Formulation of a Kit for the Preparation of $^{111}$In-BzDTPA-NLS-trastuzumab Injection under Good Manufacturing Practices (GMP): An Auger Electron-Emitting Radioimmunotherapeutic Agent for HER2-Positive Breast Cancer

by

Vanessa Prozzo

A thesis submitted in conformity with the requirements for the degree of Master of Science

Pharmaceutical Sciences
University of Toronto

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Master of Science
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2017

Abstract

BACKGROUND: To advance $^{111}$In-BzDTPA-NLS-trastuzumab to a Phase I/II clinical trial, studies were conducted to develop a kit under GMP conditions to prepare clinical quality $^{111}$In-BzDTPA-NLS-trastuzumab injection.

METHODS: Trastuzumab was buffer-exchanged into 0.1 M NaHCO$_3$, then reacted with 10-fold molar excess of BzDTPA. The ICs were then buffer-exchanged into 0.1 M NaPO$_4$ with 0.01% PS20. Purified BzDTPA-trastuzumab was reacted with 5-fold molar excess of sulfo-SMCC in 0.1 M NaPO$_4$ with 0.01% PS20. Following re-purification, the ICs were reacted with 60-fold molar excess of NLS peptides. The BzDTPA-NLS-trastuzumab ICs were buffer-exchanged into 0.05 M NH$_4$Ac with 0.01% PS20. Unit dose kits meeting specifications for pharmaceutical quality were prepared. MicroSPECT/CT was performed in mice with SK-OV-3 human ovarian cancer xenografts.

RESULTS: Three kits that met specifications for pharmaceutical quality were manufactured. Tumor xenografts were imaged by microSPECT/CT.

CONCLUSION: Careful selection of reaction conditions was required to obtain kits that met all specifications.
Acknowledgments

I am forever thankful to my supervisor, Dr. Raymond Reilly, for allowing me to pursue my graduate studies in his laboratory. Not only did he entrust me with this project, but also provided unlimited guidance, support and mentorship throughout my entire degree. I could not have asked for a better supervisor. I would also like to thank my committee members Dr. Gang Zheng and Dr. Susan Done for taking time out of their busy schedules to provide valuable input and encouragement for my research. I am thankful to Dr. Barry Bowen and Dr. Ping Lee for serving as external examiners for my defense exam.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>% ID/g</td>
<td>Percent injected dose per gram</td>
</tr>
<tr>
<td>ACS</td>
<td>American Chemical Society</td>
</tr>
<tr>
<td>ADA</td>
<td>Anti-drug antibody</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine amino-transferase</td>
</tr>
<tr>
<td>bzDTPA</td>
<td>benzyliothiocyanate DTPA</td>
</tr>
<tr>
<td>BC</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>BCS</td>
<td>Breast conserving surgery</td>
</tr>
<tr>
<td>BCT</td>
<td>Breast conserving therapy</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer gene 1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer gene 2</td>
</tr>
<tr>
<td>Cr</td>
<td>Serum creatinine</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete blood cell counts</td>
</tr>
<tr>
<td>CDK2</td>
<td>Cyclin-dependent kinase 2</td>
</tr>
<tr>
<td>COA</td>
<td>Certificate of Analysis</td>
</tr>
<tr>
<td>CTA</td>
<td>Clinical Trial Application</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma \textit{in situ}</td>
</tr>
<tr>
<td>DSB</td>
<td>Double stranded break</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ErbB</td>
<td>Erythroblastosis oncogene</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular Domain</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EP</td>
<td>European Pharmacopoeia</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>GCP</td>
<td>Good Clinical Practice</td>
</tr>
<tr>
<td>GMP</td>
<td>Good Manufacturing Practices</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphase</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HAMA</td>
<td>Human anti-mouse antibody</td>
</tr>
<tr>
<td>HCT</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>HER</td>
<td>Human epidermal growth factor receptor</td>
</tr>
<tr>
<td>HER1</td>
<td>Human epidermal growth factor receptor 1</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HER3</td>
<td>Human epidermal growth factor receptor 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HER4</td>
<td>Human epidermal growth factor receptor 4</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IC</td>
<td>Immunoconjugate</td>
</tr>
<tr>
<td>IDC</td>
<td>Invasive Ductal Carcinoma</td>
</tr>
<tr>
<td>ILC</td>
<td>Invasive Lobular Carcinoma</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Insulin growth factor 1 receptor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>ITLC</td>
<td>Instant thin layer chromatography</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular carcinoma <em>in situ</em></td>
</tr>
<tr>
<td>LET</td>
<td>Linear energy transfer</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NRG</td>
<td>Neuregulins</td>
</tr>
<tr>
<td>NHL</td>
<td>Non-Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>NR</td>
<td>Not reported</td>
</tr>
</tbody>
</table>
OCOG  Ontario Clinical Oncology Group
OCREB  Ontario Cancer Research Ethics Board
OLINDA  Organ Level Internal Dose Assessment
OS  Overall survival
p.i.  Post injection
PFS  Progression free survival
PI3K  phosphatidylinositol 3-kinase
PIP$_2$  Phosphatidylinositol biphosphate
PIP$_3$  Phosphatidylinositol triphosphate
PPI  Protein-protein interactions
PgR  Progesterone receptor
PS20  Polysorbate 20 (Tween 20)
PTEN  Phosphatase and tensin homologue
RIT  Radioimmunotherapy
RTK  Receptor tyrosine kinase
s.c.  Subcutaneous
src  sarcoma
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP  Standard Operating Procedure
SPECT  Single photon emission computed tomography
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>T-DM1</td>
<td>Trastuzumab emtansine</td>
</tr>
<tr>
<td>TGFα</td>
<td>Transforming growth factor α</td>
</tr>
<tr>
<td>TLDU</td>
<td>Terminal lobular ductal unit</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor, Node, Metastasis</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopeia</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction
1 Introduction

1.1 The Incidence, Diagnosis, and Treatment of Breast Cancer

1.1.1 Epidemiology and Etiology of Breast Cancer

According to the Canadian Cancer Society, breast cancer (BC) continues to be the leading cause of cancer diagnosis in Canadian women. An estimated 25,000 women will be diagnosed with BC in 2015, accounting for 26% of all new female cases of cancer (1). Risk factors for BC include genetic mutations in BRCA1 and BRCA2 genes (2), being a woman (male breast cancer is less common), age, race, exposure to estrogen and progesterone, and lifestyle factors such as smoking, alcohol consumption, and obesity (3). Fortunately, the female breast cancer death rate in Canada has been declining at a rate of 2.5% per year since 2001 (1). This has been attributed to a combination of increased use of mammography screening and the use of more effective therapies after BC surgery (4–6).

The mammary gland is the functional structure of the female breast. The mammary gland is made up of lobes that are subdivided into lobules. Lobules consist of glandular alveoli that are involved in producing milk. Once milk is produced it is secreted out of the alveoli through tubules, which drain into secondary tubules. These secondary tubules converge to form mammary ducts. Mammary ducts further converge into lactiferous ducts that drain at the nipple. Most BCs originate at the terminal ductal lobular unit (TDLU), the site of conversion of lobes and ducts within the breast (7). Initiation of BC is due to genetic and epigenetic changes but it is not clear whether this occurs in a single cell or in a group of cells simultaneously. Progression of BC involves ductal hyperproliferation, with successive evolution into ductal or lobular carcinoma *in situ* (DCIS or LCIS) and invasive ductal or lobular (IDC or ILC) carcinomas, which may progress further into metastatic disease (8). However, not all in situ breast
carcinomas will progress to invasive BC (9,10). The onset of BC is sporadic in nature in most patients, except those in patients with known genetic predispositions. The risk of developing the disease increases in women with atypical ductal hyperplasia, atypical lobular hyperplasia, lobular neoplasia, and LCIS or DCIS (11).

1.1.2 Diagnosis, Staging, and Treatment of Breast Cancer

Once the presence of BC is confirmed, the tumor is staged. The Union for International Cancer Control established, and the American Joint Committee on Cancer accepted the TNM classification system that is currently used to stage the disease. The TMN classification system describes the extent of many solid cancers based on tumor size and the degree of spread to nearby tissues (T), whether the cancer has spread to draining lymph nodes and the number of involved nodes present (N), and whether the disease has metastasized to distant organs (M) (12). These three factors are taken into consideration before assigning a patient a stage between 0 to IV (0 to 4). The higher the stage the more advanced the cancer (12). In Stage 0 the cancer has not invaded locally and is at an early stage, getting progressively more extensive until the cancer has spread to other organs of the body in Stage IV. The purpose of staging is two-fold: (1) to plan the most effective therapeutic strategy for patients, and (2) to inform on the prognosis of the disease in the patient (12).

Diagnostic imaging, pathology, surgery, radiation and medical oncology are all modalities used in the management of BC. All patients undergo surgery which is usually followed by local radiation treatment and systemic therapies, which may be administered in an adjuvant setting or to treat known lesions (7). Breast conserving surgery (BCS) is considered a standard treatment option for ductal carcinoma in situ (DCIS) and most invasive carcinomas of the breast. In addition to BCS, the axillary nodes draining the tumor are removed and examined
to stage the disease. Mastectomy may be recommended as a treatment option in some cases if the area of cancer is large in comparison to the area of the breast, the cancer is present in more than one area of the breast, and little or deformed breast tissue would remain if BCS were done. Local radiation therapy is provided to eradicate any remaining cancer cells in the breast or in the axillary nodes. Breast conserving therapy (BCT), consisting of BCS and radiotherapy is the preferred method of treatment for most patients and the current standard of care as opposed to mastectomy (13). Results from multiple prospective, randomized clinical trials spanning up to 20 years concluded no significant difference in overall or disease-free survival when comparing BCT with mastectomy (14–17). Additionally, BCT provides much better cosmetic effect than mastectomy resulting in a significant gain to the patient. Furthermore, combinations of hormonal therapy and chemotherapy are utilized to reduce the chance of local BC recurrence as well as progression to metastatic disease (7).

Along with assigning a stage, a sample of tissue from a biopsy will be assessed for “receptor status” of the cells. Immunohistochemistry (IHC) markers such as estrogen-receptor (ER), progesterone-receptor (PgR) and human epidermal growth factor 2 receptor (HER2) status, along with the classical TNM staging system, are conventionally used for patient prognosis and management (18). Evidence suggests that BC with different pathological and biological features display distinct behaviors resulting in different treatment responses and thus should be managed with different therapeutic strategies (19). BC tumors can be classified into 4 intrinsic subtypes, i.e. luminal A, luminal B, HER2+, and basal (Table 1). Luminal A tumors are ER+, PgR+, and HER2-. In the vast majority of cases endocrine therapy alone is recommended. Luminal B tumors are ER+, PgR+, and HER2- however part of the luminal B subtype is HER2+. Endocrine therapy and chemotherapy is recommended for the majority of cases, however if the tumor in the patient is HER2+, then anti-HER2 therapy is recommended as well (20). An important difference
between the luminal A and B subtypes is the presence of cellular proliferation marker Ki-67. Typically, Luminal A tumors express low levels of Ki-67 whereas Luminal B tumors express high levels of Ki-67. If the tumor is classified as part of the Luminal B, HER2+ subtype, then any level of Ki-67 can be expressed. High Ki-67 is indicative of high proliferation and a worse prognosis. Thus, Luminal B tumors are more proliferative and have a worse prognosis than Luminal A tumors (20). HER2+ tumors are ER-, PgR-, and HER2+ as identified by IHC for the HER2 protein or fluoresce in situ hybridization (FISH) for increased copies of the HER2 gene (21). The procedure for determining HER2-positivity is described in greater detail in section 1.1.7. The recommended treatment is chemotherapy and molecularly targeted anti-HER2 therapies such as trastuzumab and pertuzumab. These agents are discussed in greater detail in sections 1.1.4 and 1.1.5. The basal subtype is ER-, PgR-, and HER2- (triple negative) with expression profiles that resemble basal epithelial cells of other parts of the body and normal breast myoepithelial cells (22). These tumors are not eligible for HER2 targeted BC therapies or hormonal therapies, rendering chemotherapy the only option. Triple-negative tumors often have a good response to chemotherapy, but the development of resistance as well as toxicity to chemotherapy are major challenges (20). Generally, patients with luminal A and B subtypes of BC carry a good prognosis but the basal and HER2+ subtypes carry a poorer prognosis (18). However, trastuzumab and other HER2-targeted therapies have changed the outcome for women with HER2-positive BC, such that this is not considered to be a poor prognosis form of the disease any longer (23).
**Table 1.** Features of molecular subtypes of breast cancer. ER: estrogen receptors; PgR: progesterone receptor; HER2: human epidermal growth factor receptor 2. +: positive, -: negative; +/-: occasionally positive; -/+: rarely positive.

<table>
<thead>
<tr>
<th>Molecular Subtype</th>
<th>ER</th>
<th>PgR</th>
<th>HER2</th>
<th>Ki-67</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triple-negative</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NR</td>
<td>Poor</td>
</tr>
<tr>
<td><strong>HER2+</strong></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>NR</td>
<td>Poor(^a)</td>
</tr>
<tr>
<td><strong>Luminal A</strong></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Low</td>
<td>Good</td>
</tr>
<tr>
<td><strong>Luminal B</strong></td>
<td>+</td>
<td>+/-</td>
<td>-/+</td>
<td>High</td>
<td>Good</td>
</tr>
</tbody>
</table>

\(^a\)This is not considered to be a poor prognosis form of the disease any longer due to the introduction of HER2 targeted therapies

### 1.2 HER2+ Breast Cancer

#### 1.2.1 The Biology of HER2

The HER2 gene is amplified and the HER2 protein is overexpressed in 15-20% of primary BCs (21). HER2 is a member of the family of epidermal growth receptors (HER) that play a critical role in regulating cell growth and survival. This family of transmembrane tyrosine kinase receptors (RTK) is comprised of HER1, HER2, HER3, and HER4 (ErbB-1, ErbB-2, ErbB-3 and ErbB-4 respectively). Each receptor is composed of an extracellular binding domain,
a transmembrane domain, and a functional intracellular tyrosine kinase domain, except for HER3 which does not have an intracellular kinase domain (24). Tyrosine kinase domains are activated by homodimerization of HER2 or heterodimerization with other HER family members, particularly with EGFR and HER3, and typically initiated by ligand binding. Examples of ligands include epidermal growth factor (EGF), transforming growth factor α (TGFα) and neuregulins (NRG) (25). HER2 is unlike any of the other receptors in the family because it does not have a known ligand (26). It is activated by homodimerization or heterodimerization with other HER family members following ligand binding to these receptors (24,27). HER2 is the preferred binding partner for receptor dimerization with other HER family members such as HER3 (26). Specifically in the case of HER2, homo- or hetero-dimerization activates signal transduction cascades such as the RAS-mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3’-kinase(PI3K)-AKT-mammalian target of rapamysin (mTOR) pathway, resulting in cellular proliferation and survival (24).

Low expression of HER2 on the surface of epithelial cells is involved in the normal development of tissues such as the breast, ovary, lung, liver, kidney, and central nervous system. However, in HER2+ BC cells the density of the receptors can be up to 100-fold amplified (1-2 million receptors/cell) (28). This is of crucial importance to the development of BC because when there are normal levels of HER2, dimerized receptors are infrequently formed resulting in normal cell growth. However, when HER2 is overexpressed in some forms of BC, many ligands binding to other HER family members will recruit HER2 for dimerization resulting in potent growth signaling, enhanced responsiveness to growth factors and tumor aggressiveness (25).

1.2.2 Trastuzumab

HER2 is an attractive target for BC therapy due to its upregulation in certain tumors at
levels that greatly exceed that on normal epithelial tissues. Trastuzumab (or Herceptin®) is a humanized IgG1 monoclonal antibody developed by Genentech Inc. (San Francisco, CA, USA) that binds to the extracellular domain IV of HER2, thus specifically targeting HER2 (29). It was constructed by inserting only the complementary-determining regions (CDRs) from the murine monoclonal antibody (mAb) 4D5 into a human kappa IgG1 to avoid evoking a human anti-mouse antibody (HAMA) response in patients (29). The mechanism of action of trastuzumab is discussed in detail in section 1.2.6.

1.2.3 Current Treatment Guidelines

The first line of treatment for HER2+ BC is a combination of trastuzumab, pertuzumab, and a taxane (30). Pertuzumab is a humanized IgG1 mAb that targets the extracellular subdomain II of HER2, a different epitope than domain IV to which trastuzumab binds (31). Pertuzumab prevents HER2 dimerization with other ligand activated HER receptors, most notably HER3, and its mechanism of action is complementary to that of trastuzumab (32). Like trastuzumab, pertuzumab acts by stimulating antibody-dependent, cell mediated cytotoxicity (33). If the patient progresses after first line treatment, trastuzumab emtansine (T-DM1) is recommended as a second-line treatment (30). T-DM1 is an antibody drug conjugate that combines the HER2-targeted antitumor properties of trastuzumab with the cytotoxic activity of the microtubule-inhibitory agent DM1. A stable linker conjugates the antibody and the cytotoxic agent (34). Trastuzumab delivers the cytotoxic agent specifically into HER2 overexpressing cells, thereby improving the therapeutic index and minimizing normal tissue exposure (35).

1.2.4 Determination of HER2 receptor status

Patients are eligible for treatment with trastuzumab and other HER2-targeted therapies, if there is overexpression of HER2 or increased copies of the HER2 gene on tumor cells in a BC
biopsy specimen. IHC staining for HER2 protein expression is performed on the specimen and the tumor is scored 0, 1+, 2+, or 3+ based on the intensity of staining of the cell membrane. A score of 0 is assigned if no staining is observed or if membrane staining is incomplete and barely perceptible and present on ≤ 10% of tumor cells. A score of 1+ is assigned if there is incomplete membrane staining that is barely perceptible and present on > 10% of tumor cells. A score of 2+ is assigned if circumferential membrane staining is incomplete and/or weak/moderate and present on > 10% of tumor cells. A score of 2+ is also assigned if complete and circumferential membrane staining is intense and present on ≤ 10% of tumor cells. A score of 3+ is assigned if circumferential membrane staining is complete, intense, and present on > 10% of tumor cells. Scores of 0 or 1+ are considered negative for HER2, 2+ is equivocal for HER2, and 3+ represents HER2 overexpression. Patients with tumors that scored 0 or 1+ do not overexpress HER2 and are not eligible for treatment with trastuzumab. Patients with tumors that scored 3+ are considered HER2+ and are eligible for treatment with trastuzumab. Repeat testing should be considered if results seem discordant with other histopathologic findings. Patients with tumors scoring 2+ require retesting the same tumor specimen using FISH or testing a new tumor specimen with either IHC or FISH (21).

FISH detects amplification of the HER2 gene by examining the average HER2 copy number. If the tumor specimen has an average HER2 copy number ≥ 6.0 signals/cell the patient is considered positive and eligible for treatment with trastuzumab. If the tumor specimen has an average HER2 copy number < 4.0 signals/cell, the patient is considered HER2 negative and not eligible for treatment with trastuzumab. If the tumor specimen has an average HER2 copy number ≥ 4.0 and > 6.0 signals/cell, the patient is considered equivocal and retesting is needed. Retesting can be done on the same tumor specimen using IHC or dual probe FISH or on a new tumor specimen using IHC or FISH (21).
1.2.5 Clinical Trials of Trastuzumab

Early studies showed that trastuzumab alone or in combination with chemotherapy improved rates of objective response, response duration, and time to disease progression in patients with HER2+ metastatic BC (36). In a Phase II trial involving 222 women with HER2+ metastatic BC who had relapsed after 1-2 cytotoxic chemotherapy regimens, administration of single agent trastuzumab yielded an overall response rate of 15% and the median duration of these responses was 9.1 months (36). Furthermore, a Phase III trial involving 469 women with HER2+ metastatic BC who had not previously received chemotherapy, were treated with chemotherapy and trastuzumab. Chemotherapeutic agents used such as doxorubicin or epirubicin plus cyclophosphamide were administered, or paclitaxel was administered if the women had received prior adjuvant anthracycline therapy. Half of the patients were randomized to receive trastuzumab. Of the women enrolled, 143 received an anthracycline plus trastuzumab, 138 received an anthracycline alone, 92 received paclitaxel plus trastuzumab and 96 received paclitaxel alone. Results indicated that trastuzumab is active when added to chemotherapy in these patients and that combining these two therapies significantly prolonged the median time to disease progression, increased the overall response rate, increased the duration of response, and improved median survival time when compared to treatment with chemotherapy alone (36). The most significant adverse event observed in studies of trastuzumab was cardiac dysfunction, especially in women who were treated with trastuzumab plus an anthracycline. This prompted a Phase III trial evaluating the efficacy and safety of a non-anthracycline regimen with trastuzumab (37). 3,222 women with HER2+ early-stage BC received the anthracycline doxorubicin and cyclophosphamide followed by docetaxel, the same regimen plus trastuzumab, or docetaxel and carboplatin plus trastuzumab. Results indicated that non-anthracycline regimens
were more favorable than the anthracycline containing regimen given its similar efficacy, since there were fewer acute toxic effects, and lower risks of cardiotoxicity (37).

1.2.6 Trastuzumab Mechanisms of Action

The effectiveness of trastuzumab is correlated with the level of HER2 expression in BC, with a higher level of HER2 expression showing a greater response to the drug (38). Although the exact mechanisms by which trastuzumab exerts its effects are not known, many mechanisms have been proposed both in vitro and in vivo.

1.2.6.1 Promotion of HER2 Degradation

Receptor down-regulation through endocytosis and subsequent degradation has been proposed as one of the mechanisms by which trastuzumab decreases the expression of HER2 on BC cells. It has been proposed that the activity of tyrosine kinase-ubiquitin ligase c-Cbl may be important for degradation of HER2 internalized following trastuzumab binding. When trastuzumab binds HER2, c-Cbl is recruited to its docking site, Tyr112. At the docking site, HER2 is ubiquinated by c-Cbl thus leading to its degradation following internalization. It is not known exactly how c-Cbl is induced or how exactly c-Cbl mediates HER2 degradation (39).

Additionally, HER2 undergoes proteolytic cleavage when overexpressed, which results in the release of the extracellular domain (ECD) and in the production of the truncated membrane bound fragment p95. This truncated membrane bound fragment p95 has constitutive kinase activity (27). Molina et al. demonstrated that trastuzumab can block the shedding of the HER2 ECD by inhibiting metalloproteinase activity in HER2 overexpressing BC cell lines (SK-BR-3 and BT474) (40). Once cleaved, the ECD of HER2 is likely to be released in serum. Clinical studies have shown that a decline in serum HER2 ECD during trastuzumab treatment improves progression free survival and predicts tumor response (40,41). These results are evidence that
indirectly support the theory that trastuzumab may act by stabilizing the ECD of HER2.

1.2.6.2 Antibody-Dependent Cellular Cytotoxicity

A major mechanism proposed for the activity of trastuzumab is to attract immune cells to the tumor site that overexpresses HER2. This process is called antibody-dependent cellular cytotoxicity (ADCC). Upon binding of trastuzumab to BC cells, natural killer cells (NK) are activated. NK cells express the Fc gamma receptor that can be bound by the human Fc domain of trastuzumab, and initiate the lysis of BC cells bound to trastuzumab (42). In HER2 overexpressing tumor xenografts Clynes et al. demonstrated that NK cells targeted HER2 cells coated with trastuzumab via a CD16-mediated ADCC mechanism (43). The immune cell modulated activity of trastuzumab was later confirmed in samples from patients with HER2+ locally advanced BC. An increase in NK cells and cytotoxic proteins was observed in these tumor infiltrates after treating the tumor samples with trastuzumab and docetaxel (44).

1.2.6.3 PI3K/AKT and MAPK Signaling

PI3K is activated by the auto-crossphosphorylation of RTKs such as HER2 after homo- or heterodimerization (Figure 1). Once PI3K is activated, its primary role is to convert PIP₂ to PIP₃. AKT is activated following recruitment to the cell surface by PIP₃, and acts downstream of PI3K to regulate cell survival, proliferation and growth. (42). AKT is negatively regulated by phosphatase and tensin homologue (PTEN), which is in turn regulated by tyrosine kinase sarcoma (src) (27,42). It is proposed that by interfering with the dimerization of HER2, trastuzumab inhibits HER2 activation and phosphorylation, resulting in suppressed AKT phosphorylation (45). It has also been shown that trastuzumab blocks tyrosine kinase src signaling, thus increasing PTEN levels and activity and also suppressing AKT phosphorylation (Figure 1) (46).
MAPK signaling is initiated by activation of the protein Ras by receptor tyrosine kinases. Activated Ras recruits Raf to the membrane where it is activated. Raf phosphorylates the protein kinase MEK, which goes on to phosphorylate ERK. ERK can go on to directly and indirectly activate transcription factors leading to the expression of genes that code for proteins involved in regulating cell proliferation and survival. It is proposed that by interfering with the dimerization of HER2, trastuzumab inhibits HER2 activation of Ras (27,47). Through these processes, binding of trastuzumab suppresses cell growth, proliferation, and survival (Figure 1).

**Figure 1.** Diagram illustrating PI3K/AKT and MAPK signaling pathways.

### 1.2.6.4 Inhibition of Angiogenesis

HER2 overexpression in BC is associated with increased angiogenesis (48). New growth in the vascular network of tumors contributes to tumor aggressiveness because there is an association between angiogenesis and the process of tumor invasion and metastasis (49).
Angiogenic factor, vascular endothelial growth factor (VEGF) is overexpressed in the BC microenvironment, compared with normal breast tissue (50). Trastuzumab can modulate various pro- and anti-angiogenic growth factors involved in angiogenesis to achieve angiogenic suppression. For example, HER2+ mouse mammary tumors showed reduced expression of pro-angiogenic factors VEGF and transforming growth factor-α (TGF-α), while showing elevated levels of anti-angiogenic factor thrombospondin-1 after treatment with trastuzumab (51).

1.2.7 Trastuzumab Resistance

Despite the success of trastuzumab for treating HER2+ metastatic BC, only about 1 in 2 patients with HER2+ BC in clinical trials responded to treatment with trastuzumab, implying that many patients had tumors that are “inherently” resistant to the drug (28). Of these initial responders, almost all patients had disease progression within one year (52). Progression of the disease in patients being treated with trastuzumab suggests that “acquired” resistance to trastuzumab frequently develops (52). The potential mechanisms of trastuzumab resistance are described below.

1.2.7.1 Reduced Trastuzumab Binding

HER2 can undergo mutation resulting in truncation of the receptor in which the ECD is absent (53). Because there is no ECD, this prevents trastuzumab binding and effects on tumor growth inhibition. Furthermore, loss of the ECD generates the truncated p95HER2 isoform which has constitutive kinase activity (53). Results from a study analyzing the tumor cells of 46 patients revealed that those who acquired the p95HER2 mutation were less likely to respond to trastuzumab compared to those who did not have the mutation (54). Another effect that may contribute to trastuzumab resistance is steric masking of the trastuzumab binding site by mucin-4(27).
1.2.7.2 Increased expression of other tyrosine kinase receptors

Since trastuzumab does not bind to the dimerization domain of HER2 (domain II), HER2 can dimerize with other tyrosine kinase receptors and activate downstream signaling pathways (55). This compensates for HER2 signaling inhibition by trastuzumab. Examples of tyrosine kinase receptors that can undergo heterodimerization with HER2 include HER3 and insulin-like growth factor 1 receptor (IGF-1R) (27). Trastuzumab is not able to block signaling via these receptors. It was previously found in NIH-3T3 cells and human mammary epithelial cells that HER2-HER3 dimerization results in increased tyrosine phosphorylation of HER3, subsequently increasing PI3K recruitment and downstream activation (56). These findings imply that signaling as a result of HER2-HER3 dimerization compensates for HER2 signaling inhibition by trastuzumab. On the contrary, IGF-1R contributes to trastuzumab resistance by overcoming trastuzumab mediated cell cycle arrest as indicated by low levels of growth inhibitor proteins p27kip1 and p21kip1, and elevated CDK2 kinase activity (27).

1.2.7.3 Loss of PTEN

Constitutively active PI3K/AKT pathways due to PTEN loss or activating mutations in PI3K contribute to inherent and acquired resistance to trastuzumab. A study analyzing breast tumor specimens from patients with metastatic BC found 36% of patients had tumors that did not express PTEN and these patients had lower response rates to trastuzumab therapy than those who had tumors that did express PTEN (57). Another group found that patients with tumors possessing PI3K mutations that had been treated with trastuzumab had significantly shorter progression-free survival than those with tumors without these mutations (58). It has also been shown that activation of src in breast cancer cell lines contributes to intrinsic and acquired resistance by down-regulating PTEN. Furthermore, the same group demonstrated that patients with tumors with active src had a poorer response to trastuzumab than those who did not have
1.2.8 Trastuzumab emtansine (T-DM1)

T-DM1 is an antibody-drug immunoconjugate consisting of the cytotoxic agent DM1 (emtansine) conjugated to trastuzumab. It incorporates the HER2 targeted tumor growth inhibitory properties of trastuzumab with the cytotoxic activity of the microtubule-inhibitory agent DM1 (34). A stable linker connects the two molecular entities. T-DM1 specifically binds HER2+ cells and facilitates intracellular drug delivery, thereby improving the therapeutic index and minimizing normal tissue exposure. In early 2013, T-DM1 was approved by the U.S. FDA as a therapy regimen for patients with HER2+ metastatic BC. In September of 2013, Health Canada approved T-DM1 for the same indications. Leading up to approval, the efficacy and safety of the drug was examined in clinical trials. EMILIA (35) is a Phase III trial involving 991 patients with HER2+ unresectable, locally advanced, or metastatic BC that had previously been treated with trastuzumab and a taxane. Patients were randomly assigned in a 1:1 ratio to receive T-DM1 or lapatinib plus capcetabine. Results revealed that the median progression free survival (PFS), overall survival (OS), and objective response rate (ORR) were significantly higher in T-DM1 treated patients compared to patients treated with lapatinib plus capecitabine. Furthermore, the adverse events observed in patients treated with T-DM1 were generally low grade (35). Another pivotal Phase III trial was MARIANNE (59), where 1,095 patients with progressed or recurrent locally advanced or previously untreated metastatic HER2+ BC received T-DM1 plus pertuzumab (363 patients), T-DM1 plus placebo (367 patients), or docetaxel or paclitaxel (365 patients). Results revealed that T-DM1 treatment is noninferior with respect to PFS when compared to treatment with trastuzumab plus a taxane in patients with locally advanced or
metastatic BC. However, the trial did demonstrate T-DM1 had a lower toxicity profile and improved quality of life outcomes compared to the control treatment (59).

1.3 Radioimmunotherapy

1.3.1 Radioimmunotherapy

Radioimmunotherapy (RIT) employs monoclonal antibodies (mAbs) labeled with a therapeutic radionuclide to target and kill cancer cells. Conceptually, radiolabelled mAbs are able to specifically bind to antigens that are overexpressed on tumor cells resulting in an increased radiation dose delivered to the tumor cells while minimizing the radiation dose to normal tissues. RIT is advantageous over treatment with unmodified antibodies because ionizing radiation deposited by a therapeutic radionuclide provides an additional source of toxicity to targeted cells and can help to overcome resistance (60). The U.S. FDA and Health Canada have approved two RIT agents: $^{90}$Y-ibritumomab tiuxetan (Zevalin®, approved in 2002) and $^{131}$I-tositumomab (Bexxar®, approved in 2003) (61). Both were approved for treatment of refractory non-Hodgkin’s lymphoma (NHL) expressing CD20. In a study comparing Zevalin® with unlabeled CD20 mAb rituximab, both the overall response rates and complete response rates were higher for RIT, 80% vs. 56% and 30% vs. 16% respectively (62). Furthermore, an overall response rate of 80% was observed in patients with rituximab-refractory NHL in a separate study conducted by Alcindor and Witzig (63). Bexxar®, which also binds CD20, demonstrated improved therapeutic efficacy compared with that provided by the most recent chemotherapy regimen in a clinical trial involving 60 patients with previously untreated follicular lymphoma. Unfortunately Bexxar® was discontinued by the manufacturer due to limited use of the agent, making Zevalin® the only remaining clinically approved RIT agent. Nonetheless, the clinical success of these agents in treating NHL highlights the potential of RIT for treatment of
malignancies. The challenge is to extend these promising results in NHL to treatment of more common tumors such as breast, lung, pancreatic, stomach, and ovarian cancer.

1.3.2 Selection of Radionuclides for RIT

Of critical importance to RIT is selection of the radionuclide for labeling the mAb (Table 2). DNA is the main target for lethal radiation induced biologic effects with DNA double stranded breaks (DSB) being the most potent lesion, due to the difficulty in repairing these compared to single strand breaks (SSB). DNA strand breaks result from the direct ionization of DNA by radiation or more commonly by the interaction of radiation-induced free radicals with DNA (64). Beta (β)-emitting radionuclides are most commonly employed in RIT but alpha (α)-emitters and Auger electron-emitting radionuclides are also under investigation. Alpha (α)-emitters have energies ranging from 5 to 9 MeV, travel about 50-100 μm (5-10 cell diameters) in tissue and have high linear energy transfer (LET) values (~80-100 keV/μm) thus making them ideal for eradicating small clusters of tumor cells (64). LET describes how much energy an ionizing particle transfers to the material transversed per unit of distance (65). Of equal importance in the cell killing properties of α-emitters are bystander effects. The bystander effect occurs when a radiobiologically damaged cell induces death in proximal nonirradiated cells through the release of cytokines and free radicals (66). Issues for using α-emitters arise due to their limited availability and relatively short physical half-life (Table 2) (67,68). β-emitters have various energies, a distribution of ranges (Table 2) and have low LET values (~0.2 keV/μm) (64). Energy deposition takes place along a distance that greatly exceeds the location of the actual decay event, resulting in a crossfire effect which is useful in irradiating bulky tumors in which not all tumor cells will be targeted by the RIT agent. “Crossfire” describes the deposition of energy in a distant cell from decay of a radionuclide in another cell over a distance of many
millimeters (69). In the micrometastatic setting, β-emitters are less ideal because a significant proportion of the radiation dose is deposited in normal tissues surrounding the tumor cells within the millimeter range of the β-particles, or in the bone marrow due to circulating radioactivity. (70). Furthermore, some radionuclides also emit gamma (γ)-photons which permit imaging of the delivery of the RIT agent to tumors by single photon emission computed tomography (SPECT). SPECT imaging is performed using a gamma-camera that acquires images by capturing the gamma photons released by γ-emitters (Table 2) (71).
Table 2. Table of some radioisotopes commonly used in radioimmunotherapy*

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half Life (hr)</th>
<th>Emission</th>
<th>$E_{\text{max}}^a$ (MeV)</th>
<th>$R_{\text{max}}^b$ (µm)</th>
<th>Gamma Emission (keV) and Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine-131 ($^{131}$I)</td>
<td>193</td>
<td>Beta</td>
<td>0.66</td>
<td>2300</td>
<td>364 (81%)</td>
</tr>
<tr>
<td>Yttrium-90 ($^{90}$Y)</td>
<td>64</td>
<td>Beta</td>
<td>2.28</td>
<td>11300</td>
<td>-</td>
</tr>
<tr>
<td>Copper-67 ($^{67}$Cu)</td>
<td>62</td>
<td>Beta</td>
<td>0.58</td>
<td>2100</td>
<td>186 (48%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>93.3 (23%)</td>
</tr>
<tr>
<td>Bismuth-212 ($^{212}$Bi)</td>
<td>1</td>
<td>Alpha</td>
<td>5.84</td>
<td>48</td>
<td>727.2 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.8 (2.6%)</td>
</tr>
<tr>
<td>$^{212}$Pb</td>
<td>11</td>
<td>Alpha</td>
<td>0.6</td>
<td>-</td>
<td>238.6 (43%)</td>
</tr>
<tr>
<td>Astatine-211 ($^{211}$At)</td>
<td>7</td>
<td>Alpha</td>
<td>5.87</td>
<td>48</td>
<td>687 (3%)</td>
</tr>
<tr>
<td>Indium-111 ($^{111}$In)</td>
<td>67</td>
<td>Auger</td>
<td>0.26</td>
<td>17</td>
<td>171 (90%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>245 (94%)</td>
</tr>
<tr>
<td>Gallium-67 ($^{67}$Ga)</td>
<td>78</td>
<td>Auger</td>
<td>0.18</td>
<td>3</td>
<td>93 (36%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>185 (20%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>300 (16%)</td>
</tr>
<tr>
<td>Iodine-123 ($^{123}$I)</td>
<td>13</td>
<td>Auger</td>
<td>0.16</td>
<td>20</td>
<td>159 (83%)</td>
</tr>
</tbody>
</table>

$^a$ $E_{\text{max}}$ or maximum energy; maximum range of particle energy in tissue

$^b$ $R_{\text{max}}$ or maximum range of emission path length of the particle radiation in tissue

* Adapted from (64,65,70)
1.3.2.1 Auger Electron-Emitters

An attractive alternative to α- or β- particle emitters for RIT are low energy Auger electron-emitters (Table 2, Table 3). Auger electrons arise when the electronic shells of radionuclides are re-organized following decay by electron capture. During electron capture, inner shell electron vacancies are created by electron transfer from the inner shell into the nucleus. The inner shell electron vacancies are subsequently filled by electron transitions from higher energy cells, occurring in cascade (70). The energy difference of these transitions is released as either photons or low-energy electrons, called Auger electrons. Auger electrons have low energy (≤25 keV), travel subcellular ranges in tissue (< 10 μm), and have high LET values (4-26 keV/μm), which cause multiple ionizations at close proximity to the decay site (64,70).

These properties make Auger electron emitters suitable for eradicating single cells or small clusters of cells. Auger electrons deposit energy in 1-2 nm spheres around the decay site and the double stranded DNA helix has a diameter of 2 nm. So, if an Auger electron emitter were to decay in close proximity to DNA, it would cause lethal DNA DSB while minimizing damage outside the cell nucleus or the cell (70). Thus, it is important for the radionuclide to decay in close proximity to nuclear DNA so that the DNA is within the penetration range of the Auger electrons to cause lethal DNA DSB (64). For example, it has been estimated that the radiation dose to the nucleus for 111In is 2- and 35-fold greater when 111In decays in the nucleus, compared to decay in the cytoplasm or cell surface, respectively (72,73). Furthermore, free radicals will also be produced in response to Auger electron decay which can then diffuse freely within the cell to cause DNA damage or into the intracellular space to cause damage to nearby cells (bystander effect) (74).
Table 3. Additional properties of Auger electron-emitting radionuclides*  

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Physical Half-Life (days)</th>
<th>Auger electrons released per decay</th>
<th>Total energy per decay released (keV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indium-111 ((^{111})In)</td>
<td>2.8</td>
<td>14.7</td>
<td>419.2</td>
</tr>
<tr>
<td>Gallium-67 ((^{67})Ga)</td>
<td>3.3</td>
<td>4.7</td>
<td>201.6</td>
</tr>
<tr>
<td>Iodine-123 ((^{123})I)</td>
<td>0.6</td>
<td>14.9</td>
<td>100.4</td>
</tr>
</tbody>
</table>

* Adapted from Buchegger et al. (70)

1.4 Enabling Nuclear Localization of Auger Electron Emitters

1.4.1 NLS Peptides

Antibodies labeled with Auger electron-emitting radionuclides may be routed to the cell nucleus for maximum therapeutic effect by appending nuclear localization signal (NLS) peptides (75). Receptor mediated endocytosis may bring the radiolabelled antibody modified with NLS into the cytoplasm of the cell, where the NLS is then able to route the radioimmunoconjugate into the nucleus of the cell. NLS are short stretches of less than 10 amino acids that are found naturally in certain biomolecules and function to actively import large molecules (>45 kDa) into the nucleus via an energy dependent process. Molecules <45 kDa are able to diffuse passively in and out of the nucleus through nuclear pore complexes (NPC) and the use of NLS is not required. NLS peptides typically have sequences of 4 or more cationic residues consisting of lysine (K) or arginine (R) (76). For example, the sequence PKKRRKV is required for localizing simian virus 40 (SV40) large T-antigen to the nucleus (77). Localization to the nucleus is facilitated by karyopherin/importin proteins. NLS directly binds to importin-α which then forms a heterotrimer with importin-β that that enables transport into the nucleus across the NPC.
Furthermore, passage of importin-cargo complexes across the nuclear pore is regulated by a Ran GTPase. Ran binds to importin-β to induce a conformational shift resulting in release of a cargo protein into the nucleus of the cell (Figure 2) (76). The importin-RanGTP complex is then transported back into the cytoplasm of the cell where GTP is hydrolyzed by Ran. This results in release of the importin protein so that more cycles of cargo import into the nucleus can occur (76). NLS has been used to transport cytoplasmic proteins into the nucleus, and it is ideal for delivering Auger electron-emitters to the nucleus for RIT (75).
**Figure 2.** Nuclear Localization Sequence (NLS)-mediated transport of Auger-electron-emitting biomolecule through the nuclear pore complex (NPC).

*Adapted from Costantini et al, 2008 (75)*

### 1.4.2 NLS-Conjugated trastuzumab

Conjugation of NLS peptides to a mAb that is internalized following binding to a cell-surface receptor provides a unique solution to routing antibodies labeled with Auger electron-emitters directly into the nucleus of cells. Our group recently reported the internalization and nuclear importation of trastuzumab site-specifically modified with a metal chelating polymer that presents multiple BzDTPA chelators for complexing $^{111}$In without and with an appending NLS sequence. The internalization and nuclear importation of the radioimmunoconjugates was
evaluated at 1h, 4h and 24h in SK-BR-3 cells (high HER2 expression). Results revealed no significant difference in cell-surface associated radioactivity at all time points. Furthermore, there was no significant difference in the nuclear import of trastuzumab site-specifically modified with a metal chelating polymer that presents multiple BzDTPA chelators for complexing $^{111}$In without or with an appended NLS sequence at 4h (7.5±1.0 vs. 7.8±0.4% respectively) or 24 h (12.5±2.6 vs. 8.6±2.8% respectively) (78). These results suggested that the polymers may mediate nuclear importation and may not require a NLS in this example. The use of NLS peptides to route non-polymer modified trastuzumab to the nucleus is described below.

Our group also reported the cytotoxicity and HER2-targeting properties of $^{111}$In-labeled trastuzumab modified with NLS peptides. Internalization of $^{111}$In-NLS-trastuzumab and $^{111}$In-trastuzumab were compared in SK-BR-3, MDA-MB-361 or MDA-MB-231 cells expressing high, intermediate, or very low levels of HER2 respectively. In all cell lines, the uptake of radioactivity was almost completely blocked after the addition of unlabeled trastuzumab to the incubation media, thus demonstrating the specificity of $^{111}$In-NLS-trastuzumab for HER2. The nuclear importation of $^{111}$In-NLS-trastuzumab and $^{111}$In-trastuzumab was compared in SK-BR-3 cells (high HER2 expression) at 24 h, revealing that the fraction of radioactivity imported to the nucleus was greater for $^{111}$In-NLS-trastuzumab compared to $^{111}$In-trastuzumab (14.4±1.8% vs. 7.2±0.9% respectively). Additionally, $^{111}$In-NLS-trastuzumab was approximately 2-fold and 5-fold more potent at killing HER2-overexpressing MDA-MB-361 and SK-BR-3 cells respectively compared with $^{111}$In-trastuzumab, and approximately 3-fold and 6-fold more potent than unlabeled trastuzumab. Less than 10% of MDA-MB-231 cells which have very low HER2 expression were killed after exposure to $^{111}$In-NLS-trastuzumab, whereas >90% of HER2-overexpressing SK-BR-3 cells were killed, thus indicating that the toxicity of $^{111}$In-NLS-trastuzumab is HER2 specific (77).
Furthermore, the cytotoxicity of $^{111}$In-NLS-trastuzumab, $^{111}$In-trastuzumab and trastuzumab against trastuzumab-sensitive and trastuzumab-resistant breast cancer cells was evaluated by clonogenic assay. Clonogenic survival was evaluated in trastuzumab insensitive MDA-MB-231 (0.4 x $10^5$ receptors/cell) and MDA-MB-231-H2N cell lines (6.1 x $10^5$ receptors/cell), and trastuzumab resistant TrR1 (5.1 x $10^5$ receptors/cell) and TrR2 (0.6 x $10^5$ receptors/cell) cell lines. A decrease in colony formation was observed for MDA-MB-231-H2N and TrR1 cells (intermediate HER2 expression) treated with increasing amounts of $^{111}$In-NLS-trastuzumab. The EC$_{50}$ for MDA-MB-231-H2N cells was 9- and 16-fold lower than that for $^{111}$In-trastuzumab and trastuzumab, respectively. In TrR1 cells the EC$_{50}$ was reduced to <20% after treatment with $^{111}$In-NLS-trastuzumab, which was significantly lower than that of $^{111}$In-trastuzumab and trastuzumab. However, MDA-MB-231 and TrR2 cells (low HER2 expression) were less sensitive to $^{111}$In-NLS-trastuzumab or $^{111}$In-trastuzumab and did not respond to trastuzumab. These results suggested that $^{111}$In-NLS-trastuzumab could potentially be used to treat trastuzumab resistant tumors in patients. Furthermore, there was a direct correlation between the cytotoxicity of $^{111}$In-NLS-trastuzumab with HER2 cell surface receptor expression on the different cells lines, with the greatest toxicity observed for MDA-MB-231-H2N and TrR1 cell lines (intermediate HER2 expression) and the least toxicity observed for MDA-MB-231 cell lines (very low HER2 expression) (60).

The tumor growth inhibitory properties and normal tissue toxicity of $^{111}$In-NLS-trastuzumab were also evaluated. Female athymic CD-1 nude mice bearing MDA-MB-361 (5.0 x $10^5$ HER2 receptors/cell) or MDA-MB-231 (0.5 x $10^5$ HER2 receptors/cell) BC xenografts received one dose of $^{111}$In-NLS-trastuzumab (9.25 MBq; 4 mg/kg). Results revealed the growth rate of MDA-MB-361 tumors in mice receiving $^{111}$In-NLS-trastuzumab was 4-fold lower than in mice treated with saline, and significantly lower than groups treated with $^{111}$In-trastuzumab,
In-NLS-hIgG (non-specific) and trastuzumab. Furthermore, mice bearing MDA-MB-361 (5.0 x10^5 HER2 receptors/cell) subcutaneous tumors were administered 2 doses (9.25 MBq; 4 mg/kg) of ^111^In-NLS-trastuzumab or the equivalent doses of unlabeled trastuzumab and results revealed that mice treated with ^111^In-NLS-trastuzumab had a significantly greater survival time than mice treated with unlabeled trastuzumab or saline (140 d vs. 96 and 84 d respectively). No nonspecific toxicities of the hematopoietic system in non-tumor bearing normal Balb/c mice were observed at 2 weeks post injection of ^111^In-NLS-trastuzumab (9.25 MBq or less; 4mg/kg). No acute hepatic or renal toxicity was observed in these same mice for ^111^In-NLS-trastuzumab despite accumulation of high concentrations of radioactivity in the liver or kidneys (11-12 %ID/g and 7-8 %ID/g respectively) (79).

Results from this preclinical research suggest that the anti-tumor effects of ^111^In-NLS-trastuzumab observed in mouse models of BC may translate into tumor responses in HER2+ BC patients if this RIT agent could be advanced translated to Phase I clinical trial. In order to achieve clinical translation, it is necessary to formulate a radiopharmaceutical product under Good Manufacturing Practices (GMP) and conduct translational bridge studies.

### 1.5 Clinical Translation

There are four main steps in the clinical translation of a novel radiopharmaceutical required to advance the agent from preclinical studies to Phase I clinical trial (Figure 3.). The first 3 steps are closely linked. For example, the mass dose and radioactivity amount to be used in the Phase 1 trial should be selected prior to radiopharmaceutical formulation because it is required by Health Canada to conduct preclinical studies using an administered dose scaled to the human dose. Furthermore, regulatory agencies require use of the actual radiopharmaceutical
formulation in any preclinical pharmacology and toxicology studies along with testing of multiples of the dose scaled to the human dose.

**Figure 3.** Roadmap demonstrating the four steps in the clinical translation of a novel radiopharmaceutical from preclinical studies to Phase I clinical trial.

* Adapted from Reilly et al. (2015)

**1.5.1 Radiopharmaceutical Formulation**

A kit is a pre-packaged formulation of sterile ingredients designed for the preparation of a radiopharmaceutical. It contains the ligand, buffer, excipients and any other required components that when mixed with a radiometal, produces the radiopharmaceutical (80). Radiometals are typically bound to ligands, such as antibodies, through use of a bifunctional chelating agent. Bifunctional chelating agents contain a metal chelating group and a functional group that can form a covalent bond with a protein or peptide (81). They allow stable attachment of a
radioactive metal to mAbs and other biological molecules. A radiopharmaceutical kit is ideal for radiopharmaceutical preparation for human studies because most quality control tests can be done prior to preparing the final radiopharmaceutical, kits can be stored and labeled for use on demand for patient studies, and the kits routinely have a labeling efficiency of ≥90% meaning no post-labeling purification is needed (82). Manufacture of a radiopharmaceutical kit simplifies final radiopharmaceutical preparation while ensuring high quality for patient administration.

To ensure kit quality, parameters such as protein concentration, volume, pH, appearance, chelator substitution level, peptide or protein substitution level, purity and homogeneity, labeling efficiency and immunoreactivity should be tested and specifications should be established. Additionally, since most radiopharmaceuticals are injectables, sterility and apyrogenicity are determined by the USP Sterility Test and USP Bacterial Endotoxins Test, respectively, ensuring the product is acceptable for patient administration (82). Quality specifications for the final radiopharmaceutical should also be established. These specifications include limits for total radioactivity and radioactivity concentration, specific activity, pH, labeling efficiency, radiochemical purity, appearance, and sterility (retrospective). Sterility testing is done retrospectively to allow for radionuclide decay. Performing these tests retrospectively ensures that the radiopharmaceutical administered to patients was sterile and that the method used to prepare the radiopharmaceutical will consistently result in a sterile product (82).

1.5.2 GMP in Radiopharmaceutical Preparation

In order to advance $^{111}$In-BzDTPA-NLS-trastuzumab to a Phase I clinical trial the radioimmunoconjugate needs to be manufactured under GMP conditions, ideally from a kit formulation. GMP is a broad quality assurance system that documents the production of a pharmaceutical from raw materials through intermediates to the final product, including the
assays and specifications implemented to ensure quality (83). Raw materials used must be pharmaceutical quality (82). This is achieved by purchasing USP or other pharmacopoeial grade materials. When this is not possible high purity materials should be obtained (>95%). The certificate of analysis (COA) for each individual lot of raw material should always be obtained from the manufacturer. To complement the COA, identity testing by USP methods or other acceptable methods should be done on all received materials to confirm receipt of the proper material. When USP methods are not possible (i.e. for peptides or chelators), testing by analytical methods such as NMR spectroscopy that identify chemical structure combined with other analytical methods that test for functional groups (e.g. UV-visible spectroscopy) is sufficient. Radiopharmaceutical kits and buffers must be tested for quality parameters such as protein concentration, volume, pH, appearance, chelator substitution level, purity, homogeneity, and immunoreactivity (82). Kits and buffers must be filtered through a 0.22µm sterilization filter to ensure sterility of the product and to remove any particulates. Quality control assays and specifications for these parameters must be established (82). Tests for sterility and pyrogenicity are performed according to methods outlined by the USP Sterility Test and USP Bacterial Endotoxins Test thus ensuring the product is acceptable for patient administration (82). The final radiopharmaceutical is tested for radiochemical purity (>90%) and other parameters including radioactivity concentration, pH and sterility (performed retrospectively) (82).

Standard operation procedures (SOPs) should be put in place to provide a replicable system for manufacturing and quality control of pharmaceutical buffers, kits, and the final radiopharmaceutical (82). A lot numbering system must be put in place and maintained in order to document the production of the radiopharmaceutical and easily track any quality issues that may arise.
The last consideration for ensuring GMP is the environment in which the kits and final radiopharmaceutical are manufactured. GMP standards for air quality must be met (83). Typically manufacture of pharmaceutical products takes place in a clean room. However, this may not always be the case in academia where resources are limited. In the absence of a clean room, a room containing a biosafety cabinet with grade A air should be dedicated to kit and final radiopharmaceutical production. The room itself should have a minimum of grade C air as determined by a certified air quality testing service. During radiopharmaceutical preparation, grade C air must contain <3,520,000 particles per m$^3$ with diameter $\geq$ 0.5 µm and <29,000 particles per m$^3$ with diameter $\geq$ 0.5 µm. At rest, these limits are 10-fold lower (83). The biosafety cabinet should be certified annually. The cabinet and any equipment or supplies placed inside the cabinet should be disinfected with 70% alcohol. Whenever single use sterile products are available for purchase they should be used throughout the manufacturing process. Nonsterile equipment must be sterilized by autoclaving or gas sterilization. Equipment used for manufacture should be used solely for kit or radiopharmaceutical preparation and regularly calibrated. Furthermore, the personnel manufacturing the kit and preparing the final radiopharmaceutical should be trained in sterile product preparation and should wear a clean lab coat, face mask and head covering during manufacture to avoid product contamination.

1.5.3 Challenges in Kit Production

Typically, high concentrations of mAbs are used in clinical formulations for reasons such as the high dose required for treatment and ease of patient administration (84). However, the solubility and tendency of antibodies to aggregate are major challenges in the development of moderate to high concentration formulations. These concerns also extend to radiopharmaceutical kit formulations containing antibodies. Aggregation is problematic because it may interfere with product manufacturability, deliverability, stability, and shelf-life (85). Furthermore, aggregation
can evoke a protein-specific immune response, which in the case of antibody therapeutics can lead to the formation of anti-drug antibodies (ADA) (86). Most antibody formulations contain low amounts of aggregates but it is not known to what extent or the type of aggregation that could pose a safety risk (87).

The behavior and stability of proteins are primarily governed by protein-protein interactions (PPI), which are in turn governed by 3D protein structure and the solution environment (88). More specifically, antibodies are folded so that the hydrophobic domains are not exposed thus preventing hydrophobic PPI. However, normal molecular movement and shear force applied during manufacture can cause partial or complete denaturation of antibodies resulting in surface exposure of their hydrophobic domains. In an attempt to conserve energy, protein-protein contacts form on these domains resulting in small aggregates that serve as a seed for the generation of larger aggregates (84,89). Not only is protein solubility dictated by intrinsic factors, but also mandated by extrinsic factors such as solution pH, salt concentration and the presence of excipients (90). pH is important because acidic conditions can affect antibody structure, stability and folding thus leading to the exposure of hydrophobic domains and the formation of hydrophobic interactions (91). Salts play a dual role in protein solubility. At low concentrations, adding salt can increase the solubility of a protein (salting in region). However, if the salt concentration is too high, the charges on the protein tend to get masked, thereby minimizing charge repulsions between proteins and allowing proteins to come in close contact and associate through exposed hydrophobic patches (88). Furthermore, excipients can either act to stabilize and prevent protein aggregation or they can act to promote aggregation, depending on the chosen excipient (86,89). Among common excipients used in antibody formulations to reduce protein aggregation are surfactants, amino acids, and buffers to adjust the pH.
The mechanisms of aggregate formation discussed above are caused by non-covalent interactions that can be classified as either weak or strong associations. PPIs involving weak associations can be dissociated by dilution whereas strong associations are not easily disrupted. Aggregates can also form as a result of covalent binding of proteins during chemical modification and these are irreversible (92).

1.5.4 Polysorbate 20 (Tween 20®)

Polysorbate 20 (PS20) is a nonionic surfactant that consists of a mixture of esters of different polymeric head groups and various fatty acid tails with multiple degrees of esterification. It is amphiphilic in nature due to its two parts: the poly-oxyethylene and dehydrated sugar core that comprises the hydrophilic head group, and hydrophobic fatty acids as the tail group (Figure 4). PS20 is commonly used in therapeutic antibody formulations due to its biocompatibility, low toxicity, and good stabilizing properties (93,94). PS20 is known to prevent aggregate formation and protect against denaturation in protein formulations (95). Furthermore, by protecting against denaturation, surfactants such as PS20 also act to preserve the biological activity of proteins such as antibodies. This is important when these proteins undergo rigorous processes such as purification, filtration, transportation, storage and delivery.
Figure 4. Chemical structure of Polysorbate 20 (Tween 20): polyoxyethylene sorbitan monolaurate. $w + x + y + z$ refers to the total number of oxyethylene subunits in each surfactant molecule and may not exceed 20.

* Adapted from Kerwin, 2008 (96)

PS20 acts to prevent aggregation by occupying the air-solvent and/or solvent-solid (ie. container) interface of the antibody solution thereby reducing surface tension and reducing the concentration of antibody molecules at the boundary. Since the hydrophobic regions of antibodies are prone to be exposed at these boundaries potentially causing aggregation, reducing the antibody concentration at the boundary by having PS20 bind to the boundary instead may reduce aggregate formation (89,96,97). Furthermore, studies have shown that PS20 weakly interacts with antibodies in the native state at millimolar concentrations. Particularly, PS20 binds to unfolded antibodies with a greater affinity than to antibodies in the native state due to a greater proportion of hydrophobic domains being exposed (93). This may further reduce aggregation by protecting from intermolecular interactions by having PS20 bind to the exposed hydrophobic domains of incorrectly folded antibodies. Of notable interest is a study by Patapoff et al. that
found PS20 mitigated the formation of insoluble aggregates caused by mechanical shear in mAb solutions (94).

1.5.5 Clinical Translation Success in Academia

Although there are barriers to translation of promising radiopharmaceutical agents from “bench to bedside”, our lab has successfully translated three agents into GMP quality radiopharmaceutical kits and evaluated these radiopharmaceuticals in Phase I trials in academia (98–101). Of particular interest was the successful design and manufacture of a kit for the preparation of $^{111}$In-DTPA-hEGF under GMP conditions, a novel radiotherapeutic agent for EGFR+ BC, that went on to be evaluated for tumor and normal issue uptake and safety in 16 patients in a Phase 1 trial. The radiopharmaceutical clearly accumulated in one known site of the disease in 47% of patients however no objective anti-tumor responses were observed at the doses studied. Nonetheless, results in athymic mice with MDA-MB-468 human BC xenografts (high EGFR expression) showed growth arrest of these tumors by $^{111}$In-DTPA-hEGF. Lack of tumor response in patients was attributed to the relatively low doses that were studied in patients. Hematological, renal and hepatic function was not altered. The most common adverse events were flushing, chills, nausea and vomiting. One patient experienced thrombocytopenia but this was attributed to bone marrow infiltration by cancer. $^{111}$In-DTPA-hEGF was not immunogenic (100,101). In order to deeply understand and achieve the full potential of radioimmunotherapy for the future benefit of cancer patients, radiopharmaceuticals must be evaluated in the clinic.

1.6 Hypothesis of the Thesis

The hypothesis of this thesis is that a pharmaceutical quality kit for $^{111}$In-BzDTPA-NLS-trastuzumab injection can be prepared under Good Manufacturing Practices (GMP) conditions at a low dose (111-148 MBq; 5 mg) for administration to patients with HER2+ metastatic BC and
may be suitable for advancement to a Phase I clinical trial. The objective was to establish a pharmaceutical kit formulation for $^{111}$In-BzDTPA-NLS-trastuzumab under GMP conditions, establish specifications and quality control assays, and manufacture and test at least three lots as required by Health Canada for a Clinical Trial Application (CTA) submission.
Chapter 2
Formulation of a Kit for the Preparation of $^{111}\text{In-BzDTPA-NLS}$-trastuzumab Injection under Good Manufacturing Practices (GMP): An Auger Electron-Emitting Radioimmunotherapeutic Agent for HER2+ Breast Cancer
Formulation of a Kit for the Preparation of $^{111}$In-BzDTPA-NLS-trastuzumab Injection under Good Manufacturing Practices (GMP): An Auger Electron Emitting Radioimmunotherapeutic Agent for HER2+ Breast Cancer

2.1 Abstract

BACKGROUND: $^{111}$In-BzDTPA-NLS-trastuzumab is routed to the nucleus of HER2+ BC cells where the Auger electrons emitted by $^{111}$In are damaging to DNA. To advance $^{111}$In-Bz-DTPA-NLS-trastuzumab to a Phase I/II clinical trial, studies were conducted to develop a kit under GMP conditions to prepare pharmaceutical quality $^{111}$In-BzDTPA-NLS-trastuzumab injection.

METHODS: Trastuzumab (Herceptin) was buffer-exchanged into 0.1 M NaHCO$_3$, then reacted with a 10-fold molar excess of benzylisothiocyanate DTPA (BzDTPA). The immunoconjugates were then buffer-exchanged by ultrafiltration into 0.1 M NaPO$_4$ buffer with 0.01% polysorbate-20 (PS20). Purified BzDTPA-trastuzumab was modified with maleimide groups for conjugation to nuclear localization sequence (NLS) peptides by reaction with a 5-fold molar excess of sulfo-SMCC in 0.1 M NaPO$_4$ buffer with 0.01% PS20. Following re-purification, the maleimide-modified BzDTPA-trastuzumab was reacted with 60-fold molar excess of NLS peptides with a terminal thiol group. The BzDTPA-NLS-trastuzumab was purified and buffer-exchanged into 0.05 M NH$_4$Ac buffer with 0.01% PS20. Final protein concentration was determined by measuring UV absorbance at 280nm and then adjusted to 5.0 mg/mL and 1 mL aliquots were sterile filtered into single vials (kits). The kits were labeled with 111-137 MBq of $^{111}$In. Desired specifications were 2-7 BzDTPA and 5-11 NLS per trastuzumab, ≥90% labeling efficiency, <10% dimerization assessed by SDS-PAGE and $K_a$ and $B_{max}$ values within 1.0-8.0× 10$^8$ L/mol.
and $0.5-2 \times 10^6$ HER2/cell respectively. MicroSPECT/CT was performed in Balb/c nude mice engrafted subcutaneously with HER2+ SK-OV-3 human ovarian cancer xenografts.

RESULTS: Three kits were manufactured. Addition of PS20 to buffers reduced visible aggregate formation in the kits. BzDTPA substitution level ranged from 2-4 BzDTPA/trastuzumab, labeling efficiency was 92.8±0.1%, there were approximately 6 NLS peptides per trastuzumab molecule, 5±3% dimerization, and the $K_a$ and $B_{max}$ were $3.6\pm 3.7 \times 10^8$ L/mol and $1.0\pm 0.4 \times 10^6$ receptors/cell respectively. Tumor xenografts were imaged by microSPECT/CT at 72 hours post-injection of $^{111}$In-BzDTPA-NLS-trastuzumab.

CONCLUSION: Careful selection of reaction conditions was required to obtain a kit for preparation of $^{111}$In-BzDTPA-NLS-trastuzumab injection exhibiting low (<10%) high molecular weight covalently linked species. Kits met specifications for BzDTPA, NLS substitution, labeling efficiency and exhibited preserved HER2 binding.
2.2 Introduction

According to the Canadian Cancer Society, breast cancer (BC) continues to be the leading cause of cancer diagnosis, accounting for 26% of new cancer diagnoses among Canadian women in 2015 (1). Approximately 15-20% of all human breast cancers are associated with overexpression of HER2 (21). HER2 gene amplification and overexpression leads to excess HER2 signaling causing deregulation of cell proliferation and survival mechanisms, thus contributing to uncontrolled cell proliferation and/or malignancy (102). This subtype of BC is referred to as HER2+ (21). HER2+ BC is an aggressive form of the disease comprising of high-grade tumors, increased growth rates, early systemic metastases, and decreased disease-free and overall survival rates (27). Despite these negative outcomes, the introduction of trastuzumab and other HER2-targeted therapies has significantly improved the outcome of women with this poor prognosis form of the disease.

Trastuzumab (Herceptin®; Hoffman-La Roche) is a humanized mAb directed against HER2 and is currently the first line of treatment for women with HER2+ BC (30). Early trials demonstrated that trastuzumab is active when added to chemotherapy (anthracycline or paclitaxel) in women with HER2+ metastatic BC and that this combination therapy significantly prolonged the median time to disease progression, increased the overall response rate, increased the duration of response, and improved the median survival time when compared to treatment with chemotherapy alone (28). However, only about 1 in 2 patients with HER2+ BC in clinical trials responded to treatment with trastuzumab and chemotherapy, implying that many of these tumors are “inherently” resistant to the drug. Of these initial responders, almost all patients progressed within one year (28). Progression of the disease in patients being treated with this combination therapy suggests that “acquired” resistance frequently develops.
To overcome resistance, one potential strategy is radioimmunotherapy. Radioimmunotherapy utilizes monoclonal antibodies labeled with a therapeutic radionuclide to target and kill cancer cells. Thus, cell death would not solely rely on the cytotoxic properties of trastuzumab but on ionizing radiation delivered from the therapeutic radionuclide combined with trastuzumab. Moreover, the damaging effects of ionizing radiation can be enhanced by routing the radioimmunoconjugates directly into the nucleus via conjugation of a nuclear localization sequence (NLS) peptide (77).

Previous work by our group demonstrated that $^{111}$In-DTPA-NLS-trastuzumab was internalized and imported into the nucleus of HER2-overexpressing BC cells (SK-BR-3) causing frequent DNA DSBs mediated by the Auger electron emissions of $^{111}$In, which reduced their clonogenic survival to $<10\%$. $^{111}$In-DTPA-NLS-trastuzumab was 5-6-fold more potent for killing HER2-overexpressing BC cells than $^{111}$In-trastuzumab or unlabeled trastuzumab (77). In addition to these promising results, $^{111}$In-DTPA-NLS-trastuzumab efficiently decreased the clonogenic survival of a HER2+ BC cell line with acquired resistance to trastuzumab (TrR1) (60). An irrelevant $^{111}$In-labeled and NLS-modified human IgG control had no significant toxicity on these cells (77). Studies evaluating tumor growth inhibition revealed the proliferation rate of HER2-overexpressing tumors was 4-fold less than the rate of proliferation in mice treated with saline, and significantly lower than groups treated with $^{111}$In-trastuzumab, $^{111}$In-NLS-hIgG or trastuzumab. Furthermore, no non-specific toxicities of the hematopoietic system in non-tumor bearing mice were observed. No acute hepatic or renal toxicity was observed in these same mice for $^{111}$In-DTPA-NLS-trastuzumab (79). Results from this preclinical research suggest that the anti-tumor effects of $^{111}$In-NLS-trastuzumab observed in mouse models of BC may translate into tumor responses in HER2+ BC patients if this RIT agent could be advanced translated to Phase I clinical trial.
In order to advance $^{111}$In-NLS-trastuzumab to clinical evaluation in patients, it is necessary to design and formulate a kit for routine and robust preparation of the radiopharmaceutical under Good Manufacturing Practice (GMP) conditions and establish quality assays to assure its suitability for patient administration. A radiopharmaceutical kit is ideal for radiopharmaceutical preparation for human studies because most quality control tests can be done prior to preparing the agent for patient administration, kits can be stored and labeled for use on demand for patient studies, and the kits which employ radiometal chelation which routinely produces a labeling efficiency of $\geq 90\%$, meaning no post-labeling purification is needed (82). In this report we outline the process used by our radiopharmaceutical research laboratory at the University of Toronto to manufacture a pharmaceutical quality kit for the preparation of $^{111}$In-BzDTPA-NLS-trastuzumab injection.

2.3 Materials and Methods

2.3.1 Raw Materials

Trastuzumab IgG (Herceptin; Hoffman La Roche, Mississauga, ON, Canada) was reconstituted to 21 mg/mL with Bacteriostatic Water for Injection USP, following the manufacturer’s instructions. Sodium bicarbonate USP (NaHCO$_3$), sodium phosphate dibasic heptahydrate USP (Na$_2$HPO$_4$•7H$_2$O), ammonium acetate (NH$_4$Ac) American Chemical Society (ACS) grade ($C_3H_7NO_2$; $\geq 98\%$), and Polysorbate 20 European Pharmacopoeial (EP) grade (Tween 20®) were purchased from Sigma-Aldrich (St Louis, MO, USA). Sterile Water for Irrigation USP and Sodium Chloride for Irrigation USP were purchased from Baxter (Toronto, ON, Canada). 2-(4-isothiocyanatobenzyl)-diethylenetriamine-pentaacetic acid (p-SCN-BzDTPA; $\geq 94\%$) was purchased from Macrocycles, Inc. (Dallas, TX, USA). Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC; $\geq 90\%$) was purchased from
Thermo Scientific (Rockford, IL, USA). Nuclear Localization Sequence (NLS) peptides (CGYCPKKRKVGG) GMP grade (≥ 98%) was purchased from Bio Basic (Markham, ON, Canada). All other chemicals and reagents were purchased in analytical ACS grade with a purity >95%. Sterile, apyrogenic Type I glass vials (5 or 30 mL) were obtained by Jubilant HollisterStier (Montreal, QC, Canada). $^{111}\text{InCl}_3$ (>3.7 GBq/mL; <0.1% $^{114m}\text{In}$ and $^{65}\text{Zn}$) was purchased from Nordion (Kanata, ON, Canada). Certificates of actual lot analysis (COA) were obtained from the vendors. The identities of NaHCO$_3$, NH$_4$Ac and Na$_2$HPO$_4$•7H$_2$O were tested by USP methods.

### 2.3.2 Pharmaceutical Quality Buffers

Sterile 0.1 M NaHCO$_3$ buffer (pH 8.2) in Sodium Chloride for Irrigation USP was prepared. Sterile 0.1 M Na$_2$HPO$_4$ with 0.01% Tween 20® buffer (pH 7.3) and 0.05 M NH$_4$OAc with 0.01% Tween 20® buffer (pH 5.5) were prepared by dissolving the appropriate amounts of pharmacopoeial quality raw materials in Sterile Water for Irrigation USP and adding 2.5 mL of Tween 20® to each buffer (0.01% of the final volume). Removal of trace metal contaminants from the buffers was achieved by passage through a 60 mL column of Chelex-100 cation exchange resin (BioRad, Mississauga, ON, Canada) pre-hydrated for 1 hour in Sterile Water for Irrigation USP. 1N HCl and 12N HCl (Sigma-Aldrich) and glacial acetic acid (Sigma-Aldrich) were used to adjust the pH of NaHCO$_3$, Na$_2$HPO$_4$, and NH$_4$OAc respectively. The buffers were sterilized by filtration through a 0.22-µm Millex-GS filter (EMD Milipore, Billerica, MA, USA) into 30mL glass vials and stored at 2-8°C. All buffers were tested for sterility by the USP Sterility Test. Clarity and color were assessed by holding a vial of buffer against a light and dark background. The concentration of NaHCO$_3$ was assayed according to the USP method. The concentration of NH$_4$Ac was calculated based on weight added into the solution as no USP method was available.
2.3.3 Kit Formulation

Thirteen different development lots of kits were prepared with modification of various parameters such as: molar reaction ratios, buffer pH and concentration, addition of Tween 20®, and purification methods as summarized in Tables 1 and 2. After optimization of the kit formulation, 3 lots were prepared under GMP conditions meeting requirements for use in humans and conforming to all defined specifications based on the development lots.

Reconstituted trastuzumab (75 mg) was diluted with 0.1 M NaHCO₃ buffer (pH 8.2) and concentrated to approximately 4.5 mL using an Amicon Ultra-15 Centrifugal Filter Device with a nominal 30-kDa molecular weight cutoff (EMD Milipore, Billerica, MA, USA). The solution was centrifuged at 5000 × g for 10 min and the filtrate discarded. Dilution and ultrafiltration were repeated a total of 4 times. The retentate was recovered into a sterile 10 mL Reacti-Vial (Pierce-Chemical Co., Rockford, IL, USA). The concentration of trastuzumab was determined by UV-Visible spectrophotometric analysis (Ultrospec 3100 pro) at 280nm wavelength using an extinction coefficient 1.5 mL mg⁻¹cm⁻¹ (103), then adjusted to 16 mg/mL by addition of 0.1 M NaHCO₃ buffer (pH 8.2). Trastuzumab was derivatized with BzDTPA by adding a 10-fold molar excess of a 10 mg/mL solution of p-SCN-BzDTPA in 0.1 M NaHCO₃ buffer (pH 8.2). The reaction mixture was swirled for 10 secs, and allowed to incubate at room temperature for 1 hour. Two, 10 µL aliquots of the reaction mixture were removed for measurement of BzDTPA conjugation efficiency. To remove unconjugated BzDTPA, the solution was diluted with 0.1 M Na₂HPO₄ with 0.01% Tween 20® buffer (pH 7.3), centrifuged at 5000 × g for 10 min and the filtrate discarded. Dilution and ultrafiltration were repeated a total of 16 times, changing the centrifugal filter unit every 4 dilutions. At the final centrifugation step, BzDTPA-Trastuzumab was concentrated in the device to a final volume of approximately 8 mL. The retentate was
recovered into a sterile 10 mL Reacti-Vial (Pierce-Chemical Co., Rockford, IL, USA). The concentration of BzDTPA-trastuzumab was determined spectrophotometrically (Ultrospec 3100 pro) using an extinction coefficient 1.5 mL mg\(^{-1}\)cm\(^{-1}\) and a correction factor of 0.734 to correct for the contribution of BzDTPA to absorbance. Our group previously determined this correction factor by measuring the absorbance at 280 nm of IgG with and without spiking with known amounts of BzDTPA. The solution was diluted to a final concentration of 6 mg/mL. A 50 µL aliquot was removed for subsequent quality control assays. NLS peptides (CGYGPKKRKVGG; BIO Basic, Markham, ON, CA) were then conjugated to BzDTPA-modified trastuzumab using the heterobifunctional crosslinker sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC; Thermo Fischer). A 5-fold molar excess of a 10 mg/mL solution of sulfo-SMCC in 0.1 M Na\(_2\)HPO\(_4\) with 0.01% Tween 20® buffer (pH 7.3) was added to BzDTPA-trastuzumab. The reaction mixture was swirled for 10 secs, and allowed to incubate at room temperature for 1 hour. To remove free sulfo-SMCC, the solution was diluted with 0.1 M Na\(_2\)HPO\(_4\) with 0.01% Tween 20® buffer (pH 7.3), centrifuged at 5000 \(\times\) g for 10 min and the filtrate discarded. Dilution and ultrafiltration were repeated a total of 8 times, changing the centrifugal filter unit every 4 dilutions. At the final centrifugation step, BzDTPA-Trastuzumab-SMCC was concentrated in the device to a final volume of approximately 9 mL. The retentate was recovered into a sterile 10 mL Reacti-Vial (Pierce-Chemical Co., Rockford, IL, USA). The concentration of the solution was determined spectrophotometrically (Ultrospec 3100 pro) at 280nm wavelength using extinction coefficient 1.5 mL mg\(^{-1}\)cm\(^{-1}\) and a correction factor of 0.734 to correct for the contribution of BzDTPA to absorbance. The solution was diluted to a final concentration of 5 mg/mL. A 60-fold molar excess of a 20 mg/mL solution of NLS peptides dissolved in Sterile Water for Irrigation was added to BzDTPA-trastuzumab-SMCC. The reaction mixture was swirled for 10 secs, and left to incubate at 4°C overnight. To
remove free NLS peptides, the solution was diluted with 0.05 M NH₄Ac with 0.01% Tween 20® buffer (pH 5.5), centrifuged at 5000 × g for 10 min and the filtrate discarded. Dilution and ultrafiltration were repeated a total of 12 times, changing the centrifugal filter unit every 4 dilutions. At the final centrifugation step, BzDTPA-NLS-trastuzumab was concentrated in the device to a final volume of approximately 4.0 mL. The final filtrate was discarded. The retentate was drawn up in a 5 mL syringe with an attached needle and sterilized by passing through a 0.22-µm Millex-GV low protein binding filter (EMD Millipore) into a sterile 30 mL glass vial (HollisterStier). The concentration was measured by UV absorbance assay at 280 nm wavelength. The final concentration was adjusted to 5 mg/mL and 1 mL aliquots were drawn up in a 5 mL syringe with an attached needle and dispensed into single 5 mL vials (HollisterStier) to yield unit-dose kits. Kit vials were stored at 2-8°C.

2.3.4 Kit Quality Testing

The pharmaceutical quality of the kits was evaluated by assaying for protein concentration, pH, clarity and color, volume per vial, BzDTPA substitution level, protein homogeneity and dimerization (high molecular weight covalently linked species), NLS substitution, labeling efficiency, immunoreactivity, and sterility and apyrogenicity. The concentration of BzDTPA-NLS-trastuzumab was measured by UV absorbance assay at 280 nm wavelength. Interferences were accounted for by adjusting the extinction coefficient based on a known amount of trastuzumab and by using a correction factor of 0.734 to correct for the contribution of BzDTPA to absorbance. The pH of the kit formulation was assured by formulation in 0.05 M NH₄Ac with 0.01% Tween 20® buffer (pH 5.5), but was confirmed by spotting a drop of the solution on two types of narrow range pH paper: 1) pHdrion® range pH 4.5-7.5 in 0.5 unit increments (MicroEssential Laboratories, NY, USA) and 2) pHdrion® range.
pH 6-8 in 0.4 unit increments (MicroEssential Laboratories, NY, USA). Clarity and color were evaluated by examining a kit vial against a light and dark background. The volume of solution in each vial was determined by calculating the net weight of each vial before and after filling, assuming a density of 1 g/cm\(^3\). The BzDTPA substitution level was determined by trace labeling two 10 µL aliquots of unpurified BzDTPA-trastuzumab reaction mixture with 0.9 MBq of \(^{111}\)In and subsequently analyzing the radiolabeled mixture by instant thin-layer chromatography-silica gel (ITLC-SG; Pall Life Sciences, Ann Arbor, MI, USA). For these analyses, 0.5 µL from each of the samples was spotted onto an ITLC-SG strip and allowed to dry. Once the spot dried, the strip was developed in 100 mM sodium citrate pH 5.0, dried, cut into 4 sections, and counted in a \(\gamma\)-counter (Perkin Elmer 1480 Automatic Gamma counter). \(^{111}\)In-BzDTPA-NLS-trastuzumab remains at the origin (\(R_f=0.0\)) and \(^{111}\)In-BzDTPA migrates to an \(R_f\) of 0.6-0.7 and free \(^{111}\)In migrates to the solvent front (\(R_f=1.0\)) (98). The proportion of radioactivity at the origin (conjugation efficiency) was multiplied by 10 (the BzDTPA: trastuzumab molar reaction ratio used) in order to calculate BzDTPA substitution level (moles BzDTPA/trastuzumab).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad electrophoresis system) was used to analyze the purity and homogeneity of the immunoconjugates. 2 µg of trastuzumab, BzDTPA-trastuzumab, and BzDTPA-NLS-trastuzumab were electrophoresed on a 5% Tris-Glycine mini-gel. The samples were run along with a Thermo Scientific PageRuler Protein Ladder, then stained with Coomassie R-250 Blue to reveal the protein bands. This method was also used on a 4-20% SDS-PAGE gel for select lots to evaluate purity. After developing the gel, densitometry was used to analyze the band density of any visible bands (UN-SCAN-IT gel, Silk Scientific Corporation, Utah, USA). To estimate the number of NLS-peptides conjugated to BzDTPA-trastuzumab, the difference between the
molecular weight values of BzDTPA-trastuzumab and BzDTPA-NLS-trastuzumab was divided by the molecular weight of the NLS-peptide plus sulfo-SMCC (~1850 Da). Alternatively, the number of NLS conjugated to trastuzumab was determined by including trace labeled $^{123}$I-labeled NLS-peptides in the reaction. NLS-peptides were labeled using the IODO-GEN method (Pierce) with $^{123}$I-sodium iodide (MDS-Nordion, CA). Briefly, 10 mg of Iodogen (1,3,4,5-tetrachloro-3α,6α-diphenylglycouril) was transferred into a glass test tube and 200 µL of chloroform (Sigma-Aldrich, St. Louis, MO, USA) was added to dissolve the sample. The mixture was then evaporated under a gentle stream of nitrogen. Then, NLS peptides dissolved in Sterile Water for Injection USP was added to the test tube containing dry Iodogen. In a fume hood, 74 MBq of $^{123}$I sodium iodide was added to the solution and incubated for 10 minutes at room temperature. After the reaction, $^{123}$I-NLS peptide was added to BzDTPA-SMCC-trastuzumab in a 60-fold molar excess and left overnight at 4°C. The radioiodinated BzDTPA-NLS-trastuzumab was purified by ultracentrifugation. The radiochemical purity of $^{123}$I-NLS peptides was determined by paper chromatography developed in 85% methanol ($R_f$: $^{123}$I–iodide = 0 or $^{123}$I-NLS = 1.0). The two methods for NLS substitution determination were compared.

The immunoreactivity of $^{111}$In-BzDTPA-NLS-trastuzumab was determined using a saturation radioligand binding assay with SK-BR-3 human breast cancer cells (1-2x10^6 HER2/cell). The SK-BR-3 cells were grown in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS). Increasing concentrations (0.07-300 nmol/L) of $^{111}$In-BzDTPA-NLS-trastuzumab were incubated with 0.5-2x10^6 SK-BR-3 cells in phosphate buffered saline (PBS) in 1.5-mL Eppendorf tubes with gentle shaking at 4°C for 3 hours. The tubes were then centrifuged in order for the pellet and supernatant to be separated and then counted in a γ-counter. The assay was performed in the presence [non-specific binding (NSB)] or absence [total binding (TB)] of 50 fold molar excess of unlabeled trastuzumab. Specific binding (SB) was obtained by
subtracting the NSB from TB and plotted vs. the applied concentration (nmol/L) of unbound $^{111}$In-BzDTPA-NLS-trastuzumab. GraphPad Prism software (San Diego, California, USA) was used to fit the data to a one-site specific binding model by non-linear regression so both the $K_a$ (affinity constant) and $B_{max}$ (maximum number of receptors) were obtained. A 5% sample from each lot of kit was tested for sterility and pyrogenicity by the USP Sterility Test and USP Bacterial Endotoxins Test (QCL-1000 Endpoint Chromogenic LAL Assay, Lonza, Walkersville, MD, USA) respectively.

The labeling efficiency of each lot of kits was tested by adding $^{111}$InCl$_3$ to the kits and incubating for 2 hours at room temperature. ITLC-SG was used as previously described to determine the proportion of $^{111}$In bound to BzDTPA-NLS-trastuzumab ($^{111}$In-BzDTPA-NLS-trastuzumab) vs. free $^{111}$In.

2.3.5 Final Radiopharmaceutical

$^{111}$In-BzDTPA-NLS-trastuzumab was prepared by aseptically decapping a single unit-dose vial of kit in a laminar air flow hood (biosafety cabinet) and adding $^{111}$InCl$_3$ to achieve a specific activity of 20-33 MBq/mg using a micropipette and sterile pipette tip. After a 2 hour incubation period at room temperature, the radiopharmaceutical was drawn up into a lead glass-shielded syringe and filtered through a 0.22-μm Millex-GV filter into a 5 mL sterile, pyrogenic Type 1 glass vial to ensure sterility of the final product. The radioactivity of the final radiopharmaceutical was measured in a dose calibrator (Capintec Model CRC-15R, Ramsey, NJ, USA). Labeling efficiency, pH, clarity and color were determined as described previously. Sterility and pyrogenicity were assessed retrospectively by USP Sterility and USP Bacterial Endotoxins Tests after allowing 30 days for radionuclide decay.
2.3.6 Imaging and biodistribution studies

Female athymic CD1 nude mice (Charles River Laboratories, Wilmington, MA, USA) were inoculated subcutaneously with HER2-positive SK-OV3 human ovarian cancer cells. A group of 3 tumor-bearing mice was injected i.v. (tail vein) with $^{111}$In-BzDTPA-NLS-trastuzumab (14 MBq; 13 µg). A blocking study was performed in one mouse, which was injected with 1 mg of trastuzumab 24 hours before injection of $^{111}$In-BzDTPA-NLS-trastuzumab. MicroSPECT/CT images were taken 72 hours post injection using a nanoSPECT/CT tomograph (BioScan, Washington, DC, USA) equipped with 4 NaI scintillation detectors fitted with 1.4mm multi-pinhole collimators [full width half maximum(FWHM) ≤1.2mm]. A total of 24 projections were acquired in a 256 x 256 matrix with a minimum of 70,000 counts per projection. Anesthesia was induced and maintained by inhalation of 2% isofluorane in O$_2$. Cone-beam CT images were obtained (180 projections, 1 s/projection, 45 kVp) prior to the micro/SPECT images. Co-registration of microSPECT and CT images was achieved using InvivoScope software (Bioscan). At 72 hours p.i. after completion of imaging, the mice were sacrificed and a blood sample was taken by intracardiac puncture. The tumor and samples of selected tissues were collected, blotted dry, and weighed. Uptake of radioactivity by these tissues was measured in a γ-counter and expressed as the percentage injected dose per gram (%ID/g). Animal studies were conducted under a protocol (#3940.2) approved by the Animal Care Committee at the University Health Network and following the Canadian Council on Animal Care (CCAC) guidelines.

2.3.7 Statistical Analysis

Data were presented as mean ± standard deviation (SD) or standard error of the mean (SEM).
2.4 Results

2.4.1 Production of Pharmaceutical Quality Buffers

Five lots of 0.1 M NaHCO₃ buffer (pH 8.2) in Sodium Chloride for Irrigation USP were prepared. All lots passed specifications for concentration (95-105 mM), pH (8.18-8.22), appearance (clear, colorless and particulate free), and passed the USP Sterility Test.

Four lots of sterile 0.1 M Na₂HPO₄ with 0.01% Tween 20® buffer (pH 7.3) were prepared. All lots met specifications for pH (5.48-5.52), appearance (clear, pale-yellow, particulate free), and passed the USP Sterility Test.

Two lots of sterile 0.05 M NH₄Ac with 0.01% Tween 20® buffer (pH 5.5) were prepared. This buffer was not assayed for concentration due to the absence of a USP assay. Instead, the concentration of NH₄Ac was calculated based on the weight incorporated into the solution. All lots met specifications for concentration (95-105 mM), pH (5.48-5.52), appearance (clear, pale-yellow, particulate free), and passed the USP Sterility Test.

2.4.2 Kit Formulation Development

Table 4 and Table 5 summarize manufacturing methods and key quality control results from all produced kits that were critical to reach a formulation method resulting in a kit meeting all specifications. The parameters employed in Lots 1-4 resulted in aggregation of the immunoconjugates. Thus, these kits were not tested further by other quality control assays. Lots 5-7 and 10 did not show visible aggregates but quality testing by SDS-PAGE and subsequent quantification of the bands by gel densitometry revealed dimerization of the immunoconjugates as revealed by high molecular weight bands (~300 kDa for dimerized immunoconjugates vs. ~150 kDa for monomeric immunoconjugates). Furthermore, lots 8-13 had low labeling efficiency (<90%) likely due to the presence of unconjugated BzDTPA in the final formulation.
Removal of unconjugated BzDTPA was achieved by adjusting purification methods until a final labeling efficiency of >90% was obtained. This was done by changing the centrifugal filter unit every 4 dilutions and increasing the number of ultrafiltration steps through the manufacturing campaign (Table 5).
Table 4. Overview of development lots of kits for preparation of $^{111}$In-BzDTPA-NLS-trastuzumab including manufacturing parameters details and quality control results.

<table>
<thead>
<tr>
<th>Lot No</th>
<th>Molar Reaction Ratio (moles)</th>
<th>Buffer Selection</th>
<th>Quality Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SMCC: NaPO₄</td>
<td>NLS: NaPO₄</td>
<td>NH₄Ac</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>50</td>
<td>x</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>50</td>
<td>x</td>
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<tr>
<td>3</td>
<td>10</td>
<td>50</td>
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</tr>
<tr>
<td>4</td>
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<td>5</td>
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<td>5</td>
<td>75</td>
<td>x</td>
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<td>8</td>
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<td>9</td>
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<tr>
<td>10</td>
<td>5</td>
<td>60</td>
<td>x</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>60</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>60</td>
<td>×</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>60</td>
<td>×</td>
</tr>
<tr>
<td>14 (GMP)</td>
<td>5</td>
<td>60</td>
<td>×</td>
</tr>
<tr>
<td>15 (GMP)</td>
<td>5</td>
<td>60</td>
<td>×</td>
</tr>
<tr>
<td>16 (GMP)</td>
<td>5</td>
<td>60</td>
<td>×</td>
</tr>
</tbody>
</table>

§ = too many aggregates to continue

n.d. = not done

tmab = trastuzumab

PS20 = Polysorbate 20
Table 5. Overview of different purification methods used in formulating developmental lots of kits for preparation of $^{111}$In-BzDTPA-NLS-trastuzumab

<table>
<thead>
<tr>
<th>Lot</th>
<th># of Ultrafiltration Steps:</th>
<th>PD10 Size Exclusion Column Used After BzDTPA Conjugation</th>
<th>Change Amicon Filter every 4 Ultrafiltration Steps:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After BzDTPA Conjugation</td>
<td>After SMCC Conjugation</td>
<td>After BzDTPA Conjugation After SMCC Conjugation</td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>13</td>
<td>17</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>14*</td>
<td>16</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>

✔ = the purification method was performed

* = The method used in Lot 14 was used in all subsequent kits
NLS peptide substitution in Lots 5-13 was determined by including a trace-labeled amount of $^{123}$I-NLS in the reaction. However, a low molar reaction ratio of SMCC: BzDTPA-trastuzumab and high number NLS peptides conjugated to trastuzumab from these kits suggested inaccurate results because it was not likely there were more NLS peptides conjugated to trastuzumab than maleimide introduced through reaction of trastuzumab with SMCC. Results from analysis of a 4-20% SDS-PAGE gel detected unconjugated NLS peptides in the final formulation (Figure 5), which interferes with conjugated NLS quantification because the counts associated with the free peptides are assumed to be associated with peptides conjugated to trastuzumab when they are not. To facilitate removal of free NLS peptides in the final product, the purification method was changed as shown in Table 5 to result in a product with low amounts of free NLS peptides as assessed by a 4-20% SDS-PAGE gel (Figure 8). Subsequently, the number of NLS peptides conjugated to BzDTPA-trastuzumab was estimated by measuring the band shift on a 5% SDS-PAGE gel (Figure 6).
Figure 5. SDS-PAGE analysis of a representative kit for the preparation of $^{111}$In-BzDTPA-NLS-trastuzumab (Lot 13, Table 4) on a 4-20% gel under non-reducing conditions. Lane MW: Molecular weight markers (kDa). Lane 1: trastuzumab; Lane 2: BzDTPA-trastuzumab; Lane 3: BzDTPA-NLS-trastuzumab containing a proportion of unconjugated NLS peptides; Lane 4: NLS peptides. The low molecular weight band in lane 3 (red box) migrates to the same position as NLS peptides (lane 4), indicating there are unconjugated NLS peptides in BzDTPA-NLS-trastuzumab in this lot.
Figure 6. SDS-PAGE analysis of a representative kit for the preparation of $^{111}$In-BzDTPA-NLS-trastuzumab (Lot 15, Table 4) on a 5% gel under non-reducing conditions. Lane MW: Molecular weight markers (kDa). Lane 1: trastuzumab; Lane 2: BzDTPA-trastuzumab; Lane 3: BzDTPA-NLS-trastuzumab. The shift in the band for BzDTPA-NLS-trastuzumab (red line; lane 3) compared to BzDTPA-trastuzumab (blue line; lane 2) is due to NLS peptide conjugation. The number of NLS peptides conjugated per BzDTPA-trastuzumab is calculated based on the equations shown at the right.

Formulas used to calculate the shift in band between lanes 2 and 3:

\[
\frac{260 - 140 \text{ kDa}}{x \text{ mm}} = \frac{\text{ kDa}}{\text{ mm}}
\]

\[
\frac{\text{ kDa}}{\text{ mm}} \times z = \frac{\text{ kDa}}{\text{ mm}}
\]

\[
\frac{\text{ kDa} \times 1000}{1856.09 \text{ Da}} = \text{ NLS}
\]

\[x = \text{ distance between the 140 and 260 kDa MW markers in mm}\]

\[z = \text{distance between the red and blue lines in mm}\]
2.4.3 Kit Development

Three lots of kit for the preparation of $^{111}$In-BzDTPA-NLS-Trastuzumab injection were prepared and all passed quality specifications (Table 6). Each manufacturing campaign resulted in three kits per lot. These lots met specifications for concentration (4.5-5.5 mg/mL), pH (5.5-6.5), and volume per vial (0.95-1.05 mL). The solution in all kits was clear, pale yellow, and particulate free. BzDTPA substitution was determined to be 3.3±0.6 BzDTPA/trastuzumab, which falls within the target specifications (2-7 BzDTPA/trastuzumab). Densitometry on the high molecular weight bands on a 5% SDS-PAGE gel was conducted to determine any dimerization of BzDTPA-NLS-trastuzumab in the kits (Figure 7). All three lots had 5.3%±2.8% density associated with high molecular weight bands, which was within specifications (<10% band density for any high molecular weight bands) (Table 6). It was estimated that there are 6 NLS/BzDTPA-trastuzumab, which is within desired specifications (5-11 NLS/trastuzumab). The mean labeling efficiency was 92.8%±0.08%, meaning all lots of kits met specifications for this parameter ($\geq$90%; Table 6). The mean $K_a$ and $B_{max}$ values for binding to HER2 on SKBR3 cells were $3.6 \pm 3.7 \times 10^8$ L/mol and $1.0 \pm 0.4 \times 10^6$ receptors/cell respectively (Figure 9). These lots also met specifications for sterility (passed USP Sterility Test), and apyrogenicity (passed USP Bacterial Endotoxins Test).
### Table 6. Quality testing of kits for the preparation of $^{111}$In-BzDTPA-NLS-trastuzumab Injection

<table>
<thead>
<tr>
<th>Test</th>
<th>Specifications</th>
<th>Observed$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Concentration</td>
<td>4.5 – 5.5 mg/mL</td>
<td>4.8±0.3 mg/mL</td>
</tr>
<tr>
<td>Volume</td>
<td>0.95-1.05 mL</td>
<td>1.0±0.0 mL</td>
</tr>
<tr>
<td>pH</td>
<td>5.5 – 6.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Appearance</td>
<td>Clear, pale yellow, particulate-free</td>
<td>Passed</td>
</tr>
<tr>
<td>Sterility</td>
<td>Passes USP XXXIV Test</td>
<td>Passed</td>
</tr>
<tr>
<td>Endotoxins</td>
<td>Passes USP XXXIV Test</td>
<td>Passed</td>
</tr>
<tr>
<td>BzDTPA Substitution Level</td>
<td>2.0 – 7.0 BzDTPA/Trastuzumab</td>
<td>3.3±0.6 BzDTPA</td>
</tr>
<tr>
<td>Densitometry on SDS-PAGE</td>
<td>&gt; 90% Density on major band</td>
<td>95%±3%</td>
</tr>
<tr>
<td>NLS Substitution Level</td>
<td>5-11 NLS/Trastuzumab</td>
<td>6.0</td>
</tr>
<tr>
<td>Radiolabelling Efficiency with $^{111}$In Chloride</td>
<td>≥ 90%</td>
<td>92.8%±0.1%</td>
</tr>
<tr>
<td>HER2 Immunoreactivity</td>
<td>$K_a = 1.1-7.8 \times 10^8$ L/mol</td>
<td>$K_a = 3.6 \pm 3.7 \times 10^8$ L/mol</td>
</tr>
<tr>
<td></td>
<td>$B_{\text{max}} = 0.5-2 \times 10^6$ sites/cell</td>
<td>$B_{\text{max}} = 1.0 \pm 0.4 \times 10^6$ sites/cell</td>
</tr>
</tbody>
</table>

$^a$ Quality testing results of three separate lots of kits (Lot 14, 15, 16). Numerical values represent mean ± S.D.
Figure 7. SDS-PAGE analysis of a representative kit for the preparation of $^{111}$In-BzDTPA-NLS-trastuzumab (Lot 15, Table 4) on a 5% gel under non-reducing conditions. Lane MW: Molecular weight markers (kDa). Lane 1: trastuzumab; Lane 2: BzDTPA-trastuzumab; Lane 3: BzDTPA-NLS-trastuzumab. The shift in the band for BzDTPA-NLS-trastuzumab (red box lane 3) compared to BzDTPA-trastuzumab (lane 2) is due to NLS peptide conjugation. The high molecular weight band shown in lane 3 (blue box) represents <7% of the total protein based on gel densitometry.
**Figure 8.** SDS-PAGE analysis of a representative kit for the preparation of $^{111}$In-BzDTPA-NLS-trastuzumab (Lot 15, Table 4) on a 4-20% gel under non-reducing conditions. Lane MW: Molecular weight markers (kDa). Lane 1: trastuzumab; Lane 2: BzDTPA-trastuzumab; Lane 3: BzDTPA-NLS-trastuzumab; Lane 4:NLS. The low molecular weight band in lane 3 (NLS, red box) has a greatly reduced intensity compared to the equivalent band in Figure 5, indicating the amount of free NLS in BzDTPA-NLS-trastuzumab has been greatly reduced.
**Figure 9.** Saturation radioligand binding assay to assess the binding affinity of $^{111}$In-BzDTPA-NLS-Trastuzumab to HER2 on SK-BR-3 human breast cancer cells prepared from kit Lot 15, Table 4. The $K_D$ estimated by fitting the specific binding curve to a one-site receptor binding model was $0.6 \times 10^{-8}$ mols/L and the estimated $B_{max}$ value was $1.4 \times 10^6$ receptors/cell.
2.4.4 Final Radiopharmaceutical

Three lots of the final radiopharmaceutical product, $^{111}$In-BzDTPA-NLS-trastuzumab, were prepared. All lots met specifications for specific radioactivity, pH, labeling efficiency, radionuclide purity, appearance, and sterility (Table 7). All assays were conducted as previously described. Sterility was assessed retrospectively following radionuclide decay.
<table>
<thead>
<tr>
<th>Test</th>
<th>Specification</th>
<th>Results$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific Radioactivity</td>
<td>20-33 MBq/mg</td>
<td>26.7±4.1 MBq/mg</td>
</tr>
<tr>
<td>pH</td>
<td>5.5-6.5</td>
<td>6.0±0.0</td>
</tr>
<tr>
<td>Labeling Efficiency</td>
<td>≥ 90%</td>
<td>92.8%±0.1%</td>
</tr>
<tr>
<td>Radionuclide Purity</td>
<td>&gt; 99.9% (&lt;0.1% $^{114m}$ In or $^{65}$Zn)</td>
<td>Pass</td>
</tr>
<tr>
<td>Appearance</td>
<td>Clear, pale yellow, particulate-free</td>
<td>Pass</td>
</tr>
<tr>
<td>Sterility (retrospectively tested)</td>
<td>Pass USP XXXIV Test (retrospective)</td>
<td>Pass</td>
</tr>
</tbody>
</table>

$^a$ Quality testing results of three separate lots of radiopharmaceutical. Numerical values represent mean ± S.D.
2.4.5 Imaging and Biodistribution Studies

HER2+ SK-OV-3 tumor xenografts were clearly imaged by microSPECT/CT at 72 hours post-injection of $^{111}$In-BzDTPA-NLS-trastuzumab with modest uptake in normal organs (Figure 10. Not Blocked). Pre-injection of 1 mg of unlabeled trastuzumab at 24h before $^{111}$In-BzDTPA-NLS-trastuzumab reduced tumor radioactivity (Figure 10. Blocked), demonstrating that uptake of $^{111}$In-BzDTPA-NLS-trastuzumab was tumor specific. Biodistribution studies showed tumor uptake was $5.1 \pm 1.6 \%$ID/g and the tumor–to-blood ratios were $9.7 \pm 5.6 \%$ID/g (Figure 11). The highest concentration of radioactivity in normal tissues was found in the liver, kidneys, and spleen, which is similar to previously reported results (77).
Figure 10. Posterior whole-body microSPECT/CT images of Balb/c nude mice bearing s.c. HER2-positive SK-OV-3 human ovarian cancer xenografts at 72 hours post-injection of $^{111}$In-BzDTPA-NLS-trastuzumab (14 MBq; 13 µg) without blocking or after blocking HER2 by pre-administration of 1 mg of unlabeled trastuzumab. The white arrow points to the tumor and the green arrow points to the liver.
**Figure 11.** Tumour and normal tissue biodistribution of $^{111}$In-BzDTPA-NLS-trastuzumab (14 MBq; 13 µg) at 72 hours post-injection in Balb/c nude mice bearing s.c. HER2+ SK-OV-3 human ovarian cancer xenografts. Values shown are the mean ± SEM (n=3).
2.5 Discussion

In this report, we describe for the first time a kit formulation for the preparation of $^{111}$In-BzDTPA-NLS-trastuzumab suitable for preparing the radiopharmaceutical for human administration for Auger electron RIT of HER2+ metastatic BC. Kits are desirable for labeling radiopharmaceuticals with relatively short-lived radiometals because key quality control tests can be performed prior to preparing the final radiopharmaceutical agent for patients. On the day of patient administration, the kits are designed to label the radiopharmaceutical to $>$90% so that post-labeling purification is not necessary (82). Manufacture of the kits for preparation of $^{111}$In-BzDTPA-NLS-trastuzumab under GMP conditions presented unique challenges such as maintaining solubility of the immunoconjugates and ensuring efficient purification from unconjugated BzDTPA or NLS peptides.

Although we have successfully designed kits for labeling human epidermal growth factor (100) or trastuzumab Fab (99) with $^{111}$In, neither of these kits presented with the challenge of solubility. Solubility is important because the presence of insoluble aggregates in antibody formulations can lead to an immune response (86). Immunogenicity may present as no observable effect, neutralization of therapeutic effectiveness of the antibody, or serious adverse reactions such as anaphylaxis (86). To minimize the chance of any adverse events, visible particles in our formulation were deemed unacceptable. The United States Pharmacopoeia (USP 39-NSF 24) Chapter $<$1$>$ Injections (Foreign and Particulate Matter section) states that “Each final container of all parenteral preparations shall be inspected to the extent possible for the presence of observable foreign and particulate matter (“visible particulates”). The inspection process shall be designed and qualified to ensure that every lot of all parenteral preparations is essentially free from visible particulates.” This standard was also applied in the formulation of
the kits for $^{111}$In-BzDTPA-NLS-trastuzumab. Furthermore, it should be brought to the attention of the reader that these guidelines were set to regulate visible particulates introduced through the manufacturing process rather than intrinsic aggregated or dimerized proteins, which was the challenge encountered in kit formulation (104). The relationship between subvisible aggregated proteins and the magnitude of the immune response is not clear and at the time of kit development no guidelines existed regulating amounts of aggregated proteins in radiopharmaceutical injections at the time of kit development, so it was best to keep levels of these as low as possible (104). The absence of USP guidelines regulating amounts of aggregated proteins in radiopharmaceutical injections was a challenge in kit development because in house quality control tests and specifications needed to be developed without guidance from the USP. Furthermore, current compendial acceptance criteria only exists for relatively large subvisible particles (>10 and 25 µm diameter), whereas criteria for smaller particles are not well defined (104). This makes apparent the importance of filtering the final product through a 0.22 µm sterilizing filter to remove any visible or subvisible particulates with a diameter of > 0.22 µm from the solution, but these particles may reform. To quantify nonvisible aggregated particulates the light obscuration test may be conducted using commercially available analytical instruments (Hach, CO, USA) under pharmacopeial methods. This method quantifies particulates according to size based on the principle of light scattering but limitations do exist. One limitation of this method is that the presence of bubbles in the formulation can be mistaken for aggregates during counting, thus producing erroneous results (92). However, post-filtering analysis by SDS-PAGE revealed subvisible covalently linked proteins were present in our product. Therefore to ensure the quality and safety of the radiopharmaceutical, stringent specifications were established to minimize the amount of covalently linked proteins in the final product (Figure 8).
\(^{111}\)In-BzDTPA-NLS-trastuzumab was efficiently labeled with high yields under GMP conditions (≥90%) as measured by ITLC. The highest labeling efficiency was obtained by removing free BzDTPA by ultracentrifugation whereas purification using a PD-10 column and ultrafiltration reduced the labeling efficiency to 43%. An additional challenge arose from the use of PS20. Although necessary to reduce aggregate formation, PS20 interfered with the ability of ultrafiltration to efficiently remove any impurities from the product (i.e. unconjugated BzDTPA and NLS peptide). PS20 exerts its stabilizing effects on liquid-antibody formulations by occupying the air-solvent interface thereby reducing the concentration of antibody molecules at the boundary, but PS20 seemed to block the passage of small molecules through the pores of the Amicon ultrafiltration device, resulting in free BzDTPA being retained in the kit solution causing poor labeling efficiency (89). This challenge was solved by replacing the Amicon filter every four ultrafiltration steps to prevent PS20 build up on the filter and facilitate more efficient removal of free BzDTPA from the solution. This was also seen during purification after conjugation with NLS. The protein yield in lots 11 and 12 (Table 4) suggested contaminants that were interfering with absorbance at 280nm. Interference with the accuracy of protein concentration was attributed to the presence of unconjugated NLS peptides in the final solution, since the presence of a tyrosine group in the NLS peptide (CGYGPKKKRKVGG) would also be detected by measuring the absorbance at OD280 thus resulting in a falsely high protein concentration. This was confirmed by the results of a 4-20% SDS–PAGE gel (Figure 5). Efficient removal of free NLS peptides from the kit solution was achieved by replacing the Amicon every four ultrafiltration steps to facilitate removal of free NLS peptide from the product (Figure 8).

\(^{111}\)In-BzDTPA-NLS-trastuzumab demonstrated preserved immunoreactivity with HER2 on SK-BR-3 human BC cells (Figure 9). The \(K_a\) value for \(^{111}\)In-BzDTPA-NLS-trastuzumab
prepared from the kit formulation ($K_a=3.6 \pm 3.7 \times 10^8 \text{L/mol}$) was similar to previously reported values for $^{111}$In-DTPA-NLS-trastuzumab incorporating 6 NLS peptides ($K_a=3.2 \pm 0.3 \times 10^8 \text{L/mol}$) (77). However, the previously reported $K_a$ value for $^{111}$In-DTPA-NLS-trastuzumab was 1.6-fold lower than that for $^{111}$In-BzDTPA-trastuzumab IgG ($K_a=5.9 \pm 2.8 \times 10^8 \text{L/mol}$) indicating a slightly lower affinity of the NLS-modified conjugate for binding HER2 (99). Modest decreases (<10 fold) in immunoreactivity have not been associated with decreased tumor localization in mouse xenograft models for other radiolabeled mAbs (105). Nonetheless, these lower $K_a$ values did not interfere with imaging HER2 positive SK-OV-3 human ovarian cancer xenografts in female Balb/c nude mice in this study using $^{111}$In-BzDTPA-NLS-trastuzumab.

Biodistribution studies demonstrated the in vivo targeting of $^{111}$In-BzDTPA-NLS-trastuzumab of a tumor overexpressing HER2. Moderate uptake in the liver, spleen, and kidneys was expected and similar to results for other $^{111}$In-labeled antibodies (98,106) and previously reported values for $^{111}$In-BzDTPA-NLS-trastuzumab with 6 NLS peptides (77). Kidney uptake may be due to charge interactions between cationic NLS peptides and anionic charges on the membrane of renal tubular cells, while liver and spleen uptake are possibly due to the presence of Fc-receptors in the liver and spleen (107).

### 2.6 Conclusion

A kit for the preparation of $^{111}$In-BzDTPA-NLS-trastuzumab injection suitable for patient administration was formulated. Quality parameters and assays were established to assure the suitability of these kits for preparing the radiopharmaceutical for advancement to a Phase 1 clinical trial. Future studies will evaluate its pharmacokinetics, radiation dosimetry, and normal tissue toxicities required for regulatory approval of a Clinical Trial Application (CTA) by Health Canada.
2.7 Acknowledgements

This study was supported by a grant from the Canadian Breast Cancer Foundation (Ontario Branch). This research was presented at the 29th Annual European Association of Nuclear Medicine Congress in Barcelona, Spain in October 2016.
Chapter 3 Future Directions
3 Future Directions

3.1 Future Directions

To ensure the kit for the preparation of $^{111}$In-BzDTPA-NLS-trastuzumab can be stored and labeled when needed, stability testing must be performed. Three independent lots of kits and the final radiopharmaceutical must be tested monthly against all established specifications except for sterility and apyrogenicity (82). An expiry date for the kit should be assigned upon completion of these studies. Furthermore, an expiry must be assigned to the final radiopharmaceutical to ensure the kit maintains high radiochemical purity prior to patient administration, likely within 24 hours of preparation. Although a formulation was reached for $^{111}$In-BzDTPA-NLS-trastuzumab that met specifications, 13 developmental lots were needed (Table 4). Manufacturing these developmental lots consumed time and resources. To reach a final formulation meeting specifications more efficiently it may be useful to conduct studies to characterize the physicochemical properties of the radioimmunoconjugate (i.e. its physical and chemical properties) and then choose reaction conditions based on these properties, instead of choosing reaction conditions that were used to manufacture kits previously developed in our lab. For example, Tween 80 may have been a more useful solubilizer than Tween 20 due to its more hydrophobic properties (96).

The ultimate goal of the project is to conduct a Phase 1 clinical trial of $^{111}$In-BzDTPA-NLS-trastuzumab in patients with HER2+ metastatic BC. In order to do so, a Clinical Trial Application (CTA) needs to be prepared. Now that a unit dose kit for the preparation of $^{111}$In-NLS-trastuzumab has been formulated under Good Manufacturing Practices (GMP) conditions, the next step in clinical translation would be to conduct preclinical pharmacology and toxicology studies.
To predict the radiation absorbed dose to humans for the Phase I trial, more detailed normal tissue biodistribution studies and pharmacokinetic studies need to be performed to determine the uptake and elimination of radioactivity from the blood and normal tissues. From this data the cumulative radioactivity in each source organ (Å, Bq × hr) can be estimated which in turn is used to predict the radiation absorbed doses (D, mSv) to target organs in humans. Organ Level Internal Dose Assessment (OLINDA) software is used for this analysis. OLINDA estimates target organ doses as $D = \bar{A} \times S$; where $S$ is the dose (mSv/Bq × hr) to a target organ per unit of cumulative radioactivity in a source organ(108). Acute toxicity needs to be studied as well. The main purpose of the toxicology studies for the planned CTA is to determine if the toxicology profile of trastuzumab was altered by $^{111}$In and NLS peptide conjugation. These studies may be performed in female non-tumor bearing mice at 10 times the planned injected mass dose scaled down from a human to a mouse on a mg/kg basis (0.2 µg; 0.4 MBq). Body weight should be continuously measured over a 15 day period. Other measurements needed for evaluation of toxicity include complete red blood cell counts (CBC), hemoglobin (Hb), hematocrit (HCT), serum creatinine (Cr), and serum alanine amino-transferase (ALT) at 2 and 15 days post-injection. Once these measurements are complete, mice should be sacrificed and tissues should be assessed for histopathological changes. Our laboratory has previously conducted these studies for a kit for the preparation of $^{111}$In-BzDTPA-pertuzumab that was successfully translated to the clinic (109).

Next for clinical translation is the design of the clinical trial and human ethics approval. In the past, our group has partnered with the Ontario Clinical Oncology Group (OCOG) to design and conduct Phase I trials for novel molecular imaging agents under Good Clinical Practices (GCP). Before individuals can be enrolled in a clinical trial, a research ethics board
must approve the research. In our case, the Ontario Cancer Research Ethics Board (OCREB) grants approval. The role of the OCREB is to ensure that the proposed research protects the rights, well-being, and safety of the study participants (110). An application for human ethics approval must be submitted and approved by this group prior to study commencement.

The last step in clinical translation is regulatory agency submission. In Canada, a CTA must be submitted to Health Canada. Generally, to advance $^{111}\text{In-BzDTPA-NLS}$-trastuzumab to a Phase I Clinical Trial in Canada the information previously discussed needs to be organized into three clear and distinct parts (modules):

1. Chemistry and Manufacturing
2. Preclinical Pharmacology and Toxicology
3. Clinical Trial Protocol, Investigators Brochure, and Informed Consent Form

Required documents supporting these modules for the CTA include the Drug Submission Application (Form HC3011), Clinical Trial Site Application form, Quality Information Summary- Radiopharmaceuticals, and Quality Information Summary-Biologics. The additional forms provide a summary of all the standards and specifications put in place to ensure the quality of all aspects of the radiopharmaceutical from the raw materials used to the final radiopharmaceutical. Information obtained from developmental batches should also be included to give a complete understanding of the radiopharmaceutical.

Once the application is complete it can be submitted to Health Canada for review. If the CTA is approved a “No Objection Letter” will be issued within 30 days of submission and the trial can begin!
References


77. Costantini DL, Chan C, Cai Z, Vallis K a, Reilly RM. (111)In-labeled trastuzumab


