M.Sc. Thesis

Auger Electron-Emitting Radioimmunotherapeutic (RIT) Agent Specific for Leukemic Stem Cells

By

Jin Hua Gao

A THESIS SUBMITTED IN CONFORMITY WITH THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCES

Graduate Department of Pharmaceutical Sciences
University of Toronto

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Abstract

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Objective: CSL360 is a chimeric IgG1 mAb recognizing CD123+ /CD131 - LSCs responsible for acute myeloid leukemia (AML). The in vitro targeting properties of 111In-labeled CSL360 modified with nuclear localization sequence (NLS) were evaluated in AML cells. Methods: 111In-NLS-CSL360 was constructed and its binding affinity, cellular uptake and nuclear importation were analyzed on CD123 + cells. Cytotoxicity was evaluated by clonogenic assays on AML cells (CD123 +/CD131 -). Results: 111In-NLS-CSL360 exhibited preserved binding to CD123. High cellular and nuclear uptake was observed at 266 nM after 24 hour of incubation. Nuclear uptake of 111In-NLS-CSL360 (266 nM) was 2.0-fold higher than 111In-CSL360 (266 nM) after 24 hour of incubation. Clonogenic survival (CS) of AML cells was reduced to 27.5 ± 4.1%. The nuclear uptake and cytotoxicity were reduced when pre-exposed to unlabeled CSL360, indicating 111In-NLS-CSL360 was CD123-specific. Conclusion: 111In-NLS-CSL360 could be a promising radioimmunotherapeutic agent specific for LSCs.
Acknowledgements

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<tr>
<td>$^{111}$In-NLS-CSL360</td>
<td>$^{111}$In-labeled CSL360 modified with Nuclear Localization Sequences</td>
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<td>$^{111}$In-NLS-ChIgG$_1$</td>
<td>$^{111}$In-labeled ChIgG$_1$ modified with Nuclear Localization Sequences</td>
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<tr>
<td>$^{111}$In-NLS-7G3</td>
<td>$^{111}$In-labeled 7G3 modified with Nuclear Localization Sequences</td>
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<tr>
<td>$^{111}$In-NLS-BM4</td>
<td>$^{111}$In-labeled BM4 modified with Nuclear Localization Sequences</td>
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<td>“7+3” regimen</td>
<td>Intensive myelosuppressing standard-dose cytarabine (100 to 200 mg/m$^2$) administered by a continuous infusion for 7 days and combined with daunorubicin, an anthracycline (45 to 60 mg/m$^2$) administered intravenously for 3 days</td>
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<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antigen Dependent Cell-mediated Cytotoxicity</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute Lymphoblastic Leukemia</td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic Lymphoma Kinase</td>
</tr>
<tr>
<td>AlloSCT</td>
<td>Allogeneic Stem Cell Transplantation</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myeloid Leukemia</td>
</tr>
<tr>
<td>APL</td>
<td>Acute Promyelocytic Leukemia</td>
</tr>
<tr>
<td>ASCT</td>
<td>Autologous Stem Cell Transplantation</td>
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<tr>
<td>Bs-scFv</td>
<td>Bispecific Single Chain Fv fragments</td>
</tr>
<tr>
<td>BCRP$_1$</td>
<td>Breast Cancer Resistance Protein</td>
</tr>
<tr>
<td>C$_L$</td>
<td>Constant domain of Light chain</td>
</tr>
<tr>
<td>C$_H$</td>
<td>Constant domain of Heavy chain</td>
</tr>
<tr>
<td>CBF</td>
<td>Core Binding Factor</td>
</tr>
<tr>
<td>CBEP_A</td>
<td>CCAAT/Enhancer binding Protein a</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity Determining Regions</td>
</tr>
<tr>
<td>CDC</td>
<td>Complementary Dependent Cytotoxicity</td>
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<tr>
<td>CFU-S</td>
<td>Spleen Colony-Forming Unit</td>
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<tr>
<td>CHX-A”</td>
<td>2-(p-isothiocyanato-benzyl)-CycloHexyl</td>
</tr>
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<td>C-kit</td>
<td>Receptor for SCF, the stem cell factor</td>
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<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
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<tr>
<td>CLL-1</td>
<td>C-type Lectin Like molecule 1</td>
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<td>CML</td>
<td>Chronic Myeloid Leukemia</td>
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<tr>
<td>CR</td>
<td>Complete Remission</td>
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<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
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<tr>
<td>CXCR4</td>
<td>CXC-chemokine Receptor 4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria Toxin</td>
</tr>
<tr>
<td>DT_{388}IL3</td>
<td>Fusion protein made of the first 388 amino acid residues of diphtheria toxin catalytic and translocation domains (DT_{388}) fused to human IL-3</td>
</tr>
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<td>DTPA</td>
<td>Diethylenetriaminepentaacetic Acid</td>
</tr>
<tr>
<td>FAB</td>
<td>French-American-British classification of AML</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FLT3</td>
<td>Fms-Like Tyrosine Kinase 3</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GO</td>
<td>Gemtuzumab Ozogamicin</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-Triphosphate</td>
</tr>
<tr>
<td>GVL</td>
<td>Graft-Versus-Leukemia</td>
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<tr>
<td>HAMA</td>
<td>Human Anti-Mouse Antibodies</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal growth factor Receptor-2</td>
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<tr>
<td>HD AraC</td>
<td>High-Dose Cytarabine</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<tr>
<td>HOX</td>
<td>Homeobox</td>
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<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cells</td>
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<td>HSCT</td>
<td>Hematopoietic Stem Cell Transplantation</td>
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<tr>
<td>HuM195</td>
<td>Humanized anti-CD33 mAb</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulins</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulins Gamma</td>
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<tr>
<td>IL-3</td>
<td>Interleukin 3</td>
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<tr>
<td>IL-5</td>
<td>Interleukin 5</td>
</tr>
<tr>
<td>ITD</td>
<td>Internal Tandem Duplications</td>
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<td>ITLC-SG</td>
<td>Instant Thin-Layer Silica Gel Chromatography</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>IUdR</td>
<td>5-ido-2’-deoxyuridine</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>K_{d}</td>
<td>Equilibrium Dissociation Constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LSC</td>
<td>Leukemic Stem Cell</td>
</tr>
<tr>
<td>Lin</td>
<td>Markers for differentiated hematopoietic Lineages</td>
</tr>
<tr>
<td>LET</td>
<td>Linear Energy Transfer</td>
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<td>M195</td>
<td>Murine anti-CD33 mAb</td>
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</table>
mAb  Monoclonal antibody
MAPK  Mitogen-Activated Protein Kinase
MDR$_1$  Multidrug Resistance phenotype
MDS  Myelodysplasia
MG-132  Carbobenzoxy-L-Leucyl-L-Leucyl-L-Leucinal
MLF1  Myeloid Leukemia Factor 1
MRC  Medical Research Council
MRD  Minimal Residual Disease
NF-κβ  Nuclear Factor κB
NHL  Non-Hodgkin’s Lymphoma
NK  Natural Killer
NLS  Nuclear Localization Sequences
NOD/SCID  Nonobese Diabetic/Severe Combined Immunodeficiency
OR  Overall Response
ORR  Objective Response Rate
OS  Overall Survival
PAGE  Polyacrylamide Gel Electrophoresis
PBS  Phosphate-Buffered Saline
PDGF  Platelet-Derived Growth Factors
PE38  a 38 kDa fragment of Pseudomonas Exotoxin A
PET  Positron Emission Tomography
PFS  Progress Free Survival
PgP  Permeability glycoprotein
PI3  Phosphatidylinositol 3-kinase
PR  Partial Remission
QR-PCR  Quantitative Reverse transcriptase Polymerase Chain Reaction
RARα  Retinoic Acid Receptor α
RCHOP  combination chemotherapy of Rituximab, Cyclophosphamide, Hydroxydaunorubicin, Oncovin, and Prednisone
RNA  Ribonucleic Acid
RIT  Radioimmunotherapy
RTK  Receptor Tyrosine Kinase
s.c.  Subcutaneous
SCF  Stem Cell Factor
ScFv  Single Chain Fv Fragments
SCID  Severe Combined Immunodeficiency
SD  Standard Deviation
SDAraC  Standard Dose of Cytarabine
SDF1  Stromal cell-Derived Factor-1
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfonate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SGN-33</td>
<td>Sialoglycoprotein 33</td>
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<td>SIRPα</td>
<td>Signal Regulatory Protein α</td>
</tr>
<tr>
<td>SL-IC</td>
<td>SCID Leukemia-Initiating Cells</td>
</tr>
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<td>SMCC</td>
<td>Sulfosuccinimidyl-4-(N-maleimidomethyl) Cyclohexane-1-Carboxylate</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single Photo Emission Computed Tomography</td>
</tr>
<tr>
<td>SRC</td>
<td>SCID-Repopulating Cells</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer Activator of Transcription</td>
</tr>
<tr>
<td>Sv-40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td>TBI</td>
<td>Total Body Irradiation</td>
</tr>
<tr>
<td>TRM</td>
<td>Treatment-Related Mortality</td>
</tr>
<tr>
<td>TTP</td>
<td>Time To Progression</td>
</tr>
<tr>
<td>V\textsubscript{H}</td>
<td>Variable domain of Heavy chain</td>
</tr>
<tr>
<td>V\textsubscript{L}</td>
<td>Variable domain of Light chain</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Chapter 1

Introduction
1.1 The Incidence, Diagnosis, Prognosis, and Treatment of AML

1.1.1 Epidemiology and Etiology

Acute Myeloid Leukemia (AML) is a subdivision of a range of cancers known as leukemias. The word leukemia is derived from the Greek words \textit{leukos} meaning white and \textit{haima} meaning blood (1). Leukemia is a disorder or cancer of the blood producing cells of the bone marrow that is characterized by an unusual increase of abnormal white blood cells. The other major subdivisions are: acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML) (2). AML refers to the disorder or cancer arising in the immature myeloid cell compartment. In adults, AML accounts for \~30\% of all leukemias. AML generally occurs in early childhood (less than 1 year) and later adulthood (older than 65 years) (3). In 2011, an estimated 44,600 new cases of leukemia will be diagnosed in the US (2), and 4,800 new cases in Canada (4). It has been estimated that 12,950 and 1,114 respectively of these new cases will be AML. The median age of newly diagnosed cases is 65 years and the highest incidence (23.0 per 100,000 persons) occurs in people older than 80 years (5). AML is therefore considered to be a disease of later adulthood. The awareness of AML has increased and the incidence of this disease is expected to further rise due to our aging population.

Despite the fact that AML is a rare disease that accounts for less than 1.2\% of all cancers, it continues to affect the overall cancer survival statistics as it is responsible for a large number of cancer-related deaths (5). According to the Leukemia and Lymphoma Society, approximately 21,840 patients died of leukemia in the US in 2010 and an estimated 8,950 of these deaths were attributed to AML (3). In children and adolescents younger than 19 years old, acute leukemia is the leading cause of death due to cancer, accounting for 65\% of the deaths in pediatric cancers (6). In spite of treatment with standard chemotherapy, the relative survival rate for AML is still
the lowest among all types of adult leukemia but varies greatly among age groups. The most recent data in 2007 reported that the five-year survival rate for AML was 24.2% in adults of all ages. However, it ranged from ~40.0% for patients aged 18-60 years, to <10% for patients older than 65 years (2). With the increasing incidence and high mortality rate of AML, new therapeutic approaches are in great need to improve the treatment of AML patients and to eventually eradicate the disease.

1.1.2 Pathogenesis

AML is characterized by clonal growth from a cancerous initiating cell that confers a proliferative and survival advantage and impairs differentiation in normal hematopoiesis. Hematopoiesis originates from hematopoietic stem cells (HSCs) capable of reproducing themselves through a process known as “self-renewal” and producing a hierarchy of downstream multilineage progenitor cells. These HSCs give rise to myeloid and lymphoid progenitor cells. The myeloid lineage further differentiates to all myeloid cells: erythrocytes, granulocytes, monocytes and platelets; whereas the lymphoid lineage differentiates to natural killer cells, T-lymphocytes and B-lymphocytes (7). The process of differentiation and proliferation of myeloid lineage is tightly controlled and regulated by a set of early and lineage-specific growth factors and their receptors, as well as a network of downstream signaling machinery. Examples of these receptors are receptors with intrinsic tyrosine kinase activity (RTK). The most studied RTKs in progenitor cells belong to the type III family, which include the PDGF (platelet-derived growth factors) receptor (PDGFR α and β), c-Fms/M-CSF (macrophage colony stimulating factor) receptor, FLT3 (Fms-like tyrosine kinase3), and c-Kit (receptor for SCF, the stem cell factor) (8). These growth factor receptors activate a network of similar intracellular signaling pathways which
have been shown to be important in myeloid differentiation, proliferation and survival. These downstream signaling pathways include the Ras/MAPK (mitogen-activated protein kinase), the PI3K (phosphatidylinositol 3 kinase)/Akt, and the JAK (Janus kinase)/STAT (signal transducer activator of transcription) signaling pathways (6). In addition, myeloid transcription factors regulate the expression of a cell type-specific pattern of genes that are required to direct the HSCs and early progenitors to fully differentiated cells of various myeloid lineages (8). Taken together, normal myelopoiesis is maintained through the network of the myeloid-specific growth factor, the corresponding receptor, the main signaling pathways involved, and the transcription factors governing myeloid differentiation. Given this reason, it is not surprising that activating mutations in any part of the network could cause subtle disruption of the normal myeloid differentiation and contribute to the pathogenesis of AML.

The current accepted theory of leukemogenesis is based on the cancer stem theory, which proposes that leukemia arises from a subpopulation of AML cells called leukemic stem cells (LSCs) (9). It is generally believed that leukemogenesis involves the acquisition of a series of genetic alterations, which ultimately convert a HSC into a LSC capable of propagating the disease clone. These alterations result in the generation of a highly proliferative clone of immature leukemic blast cells with intrinsic survival and proliferation advantage (10). Although the existence of LSCs is fundamentally an experimental concept, the clinical significance of LSCs has been well recognized and will be discussed later in this chapter.

At a molecular level, leukemic transformation is characterized by recurring chromosomal aberrations and genetic or epigenetic mutations. AML results when hematopoietic cells acquire two types of genetic mutations. Type 1 involves gene mutations that confer a proliferative and survival advantage to hematopoietic progenitors but do not affect cell differentiation; while Type
2 involves gene rearrangements resulting in the generation of chimeric fusion proteins and transcription factors that impair differentiation and apoptosis (11). A number of clinically relevant gene mutations have been recently identified in AML and these include: NPM (nucleophosmin) mutant, FLT3-ITDs (FLT3-internal tandem duplications), RUNX1/CBFβ (a heterodimeric transcriptional regulator) mutations and CEBPA (CCAAT/enhancer binding protein α) gene mutations (8).

More than 85% of AML patients are diagnosed with genetic alterations, and the most frequent are NPM mutations that occur in 55% of all normal karyotype cases (6). NPM is a multifunctional protein initially characterized as a nucleolar protein functioning in the ribosomal ribonucleic acid (RNA) and protein processing and transport (12). However, NPM exon 12 mutations alter the NPM protein at the C-terminus causing its aberrant cytoplasmic localization and its altered function in nucleo-cytoplasmic transport, which is linked to leukemia development (13). Moreover, the NPM gene fuses with other gene partners to generate chimeric proteins which are thought to also play a role in leukemogenesis. These fusion proteins are namely NPM-ALK (anaplastic lymphoma kinase), NPM-RARα (retinoic acid receptor α), and NPM-MLF1 (myeloid leukemia factor 1) (14). NPM appears to contribute to leukemogenesis by activating the oncogenic potential of the fused protein partners (ALK, RARα, and MLF1). Following the NPM mutations, FLT3-ITD is the second most characterized genetic mutation associated with AML. Clinical studies have identified the FLT3-ITD mutations in 17%-26% of AML cases and the aberrantly activated FLT3 pathway is observed in about 30% of patients in AML (15). In normal hematopoiesis, FLT3 has important functions in the recruitment of early hematopoietic progenitors to the B-cell, granulocytic and monocytic lineages (16). Internal tandem duplications (ITD) of varying lengths resulted in the repetition of a stretch from 4 to up to 50 amino acids in
the juxtamembrane region of the FLT3 receptor. FLT3-ITDs cause ligand-independent dimerization and constitutive autophosphorylation of FLT3 receptor, leading to constitutive activation of many downstream effectors (Ras, AKT, and STAT) (17). The biological consequences of autophosphorylation of FLT3 could be factor-independent growth and survival in myeloid cell lines and self-renewal of leukemic progenitor cells (17-19).

Besides genetic mutations, epigenetic alterations are another important factor in the process of AML development. These epigenetic alterations result in a loss of gene function but do not modify the deoxyribonucleic acid (DNA) coding sequence and can be reversed pharmacologically. One example is the aberrant DNA methylation in the leukemia genome (20). Hypermethylation inactivates gene transcription and disrupts the well-established function in cell cycle control, apoptosis, and DNA repair.

AML could also arise from many other factors, including age related antecedent hematologic disease, myelodysplastic syndrome (also known as secondary AML), as well as exposure to radiation, chemical, or viruses and other occupational hazards (21). Patients may develop treatment-related AML after therapeutic radiation and chemotherapeutic agents, especially when treated with alkylating agents that inhibit DNA repair enzymes, or damage bone marrow (22).

### 1.1.3 Clinical Diagnosis

Leukemogenesis results in rapid growth of abnormal white blood cells (blasts) that accumulate in the bone marrow and interfere with the production of other normal blood cells. Patients who develop leukemia may have common symptoms including fatigue, bruising or bleeding, fever, and infection. The clinical diagnosis of AML is made by a cumulative evaluation
and decision based on complete blood cell counts and subsequent bone marrow aspiration and biopsy evaluating the percentage of AML blasts relative to normal white blood cells (23). According to the World Health Organization (WHO), acute leukemia is diagnosed when a 200-cell differential reveals the presence of 20% or more blasts in the blood and/or bone marrow aspirate (24).

Immunophenotyping can also be used to confirm myeloid lineage by the presence of surface antigens, including CD13, CD33, C-KIT, CD14, CD64, Glycophorin A, and CD41. Microscopic, histochemical and cell-surface phenotype all aid in the identification of the distinct subtype of AML at the time of diagnosis which determines the appropriate treatment option for the patients. The conventional classification of AML into eight distinct subsets (M₀ to M₇) by the French-American-British (FAB) system is based on the morphological characteristics, predominant differentiation pathway (monocytic, erythroid, megakaryotic) and the degree of blast maturation (24). In the case of minimally differentiated (M₀) and megakaryoblastic (M₇) leukemia, where blasts are stained negative, immunophenotyping must be used to define the presence of the disease.

In addition to FAB criteria, new and important genetic classifications are now being incorporated into the standard AML diagnostic techniques. The WHO classification includes the recurrent cytogenetic information that is commonly observed with distinct subsets of AML. Patients with these distinct chromosomal abnormalities are diagnosed with the respective acute leukemias even when blasts are less than 20% (19). The WHO’s classification of AML provides important prognostic information and divides AML into four subgroups. The first group is characterized by recurrent chromosomal abnormalities, the second group is characterized by myelodysplasia-related changes, and the third group is characterized by therapy-related myeloid
neoplasms, while the last group is not otherwise categorized. Therefore, the current diagnosis of AML requires a multidisciplinary approach, including clinical assessment, morphologic and cytochemical examination, flow cytometric immunophenotyping, cytogenetics, and molecular genetics.

1.1.4 Clinical Prognosis

A risk stratification based on cytogenetic and genetic abnormalities has been used to facilitate optimal therapy selection and determine the prognosis for clinical remission, overall survival, and disease-free survival. AML patients can be classified into several groups depending on their prognosis. These classifications are: favourable, intermediate, and unfavourable. The favourable risk group accounts for approximately 20% of all AML cases. These patients have chromosomal and genetic aberrations such as t (15; 17) translocations and core binding factor (CBF) disruption: t (8; 21) translocation and inv 16 (22) inversion. The intermediate risk group accounts for 45%-50% of all AML cases and these patients can have normal karyotypes, or present with +6, +8, -Y, or 12p chromosome abnormalities. The unfavorable risk group features a complex karyotype (three or more chromosomal abnormalities), or the possession of -5, -7 abnormalities, and abnormalities of 3q, 9q, 11q, 20q, 21q or 17p, t (6; 9) or t (9; 22). The five-year survival rates for these groups are 65%, 41%, and 14% respectively (25).

A number of other factors predicting poor outcome have been described for AML, including advanced age, performance status, and multidrug resistance phenotype (MDR1). Typically, older patients are considered to be those who are 60 years of age and over. Older patients usually have a higher percentage of unfavorable cytogenetic abnormalities which are associated with poor treatment outcome. In addition, they are more likely to have leukemia with intrinsic drug resistance and secondary AML or treatment-related AML, which is less responsive
to standard chemotherapies. About 58% to 71% of older patients have AML with MDR\textsubscript{1}, while 21% to 34% have secondary AML (26). For those patients, the complete remission (CR) rate is only 12%, compared with a CR rate of 81% in a same age-matched group with de novo AML without MDR\textsubscript{1} (26). Moreover, age-related changes in physiology are a major determinant of poor treatment outcome in older patients. Their general poor health status leads to increased treatment-related mortality (TRM) and toxicity, affecting their quality of life and overall survival. Therefore, age and MDR\textsubscript{1} phenotype are additional complexities in AML treatment.

1.1.5 Standard Treatment

From a biologic and clinical viewpoint, AML is an extraordinarily heterogeneous disease. The molecular heterogeneity of AML is the key determinant of treatment difficulty and complexity. Yet, except for acute promyelocytic leukemia (APL), the therapeutic approach for most AMLs has been similar and the standard treatment has changed little over the past 20 years. The conventional treatment of AML is divided into remission induction therapy and post-remission therapy. The standard induction therapy for adult AML patients is widely known as the “7+3” regimen. This regimen involves at least one course of intensive myelosuppressing standard-dose cytarabine (SD\textsubscript{A}raC) 100 to 200 mg/m\textsuperscript{2} that is administered by a continuous infusion for 7 days. This is combined with daunorubicin, an anthracycline (45 to 60 mg/m\textsuperscript{2}), which is administered intravenously for 3 days (27). With the standard induction therapy, complete remission (CR) rates of 65% to 75% and a long-term disease-free survival rate of ~30% were achieved in younger patients (28). However in older patients, the CR rate is 40% to 60% and the long-term survival rate is less than 10% (26). Alternative chemotherapies to improve CR rates by the incorporation of high-dose cytarabine (HD\textsubscript{A}raC), using various anthracylines (e.g.
idarubicin) or the addition of other agents (e.g. etoposide, mitoxantrone, fludarabine, or cladribine) have failed to show clinical improvement for both younger and older patients (28).

Post-remission therapy is given to those patients who achieve CR by induction chemotherapy, to prevent relapse. The intensity of post-remission therapy varies and includes: consolidation, maintenance, intensification therapy, and/or a hematopoietic stem cell transplant (HSCT). Post-remission therapy is guided by risk-stratification that is based on the persistence of blasts and the cytogenetic profile. For example, the intensity of the consolidation therapy and the decision to proceed to HSCT are strongly dependent on AML patient’s cytogenetic profile and age (29). For patients who fail to achieve CR with standard therapy, cytogenetic information becomes extremely important for planning secondary attempts, or new investigational treatments. Consolidation treatment strategies are the most frequent option for AML patients to eliminate minimal residual disease (MRD) after the first CR. For younger patients with favorable and intermediate cytogenetic risk, the standard consolidation therapy is high-dose cytarabine (HD AraC) (3g/m²) (30). Standard induction therapy “7+3” regimen followed by consolidation therapy with HD AraC result in overall survival (OS) rates between 60% to 75% in the favourable risk group, and approximately 40% in the intermediate risk group (31). Compared to consolidation therapy, maintenance therapy is considered less myelosuppressive and employs a lower dose of AraC, used to further reduce the number of residual leukemic cells and maintain disease-free status. The treatment outcome of maintenance therapy in AML patients is controversial and has not convincingly been shown to be effective in most AML subtypes except for APL (31).

HSCT is recommended for patients who are experiencing their first CR, if the stem cell can be harvested from the same patient (autologous, ASCT) or a human leukocyte antigen (HLA)
matched donor (allogeneic). In some cases, allogeneic stem cell transplantation (alloSCT) is considered to be the standard post-remission treatment option with a potential for producing long-term survival in younger adults with unfavourable prognostic markers. For these patients, AlloSCT may provide a better outcome than ASCT, as it exploits an immunological reaction known as graft-versus-leukemia (GVL) effect, wherein the donated allogeneic cells recognize the recipient’s leukemic cells as foreign (29). This has led to the development of less toxic alloSCT regimens that rely on GVL effect rather than the conventional myeloablative chemotherapy for complete eradication of malignant cells. For example, non-myeloablative chemotherapy using fludarabine combinations followed by alloSCT are immunosuppressive enough to allow engraftment of allogeneic blood progenitor cells (32). A recent review demonstrated that patients with unfavourable and some patients with intermediate cytogenetics (with the exception of those are characterized by the NPM mutation without FLT3-ITD) are candidates for alloSCT (28), while patients in the favourable group do not generally benefit from alloSCT. However, alloSCT is often associated with a high treatment-relative mortality (TRM) (15%-25%) (2, 30, 33), depending on the applied conditioning regimens. Therefore, selection for allogeneic transplantation among patients with unfavourable cytogenetic risk must be based on individual clinical characteristics, as it only benefits a small percentage of older patients.

1.1.6 Current Challenges in AML Treatment

As mentioned previously, older AML patients have a very poor prognosis due to the presence of unfavourable cytogenetics, MDR1 phenotype, secondary AML, and poor performance. The majority of older AML patients do not benefit from the standard induction treatment, and clinical investigational therapy is therefore recommended. Unfortunately, clinical
trials investigating various anthracyclines (such as mitoxantrone or idarubicin) or hematopoietic growth factors (such as G-CSF, granulocyte colony-stimulating factor) have not consistently been shown to improve response rates or CR rates (26). Attenuation of the intensity of induction therapy (low-dose cytarabine) to reduce toxicity results in lower CR rates (17%) (34). To conclude, no induction chemotherapy regimen for older AML patients has demonstrated superior clinical outcomes. In addition, the optimal post-remission therapy for older adults is also unclear, due to a higher likelihood that patients would have to withdraw from any additional treatment because of functional impairment or a poor performance status resulting from induction therapy (34).

The treatment of acute myeloid leukemia (AML), in spite of steady progress is still associated with considerable failure rates, due to high relapse and low response rates. As discussed above, for patients of any age with unfavourable risk and the majority of older patients, treatment outcome has remained most unsatisfactory. Current chemotherapeutic options provide a low chance for durable remission. Over 50% of patients who achieve first CR are expected to relapse within 3 years of diagnosis (11, 28). Unfortunately, for patients with relapsed and refractory AML, there is no single regimen or approach currently available as the standard treatment. Use of alloSCT may be curative for a minority of patients who achieve a second CR, but for the majority of relapsed patients, salvage therapy consisting of high-dose cytarabine is given in the hope of achieving second remission. For patients who continue to relapse beyond salvage therapy, achieving CR is nearly impossible (29). For this group of patients, investigational studies have been conducted to evaluate the use of novel agents and to explore new ways of using conventional approaches. Emerging agents that are potentially useful in the
treatment of patients with AML include: FLT3 inhibitor, hypomethylating agents, immunotoxins, monoclonal antibodies, and radioimmunotherapeutic agents.

1.2 Targeting AML Leukemic Stem Cells (LSCs)

Cancer stem cells have been shown to exist in some cancer types, including breast cancer, ovarian cancer and leukemia. The CSC model proposes that many cancers are organized hierarchically and sustained by a subpopulation of CSCs at the apex that has self-renewal capacity (10). The concept of cancer stem cells is most evident in leukemia, in which LSCs arise from a leukemic transformation of the HSC after acquiring multiple genetic mutations (35). LSCs may have the same potential as HSCs to self-renew and sustain the AML blasts, while most are quiescent in the microenvironment niche and protected from cell-cycle-specific chemotherapeutic drugs (36-38). Thus, understanding the biological properties of HSCs and LSCs, and the similarity and difference between them will be helpful in developing novel therapeutic agents that target LSCs while sparing normal HSCs. More importantly, targeting AML LSCs could potentially provide a cure for the disease, as so far no further improvement has been obtained by novel chemotherapeutic entities that target mainly the leukemic blasts.

1.2.1 AML Arises from a Leukemic Stem Cell (LSC)

HSCs are defined by their potential for self-renewal and ability to proliferate and differentiate into various cell types. HSCs comprise a very small sub-population of the total number of hematopoietic cells, and less than 0.01% of the total cells in the bone marrow (7). The self-renewal and differentiating capability of human HSCs are most evident in murine xenotransplantation experiments. The first study of normal HSCs described by Till and
McCulloch in 1961 demonstrated that a spleen colony is generated from a single cell called a spleen colony-forming unit (CFU-S) (39). Subsequent studies demonstrated that cells arising from a CFU-S can rescue a lethally irradiated mouse and repopulate the entire hematopoietic system (40, 41). Similar results were obtained by transplanting human bone marrow cells into sublethally irradiated, severe combined T- and B- cell immune deficiency (SCID) mice, or the nonobese diabetic (NOD)-SCID mouse, which yielded a higher engraftment efficiency (9). These studies defined the human cells repopulating the murine recipients as “human SCID-repopulating cells (SRCs)” and these cells were considered to be the engrafting human HSCs. Further studies demonstrated that human HSC pool contains two classes of cells: long-term SRCs and short-term SRCs, which have long-term and short-term repopulating capacity, respectively (42). The existence of HSC classes is thought to be the basis for HSC heterogeneity. Detecting and isolating different SRC classes of HSC have formed the basis for determining the biological function of HSCs.

Isolation of HSCs has been done with phenotypic cell-surface markers associated with defined lineages and development stages of hematopoietic cells. A variety of cell-surface markers have been discovered and defined by monoclonal antibodies that recognize these markers present on differentiated hematopoietic lineages (Lin) as well as other markers such as CD34, CD38, and CD90. Using fluorochrome-conjugated mAb and high-speed multi-parameter flow cytometry, subpopulations of cells were purified and collected for functional analysis. CD34 is a cell-surface marker normally expressed on a small population of bone marrow cells, including progenitor cells and pluripotent stem cells. An antibody against CD34 became a key in studying both normal and malignant human marrow samples. For normal human HSCs, CD34 serves as an effective positive selection marker for the enrichment of HSC activity. Further isolation of HSC was
accomplished by combining CD34 staining with removal of cells expressing antigens (such as CD38) found on the surface of lineage-committed cells. CD38 is a single chain transmembrane glycoprotein predominantly expressed by progenitors and early hematopoietic cells. CD34+ cells lacking expression of CD38 (CD34+/CD38−) were shown to contain a population of early progenitor HSC, whereas CD34+/CD38+ cells constituted a committed myeloid progenitor population (43). Therefore, the immunophenotype CD34+/CD38− defines primitive HSCs. Subsequent studies showed that CD90 (thymocyte differentiation antigen 1, Thy1) was also expressed on a subset of CD34+ CD38− Lin− human hematopoietic cells (44). The current phenotype of CD90+ CD34+ CD38− Lin− allows for isolation of cells with highly enriched HSC activity.

LSCs are similar to HSCs as they are also rare, have self-renewal ability and exert hierarchical control over leukemic blasts (9). The models and techniques employed in the study of HSCs have also been employed in the study of LSCs. The first demonstration about the existence of LSCs comes from the repopulating assay with AML cells in SCID mice by John Dick’s laboratory in 1994 (45). SCID leukemia-initiating cells (SL-IC) isolated from patient AML specimens homed to the bone marrow and proliferated extensively in response to in vivo cytokine treatment, resulting in a pattern of dissemination and leukemic cell morphology similar to that seen in the original patients. AML cells were furthered fractionated on the basis of cell-surface-marker expression and only SL-IC cells with a phenotype of CD34+CD38− could engraft in SCID mice. Upon transplantation of the CD34+ CD38− fraction, the entire leukemia population was recapitulated, whereas the CD34+CD38+ and CD34− fractions contained no cells with these properties (45). Furthermore, this population of SL-ICs resides in 0.1% to 1% of the AML cell population (1 engraftment unit in 2.5 × 10^5 cells), a very low frequency similar to that of normal
HSCs. Following this initial publication, a series of studies using an improved xenotransplantation model (NOD/SCID) provided direct experimental proof that leukemia is a hierarchy sustained by rare LSCs in a process closely resembling normal development (9, 46, 47). The hierarchy model of leukemogenesis predicts that LSC and HSC share many of the same properties that render them stem cells. In a separate study, individual LSCs were found to differ widely in self-renewal potential and only a minority of LSCs was found to possess high self-renewal capability for enabling the initiation of AML following serial transplantation in NOD/SCID mice (48). This has further proven that LSC is not homogeneous and still retains aspects of normal HSC organization.

1.2.2 LSC-Specific Niche

Repopulating stem cells reside in a highly complex hematopoietic niche that is regulated by a multifunctional network of hematopoietic growth factors, signaling pathways, cell cycle regulators and transcription factors. For example, Homeobox (Hox), Notch/Jagged, Hedgehog and Wnt/β-catenin signaling pathways have well-described roles in regulating HSC self-renewal (36, 38). Upon binding of these regulators to the cell-surface receptor, signaling pathways are active, leading to the translocation of transcription factors from the cytoplasm to the nucleus, which in turn recruits other cell-specific transcription factors to regulate the self-renewal of HSCs. Similarly, the molecular pathways also regulate the self-renewal of LSCs. For example, Wnt/β-catenin pathway is constitutively activated in leukemic cells, leading to the elevated expression of transcription factors and cell-cycle regulators that govern LSC self-renewal (36). In addition, cell cycle regulator deregulations are involved in the anti-apoptotic properties of LSCs. For example, the protein complex nuclear factor kB (NF-kB) is important in inflammation, anti-apoptotic
response, and immune response to stress or infection (49). High level of NF-κB was found in AML cell populations enriched in LSCs but not in HSC. Inhibiting NF-κB by a proteasome inhibitor carbobenzoxyL-Leucyl-L-Leucyl-L-Leucinal (MG-132) was effective at inducing preferential apoptosis of leukemic cells and interfering with their ability to engraft in NOD/SCID mice (50).

Besides the self-renewal signaling pathway, the microenvironment plays an important role in the abnormal migration and proliferation of LSCs. Substantial evidence shows that LSCs do not exist primarily in the blood circulation, but home to and engraft in the osteoblast-rich area of the bone marrow, where they are protected from chemotherapy induced apoptosis (10, 37, 51). The interaction between stromal cell-derived factor-1 (SDF-1) and CXCR4 (CXC-chemokine receptor 4 which is specific for SDF-1) is critical for the homing and retention of LSCs and other progenitor cells to the hematopoietic niche (52). The protein complex CXCR4/SDF-1 promotes leukemic cell homing as well as in vivo growth. Novel therapeutics such as a small molecule inhibitor of CXCR4 prevented the transmigration and colony formation of AML blasts (52). After becoming established in their niche, LSCs become quiescent, spend the majority of their time in the G0 phase of the cell cycle, and are resistant to endogenous or exogenous apoptotic stimuli (53). Hematopoietic grow factors such as G-CSF can mobilize resting G0-phase cells into the G1 phase and promote the release of leukemia cells from the bone marrow to peripheral blood (54). Clinical studies have indicated that the simultaneous exposure of leukemic cells to chemotherapy and G-CSF (referred to as growth-factor priming) increases the susceptibility of these cells to be destroyed by chemotherapy, especially by cytarabine (55).

1.3 Monoclonal Antibody-Targeted Therapy of AML
1.3.1 Monoclonal antibody (mAb)

The use of antibodies has provided an effective modality in the diagnosis and treatment of many cancers, including hematological malignancies. Antibodies are immunoglobulins (Ig) that are secreted by antigen-reactive B-cells to mount an immune response. There are five classes of antibodies that are heterogeneous but share a common basic structure: IgA, IgD, IgE, IgG, and IgM (56). Among them, IgG is the subtype of antibodies most commonly used in diagnostics and medical treatment (57). Furthermore, IgG is divided into 4 isotypes: IgG1, IgG2, IgG3, and IgG4. The four human IgG isotypes each have different complement-activating functions. (57).

Structurally, IgG is a Y-shape molecule made up of two identical heavy chains (50-70 kDa) and two identical light chains (25 kDa). The two heavy chains are linked to each other and bound to the light chains through disulfide bonds. Each light chain consists of one constant domain (C_L) and one variable domain (V_L); each heavy chain consists of three constant domains (C_H1, C_H2 and C_H3) and one variable domain (V_H). Functionally, IgG consists of two Fab (antigen-binding fragments) regions and one Fc (constant) region connected by a hinge region. The Fab region is composed of the V_L and V_H domain (56). The variable region of the Fab region contains the unique epitope-binding domains, referred to as complementarity determining regions (CDRs), which gives each antibody its specificity. Two Fab regions bind to two identical epitopes at the same time (a process called bivalent epitope binding). The Fc portion of the IgG binds to Fc receptor, which is found on white blood cells, monocytes, macrophages, and natural killer cells. In humans, there are three different classes of Fc receptors that mediate the host effector cell function: FcR\textsubscript{1}, FcR\textsubscript{2} and FcR\textsubscript{3}. The Fc domains of human IgG\textsubscript{1} and IgG\textsubscript{3} have high affinity to all three FcRs. The binding of Fc to FcR activates host effector functions, such as antigen dependent cell-mediated cytotoxicity (ADCC) and complementary-dependent cytotoxicity (CDC). In
ADCC, the binding of antibodies to Fc receptors on the surface of effector cells triggers phagocytosis or lysis of the targeted cells, whereas in CDC, antibodies kill the targeted cells by triggering the complement cascade (57).

Monoclonal antibodies (mAbs) are identical antibodies specific for a given antigen first produced by hybridoma cells discovered by Kohler and Milstein in 1975 (58). Ever since then, mAbs have proven to be extensively useful in both medical diagnostics and laboratory-based immunoassays, and more recently, as a potential treatment for various cancers and autoimmune disease. MAbs were initially made from hybridoma cells that were generated from the B-cells of an immunized mouse and are thus termed “murine” antibodies (56). The immunogenicity of murine antibodies was quickly found to be one of the limitations of repeatedly administering a therapeutic mAb. The host response to the murine Fc portion of the antibody caused the formation of human anti-mouse antibodies (HAMA). To reduce this immunogenicity, chimeric, humanized and fully human antibodies have been produced through more advanced recombinant technologies. Chimeric antibodies consist of murine-derived variable domains fused to human constant regions. Replacement of murine constant domains with human constant domains would retain the antibody binding affinity for the antigen but make the chimeric antibody less immunogenic. To further reduce the murine content and immunogenicity, a humanized antibody can be produced by substituting human framework sequences for the murine sequences in the variable region while maintaining the murine CDRs. Lastly, a fully human antibody with little or no immunogenicity can be achieved by using human hybridomas, transgenic animals and by selection from a phage display human sequence antibody library. More recently, several types of antibody-derived fragments have been developed to improve avidity and/or in vivo targeting,
including single-chain Fv (scFv) fragments, F(\(ab\)')\(_2\) fragments, diabodies, triabodies, tetrabodies and affibodies.

Therapeutic mAbs have emerged as one of the most successful class of drugs for treating cancer. The most successful example is trastuzumab (Herceptin®, a humanized IgG\(_1\) mAb that is used in the treatment of patients with human epidermal growth factor receptor-2 (HER2)-overexpressing breast cancer. In hematological malignancies, rituximab (Rituxan®), a chimeric IgG\(_1\) anti-CD20 mAb, has been approved to treat non-Hodgkin’s lymphoma (NHL) and chronic lymphocytic leukemia (CLL) (59). Alemtuzumab (Campath®), a humanized IgG\(_1\) anti-CD52 mAb and ofatumumab (Arzerra®), a humanized IgG\(_1\) anti-CD20 are approved to treat CLL (58, 60). In the case of AML, gemtuzumab ozogamicin (Mylotarg®), a humanized anti-CD33 mAb conjugated with a cytotoxic drug was initially approved for the treatment of CD33\(^+\) AML in the first relapse in elderly patients, who could not tolerate chemotherapy (61). However, gemtuzumab ozogamicin has been withdrawn from the U.S. market since June 2010 because the post-approval trials showed little or no benefit to patients. Its clinical efficacy and mechanism of action will be discussed in more detail later in this chapter.

Several lessons have been learned from the development of these antibodies and their derivatives. In particular, IgG\(_1\), chimeric or humanized antibodies are the most suitable for administering in humans, with respect to their high binding affinity for FcRs, and induction of target cell killing with a favorable pharmacokinetic profile and low immunogenicity. The success of the current therapeutic mAbs has encouraged studies to identify LSC-specific antigens and explore the use of antibodies directed against these antigens.

### 1.3.2 LSC-Specific Antigens
The effectiveness of mAbs for treatment of AML depends primarily on their ability to recognize an antigen preferentially expressed on LSCs as compared to non-LSCs. Although normal HSCs and LSCs share a phenotype of CD34+/CD38−, a number of distinct markers have been reported to help distinguish these two groups of cells. For example, CD90, CD117, and HLA-DR are usually expressed on HSCs but are absent from LSCs, whereas CLL-1, CD96, CD44, CD47 and CD123 are expressed more frequently on LSCs (43, 62). Their functions and expression level on AML LSCs are summarized in Table 1.3.2. The expression level of these antigens is assessed by fluorescence-activated cell sorting (FACS) from human AML samples and their LSC-properties have been studied by engrafting leukemia-initiating cells in xenotransplantation assays. There is also substantial overlap between the LSC and HSC surface antigen expression, such as CD33 which is highly expressed on most leukemic blasts, but is also expressed on some normal HSCs but at a much lower level (63). Theoretically, these surface antigen molecules preferentially expressed in subsets of AML LSCs would be ideal LSC targets.

Table 1.3.2 Cell surface antigens preferentially expressed on AML and the corresponding antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Molecular Identity, Function and expression</th>
<th>Targeted antibodies or immunotherapeutics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL-1</td>
<td>C-type lectin like molecule 1, unknown function, expressed on approximately 90% of AML samples</td>
<td>Under development</td>
<td>(63)</td>
</tr>
</tbody>
</table>
1.3.3 Mechnism of mAb Targeting of LSC-Specific Antigens

Recently three potential mechanisms have been proposed for using mAbs to target LSC-specific antigens. Currently approved antibody therapies for cancer are believed to act via antibody-mediated recruitment of effector cells to the tumour cells, or via disruption of critical receptor-ligand interactions. This involves binding of the “naked” or unconjugated mAb to the antigen which in some cases may be a peptide growth factor receptor, thus sterically blocking the mitogenic signal, or alternatively causing internalization of the receptor, downregulating cell surface expression. In other instances, binding of the mAb to the cells may result in activation of immune mediated responses, such as ADCC or CDC, resulting in cell death. Similarly, an antibody conjugated to cytotoxic agent can result in cell death by the delivery of these agents to

| CD123 | Interleukin 3 receptor α chain, High affinity IL-3 receptor, expressed on 100% of AML samples | DT 388IL-3, 26292(Fv)-PE38, Bs-scFv[123 x ds 16] 7G3, CSL360 | (64, 65) |
| CD44 | Cell surface receptor, expressed on 100% of AML samples | H90 | (66) |
| CD47 | Surface protein, interacts with macrophage receptor signal regulatory protein α (SIRPα) to inhibit phagocytosis, expressed on 100% of AML samples | B6H12 | (67)(68, 69) |
| CD96 | May have function in NK cell adhesion and/or activation, expressed on 66% of AML samples | Under development | (70) |
| CD33 | Glycoprotein, present on over 90% on AML samples | Gemtuzumab Ozogamicin, M195, HuM195 | (61, 71) |
the cancer cell (64). Functional antibody fragments and intact mAbs conjugated to toxins (such as diptheria toxin), chemotherapeutic agents (such as gemtuzumab ozogamicin), or radioactive isotopes belong to this category. This is the most widely used approach for using antibodies to target LSC by exploiting anti-CD33 and anti-CD123 mAbs, and will be discussed in more detail later.

A second potential mechanism involves the binding of a mAb to an LSC antigen that mediates interactions with cells in the LSC microenvironment, and thereby interfering with the homing of LSC to its protective niche. As mentioned earlier, LSC-niche interactions are very important for the proper engraftment of LSC. The binding of the mAb to the antigen causes disruption of the LSC-specific niche and results in the loss of the LSC’s ability to home to these essential microenvironments as well as increasing the sensitivity to apoptotic stimuli (10). For example, CD44 is a transmembrane glycoprotein mediating cell-cell and cell-extracellular matrix interactions. It is a receptor for osteopontin, which is an extracellular matrix component of the endosteal niche (65). Increased expression of CD44 was identified on the engrafting CD34+/CD38− cells from multiple AML samples compared to normal human cord blood which contains HSCs (66). An activating CD44-specific antibody (H90) was demonstrated to induce the differentiation of primary AML blasts in vitro. Sequent experiments showed that H90 eradicates LSCs in NOD/SCID mice by preventing their trafficking to the supportive microenvironment in the bone marrow and spleen (66).

Another potential mechanism involves using an antibody to block the CD47-mediated inhibition of LSC phagocytosis, thereby enabling the phagocytosis of LSCs by the innate immune system (67). CD47 is a transmembrane protein that serves as a ligand for signal regulatory protein α (SIRPα) that is expressed on phagocytic cells (68). When CD47 binds to SIRPα, the complex
initiates a signal transduction cascade that results in the inhibition of phagocytosis, referred as the “do not eat me” signal (69). Since CD47 is more highly expressed on LSCs from primary specimens of AML as compared with the normal HSCs (67), the upregulation of CD47 could contribute to AML by inhibiting phagocytosis of LSCs through CD47-SIRPα interaction. Therefore, a mAb directed against CD47 could block the CD47- SIRPα interaction and remove the “do not eat me” signal, resulting in phagocytosis of LSCs. Majeti et al. conducted an in vivo experiment to establish CD47 targeting as a viable therapeutic strategy (67). In this study, human AML LSCs-engrafted mice were treated with an anti-CD47 antibody. Eight out of eight mice had marked decreased AML burden and three out of eight mice had undetectable levels of human AML. In another experiment, an anti-CD47 murine mAb was studied in a mouse model of AML with high CD47 expression. It was demonstrated that this antibody enabled phagocytosis of mouse AML cells in vitro, and resulted in a survival benefit with minimal toxicity in vivo (67). In addition, a combination of anti-CD47 antibody with chemotherapy or antibodies targeting several different antigens may offer a synergized effect and improve the efficacy of anti-CD47 antibody therapies against LSC. A synergistic effect was observed in one study in which human NHL-engrafted mice treated with a combination of anti-CD47 antibodies and rituximab resulted in eradication of established NHL (70). However, the exact mechanism responsible for this selective targeting of LSCs by anti-CD47 antibodies is still unclear.

1.4 Monoclonal Antibodies Targeting CD33+ LSCs

CD33 is one of the most widely-studied antigens that is highly expressed on leukemic blasts and on LSCs (63). CD33, also known as siglec-3, is a 67 kDa cell surface sialoglycoprotein that is specific for myeloid cells. CD33 may be involved in cell activation processes and cell adhesion.
In normal hematopoiesis, CD33 is expressed on myeloid cells and their progenitors, but not on lymphoid cells (63). CD33 is expressed on blast cells in approximately 90% of AML patients (71). In one study, a subset of LSCs (defined as CD34+/CD38−/CD123+) was found to express CD33 in patients with CD33+ AML, whereas CD33 was not detectable in normal bone marrow cells with the CD34+/CD38− stem cell phenotype and on LSCs in patients with CD33−AML. However, the expression level of CD33 in normal HSCs is still controversial, and the level of CD33 and the percentage of CD33+ blasts may vary among patients (63). In addition, not all AML stem cells express CD33, and this would limit CD33-targeted therapy administered as a single agent.

1.4.1 Anti-CD33 mAb Lintuzumab

The CD33 antigen provides one possible target of intervention. Lintuzumab, also known as SGN-33 or HuM195, is a humanized anti-CD33 mAb which was shown to have anti-leukemic activity in pre-clinical studies. In one study by Sutherland et al., disseminated models of multidrug resistance (MDR)-negative and MDR-positive AML were developed by intravenous administration of commercially available human CD33+ AML cell lines (MDR-negative HL60, MDR-positive HHL9217 and TF1-α cell lines) into severe combined immunodeficiency (SCID) mice (72). Lintuzumab demonstrated significant anti-tumour activity in all models through its ability to mediate ADCC and phagocytosis, and prolonged the survival of mice regardless of MDR status. This suggests that lintuzumab represents a valid targeted therapy for the treatment of CD33+ myeloproliferative diseases. However, a Phase II clinical study of lintuzumab with low-dose cytarabine chemotherapy versus cytarabine alone failed to show a statistically significant difference in overall survival between treatment arms in AML patients 60 years or older (73).
This was suspected to be due to the low expression of CD33 on human leukemic cells as well as rapid internalization that may have prevented activation of ADCC. Collectively, the use of lintuzumab as an unconjugated mAb-targeted therapy still needs to be optimized in the treatment of AML.

1.4.2 Clinical Status of Anti-CD33 mAb Gemtuzumab Ozogamicin

The most successful clinical study of anti-CD33 mAb-targeted therapy in AML is calicheamicin conjugate gemtuzumab ozogamicin (GO, Mylotarg ®). GO is a Food and Drug Administration (FDA)-approved humanized IgG4 anti-CD33 monoclonal antibody (gemtuzumab) conjugated to a cytotoxic drug, N-acetyl-γ calicheamicin dimethyl hydrazide (a derivative of calicheamicin) (74). The constant and framework regions of GO contain human IgG4 sequences, whereas the CDR regions contain murine CD33-binding sites. The toxin calicheamicin can induce site-specific double-strand breaks when it binds to the minor groove in the DNA (75). Calicheamicin is linked to gemtuzumab by covalent linkage of a bifunctional linker, which is stable in physiological buffers (pH 7.4) but allows efficient drug release at low lysosomal pH (approximately pH= 4) (74). Pre-clinical studies indicated that when GO binds to CD33 through CD33 recognition and complex formation, the complex is rapidly internalized and taken up in lysosomes in AML blast cells, releasing calicheamicin, which in turn results in apoptosis of leukemic cells (74, 76).

GO has been approved for treating patients aged 60 years or older with CD33+ AML in their first relapse who are unable to tolerate cytotoxic chemotherapy and have an overall response rate of 30% in clinical studies (77, 78). A number of clinical studies have explored the use of GO in a monotherapy or in combination therapy in AML patients (76). A Phase II study (79)
conducted on 277 CD33+ AML patients in first relapse who received GO at 9 mg/m² demonstrated an overall response (OR) rate of 26%, and over 50% of these patients had blast cell clearance after the first dose of GO. However, a long duration of pancytopenia and impaired liver function were observed in some of the patients, which may be due to the expression of CD33 on distinct vascular cells in the liver and other organs. Taksin et al. demonstrated an excellent efficacy/safety trial through administration of fractionated doses of GO (3 mg/m² on day 1, 4, and 7) to 57 patients with AML in first relapse, with a CR of 26% and a much lower toxicity profile compared to the study above that had administered 9 mg/m² (80). The mechanism leading to resistance in the other 74% of patients is incompletely understood. Intracellular membrane-associated transporter proteins such as permeability glycoprotein (Pgp or MDR₁) are likely important in modulating GO susceptibility (81, 82). Residual marrow leukemia persisted after GO treatment and lower CR rates were highly correlated with blast cell Pgp expression and low in vitro drug-induced apoptosis (83). In summary, GO can be used as a single agent for AML therapy but is associated with drug resistance.

On the other hand, the results of clinical trials that combined GO and chemotherapy are heterogeneous due to the variable activity of these regimens used and the different characteristics of the patient populations. In a highly influential study, the British Medical Research Council (MRC) reported a large randomized trial testing the addition of GO to the “7+3” regimen induction and/or consolidation chemotherapy in 1,115 untreated younger patients (84). The addition of GO had no effect on the CR rates but improved survival in patients with core binding factor (CBF) AML who belonged to the favourable prognostic group, but had no benefit for patients with poor-risk disease, and showed a trend for some benefit in the intermediate-risk patients. Post-approval clinical studies also demonstrated no overall improvement in survival
outcomes with the addition of GO to induction or maintenance therapy. Therefore, GO was removed from the U.S. market in June 2010 and can be only used as an investigational drug (85).

1.5 Novel Targeted Therapy against CD123\(^+\) LSCs

1.5.1 Biological Activity of CD123

Another important LSC-specific antigen is CD123, the alpha chain of the interleukin-3 receptor (IL-3R\(\alpha\)). Human interleukin-3 (IL-3) is a cytokine that stimulates production of hematopoietic cells from multiple lineages. IL-3 exerts its biological activity through interaction with its cell surface receptor (IL-3R) which consists of two subunits: the \(\alpha\)-chain (IL-3R\(\alpha\), CD123) and the \(\beta\)-chain (\(\beta\)c, CD131). Both receptor chains belong to the cytokine superfamily, and are closely related to the receptors for GM (granulocyte-macrophage)-CSF and interleukin 5 (IL-5) (86). IL-3 binds to CD123 alone with high specificity but low affinity. CD131 alone, in the absence of CD123, also confers little binding affinity to IL-3, but it converts low-affinity ligand binding to CD123 to high-affinity ligand binding when co-expressed with CD123, and acts as a signal transducer. After ligand binding, the CD123/CD131 complex becomes phosphorylated, and through the recruitment of adaptor proteins, activates the Ras signaling pathway followed by the downstream induction of the MAPK (mitogen-activated protein kinase) pathway (86). In addition, activation of IL-3R is also known to stimulate the PI3K (phosphatidylinositol 3-kinase)/AKT pathway. Ultimately, activation of these transcription pathways in turn leads to the activation of transcription factors for specific genes that control cell cycle progression, proliferation or apoptosis. In normal hematopoiesis, the binding of IL-3 to IL-3R stimulates the survival and development of multilineage colonies from normal bone marrow. In leukemia, IL-3 elicits a stimulation effect on most human AML blasts which proliferate in response to IL-3 (86-88). Also,
mutation of either the CD123 or CD131 chains may contribute to the development of leukemia (87).

1.5.2 Expression of CD123 and CD131 on LSCs

CD123 is widely expressed on a variety of hematopoietic cells including myeloid cells and a subpopulation of B lymphocytes (87, 89-91). As the technique of fluorescence activated cell sorting (FACS) and quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) advanced, the screening of hematological malignancies has provided evidence that an elevated expression of CD123 is mainly observed in AML and B-ALL. Its role as a unique marker for human AML LSC was first discovered by Jordan et al. in 2000 (90). In that study, all 18 primary AML cells of all subtypes were CD123+. Particularly, in the more primitive AML subpopulation enriched in LSC (CD34+/CD38−), greater than 99% of the cells were positive for CD123. In contrast, the normal HSC fraction (CD34+/CD38−) showed no significant expression of CD123. Yalcintepe et al. (92) reported that the expression of CD123 was significantly higher among CD34+/CD38−/CD71− cells, enriched for LSCs than among cell fractions depleted of such progenitors. Similar to these studies, recent work by Jin et al. (93) also demonstrated that CD123 is highly expressed on the bulk of AML cells as well as the CD34+/CD38− fraction compared to normal counterparts. Interestingly, overexpression of CD123 was also detected on CD34+/CD38− cells from Fanconi anemia patients with AML when compared to normal HSCs (94). According to these findings, it is clear that the antigen CD123 represents an appropriate target for cytotoxic agents designed to selectively kill AML progenitor cells while sparing their normal hematopoietic-cell counterparts.
Since both α and β chains are necessary to form the high affinity receptor for IL-3, lack of CD131 may slow internalization of IL-3R in AML stem cells, resulting in slow release from lysosome. Thus, the expression of CD131 was also examined. Interestingly, CD131 was never detected in the bulk AML populations in the study by Jordan et al. (90), whereas in Yalcintepe’s study (92), the level of CD131 was much lower (4-to-15 fold) than that of CD123 in each isolated subpopulations and was relatively the same for both normal and malignant cells. This suggested that the expression of CD131 is more likely to be a limiting factor in the formation of high-affinity IL-3R-binding site. Consequently, the targeting of CD123 with cytotoxic agents requiring high-affinity interaction with the fully functional CD123/CD131 complex may not be effective in the absence of CD131, and may cause a low response rate. This was observed in study of diphtheria toxin (DT_{388})-IL-3 toxin fusion proteins as discussed below.

1.5.3 Targeting CD123⁺ LSCs with Cytokine-Toxin Fusion Proteins

Based on the studies of IL-3 and IL-3R expression in AML, several investigators have explored anti-leukemic activity of a genetically engineered fusion protein (DT_{388}IL-3). This immunotoxin DT_{388}IL-3 is composed of the first 388 amino acid residues of diphtheria toxin catalytic and translocation domains (DT_{388}) fused to human IL-3 (92). DT_{388}IL-3 interacts with the leukemic blasts expressing high levels of IL-3R, is internalized and exerts its highly potent cytotoxic effect. The \textit{in vivo} anti-leukemic efficiency of DT_{388}IL-3 has been evaluated in immunocompromised mice engrafted with human IL-3R positive AML blasts (95). DT_{388}IL-3 induced substantial killing of malignant progenitors \textit{in vivo} with a potency similar to anti-CD33 cytotoxic agent gemtuzumab ozogamicin. Moreover, a phase I/II clinical trial of DT_{388}IL-3 for treatment of patients with relapsed and refractory AML or myelodysplasia (MDS) showed that
DT\textsubscript{388}IL-3 was well tolerated and induced one CR in 40 evaluable AML patients and one partial remission (PR) in 5 MDS patients (96).

However, other studies indicated that the expression of high affinity IL-3R (CD123/CD131) on the surface of AML leukemic blasts represent a major determinant in their sensitivity to DT\textsubscript{388}IL-3 (92, 97). Particularly, DT\textsubscript{388}IL-3 exhibited a low \textit{in vitro} cytotoxicity in AML cases expressing low IL-3R levels, but a more pronounced cytotoxicity in AML cases expressing high IL-3R levels (97). Therefore, information on CD123 and CD131 expressions might explain low response rate of DT\textsubscript{388}IL-3 in the clinical trials. Furthermore, DT\textsubscript{388}IL-3[K116W], a variant of DT\textsubscript{388}IL-3 with a lysine (K) in the IL-3 moiety mutated to a tryptophan (W), was constructed to improve the sensitivity and potency of DT\textsubscript{388}IL-3 (96). The variant exhibited a 15-fold more potent anti-leukemic activity \textit{in vitro} and \textit{in vivo} than its wild-type form. Further studies using the variant toxin to target AML are still ongoing.

1.5.4 Targeting CD123\textsuperscript{+} LSCs with Novel Immunoconjugates of Single-Chain Fv Antibody Fragments

To bypass the need for the high affinity IL-3R complexes, the use of high affinity antibodies was exploited. Improved affinity can be achieved by \textit{in vitro} affinity maturation, for example, the cDNA coding for single-chain Fv antibody fragments (scFv) with the highest affinity for CD123 was used for the construction of novel immunoconjugates targeting CD123\textsuperscript{+}LSCs. These immunoconjugates can be selected to target only the most abundant subunit in the complex (CD123) to bypass the need for other components such as CD131. In addition, the use of scFv instead of full-length immunoglobulin prevents the molecule from binding to Fc receptors on noncytotoxic cells and thus avoids the induction of a nonspecific immune response.
In one study, three recombinant immunotoxins were made by fusing CD123-directed scFv with a 38 kDa fragment of *Pseudomonas* Exotoxin A (PE38) (98). The Fv moiety confers the specific binding to CD123⁺ AML cells and the toxin moiety kills the cells. One of the immunotoxins 26292(Fv)-PE38 showed high cytotoxic activity on the CD123⁺ leukemia cell line TF-1. Its variant 26292(Fv)-PE38-KDEL was made by mutating the REDLK sequence at the C-terminus to KDEL and was cytotoxic to a panel of cell lines with moderate CD123 expression but not to cell lines with low or absent-expression (98). Due to their relatively small molecular weight, scFv are rapidly cleared by the kidneys and this limits the general therapeutic application of scFv monomers in RIT against CD123⁺LSCs.

Another approach is the generation of bispecific single chain Fv fragments (bs-scFv) consisting of one binding site for the target antigen CD123 and a second binding site for an activating trigger molecule on an effector cells, such as CD16 (FcγRIII) on natural killer cells (NK cells). The common goal of bis-cFv is to bind and kill tumour cells more selectively over other populations by requiring both binding sites to be present (99). These bispecific antibodies offer distinct advantages over immunotoxins, including specific recruitment of the preferred effector cells and avoiding induction of an immune response and production of neutralizing antibodies that often interfere with repeated administering of the agent. Stein *et al.* reported a bs-scFv directed against CD123 and trigger molecule CD16 [123 x ds 16] that was capable of triggering lysis of cultured AML-derived cells by recruiting NK-cells (100). However, this bs-scFv may be limited by rapid renal clearance due to its lower molecular mass (60 kDa) (100). To improve anti-leukemia activity of bs-scFv, a “trispecific” scFv [123 x ds 16 x 33] was constructed with a molecular mass of 90 kDa and an additional antigen (CD33) binding site to increase avidity (101). It has one binding site for effector cells and two different binding sites for antigens,
CD123 and CD33. Due to the dual targeting of the tumour cell, this trispecific scFv produced much stronger lysis than the mono-targeting agent and was more potent in mediating ADCC of primary leukemia cells isolated from peripheral or bone marrow of 7 patients with AML (101). Future studies still need to find out whether preferential dual-targeting by trispecific scFv via ADCC mechanism is feasible in an animal model.

### 1.5.5 Targeting CD123+ LSCs with Monoclonal Antibodies

Monoclonal antibody 7G3 is a murine IgG2a directed against CD123 (102). As a specific IL-3 receptor antagonist, 7G3 specifically binds to the IL-3R α chain (CD123) and completely abolishes its function. 7G3 has an affinity (Kd = 0.9 nM) for the IL-3R α chain that is 100-300 fold greater than IL-3 itself, although its binding to the fully functional IL-3 α/β receptor (CD123/CD131) is 3-10 fold lower than IL-3 (Kd = 0.1 nM) (102). Jin et al. reported that in vitro exposure of AML specimens to 10 µg/mL of mAb 7G3 diminished their ability to engraft in NOD/SCID mice by 10-fold by interfering with homing to the bone marrow and Fc-activation of residual NK cell activity (93). Moreover, treatment of AML-engrafted mice at 28 days post-cell inoculation with 3×300 µg doses weekly for 5 weeks decreased leukemia in the bone marrow for 2 out of 5 specimens. Also it was found that treatment at 24 hours post-cell inoculation reduced engraftment for 2 out of 3 specimens and its administration 6 hours prior to cell inoculation abrogated AML engraftment. These results suggest mAb targeted to CD123 may represent one potential way to eradicate AML.

In addition, CSL360 is a human IgG1 chimeric variant of 7G3 that neutralizes IL-3 and has anti-leukemic activity in vitro and in vivo (103). By having a human IgG1 Fc domain, CSL360 is less likely evoke an immune reaction if administered to humans. The mechanisms of
action of 7G3 or CSL360 for treatment of CD123 expressing leukemias may involve: 1) inhibition of the IL-3-mediated signaling pathway by blocking IL-3 from binding to its receptor, 2) recruitment of complement after the antibody has bound to a target cell and caused CDC, or 3) recruitment of effector cells after the antibody has bound to a target cell and caused ADCC (104).

Recently, CSL360 has been investigated in a Phase I clinical trial of relapsed, refractory high-risk AML patients (105). Patients were administered 12 weekly intravenous infusions of 0.1 to 10 mg/kg of CSL360. Out of the 11 patients who received 12 doses, only one complete response (CR) was observed. This indicated that anti-CD123 mAb therapy with CSL 360 does not provide definite clinical benefit in high-risk AML patients. These studies suggested that blockade of IL-3 signaling alone with anti-CD123 mAbs may be insufficient to eradicate LSC.

In summary, monoclonal antibodies that target specific antigens on leukemic cells present a promising strategy to eradicate the leukemic blasts. The effectiveness of immunotherapy has been proven both in preclinical studies and clinical trials. However, there are remaining limitations in using immunotherapy to target AML. Some obstacles to the successful targeting and elimination of the disease by immunotherapeutic agents include: rapidly internalization of antigen-antibody complexes which prevent activation of the immune response, low expression of antigens or receptors on leukemic cells, as well as high immunogenicity which prevents repeated application of the agent. An alternative approach is to use antibodies to target radionuclides directly to leukemic cells, especially to the leukemic stem cells.

1.6 Monoclonal Antibody-Targeted Radioimmunotherapy of AML

The radioimmunotherapy approach (RIT) using monoclonal antibodies conjugated with a radioisotope, has been developed to deliver targeted radiation to cancer cells while potentially
sparing normal tissues. Currently, total body irradiation (TBI) is the conventional preparative regimen used prior to hematopoietic stem cell transplantation (HSCT) and it is applied to eradicate or reduce the leukemic burden and to facilitate the engraftment of hematopoietic cells in the marrow, or to prevent graft rejection (106). High dose TBI significantly reduces the relapse rate in AML and CML; however, survival is not improved because of higher normal organ toxicity and higher regimen-related nonrelapse mortality (107, 108). Therefore, the intensifying conditioning regimen by radioimmunotherapy (RIT) has greater advantages over TBI, as it delivers more selective irradiation of the bone marrow to reduce the risk of relapse while minimizing toxicity in nontargeted organs such as liver and kidney.

RIT is a promising approach for increasing the specific radiation dose to the bone marrow without increasing nonspecific cytotoxicity to extramedullary sites (109). The basic principle of RIT of cancer cells is that molecular transformations in these cells present potential targets (such as tumour-associated antigens) for specific interaction with antibodies carrying radionuclides, thus permitting selective deposition of lethal doses of DNA-damaging radiation to malignant cells, while sparing normal tissues (110). The successful targeting and elimination of malignant cells by RIT is therefore dependent on the identification of an appropriate target and the optimal design of a targeting vehicle-radionuclide conjugate, as well as selection of a suitable radionuclide (110).

Leukemias are suited for RIT as the leukemic blasts are easily accessible to circulating antibodies, and the target antigens on blasts can be characterized for individual patients (111). Particularly, the target antigens of AML that have been investigated more extensively for RIT include: CD33, CD45, and CD66 (109, 111, 112). Monoclonal antibodies (mAb) recognize these antigens and can be used as targeting vehicles to selectively deliver radionuclides to leukemic
cells. As discussed earlier, CD33 is a glycoprotein expressed on myeloid leukemic blasts, and an anti-CD33 mAb M195 and HuM195 have been used for immunotherapy as well as RIT of AML (63, 74, 113-115). CD45 is a pan-leukocyte antigen expressed on virtually all leukocytes and a wide range of myeloid and lymphoid blasts. RIT using anti-CD45 mAb BC8 radiolabeled with β particle-emitting Iodine-131 (\(^{131}\text{I}\)) has been shown to eliminate not only leukemic blasts but also normal leukocytes in the marrow (110, 116, 117). CD66 is a glycoprotein expressed at a high density of normal myeloid cells from the promyelocyte onward up to mature granulocytes, but not leukemic blasts. RIT using anti-CD66 mAb BW250/183 radiolabeled with β particle-emitting Rhenium-188 (\(^{188}\text{Re}\)) or Yttrium-90 (\(^{90}\text{Y}\)) has been explored in phase I and II studies (118). Both \(^{188}\text{Re}\)- and \(^{90}\text{Y}\)-labeled anti-CD66 RITs were shown to be safe as part of a reduced intensity preparative regimen prior to HSCT in older patients with AML and MDS with a 2-year survival of 52%. However, currently there is no radiolabeled antibody with FDA approval for the treatment of AML, and the only approved RIT for hematological malignancies is for the treatment of Non-Hodgkin’s B-cell Lymphoma, as discussed later.

### 1.6.1 Radionuclides used for RIT of AML

In general, radionuclides suitable for targeted radiotherapy of tumours include α-emitters, β-emitters and low-energy Auger electron-emitters. The selection of the optimal radionuclide for RIT of cancer is based on the physical characteristics of the radioisotopes, such as physical half-life, mean range of particulate emission and the linear energy transfer (LET) (111). LET is defined as the ratio of the amount of energy transferred by a charged particle to the target atoms in the immediate vicinity of its path in traversing a small distance. Alpha particles (α-particles) such as \(^{211}\text{At}\), \(^{213}\text{Bi}\), \(^{212}\text{Bi}\) or \(^{225}\text{Ac}\) are doubly positively charged with a mass and charge equal to the
helium nucleus, and their emission leads to a daughter nucleus with 2 fewer protons and 2 fewer neutrons. Alpha particles are known as energetic particles with high LET. They have energies ranging from 5-9 MeV with ranges in tissue from 50-100 µm, and they deposit as much as 80-100 eV/µm along their track length. Owing to their short track length (5-10 cell diameters) and high LET, α-particles have great advantage for treatment of single cells or small clusters of cells (119). However, the use of α-particles as the type of RIT for hematological malignancies is limited by their tissue penetration of only a few cell diameters and the short half-life of the most available α-emitters.

Current radionuclide therapy is based almost exclusively on β-particle-emitting radioisotopes. β-particle emitting radioisotopes include $^{131}$I, $^{90}$Y, $^{67}$Cu, $^{177}$Lu or $^{188}$Re. β-particles are negatively charged particles with the same mass as an electron, and their emission leads to a daughter nucleus with 1 more proton and 1 less neutron. They are known as energetic particles (50-2,300keV) with low LET (0.2-0.5 keV/µm) (119). The range of β-particles (2-12 mm) in tissues is directly proportional to their energy, and most of their energy is deposited at the end of their track length (111). Therefore, the long track length (200-1200 cell diameters) of β-particles makes them most suitable for treating solid tumours and larger lesions. Furthermore, the long-range β-particles creates a field effect called the “cross-fire” effect, where each emitted electron traverses neighboring cells and irradiates a larger proportion of tumour cells than if the β-particle was only deposited in the cell to which the radioisotope is bound. Owning to this long-range tissue penetration, β-particles have major advantages in targeting a high percentage of cancerous cells rather homogenously even in a solid tumour and can overcome the limitation of inhomogeneous radiopharmaceutical distribution (110). However, the cross-fire effect from β-particle emitters is known to cause non-specific toxicity to bone marrow stem cells, due to
perfusion of the marrow by circulating radioactivity. In addition, the low LET makes β-particles less efficient for killing single cells or small tumour clusters.

Auger electron-emitting radioisotopes include $^{125}$I, $^{123}$I, $^{111}$In, $^{67}$Ga, or $^{99m}$Tc. Auger electrons are very low-energy electrons emitted by radionuclides that decay by electron capture. Electron capture processes create inner shell electron vacancies by an electron transfer from this shell into the nucleus. The inner shell electron vacancies are subsequently filled by the decay of an electron from a higher shell. The energy difference of these transitions can be released either as photons or low-energy electrons, i.e. Auger electrons (111). Compared to α-particles and β-particles, Auger electrons are of much lower energy (<30 keV) but with high LET (4-26 keV/µm). The larger majority of Auger electrons have a very short range (nm to µm, less than one cell diameter). Similar to α-emitting radiation, their high LET and subcellular range render Auger-electron radiation more effective against single tumour cells or small cell clusters. Owing to their subcellular range, Auger electron emitters are required to be internalized and ideally translocated to the nucleus for the electrons to become highly efficient in damaging DNA. The internalization of Auger electron emitters can be facilitated by conjugating them to a mAb or peptide that specifically binds and is internalized by malignant cells. Moreover, Auger electron radiation therapy approaches have major advantages over α or β-emitters because of their selective toxicity for these cells that specifically bind and internalize the radiolabeled mAbs or peptides. There is no cross-fire effect from Auger electrons. While circulating in the blood or bone marrow, the Auger electron emitters exhibit low toxicity but become highly efficient for cell killing when in close proximity to the DNA of target cells (119).

In the case of RIT targeting AML, the radioisotopes that are widely used for clinical use are β-particle emitters such as iodine-131 ($^{131}$I), yttrium-90 ($^{90}$Y) or rhenium-188 ($^{188}$Re). The
crossfiring β-particle makes them useful for treating bulky disease and irradiating the entire bone marrow before HSCT. $^{131}$I is attractive for its relatively long half-life (8.1 days) and low-energy β-particle (~600 keV) (109, 111). Its emission of γ photons (Eγ= 364 keV) can also be useful for imaging using a gamma camera which allows a dosimetry study to be performed easily, whereas a disadvantage is that high doses of $^{131}$I require patient isolation due to the penetrating nature of the gamma emissions. $^{90}$Y is a pure β-emitter with a half-life of 2.7 days and no γ-emissions, allowing outpatient administration of high doses. $^{188}$Re is suitable for biodistribution and dosimetry studies but is limited by its short half-life (17h). Compared to β-particles, RIT with α-particle-emitters may result in more effective treatment of minimal residual disease (MRD) in AML patients who achieve complete remission (111). The most commonly used α-particle emitters for AML are Bismuth-213 ($^{213}$Bi), Actinium-225 ($^{225}$Ac) and Astatine-211 ($^{211}$At). Particularly, $^{225}$Ac is considered to be highly effective because it generates daughter decay products that are themselves α-emitters or β-emitters, thus amplifying DNA damage. However, the toxicity of $^{225}$Ac caused by redistribution and renal uptake of these decay products are likely to limit its use for treatment of AML in humans (111). Finally, the most important Auger electron emitters of RIT for AML are Indium-111 ($^{111}$In) and Iodine-125 ($^{125}$I), as discussed later.

1.6.2 Current Clinical Status of RIT of Leukemia and Lymphoma

There are only two agents in current clinical practice that are FDA-approved for RIT of hematological malignancies and these are for the treatment of Non-Hodgkin’s B-cell lymphomas (NHL): $^{131}$I-tositumomab (Bexxar®), and $^{90}$Y-ibritumomab tiuxetan (Zevalin®) (120). $^{131}$I-tositumomab is a murine IgG2a anti-CD20 mAb conjugated to β particle-emitting, $^{131}$I for the treatment of relapsed or refractory CD20 antigen-expressing follicular NHL or transformed B-cell
NHL including patients with rituximab refractory NHL (121). $^{90}$Y-ibritumomab tiuxetan (Zevalin®) is a murine IgG$_{1k}$ anti-CD20 mAb linked to the radiometal chelator isothiocynatobenzyl MXDTPA (tiuxetan) which strongly binds to β particle-emitting, $^{90}$Y (122). CD20 is a 35 kDa transmembrane glycoprotein displayed by 95% of B-cell lymphomas and normal mature B-cells, but not present on early progenitor B cells (110). CD20 has proven to be an excellent target for RIT because it is stably expressed on almost all B-cell NHL patients and is not internalized after mAb binding to the antigen (123, 124). The β-emitters, $^{131}$I or $^{90}$Y do not require internalization for their cytotoxic effects.

The efficacy of $^{131}$I-tositumomab and $^{90}$Y-ibritumomab tiuxetan administered as either a single treatment or multiple treatments for patients is demonstrated by the significant increased objective response rate (ORR) and completed remission (CR) rates in comparison with immunotherapy using rituximab, a non-radiolabeled antibody directed against CD20. In the majority of RIT trials of NHL, unlabeled anti-CD20 mAbs are given a week prior and on the day of administering the RIT agent to saturate CD20 antigen sites on normal B-cells in the blood and spleen, thereby enhancing tumour uptake of the RIT agents. Dosimetry studies are also performed to calculate the amount of radioactivity which must be administered to deliver a total body radiation dose of 65 cGy to 75 cGy (110). The ORR of $^{90}$Y-ibritumomab tiuxetan or $^{131}$I-tositumomab in relapsed refractory indolent lymphoma as a single treatment was about 74%-92% with a CR of 15% to 51%. As compared to treatment with single agent rituximab, a significant higher ORR and CR was achieved for a single treatment of RIT in patients with follicular lymphoma (ORR = 86% vs. 55%, CR = 34% vs. 20%, P<0.05), respectively, and a similar trend was observed in patients with transformed lymphoma and rituximab-refractory disease (120, 121, 125). In particular, the ORR for treatment with $^{90}$Y-ibritumomab tiuxetan and $^{131}$I-tositumomab
in rituximab-refractory patients was 74% with time to progression (TTP) of 8.7 months and 65% with TTP of 24.5 months, respectively (120). In addition, multiple treatments of RIT demonstrated an improved response rate and durable remission compared to chemotherapy (68% vs. 28%) (120).

The use of RIT monotherapy as first line therapy for patients at advanced stage has also been investigated and the results were comparable to the standard combination treatment of immunotherapy and chemotherapy (120, 122). The ORR of 95%-100% and mean progression free survival (PFS) of 6.1 years for RIT monotherapy was comparable to the PFS (6.9 years) achieved by standard treatment of RCHOP (combination chemotherapy of rituximab, cyclophosphamide, hydroxyldaunorubicin, oncovin, and prednisone)(120). A recent Phase II clinical study was conducted to evaluate the efficacy and safety of administering $^{90}$Yttrium ibritumomab as first line treatment with standard single dose of 15 MBq/kg in previously untreated patients with an advanced stage of follicular lymphoma (126). A CR rate of 52% and PR rate of 9% were achieved among the 33 evaluative patients who were followed up for more than 18 months, while 36% of the patients progressed and were off study, either in observation or under a new treatment. The results indicated high percentages of clinical responses to $^{90}$Yttrium ibritumomab when given as a first line treatment to patients. Remission rates were similar to those achieved by standard chemoimmunotherapy protocols, but the absence of severe side effects compared extremely well with the much greater toxicity of chemotherapy regimens. RIT with $^{90}$Yttrium ibritumomab tiuxetan was also shown to be very safe and well accepted by patients (126).

In the case of AML, most clinical RIT trials to date have used $\beta$-particle-emitting $^{131}$I or $^{90}$Y-labeled anti-CD33 antibodies, and more recently, $\alpha$-particle RIT has also been studied in
patients with myeloid leukemias. The studies using radiolabeled anti-CD33 mAbs as RIT agents for AML focused on the use of anti-CD33 mAbs initially with the murine antibody (M195), and subsequently with the humanized form of this antibody (HuM195 or lintuzumab) to minimize immunogenicity in humans. The group at Memorial Sloan-Kettering Cancer Center showed that $^{131}$I-M195 and $^{131}$I-HuM195 could be added to standard conditioning regimens in order to intensify treatment prior to HSCT in patients with relapsed and refractory AML and CML (114). Thirty-one patients were treated with a dose of 4440-8510MBq/m$^2$ followed by busulfan (16 mg/kg), cyclophosphamide (90 or 120 mg/kg), and an infusion of related-donor bone marrow. The results of this Phase I study showed that 28 of 30 evaluable patients achieved remission, and there was a 20% long-term survival rate for patients with AML, whereas none of the CML patients survived long term (114).

More recently, $\alpha$-particle RIT with HuM195 has shown some promising results for RIT of AML, as it has greater advantages in the treatment of small-volume disease over $\beta$-particles due to its shorter range and higher LET. In a Phase I/II study, thirty-one patients with newly diagnosed or relapsed/ refractory AML were treated with cytarabine for 5 days followed by $^{213}$Bi-HuM195 (18.5-46.3MBq/kg) (127). The clinical response rate was 24% in those who received a dose of at least 37MBq/kg and the mean response duration was 7.7 months. However, as previously discussed there are limitations of $\alpha$-particle RIT associated with short half-lives of the radionuclides and dose-limiting toxicity to the bone marrow and other normal tissues (117, 128,129).

1.6.3 Auger Electron RIT of AML
In contrast to α and β-particle radiation, Auger electrons are better adapted for the treatment of AML due to their high LET as well as lower nonspecific toxicity to normal organs, especially the bone marrow. Radiolabeled mAbs conjugated to low-energy Auger electron-emitters cause low toxicity while circulating in blood or bone marrow but become extremely toxic when bound and internalized and transported to the nucleus of target tumour cells (119). From a radiobiologic prospective, the toxicity of Auger-electron-emitting radionuclides in close proximity to DNA is extremely high (130). As illustrated by Kassis et al., the complex organization of chromatin within the mammalian cell nucleus involves multiple structural level compactions such as nucleosomes with dimensions that are within the range of the Auger electrons (nanometer to micrometer) (119).

Previous studies have demonstrated that the decay of Auger-electron emitters covalently bound to nuclear DNA leads to an extremely high degree of double-strand breaks and cytotoxicity. The efficacy of such a DNA-incorporated Auger-electron emitter was first demonstrated in the study by Bloomer et al. where $^{125}$I–radiolabeled thymidine analogue 5-iodo-2’-deoxyuridine (IUdR) showed excellent therapeutic efficacy when injected into mice bearing an intraperitoneal ascites ovarian cancer, leading to 5-log reduction in tumour cell survival (131). Ever since then, the therapeutic efficacy of DNA-incorporated Auger-electron emitters have been investigated extensively (130, 132-134). Moreover, Auger-electron-DNA-targeted therapy is a promising treatment approach for breaking chemoresistance and radioresistance in leukemia cells (135). In one study, it has been shown in principle that a thymidine analogue labeled with the Auger electron emitter Iodine-123 ($^{123}$I) would be able to kill leukemia cells that were resistant to doxorubicin (136).
1.6.4 Auger Electron RIT of AML Using Antibodies Modified with Nuclear Localization Sequence Peptide

Based on the same principle, using antibodies as a vehicle to deliver Auger-electron radiation to the nucleus of the cell is potentially feasible and would be expected to cause high cytotoxicity once inside the nucleus. However, intranuclear importation of a mAb with a molecular weight of 150 kDa via passive diffusion may be difficult due to the mechanism governed by the nuclear pore complex that regulates the nuclear import of proteins from the cytoplasm to the nucleus. The nuclear pore complex is a complex of nucleoporins with an inner channel with a diameter of about 9 nm which allows the passive diffusion of molecules with molecular weight cut-off of 40-60 kDa (137). Therefore, transport of larger proteins (> 65 kDa) into the nucleus requires active transport mediated by both a nuclear-localization signal (NLS) and exposure of that signal to components of the transport machinery (138). NLS is recognized by importin (karyopherin) α and β heterodimers, which subsequently shuttle the protein across the nuclear pore complex into the nucleus. Once in the nucleus, the importin encounters RanGTP (Guanosine-5´-triphosphate), and the ensuing importin/RanGTP complex leads to dissociation of the cargo from the importin, whereby the importin is recycled back to the cytoplasm.

The simian virus 40 large tumour antigen (SV-40 large T antigen) contains a nuclear localization signal (NLS) sequence of Pro-Lys-Lys-Lys-Arg-Lys-Val (PKKKRKV), which has been studied in great detail (139-142). Our group has developed a strategy to conjugate a peptide harboring the NLS of SV-40 T antigen onto $^{111}$In-labeled mAbs in order to promote the nuclear translocation of these Auger electron-emitting RIT agents to cause high specific cytotoxicity to the DNA of the tumour cells binding the radioimmunoconjugates (115, 143-145).
The first application of NLS-containing peptides to insert radionuclide antibodies into the nucleus of cancer cells for Auger electron radiotherapy of AML was reported by Chen et al. (146). The nuclear translocation and cytotoxicity of $^{111}$In-HuM195 in HL-60 leukemia cells and AML patient specimens was enhanced by its conjugation to synthetic 13-mer peptides (CGYGPKKKRKVG) harboring the NLS of SV-40 large T antigen (underlined). Following this study, conjugation of this NLS peptide to $^{111}$In-labeled trastuzumab (Herceptin) demonstrated increased nuclear uptake of $^{111}$In and subsequently decreased both the clonogenic survival of human epidermal growth receptor-2 (HER2)-overexpressing tumour cells in vitro as well as inhibited the growth of tumour xenografts in vivo (144, 147). This same strategy has now been explored in the current thesis research in which $^{111}$In-labeled anti-CD123 mAb CSL360 was linked to the NLS-peptide to promote high nuclear uptake and anti-leukemic cytotoxicity in AML cells (148). The mechanism of action is shown in Figure 1.6.5.
Figure 1.6.5 The proposed mechanism of nuclear importation of $^{111}$In-NLS-7G3 or $^{111}$In-NLS-CSL360 targeting CD123$^+$ leukemic stem cells and induction of lethal DNA double-strand breaks caused by the emission of Auger electrons. $^{111}$In-NLS-7G3 or $^{111}$In-NLS-CSL360 binds to the cell surface antigen CD123 irreversibly and is gradually transported inside the cytoplasm of the cell through receptor-mediated internalization. After internalization, the active transport of mAbs into the nucleus is facilitated upon exposure of the NLS signal to the transport machinery. NLS is recognized by importin α and β heterodimers, which subsequently shuttle the protein across the nuclear pore complex into the nucleus. Once in the nucleus, the cargo is disassociated, whereby the importin is recycled back to the cytoplasm. When close to the DNA molecule, Auger electrons emitted from $^{111}$In can cause lethal DNA double-strand breaks and eventually cell-death.
1.7 Hypothesis of the Thesis

Only about 20-30% of patients with high-risk AML become long-term survivors after intensive chemotherapy and HSCT with standard conditioning. The most common cause of treatment failure is relapse. The frequent relapses of AML patients are believed to be caused by the repopulation of leukemic blasts by leukemia stem cells (LSCs). In order to eradicate the disease and to potentially cure the patient, the LSC population must be targeted. One such approach to target LSCs is the use of a RIT agent that can specifically recognize the LSCs by exploiting their unique phenotype of CD123+/CD131−. Therefore, it was hypothesized in this thesis that 111In-labeled anti-CD123 CSL360 mAbs modified with peptides harboring the NLS of SV40 large T antigen (CGYGPKKKRKVG) to promote nuclear importation would cause specific cytotoxicity in AML cells that share the CD123+/CD131− phenotype with the LSC through the emission of subcellular range Auger electrons.

1.8 Specific Aims

The specific aims of the thesis designed to test the hypothesis were:

**Aim I**: To construct and characterize 111In-NLS-CSL 360 by analytical techniques to measure its purity, homogeneity and immunoreactivity for CD123.

**Aim II**: To characterize the cell binding properties and internalization and nuclear importation properties of 111In-NLS-CSL360 in CD123-positive cells.

**Aim III**: To evaluate the *in vitro* cytotoxicity of 111In-NLS-CSL360 in AML cells that share a leukemic stem cells phenotype (CD123+/CD131−) using a clonogenic survival assay.
Chapter 2

111In-labeled anti-CD123 Monoclonal Antibody CSL360 Modified with Nuclear Localization Sequences (NLS) for Auger Electron Radioimmunotherapy of AML

All experiments and analysis of data were carried out by Jin Hua Gao, except for the analysis of flow cytometry performed by Dr. Jeffrey Leyton.
2.1 Introduction

In 2010, an estimated of 90,000 people in Canada were living with or in remission from various types of leukemias, Hodgkin’s and non-Hodgkin’s lymphoma or myeloma (4). Of the leukemias, acute myeloid leukemia (AML) is a more common adult leukemia and is characterized by an uncontrolled proliferation of myeloid progenitors in the bone marrow (BM) (24). Despite advances in therapy, only about 20%-30% of patients with high-risk AML become long-term survivors after hematopoietic stem cell transplantation (HSCT) with standard conditioning regimens (28). Unfortunately, most of the patients relapse within 3 years of diagnosis (5).

The frequent relapses of AML patients are believed to be caused by the repopulation of leukemic blast cells by leukemic stem cells (LSCs) (35). These LSCs are capable of self-renewal as well as sustaining progenitor cells and leukemic blasts in a hierarchical fashion (9). They reside in the bone marrow at low population numbers and share the CD34+/CD38- phenotype with normal hematopoietic stem cells (HSCs) (45, 51,149). In addition, LSCs possess unique biological properties that are believed to render them resistant to chemotherapy (37). In most cases, standard chemotherapy mainly ablates highly-proliferating leukemic blasts while sparing the often quiescent LSCs and this leads to re-establishment of the disease (53). LSCs are also thought to have high levels of multidrug resistance transporters (53). The implication is that LSCs must also be eliminated in order to eradicate the disease. Therefore, novel therapeutic approaches that target LSCs have great potential to produce more durable remissions in patients with AML, and potentially cure this disease.

One such promising novel strategy to target LSCs is the use of therapeutic monoclonal antibodies (mAbs) that target antigens displayed by these cells. One such antigen is the interleukin 3 receptor (IL-3R) α subunit (CD123). Jordan et al. reported that CD123 was uniquely
expressed on >99% of CD34+/CD38- leukemic cells from 16 of 18 AML patients, but was absent on normal BM cells with the CD34+/CD38- stem cell phenotype (90). In normal hematopoiesis, CD123 is essential for IL-3 ligand binding, and together with the β subunit (CD131), forms the high affinity functional IL-3R which causes phosphorylation and subsequent rapid internalization of the ligand-receptor complex (86). Unlike normal HSCs, LSCs display CD123 in the absence of CD131 (90, 150). Therefore, because of the elevated expression of CD123 and the ablated IL-3R function on LSCs, CD123 is an attractive target for delivering a cytotoxic agent to LSCs.

A number of monoclonal antibodies (mAbs) that are specific for CD123 have been reported. One is 7G3, which is a murine IgG2a mAb that binds to CD123 and antagonizes IL-3 activity (102). More importantly, 7G3 preferentially binds to CD123 in the absence of CD131, with a 100-300 fold higher affinity ($K_d=0.9$ nM) than IL-3 itself, whereas its binding to the fully functional IL-3R is 3-10 fold lower than IL-3 (102). Since LSCs display elevated CD123 in the absence of CD131 (CD123+/CD131−), this unique and preferential binding of 7G3 makes it an excellent vehicle to deliver cytotoxic agent to LSCs. In addition, CSL360 is a human IgG1 chimeric variant of 7G3 that neutralizes IL-3 and has anti-leukemic activity in vitro and in vivo (105). The CD123 binding properties of CSL360 were confirmed to be equivalent to those of the original parent 7G3 mouse mAb. Owing to its human Fc region, CSL360 is less immunogenic when administered to humans and therefore more suitable for development as a novel immunotherapy for AML.

Recently, Auger electron radioimmunotherapy (RIT) of AML has been explored to target CD123 displayed by LSC populations (148, 150). This RIT approach exploits the low energy but very high linear energy transfer (LET) Auger electrons emitted by $^{111}$In, which offers important advantages over conventional RIT using β-emitters. The β-emitters $^{131}$I or $^{90}$Y have low LET and
deposit most of their energy at the end of their track length (2 or 12 mm, respectively), making them less effective for single cell killing (151). Furthermore, these long range β-emitters irradiate non-targeted cells (known as “cross-fire” effect) which cause dose-limiting myelosuppression due to high levels of circulating radioactivity perfusing the bone marrow (111). For example, $^{131}$I-labeled anti-CD33 HuM195 mAb has proven to be effective as a conditioning regimen prior to HSCT in patients with relapsed/refractory AML, because it causes major radiotoxicity to the bone marrow (152). In contrast to β-radiation, Auger electron radiation provides high LET (4-26 keV/µm) within a nanometer-to-micrometer range (111). However, this requires delivery of Auger electron-emitting radionuclides into the cytoplasm of tumour cells and ideally, into the cell nucleus. Auger electron radiation is most effective for killing cancer cells when the electrons are emitted in close proximity to DNA (135). Owing to their ultrashort range, Auger electron emitters produce low toxicity while circulating in the blood or perfusing the bone marrow (111). In this study, indium-111 ($^{111}$In) that has a physical half-life of 2.8 days and emits approximately 15 Auger electrons per decay was studied conjugated to CSL360 as a potential RIT agent for AML. In addition, $^{111}$In is a γ emitter, which makes it a useful radionuclide for microSPECT imaging.

To enable the intranuclear localization of $^{111}$In, the $^{111}$In-labeled CSL360 mAbs were modified with 13-mer peptides [CGYGPKKKRKKVGG] harboring the nuclear translocation sequence (NLS) of simian virus 40 (SV40) large T-antigen (underlined) (148). These NLS peptides have been previously used to promote the translocation of macromolecules into the cell nucleus without interfering with their receptor-binding affinity (145, 147, 153-155). NLS-modified IgG is recognized by the importin (karyopherin) family of transporters and shuttled into the nucleus across the nuclear pore complex (140). In one example, conjugation of $^{111}$In-labeled anti-CD33 murine mAb M195 or its humanized form HuM195, to NLS-peptides enhanced its
nuclear importation and antiproliferative effects in HL-60 leukemia cells and primary AML specimens (115). A recent study from our group indicated that combining 7G3 and this nuclear localization strategy improved the effectiveness of $^{111}$In for diminishing the viability of patient-derived AML cells that share the CD123$^+$/CD131$^-$ phenotype with the LSC (148). NLS modification of $^{111}$In-7G3 significantly increased its nuclear uptake, and resulted in increased DNA double-strand breaks caused by Auger electrons emitted within striking distance of the DNA.

Based on these findings, in this study, the effectiveness of $^{111}$In-NLS-CSL360 for decreasing the clonogenic survival of AML cells with the CD123$^+$/CD131$^-$ was examined. Firstly, $^{111}$In-NLS-CSL360 was constructed and analyzed for its purity and homogeneity. The cell binding, internalization and nuclear importation properties of $^{111}$In-NLS-CSL360 were then evaluated using CD123-expressing cells. Finally, its cytotoxic effects were evaluated on AML cells with the CD123$^+$/CD131$^-$ phenotype using a clonogenic survival assay.

### 2.2 Materials and Methods

#### 2.2.1 Cell culture

Wild-type Raji cells, a CD123-transfected subclone (Raji-CD123), and Chinese Hamster Ovary cells transfected with CD123 [(CHO)-CD123] were provided by CSL Ltd (Parkville, Australia). For subsequent descriptions, Raji-CD123 cells transfected with a high or low copy number of CD123 or wild-type Raji cells will be represented as Raji-Hi, Raji-Low and Raji-Wild cells, respectively. Patient-derived AML cell lines (AML 3, 4, and 5) and primary AML samples were provided by Dr. Mark Minden (Ontario Cancer Institute, Toronto, ON). Raji-Wild and Raji-CD123 cells were cultured at 37 °C in a 5% CO$_2$ atmosphere in Roswell Park Memorial Institute
(RPMI) 1640 medium (Sigma, Oakville, ON), supplemented with 10% (v/v) heat-activated fetal bovine serum (FBS; Sigma, Oakville, ON) and 1% (v/v) penicillin/streptomycin (pen strep; Sigma, Oakville, ON). CHO-CD123 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma, Oakville, ON) with 10% FBS and 1% pen strep. AML cells were maintained in alpha minimum essential medium (α-MEM; Invitrogen, Burlington, ON) with 10% FBS, 10% conditioned media (from incubation with 5637 cells) and 1% pen strep. The 5637 cells are the adherent bladder carcinoma cells known to produce and secrete large quantities of various growth factors including G-CSF, GM-CSF, SCF and others (but not IL-3). Approximately 1×10⁶ 5637 cells/80 cm² in 10 mL of RPMI 1640 medium was incubated at 37°C in 5% CO₂ until cell monolayer became confluent. The medium was then collected and filtered by 0.2µm to exclude nonadherent cells before mixing with α-MEM.

2.2.2 Preparation of ¹¹¹In-NLS-CSL360 Immunoconjugates

The overall conjugation steps for preparing ¹¹¹In-NLS-CSL360 are illustrated in Figure 2.2.2. CSL360, a chimeric IgG₁ monoclonal antibody (mAbs) and isotype-matched mAb ChIgG₁ were provided by CSL Ltd. CSL 360 or ChIgG₁ were first derivatized with a chelator for labeling with ¹¹¹In. The chelator 2-(p-isothiocyanato-benzyl)-cyclohexyl diethylenetriamine-pentaacetic acid (CHX-A’’DTPA) was provided by Dr. Martin Brechbiel at the U.S. National Institutes of Health (NIH). Briefly, CSL360 or ChIgG₁ (500 µg, 5 mg/mL in 50 mM sodium bicarbonate buffer, pH =7.5) was reacted with 30-fold molar excess of CHX-A’’DTPA for at least 4 hours at 30 °C. The final DTPA immunoconjugate was purified on a Sephadex-G50 minicolumn (Biorad, Mississauga, ON) eluted with phosphate-buffered saline (PBS, pH 7.6). Synthetic 13-mer NLS-peptides (CGYGPKKKRKVGG) were then conjugated to CHX-A’’DTPA-modified
mAbs by introducing maleimide groups through a cross-linking agent sulfo-succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC; Pierce, Waltham, MA) that provides a maleimide group for reaction with the terminal thiol on the cysteine residue of the peptide. Briefly, CHX-A''DTPA-conjugated mAb (1.0-2.5 mg/ml in PBS, pH 7.6) was reacted with 25-fold molar excess of sulfo-SMCC at room temperature (RT) for 1 hour, and then purified on a Sephadex-G50 minicolumn eluted with 25 × 100 uL of PBS, pH 7.0. Fractions containing maleimide-derivatized CHX-A''DTPA antibodies were concentrated to 1.0 to 2.5 mg/ml, in PBS (pH 7.0) using a YM-50 Microcon ultrafiltration device (M, cutoff, 50 kDa; Millipore, Billerica, MA), and subsequently reacted with a 50-fold molar excess of NLS-peptides (20mg/mL in PBS, pH 7.0; Advanced Protein Technology Center, Hospital for Sick Children, Toronto, ON) overnight at 4 ºC. The excess unconjugated NLS peptides were removed by purification on a Sephadex-G50 minicolumn eluted with PBS, pH 7.0.
Figure 2.2.2 Scheme for the preparation of $^{111}$In-NLS-CSL360. The scheme only shows the conjugation occurring at the lysine residues of the Fc portion of the antibody and it should be noted that the conjugation may also occur at the lysine residues present on the Fab portion of the antibody. Monoclonal antibody CSL360 is first derivatized with a chelator CHX-A"DTPA for labeling with $^{111}$In. Synthetic 13-mer NLS-peptides (CGYGPKKKRKVGG) are then conjugated to CHX-A"DTPA-modified mAbs through a cross-linking agent sulfo-SMCC that provides a maleimide group for reaction with the terminal thiol on the cysteine residue of the peptide. Finally, immunoconjugates CHX-A"DTPA-CSL360-NLS (20-50 µg) is incubated with (15-37 MBq) of $^{111}$In-acetate for radiolabeling with $^{111}$In.
For radiolabeling, 20-50 µg of DTPA-NLS-CSL360 immunoconjugates (in 1.0 M sodium acetate buffer, pH 6.0) was incubated with (15-37 MBq) of $^{111}$In-acetate for 1 hour at RT. $^{111}$In-acetate was prepared by mixing $^{111}$In-chloride (MDS-Nordion, Inc., Vancouver, BC) with 1.0 M sodium acetate buffer, pH 6.0 in a ratio of 1:1 (v/v). This amount of $^{111}$In was used to maximize the specific activity, with the remaining free $^{111}$In removed by size-exclusion chromatography on a Sephadex-G50 minicolumn. Fractions containing the pure radioimmunoconjugates (~500 µL) were combined and concentrated to 100 µL using an YM-50 Microcon ultracentrifugation device. $^{111}$In-NLS-CSL360 preparations were analyzed by instant thin-layer silica gel chromatography (ITLC-SG; Pall Cop., Port Washington, NY) developed in 100 mM sodium citrate buffer, pH 5.0 ($R_f$ $^{111}$In-CSL360 = 0.0; $R_f$ free $^{111}$In = 1.0) or by size-exclusion high-performance liquid chromatography (SE-HPLC). After removal of free $^{111}$In, the radiochemical purity was >95%. Depending on the purity and concentration of the $^{111}$In in the stock solution, the specific activity of the final radioimmunoconjugates was generally 0.37-0.74MBq/µg. All radioactivities were measured by an automatic γ-counter (Wallac Wizard-1480; Perkin Elmer, Waltham, Massachusetts). For subsequent descriptions, $^{111}$In-labeled CHX-A”DTPA-conjugated CSL360 and $^{111}$In-labeled CHX-A”DTPA-CSL360-NLS will be represented as $^{111}$In-CSL360 and $^{111}$In-NLS-CSL360, respectively. Similarly, the isotype-matched controls $^{111}$In-labeled CHX-A”DTPA-conjugated ChIgG₁ and $^{111}$In-labeled CHX-A”DTPA-conjugated ChIgG₁-NLS will be represented as $^{111}$In-ChIgG₁ and $^{111}$In-NLS-ChIgG₁.

2.2.3 Characterization of $^{111}$In-NLS-CSL360

The purity and homogeneity of CSL360, maleimide-activated CHX-A”DTPA-CSL360, CHX-A”DTPA-CSL360-NLS, NLS peptides and final radioimmunoconjugates ($^{111}$In-NLS-
CSL360) were determined by non-reducing sodium dodecyl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE) on a 5% Tris HCl mini-gel stained with Coomassie R-250 brilliant blue (BioRad), or by SE-HPLC. For SE-HPLC, samples were eluted with 100 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.7 mL/min through a Biosep SEC-S2000 column (Phenomenex Inc., Torrance, CA), and detected by a Perkin-Elmer model 218 diode array detector set at 280 nm and a radioactivity detector flow scintillation analyzer (FSA; Perkin Elmer, Waltham, MA). The percentage of impurities, such as intermolecular crosslinking between two antibodies, was calculated by measuring the density of the bands on a SDS-PAGE gel by Spot Densitometry (FluorChem® FC2 Imager, Alpha Innotech, San Leandro, CA).

The chelator substitution level (i.e. the number of DTPA per mAb molecule) was quantified by adding a trace amount of $^{111}$In-chloride to the reaction mixture, measuring the proportion of radioactivity incorporated and multiplying by CHX-A”DTPA-to-IgG molar ratio used in the reaction. The number of NLS-peptides introduced per mAb molecule was quantified by adding tracer quantities of $^{123}$I-NLS-peptides into the conjugation reaction and measuring the proportion of radioactivity incorporated into the antibodies and then multiplying by the NLS peptides-to-IgG molar ratio used in the reaction. Briefly, NLS-peptides were radiolabeled to a specific activity of 1-2 MBq/µg with $^{123}$I-sodium iodide using the Iodogen method (Thermo Fisher Scientific, Rockford, IL). The radiochemical purity of $^{123}$I-NLS-peptides was 95%, as determined by paper chromatography developed in 85% methanol. ($R_f$: $^{123}$I-NLS-peptides=0.0; $R_f$ $^{123}$I iodide=1.0). Furthermore, NLS peptide modifications were also assessed and quantified by a band shift assay on a 6% SDS-PAGE gel. The migration distance in the gel relative to the bromphenol blue dye front ($R_f$) was measured and the number of NLS-peptides introduced into CSL360 was estimated by reference to a plot of the logarithm of $M_f$ versus $1/R_f$ for broad-range
M_r standards (Precision Plus Standards, M_r 10–250 kDa; Bio-Rad). The number of NLS-peptides conjugated to CHX-A”DTPA-CSL360 was calculated by dividing the difference between the M_r values for maleimide-activated CHX-A”DTPA-CSL360 and CHX-A”DTPA-CSL360-NLS by the M_r of the NLS-peptide (~1,418 Da).

2.2.4 Cell-Binding Assays

The immunoreactivity of CHX-A”DTPA-CSL360 and CHX-A”DTPA-CSL360-NLS was evaluated in a competition binding assay. Approximately 1 x 10^5 CHO-CD123 cells were incubated in 24-well plates for 24 hours in culture medium. After a gentle rinse with PBS (pH 7.5), the cells were incubated with 0.4 nM ¹¹¹In-CSL 360 for 1 hour at 4 °C in the presence of 0.2-225 nM of CHX-A”DTPA-CSL360 (without NLS) or CHX-A”DTPA-CSL360-NLS. The cells were rinsed twice with PBS, pH 7.5. Cell suspensions were collected and the radioactivity was measured in a γ-counter. The proportion of ¹¹¹In-CSL360 initially bound to CHO-CD123 cells (in the absence of competitor) that was displaced (B/Bo) vs. increasing concentrations (nM) of the competitors was plotted, and fitted to a 1-site competition model using Prism Ver. 5.0 software (GraphPad Software, San Diego, CA). The dissociation constant (K_d) and IC_{50} were estimated. The equation used to model this curve is \( Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{(X - \text{LogEC}_{50})})} \), where x represents the concentration of the unlabeled ligands (nM) and Y represents the proportion of radioligands bound to the cells (B/Bo).

Alternatively, a saturation receptor-binding assay was used to evaluate CD123 binding affinity as well as to measure the number of CD123 epitopes on the CHO-CD123 and the AML-5 cell lines. Briefly, approximately 1 x 10^5 cells were incubated with ¹¹¹In-NLS-CSL360 in concentrations ranging from 0-220 nM for CHO-CD123 cells and 0-20 nM for AML-5 cells in
250 µL of normal saline for 2 hours. After incubating, the cells were gently rinsed with cold normal saline to remove unbound radioactivity. The cells were then dissolved using 0.1 M NaOH and collected to measure the cell-bound radioactivity in a γ-counter. The assays were performed in the absence (total binding) or in the presence of unlabeled CSL360 (nonspecific binding) at a 50-fold molar excess compared to the radioimmunoconjugates (0-11,000 nM for CHO-CD123 and 0-1,000 nM for AML-5 cells). Specific binding was obtained from subtraction of nonspecific binding from total binding, which at saturation represented the maximum number of binding sites on the cell (B_max) assuming 1:1 binding of 111In-labeled CSL360-to-CD123. The direct receptor binding curve was constructed by plotting the bound amount of radioimmunoconjugates (total, nonspecific and specific) vs. increasing concentrations (nM). The curve was further fitted by Prism Ver. 5.0 (GraphPad) to a 1-site saturation binding model to estimate the dissociation constant (K_d) and B_max. The equation used for corresponding specific binding curve was

\[ Y = \frac{B_{\text{max}}X}{K_d + X} \]

where X presents the concentration (nM) and Y represents bound radioligand (pmol); for the nonspecific binding curve (a straight line) was \[ Y = NS \cdot X \] where X presents the concentration (nM) and Y represents bound radioligand (pmol); for the total binding curve was

\[ Y = \frac{B_{\text{max}}X}{K_d + X} + NS \cdot X \]

Specific binding of 111In-CSL360 and 111In-NLS-CSL360 was also analyzed at a single excess concentration (266 nM) on Raji lymphoma cells engineered to express high (Raji-Hi) or intermediate (Raji-Low) levels of CD123 as well as wild-type Raji cells that have no expression of CD123 (Raji-Wild). Approximately \(1 \times 10^6\) Raji-Hi, Raji-Low and Raji-Wild cells were incubated with 200 µL of PBS containing 266 nM of 111In-CSL360 or 111In-NLS-CSL360 (0.037-0.074 MBq/µg) at 4°C for 2 hours. The cell suspensions were then centrifuged for 5 min at 420 \(\times\)
g, and the supernatant was collected. The cell pellet was rinsed twice in 500 µL of ice-cold PBS and collected. The amount of radioactivity in the supernatant and cell pellet was measured in a γ-counter. The percentage of total radioactivity in the cell pellet was calculated with respect to the total amount added in the incubation media. Finally, the percentages of total radioactivity in Raji-Low and Raji-Wild cells were normalized with respect to that in Raji-Hi cells.

The CD123 and CD131 expression on the AML-5 cells were evaluated by standard flow cytometric techniques. Briefly, AML-5 cells were pelleted at 500 × g for 5 minutes and re-suspended in PBS, pH 7.5. The cells (1 × 10^6 cells in 100 µL) were then added to 12 × 75 mm polypropylene tubes (Falcon Labware, Franklin Lakes, NJ) along with 2 µL of PE-conjugated mouse anti-human CD123 (CD123-PE) antibodies and 2 µL of PE-conjugated mouse anti-human CD131 (CD131-PE) immunoconjugates (BD Biosciences, San Jose, CA). CD123-PE mAbs recognize the same binding site on CD123 as CSL360. An irrelevant fluorescently-labeled antibody was used as a control for background fluorescence in the assay. To evaluate the specific binding to AML-5 cells, 1 × 10^6 AML-5 cells were incubated in 100 µL of PBS with 10 µg of unlabeled CHX-A’DTPA-CSL360-NLS at 4 °C for 30 minutes to block the CD123 binding site prior to the addition of immunofluorescence antibodies. The cells were then rinsed with ice-cold PBS, centrifuged, and incubated with 2 µL of CD123-PE mAb and 2 µL of CD131-PE mAb on ice for 30 minutes. Following 1 hour incubation at 4 °C, the cells were washed three times with 3 mL of PBS. Finally, cells were pelleted at 500 × g for 5 minutes, re-suspended in 500 µL of PBS and analyzed using a flow cytometer (Beckman Coulter LSR II). A minimum of 10,000 events were recorded and the flow cytometry data was analyzed using CellQuest Pro software (Becton Dickenson).
2.2.5 Cellular uptake, Internalization and Nuclear Localization Studies

The effect of varying the concentration of \(^{111}\)In-NLS-CSL360 on the uptake by AML-5 cells was evaluated. Approximately 1 x 10^6 AML-5 cells were incubated with \(^{111}\)In-NLS-CSL360 at increasing concentrations ranging from 33 nM to 266 nM. An irrelevant mAb \(^{111}\)In-NLS-ChIgG\(_1\) was used as a control. In addition, the uptake of \(^{111}\)In-NLS-CSL360 at a saturating concentration (266 nM, 2.95MBq) at 37 °C after 4 hours of incubation was also measured on a panel of AML cells: AML-3, AML-4 and AML-5. In a separate experiment, the effect of varying lengths of incubation on the cellular uptake of the radioimmunoconjugates on AML-5 cells was evaluated by incubating approximately 1 x 10^6 AML-5 cells at 37 °C with 200 µL PBS (pH 7.5) containing 266 nM of \(^{111}\)In-NLS-CSL360 or \(^{111}\)In-CSL360 for 4 hours or 24 hours. A blocking study was performed by adding an excess of unlabeled CSL360 (13,300 nM) to the incubation media. After incubation, the cells were centrifuged for 5 minutes at 420 x g to remove the medium. The cells were then rinsed three times with ice-cold PBS to remove unbound radioactivity. The cells were then re-suspended in 200 µL of PBS pH 7.0. The amount of \(^{111}\)In in the incubation medium and in the cells (bound and internalized) was measured in a \(\gamma\)-counter (Wizard, PerkinElmer). From the specific activity of the radioimmunoconjugates used, and correcting for the percentage of the unbound \(^{111}\)In in the radioimmunoconjugates (<5%), the number of antibody molecules bound to 1 x 10^6 cells was calculated and expressed as picomoles (pmol) bound.

To further determine the amount of radioimmunoconjugates (pmol) imported into the cell nucleus, subcellular fractionation was performed by selective lysis of the cell membrane as described previously (148). Briefly, approximately 1 x 10^6 AML-5 cells were incubated with \(^{111}\)In-NLS-CSL360 or irrelevant control antibody \(^{111}\)In-NLS-ChIgG\(_1\) (33 - 266 nM) in 200 µL at
37 °C for 24 hours. Following incubation, the cells were washed three times with cold PBS pH 7.5 to remove unbound radioactivity. The cells were then lysed with 200 µL of cell membrane lysis buffer (Biovision, Mountain View, CA) for 10 minutes on ice, followed by the addition of 11 µL of cytosol extraction buffer (Biovision). The cells were centrifuged again to separate the cytoplasmic fraction of radioactivity (supernatant) and nuclear fraction (pellet). Finally, the radioactivity in the membrane/cytoplasm fraction and nuclear fraction were measured in a γ-counter. The percentage of total radioactivity in the nucleus was calculated and converted to picomoles (pmol) of antibody as described above. In a subsequent experiment, cell fractionation procedures were carried out by incubating AML-5 cells with 266 nM of radioimmunoconjugates in the presence or absence of 50-fold excess of unlabeled CSL360 to determine if the nuclear uptake was receptor-mediated. In addition, to study the role of NLS-peptide in facilitating nuclear-uptake, AML-5 cells were incubated with 266 nM of $^{111}$In-NLS-CSL360 or $^{111}$In-CSL360 (without NLS) for 4 hours or 24 hours. After incubation, cell fractionation was then performed as described above.

### 2.2.6 Clonogenic Survival Assays

The cytotoxicity of $^{111}$In-NLS-CSL360 towards AML-5 cells was studied by a clonogenic survival assay. Briefly, 1 x $10^6$ cells were incubated with increasing amounts (0.4 -266 nM, 0.04 -2.95 MBq) of $^{111}$In-NLS-CSL360 or isotype-matched irrelevant $^{111}$In-NLS-ChIgG$_1$ radioimmunoconjugates in a 96-well plate at 37 °C for 24 hours. To determine if the cytotoxic effect was receptor-mediated, AML-5 cells were pre-incubated with 50-fold molar excess (13,300nM) of unlabeled CSL360 before being exposed to radioimmunoconjugates (266 nM, 2.95 MBq). To determine if a longer incubation period increases specific killing, AML-5 cells were
exposed to 266 nM of $^{111}$In-NLS-CSL360 for 4 hours or 24 hours. The cytotoxicity of $^{111}$In-NLS-CSL360 was compared to that of $^{111}$In-CSL360 (266 nM, 2.95MBq). Controls consisted of cells that were cultured in medium only for 24 hours or in medium containing unlabeled CSL360 (266 nM), unlabeled CHX-A^−DTPA-CSL360-NLS (266 nM), $^{111}$In acetate (5.55MBq), and $^{111}$In-NLS-7G3 (266 nM, 3.0MBq). $^{111}$In-NLS-7G3 was constructed as previously described (148).

After incubation, the cells were rinsed twice and resuspended in PBS, pH 7.5. The cells were then resuspended in fresh medium to a concentration of 1-2 x10^4 cells/mL. Special cell culture conditions using complete Methocult medium were used for the clonogenic assays of AML cells exposed to $^{111}$In-NLS-CSL360. Complete Methocult medium was prepared by mixing 40 mL of Methocult-4100 (Stem Cell Technologies, Vancouver, BC) with 39 mL of α-MEM, 10 mL of conditioned media, 10 mL of FBS, and 1mL of pen strep. Approximately 6,000 cells (in 300 µL) were mixed with 2.7 mL of complete Methocult medium. Of this mixture, 1 mL was plated in duplicate into a gridded 35 mm dish (Sarstedt, Montreal, QC). The cells were cultured at 37 °C and 5% CO₂, for 7 to 10 days, and the number of colonies (>50 cells) were counted under a microscope. The clonogenic survival was calculated by dividing the number of colonies formed for treated cells by the number for untreated cells. The experiments were repeated three times.

2.2.7 Statistical Analysis

Error bar represents the standard deviation of a representative experiment performed in triplicate. Data were analyzed for statistical significance using Student’s t-test and one-way analysis of variance (ANOVA). Differences were considered statistically significant at P<0.05.
2.3. Results

2.3.1 Characterization of $^{111}$In-NLS-CSL360

The number of CHX-A’’DTPA incorporated into each molecule of CSL360 was 3.5 ± 0.2 at a CHX-A’’DTPA-to-CSL360 ratio of 30:1 (Table 2.3.1). As determined by SDS-PAGE, the $M_r$ for CSL360 IgG increased from 190 ± 10 kDa to 210 ± 15 kDa following reaction with a 25-fold excess of SMCC and then with a 50-fold molar excess of NLS peptides (Figure 2.3.1A). The NLS-peptide substitution level was determined by $M_r$ analysis on a 6% SDS-PAGE gel (Figure 2.3.1B) or by measuring the proportion of tracer $^{123}$I-labeled NLS peptides incorporated into the mAb. Using the $^{123}$I-NLS incorporation method, the number of NLS peptides on CSL360 was determined to be 6.2 ± 0.1 (Table 2.3.1). The result was close to that determined by $M_r$ analysis on a 6% SDS-PAGE gel (5.0 ± 0.6). Higher molecular-weight dimer and polymer complexes (>300 kDa) were also observed (Figure 2.3.1A), indicating the formation of IgG-crosslinked species upon modification with the maleimide group. The proportion of polymeric forms was not significantly different for CHX-A’’DTPA-CSL360-NLS (15.2% ± 0.2%) than for maleimide-activated CHX-A’’DTPA-CSL360 (14.7% ± 0.3%).

SE-HPLC analyses revealed a high protein purity of the unlabeled CHX-A’’DTPA-CSL360-NLS. The unmodified CSL360 (apparent $M_r$ =190 ± 10 kDa) and the unlabeled CHX-A’’DTPA-CSL360-NLS (apparent $M_r$ = 210 ± 15 kDa) eluted at $t_R$=8.6 mins, whereas the unconjugated NLS-peptide ($M_r$ =1.4 kDa) and $^{111}$In ($M_r$ < 1 kDa) eluted at 13.2 mins and 14.0 mins, respectively (Figure 2.3.1 C and Figure 2.3.1 D). The high molecular-weight IgG-crosslinking species was also detected by SE-HPLC at $t_R$= 6.0 min and percentage of this crosslinking species was about 10% estimated from the area under curve. Radiolabeling of CHX-A’’DTPA-CSL360-NLS with $^{111}$In was efficient with an average specific activity (SA) of 371.5 ±
1.8 MBq/mg and a radiochemical purity of $97.5\% \pm 0.6\%$ determined by instant thin layer chromatography developed in 100 mM sodium citrate, pH 5.0 (Table 2.3.1). The radiochemical purity of $^{111}$In-NLS-CSL360 was confirmed by SE-HPLC analysis. The radioactivity of $^{111}$In-NLS-CSL360 was associated with a retention time consistent with intact CSL360 and the portion of free $^{111}$In was less than 5% (Figure 2.3.1D).

Table 2.3.1: Characteristics of $^{111}$In-NLS-CSL360.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>$^{111}$In-NLS-CSL 360</th>
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<tbody>
<tr>
<td>CHX-A''DTPA/mAb</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>NLS-peptides/mAb ($^{123}$I-NLS method)</td>
<td>6.2 ± 0.1</td>
</tr>
<tr>
<td>Degree of crosslinking</td>
<td>15.2 ± 0.2%</td>
</tr>
<tr>
<td>Specific activity (MBq/mg)</td>
<td>371.5 ± 1.8</td>
</tr>
<tr>
<td>Radiochemical purity</td>
<td>97.5 ± 0.6%</td>
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</tbody>
</table>
Figure 2.3.1: (A) SDS-PAGE using 6% Tris-HCl gel shows a band shift associated with the conjugation of NLS-peptides to CSL360, as indicated by the arrow. Molecular Weight standards (kDa) are indicated at the left. Lane 1, protein ladder; Lane 2, unmodified mAb CSL360; Lane 3, maleimide-activated CHX-A’DTPA-CSL360; Lane 4, NLS-conjugated CHX-A’DTPA-CSL360. Approximately 5 µg of protein was loaded in each lane. Higher molecular-weight dimer and polymer complexes (>300 kDa) were also observed, indicating formation of IgG-crosslinked species upon modification with the maleimide group. The percentage of the crosslinking species was 15.2% ± 0.2% and 14.7% ± 0.3% for maleimido-CHX-A’DTPA-CSL360 and CHX-A’DTPA-CSL360-NLS, respectively. (B) Calibration curve showing the plot of migration distance of the bands on the SDS-PAGE gel relative to the dye front (Rf) versus Log molecular weight (Mw) for the standard reference markers (30-250 kDa). (C) SE-HPLC chromatograms
showing elution profiles of CHX-A’’DTPA-CSL360-NLS [retention time ($t_R$) = 8.6 mins] and free NLS peptides ($t_R$=13.2 mins). This was detected using UV detector at 280 nm. (D) SE-HPLC chromatograms of $^{111}$In-NLS-CSL360 (1 µg/µL, 0.4 MBq) ($t_R$= 8.6 min) and the intermediate immunoconjugates: CHX-A’’DTPA-CSL360-NLS (1.2 µg/µL) ($t_R$= 8.6 min) and unmodified mAb CSL360 (2.5 µg/µL) ($t_R$=8.6 min). The high molecular weight aggregate peak ($t_R$= 6.0 min) and unbound $^{111}$In ($t_R$=14.0 min) were also detected. The radioactivity was detected by Radiomatic flow scintillation analyzer (FSA) detector.
2.3.2 Immunoreactivity for CD123

The immunoreactivity of $^{111}$In-NLS-CSL360 was evaluated using CD123-transfected CHO cells and a saturation receptor-binding assay (Figure 2.3.2A). The dissociation constant ($K_d$) of $^{111}$In-NLS-CSL 360 for binding to the CHO-CD123 cells was $2.49 \pm 0.16$ nM and the maximum binding ($B_{max}$) to $1 \times 10^5$ CHO-CD123 cells was $0.386 \pm 0.005$ pmol. Based on this $B_{max}$ value, the binding site density on the CHO-CD123 cells was $2.32 \pm 0.32 \times 10^6$ receptors per cell. Similarly, saturation receptor-binding assays of $^{111}$In-NLS-CSL 360 conducted on patient-derived AML-5 cells showed that the $K_d$ was $0.92 \pm 0.23$ nM and the $B_{max}$ for approximately $1 \times 10^5$ AML-5 cells, was $0.00463 \pm 0.0003$ pmol (Figure 2.3.2B). The binding site density on AML-5 cells was $2.78 \pm 0.18 \times 10^4$ receptors per cell, which was almost 100-fold lower than that on CHO-CD123 cells.

In competition receptor-binding assays, unmodified CHX-A''DTPA-CSL360 and unlabeled CHX-A''DTPA-CSL360-NLS both competed with $^{111}$In-CSL360 for binding to CHO-CD123 cells (Figure 2.3.2C). The dissociation constants ($K_d$) measured in the competition binding assays were $6.0 \pm 0.5$ nM and $11.1 \pm 0.7$ nM for CHX-A''DTPA-CSL360 and CHX-A''DTPA-CSL360-NLS, respectively. The $K_d$ values were comparable to the previously reported $K_d$ for anti-CD123 murine mAb 7G3, which shares the same binding site as mAb CSL360 ($K_d = 0.9$ nM). These $K_d$ values obtained from both the competition binding assays and saturation binding assays indicated that the binding of CSL360 to CD123 was conserved after modification with CHX-A''DTPA, and NLS-peptides, as well as radiolabeling with $^{111}$In.

The binding of $^{111}$In-CSL360 or $^{111}$In-NLS-CSL360 at a saturating concentration of radiolabeled antibody were compared in Raji-Hi and Raji-Low cells expressing high or low levels of CD123, and Raji-Wild cells lacking CD123. The fractions of cell-bound radioactivity in Raji-
Low and Raji-Wild cells were normalized with respect to that of Raji-Hi cells. As shown in Figure 2.3.2D, the relative binding of $^{111}$In-CSL360 in the Raji-Hi and the Raji-Low cells were 4.4-fold and 3.7-fold higher than that of the Raji-Wild cells (100.0% ± 23.5% and 82.5% ± 25.6% vs. 22.6% ± 7.1%, P<0.05). Similarly, the relative binding of NLS-modified $^{111}$In-CSL360 in Raji-Hi and Raji-Low cells were 3.5-fold and 2.5-fold higher than in Raji-Wild cells (100% ± 2.5% and 70.2% ± 4.2% vs. 28.5 ± 2.5%, P <0.05). This suggested that the binding of $^{111}$In-CSL360 and $^{111}$In-NLS-CSL360 was CD123-specific. The nonspecific uptake of $^{111}$In-CSL360 (22.6% ± 7.1%) and $^{111}$In-NLS-CSL360 (28.5% ± 2.5%) in the wild-type Raji cells were probably due to the presence of Fc receptor on the Raji cells which are known to interact with IgG Fc domain (169). Finally, the flow cytometry analysis confirmed that AML-5 cells were CD123⁺/CD131⁻ (Figure 2.3.2E). The binding of PE-conjugated anti-human CD123 antibodies to AML-5 cells was blocked by an excess of CHX-A’’DTPA-CSL360-NLS, indicating the binding of CHX-A’’DTPA-CSL360-NLS to CD123.
Figure 2.3.2: Saturation receptor-binding assay of $^{111}$In-NLS-CSL360 using CD123-transfected CHO cells (A) or on patient-derived AML-5 cells (B). The total binding curve is obtained by incubating increasing concentrations of $^{111}$In-NLS-CSL360 (0-220 nM for CHO-CD123 cells or 0-20 nM for AML-5 cells) with these cells. The nonspecific binding curve is obtained by repeating the assay in the presence of a 50-fold excess of unlabeled CSL360 (0-11,000 nM for CHO-CD123 cells or 0-1,000 nM for AML-5 cells). The specific binding curve was obtained by subtracting nonspecific binding from the total binding. For CHO-CD123 cells, $K_d = 2.49 \pm 0.16$
nM and $B_{\text{max}} = 0.386 \pm 0.005$ pmol; and for AML-5 cells $K_d = 0.92 \pm 0.23$ nM and $B_{\text{max}} = 0.00463 \pm 0.0003$ pmol. (C) The displacement of binding of $^{111}$In-CSL360 to CD123-transfected CHO cells by increasing concentrations of CHX-A''DTPA-CSL360 (open squares) or CHX-A''DTPA-CSL360-NLS (filled circles). The y-axis is plotted as the fraction of radioligand bound (B) in the presence of the competitors divided by total radioligand bound in the absent of competitors ($B_0$). Both immunoconjugates competed for binding of $^{111}$In-CSL360 to CHO-CD123 cells. The $K_d$ value for CHX-A''DTPA was 6.0 ± 0.5 nM and the $K_d$ value for CHX-A''DTPA-CSL360-NLS was 11.1 ± 0.7 nM, respectively. (D) Comparison of the binding of $^{111}$In-CSL360 or $^{111}$In-NLS-CSL360 (266 nM, 3MBq) to a panel of human Raji lymphoma cells: Raji-Hi and Raji-Low transfected with high or low levels of CD123 and Raji-Wild cells which do not express CD123. The percentage of total radioactivity (%) bound to the Raji-Low cells and Raji-Hi cells were normalized with respect to that bound to Raji-Hi cells. Values shown are the mean ± SEM (standard error of the mean) of triplicate determinations (*significantly different compared to Raji-Wild; P<0.05).
**Figure 2.3.2 (E):** Flow cytometry analysis showing CD123 and CD131 expression on AML-5 cells and binding to CD123 by CHX-A’DTPA-CSL360-NLS. AML-5 cells were incubated with phycoerythrin-conjugated anti-CD123 mouse anti-human fragments (CD123PE, red colour) and phycoerythrin-conjugated anti-CD131PE mouse anti-human fragments (CD131PE, blue colour) for 1 hour. The cells were subsequently rinsed with cold PBS pH 7.0 and analyzed by flow cytometry. The phenotype of AML-5 cells was determined to be CD123⁺ and CD131⁻. To test if the immunofluorescence staining can be blocked by CHX-A’DTPA-CSL360-NLS, AML-5 cells were pre-incubated with 10 µg of unlabeled CHX-A’DTPA-CSL360-NLS on ice for 30 mins before adding CD123PE and CD131PE fragments. Fluorescence intensity of CD123PE was reduced by 10-fold by pre-incubation with these unlabeled immunoconjugates (green colour).

### 2.3.3 Cellular Uptake, Internalization and Nuclear Importation

To further evaluate the CD123-targeting properties of ^11^In-NLS-CSL360 *in vitro*, the cellular uptake of ^11^In-NLS-CSL360 was studied on patient-derived AML cells that share the
CD123\(^+\)/CD131\(^-\) phenotype with LSCs. As shown in Figure 2.3.3A, the uptake of \(^{111}\)In-NLS-CSL360 increased as the radioimmunoconjugate concentration was increased from 33 nM to 266 nM. Incubation at the highest antibody concentration (266 nM) yielded the largest significant difference in the cellular uptake between \(^{111}\)In-NLS-CSL360 (3.3 ± 0.1 pmol) and the nonspecific uptake of the isotype-matched control \(^{111}\)In-NLS-ChIgG\(_1\) (0.8 ± 0.1 pmol). This difference was decreased to 0.4 pmol at 133 nM but remained significant, whereas at concentrations lower than 133 nM, the differences between the binding of \(^{111}\)In-NLS-CSL360 and \(^{111}\)In-NLS-ChIgG\(_1\) were not significant. Therefore, 266 nM was considered the optimal concentration for subsequent experiments. More importantly, the cellular uptake of \(^{111}\)In-NLS-CSL360 was shown to be CD123-specific in a panel of AML cells. The amount of radioimmunoconjugate binding to \(1 \times 10^6\) AML 3, AML 4 and AML 5 cells (0.72 ± 0.68 pmol, 0.67 ± 0.14 pmol, and 0.59 ± 0.22 pmol, respectively) was significantly higher than for \(^{111}\)In-NLS-ChIgG\(_1\) (0.38 ± 0.12 pmol, 0.24 ± 0.01 pmol, 0.22 ± 0.01 pmol) (all \(P<0.05\)) (Figure 2.3.3B).

Prolonged radioimmunoconjugate incubation yielded significantly greater cellular uptake in AML-5 cells. The uptake of \(^{111}\)In-CSL360 and \(^{111}\)In-NLS-CSL360 by \(1 \times 10^6\) AML-5 cells increased significantly from 0.46 ± 0.06 pmol and 0.59 ± 0.01 pmol, respectively, after 4 hours of incubation to 1.2 ± 0.10 pmol and 3.3 ± 0.10 pmol, respectively, after 24 hours (\(P<0.05\)) (Figure 2.33C). In comparison to unmodified \(^{111}\)In-CSL360, \(^{111}\)In-NLS-CSL360 had a significantly higher cellular uptake after 24 hours of incubation (3.3 ± 0.10 pmol vs. 1.2 ± 0.10 pmol, \(P<0.05\)), whereas this difference did not occur at 4 hours. The uptake of radioactivity was blocked in the presence of excess unlabeled CSL360, demonstrating that uptake was CD123-mediated (Figure 2.33C).
Subcellular fractionation was performed to measure the amount of radioactivity deposited by $^{111}$In-NLS-CSL360 in different cell compartments, especially the nucleus where the Auger electron radiation is the most effective in causing DNA damage. As shown in Figure 2.3.3D, the cytoplasmic and nuclear fraction of $^{111}$In-NLS-CSL360 increased as the radioimmunoconjugate concentration increased. The cytoplasmic fractions of $^{111}$In-NLS-CSL360 contained 2.2-fold significantly higher amounts of radioactivity than that of $^{111}$In-NLS-ChIgG$_1$ at both the 133 nM (0.47 ± 0.14 pmol vs. 0.20 ± 0.10 pmol, P<0.05) and 266 nM concentrations (1.55 ± 0.05 pmol vs. 0.69 ± 0.05 pmol, P<0.05). Similarly, in comparison with the irrelevant radioimmunoconjugates, the nuclear uptake of $^{111}$In-NLS-CSL360 was 3.7-fold significantly higher at 133 nM concentration (0.12 ± 0.03 pmol vs. 0.03 ± 0.01 pmol, P<0.05) and 5.1-fold significantly higher at 266 nM (0.70 ± 0.14 pmol vs.0.14 ± 0.01 pmol, P<0.05), respectively. Moreover, the nuclear uptake of $^{111}$In-NLS-CSL360 was blocked in the presence of an excess of unlabeled CSL360, whereas the blocking effect was not observed for the irrelevant $^{111}$In-NLS-ChIgG$_1$ radioimmunoconjugates (Figure 2.3.3E). This clearly demonstrated that the nuclear uptake of $^{111}$In-NLS-CSL 360 was CD123-specific.

As mentioned previously, prolonged radioimmunoconjugate incubation resulted in a significantly increased cellular uptake of $^{111}$In-NLS-CSL360 in AML-5 cells at 24 hours of incubation but not with 4 hours incubation. Similarly, higher nuclear and cytoplasmic uptake of $^{111}$In-NLS-CSL360 was observed at 24 hours than at 4 hours (Figure 2.3.3F). At 24 hours, the antibody-equivalent nuclear uptake of $^{111}$In-NLS-CSL360 by $1 \times 10^6$ AML-5 cells was 7.0-fold significantly higher than that seen at 4 hours (0.70 ± 0.04 pmol vs. 0.10 ± 0.01 pmol P<0.05). The corresponding amounts in the nucleus for $^{111}$In-CSL360 without NLS modification were 0.10 ± 0.02 pmol at 4 hours and 0.35 ± 0.03 pmol at 24 hours (Figure 2.3.3F). Therefore, in comparison
with $^{111}$In-CSL360, NLS-modification achieved 2.0-fold significantly increased nuclear uptake at 24 hours (P <0.05). Interestingly, $^{111}$In-NLS-CSL360 had also significantly higher distribution to the cell membrane and in the cytoplasm when compared to $^{111}$In-CSL360 at 24 hours (1.2 ± 0.09 pmol vs. 0.53 ± 0.07 pmol, P< 0.05), but this difference was not seen at the 4 hour incubation time point (P> 0.05) (Figure 2.3.3F).

![Graph](image)

**Figure 2.3.3(A):** Cellular uptake of $^{111}$In-NLS-CSL360 on AML-5 cells at increasing concentrations after 24 hours of incubation. 1x $10^6$ AML-5 cells were incubated with $^{111}$In-NLS-CSL360 at concentrations ranging from 33 nM (0.37 MBq) to 266 nM (2.95 MBq). After 24 hours of incubation, the cells were centrifuged and rinsed three times with ice-cold PBS to remove unbound radioactivity. The cell-bound cpm was measured in a γ-counter. From the specific activity of the radioimmunoconjugate used, and correcting for the percentage of the...
unbound $^{111}$In (<5%), the amount bound to $1 \times 10^6$ cells was calculated and expressed in picomoles (pmol). The isotype-matched irrelevant $^{111}$In-NLS-ChIgG$_1$ radioimmunoconjugate was used as a control. Values shown are the mean ± SEM of triplicate determinations. Differences in the cellular uptake of the $^{111}$In-NLS-CSL360, relative to the isotype matched control that reached statistical significance (P<0.05) are indicated by an asterisk (*).

Figure 2.3.3 (B): Cellular uptake of $^{111}$In-NLS-CSL360 in a panel of AML cells: AML-3, AML-4, and AML-5 cells. $1 \times 10^6$ AML cells were incubated with $^{111}$In-NLS-CSL360 (266 nM, 2.95 MBq) in 200 µL of PBS at 37 °C for 4 hours. The cell-bound cpm was measured in a γ-counter. From the specific activity of the radioimmunoconjugate used, and correcting for the percentage of unbound $^{111}$In (<5%), the amount bound to $1 \times 10^6$ cells was calculated and expressed in picomoles (pmol). The amount of $^{111}$In-NLS-CSL 360 bound to $1 \times 10^6$ cells were compared to that of isotype-matched $^{111}$In-NLS-ChIgG$_1$ irrelevant radioimmunoconjugates. Values shown are the mean ± SEM of triplicate determinations. Differences in the cellular uptake of $^{111}$In-NLS-
CSL360 relative to the isotype matched control reaching statistical significance (P<0.05) are indicated by an asterisk (*).

Figure 2.3.3 (C): Comparison of the cellular uptake of \(^{111}\text{In}\)-NLS-CSL360 or \(^{111}\text{In}\)-CSL360 by AML-5 cells at 4 hours and 24 hours of incubation. 1 x 10^6 AML-5 cells were incubated at 37°C with 200 μL PBS (pH 7.5) containing 266 nM (2.95 MBq) of \(^{111}\text{In}\)-NLS-CSL360 or \(^{111}\text{In}\)-CSL360 for 4 hours or 24 hours. For the 24 hours incubation period, the cellular uptake was also performed in the presence of an excess of unlabeled CSL360 (13,300 nM; blocked). After incubation, the cells were centrifuged and then rinsed three times with ice-cold PBS to remove unbound radioactivity, and the cell-bound radioactivity was measured. From the specific activity of the radioimmunoconjugates used, and correcting for the percentage of the unbound \(^{111}\text{In}\) (<5%), the amount bound to 1 x 10^6 cells was calculated and expressed in picomoles (pmol).
Values shown are the mean ± SEM of triplicate determinations. Differences in the cellular uptake of radioimmunoconjugates at 24 hours relative to 4 hours or with or without blocking, reaching statistical significance (P<0.05) are indicated by an asterisk (*).

**Figure 2.3.3 (D):** Cytoplasmic and nuclear accumulation of $^{111}$In-NLS-CSL360 in AML-5 cells incubated in the presence of increasing concentrations (33 nM-266 nM) of $^{111}$In-NLS-CSL360 or isotype-matched irrelevant $^{111}$In-NLS-ChIgG1 radioimmunoconjugates for 24 hours at 37 °C. After incubation, subcellular fractionation was performed to isolate the membrane and cytoplasm fraction and nuclear fraction of radioactivity. Radioactivity associated with these different cellular compartments was measured and expressed as amount of radioimmunoconjugates (pmol) calculated from the specific activities. Values shown are the mean ± SEM of triplicate determinations. The differences in the cytoplasmic fractions and nuclear fractions of $^{111}$In-NLS-CSL360, relative to the isotype matched control, reaching statistical significance (P<0.05) are indicated by an asterisk (*).
Figure 2.3.3 (E): Comparison of amount of radioimmunoconjugates in the nucleus of AML-5 cells incubated with 266 nM of $^{111}$In-NLS-CSL360 or $^{111}$In-NLS-ChIgG$_1$ with or without blocking with a 50-fold excess of unlabeled CSL360 (13,300 nM) in the incubation medium. After incubation, cellular fractionation was performed to isolate the nuclear radioactivity. Nuclear radioactivity was measured in a γ-counter and expressed as the amount of antibody (radioimmunoconjugates) accumulated in the nucleus. Values shown are the mean ± SEM of triplicate determinations. Differences in the nuclear uptake of the “no blocking” group relative to “blocked” group reaching statistical significance (P<0.05) are indicated by an asterisk (*).
Figure 2.3.3 (F): Comparison of the cytoplasmic and nuclear radioactivity fractions of $^{111}$In-NLS-CSL360 (266 nM, 2.95MBq) and $^{111}$In-CSL360 (266 nM, 2.95MBq) incubated with AML-5 cells for 4 hours vs. 24 hours. After incubation, the cells were rinsed three times with cold PBS, pH 7.5 to remove unbound radioactivity. The cells were then lysed with cell membrane lysis buffer and the cytoplasmic fraction was extracted by addition of cytosol extraction buffer. The cells were centrifuged again to separate the cytoplasmic (membrane and cytoplasm) radioactivity (supernatant) from the nuclear radioactivity (pellet). Cytoplasmic and nuclear radioactivity was measured in a $\gamma$-counter and expressed as the amount of antibody (radioimmunoconjugates) based on the specific activity. Significant differences between $^{111}$In-NLS-CSL360 and $^{111}$In-CSL360 at 4 hours or 24 hours are indicated by the double asterisk (**, $P<0.05$). Differences in radioactivity in the cytoplasmic fraction and nuclear fraction, at 24 hours relative to that at 4 hours, reaching statistical significance are indicated by an asterisk (*$P<0.05$).
2.3.4 *In vitro* Cytotoxicity of $^{111}$In-NLS-CSL360

Clonogenic progenitors for patient-derived AML-5 cells can be assayed on the basis of colony formation in methycellulose cultures. The clonogenic survival (CS) of AML-5 cells was determined following treatment with increasing concentrations of the radioimmunoconjugates for various incubation periods. As shown in Figure 2.3.4A, the CS of AML-5 cells was inhibited by $^{111}$In-NLS-CSL360 in a dose-dependent manner, with the maximum inhibition found at the highest concentration tested (266 nM). At 266 nM, $^{111}$In-NLS-CSL 360 decreased the survival of AML-5 cells by more than 70% (CS = 27.5% ± 4.1%) and was 2.0-fold significantly more potent than the nonspecific, irrelevant $^{111}$In-NLS-ChIgG1 immunoconjugates (CS = 60.0% ± 3.6%, P<0.05). At lower concentrations (< 266nM), there were no apparent significant differences in the cytotoxicity of CSL360 and the irrelevant control radioimmunoconjugates (Figure 2.3.4A). This inhibition of the survival of AML-5 cells treated with $^{111}$In-NLS-CSL360 was reduced to approximately 10% when the cells were pre-incubated with an excess of unlabeled CSL360 (CS = 90.0% ± 8.5%), whereas no significant change was observed for the pre-incubation with the irrelevant control radioimmunoconjugates (P>0.05) (Figure 2.3.4 B). This clearly demonstrated that the decreased CS at a concentration of 266 nM was specific for CD123. The effect of incubation time on the survival of AML cells was also studied (Figure 2.3.4C). The CS of AML-5 cells exposed to 266 nM of $^{111}$In-NLS-CSL360 was 82.1% ± 13.1% after 4 hours of incubation and 27.5% ± 4.1% after 24 hours, demonstrating 3.0-fold increased cytotoxicity by the radioimmunoconjugate with a prolonged incubation period. In comparison with $^{111}$In-CSL360, NLS-modification achieved 1.5-fold greater inhibition on survival of cells at 24 hours (CS = 27.5% ± 4.1% vs. 50.0% ±16.4%, P<0.05).
To show that the cytotoxicity observed was a result of radiation delivered to AML cells, control experiments were done with unlabeled antibody CSL360 and unlabeled immunoconjugates CHX-A”DTPA-CSL360-NLS, at 266 nM, and with free $^{111}$In-acetate, 5.55MBq, which is higher than the highest amount of radioactivity that was used. The CS of AML-5 cells exposed to unlabeled CSL360, unlabeled CHX-A”DTPA-CSL360-NLS, and free $^{111}$In-acetate was 111.0% ± 14.7%, 128.0% ± 12.2% and 90.0% ± 11.6%, respectively (Figure 2.3.4D). The CS of these control groups was significantly higher than for cells exposed to $^{111}$In-NLS-CSL360 (27.5% ± 4.1%) or $^{111}$In-NLS-7G3 (36.8% ± 3.0%, P<0.05). Finally, the cytotoxicity of $^{111}$In-NLS-CSL360 was compared with that of $^{111}$In-NLS-7G3, the murine form of these radioimmunoconjugates, and no significant difference was observed between the two groups (P>0.05).
Figure 2.3.4 (A): Clonogenic survival of AML-5 cells exposed to increasing concentrations of $^{111}$In-NLS-CSL360 (0.4 nM to 266 nM, 0.04-2.95MBq). Percentage of clonogenic survival was determined by dividing the number of colonies formed for cells exposed to $^{111}$In-NLS-CSL360 by the number of colonies formed for untreated cells. The cytotoxicity of $^{111}$In-NLS-CSL360 was compared to that of $^{111}$In-NLS-ChIgG$_1$ (irrelevant radioimmunoconjugates). (B) Comparison of the clonogenic survival of AML-5 cells exposed to $^{111}$In-NLS-CSL360 (266 nM) or $^{111}$In-NLS-ChIgG$_1$ (266 nM) for 24 hours in the presence (blocked) or absence (no blocking) of an excess of unlabeled CSL360 (13,300 nM) in the medium. Values shown are the mean ± SEM of triplicate determinations. Significant differences in the clonogenic survival between “blocked” and “no blocking” are indicated by an asterisk (P<0.05).
Figure 2.3.4 (C): Comparison of the clonogenic survival of AML-5 cells treated with 266 nM (2.95 MBq) of $^{111}$In-CSL360 and $^{111}$In-NLS-CSL360 after 4 hours and 24 hours incubation. At the 24 hours incubation period, both radioimmunoconjugates showed significantly higher cytotoxicity on AML-5 cells. In addition, $^{111}$In-NLS-CSL360 showed a significantly higher cytotoxicity than $^{111}$In-CSL360 without NLS peptide modification. Values shown are the mean ± SEM of triplicate determinations. Significant differences are indicated by an asterisk ($P < 0.05$) (*).
Figure 2.3.4 (D): Comparison of the clonogenic survival of AML-5 cells treated with unlabeled CSL360, CHX-A”DTPA-CSL360-NLS, $^{111}$In-acetate (5.55MBq), $^{111}$In-NLS-7G3 (2.95MBq) or $^{111}$In-NLS-CSL360 (2.95MBq) after 24 hours incubation period. The concentration of the antibody incubated for all immunoconjugates was 266 nM. $^{111}$In-NLS-CSL360 and $^{111}$In-NLS-7G3 showed a significantly higher cytotoxicity than the control treatments in AML-5 cells. Values shown are the mean ± SEM of triplicate determinations. Significant differences are indicated by an asterisk (P< 0.05) (*).
2.4 Discussion

The results of this study demonstrated that chimeric IgG\textsubscript{1} anti-CD123 mAb CSL360 labeled with the Auger electron-emitter \(^{111}\text{In}\) was toxic to AML cells that share the CD123\textsuperscript{+}/CD131\textsuperscript{−} phenotype of LSCs. Furthermore, the nuclear translocation and cytotoxicity of \(^{111}\text{In-}	ext{CSL360}\) was enhanced by its conjugation to synthetic 13-mer peptides (CGYGPKKKRKVGG) harboring the NLS-sequence of SV-40 large T-antigen (underlined). The cytotoxicity of \(^{111}\text{In-NLS-CSL360}\) was shown to be CD123-specific and was correlated with the delivery of radioactivity to the nucleus that was facilitated by the NLS-peptides.

The poor survival of AML patients and high relapse rates raises the prospect that LSC-targeted therapies might be able to attain long-lasting remissions and potentially cure these patients of this disease. AML-LSCs can be targeted with the CD123-specific CSL360 mAb and its murine form, 7G3 mAb. These anti-CD123 mAbs are attractive for their potential dual benefit of being a CD123-blocking antibody and mediating antibody-dependent cell-mediated cytotoxicity (ADCC) against leukemic cells. Jin et al. reported 7G3 treatment was most effective in reducing AML-LSC engraftment under conditions where the leukemic burden was low. This study found that administering 7G3 at 4 weeks post-transplantation in mice with established AML, impaired bone marrow engraftment in only two out of the five primary AMLs (93). In addition, CSL360 produced no definite clinical benefit in relapsed and refractory high-risk AML patients. Out of the 11 patients who received 12 doses of 0.1 to 10 mg/kg of CSL360, only one complete response (CR) has been observed (105). Taken together, LSC-targeted therapies with naked anti-CD123 mAbs may be insufficient. This suboptimal killing of leukemic cells was believed to be due to low levels of CD123 expressed on the surface of the LSCs (98). In this study, the cytotoxicity of these naked CSL360 mAbs was enhanced by conjugating to an Auger electron emitter, \(^{111}\text{In},\)
particularly if the $^{111}$In-anti-CD123 mAbs were imported into the nucleus, where the electrons are most damaging to DNA.

$^{111}$In-CSL360 reduced the CS of AML-5 cells in vitro by 50% at a prolonged incubation period (24 hours) and further down to less than 30% when modified with NLS-peptides (Figure 2.3.4C). Moreover, the enhanced cytotoxicity of $^{111}$In-NLS-CSL360 was correlated with 2.0-fold higher nuclear uptake than $^{111}$In-CSL360 (Figure 2.3.3F). This clearly demonstrated that $^{111}$In-NLS-CSL360 was more potent at killing AML-5 cells and that the increased cytotoxicity was mainly due to the higher amount of radioactivity delivered to the nucleus by NLS-peptides. When compared to its murine form $^{111}$In-NLS-7G3, $^{111}$In-NLS-CSL360 exhibited equivalent cytotoxicity and in both cases it was primarily an effect of the CD123-specific radiation delivered, as shown by results obtained with the unlabeled antibodies and unlabeled immunoconjugates as well as noninternalized $^{111}$In, which produced either apparent growth stimulation, or a small insignificant reduction in the CS (Figure 2.3.4D). These findings are consistent with our previous report in which NLS-modified $^{111}$In-7G3 diminished the viability of AML-5 cells to less than 30%, and exhibited increased unrepaired DNA double-strand breaks than the unmodified $^{111}$In-7G3 (148).

On the other hand, decreased survival of AML cells treated with $^{111}$In-NLS-ChIgG$_1$ was observed in this study (Figure 2.3.4A), particularly at lower concentrations (33 to 133 nM). Nonetheless, at the higher concentration (266 nM), there was a significantly decreased CS with $^{111}$In-NLS-CSL360 than the chimeric control radioimmunoconjugates. Also, the cytotoxicity of $^{111}$In-NLS-CSL360 could be diminished by pre-treating the cells with an excess of unlabeled CSL360 to block uptake of the radioimmunoconjugates (Figure 2.3.3C), whereas blocking was ineffective for decreasing the cytotoxicity of the chimeric control radioimmunoconjugates (Figure
2.3.4B), indicating that the toxicity of $^{111}$In-NLS-CSL360 was specific for CD123. The nonspecific cytotoxicity of $^{111}$In-NLS-ChIgG$_1$ was correlated with cellular uptake and cytoplasmic internalization but not nuclear uptake at 24 hours of incubation. The observed cytotoxicity of $^{111}$In-NLS-ChIgG$_1$ on AML-5 cells might be due to interaction of the Fc-domain on the radioimmunoconjugates with the Fc gamma receptor expressed on AML-5 cells. For example, CD16, which is identified as an Fc gamma receptor and is normally expressed on natural killer cells, was found on AML cells with M5 subtype (156). Interaction with CD16 might be the cause of nonspecific cellular uptake and cytotoxicity of $^{111}$In-NLS-ChIgG$_1$. Nevertheless, Auger electron RIT with $^{111}$In-NLS-CSL360 is not expected to be severely limited by normal-tissue toxicity, because CD123-mediated internalization and NLS-promoted nuclear importation are required for manifestation of the cytotoxic effects of the Auger electrons.

$^{111}$In-NLS-CSL360 was constructed with a high protein homogeneity and radiochemical purity and preserved immunoreactivity for CD123. As illustrated in the scheme for NLS-attachment to the antibody (Figure 2.2.2), the lysine residues on the antibody are first modified via reaction with SMCC to obtain the intermediate maleimido-CSL360-CHX-A''DTPA. In the subsequent step, the maleimide of the intermediate species reacts with the free thiol of cysteine on the C-terminal of the NLS-peptide via a Michael addition mechanism (157, 158). However, the existence of a small proportion of higher molecular-weight dimer or polymer complexes indicated that IgG-crosslinked species were formed upon modification with maleimide (Figure 2.3.1A). Nonetheless, apparently the formation of these cross-linked species did not diminish CD123 immunoreactivity or internalization and nuclear importation in AML cells (Figure 2.3.3B and Figure 2.3.3E). One explanation for this aggregation would be the formation of intermolecular cross-links involving the maleimidyl moiety and the side chains of nucleophilic amino acids on the
antibody molecule, such as cysteine, serine or tyrosine (157). Although optimization of the conjugation reaction to diminish cross-linking was not performed, further studies would be useful to explore possible ways to reduce the amount of aggregation formation which has been associated with nonspecific binding and nontargeted cytotoxicity for other antibodies (159). In this study, additional modification of CSL360 with multiple NLS-peptides did not have a major deleterious effect on its ability to bind CD123. The $K_d$ value for CSL360 substituted with 6 NLS-peptides ($11.1 \pm 0.7$ nM) was not significantly different than that of $^{111}$In-CSL360 without NLS-peptides ($K_d= 6.0 \pm 0.5$ nM) (Figure 2.3.2C). Furthermore, the specificity of $^{111}$In-CSL360 and $^{111}$In-NLS-CSL360 for CD123-positive leukemia cells was illustrated by their differential binding toward the Raji-Hi (2.44 $\times 10^4$ receptors per cell), Raji-Low (0.48 $\times 10^4$ receptor per cell) and wild-type Raji lymphoma cells (lacking CD123) (Figure 2.3.2D), respectively. In addition, the specificity of $^{111}$In-NLS-CSL360 was further demonstrated by its higher uptake in a panel of AML cells than the nonspecific irrelevant $^{111}$In-NLS-ChIgG$_1$ radioimmunoconjugates (Figure 2.3.3 B) as well as the blocking effect of an excess of the intact unlabeled antibody on its cellular uptake (Figure 2.3.3C).

An important aspect of this study was the examination of the CD123-mediated internalization and subsequent NLS-peptide mediated nuclear importation of $^{111}$In-NLS-CSL360 at various concentrations and incubation periods. At a saturating antibody concentration, a prolonged incubation period (24 vs. 4 hours) markedly increased the cellular uptake and nuclear importation of $^{111}$In-NLS-CSL360 compared to that of $^{111}$In-CSL360 without NLS-peptides (Figure 2.3.3C and Figure 2.3.3.F). These findings are in agreement with previous observations that these NLS-peptides were able to mediate the translocation of $^{111}$In-labeled trastuzumab molecules into the nuclei of HER2/neu-expressing breast cancer cells after their receptor-
mediated-internalization (144), or that NLS-peptide conjugation promoted the nuclear translocation of $^{111}$In-anti-CD33 mAb HuM195 in HL-60 leukemic cells (115). Furthermore, the increased cellular uptake of $^{111}$In-NLS-CSL360 and $^{111}$In-CSL360 with a prolonged period of incubation (Figure 2.3.3C) may be due to the recycling and repopulation of CD123 epitopes on the surface of the cells due to *de novo* synthesis, which may allow more $^{111}$In-NLS-CSL360 to bind, internalize and be transported into the nucleus, resulting in enhanced cytotoxicity seen at 24 hours incubation. Griffiths *et al.* reported that recycling of receptors enhanced the cytotoxicity of $^{111}$In-anti-CD74 mAb on lymphoma cells (160). Other argued that the enhanced therapeutic response could be caused by nuclear localization of the interleukin receptors which have been found in the nucleus (161). Elucidation of these mechanisms would require more detailed analysis of AML cells and time-course of CD123 expression over a prolonged incubation period.

The inability of $^{111}$In-NLS-CSL360 to inhibit the survival of the AML cells to by more than 3-fold at a saturating concentration was probably due to the low expression level of CD123. The expression level of CD123 on AML-5 cells was $2.71 \pm 0.18 \times 10^4$ receptors/cell, based on the $B_{\text{max}}$ value obtained from the direct saturation binding assay (Figure 2.3.2A). This number is close to the number of CD123 (0.19-1.4 x10$^4$ sites per cell) on four CD123-positive cells reported by Du *et al.* or on patient primary AML cells (1.1-2.1x10$^4$ sites/cell) by Feuring-Buske *et al* (162). As previous studies have shown, the cytotoxicity of NLS-modified $^{111}$In-labeled mAbs was directly corrected with the receptor expression densities of the cells, and the Auger electron radiation approach was the most effective for cells that express high levels of receptors (115, 144, 145, 161). Future studies should therefore explore the effect of increased specific activity on the cytotoxicity of $^{111}$In-NLS-CSL360 since this could deliver more $^{111}$In per receptor recognition
event. Moreover, the potency of $^{111}$In-NLS-CSL360 should also be evaluated on a panel of primary AML samples which usually have heterogeneous CD123 expressions.

2.5 Conclusion

$^{111}$In-NLS-CSL360 was successfully constructed with retained immunoreactivity to CD123 and was specifically bound, internalized, and translocated to the nucleus of AML cells that share the CD123$^+/CD131^-$ phenotype of LSCs. NLS delivery enhanced the cytotoxicity of the Auger electrons emitted by $^{111}$In-NLS-CSL360. Substantially decreased CS was achieved in AML cells by exposure to $^{111}$In-NLS-CSL360. These results suggest that $^{111}$In-NLS-CSL360 could be a promising Auger electron RIT agent for treatment of AML.
Chapter 3

Overall Discussion and Future Directions
3.1 Research Implications for Auger Electron RIT Targeting Leukemic Stem Cells (LSC)

3.1.1 Auger Electron RIT Overcomes AML Multidrug Resistance

One reason for the relative ineffectiveness of current AML chemotherapy regimens is the acquisition of multidrug resistance (MDR). Most conventional chemotherapy only targets the rapidly proliferating leukemic blast cells, while sparing the quiescent LSCs that take refuge in the bone marrow. As described in Chapter 1, LSCs are niche-dependent and sheltered in a neoplastic niche bearing resemblance to its normal hematopoietic stem cell (HSC) counterpart. Moreover, LSCs express a number of membrane transporters with broad specificity that are linked to drug resistance and thus escape from the cytotoxic effects of chemotherapy. These drug transporters have been found to be highly expressed on both normal HSCs and LSCs but not in the committed progenitor cells. Extrusion of chemotherapeutic drugs by the adenosine triphosphate (ATP)-dependant drug efflux pumps (ATP-binding Cassette [ABC] transporters) is commonly implicated in MDR. Some of the ABC transporters are expressed in immature CD34+/CD38- sub-populations but are down-regulated when differentiated into the more committed CD34+/CD38+ sub-populations (53, 64, 163). One study has shown that 22 of the 49 ABC transporters were differentially expressed in AML LSCs versus AML blasts, and all were expressed at higher level in the CD34+/CD38- cells in comparison with the CD34+/CD38+ cells (163, 164). The common subtypes of the ABC family are MDR1, BCRP1 (breast cancer resistance protein) and the multidrug resistance associated protein (MRP1) (164).

Because the chemotherapy-resistant AML LSCs are believed to underlie the disease relapse, Auger electron radioimmunotherapy (RIT) specially targeting these cells could provide a new opportunity to potentially circumvent MDR by delivering the low-energy radiation to the
tumour cells and possibly achieve remission in patients who are refractory to chemotherapy, which is a major obstacle in the effective clinical management of this disease. The study by Kersemans et al. demonstrated that the Auger electron emissions from the $^{111}$In-labeled anti-CD33 murine monoclonal antibody (mAb) M195 and humanized mAb HuM195 modified with NLS peptides significantly diminished the survival in vitro of mitoxantrone-resistant HL-60-MX-1-myeloid leukemia cells as well as a panel of primary AML specimens that express ABC transporters (168). As discussed in Chapter 2, $^{111}$In-labeled anti-CD123 mAb CSL360 modified with NLS peptides was shown to inhibit the clonogenic survival of AML cells that share LSC phenotype (CD123$^+$/CD131$^-$).

With the discoveries of new surface proteins or markers that are highly expressed on LSCs, Auger electron RIT recognizing these molecules as drug targets would remain an attractive approach to overcome MDR in AML treatment. For example, two promising surface proteins-CD32 and CD25, the alpha chain of the interleukin-2 receptor, are more frequently expressed in LSCs than others in over half of the 61 samples of cells from AML patients (53). They remain on the LSCs even after treatment with the common chemotherapeutic drug cytarabine. Therefore, the development of new Auger electron RIT agents targeting these two new markers may be feasible to overcome MDR. In addition, it is also suggested that the use of Auger electron RIT in combination with chemotherapeutic drugs could improve the effectiveness of treatment, than when using chemotherapy alone, as Auger electron RIT could potentially eradicate the remaining LSCs that survive chemotherapy.
3.1.2. Auger Electron RIT Improves the Targeting and Detection of Minimal Residual Disease

The existence of LSCs could also explain the cause of minimal residual disease (MRD) and subsequent AML relapse. Most patients who attain CR are thought to harbour residual leukemia cells, a clinical indication of MRD. These residual leukemia cells survive conventional chemotherapy and have disease-sustaining capacity (165). Given the possible intrinsic resistance properties of LSCs, it is likely that the residual leukemia cells reside in the pool of LSCs and eventually cause AML relapse. The primary reason why residual leukemia cells can survive chemotherapy is drug resistance and impaired apoptosis-related mechanisms caused by aberrant signal transduction pathways. As mentioned in Chapter 1, the same mechanisms probably contribute to the survival of the LSCs. Monitoring LSC-associated properties would provide a direct link between MRD detection and prediction of potential relapse of leukemia (166). Indeed, evidence indicates that increased MRD correlate with the LSCs frequency and lower survival rates after treatment in patients (167). Therefore, it may be important to detect LSCs after chemotherapy to predict relapses prior to initiating clinical management of patient-adapted post-remission therapies. In addition to its potential as a RIT agent to eradicate MRD, $^{111}$In-NLS-CSL360 is also useful for single photo emission computed tomography (SPECT) and will provide improved detection of LSCs in MRD through SPECT imaging, as discussed below.

The origin of the LSC and its role in AML relapse has implications in leukemia therapy and more importantly, has great impact on the current perception regarding therapeutic options. In Chapter 1, the current therapeutic strategies that mainly focus on inhibiting the molecular pathogenesis of leukemia and target the bulk cells that make up the majority of the disease was discussed. As the concept and experimental evidence for LSCs emerges, research is now focusing
more on targeting LSCs, since this will not eradicate the bulk tumour cells as well as the malignant stem cells that sustain these bulk populations. Given the similarity between HSCs and LSCs, challenges remain to develop novel agents that spare the normal HSCs while exerting the desired effect on LSCs. In most of the cases, the development of such agents against LSCs relies on biological features that distinguish LSCs from HSCs. As described in Chapter 1, novel agents targeting the LSC-specific cell cycle, drug resistance mechanism or microenvironment would be one of the potential strategies to interfere with LSC survival mechanisms and overcome resistance; however, the challenge of this approach remains that it must not disrupt the delicate relationships between the niche and normal hematopoietic cells. Another approach to LSC-targeted therapies is to exploit the aberrant cell surface antigen expression on LSCs in comparison with HSCs. In addition, monoclonal antibodies targeting several of these LSC-specific antigens have been developed and investigated in recent clinical trials.

Taking advantages of these unique properties and the known biology of LSCs, the strategy of using an Auger electron-emitting RIT agent for AML presented in this thesis could be one promising strategy to eradicate the MRD while sparing the normal HSCs. As shown in Chapter 2, $^{111}$In-NLS-CSL360 is an Auger electron-emitting RIT that specifically targets AML cells that display CD123$^+$/CD131$^-$ LSC phenotype and demonstrates specific cytotoxicity for human AML cells. This study provided the first preclinical evaluation of $^{111}$In-NLS-CSL360 for treatment of AML, and if successful in future studies, this could ultimately provide a new RIT agent for treating AML patients.
3.2 Future Directions

In addition to its potential as a RIT agent, $^{111}$In-NLS-CSL360 is useful for single photon emission computed tomography (SPECT). SPECT imaging is performed by using a gamma-camera that acquires images by capturing gamma photos emitted by $^{111}$In to form three-dimensional images. As previously shown by Leyton et al. (148), SPECT imaging of $^{111}$In-NLS-7G3 was able to visualize s.c. Raji-CD123 tumour xenografts as well as AML cells engrafted into the bone marrow, spleen and at extramedullary sites in NOD/SCID mice. This suggests that as its chimeric form, $^{111}$In-NLS-CSL360 would similarly be useful as an imaging agent to detect the CD123$^+$ cells as well as monitoring therapeutic response.

In attempt to study its *in vivo* targeting properties, I had collaboratively conducted a preliminary study that looked at the ability of microSPECT/CT imaging with $^{111}$In-NLS-CSL360 to localize in Raji-CD123 xenografts. Raji-CD123 tumours were established by s.c. inoculation of $5 \times 10^6$ cells into NOD/SCID mice. Tumour-bearing mice were imaged on a Nano-SPECT/CT tomography. Mice were injected i.v. (tail vein) with 66-257 ug (2.72-3.91 MBq) of $^{111}$In-NLS-CSL360. Tomographic images were obtained for a total of 50,000 counts per frame (mice injected typically a 200-count per second rate) for 16 frames (66.7 min/mouse scan time). Images were reconstructed using In Vivo Scope software v1.40 (Bioscan). The uptake of radioactivity in blood, tumour, and normal organs were measured at 72 h post injection (p.i). This study was conducted in collaboration with Dr. Leyton, a post-doctoral fellow in Dr. Reilly’s laboratory. The preliminary study showed that Raji-CD123 tumours in NOD/SCID mice could be visualized with $^{111}$In-NLS-CSL360 at 72 h p.i (Figure 3.2). The tumour uptake at 72 h p.i. was $2.77 \pm 1.17 \%$ ID/g and the tumour-to-blood ratio was 4.17 (Table 3.2). High spleen and liver uptake ($4.88 \pm 0.49 \%$ID/g, $14.33 \pm 3.72 \%$ID/g) at 72 h p.i. could be caused by sequestration of mAbs of this
isotype by FcγRI receptors in the liver and spleen. Further studies are required to improve the tumour uptake of $^{111}$In-NLS-CSL360 by pre-blocking the nonspecific site with unlabelled CSL360 prior to the injection of radioimmunoconjugates. This study would provide important preclinical evaluation of $^{111}$In-NLS-CSL360 for future studies of AML engrafted into NOD/SCID mice with microSPECT/CT tool used to monitor the response to these RIT agents. Ultimately, this new RIT agent could be used to more effectively treat AML as well as more invasively follow response to treatment.

Figure 3.2 Coronal micro-SPECT/CT images of subcutaneous Raji-CD123 xenografts (solid arrow) in NOD/SCID mice injected with $^{111}$In-NLS-CSL 360 at 72 h after injection (p.i.). This study was performed in collaboration with Dr. Jeffrey Leyton.
Table 3.2 the % ID/g in the major organs in NOD/SCID mice injected with $^{111}$In-NLS-CSL360 after 72 p.i.

<table>
<thead>
<tr>
<th>Organs</th>
<th>% ID/g (mean ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.67 ± 0.45</td>
</tr>
<tr>
<td>Liver</td>
<td>4.88 ± 0.49</td>
</tr>
<tr>
<td>Spleen</td>
<td>14.33 ± 3.72</td>
</tr>
<tr>
<td>Kidneys</td>
<td>6.35 ± 1.21</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.82 ± 0.38</td>
</tr>
<tr>
<td>Raji-CD123</td>
<td>2.77 ± 1.17</td>
</tr>
</tbody>
</table>

Tumor/blood: 4.15
Tumor/muscle: 3.38


References:


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