52-TARGETED IMMUNOTHERAPEUTIC AGENTS

Abstract:

CD52 is a glycosyl-phosphatidylinositol (GPI) anchored surface protein that has been targeted by humanized antibodies such as alemtuzumab (Campath 1-H). Clinical activity has been shown by these antibodies in depleting normal and malignant CD52+ B cells as well as T cells \textit{in vivo}. To date, efforts to study both mechanism of action and potential combination strategies with alemtuzumab have been limited due to the absence of stable expressing CD52 transformed B-cell lines and animal xenograft models. We herein describe generation of isolated Raji Burkitt’s lymphoma cell line clones that stably express high levels of CD52 \textit{in vitro} and \textit{in vivo}. \textit{In vivo} usefulness of the CD52\textsuperscript{high} cell line to evaluate the therapeutic efficacy of CD52 directed antibody was demonstrated using a SCID mouse model. Compared to control antibody, alemtuzumab (5mg/Kg) treatment in CD52\textsuperscript{high} inoculated mice resulted in significantly increased median survival (p=0.0005). Based on these findings, we conclude that the CD52\textsuperscript{high} Raji cell line and the disseminated leukemia-lymphoma mouse model described here can serve as an excellent system for \textit{in vitro} and \textit{in vivo} therapeutic and mechanistic evaluation of existing and novel antibodies directed against CD52 molecule.
Introduction

CD52 is a 21- to 28 KDa glycosyl-phosphatidylinositol (GPI) anchored structure surface glycoprotein \(^1\) that is highly expressed on all normal B and T lymphocytes, monocytes, macrophages, eosinophils, natural killer cells and dendritic cells \(^2\)-\(^5\). Hematopoietic stem cells, erythrocytes, plasma cells and platelets do not express this antigen \(^6\). Although the biological function of CD52 in these cells is elusive, recently a co-stimulatory role for CD52 in T cells contributing to the induction of regulatory T cells has been proposed \(^7\).

Corresponding to normal hematopoietic expression, the CD52 antigen is also expressed on varying subsets of tumor cells, especially T-prolymphocytic leukemia (T-PLL), hairy cell leukemia, multiple myeloma, non-Hodgkin lymphomas (NHL), acute lymphoid leukemia and B-chronic lymphocytic leukemia (CLL). \(^8\),\(^9\) CD52 has been shown to be expressed at higher density in leukemic B and T cells compared with normal T and B-lymphocytes and at least for CLL, the expression of the CD52 is virtually uniform on all tumor cells \(^9\).

Alemtuzumab (Campath 1-H) is a humanized IgG1 kappa antibody directed against CD52 that is remarkably effective at mediating depletion of both normal and transformed lymphocytes \textit{in vivo} \(^10\),\(^11\). Initially used in clinical practice in treatment of autoimmune disease, it was subsequently found to be highly effective in treating lymphoproliferative disorders such as B-CLL \(^12\)-\(^14\). Despite the extensive use in clinical trials, it is unclear how cross-linking CD52 by alemtuzumab mediates growth inhibition and apoptosis. Preclinical studies have demonstrated that alemtuzumab acts
mostly through immunological mechanisms, such as complement-mediated cytotoxicity (CDC)\textsuperscript{15-17} and/or antibody-dependent cellular cytotoxicity (ADCC)\textsuperscript{18-20} by virtue of its IgG1 Fc region. Two recent studies have shown that this agent induced enhanced apoptosis in primary CLL cells \textit{in vitro}, alone or in combination with a cross-linking anti-Fc antibody, in absence of complement or immune effector cells, through a non-classic caspase-independent pathway\textsuperscript{21,22}. There is also evidence to suggest that alemtuzumab may trigger caspase-dependent cell death in CLL cells\textsuperscript{23-24}, as well as B-lymphoid Wien 133\textsuperscript{25} and Ramos cell lines\textsuperscript{22}. Contrasting with these, another study has shown that alemtuzumab alone did not induce apoptosis in serum-free medium\textsuperscript{15}.

Detailed analysis of \textit{in vivo} and \textit{in vitro} mechanistic studies to elucidate CD52-mediated killing of malignant B cells and testing of novel combination strategies have been limited due to the absence of valuable cell lines and animal models. Although many of \textit{in vitro} maintained lymphoid-derived tumor cells express CD52, the levels of expression is very low and the stability of the expression is unpredictable. To overcome this and to develop a model system to evaluate the CD52 directed antibody reagents, we describe isolation and evaluation of a Raji Burkitt’s lymphoma B cell line that stably expresses high level of CD52 (CD52\textsuperscript{high} Raji) \textit{in vitro} and \textit{in vivo}. The functional integrity of the expressed CD52 molecule was demonstrated using alemtuzumab, which induced cytotoxic effects in the CD52\textsuperscript{high} Raji clone. \textit{In vivo} usefulness for the CD52\textsuperscript{high} cell line to evaluate the therapeutic efficacy of CD52 directed antibody is demonstrated using a xenograft SCID mouse model of disseminated leukemia/lymphoma.
Material and Methods

Analysis of direct cytotoxicity: Cells (1x10^6 cells/mL) were treated with alemtuzumab, rituximab or trastuzumab at a concentration of 10 μg/mL. The cross-linker, goat anti-human IgG (Fc specific; Jackson ImmunoResearch Laboratories, West Grove, PA), was added to the cell suspension 5 min after adding the primary antibodies at a concentration of 50 μg/mL. In addition, a group of samples with no treatment was collected as media control. The apoptosis of cells 24 and 48 hours post treatment was measured using annexin V-FITC/propidium iodide (PI) staining followed by FACS analysis according to the supplier's instructions (BD Biosciences). Results were analyzed with percentage (%) of total positive cells over media control = (% annexin-V and/or PI positive cells of treatment cell sample) – (% total annexin-V and/or PI positive cells of media control). FACS analysis was performed using a EPICS XL cytometer (Beckman-Coulter).

Development of a disseminated leukemia-lymphoma model using Raji cell clones. Female C.B.-17 SCID mice (Taconic Farm, Germantown, NY), 4-6 weeks of age were housed in pathogen-free, isolated cages. Raji cell clones frozen in cryovials (1x10^7 cells each) and stored in liquid nitrogen (-180°C) were thawed 10 days before the in vivo engraftment. To ensure the consistency of engraftment, cells were examined for the viability, CD52/CD19/CD20 expression and in vitro sensitivity to antibody treatments on the same day of inoculation. Only cells with > 90% viability were used for injection. Cells were resuspended in 0.9% sodium chloride injection
(USP, Hospira, Inc., Lake Forest, IL) at a density of $10^7$ cells/mL at room temperature, and 200μL (2x10⁶ cells) were inoculated through tail vein using a mouse tail illuminator (Braintree Scientific, Braintree, MA). Tissue samples obtained from tumor-bearing SCID mice that showed early signs of paralysis were submitted to OSU Pathology Core Facility for histological analysis. The presence of disease was confirmed by presence of human leukemic cells in the tissue sections. Bone marrow cells (1x10⁶ cells/mL) flushed from femurs with cold PBS within 0-3 days after hind limb paralysis were stained with PE-labeled anti hCD52 and FITC-labeled anti hCD19 to confirm the existence of human leukemia cells. Antibody treatment was started 3 days post inoculation of target Raji cells. Alemtuzumab dissolved in PBS 1mg/mL were injected via tail vein and maintained every other day i.v. schedule for 2 weeks (5mg/kg/injection, 7 injections each mouse). Isotype control antibody (trastuzumab) was administered at the same schedule and dose. Animals were monitored daily for sign of illness and sacrificed immediately if hind limb paralysis, respiratory distress or 30% body weight loss was noted. Body weight was measured once every week. Survival time (paralysis time) was used as primary endpoint for evaluation.

**Statistical analysis of data:** All the analysis was performed by statisticians in Center for Biostatistics, the Ohio State University. Mixed effects models were fitted to the cytotoxicity data. Kaplan-Meier estimates of the median survival times for each treatment engraftment combination were presented with 95% confidence intervals. A Cox proportional hazards model was applied to the data with the factors treatment,
engraftment, and their interaction in the model. A significance level of \( a = 0.05 \) was used for all tests. SAS software version 9.1, (SAS Institute Inc., Cary, NC) was used for all the statistical analysis.

RESULTS

Cell surface expression of CD52 in Raji clones.

The Raji Burkitt’s lymphoma cell line expresses high levels of surface CD52 only in a small proportion of cells (40\% \pm 5\%) that appears to diminish with serial culturing. By limiting dilution cloning, we have established clones that stably express high or low level of surface CD52. Cells of different clones were collected and analyzed for different surface protein expression by immunostaining. The immunophenotypic characteristics of the original Raji Burkitt’s lymphoma cell line (parental cell line) compared with 2 selected clones expressing high or low levels of CD52 (CD52\textsuperscript{high} and CD52\textsuperscript{low} Raji clones) are reported Figure B.1.

Direct cytotoxicity

In order to determine if CD52\textsuperscript{high} Raji clone exhibited increased sensitivity to alemtuzumab-mediated direct cytotoxicity, parental, CD52\textsuperscript{high} and CD52\textsuperscript{low} Raji clones were subjected to alemtuzumab or trastuzumab treatment in media without plasma or effector cells. Alemtuzumab treatment of CD52\textsuperscript{high} Raji clone resulted in 64\% \pm 1.3\% cell death compared to 6.2 \% \pm 0.6\% and 8.5\% \pm 0.4\% respectively in parental and CD52\textsuperscript{low} Raji clones after 24h of treatment, in presence of a secondary cross-linking antibody (\( P <.0001 \)). (Fig. B.2). A significant synergy with crosslinking
for CD52\textsuperscript{high} vs. CD52\textsuperscript{low} was found. Specifically, without crosslinking, the average %
cytotoxicity for CD52\textsuperscript{high} was 16.4\% higher than for CD52\textsuperscript{low} and with the addition of
crosslinking, the difference between CD52\textsuperscript{high} and CD52\textsuperscript{low} increased by 46\%.
Extended incubation up to 48h resulted in a similar trend (data not shown).

\textit{In vivo applicability of CD52\textsuperscript{high} for evaluation of CD52 targeted therapy}

In order to evaluate the expression stability of CD52 molecules and the
usability of the Raji variants for \textit{in vivo} evaluation of CD52 targeted reagents, we
established xenograft leukemia/lymphoma SCID mouse model using CD52\textsuperscript{high} and
CD52\textsuperscript{low} Raji clone cells. Inoculation of each of the cell lines demonstrated
tumorigenic activity in mice characterized by symptomatic metastasis at multiple
sites, including central nervous system, liver and lung, resulting in a progressive body
weight loss as well as hind limb paralysis followed by death of nearly 100\% mice
from massive tumor burden 17-30 days post inoculation. Mice inoculated with
CD52\textsuperscript{high} and CD52\textsuperscript{low} Raji cells were randomly divided into 3 groups and treated with
alemtuzumab, trastuzumab or saline solution at an early stage of disease, i.e. on day 3
after cell inoculation. All the CD52\textsuperscript{low} Raji cells inoculated mice treated with saline
(data not shown) or trastuzumab died within 17-20 days (median survival times 20
days) whereas >93\% of the CD52\textsuperscript{high} Raji cells inoculated mice receiving the same
treatment died within 20-40 days (median survival times 34 days) (Figure B.3). No
difference were found between trastuzumab and alemtuzumab treated groups in
CD52\textsuperscript{low} Raji clones inoculated mice, which further indicated the lack of CD52
expression in these cells. Mice inoculated with CD52\textsuperscript{high} Raji clones and treated with
alemtuzumab survived more than 80 days longer (note that only 29\% of the CD52\textsuperscript{high}
Raji mice given alemtuzumab died by the end of the study, so we cannot provide an estimate of the median survival time for that group) than mice inoculated with CD52\text{low}\ Raji clones receiving the same treatment (p = 0.0024). Tissue obtained from sacrificed trastuzumab-treated tumor-bearing SCID mice showed massive distribution of neoplastic cells in multiple sites including liver, kidney, mesenteric and tracheobronchial lymph node and spinal cord (Fig. B.4.A.). Corresponding tissue obtained from alemtuzumab-treated CD52\text{high}\-inoculated mice showed absence of neoplastic cells except for spinal cord where a few residual neoplastic cells (Fig. B.4.B.) were observed in the meninges compared to control mice.

Bone marrow obtained within 0-3 days of hindlimb paralysis from trastuzumab treated mice which had received CD52\text{high}\ Raji cell inoculation had a 10% CD19\text{+}/CD52\text{+}\ cell population (Fig. B.4.C.). In contrast, no CD19\text{+}/CD52\text{+}\ cell population (<0.5%) was observed in alemtuzumab treated CD52\text{high}\ Raji inoculated mice (Fig. B.4.D.). Analysis of bone marrow cells from similarly treated CD52\text{low}\ Raji clone inoculated mice exhibited presence of human CD19\text{+}/CD52\text{−}\ cells (data not shown).

**DISCUSSION**

Herein we have established Raji cell line models expressing high or low levels of CD52 useful for evaluating both \textit{in vitro} and \textit{in vivo} effects of CD52 targeted antibody reagents such as alemtuzumab. By limiting dilution of the Raji Burkitt’s lymphoma cell line, which expresses high levels of CD52 only in a small percentage
of cells (43%), we isolated clones expressing either high or low levels of CD52 in more than 90% of the cell population and comparable high level of HLA-DR, CD19, CD40, CD32 and CD20. The pattern of surface expression of CD52 as well as other GPI linked protein was found stable with continuous cell culture over several months. The data clearly show that alemtuzumab as well as rituximab can induce in vitro cell lysis in CD52\textsuperscript{high} Raji cells. Furthermore these cells are sensitive to complement or antibody mediated cytotoxicity. In addition, either CD52 or CD20 targeted direct-antibodies induced direct cytotoxicity in the presence of a cross-linking antibody. Clearly, the CD52 density was important in alemtuzumab mediated killing as it was able to induce cytotoxic effects in vitro on the CD52\textsuperscript{high} clone but not on the parental and CD52\textsuperscript{low} Raji clone. Alemtuzumab treatment in CD52\textsuperscript{high} inoculated mice increased significantly the median survival (71% of the mice were still alive by the end of the study). Human-CD19\textsuperscript{+}/CD52\textsuperscript{+} cells were found in bone marrow from trastuzumab- but not alemtuzumab-treated CD52\textsuperscript{high} inoculated mice, after 24 days in vivo. Consistent with the elimination of alemtuzumab targeted cells, no sign of hCD19\textsuperscript{+}/CD52\textsuperscript{+} cells were found in bone marrow of similarly treated CD52\textsuperscript{low} inoculated mice.

Alemtuzumab has been proved very effective in eliminating CD52\textsuperscript{+} cells and is currently used to target leukemic B and T cells as well as in immunosuppressive treatment\textsuperscript{40-43}. Despite large employment of alemtuzumab in clinical trials, the mechanism of its action remains to be clarified due mostly to the lack of reproducible model cell line and animal models to study the in vitro and in vivo effect of this monoclonal antibody. In fact, many of in vitro maintained lymphoid-derived tumor
cells only express CD52 at very low levels \(^1\) and the stability of the molecule in vivo is less predictable. Recently a xenotransplant model of multiple myeloma (MM) in NOD/SCID mice using KMS-11 cells has been developed to investigate the in vivo activity of alemtuzumab. In this experimental condition, probably due to the low expression of CD52 on KMS-11 cells, alemtuzumab treatment initiated at an early stage of disease substantially induced a delay in the in vivo growth of myeloma cells rather than eradicate the disease \(^{44}\).

The Raji Burkitt’s lymphoma cell clones expressing high levels of CD52 stably both in vitro and in vivo will overcome the limitations of previously described Raji-xenograft models that showed drastic modulation of the expression of the CD52 molecules in vivo. The stable CD52\(^{\text{high}}\) B cell lines and the disseminated leukemia-lymphoma mouse model described here can serve as an excellent system for in vitro and in vivo therapeutic and mechanistic evaluation of existing and novel antibodies directed against CD52 molecule.
LIST OF REFERENCES


Figure B.1. Expression of CD52 and CD20 on Raji clones. Cells from parental, CD52$_{\text{low}}$ and CD52$_{\text{high}}$ Raji clones were stained with PE-labeled anti CD52 (A), CD20 (B) or isotype matched antibodies and analyzed on FACS. Shaded histogram profile indicates the isotype control and open histogram indicates the specific antibody.
Figure B.2. Alemtuzumab mediated cytotoxicity. Direct cytotoxicity determined by annexin-V/PI staining after 24 h. For alemtuzumab, there was significant synergy with crosslinking for CD52\textsuperscript{high} vs. CD52\textsuperscript{low} (P < 0.0001). White bars: Parental Raji cells; Gray bars: CD52\textsuperscript{high} Raji clone; Dark bars: CD52\textsuperscript{low} Raji clone.
Figure B.3. *In vivo* therapeutic efficacy of alemtuzumab towards CD52+ Raji cells. SCID mice were inoculated with CD52^{high} (N=29) or CD52^{low} (19) Raji cells and treated with alemtuzumab (N=14 and N=10 respectively) in parallel with trastuzumab (N=15 and N=9 respectively). A Cox model was applied to the data with the factors treatment, engraftment, and their interaction in the model (P < 0.0001 for engraftment $\times$ treatment interaction between CD52^{high} and CD52^{low} alemtuzumab treated mice; p = 0.0005 between CD52^{high} alemtuzumab and trastuzumab treated mice)
Figure B.4. CD52+ cells in bone marrow. Neoplastic cells in spinal cord of (A) a trastuzumab treated CD52\textsuperscript{high} inoculated mouse and (B) alemtuzumab treated CD52\textsuperscript{high} inoculated mouse with only a few neoplastic cells in the meninges (arrow). (C) Expression of human CD52 in mouse bone marrow cells from CD52\textsuperscript{high}-inoculated mice treated with trastuzumab (left panel) or alemtuzumab (right panel).
A NOVEL PROCESSING METHOD FOR FR-TARGETED LIPOSOMAL DOXORUBICIN PRODUCTION

The large-scale manufacture of the liposomal vesicles involves complex processes that are difficult to optimize, presenting a challenge to the industry. Development of large scale production methods is, therefore, critical for the translation of folate liposomal doxorubicin (FLD) from the bench to the bedside. A novel processing method combining solvent exchange, tangential flow diafiltration, high-pressure homogenization and remote loading for the reliable and scalable production of FLD was designed and optimized. Production of sterile homogeneous small unilamellar liposomal suspensions at a relevant scale for clinical studies was validated. Reproducible production of sterile homogeneous small unilamellar liposomal suspensions, with defined particle size of 120nm ± 30nm and encapsulation efficiency > 95%, at batch of 600 mL each, was validated. This method provides the basic feasibility of targeted liposome product for potential clinical application.