Bispecific Radioimmunoconjugates for Molecular Imaging and Radioimmunotherapy of HER2 Overexpressing Breast Cancer

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
University of Toronto

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Abstract

The HER2 receptor is overexpressed in approximately 20% of breast cancer (BC) cases, and is the target for multiple therapies. One challenge in treating HER2+ BC is that a large proportion of patients demonstrate resistance to therapy. Upregulation of other members of the HER family, plays an important role in resistance to HER2-targeted therapies. The hypothesis was that bispecific radioimmunoconjugates (bsRICs) developed against HER2 and another member of the HER receptor family would be able to successfully target cells expressing one or more receptor for molecular imaging and radioimmunotherapy of BC.

Trastuzumab Fab fragments were conjugated to heregulin (HRG) or epidermal growth factor (EGF) with a 24-mer polyethylene glycol (PEG) linker, and labeled with $^{111}$In or $^{177}$Lu. $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG and $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF demonstrated the ability to specifically bind to one or both receptors on cancer cell lines, and demonstrated higher binding than monospecific agents recognizing HER2, HER3 or EGFR. SPECT imaging was performed on mice bearing tumour xenografts, and optimal tumour uptake was found 48 hr after injection.
Biodistribution studies showed tumour: blood uptake ratios of 6-8. The cytotoxicity of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF and $^{177}$Lu- DOTA-Fab-PEG$_{24}$-EGF was evaluated through clonogenic assays which revealed specific cell killing. Radiation absorbed doses to tumours and normal tissues were estimated and compared for $^{111}$In and $^{177}$Lu-labeled bsRICs. The maximum injected amount of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF which caused no observable adverse effects (NOAEL) was identified to be 11.1 MBq. Mice bearing trastuzumab-sensitive or resistant tumours were treated with $^{111}$In and $^{177}$Lu-labeled bsRICs and $^{177}$Lu-bsRICs were found to inhibit tumour growth more effectively than $^{111}$In-bsRICs due to a 9.3-fold higher radiation absorbed dose (55.0 vs. 5.9 Gy, respectively).

These results suggest that bsRICs may be useful for imaging and radioimmunotherapy of HER2-positive BC co-expressing HER3 or EGFR. Co-expression of these receptors in HER2+ tumours is associated with resistance to HER2-targeted therapies.
Acknowledgments

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List of Abbreviations

%ID/g  percent injected dose per gram of tissue
AC  anthracycline
ADC  antibody drug conjugate
ADCC  antibody-dependent cellular cytotoxicity
AE  adverse event
AI  aromatase inhibitor
AJCC  American Joint Committee on Cancer
AMU  atomic mass units
ASCO  American Society of Clinical Oncology
BBN  bombesin
BC  breast cancer
BCIRG  Breast Cancer International Research Group
BCS  breast conserving surgery
BGO  bismuth germanate
BSIG  breast-specific gamma imaging
bsRIC  bispecific radioimmunoconjugate
CAP  College of American Pathologists
CCO  Cancer Care Ontario
CEM  contrast enhanced mammography
CHO  chinese hamster ovary
CLEOPATRA  CLinical Evaluation Of Pertuzumab And TRAStuzumab
CNB  core needle biopsy
CT  computed tomography
CTA  clinical trial application
DCE-MRI  dynamic contrast-enhanced MRI
DCIS  ductal carcinoma in situ
DFS  disease free survival
DOTA  1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DTPA  diethylene triamine pentaacetic acid
EBC  early breast cancer
EC  electron capture
ECD  extracellular domain
ECOG  Eastern Cooperative Oncology Group
EGF  epidermal growth factor
EGFR, erbB1  epidermal growth factor receptor
ER  estrogen receptor
Fab  fragment antigen binding
FDA  Food and Drug Administration
FDG  fluoro-2-deoxy-D-glucose
FNA  fine needle aspiration
FWHM  full width at half maximum
GLUT  glucose transporter
GMP  Good Manufacturing Practices
HDI  HER dimerization inhibitor
HER2, erbB2  human epidermal growth factor receptor 2
HER3, erbB3  human epidermal growth factor receptor 3
HER4, erbB4  human epidermal growth factor receptor 4
HERA  HERceptin Adjuvant Trial
HR  hazard ratio
HRG  heregulin
i.v.  intravenous
IBC  invasive breast carcinoma
IC  internal conversion
IGF-IR  insulin-like growth factor-I receptor
IHC  immunohistochemical
ISH  in situ hybridization
LABC  locally advanced breast cancer
LCIS  lobular carcinoma in situ
LET  linear energy transfer
LOR  line of response
LSO  lutetium oxyorthosilicate
LVI  lymphovascular invasion
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MBC</td>
<td>metastatic breast cancer</td>
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<tr>
<td>MCC</td>
<td>4-[N-maleimidomethyl] cyclohexane-1-carboxylate</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MRM</td>
<td>modified radical mastectomy</td>
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<tr>
<td>NCCTG</td>
<td>North Central Cancer Treatment Group</td>
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<tr>
<td>NHL</td>
<td>non-Hodgkin’s lymphoma</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NOAEL</td>
<td>no observable adverse effect limit</td>
</tr>
<tr>
<td>NPV</td>
<td>negative predictive value</td>
</tr>
<tr>
<td>NSABP</td>
<td>National Surgical Adjuvant Breast and Bowel Project</td>
</tr>
<tr>
<td>OBSP</td>
<td>Ontario Breast Screening Program</td>
</tr>
<tr>
<td>ORR</td>
<td>overall response rate</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<tr>
<td>PEM</td>
<td>positron emission mammography</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<tr>
<td>PFS</td>
<td>progression free survival</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PK</td>
<td>pharmacokinetic</td>
</tr>
<tr>
<td>PPV</td>
<td>positive predictive values</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
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<tr>
<td>RBE</td>
<td>relative biological effectiveness</td>
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<tr>
<td>RCC</td>
<td>renal cell carcinoma</td>
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<tr>
<td>RECIST</td>
<td>Response Evaluation Criteria In Solid Tumours</td>
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<td>RGD</td>
<td>arginine-glycine-aspartate</td>
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<tr>
<td>RIT</td>
<td>radioimmunotherapy</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SPECT</td>
<td>single photon emission computed tomography</td>
</tr>
<tr>
<td>TDLU</td>
<td>terminal ductal-lobular unit</td>
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<tr>
<td>T-DM1</td>
<td>trastuzumab emtansine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-------------------------------------</td>
</tr>
<tr>
<td>TGI</td>
<td>tumor growth index</td>
</tr>
<tr>
<td>TKI</td>
<td>tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TNM</td>
<td>tumour, node, metastasis</td>
</tr>
<tr>
<td>UICC</td>
<td>Union for International Cancer Control</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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CHAPTER 1

Introduction
1.1 Current Status in the Detection, Diagnosis and Treatment of Breast Cancer

1.1.1 Breast Cancer Epidemiology and Etiology

In 2014 approximately 93,600 new cases of cancer will be diagnosed in Canadian women, with Breast Cancer (BC) accounting for over a quarter of these (26.1%). Canadian females are most likely to develop BC, with 1 in 9 females expected to develop the disease within their lifetime. Since 2004, BC rates have stabilized in Canada, a trend consistent with recent reports from the United States. By 2009 close to 24,000 deaths from BC will have been avoided in Canada since the female BC death rate peaked in 1986. This is in part due to the role of BC screening in women and advances in BC treatment. However, despite such advances, BC remains the second most common cause of cancer death in females, accounting for 13.8% of all cancer deaths in 2014, and the chances of a female Canadian dying from BC remain 1 in 30. The risk of being diagnosed with BC increases with age. In 2015, it is estimated that 82% of new BC cases will occur in Canadian women over the age of 50. About 1 in 5 BCs (18%) will be diagnosed in women less than 50 years of age. For women 30 to 49 years of age, 36 percent of all cancers diagnosed will be BC.

While the cause of BC in individual patients is not always known, there are many risk factors associated with an increased incidence of the disease. Some of the most common risk factors include age, a family history of BC, obesity, radiation exposure, late first pregnancy, genetic predisposition (e.g. mutations in genes such as BCRA1 and BCRA2) and lifestyle factors such as excessive alcohol consumption.

1.1.2 Detection, Diagnosis and Staging of Breast Cancer

The mammary gland is the functional structure of the female breast, and this is where BC originates. In female adults, each mammary gland is composed of lobes divided by adipose tissue. Each lobe is subdivided into lobules which contain the alveoli that secrete milk when a female is lactating, into a series of secondary tubules. The tubules converge to form a series of mammary ducts. Most BCs are believed to occur in the terminal ductal-lobular unit (TDLU), consisting of the lobule and its adjacent ducts. There are many theories as to how BC develops,
however the two most common postulate that BC originates either from a single cell, whose progeny spread through the duct system accumulating genetic changes towards malignant transformation; or that BC originates from a cluster of cells that are genetically unstable. While the development of BC is still not completely understood, current evidence suggests that atypical ductal hyperplasia, atypical lobular hyperplasia, lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS) increase the risk of developing invasive breast carcinoma (IBC). Specifically, DCIS and IBC are thought to be progressive stages of an evolutionary continuum, with DCIS being found adjacent to the vast majority of IBC, and accounting for up to 40% of newly diagnosed cases of BC.

The diagnosis of BC most commonly occurs through a combination of clinical examination, mammography or other diagnostic imaging (see Section 1.5) and histopathological analysis of a primary lesion biopsy sample, as collected by core needle biopsy (CNB), fine needle aspiration (FNA) or other methods. Following diagnosis, the treatment and prognosis of the patient is determined by staging of the disease and determination of the pathological features including tumour grade and receptor status. BC is staged by the tumour, node and metastasis (TNM) system published by the Union for International Cancer Control (UICC) and American Joint Committee on Cancer (AJCC). The TNM system characterizes cancers based on the tumour size (T), number and location of lymph nodes involved (N) and the presence or absence of metastases (M). Additional detail is provided to each factor to stage the disease, for example X (cannot be assessed), 0 (no evidence), is (carcinoma in situ) or 1-4 (representing the size of the primary tumour or degree of spread to the lymph nodes). The stages progress from Stage 0 to Stage IV, classifying tumours with increasingly poor prognostic risk. Stage 0 is the earliest stage (Tis, N0, M0) while Stage IV (any T, any N, M1) represents metastatic disease, the most advanced stage. Between these extremes are stages I, IIA, IIB, IIIA, IIIB and IIIC. For patients with stage II and III BC, evaluation of lymph node involvement is particularly important as those with node-positive BCs have a significantly higher likelihood of disease recurrence and metastases formation. Other pathological features, such as histological grade, are also evaluated and are recognized as an important prognostic factor for BC. The most commonly used system is the Nottingham combined histologic grade where tumours are graded for percentage of tubule formation, degree of nuclear pleomorphism and mitotic index. Each feature is graded out of 3
and the scores are summed to give a total out of 9, where 3-5 is low, 6-7 is intermediate and 8-9 is high.\textsuperscript{12}

\subsection*{1.1.3 Treatment of Breast Cancer}

Once the diagnosis of BC is confirmed and the disease has been staged, the choice of treatment can begin. This is done primarily based on features of the disease including the stage and pathological features such as receptor status and tumour grade, but can also take into account other factors such as the patient’s age, health and personal preference. Most treatment regimes will begin with some form of surgery for local disease control, followed by single or multiple adjuvant therapies.

DCIS (Stage 0) is the earliest stage of BC, and usually has a very good prognosis. DCIS can be classified into similar molecular subtypes based on expression patterns of estrogen receptor (ER), progesterone receptor (PR), HER2 or EGFR,\textsuperscript{7} although these are rarely measured at this stage. Current practice guidelines for treatment of DCIS suggest breast-conserving surgery (BCS), or total mastectomy with the option of reconstruction for women preferring to maximize local control. Post surgery adjuvant breast irradiation is also recommended.\textsuperscript{13,14} While there is some evidence suggesting tamoxifen may be effective in preventing progression of ER-positive DCIS to IBC, a review of the evidence suggests the absolute benefit is considered to be small.\textsuperscript{13}

Treatment of early stages (I and II) of IBC often involves BCS and axillary node dissection. In some cases, such as large tumour size or multifocal disease, mastectomy may be recommended, or alternatively preoperative chemotherapy can be used to shrink a large primary tumour to allow for BCS instead.\textsuperscript{15} To further reduce the risk of local recurrence, patients may receive radiation therapy with or without tamoxifen, depending on ER status.\textsuperscript{16,17} Adjuvant chemotherapy is also recommended, especially for women who are likely to tolerate it, and who have tumours that are lymph node positive, ER negative with tumour size > 5mm, HER2 positive, or high risk lymph node negative with tumour size > 5mm. High risk lymph node negative tumours include those with grade 3, triple negative, lymphovascular invasion (LVI) positive or HER2 positive. An anthracycline-taxane containing regimen is considered the optimal strategy for adjuvant chemotherapy of IBC, particularly in patients deemed to be high risk. If taxanes are contraindicated, optimal dose anthracyclines should be used.\textsuperscript{18–20} Sentinel lymph node biopsy is
recommended as the preferred method of axillary staging for all patients with a clinical presentation of early-stage BC, except in women for whom there is no obvious clinical or pathological evidence of node involvement.\textsuperscript{21}

While the incidence of locally advanced breast cancer (LABC; stage III) has diminished due to improved screening techniques which detect BC at an earlier stage, there are still cases with this form of locally advanced disease that are clinically challenging to treat. Patients with LABC generally have a worse prognosis and long term survival rates are lower. Stage III disease is divided into operable (IIIA) and inoperable (IIIB & IIC) tumours.

Currently the standard of care for treatment of patients with stage IIIA operable tumours includes modified radical mastectomy (MRM) followed by locoregional radiotherapy encompassing the breast/chest wall and local node bearing areas. BCS may be considered for patients with non-inflammatory LABC on a case-by-case basis.\textsuperscript{22} Patients should also receive adjuvant chemotherapy following surgery. Alternatively, primary (neoadjuvant) chemotherapy can be used for tumour shrinkage followed by locoregional management. Chemotherapy should contain an anthracycline (AC) and taxanes,\textsuperscript{22} and tamoxifen should be recommended to pre- and postmenopausal women whose tumours are hormone responsive.\textsuperscript{23}

Patients with stage IIIB or IIC disease (inoperable tumours), including those with inflammatory LABC should be treated with neoadjuvant AC-based chemotherapy until the response plateaus or up to a maximum of 6 cycles.\textsuperscript{23} For patients with stage IIIB disease who respond to chemotherapy, definitive surgery and locoregional radiotherapy should be used. Patients whose tumours do not respond to primary chemotherapy can be treated with taxane chemotherapy or can proceed to irradiation followed by MRM (if feasible). Patients with stage IIIC disease who respond to chemotherapy should be individualized for locoregional management. If patients do not respond to neoadjuvant chemotherapy, locoregional radiotherapy should be given with subsequent surgery, if possible. For both IIIB and IIC patients, if maximum response is achieved with fewer than 6 cycles, further adjuvant chemotherapy can be given following surgery and irradiation. Tamoxifen for 5 years should be recommended to pre- and postmenopausal women whose tumours are hormone responsive, including those who are not candidates for chemotherapy.\textsuperscript{23} While effective, tamoxifen does carry the risk of increased adverse events (AEs) including gynecological symptoms (vaginal bleeding, discharge and
endometrial neoplasia), venous thromboembolic events and hot flushes.\textsuperscript{24} It has also been shown to increases the risk of blood clots and cancer of the uterus in some women.

Because of the potential safety risk with tamoxifen, there has been expanding use of aromatase inhibitors (AIs) in recent years. AIs such as anastrazole have been shown to be more effective and safe than tamoxifen and are now recommended as the preferred hormonal approach to postmenopausal hormone-sensitive patients.\textsuperscript{24} Treatment choices with these agents include the use of an AI as an upfront strategy for 5 years, as a sequential approach after 2–3 years of tamoxifen, or as an extended use after the classical 5 years of tamoxifen. The improved efficacy of AIs over tamoxifen has been largely demonstrated in terms of better disease-free survival, reductions in the occurrence of early distant metastasis as well as improvement of overall survival.\textsuperscript{24}

Stage IV or advanced BC is defined as disease that has metastasized to organs outside the breast including lymph nodes, bones, lungs, liver or brain.\textsuperscript{25} Advanced BC is associated with a median survival of 2-3 years, with the prognosis dependent on the size of metastases and the presence of visceral involvement.\textsuperscript{5} Treatment of advanced BC focuses on palliative care, with a cure not expected, but managing symptoms and improving quality of life for the patient. The primary treatment is systemic chemotherapy, with potential endocrine therapy for hormone responsive tumours.\textsuperscript{25} The American Society of Clinical Oncology (ASCO) recently released a new set of evidence based guidelines on the use of biomarkers in women with metastatic breast cancer (MBC),\textsuperscript{26} which recommend testing for ER, PR and HER2 in both accessible metastasis and primary tumours via biopsy and using these markers to guide therapy. If there is discordance between the status of the primary and metastatic tissues, the ER, PR and HER2 status of the metastasis should be used preferentially to direct treatment.

In recent years, biological therapies have dominated new BC treatment approvals. These innovative agents are designed to mimic endogenous macromolecules such as proteins or antibodies, and are able to target specific biological pathways leading to better efficacy and outcomes.\textsuperscript{27} One of the most common targets of new biological therapies is the HER2 receptor.
1.2 Monoclonal Antibodies in the Management of HER2 Overexpressing Breast Cancer

1.2.1 HER2 Biology

The human epidermal growth factor receptor 2 (HER2) is a 185 kDa type 1 transmembrane glycoprotein, encoded by the HER2 gene: a proto-oncogene located in the long arm of chromosome 17 (17q21).\(^{28}\) HER2 is a member of the ErbB family of cell surface receptor tyrosine kinases (RTKs) involved in the transmission of signals that control cell growth and differentiation.\(^{29,30}\) There are four receptors in this family, all of which share a high degree of homology: HER1 (EGFR, ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). The ErbB family all have a similar structure, comprised of a cysteine-rich extracellular domain, a hydrophobic transmembrane region, an intracellular tyrosine kinase domain and a carboxy terminal domain that is autophosphorylated upon receptor activation.\(^{31}\) The receptors exist as monomers, which are activated upon ligand binding which causes homo- or heterodimerization. This dimerization activates the intrinsic kinase activity and leads to stimulation of intracellular signaling cascades.\(^{32}\)

X-ray crystallography of the HER family have shown that HER1, HER3 and HER4 exist in a closed conformation where domain II (the dimerization domain) is tethered to domain IV, preventing the formation of dimers. Upon binding of a ligand, domains I and III of the receptors undergo conformational rearrangement, exposing the previously concealed dimerization domain and allowing for interaction with other HER receptors in the active state.\(^{33-35}\) HER2 is an “orphan receptor” that does not require ligand activation, and exists in an open “dimerization ready” conformation with domain II exposed (Figure 1-1).\(^{34,36}\) This open-confirmation facilitates dimerization with other HER family receptors, defining a key role for HER2 in the signal transduction of ligand-driven heterodimeric complexes.\(^{33,35}\) As such it is the preferred dimerization partner of the other receptors in the ErbB family.\(^{37}\) HER2 containing heterodimers produce potent mitotic signaling, especially HER2:EGFR and HER2:HER3 heterodimers.\(^{37,38}\)
Figure 1-1. Mechanism of dimerization of the HER family of receptors.
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In normal adult cells including the breast epithelium, central nervous system, bone, muscle, skin, heart, lungs and intestinal epithelium, HER2 is normally expressed in low levels. The receptor plays a role in the proliferation and differentiation of epithelial cells and protects cardiomyocytes against apoptosis. HER2 has been found to be widely expressed in fetal tissue, and plays a critical role in normal development.

1.2.2 Clinical Significance of HER2

The importance of HER2 in BC was first realized in the early 1980s when the neu oncogene, the mutationally active rat homologue of HER2, was identified. The human homolog HER2 was soon identified and found to be overexpressed in a variety of mammary carcinoma cell lines. Further studies examined the expression of HER2 in a panel of human primary BC tumour samples and reported an association between HER2 overexpression and patient relapse and survival. Approximately 20% of BC cases are known to over express HER2, and overexpression has also been reported in other diseases including lung, ovarian, prostate and gastric cancers.

The mechanism for HER2 overexpression in BC is primarily due to gene amplification, resulting in increased transcription and expression of the protein. Post-transcriptional dysregulation such as increased protein translation may also play a role. BCs that overexpress HER2 may contain levels of the protein that are 10- to 100- fold greater than in normal epithelial tissues. This high density allows for formation of ligand independent, constitutively active HER2 homodimers as well as HER2 heterodimers. HER2 homodimers persist longer on the cell surface due to slower internalization, and as such lead to more persistent signaling and dysregulated cell growth and proliferation. The formation of HER2 heterodimers, such as those formed between HER2 and HER3, also play an important role in driving breast tumour cell proliferation. Clinically, a HER2 overexpressing BC phenotype is associated with more aggressive disease, increased risk of relapse and poor long term survival. HER2 is the target for trastuzumab and other molecularly targeted therapies, and thus, this provides effective treatment options for women with HER2-overexpressing BC (see Section 1.2.3)
1.2.3 Targeted Therapies for HER2 Breast Cancer

Due to its attractiveness as a therapeutic target and the critical role it plays in oncogenesis and progression, multiple therapies have been developed targeting HER2 consisting of both small molecules and biological therapies (Figure 1-2). In particular, there are several monoclonal antibodies (mAbs) or mAb conjugates that have been approved by the U.S. Food and Drug Administration (U.S. FDA) and Health Canada for treatment of HER2 overexpressing BC.

The most widely used small molecule agent for targeting HER2 is lapatanib (Tykerb®, GlaxoSmithKline), a dual tyrosine kinase inhibitor (TKI) first approved in 2007 by the U.S. FDA. It functions by binding to the intracellular ATP-binding pocket of the protein kinase domain of HER2, thereby preventing autophosphorylation of the cytoplasmic domain and inhibiting downstream signaling and tumour cell growth (Figure 1-2B). Lapatanib is indicated for use in combination with capecitabine for patients with metastatic BC whose tumours overexpress HER2. It is used for patients who have already progressed on taxanes and anthracycline, and is used in combination with trastuzumab in the metastatic setting. It is also indicated in combination with letrozole for the treatment of post-menopausal patients with hormone receptor positive metastatic BC, whose tumours overexpress the ErbB2 (HER2) receptor, and who are suitable for endocrine therapy. Lapatanib treatment is associated with an unfavorable AE profile, including diarrhea, nausea and vomiting in many patients.

The first mAb to be approved for HER2 BC was trastuzumab (Herceptin®), which was developed by Genentech and now distributed by Hoffmann-La Roche. Trastuzumab is a recombinant humanized mAb that binds to the C-terminal region of the extracellular domain of HER2 (Figure 1-2A). The antibody is an IgG₁ isotype that contains human framework regions but with complementarity-determining regions of the murine anti-p185 HER2 antibody 4D5 that binds to human HER2 with high affinity (Kₐ = 5 nM). It is produced in Chinese Hamster Ovary (CHO) cells. Trastuzumab is indicated for treatment of both EBC and MBC (See Section 1.2.5.1)

Another antibody therapy targeted at HER2 is pertuzumab (Perjeta®, Hoffmann-La Roche), which is also a recombinant, humanized mAb. It is the first of its class of agents known as HER dimerization inhibitors (HDIs). Pertuzumab binds to the extracellular dimerization domain (subdomain II) of HER2, thus blocking dimerization of the receptor with itself or other members
of the HER family and inhibiting downstream signaling (Figure 1-2C). It received U.S. FDA approval for the treatment of HER2-positive MBC in 2012.\textsuperscript{55}

Most recently, the first antibody-drug conjugate (ADC) for treating HER2 positive metastatic BC was approved. Ado-trastuzumab emtansine (TDM1, Kadcyla®, Hoffmann-La Roche) received U.S. FDA approval in 2013\textsuperscript{56} and consists of the antibody trastuzumab covalently linked to the microtubule inhibitory drug DM1 (a derivative of maytansine) via the stable thioether linker, 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (MCC). Emtansine refers to the MCC-DM1 complex. An average of 3.5 DM1 molecules are conjugated to each molecule of trastuzumab. It binds to the same domain of HER2 as trastuzumab (Figure 1-2D). The clinical experience with treatment of BC using trastuzumab, pertuzumab and TDM1 is described in the following sections.
Figure 1-2. Binding sites and mechanisms of action of therapeutic agents targeting HER2.
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1.2.4 Summary of Clinical Data with Monoclonal Antibodies

1.2.4.1 Trastuzumab

The safety and efficacy of trastuzumab has been evaluated in both patients with MBC as well as for treatment of EBC.

In patients with MBC trastuzumab was studied in a multicentre, randomized, controlled clinical trial conducted in 469 patients with HER2 overexpressing MBC who had not been previously treated with chemotherapy for metastatic disease.\textsuperscript{57,58} Patients were randomized to receive chemotherapy alone or in combination with trastuzumab. The patients randomized to trastuzumab and chemotherapy experienced a significantly longer median time to disease progression (7.6 vs. 4.6 months, $P = 0.0001$), a higher overall response rate (ORR) (49\% vs. 32\%, $P = 0.0002$), a longer median duration of response (9.3 vs. 5.9 months, $P = 0.0001$), and a longer median survival (25.4 vs. 20.3 months, $P < 0.025$) compared with patients randomized to receive chemotherapy alone.\textsuperscript{57} The degree of HER2 overexpression was a predictor of treatment effectiveness.

In EBC, the efficacy and safety of trastuzumab was investigated in 4 large multicenter randomized trials. The HERceptin Adjuvant (HERA) trial compared treatment with trastuzumab for 1 and 2 years with observation after standard neoadjuvant chemotherapy, adjuvant chemotherapy, or both in 5,102 patients with HER2-positive EBC following surgery, chemotherapy and radiotherapy (if applicable)\textsuperscript{58,59} The primary endpoint was disease-free survival (DFS). After a median follow-up of 12 months, 1 year of trastuzumab treatment was associated with a hazard ratio (HR) for DFS of 0.54 (adjusted 99.9\% CI: 0.38-0.78) versus observation. After an 8 year follow up, 1 year of trastuzumab treatment was associated with a 24\% risk reduction of DFS compared to observation only (HR = 0.76, 95\% CI: 0.67-0.86). This translates into an absolute benefit in terms of an 8 year disease free survival rate of 6.4\% in favor of trastuzumab treatment.\textsuperscript{58} When DFS was compared in the 1 year group versus the 2 year group, a HR of 0.99 (95\% CI 0.85–1.14, $P=0.86$) was found, showing that 2 years of adjuvant trastuzumab was not more effective than 1 year of treatment for patients with HER2-positive EBC, and that 1 year of treatment provides a significant improvement in DFS and overall survival (OS) benefit compared with observation establishing adjuvant trastuzumab as the standard of care.\textsuperscript{59}
Two trials, the National Surgical Adjuvant Breast and Bowel Project (NSABP) trial B-31 and North Central Cancer Treatment Group (NCCTG) trial N9831, examined the clinical utility of combining trastuzumab with paclitaxel following AC chemotherapy in HER2 positive EBC following surgery.\textsuperscript{58,60–62} NCCTG N9831 also examined adding trastuzumab sequentially after AC-paclitaxel chemotherapy.\textsuperscript{58,62} Patients were randomized to receive doxorubicin and cyclophosphamide followed by paclitaxel or doxorubicin and cyclophosphamide followed by paclitaxel plus trastuzumab. DFS was the pre-specified primary endpoint of the combined efficacy analysis of these studies. Median follow-up from the time of randomization was 1.8 years for the chemotherapy alone arm and 2.0 years for the trastuzumab and chemotherapy arm for both studies. For the primary endpoint, addition of trastuzumab to chemotherapy reduced the risk of a first recurrence event by 52\% (HR 0.48, 95\% CI 0.39–0.59, \(P<0.0001\)) compared to chemotherapy alone.\textsuperscript{58,60}

Finally, the Breast Cancer International Research Group study 006 (BCIRG-006) was designed to investigate combining trastuzumab treatment with docetaxel either following AC chemotherapy, or in combination with docetaxel and carboplatin in patients with HER2 positive EBC following surgery.\textsuperscript{58,63} The study enrolled 3222 women with HER2-positive early-stage BC who were randomized to receive doxorubicin and cyclophosphamide followed by docetaxel every 3 weeks, the same regimen plus 52 weeks of trastuzumab, or docetaxel and carboplatin plus 52 weeks of trastuzumab. The primary study end point was DFS. At a median follow up of 65 months, patients treated with chemotherapy plus trastuzumab compared to chemotherapy alone showed a HR of 0.61 (95\% CI 0.44–0.84, \(P<0.0001\)) favoring trastuzumab treatment in combination with chemotherapy.\textsuperscript{63}

1.2.4.2 Pertuzumab

In MBC, pertuzumab was evaluated in The CLinical Evaluation Of Pertuzumab And TRAStuzumab (CLEOPATRA) study.\textsuperscript{64,65} This study assessed the efficacy and safety of pertuzumab plus trastuzumab plus docetaxel, as compared with placebo plus trastuzumab plus docetaxel, as first-line treatment for patients with HER2-positive MBC. The primary endpoint was progression free survival (PFS), and the results showed a HR of 0.62 (95\% CI 0.51–0.75; \(P<0.001\)) favoring the pertuzumab group compared to the control group. While the results for OS
were non-significant, the data showed a strong trend toward a survival benefit with pertuzumab–trastuzumab–docetaxel therapy. The response rate was 69.3% in the control group, as compared with 80.2% in the pertuzumab group for a difference of 10.8% (95% CI, 4.2 to 17.5; \( P=0.001 \)).

### 1.2.4.3 Trastuzumab Emtansine

The pivotal trial for trastuzumab emtansine (T-DM1) was the EMILIA study, a randomized, open-label, international trial involving patients with HER2-positive, unresectable, LABC or MBC that were previously treated with trastuzumab and a taxane. Patients were randomized to receive T-DM1 or lapatinib plus capecitabine, with a primary endpoint of PFS and OS. The median duration of follow up was 19 months, with T-DM1 significantly improving PFS (HR 0.65, 95% CI 0.55-0.77; \( P<0.0001 \)) and OS (HR 0.68, 95% CI 0.55-0.85, \( P=0.0006 \)) compared to lapatinib plus capecitabine. The objective-response rate was higher in the T-DM1 group (43.6%; 95% CI, 38.1-48.6%) than in the lapatinib–capecitabine group (30.8%; 95% CI, 26.6-35.7%; \( P<0.001 \)), and the median duration of response was longer (12.6 months vs. 6.5 months).

Another study examining the efficacy of T-DM1 was the TH3RESA study. This open label study compared T-DM1 to the physicians treatment of choice in patients with progressive HER2-positive MBC who had received two or more HER2-directed regimens in the advanced setting, including trastuzumab and lapatinib, and previous taxane therapy in any setting. After a median follow up of 7.2 months, the results showed significant improvements in PFS (HR 0.528, 95% CI 0.42-0.66, \( P<0.0001 \)) and OS favoring T-DM1 (HR 0.552, 95% CI 0.37-0.83, \( P=0.0034 \)).

There are a number of trials ongoing that aim to examine the use of T-DM1 in combination with pertuzumab in patients with both MBC and EBC, specifically comparing the efficacy of this regimen to T-DM1 or trastuzumab for the treatment of HER2 BC. Most recently, preliminary results from the MARIANNE trial showed that T-DM1 treatment in combination with pertuzumab resulted in non-inferior (but not superior) PFS compared with trastuzumab plus a taxane (HR 0.87, 95% CI 0.73-1.13, \( P=0.14 \)) in patients with HER2 positive LABC or MBC as first line treatment.
1.2.5 Current Status of Monoclonal Antibodies for Treatment of HER2+ Breast Cancer

1.2.5.1 Approved Indications

In Canada, trastuzumab is indicated for the treatment of patients with EBC with Eastern Cooperative Oncology Group (ECOG) 0-1 status, whose tumours overexpress HER2, following surgery and after chemotherapy, following adjuvant chemotherapy consisting of doxorubicin and cyclophosphamide, in combination with paclitaxel or docetaxel, and in combination with adjuvant chemotherapy consisting of docetaxel and carboplatin. It is also indicated for the treatment of patients with MBC whose tumours overexpress HER2. Pertuzumab is indicated in combination with trastuzumab and docetaxel for the treatment of patients with HER2-positive MBC who have not received prior anti-HER2 therapy or chemotherapy for metastatic disease. T-DM1, as a single agent, is indicated for the treatment of patients with HER2-positive, MBC who received both prior treatment with trastuzumab and a taxane, separately or in combination. Patients should have either received prior therapy for metastatic disease, or developed disease recurrence during or within 6 months of completing adjuvant therapy.

1.2.5.2 Recommended Guidelines for Treatment of HER2+ Breast Cancer

Guidelines from ASCO and the College of American Pathologists (CAP) on HER2 testing recommend that HER2 status (HER2 negative or positive) be defined in all patients with invasive (early stage or recurrent) BC on the basis of one or more HER2 test results (negative, equivocal, or positive). Positive HER2 status is based on either increased protein expression (immunohistochemical [IHC] assay) or HER2 gene expression (fluorescence in situ hybridization [FISH] assay). A sample is defined as HER2 positive if IHC is 3+ (circumferential membrane staining that is complete, intense, and within > 10% of tumor cells) or FISH amplified (Single-probe average HER2 copy number ≥ 6.0 signals/cell, dual-probe HER2/CEP17 ratio ≥ 2.0 with an average HER2 copy number ≥ 4.0 signals per cell, dual-probe HER2/CEP17 ratio ≥ 2.0 with an average HER2 copy number < 4.0 signals/cell or dual-probe HER2/CEP17 ratio < 2.0 with an average HER2 copy number ≥ 6.0 signals/cell). Equivocal for HER2 is defined as: IHC 2+ (based on circumferential membrane staining that is incomplete and/or weak/moderate and within > 10% of the invasive tumor cells or complete and circumferential membrane staining that is intense and within ≤ 10% of the invasive tumor cells) or ISH equivocal (single-probe ISH
average HER2 copy number ≥ 4.0 and < 6.0 signals/cell).\textsuperscript{72} HER2 testing should especially be considered for patients who may have previously tested HER2 negative and present with disease recurrence. If HER2 testing is shown to be equivocal, testing should be performed using an alternate test or on an alternate specimen if available. The decision to pursue HER2 targeted therapy is based on a positive HER2 test result, if there is no apparent histopathologic discordance with HER2 testing and if clinically appropriate.\textsuperscript{72}

The most recent ASCO guidelines recommend HER2-targeted therapy for patients with HER2-positive advanced BC.\textsuperscript{73} The only exception is for patients with clinical congestive heart failure or other safety concerns, who should be evaluated on a case-by-case basis. First line treatment recommendations are trastuzumab, pertuzumab, and taxane. If patients progress on first line treatment, T-DM1 for second-line treatment is recommended. If the patient continues to progress, third-line treatment should consist of other HER2-targeted therapy combinations (trastuzumab, pertuzumab, lapatanib) or T-DM1 (if not previously administered). Optimal duration of chemotherapy is at least 4 to 6 months or until maximum response, depending on toxicity. HER2-targeted therapy can continue until time of progression or unacceptable toxicities. For patients with HER2-positive and ER–positive/PR–positive BC, standard first-line therapy or endocrine therapy plus HER2-targeted therapy may be used.\textsuperscript{73}

For treatment of HER2 positive EBC, Cancer Care Ontario (CCO) guidelines recommend that trastuzumab should be offered for one year to all patients with HER2-positive node-positive or node-negative tumour greater than 1 cm in size, and primary breast cancer and who are receiving or have received (neo)adjuvant chemotherapy. Trastuzumab should be offered after chemotherapy.\textsuperscript{74}

1.3 Mechanisms of Action of HER2 Monoclonal Antibodies

1.3.1 Mechanism of Trastuzumab

While the clinical benefit of trastuzumab therapy has been clearly demonstrated (see Section 1.2.4.1), its mechanisms of action are still not fully understood, despite significant study.\textsuperscript{52} Studies on BC cell lines support various mechanisms that lead to inhibition of tumour cell proliferation and tumour angiogenesis. Some of those proposed include activation of antibody-
dependent cellular cytotoxicity (ADCC), inhibition of extracellular domain (ECD) cleavage, inhibition of downstream signaling and reduction of angiogenesis.\textsuperscript{75–77}

One of the proposed mechanisms of trastuzumab is its ability to activate ADCC, as demonstrated in numerous BC cell lines.\textsuperscript{78} ADCC is mainly due to the activation of natural killer cells (NK), expressing the Fc gamma receptor. The NK cells can be bound by the Fc domain of trastuzumab, which activates the lysis of cancer cells bound to trastuzumab.

Another proposed mechanism is the inhibition of ECD cleavage of HER2 by trastuzumab. When overexpressed, HER2 undergoes proteolytic cleavage which results in the release of the ECD and in the production of a truncated membrane-bound constitutively active receptor. In HER2 overexpressing BC cell lines, trastuzumab has been shown to block the shedding of the ECD by inhibiting metalloprotease activity.\textsuperscript{79} Once cleaved, the ECD of HER2 is likely to be released into the serum. Several clinical studies show a decline in serum HER2 ECD during trastuzumab treatment, and these lower levels predicted tumour response and improved progression-free survival.\textsuperscript{80,81}

When HER2 is activated, the phosphoinositide 3-kinase (PI3K)/AKT pathway is activated leading to increased cell proliferation. A proposed mechanism of action of trastuzumab is the reduction of signaling from this pathway, thus promoting apoptosis and arresting cell proliferation.\textsuperscript{82} Diminished receptor signaling may be the result of trastuzumab-mediated internalization and degradation of the HER2 receptor, however currently it is unclear whether trastuzumab actually down-regulates HER2.\textsuperscript{76}

Overexpression of HER2 in BC cells is closely associated with increased angiogenesis\textsuperscript{83}, and trastuzumab has been shown to reduce this process.\textsuperscript{84} In mice, treatment with trastuzumab induced normalization and regression of the vasculature in HER2-overexpressing BC xenografts, and a correlation between tumour response to trastuzumab and reduction of microvessel density was observed.\textsuperscript{85}

**1.3.2 Mechanism of Pertuzumab**

The mechanism of pertuzumab is mainly through HER2 dimerization inhibition.\textsuperscript{86} The antibody binds to the extracellular dimerization domain of HER2, and as such blocks the ability of HER2
to associate with itself or other members of the HER family (particularly EGFR and HER3). This leads to blocking of ligand dependent signaling through the MAP kinase and PI3K pathways, and leads to cell cycle arrest in cells that overexpress HER2.\textsuperscript{87} Because the two antibodies bind in different locations on the HER2 receptor, the combination of trastuzumab and pertuzumab are found to act synergistically.\textsuperscript{88} The combination leads to complimentary inhibition of HER signaling, and blocking of ligand dependent and independent pathways as well as homo and heterodimerization.\textsuperscript{89} Similarly to trastuzumab, pertuzumab is also thought to induce ADCC.\textsuperscript{87}

### 1.3.3 Mechanism of T-DM1

Trastuzumab emtansine is an antibody drug conjugate whose primary mechanism of action is to deliver cytotoxic drugs directly to targeted tumour cells.\textsuperscript{90} In the case of T-DM1 the cytotoxic moiety DM1 is an anti-microtubule agent derived from maytansine linked to trastuzumab, with an average of 3.5 DM1 molecules per molecule of trastuzumab.\textsuperscript{68} Linkage of DM1 to trastuzumab does not affect the binding affinity of trastuzumab to HER2, nor does it reduce the inherent anti-tumour effects of trastuzumab\textsuperscript{91} (see Section 1.3.1). Binding of T-DM1 to HER2 triggers entry of the HER2-T-DM1 complex into the cell via receptor-mediated endocytosis.\textsuperscript{90,92} Since the non-reducible thioether linker MCC is stable in both the circulation and the tumour microenvironment, active DM1 release occurs only as a result of proteolytic degradation of the antibody part of T-DM1 in the lysosome.\textsuperscript{93,94} Following release from the lysosome, DM1-containing metabolites inhibit microtubule assembly, eventually causing cell death.\textsuperscript{91,95} The cytotoxic effect of T-DM1 likely varies depending on the intracellular concentration of DM1 accumulated in cancer cells. High intracellular levels result in rapid apoptosis, while somewhat lower levels lead to impaired cellular trafficking and mitotic catastrophe. Low levels of uptake lead to poor response to T-DM1.\textsuperscript{95}
1.4 Resistance of HER2+ Breast Cancer to Trastuzumab and Other Monoclonal Antibody Therapies

1.4.1 Resistance to Trastuzumab

One of the major challenges in the treatment of HER2+ BC with trastuzumab is resistance to therapy. Resistance can be classified into two categories: primary resistance, where patients do not respond to first line treatment with trastuzumab, and acquired resistance, where a patient stops responding to treatment. Despite showing significant efficacy in patients with HER2+ MBC, a large number of patients in early trials did not respond to first-line trastuzumab, both as monotherapy or when used in combination with chemotherapy. Furthermore, some patients that initially responded to trastuzumab treatment relapsed after a year of initial treatment. Several mechanisms for this resistance have been proposed and investigated, and significant research is being done to provide therapeutic alternatives to patients that are resistant.

The uncertainties in the mechanisms responsible for the efficacy of trastuzumab are mirrored by uncertainties in why cells are resistant to trastuzumab treatment, however understanding these mechanisms is critical to improving treatment and survival for women with HER2+ BC. Multiple mechanisms contributing to trastuzumab resistance have been proposed, the most prominent of which include loss of phosphatase and tensin homolog (PTEN), activation of insulin-like growth factor-I receptor (IGF-IR) signaling, receptor masking, competition for binding, or increased ErbB family receptor signaling.

The decreased expression of PTEN has been suggested as playing an important role in conferring trastuzumab resistance. In cells sensitive to trastuzumab, the antibody was shown to cause a disruption of the binding of Src to HER2, allowing PTEN to inhibit AKT and arrest growth. When PTEN levels were low, AKT remained active and trastuzumab efficacy was impaired. These results were validated using tumours from HER2+ positive patients, where the absence of PTEN expression was associated with a poorer response to trastuzumab therapy compared to tumours with normal PTEN levels.

Another potential mechanism proposed for trastuzumab resistance is activation of IGF-IR. IGF-IR is a transmembrane tyrosine kinase receptor that is frequently overexpressed in human BCs, and which stimulates mitotic signaling pathways involved in cell proliferation and metastasis. Overexpression of IGF-IR has also been shown to confer resistance to trastuzumab. A study on
HER2 overexpressing BC cells showed that when cells responsive to trastuzumab were transfected with the IGF-IR gene, trastuzumab activity was significantly reduced.\textsuperscript{103} Furthermore, this resistance was reversed when the IGF-IR was blocked with an IGF-IR antagonist. Similar results were found when cells that overexpress IGF-IR were transfected to express HER2.\textsuperscript{103} There is evidence to show that IGF-IR and HER2 form heterodimers, and that in the presence of these heterodimers, trastuzumab was unable to block cell proliferation.\textsuperscript{104} Combined, these results suggest IGF-IR as a possible mediator of trastuzumab resistance and a potential target for patients with trastuzumab resistant BC.

Potential masking of the HER2 receptor leading to an inability of trastuzumab to bind its epitope has also been proposed as one of the mechanisms of resistance. MUC4, a membrane associated mucin which contributes to masking of membrane proteins, was found to contribute to trastuzumab resistance in BC cells by binding epitopes of HER2 and subsequently blocking trastuzumab.\textsuperscript{105} Specifically, cell surface expression levels of MUC4 were inversely correlated with the ability of trastuzumab to bind HER2. When MUC4 expression was downregulated by RNA interference (RNAi), trastuzumab binding and sensitivity were restored.\textsuperscript{105}

There is evidence to show that matrix metalloproteases cleave the full length 110 kDa HER2 protein into a 100 kDa ECD and 95 kDa amino-terminally truncated membrane associated fragment.\textsuperscript{106} The ECD can be found circulating in the serum of patients with HER2+ BC, and elevated levels have been found to be correlated with a poor prognosis in advanced BC.\textsuperscript{107} Antibodies that target the HER2 are found to be able to bind to circulating HER2 ECD,\textsuperscript{108} and this binding may limit the ability of those antibodies to bind membrane associated HER2. This has been proposed as a mechanism of resistance, particularly in patients with high levels of HER2-ECD, where the efficacy of trastuzumab has been found to be limited.\textsuperscript{81,109}

While trastuzumab is believed to mediate HER2 signaling, it may not reduce signaling mediated through other receptors of the HER family.\textsuperscript{99} For example, heterodimerization of HER2 with other receptors of the erbB2-family can be induced by ligands of EGFR, HER3 and HER4. In the presence of an excess of ligands for these receptors (eg. EGF for EGFR, and heregulin for HER3), the resulting heterodimers may initiate mitogenic signaling even in the presence of trastuzumab.\textsuperscript{99,110} An increase in expression of the ErbB family of ligands have been associated with increased signaling from HER2/EGFR and HER2/HER3 complexes, and blocking of
trastuzumab mediated growth inhibition. The hypothesis of EGFR amplification as a mechanism for trastuzumab resistance is further supported by data that shows that EGFR TKIs erlotonib and gefitinib, and the EGFR and HER2 TKI lapatinib were able to induce apoptosis in HER2 overexpressing cells that were resistant to trastuzumab. Clinically, EGFR overexpression has been correlated with disease progression in patients receiving trastuzumab. Tumour growth factor-α (TGF-α), a ligand for the EGFR has also been found at increased levels in samples obtained from patients with HER2 positive BC who had been treated with trastuzumab and whose disease has progressed, and is thought to play a role in EGFR signaling in trastuzumab-resistant tumours.

1.4.2 Resistance to Trastuzumab Emtansine

To overcome resistance to trastuzumab, other HER2 targeting therapies such as T-DM1 have been developed. Unfortunately, despite showing favorable efficacy results, many patients treated with these therapies do not respond and continue to progress. In clinical studies of T-DM1 in patients with HER2 positive MBC, approximately 20% of patients showed primary resistance to T-DM1 despite prior exposure to HER2-directed therapy. Despite this, most patients that initially responded to T-DM1 eventually progressed, suggesting that acquired resistance may be an issue with this therapy.

Beyond the hypotheses relating to trastuzumab resistance, factors related to poor efficacy of T-DM1 are not yet understood. Most of the factors that are thought to contribute to T-DM1 resistance are related to mechanisms that do not allow for adequate accumulation of the DM1 metabolite in cancer cells, thus meaning the drug is unable to achieve the necessary concentrations to evoke cell death. Factors that would affect this ability to accumulate DM1 in cells include low HER2 expression, poor internalization of the HER2-T-DM1 complex, defects in intracellular and endosomal trafficking of the HER2-T-DM1 complex, defects in lysosomal degradation of T-DM1, rapid recycling of HER2-T-DM1 or drug efflux pumps that export DM1.

Of particular interest is the role that other members of the ErbB family play in resistance to T-DM1, particularly HER3. Presence of the HER3 ligand neuregulin-1β (heregulin, HRG)
suppressed the cytotoxic activity of T-DM1 in four out of six BC cell lines tested, but this effect could be reversed by pertuzumab as it blocked dimerization of HER2 with HER3 and neutralized the effect of HRG.\textsuperscript{122} Additionally, activating mutations were present on the gene that encodes PI3K in the two BC cell lines where HRG did not inhibit T-DM1 activity, while the four cell lines where T-DM1 activity was reduced did not harbor these mutations.\textsuperscript{122} Dual targeting of HER2 with the combination of T-DM1 and pertuzumab in both cell culture and mouse xenograft models has shown enhanced antitumour activity, suggesting an important role of HER2 heterodimerization with other ErbB family members in conferring resistance.\textsuperscript{95,122}

1.5 Diagnostic Imaging Modalities for Breast Cancer

Imaging techniques are used for screening, detection, diagnosis and management of BC. Currently the most common imaging modalities include mammography, ultrasonography, magnetic resonance imaging (MRI) and nuclear medicine. Each technique has advantages, disadvantages and applications to which it is best suited. Below, a brief overview of each technique is provided, with a focus on use in BC.

1.5.1 Mammography

Mammography is the primary imaging modality for screening and diagnosis of BC. With mammography, low dose X-rays are used to examine the internal structure of the breast in order to reveal abnormalities such as cancer. The sensitivity of mammography for detecting malignant lesions is about 80-90\%, however its sensitivity for detecting abnormalities in dense breasts is suboptimal at 40-60\%.\textsuperscript{123} Recent advances in digital mammography have improved the sensitivity in dense breasts to overcome this challenge.\textsuperscript{124} While film mammography was previously the standard technique used, it has been almost completely replaced by digital mammography, which is significantly better for detection of BC in dense breasts and which can be interpreted more quickly and easily.\textsuperscript{125,126} Another limitation of mammography is the false-negative rate, which has been reported to be between 10-30\%, indicating that a significant proportion of BCs will be occult in mammographic imaging.\textsuperscript{127} Furthermore, mammography has low-specificity, a high false-positive rate and low positive predictive value. Mammography can
also be unreliable following surgery or radiation therapy due to limitations in distinguishing between recurrent disease and scar tissue.\textsuperscript{123}

Recently, advances in contrast enhanced mammography (CEM) have increased the potential utility of this technique in BC. In CEM, digital mammography is performed after iodinated contrast administration.\textsuperscript{128} Early studies suggest that CEM performs significantly better than digital mammography in lesion detection, and that while the sensitivity is not as high as MRI, there are fewer false positives than with MRI.\textsuperscript{129}

Mammography is the primary screening tool used to detect BC in the absence of abnormal signs or symptoms, such as lumps or breast pain.\textsuperscript{125} In Ontario, the Ontario Breast Screening Program (OBSP) provides mammography every two years for women aged 50 to 74 who are at average risk for BC, and annual mammography and breast MRI for women age 30 to 69 years who are identified as being at high risk.\textsuperscript{130} In 2010, 467,531 women were screened for BC with mammography in Ontario, resulting in detection of 2,233 BC cases (1,872 invasive and 361 DCIS).\textsuperscript{130} Recommendations for screening are based on meta-analyses and systematic reviews of prospective trials, and overall show that for women of all ages at average risk, screening is associated with a reduction in BC mortality of approximately 20%.\textsuperscript{131} Furthermore, BC that is detected with screening mammography is generally smaller, less likely to have metastasized, and can usually be treated with less aggressive therapy.\textsuperscript{132}

1.5.2 Ultrasonography

Ultrasonography is an integral part of diagnostic breast imaging. Ultrasound works by using high frequency reflected sound waves to generate real time images of internal structures. In the breasts, ultrasound is used to distinguish between cysts and solid masses, and is able to do so with high specificity.\textsuperscript{123} Ultrasound is often used in combination with mammography to differentiate between benign and malignant breast masses as it is able to discriminate features such as shape, margins and echogenicity. When combined with mammography, ultrasound has shown improved detection sensitivity when compared to mammography and physical examination.\textsuperscript{133} This improved sensitivity was also present for women with dense breasts.\textsuperscript{133} Advances in transducer technology have also lead to the frequent use of ultrasound in the guidance of breast biopsies.\textsuperscript{134} Other advancements, such as 3D Doppler Ultrasound, which is
especially useful at characterizing blood flow and pressure in lesions, which often exhibit increased blood flow. Limitations of ultrasound include the increased false positive rates when used as an adjunct to mammography, its inability to distinguish between some benign and malignant masses, its poor ability to visualize deep lesions, a lower sensitivity than mammography for detection of microcalcifications and the dependence of the effectiveness of the modality on the experience of the radiologist. 

1.5.3 Magnetic Resonance Imaging

MRI measures the emission of radiowaves emitted by tissues in the body caused by relaxation of protons in biological molecules following exposure to a strong magnetic field in combination with a radiofrequency pulse, and uses these measurements to visualize internal structures of the body with high soft tissue resolution. In BC, the use of MRI has advanced significantly as MRI technology has grown to deliver better contrast between malignant lesions, benign breast lesions and normal tissues. Specifically, use of dynamic contrast-enhanced MRI (DCE-MRI) using gadolinium-diethylenetriaminepentaacidic acid (Gd-DTPA) as a contrast agent has been instrumental in the increased use of MRI for detection of BC. DCE-MRI for detection of BC works based on the principle that malignant lesions have increased perfusion and vascular permeability, identified by a unique washout pattern of Gd-DTPA. Other anatomical features also help identify malignancies, including spiculated margins, irregular shape, rim enhancement in the presence of central enhancement, and segmental or clumped ductal enhancement. The presence and kinetics of enhancement and the morphology of the lesion can be used to distinguish benign and malignant lesions, with positive predictive values (PPV) ranging from 76-92%. Limitations of MRI include low sensitivity for detection of in situ cancers, and the inability to distinguish between some benign diseases such as fibroadenomas and granulation tissue.

MRI has high sensitivity for detecting BC (88-100%) although most studies show relatively poor specificity. However, in studies of high-risk women, MRI has a higher sensitivity than mammography (71%-77% vs. 36%-40%) but a lower specificity (71%-77% vs. 81%-95%). As the sensitivity of the two methods combined is over 90%, and mammography has low sensitivity
in women with dense breasts, screening with MRI has gained popularity as an adjunct to mammography in high-risk women.\textsuperscript{128} For example, the OBSP recommends annual MRI screening in addition to annual mammography for women aged 30-69 who are identified as high risk for BC.\textsuperscript{130} The use of MRI as an adjunct screening modality in women of average risk is limited by cost, access, and exclusion criteria such as claustrophobia and pacemakers which may be affected by the radiofrequency energy used, in addition to concerns about increasing false positives.\textsuperscript{128}

MRI is also been useful in planning surgical therapy in women with known BC. MRI can be used to determine the size of tumours, extent of disease, multicentricity, and multifocality, which could greatly affect the surgical approach. Studies in women with localized BC have demonstrated that preoperative MRI identifies additional tumour foci in 11% to 31% of cases and that these findings often alter the original surgical plan.\textsuperscript{136} Additionally MRI may alter treatment planning by depicting occult, contralateral BC. MRI has been shown to detect contralateral BC that was occult mammographically and by physical examination in between 3-5% of women with known BC.\textsuperscript{137,138} Often these occult lesions are detected in patients with dense breast tissue. While the sensitivity of mammography is inversely proportional to breast tissue density, MRI is extremely useful in this subpopulation, especially since bilateral cancer has been found to be twice as prevalent in patients with dense breast tissue.\textsuperscript{134} MRI is also increasingly used to characterize indeterminate findings on mammography. MRI has a negative predictive value (NPV) of 97% to 100% for mammographic abnormalities that lack Gd-DTPA uptake.\textsuperscript{139}

1.5.4 Nuclear Medicine Imaging

Nuclear medicine imaging is another modality that has been investigated for use in the detection, diagnosis, staging and management of BC. In contrast to other imaging modalities such as mammography, ultrasound, computed tomography (CT), and MRI which detect mostly anatomical abnormalities in the breast, nuclear medicine imaging uses small concentrations of radioactively-labeled imaging probes (radiopharmaceuticals) to non-invasively detect biological characteristics on a molecular level or sometimes physiological processes of tumours. As such, the term ‘molecular imaging’ has been recently applied to describe nuclear medicine imaging
techniques used for cancer detection. In cancer, radiopharmaceuticals used in nuclear imaging are able to detect biomarkers such as growth factor receptors, protein kinases, cell adhesion molecules and proteases, as well as biological processes such as hypoxia, apoptosis and angiogenesis. Nuclear medicine imaging is also widely used for the detection and diagnosis of other diseases, including cardiovascular disease, infections and neurological disorders.

Radiopharmaceutical imaging probes are usually administered intravenously (i.v.), where they circulate systemically and accumulate in target sites. Patients are then imaged using dedicated nuclear medicine tomographic cameras to detect gamma-photons emitted from sites of radiopharmaceutical accumulation. Abnormal accumulation of radiopharmaceutical probes designed for cancer imaging may be indicative of malignancy. The sensitivity and specificity of nuclear medicine imaging is dependent on the targeting moiety of the radiopharmaceutical probe, the properties of the radionuclide and the characteristics of the imaging modality.

Radiopharmaceuticals are designed either for single-photon emission computed tomography (SPECT) using single gamma-photon emitting radionuclides (e.g. technetium-99m [99mTc], indium-111 [111In] or iodine-123 [123I]) or positron emission tomography (PET) using positron-emitting radionuclides (e.g. fluorine-18 [18F], oxygen-15 [15O], carbon-11 [11C], copper-64 [64Cu], gallium-68 [68Ga] or iodine-124 [124I]).

Nuclear medicine can be used to detect both primary and metastatic disease, however due to the limited sensitivity for detection of lesions less than 1 cm in diameter and the relatively higher radiation absorbed doses associated with radiopharmaceuticals compared to other types of radiological imaging, nuclear medicine is not commonly used for screening. Currently, nuclear medicine is most useful for diagnosing clinically palpable or mammographically detectable primary BC lesions. Furthermore, because nuclear medicine imaging often visualizes the whole body, it is useful for detecting local recurrence, as well as distant metastases.

Nuclear medicine imaging is unique to other imaging modalities in that it is able to characterize the biology of tumours, select patients for targeted therapies, and monitor response to treatment. As such, it can aid oncologists in noninvasively assessing the phenotypic signature of a tumour in a patient, making therapeutic decisions, and monitoring a patient’s response to optimize treatment. Nuclear imaging is also a useful tool in the discovery and development of new targeted therapies to improve personalized therapy for cancer patients. Furthermore, nuclear
medicine techniques are up to 1 million times more sensitive than other imaging modalities and have the greatest promise for rapid clinical translation of imaging strategies for detection of cancer-associated molecular phenotypes.140

1.5.4.1 Single-photon emission computed tomography (SPECT)

Single-photon emission computed tomography (SPECT) is a nuclear medicine technique for producing 3D cross-sectional images of the distribution of radiopharmaceuticals labeled with single gamma-photon emitting radionuclides such as $^{99m}$Tc, $^{111}$In or $^{123}$I. SPECT is performed using a gamma camera fitted with one to three NaI(TI) crystal detector heads which are rotated in small angular steps around the patient. A sufficient number of 2D projections are acquired to generate a tomographic data set. The sensitivity and resolution of SPECT are governed by the same mechanisms as in planar (2D) scintigraphy. Following image acquisition, data are reconstructed by filtered back projection or by iterative algorithms to generate a 3D dataset often presented as cross-sectional slices. An advantage of SPECT is that it separates radioactivity distributions that project on top of each other in planar imaging, allowing higher contrast.143 Recently, advances in hardware (e.g. small pinhole collimators) and software (e.g. attenuation correction) have improved the sensitivity and spatial resolution of SPECT.144 Current clinical SPECT cameras have spatial resolution of approximately 1 cm full width at half maximum (FWHM), depending on the type of collimator and distance from the source.143,145 Pinhole collimators can achieve sub-millimeter resolution in the case of small animal imaging; however pinhole collimation further reduces the sensitivity of SPECT imaging.144 Additionally, the development of a SPECT/CT combined imaging modality allows the co-registration of molecular features visualized using radiopharmaceutical probes by SPECT with the precise anatomical depiction provided by CT.

1.5.4.2 Positron emission tomography (PET)

Positron-emission tomography (PET) is another tomographic nuclear medicine modality that generates 3D data sets of the in vivo distribution of radiopharmaceuticals labeled with positron emitting radioisotopes. PET detects the two coincidence gamma photons with energy of 511 keV simultaneously emitted in virtually opposite directions (angle of 180 degrees) upon annihilation
of a positron by collision with an electron. Most PET cameras are composed of a 360-degree ring of inorganic scintillation crystal detectors (usually bismuth germanate [BGO] or lutetium oxyorthosilicate [LSO]) and affixed photomultiplier tubes. The symmetry of the photons allows electronic collimation using a coincidence-timing window of a few nanoseconds to record coincident photon events. This negates the need for physical collimation, improving the sensitivity of PET, which is typically 10 to 100-fold higher than SPECT. Detection of coincidence photons in opposite detectors localizes the annihilation event to a line of response (LOR). During a PET scan, several million coincidence events are recorded generating many intersecting LORs providing information on the spatial location of radiopharmaceutical uptake in the body. The improved sensitivity of PET provides better image quality with a reduction in noise compared to SPECT using similar administered amounts of radioactivity. While the spatial resolution of SPECT is limited only by technology, the spatial resolution of PET is limited by two intrinsic physical phenomenon, namely the positron range and photon non-collinearity.

Positron range is the short distance the positron travels before collision with an electron. This distance depends on the energy distribution of the positron, in which higher energy positrons exhibit a longer range before annihilation. Photon non-collinearity is the slight (~0.25 degrees) deviation from 180 degrees between the trajectories of the emitted gamma photons resulting in resolution blurring that depends on the ring diameter of the detector. Current PET scanners have spatial resolution of 4-6 mm at FWHM. Combined modality PET/CT has received wide clinical acceptance with the obvious advantage of co-registration of the molecular and physiological features imaged by PET with the precise anatomical information provided by CT.

Whole body PET using [$^{18}$F]-fluoro-2-deoxy-D-glucose ($^{18}$F-FDG) has good specificity for detecting primary BC, however the intrinsic spatial resolution of the PET technology is poor compared to other radiographic imaging resulting in low detection sensitivity, especially for small lesions. Dedicated breast PET [positron-emission mammography (PEM)] detectors have also been developed. These are small, breast-specific PET tomographs with detectors that are mounted on two compression paddles. Spatial resolution is approximately 1.5 mm FWHM, improving the visualization of small lesions.
1.6 Nuclear Medicine Imaging in Breast Cancer

Nuclear medicine techniques have been studied for the detection, diagnosis, staging and management of both primary BC lesions and metastatic disease as described in section 1.6.2. The modality has some advantages over other radiological imaging methods for BC including increased specificity. Furthermore, nuclear imaging has the unique ability to assess the phenotypic signature of a tumour non-invasively. It is also able to visualize biological processes such as tumour metabolism and angiogenesis, as well as receptor expression. These properties have led to the investigation of nuclear medicine imaging techniques for a variety of applications in BC including drug development, diagnosis, staging, and monitoring response to therapy.

1.6.1 Nuclear Imaging for Drug Development

While chemotherapy historically has been the mainstay of cancer management, recently biologic therapies have dominated research and development efforts for new treatments for cancer. Of particular note is the role of mAbs, due to their targeting specificity and generally minimal safety risks. Currently more than 50 mAbs, including several ADCs, are in advanced clinical development. However, drug development is an expensive process that limits the number of promising agents that can be moved into later stages of testing. Nuclear medicine imaging techniques such as SPECT and PET can play a key role in this field by enabling development decisions to increase the power and value of early trials.

Patients that are most likely to benefit from targeted cancer therapy are logically those that display tumour expression of the molecular target. Here, nuclear medicine imaging can be used to assess the expression of the target on tumours non-invasively to help properly select patients for early trials. Nuclear medicine imaging has many advantages over standard ex vivo techniques that are currently used such as IHC on biopsy samples, including the ability to non-invasively visualize target expression on tumours throughout the whole body. In a tumour visualization study of patients with HER2 MBC with $^{89}$Zr-trastuzumab for PET imaging, uptake of the radiotracer was able to confirm known HER2 positive lesions in the liver, lung and bone while also identifying the presence of brain metastasis that were previously unknown. This allowed for determination of HER2 status in a setting where it was clinically important but biopsy was impractical or impossible.
Another use of nuclear medicine imaging in drug development is the ability to assess the target levels before and after treatment with a therapeutic agent. A study in patients with renal cell carcinoma (RCC) showed that $^{111}$In-bevacizumab SPECT was able to visualize tumours 7 days after injection of the radiopharmaceutical probe, and reduced uptake was observed after 4 weeks of neoadjuvant sorafenib treatment which is expected to interfere with angiogenesis. When examined in patients with melanoma, $^{111}$In-bevacizumab SPECT detected all known lymph node lesions and showed a reduction in uptake after one therapeutic dose of bevacizumab, which correlated with lower vascular endothelial growth factor (VEGF)-A levels measured in tumour tissue by IHC. Treatment was shown to result in reduced VEGF activity and consequent reduction in vessel density and macromolecule permeability, biomarkers of decreased angiogenesis.

Nuclear imaging is also a valuable tool for evaluating the pharmacokinetics of mAbs to help determine optimal dosing. Pharmacokinetic (PK) studies of trastuzumab have shown that clearance from the blood is dose dependant, and slowed in patients with high tumour burden. PET imaging with $^{89}$Zr-trastuzumab in patients with HER2+ MBC has added to the knowledge of tumour tissue kinetics of trastuzumab, and provides evidence that current clinical practice may be under dosing trastuzumab in patients with a high burden of antibody internalizing tumours. This utility also extends to evaluating ADC therapies, as molecular imaging of mAbs can help quantify tumour uptake and confirm adequate dosing. Recent results of an ongoing trial in patients with HER2 MBC showed $^{89}$Zr-trastuzumab PET imaging had an 88% NPV for Response Evaluation Criteria In Solid Tumours (RECIST) response to one cycle of T-DM1 treatment, which was increased to 100% when combined with $^{18}$F-FDG PET.

1.6.2 Nuclear Medicine Imaging for Diagnosis and Staging of Breast Cancer

Perfusion imaging with $^{99m}$Tc-sestamibi was the first approved radiopharmaceutical for detection of primary BC (scintimammography) in the U.S. and Canada. $^{99m}$Tc-sestamibi [hexakis(2-methoxyisobutylisonitrile)] was first developed for myocardial perfusion imaging and the first cases of BC detection using the radiopharmaceutical were described in 1992 by Aktolun and coworkers. The exact mechanism of $^{99m}$Tc-sestamibi uptake and retention is not fully understood, however it appears to be mediated by increased tumour blood flow and increased
cellular uptake and retention by cancer cells. Studies have shown that the uptake of $^{99m}$Tc-sestamibi is associated with increased perfusion of malignant lesions possibly related to the degree of angiogenesis and the percentage of poorly formed blood vessels. The cumulative sensitivity in detecting primary BC from a meta-analysis of published studies with over 3,000 patients was 83% and the average specificity, PPV and NPV were 85%, 85% and 82%, respectively. Most of these studies, however, were biased towards larger palpable lesions. The sensitivity for lesions less than 1 cm was low (~50%), and no lesions less than 5 mm were detected. Additionally, the benefit of SPECT in detection of primary BC using $^{99m}$Tc-sestamibi is uncertain.

PET using $^{18}$F-FDG has also been explored for imaging BC. $^{18}$F-FDG is a glucose analogue that is taken up by cells in proportion to their glucose utilization. PET imaging using $^{18}$F-FDG (FDG-PET) is based on the premise that cancer cells have a higher glycolytic rate and higher glucose avidity compared to normal tissue, thus allowing visualization of malignant lesions. $^{18}$F-FDG is actively transported into cells by glucose transporters (GLUTs) and is phosphorylated by hexokinases to $^{18}$F-FDG-6-phosphate ($^{18}$F-FDG-6P). $^{18}$F-FDG-6P is not a suitable substrate of glucose-6-phosphate isomerase and therefore cannot be converted to fructose-6-phosphate for entry into the glycoytic pathway. Furthermore, $^{18}$F-FDG-6P undergoes limited dephosphorylation due to low levels of glucose-6-phosphatase in tumours, thereby becoming metabolically trapped within cells. $^{18}$F-FDG accumulation, however, is not specific to tumour cells, since inflammation also shows elevated levels of $^{18}$F-FDG, likely due to uptake by macrophages. Additionally, organs with high physiological glucose utilization (e.g. heart and brain) or excretion (e.g. kidneys and liver) also show high normal uptake of $^{18}$F-FDG.

FDG-PET has been investigated for detection of primary BC, for detection of recurrence and metastatic disease, and for staging and monitoring of treatment response. Samson et al. reviewed the use of whole body FDG-PET in the detection of primary BC and found that in 13 studies, FDG-PET had a sensitivity of 89%, a specificity of 80% and a NPV of 87.9%; thus, a false-negative risk of 12.1%. Many of these studies, however, were biased towards large palpable lesions and smaller lesions are more difficult to detect by FDG-PET. It has also been demonstrated that FDG-PET has poor sensitivity for detecting lobular carcinomas. A study by Kumar et al showed that tumour size less than 1 cm and low tumour grade were significant predictors of false-negative FDG-PET results. Primary BC detection and especially in a
screening context requires the ability to demonstrate small (<1 cm) non-palpable, invasive and *in situ* lesions. Currently, these requirements are beyond the capabilities of whole-body FDG-PET and therefore this modality has limited utility in detecting small primary BC tumours at this time.

Recently there have also been efforts focused on developing radionuclide imaging systems that are dedicated specifically to breast imaging. These systems are commonly known as positron emission mammography (PEM) for PET radiopharmaceuticals, and breast-specific gamma imaging (BSIG) for SPECT radiopharmaceuticals. The goal of these systems is to overcome the limited spatial resolution of whole body PET and SPECT scanners in detecting small lesions by positioning the detectors closer to the breast.\textsuperscript{164} Dedicated breast scanners provide higher spatial resolution and higher sensitivity, while reducing the costs associated with clinical whole-body scanners.\textsuperscript{164} A clinical study that evaluated the effect of PEM in BC pre-surgical planning found that PEM imaging with \textsuperscript{18}F-FDG was more sensitive and specific than MRI, and was able to detect new malignant lesions that had previously not been identified.\textsuperscript{165} Similar results have been observed for BSIG. A clinical study with \textsuperscript{99m}Tc-sestamibi found that in patients with dense breasts, BSIG was able to detect lesions not found by mammography, particularly those of small size.\textsuperscript{166}

### 1.6.3 Nuclear Medicine Imaging for Monitoring Response to Therapy

While personalized medicine for BC is improving with the development and approval of new targeted therapies, there are still major limitations in achieving positive outcomes in patients due to overall low response rates observed in clinical studies.\textsuperscript{167} As such, nuclear imaging can play a critical role in monitoring treatment effectiveness, particularly early during therapy.

One of the most studied approaches for monitoring response to therapy has been the use of \textsuperscript{18}F-FDG for PET imaging of glucose metabolism. Because glucose metabolic changes occur earlier than changes in tumour size and are a biomarker of tumour viability, FDG-PET has proven an effective way to predict treatment response to chemotherapy.\textsuperscript{168} Multiple studies have been performed with FDG-PET on BC patients with a variety of stages of disease and have generally shown that FDG-PET is able to predict a pathological response to chemotherapy with high sensitivity and specificity.\textsuperscript{169} Unfortunately when the utility of FDG-PET was tested specifically in women with HER2+ BC, the results were conflicting. Two clinical studies found value for
FDG-PET to predict early response to treatment in women with HER2+ BC, with levels of \( ^{18}\text{F}-\text{FDG} \) uptake able to predict residual disease at completion of neoadjuvant chemotherapy after two cycles\(^{170} \) or after one cycle of neoadjuvant chemotherapy in patients treated with trastuzumab and taxanes.\(^{171} \) In contrast, two clinical studies by Koolen et al found that FDG PET during neoadjuvant chemotherapy may predict response in ER+/HER2- and triple negative BC, but not in HER2+ BC.\(^{172,173} \) Pre-clinical work by McLarty et al. showed that in athymic mice, FDG-PET accurately identified responding and non-responding human BC xenografts expressing various levels of HER2 after treatment with trastuzumab; however diminishing glucose utilization did not precede changes in tumor volume.\(^{174} \)

Since there is no clear evidence on the benefit of using FDG-PET alone for monitoring response to treatment in HER2 BC, there is a need for additional non-invasive methods to evaluate response. One attractive strategy would be to employ probes that would be able to monitor HER2 status, and in this regard, HER2 monoclonal antibodies have been labeled with a variety of radioisotopes for SPECT and PET imaging for this purpose.\(^{167} \)

1.6.4 Nuclear Medicine Imaging of HER2 in Breast Cancer

As the importance of targeting HER2 in BC has been well established, there is a need to develop companion diagnostics that can report on tumour HER2 status. Most of the work in developing imaging probes to visualize HER2 expression has focusing on anti-HER2 immune based agents. These agents include intact immunoglobulins such as trastuzumab and pertuzumab, immunoglobulin fragments (e.g. Fab and F(ab’)_2), and novel constructs such as affibodies.\(^{175} \)

Intact antibodies such as trastuzumab and pertuzumab have been labeled with radionuclides for both SPECT (\(^{111}\text{In}, ^{131}\text{I} \)) and PET (\(^{64}\text{Cu}, ^{68}\text{Ga}, ^{89}\text{Zr} \)), and have shown promising results in early phase clinical studies.\(^{175} \) In patients with HER2+ MBC, PET imaging with \(^{98}\text{Zr}-\text{trastuzumab} \) has shown the ability to detect most known lesions, as well as some that have not been previously detected by other imaging modalities. In addition, relative uptake in most normal tissues was low.\(^{148} \) Promising results have also been observed in early trials of \(^{64}\text{Cu}-\text{labeled trastuzumab}, \) where PET imaging was shown to be a feasible method to identify HER2+ lesions in patients with both primary and metastatic BC. PET was performed on patients with primary or metastatic HER2 BC at 1, 24 and 48 h after injection of approximately 130 MBq of \(^{64}\text{Cu-DOT-trastuzumab}, \)
and the best images were observed 48 h p.i. While at this time point the radioactivity in the blood was high for this probe, uptake in normal tissues was low and the radiation dose for \(^{64}\text{Cu}\)-trastuzumab PET was equivalent to that for \(^{18}\text{F}\)-FDG PET.\(^{176}\) While able to effectively target HER2+ BC lesions, there are some drawbacks to using intact antibodies for imaging. One of the challenges is posed by the slow clearance of these molecules, meaning that a prolonged interval (usually 48-72 hours) is required between radiopharmaceutical injection and imaging. Due to these longer times, longer-lived radionuclides are often used, which lead to increased radiation doses.

The challenges associated with using intact antibodies for imaging has spurred development of smaller molecules to overcome some of the barriers. Particularly, antibody fragments have been explored as they are able to maintain the immune-recognition capabilities of the antibodies, while providing pharmacokinetic properties more favorable for imaging. Fab fragments of trastuzumab have been developed labeled with \(^{111}\text{In}\)^{177,178} and \(^{64}\text{Cu}\)^{178}, and \(^{68}\text{Ga}\) has been used to label F(\(\text{ab}'\))\(_2\) fragments of the antibody.\(^{179–181}\) An early clinical study from Beylergil \textit{et al.} examined \(^{68}\text{Ga}\)-F(\(\text{ab}'\))\(_2\)-trastuzumab for PET imaging in patients with both HER2+ and HER2-BC.\(^{179}\) Briefly, DOTA-F(\(\text{ab}'\))\(_2\)-trastuzumab was labeled with \(^{68}\text{Ga}\) (\(t_{1/2}\sim68\) mins) and the median immunoreactivity was found to be 51.3%. Patients were administered approximately 236 MBq/5 mg of \(^{68}\text{Ga}\)-DOTA-F(\(\text{ab}'\))\(_2\)-trastuzumab via i.v. infusion, and imaged 1.1, 1.8 and 2.7 h later. Blood samples were also taken at 5, 10, 30 and 60 min, and 3-6 h after infusion to determine blood plasma clearance. The results showed tumor targeting was achieved in half of patients with HER2+ BC, however high concentrations of \(^{68}\text{Ga}\)-DOTA-F(\(\text{ab}'\))\(_2\)-trastuzumab remained in the blood pool. Targeting was not observed in patients with trastuzumab therapy ongoing at time of imaging, probably due to high levels of circulating trastuzumab that interfered with tumor targeting of the probe in the short time between infusion and imaging. While this study did show promising initial results, further assessment is required using larger administered masses of antibodies with a better immunoreactive fraction to improve tumour targeting. Alternatively, a different isotope should be considered for imaging with F(\(\text{ab}'\))\(_2\)-trastuzumab, as the short half life of \(^{68}\text{Ga}\) is not well matched to the pharmacokinetic properties of the targeting agent.

Another area of study has been the development of special constructs such as affibodies and diabodies for HER2 targeting, with some showing positive results in clinical trials. Specifically, \(^{111}\text{In}\) or \(^{68}\text{Ga}\) labeled HER2:342-pep2 (ABY-002) affibodies have been tested in patients with
recurrent MBC, and it was found that these agents were able to visualize HER2 expressing metastatic lesions by SPECT and PET imaging. The imaging properties of both $^{111}$In and $^{68}$Ga ABY-002 were found to be favorable, with the probes having half-lives of 4-11 min and 10-14 min, respectively, and with high contrast imaging performed as soon as 2-3 hours after injection. Another affibody, ABY-025, has been engineered to target a unique epitope of HER2 not currently occupied by other therapeutic agents. ABY-025 has been labeled with $^{111}$In and used for SPECT imaging of HER2 in patients with MBC. Fast blood clearance allowed high-contrast images to be obtained within 4-24 h. Furthermore, in one patient with a HER2-positive primary tumour, $^{111}$In-ABY-025 imaging correctly suggested the HER2-negative status of metastases.

There have been significant advances in the use of nuclear medicine imaging for the diagnosis, staging and monitoring of BC, particularly for HER2+ BC. One of the unique properties of using radiolabeled probes for imaging is the ease with which they can be adapted for therapeutic applications. Often by simply increasing the dose or altering the isotope used, imaging probes based on radiolabeled mAbs can be extended to radioimmunotherapy (RIT) applications.

### 1.7 Radioimmunotherapy of Breast Cancer

RIT is a branch of nuclear medicine in which an antibody, or antibody fragment linked to a radionuclide, is used to deliver a therapeutic dose of radiation to selectively kill cancer cells. In RIT, this means targeting antigens that are expressed uniquely or preferentially on cancer cells, so that the radiotherapeutic agent is able to selectively target the malignancy. By doing so, the radiopharmaceutical can achieve maximum uptake in the tumour, while limiting uptake and toxicity to normal tissues. RIT differs from conventional external beam radiation therapy in that RIT is a form of systemically delivered and targeted radiotherapy. The anti-tumour effect of RIT is primarily due to the radioactivity delivered by the antibody to tumour cells, which provides continuous, exponentially decreasing, low-dose-rate radiation that is sufficient to cause lethal DNA damage in cancer cells. One advantage of RIT is that the antibody itself may also contribute to tumour cell killing by eliciting the same mechanisms as it would when used therapeutically. Several factors that are critical in developing an effective RIT regimen include the selection of an optimal radionuclide; identification of a promising tumour-associated...
antigen; and the design of an antibody-radionuclide immunoconjugate with high specificity and low immunogenicity.\textsuperscript{184}

1.7.1 Radionuclides used for RIT

Radionuclides that are commonly conjugated to antibodies for use in RIT of cancer include alpha (\(\alpha\))-emitters such as \(^{211}\)At, \(^{213}\)Bi or \(^{225}\)Ac, beta (\(\beta\))-emitters such as \(^{177}\)Lu, \(^{131}\)I or \(^{90}\)Y, or low energy Auger electron emitters such as \(^{111}\)In, \(^{123}\)I, \(^{125}\)I, \(^{99m}\)Tc and \(^{67}\)Ga.\textsuperscript{185} The choice of the optimal radionuclide for RIT depends on both its intended use and the practical considerations related to its particular application.\textsuperscript{186}

Radionuclides that emit \(\alpha\) particles contain a proton to neutron ratio that exceeds that for stable elements of similar atomic number. Emission of an \(\alpha\)-particle (i.e., two protons and two neutrons identical to a helium nucleus with a mass of two atomic mass units [AMU]) reduces the repulsive forces between the positively charged protons, and brings the decaying radionuclide to a more stable configuration. \(\alpha\)-Particle emitting radionuclides have very high potency, due to the fact that the emission of an \(\alpha\)-particle releases a large amount of energy in a linear manner within a few cell diameters (50–90 \(\mu\)m). The high linear energy transfer (LET) of \(\alpha\)-emitters (~100 keV per \(\mu\)m) confers a high relative biological effectiveness (RBE) for cell killing.\textsuperscript{187,188} High RBE derives from the fact that the extent of damage (for example, DNA double-strand breaks) to the cell from \(\alpha\)-particle exposure is so great that cell repair mechanisms are not effective and the cell undergoes apoptosis or necrosis.\textsuperscript{190} Furthermore, the effect of oxygen on cell killing is minimal for high LET radiation such as \(\alpha\)-particles, and thus effective cell killing can be expected even in areas of the tumour that are hypoxic.\textsuperscript{187,188}

In \(\beta\)-decay, an unstable atomic nucleus that has an excess of neutrons converts one neutron into a proton by emitting a \(\beta\)-particle (i.e., an electron with a single negative charge and mass that is \(1/1837\)th of an AMU) as well as an antineutrino. The range of \(\beta\)-particles in tissue is much longer than for \(\alpha\)-particles or Auger electrons, but is directly proportional to their energy. For example, the range in tissue of the \(\beta\)-particles emitted by \(^{131}\)I (\(E_\beta = 0.6\) MeV) is about 2 mm, for \(^{90}\)Y (\(E_\beta = 2.3\) MeV) it is about 12 mm, and for \(^{177}\)Lu (\(E_\beta = 0.5\) MeV) it is about 0.3 mm.\textsuperscript{185,191} \(^{177}\)Lu, in addition to \(\beta\)-particles, emits low energy gamma radiation of 113 and 208 keV allowing for gamma imaging of tumours.\textsuperscript{191} \(\beta\)-particles transfer their energy to the surrounding matter over a
long distance (several millimeters). Moreover, β-particles deposit most of their energy at the end of their track length (Bragg peak), and are therefore considered to be low LET radiation. Low LET radiation is dependent on oxygen to generate oxygen free radicals and thus, hypoxia decreases the effectiveness of this form of radiation. This property makes them most suitable for treating tumours with a diameter of 2-12 mm (200-1200 cell diameters) since for smaller tumours, most of the radiation energy would be deposited outside the target volume due the Bragg peak phenomenon. Nevertheless, the long range β-particles addresses the issue of heterogeneity in tumour uptake of the radionuclides, since radioactivity targeted to tumour cells can also kill neighboring non-targeted cells within striking distance of the β-particles (“cross-fire” effect).

Currently ¹³¹I and ⁹⁰Y, both β-particle-emitting isotopes, have been used in >95% of clinical RIT trials and represent the current standard to which all other radionuclides are compared. ¹³¹I and ⁹⁰Y are favorable for RIT because of their emission characteristics, availability, and the reliability and stability with which they can be attached to antibodies. Furthermore, hundreds of published clinical trials attest to their efficacy, especially for the treatment of haematological and in some cases for solid malignancies. Both isotopes have certain advantages: ¹³¹I is relatively inexpensive, can be used for both imaging and therapy, and has a long successful history of treating malignancies. However, ¹³¹I-labelled proteins degrade rapidly if endocytosed into tumour cells, resulting in the release of ¹³¹I-tyrosine and free ¹³¹I into the blood. In addition, the γ-rays emitted by ¹³¹I may pose a radiation risk to family members and healthcare personnel, and patient hospitalization for radiation isolation may be required if large amounts are injected. ⁹⁰Y emits β-particles almost exclusively, and as this form of radiation has a very short millimeter range, it does not penetrate the patient's body, and therefore, caregivers and family members are exposed to lower levels of radiation. ⁹⁰Y emits β-particles that are 5 times more energetic than those of ¹³¹I, also emits relatively weak electromagnetic radiation resulting from interaction of the β-particles with tissues (Bremsstrahlung), is easily administered to outpatients and is stably retained by tumour cells even after endocytosis. For RIT with both ¹³¹I and ⁹⁰Y, dose-limiting myelosuppression at conventional doses and cardiopulmonary toxicities at much higher myeloablative doses used in the setting of stem cell transplantation may be observed.

Auger electrons are emitted by radionuclides that decay by electron capture (EC). In EC, a proton in the nucleus captures an inner orbital electron, decreasing the number of protons by one,
and creating a vacancy in the shell. An electron from a higher energy level decays to fill the vacancy, resulting in a release of energy. Although sometimes this energy is released in the form of an emitted X-ray, the energy can also be transferred to an outer orbital electron, which is ejected from the atom. This ejected electron is called an Auger electron, named after Pierre Auger who first discovered them in 1925. The EC decay process causes the release of a shower of Auger electrons of discrete but low energies that travel nanometer to micrometer distances in tissues (i.e., less than one cell diameter). Internal conversion (IC) electrons are created by collision of photons (from the nuclear decay) with inner shell electrons that are ejected. Because Auger electrons travel very short distances in tissue (nanometer-to-micrometer distances), their energy deposition is high (4-26 keV/μm). Auger electrons are therefore considered to be high LET radiation. IC electrons have discrete energies in the keV range. This energy is higher than that of most Auger electrons, but lower than that of the β-particles, such as those emitted by $^{131}$I or $^{90}$Y. For therapeutic purposes the decay of an Auger electron-emitting radionuclide should ideally occur inside the cell nucleus to have the strongest lethal effect against cancer cells. The short path length and consequent high LET of α-particles and Auger electrons have therefore been proposed as ideal for the treatment of small tumour deposits, micrometastatic disease, and for eradication of malignant single cells.

1.7.2 Current use of Radioimmunotherapy in the Clinic

Currently the only radiolabeled antibodies that have been approved for the treatment of cancer are $^{90}$Y-ibritumomab tiuxetan (Zevalin®, Spectrum Pharmaceuticals) and $^{131}$I-tositumomab (Bexxar®, GlaxoSmithKline Inc). Both are indicated for treatment of chemotherapy-refractory non-Hodgkin’s lymphoma (NHL), and both agents target the CD20 antigen, a 35-kDa transmembrane glycoprotein that is abundantly expressed on a high percentage (>95%) of both normal and malignant B-cells, but not on early progenitor B cells in the bone marrow. Encouraging results have been reported at low radiation absorbed doses (25-45 cGy to the whole body), and at higher doses (65-75 cGy to the whole body) with bone marrow support. In several phase III clinical trials, the efficacy of Bexxar and Zevalin were evaluated in patients with chemo-refractory, low-grade or transformed NHL (Bexxar study), or with relapsed/refractory low-grade, follicular or transformed NHL (Zevalin study). In the Bexxar study, the ORR was greater in patients receiving RIT with Bexxar (1.7-7.8 GBq; ORR:
approximately 65%) whereas only 28% of patients responded to chemotherapy. In the Zevalin study, the ORR was significantly higher for patients receiving RIT with Zevalin (14.8 MBq/kg, maximum dose of 1.4 GBq; ORR: approximately 80%) than for patients treated with rituximab (Rituxan; Roche Pharmaceuticals) a non-radiolabeled chimeric anti-CD20 mAb or chemotherapy alone (ORR approximately 56%), thus demonstrating that the response to immunotherapy of NHL may be enhanced by incorporation of a therapeutic radionuclide.

Zevalin and Bexxar represent the most successful forms of RIT described to date. However, other radioimmunoconjugates being tested clinically could potentially improve response or expand indications in patients with NHL or other forms of cancer. For example, an approach to increase radiosensitivity by combining $^{90}$Y-clivatuzumab tetraxetan (hPAM4), an antibody that recognizes pancreatic cancer, with low-dose gemcitabine has shown objective tumour responses by RECIST, with 16% partial remission and 42% disease stabilization. This strategy is currently being tested in an ongoing Phase III clinical trial.

1.7.3 HER2-Targeted Radioimmunotherapy

HER2 is an attractive target for systemic RIT of BC due to the high levels of expression of this receptor on tumour cells compared to normal cells, and because of the promising results that have already been achieved with non-radiolabeled forms of anti-HER2 mAbs. Trastuzumab has been the primary focus for development of RIT against HER2+ BC, and has been labeled with α-emitters ($^{213}$Bi or $^{211}$At), β-emitters ($^{177}$Lu, $^{131}$I, $^{90}$Y) and Auger electron emitters ($^{111}$In). Other antibodies, antibody fragments, or nanobodies targeting HER2 have also been investigated for RIT with varying degrees of success in preclinical studies.

So far, one agent is being investigated in clinical trials for RIT of HER2 positive cancer. Meredith et. al. have labeled trastuzumab with $^{212}$Pb using the chelator 2-(4-isothiocyanotobenzyl)-1, 4, 7, 10-tetraaza-1, 4, 7, 10-tetra-(2-carbamonyl methyl)-cyclododecane (TCMC). While not an α-emitter itself, the physical decay of $^{212}$Pb by β-particle emission produces several daughter radionuclides including $^{212}$Po which emits α-particles. Imaging studies with $^{212}$Pb-TCMC-trastuzumab using a planar gamma imaging showed suitable pharmacokinetics in patients with ovarian cancer, and minimal distribution to other tissues after
intraperitoneal (i.p.) injection for treatment of peritoneal lesions.\textsuperscript{213} A safety study in patients with HER2 positive lesions is ongoing (ClinicalTrials.gov identifier: NCT01384253).

1.8 Bispecific Agents for Imaging and Therapy of Cancer

As more is learned about the complex signaling pathways that lead to the aberrant growth in cancer, dual targeting with bispecific agents is emerging as a new strategy for both imaging and therapy of cancer patients. Currently there are over 50 ongoing or completed trials to evaluate bispecific agents for the treatment of a variety of cancers.\textsuperscript{214} The advantage to such probes is that they are able to bind multiple different targets simultaneously with better affinity, avidity and efficacy compared to monomeric agents, making them favorable as probes for nuclear imaging and radioimmunotherapy.\textsuperscript{215,216} A wide variety of bispecific agents have been developed, with the binding domains based either on small binding peptides or antibody fragments.

Bispecific peptides are also being investigated. In prostate cancer, a bispecific peptide was developed composed of arginine-glycine-aspartate (RGD), a peptide targeting the $\alpha_v\beta_3$ integrin, crosslinked with a glutamate linker to bombesin (BBN), which can bind to the gastrin releasing peptide receptor (GRPR), and labeled with $^{18}$F for PET imaging. When tested in PC-3 prostate cancer tumour xenografts, $^{18}$F-RGD-BBN showed tumour uptake that was significantly higher than $^{18}$F-RGD or $^{18}$F-BBN, while demonstrating substantially lower liver and renal accumulation.\textsuperscript{217} To further improve on these properties, the investigators modified the linker used from a short glutamate linker a longer and more flexible Fmoc-Glu-Oall, which allowed for higher tumour uptake and better binding to the target receptors.\textsuperscript{218}

Bispecific antibody fragments or constructs have also shown success for potential as nuclear imaging agents in cancer. Specifically, relating to HER2 targeting, bispecific single chain Fv fragments (bs-scFv) have been developed to target HER2 and HER3.\textsuperscript{219} These bs-scFv fragments were engineered to have similar selective binding capacity to the two target receptors. While the biodistribution of this antiHER2-antiHER3 A5-linker-ML3.9 bs-scFv (ALM) was not studied with imaging, it was radioiodinated with $^{125}$I and tumour accumulation measured in mice with xenografts that were HER2+/HER3+ (SK-OV-3), HER2+/HER3- (MVM2) or HER2-/HER3+ (MDA-MB-468). The results showed higher accumulation of the bs-scFv in tumour xenografts expressing both target receptors, and uptake of the bispecific agent in these tumours was much
higher than either of the monospecific agents. Biodistribution into other tissues was generally low. Another sc-bsFv targeting HER2/HER3, MM-111 (Merrimack Pharmaceuticals) has been shown to bind with both avidity and specificity to tumour cells expressing ErbB2 and ErbB3, and is able to block ligand-induced signaling and tumour growth in several preclinical models. This agent is currently in Phase I clinical studies in combination with trastuzumab for patients with advanced HER2 overexpressing, heregulin positive BC (ClinicalTrials.gov Identifier NCT01097460, NCT00911898).
1.9 Hypothesis

BC continues to be a terminal disease for women, and approximately 20% of cases overexpress HER2. Multiple therapies have been developed to target HER2+ BC; however, efficacy is limited by high incidence of resistance, either de novo or acquired during treatment. Upregulation of other members of the HER family, such as HER3 and EGFR, has been found to play an important role in resistance to HER2 targeted therapies.

Molecular imaging (nuclear medicine) techniques have the ability to non-invasively assess the phenotype of receptor expression in BC, as well as monitor the response and resistance to HER2 targeted therapies. These procedures could provide a means to more accurately select patients for treatment with these drugs, and report on the mechanisms of resistance when it develops. Furthermore, by altering the radionuclides used or the amount of radioactivity administered, these probes could potentially be used for RIT.

The hypothesis of this thesis is that bispecific radioimmunoconjugates could be developed to selectively target multiple receptors of the HER family, and be used to effectively report on receptor status through molecular imaging techniques such as SPECT. Furthermore, these same probes could be used to selectively target cancer cells for radioimmunotherapy, and would be able to effectively kill a wide population of breast cancer cells, including those that were resistant to HER2 therapies such as trastuzumab.

1.10 Specific Aims

To test this hypothesis, the thesis research included the following specific aims:

I. To develop $^{111}$In labeled bsRICs consisting of a domain that binds to HER2 covalently crosslinked to a domain that recognizes HER3, optimize the linker properties to bridge these domains, demonstrate that these bsRICs are able to retain specificity for the individual receptors both in vitro and in vivo, and can be used for SPECT imaging of tumour xenografts expressing these single receptors or combination of receptors in athymic mice.
II. To develop analogous $^{111}$In labeled bsRICs consisting of a domain that binds to HER2 covalently crosslinked to a domain that recognizes EGFR that would be capable of interaction with both HER2 and EGFR through bivalent receptor recognition and to demonstrate that these bsRICs may be useful for imaging tumours that express these single receptors or combination of receptor in mice bearing BC xenografts by SPECT imaging.

III. To extend the application of bsRICs targeting HER2 and EGFR for radioimmunotherapy of BC by labeling them with $^{177}$Lu or $^{111}$In and demonstrating that both $^{111}$In and $^{177}$Lu-bsRICs would cause cytotoxicity in vitro to BC cells expressing HER2 and EGFR receptors, and inhibit tumour growth in vivo in athymic mice bearing trastuzumab-sensitive or resistant BC xenografts with acceptable normal tissue toxicity.

1.11 Overview of Chapters 2, 3, 4 and 5

The studies addressing the above aims are described in Chapters 2-4 of the thesis. Chapter 2 describes the synthesis and in vitro characterization of $^{111}$In-bsRICs targeting HER2 and HER3, and demonstrates that $^{111}$In-labeled bsRICs composed of trastuzumab Fab fragments conjugated to HRG through a PEG spacer of appropriate length bound specifically to both HER2 and HER3. Examination of these bsRICs in athymic mice bearing tumour xenografts expressing HER2 or HER3 or both receptors showed good tumour uptake and low normal tissue accumulation, and the optimal time for SPECT imaging was 48 h post injection. In Chapter 3 an analogous strategy is explored where novel bsRICs that recognize HER2 and EGFR are synthesized and characterized. The results demonstrate that these bsRICs bind in higher amounts to cells that express both HER2 and EGFR, as compared to cells that only express one of these receptors, and can be used for tumour imaging by SPECT. In Chapter 4, bsRICs targeting HER2 and EGFR are labeled with $^{177}$Lu or $^{111}$In and it was demonstrated that both $^{111}$In and $^{177}$Lu labeled bsRICs were effective for killing tumour cells in vitro that displayed HER2 or EGFR or both receptors and that a single dose of the bsRICs, and at the NOAEL yielded moderate to strong tumour growth inhibition in vivo in mice bearing s.c. trastuzumab-sensitive or -resistant human BC xenografts. In Chapter 5, the overall findings of the thesis are summarized, conclusions are presented, and future research is proposed.
CHAPTER 2

MicroSPECT/CT Imaging of HER2 and HER3 Expression in Tumour Xenografts in Athymic Mice Using Trastuzumab Fab-Heregulin Bispecific Radioimmunoconjugates


All experiments and analyses of data were completed by Eva J. Razumienko in this chapter. SPECT/CT imaging was performed with the assistance of D.A. Scollard.
2.1 Abstract

Heterodimerization of HER2 with HER3 initiates aberrant downstream growth signalling pathways in tumours. Our objective was to construct bispecific radioimmunoconjugates (bsRICs) that recognize HER2 and HER3 and evaluate their ability to image tumours in athymic mice that express one or both receptors by microSPECT/CT. **Methods:** bsRICs were constructed by reaction of maleimide-derivatized trastuzumab (Herceptin) Fab fragments that bind HER2 with a thiolated form of the HER3-binding peptide of heregulin-β1 (HRG) with/without a 12 or 24 mer polyethyleneglycol (PEG) spacer. bsRICs were derivatized with diethylenetriaminepentaacetic acid (DTPA) for labeling with \(^{111}\)In. The ability of \(^{111}\)In-bsRICs to bind HER2 or HER3 was determined in competition assays with unlabeled Fab or HRG on cells expressing one or both receptors. Tumour and normal tissue uptake were examined in CD1 athymic mice bearing subcutaneous tumour xenografts that expressed HER2, HER3 or both receptors, with or without pre-administration of unlabeled Fab or HRG to determine the specificity of uptake. **Results:** Conjugation of Fab to HRG was confirmed by SDS-PAGE/Western blot and size-exclusion HPLC. Improved HER2 and HER3 binding and greater displacement of binding by competitors was found for \(^{111}\)In-bsICs that incorporated a PEG spacer, with the PEG\(_{24}\) spacer being optimal. The highest uptake of \(^{111}\)In-bsRICs [7.8 ± 2.1% injected dose/g (% i.d./g)] in BT-474 human breast cancer xenografts (HER2+/HER3+) occurred at 48 h post-injection (p.i.). Pre-administration of trastuzumab Fab decreased uptake in SK-OV-3 (HER2+/HER3-) human ovarian cancer xenografts from 7.0 ± 1.2 to 2.6 ± 1.5% i.d./g (p<0.001). Pre-administration of excess HRG decreased uptake in MDA-MB-468 (HER2-/HER3+) human breast cancer xenografts from 4.4 ± 0.9% to 2.6 ± 0.5% i.d./g (p<0.05). All tumours were imaged by microSPECT/CT. **Conclusion:** \(^{111}\)In-bsRICs composed of trastuzumab Fab and HRG exhibited specific binding *in vitro* to tumour cells displaying HER2 or HER3, and were taken up specifically *in vivo* in tumours expressing one or both receptors, permitting tumour visualization by microSPECT/CT. These agents could be useful for imaging heterodimerized HER2-HER3 receptors since their bivalent properties may result in preferential binding to the heterodimerized forms. The approach may also be extended to constructing bsRICs for visualizing other peptide growth factor receptors.
2.2 Introduction

Approximately 20% of all breast cancers show overexpression of HER2 as a result of gene amplification. This receptor is the target for trastuzumab (Herceptin®), a therapeutic monoclonal antibody that binds to the extracellular domain. However, HER2 must homodimerize or heterodimerize with another receptor in the epidermal growth factor receptor (EGFR) family before a cellular signalling pathway can be initiated. Recent work has shown that the heterodimerization of HER2 with HER3, its preferred dimerization partner, has an important function in the growth of breast cancer, as this heterodimer functions as an oncogenic unit and is associated with a more aggressive phenotype. HER3 binds its preferred ligand, heregulin, which promotes recruitment of HER2 to the complex. There is no known ligand for HER2, but the receptor is locked in a “dimerization ready” conformation. When the HER2/HER3 heterodimer forms, the intracellular kinase domains of the two receptors autophosphorylate their C-terminal ends leading to activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway. This pathway causes a cascade of signals that are involved in the regulation of cellular processes responsible for proliferation of tumour cells and thus a more aggressive phenotype.

New classes of therapeutic drugs against breast cancer, known as HER dimerization inhibitors (HDIs), are being developed and are intended to block HER2/HER3 heterodimerization. The most well known example is the monoclonal antibody pertuzumab (Perjeta®). Recent clinical trial data has shown that pertuzumab, which blocks HER2 dimerization, in combination with trastuzumab and docetaxel, significantly prolonged progression free survival for HER2 positive metastatic breast cancer patients as compared to trastuzumab and docetaxel alone. Preclinically, agents that will directly target these HER2/HER3 heterodimers have also been reported, including bispecific antibodies and bispecific scFvs that recognize HER2 and HER3 and were developed as novel immunotherapeutics.

Our objective was to develop a novel bispecific radioimmunoconjugate (bsRIC) consisting of a domain capable of binding to HER2 covalently crosslinked to a ligand for HER3 that may be capable of selectively imaging HER2/HER3 heterodimers. There is great value in targeting HER2 and HER3, as HER3 is the preferential binding partner of HER2 and the heterodimer formed by these two receptors constitutes one of the most potent signalling pairs of the HER
family. In addition, the two receptors have been shown to be frequently co-expressed in human breast cancer, and such coexpression is associated with a poor prognosis for patients.

Here we show that by designing these bsRICs with flexible crosslinkers of varying lengths we can provide greater mobility to the individual components of the imaging probe, thus allowing it to better bind both receptors simultaneously. Our results show that such bsRICs are able to retain specificity for the individual receptors both in vitro and in vivo, and show good tumour uptake in mouse xenografts models that express either HER2, HER3 or both receptors. These properties render the bsRICs capable of interacting with and potentially imaging heterodimerized HER2-HER3 receptors.

2.3 Materials and Methods
2.3.1 Tumour Cells
SK-OV-3 human ovarian carcinoma cells and MDA-MB-468 and BT-474 human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA). SK-OV-3 and MDA-MB-468 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY). BT-474 cells were cultured in Dulbecco’s Modified Eagle medium supplemented with 10% fetal bovine serum. All cells were cultured under a 5% CO₂ atmosphere at 37ºC. Cells were selected based on their reported expression levels of HER2 or HER3, which was confirmed through radioligand binding assays.

2.3.2 Preparation of ¹¹¹In-DTPA-Fab- HRG
Bispecific radioimmunoconjugates (bsRICs) were prepared by crosslinking trastuzumab (Herceptin) Fab fragments (50 kDa) to human Heregulin-β1 (rhHRG-β1, 7.5 kDa, Peprotech, Rockey Hill, NJ) by the reaction scheme shown (Figure 2-1). Fab fragments were generated by digestion of trastuzumab IgG using immobilized papain (Pierce, Rockford, IL) and purified by centrifugal ultrafiltration on an Amicon Ultra Centrifugal Filter Unit with a 10 kDa molecular weight cut off (Millipore, Billerica, MA), as previously described. A maleimide functional
group was introduced onto Fab fragments by treatment with a 10 fold molar excess of succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (sulfoSMCC, Pierce) or analogs containing polyethylene glycol (PEG) chains with either 12 or 24 repeating units (PEG_{12} or PEG_{24}) providing a spacer arm length of 53.4 or 95.2 Å, respectively. Fab fragments at a concentration of 1 mg/mL were reacted with the appropriate linker in PBS (pH 7.2) with 5 mM EDTA for 1 h at room temperature (RT). The reaction mixture was purified on a Sephadex G25 minicolumn (Sigma, St. Louis, MO) eluted with PBS (pH 7.4). Primary amines on the HRG peptide were modified by reacting with a 10 fold molar excess of 2-iminothiolane HCl (Pierce) in PBS (pH 8.0) with 5 mM EDTA for 1 h at RT to insert a free sulfhydryl group, and the reaction mixture was purified on a polyacrylamide P6 minicolumn (Bio-Rad, Mississauga, ON). Fab fragments were immediately reacted with a 2 fold molar excess of thiolated HRG, and purified by ultrafiltration on a Microcon 30K device (Millipore). bsRICs were reacted with a 4-fold molar excess of DTPA dianhydride (Sigma-Aldrich) in 50 mM NaHCO_3 (pH 8.0) for 1 h at RT and excess DTPA was removed on a Sephadex G25 minicolumn eluted with 1 M sodium acetate (pH 6.0). Both the thioether linkage used to cross-link thiolated heregulin to maleimide-derivatized trastuzumab Fab and the ^{111}\text{In}-DTPA complex have been shown to be very stable, \cite{229,230} and as such no stability studies were conducted. Purified DTPA-Fab-HRG was labeled with ^{111}\text{InCl}_3 (MDS Nordion, Vancouver, BC) for 1 h at RT. The final radiochemical purity was >95% as measured by instant thin layer silica gel chromatography (ITLC-SG, Pall Life Sciences, Port Washington, NY) developed in sodium citrate (100 mM, pH 5.0).

2.3.3 Characterization of ^{111}\text{In}-DTPA-Fab-HRG

The successful conjugation of Fabs to HRGs and purity of the bsRICs was assessed by Western blot and size exclusion high performance liquid chromatography (SE-HPLC). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 7.5% Tris-HCl minigel (Bio-Rad) under non-reducing conditions. Western blot was then performed by transferring electrophoresed proteins onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). Membranes were probed with anti-HRG and anti-Fab antibodies. Reactive bands were detected using the chromogenic substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma). To confirm conjugation to Fab, HRG was labeled with ^{111}\text{In} to be used as a radiotracer for size exclusion HPLC (SE-HPLC) analysis. HRG was reacted with a 4-fold molar excess of...
DTPA dianhydride (Sigma-Aldrich) in 50 mM NaHCO$_3$ (pH 8.0) for 1 h at RT and excess DTPA was removed on a polyacrylamide P6 minicolumn. Purified DTPA-HRG was labeled with $^{111}$InCl$_3$ (MDS Nordion) for 1 h at RT. The final radiochemical purity was measured by ITLC-SG developed in sodium citrate (100 mM, pH 5.0). This radiotracer was then reacted with Traut’s reagent as described above to introduce a free sulphydryl group. Combinations of $^{111}$In-HRG (without SH) and Fab-SMCC or $^{111}$In-HRG-SH and Fab were reacted for 1 h at RT. SE-HPLC was performed on a BioSep SEC-S2000 column (Phenomenex, Torrance, CA) eluted with 100 mM NaH$_2$PO$_4$ buffer (pH 7.0) at a flow rate of 0.8 mL/min using a g a Series 200 pump (PerkinElmer) interfaced with a diode array detector (UV, PerkinElmer) set at 280 nm and a Radiomatic 610TR flow scintillation analyzer (FSA, PerkinElmer).

### 2.3.4 Competition Receptor Binding Assays

The ability of the bsRIGs to bind to HER2 and HER3 was measured by competition binding assays using SK-OV-3 (HER2+), MDA-MB-468 (HER3+) and BT-474 (HER2+/3+) cells. Approximately 1.5 × 10$^5$ cells were seeded into wells in 24-well plates and cultured overnight. The medium was removed and the cells were rinsed in cold PBS. The cells were then exposed to 10 nM (4.5 MBq/µg) $^{111}$In-Fab-HRG, $^{111}$In-Fab-PEG$_{12}$-HRG or $^{111}$In-Fab-PEG$_{24}$-HRG in PBS in the presence of 1000 nM of unlabeled Fab, HRG or both for one point assays, or increasing concentrations (0-1200 nM) of unlabeled Fab, HRG or both for competition assays, all in a total volume of 500 µL PBS for 3 h at 4 ºC to measure only cell surface binding and prevent internalization of the radioimmunoconjugate. Unbound radioactivity was removed and the cells were rinsed 2 times with PBS and then solubilized in 200 µL of 100 mM NaOH for 30 mins at 37ºC. The solubilized cells were transferred to γ-counting tubes and the cell bound radioactivity was measured in a γ-counter. For competition assays the cell bound $^{111}$In-labeled bsRIGs in the presence of competitor was normalized against cell bound bsRIGs in the absence of competitor (B/B$_0$) and plotted versus the logarithm of the competitor concentration (log C). The data was fitted to a one site competition model by Prism software version 4.0 (GraphPad, San Diego) and the effective concentration that caused 50% displacement of the binding of the $^{111}$In-labeled bsRIGs to the cells (EC$_{50}$) was calculated.
2.3.5 SPECT/CT Imaging

MicroSPECT/CT was performed on female athymic CD1 nu/nu mice (Charles River, Boston, MA) bearing s.c. BT-474 (HER2+/3+) tumour xenografts at 4, 24, 48 and 72 h post injection (p.i.), and in mice bearing s.c. SK-OV-3 and MDA-MB-468 tumour xenografts at 48 h p.i. For BT-474 xenografts, mice were inoculated with a 0.72 mg, 60 day sustained release 17β-estradiol pellet (Innovative Research of America, Sarasota, FL) and 48 h later were inoculated s.c. in the thigh with $1 \times 10^7$ cells in 200 µL of a 1:1 mixture of Matrigel (BD Biosciences) and serum free growth medium. For SK-OV-3 and MDA-MB-468 xenografts, mice were inoculated with $1 \times 10^7$ cells in 100 µL of serum free growth media. After 4-6 weeks, groups of 3 mice with 200 ± 30 mm³ tumours (measured with external callipers and calculated using length × width² × 0.5) were injected i.v. (tail vein) with $^{111}$In-DTPA-Fab-PEG₂₄-HRG (10 µg, 35-40 MBq per mouse) in 100 µL of normal saline. For blocking studies 1 mg of unlabeled Fab or HRG was injected i.p. 3 h prior to $^{111}$In-DTPA-Fab-PEG₂₄-HRG injection. The tumour and normal tissue uptake of $^{111}$In-DTPA-Fab (10 µg, 35-40 MBq per mouse) and $^{111}$In-DTPA-HRG (10 µg, 35-40 MBq/mouse) was also evaluated in SK-OV-3 and MDA-MB-468 xenografts, respectively. Mice were anaesthetized by inhalation of 2% isoflurane in O₂. Imaging was performed on a NanoSPECT/CT tomograph (BioScan, Washington, DC) equipped with 4 NaI detectors and fitted with 1.4-mm multipinhole collimators (full width at half maximum ≤ 1.2mm). A total of 24 projections were acquired in a 256 × 256 acquisition matrix with a minimum of 90,000 counts per projection. Images were reconstructed using an ordered-subset expectation maximization (OSEM) algorithm (9 iterations). Cone beam CT images were acquired (180 projections, 1s/projection, 45 kVp) before micro-SPECT images. Co-registration of micro-SPECT and CT images was performed using InVivoScope software (BioScan).

2.3.6 Tumour and Normal Tissue Uptake of bsRICs

Biodistribution studies were performed immediately after microSPECT/CT imaging. Mice were sacrificed and the tumour and samples of blood and selected normal tissues collected, weighed and their radioactivity counted in a γ-counter. Tumour and normal tissue uptake were expressed as mean ± SD percentage injected dose (%ID) per gram and as tumour-to-blood (T/B) and tumour-to-normal tissue (T/NT) ratios. The Principles of Laboratory Animal Care (NIH
publication no 86-23, revised 1985) were followed, and all animal studies were conducted under a protocol (No. 989.1) approved by the Animal Care Committee at the University Health Network in accordance with Canadian Council on Animal Care (CCAC) guidelines.

2.4 Results

2.4.1 Synthesis and Characterization of Bispecific Radioimmunoconjugates

Successful conjugation of Fab-HRG, Fab-PEG\textsubscript{12}-HRG and Fab-PEG\textsubscript{24}-HRG was achieved (Figure 2-1).

All immunoconjugates (ICs) migrated on SDS-PAGE under non-reducing conditions primarily as single bands (Figure 2-2A), with minor smearing observed, probably due to the PEG chains which affected migration through the gel. Western blotting of all three ICs showed immunoreactivity of the bands with both anti-Fab and anti-HRG antibodies (Figure 2-2B) confirming conjugation. Increasing the PEG linker length caused an increase in overall estimated molecular weight (MW) between 60 and 65 kDa.

Conjugation was further confirmed though use of \textsuperscript{111}In-HRG as a radiotracer for SE-HPLC analysis (Figure 2-3A). A migration of the radioactivity peak from the HRG retention time (t\textsubscript{R}) of 13.0 mins to the Fab retention time of 9.8 mins was only observed when both components were modified with crosslinking agents. SE-HPLC analysis of Fab-HRG ICs showed major peaks with t\textsubscript{R} of 9.9, 9.6, 9.3 and 9.0 mins for Fab, Fab-HRG, Fab-PEG\textsubscript{12}-HRG and Fab-PEG\textsubscript{24}-HRG, respectively (Figure 2-3B).
bsRICs consisting of trastuzumab Fab fragments chemically crosslinked to a peptide of the EGF like domain of heregulin β1 (HRG) via maleimide derivitization of Fab fragments were then coupled to HRG thiolated with Traut’s Reagent. Various length polyethylene glycol (PEG) spacers (0, 12 or 24 repeating units) were incorporated into the bsRICs.
Figure 2-2. SDS-PAGE of bispecific immunoconjugates (bsICs).

SDS-PAGE of bsRICs containing 1, 12 or 24 length polyethylene glycol spacers between Fab and heregulin (HRG) on a 7.5% Tris-HCl minigel under non-reducing conditions stained with Coomassie. (A) Expansion of boxed area showing western blotting of bsICs probed with a 1:10,000 dilution of goat anti-human Fab antibody conjugated to horseradish peroxidase (A0293, Sigma-Aldrich, St. Louis, MO) or a 1:500 dilution of primary goat anti-hereregulin-β1 antibody (sc1792, Santa Cruz Biotechnology, Santa Cruz, CA) followed by a 1:5000 dilution of rabbit anti-goat antibody conjugated to horseradish peroxidase (sc2020, Santa Cruz). All antibody treatments were for 1 h at RT, and the membrane was then washed 3 times with TBS with 0.5% Tween 20. HRG and Fab were included as positive controls. Good overlap of immunoreactive bands, as well as an increase in the overall size of the immunoconjugate is observed when increasing length PEG chains are incorporated.
Figure 2-3. SE high-performance liquid chromatograms of bsRICs.
bsRICs were analyzed on BioSep-SEC-S2000 column eluted with 0.1 M NaH₂PO₄ (pH 7.0) at flow rate of 0.8 mL/min with ultraviolet detection at 280 nm and radioactivity (FSA) detection using a flow scintillation analyzer. (A)¹¹¹In-HRG was used as radiotracer to confirm that conjugation occurred only when both Fab and HRG were modified with appropriate cross-linkers (SH = sulfhydryl; SMCC = succinimidyl 4-\{N-maleimidomethyl\}cyclohexane-1-carboxylate). (B) Ultraviolet trace of all bsRICs. Fab was eluted at retention time of 9.9 min. Peaks at earlier retention times represent higher-molecular-weight aggregates (<10%). Retention time gradually decreased with increasing PEG linkers. FSA= flow scintillation analyzer; SH = sulfhydryl; SMCC = succinimidyl 4-\{N-maleimidomethyl\}cyclohexane-1-carboxylate; UV = ultraviolet.
2.4.2 Binding of $^{111}\text{In}$-bsRICs in vitro to Tumour Cells

The binding of $^{111}\text{In}$-labeled Fab-HRG, Fab-PEG$_{12}$-HRG and Fab-PEG$_{24}$-HRG to HER2 and HER3 was displaced by a 50 fold molar excess of unlabeled Fab, HRG or both on cells that expressed only HER2 (SK-OV-3), HER3 (MDA-MB-468) or both HER2 and HER3 (BT-474). In one point binding assays, an overall increase in total binding (in the absence of competitor) of the bsRICs was observed when the length of the PEG chain was increased for cells expressing only HER2 (SK-OV-3) or HER3 (MDA-MB-486) (Figure 2-4).

In addition, insertion of a PEG spacer between Fab and HRG improved displacement of binding of $^{111}\text{In}$-bsICs to HER2 and HER3 in BT-474 cells that express both receptors. For $^{111}\text{In}$-bsICs with the longest spacer (PEG$_{24}$), the binding to BT-474 cells (HER2+/HER3+) was reduced to $32.7 \pm 0.3\%$, $51.2 \pm 1.3\%$ and $15.0 \pm 0.4\%$, respectively by competition with trastuzumab Fab, HRG or both ligands. The binding to SK-OV-3 cells (HER2+/HER3-) was reduced to $16.7 \pm 0.8\%$, $88.8 \pm 5.3\%$ or $13.4 \pm 0.1\%$ by trastuzumab Fab, HRG or both ligands. The binding to MDA-MB-468 cells (HER2-/HER3+) was reduced to $98.5 \pm 1.5\%$, $68.8 \pm 0.3\%$ or $64.8 \pm 4.5\%$ by trastuzumab Fab, HRG or both ligands. Less efficient competition for HER3 was observed for the PEG$_{12}$ spacer and little competition for $^{111}\text{In}$-bsICs was observed without a PEG spacer.
Figure 2-4. Binding of $^{111}$In-labeled bsRICs to HER2 and HER3 on tumour cells in 1-point competition binding assays.

Binding of bsRICs was evaluated using 10 nM $^{111}$In-Fab-HRG, $^{111}$In-Fab-PEG$_{12}$-HRG, or $^{111}$In-Fab-PEG$_{24}$-HRG alone or competed with 50-fold molar excess of unlabeled trastuzumab Fab, HRG, or both in cell lines that are HER2+/HER3+ (BT-474) (A), HER2+/HER3− (SK-OV-3) (B), or HER2−/HER3+ (MDA-MB-468) (C).
In order to examine HER2 or HER3 binding of the $^{111}$In-bsICs with a PEG$_{24}$ spacer in more detail, competition binding curves were obtained with displacement of radioligand binding by increasing concentrations of trastuzumab Fab or HRG (Figure 2-5).

**Figure 2-5. Displacement of binding of 10 nM $^{111}$In-Fab-PEG$_{24}$-HRG bsRICs to HER2 and HER3.**

$^{111}$In-Fab-PEG$_{24}$-HRG bsRICs were displaced by increasing concentrations of trastuzumab Fab (A) and HRG (B) in cell lines that are HER2+/HER3+ (BT-474), HER2+/HER3− (SK-OV-3), or HER2−/HER3+ (MDA-MB-468). By fitting curves to 1-site competition binding model, EC$_{50}$ values were estimated to be 9.1 and 6.7 nM with Fab on BT-474 and SK-OV3 cells and 41.1 and 32.7 nM with HRG on BT-474 and MDA-MB-468 cells, respectively.
Fitting of the curves to a 1-site competition binding model, estimated that the EC$_{50}$ values were 9.1 and 6.7 nM with Fab on BT-474 and SK-OV3 cells, and 41.1 and 32.7 nM with HRG on BT-474 and MDA-MB-468 cells, respectively. These assays were also performed using $^{111}$In-DTPA-Fab and $^{111}$In-DTPA-HRG, which were found to have EC$_{50}$ values of 1.4 and 21.3 nM, respectively, on BT-474 cells (not shown). It should be noted that even at the highest concentrations of HRG, it was not possible to completely displace the binding of the bsRICs to HER3 on MDA-MB-468 cells. Because of its optimal HER2 and HER3 binding properties, $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG was selected for tumour imaging studies in vivo.

### 2.4.3 Uptake of $^{111}$In-bsRICs and MicroSPECT/CT imaging in vivo

MicroSPECT/CT images of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG in mice bearing BT-474 (HER2+/HER3+) xenografts showed accumulation in the tumour and low normal tissue uptake at 24, 48 and 72 h p.i. (Figure 2-6), with the exception of the kidneys. Maximum tumour uptake was seen at 48 h p.i., and was confirmed by biodistribution studies which showed uptake of 7.8 ± 2.1% ID/g (Table 2-1) and a tumour:blood (T:B) ratio of 11.1 ± 2.9 (Table 2-2).

The uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG was then examined in mice with SK-OV-3 (HER2+/HER3-) and MDA-MB-468 (HER2-/HER3+) tumours at 48 h p.i. Tumour uptake was seen in both xenografts on microSPECT/CT images (Figure 2-7) and confirmed in biodistribution studies (Table 2-3). When pre-administered an excess of Fab, $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG uptake in SK-OV-3 tumours was significantly decreased 2.6 fold (from 7.0 ± 1.2 %ID/g to 2.6 ± 1.5 %ID/g, p<0.001). Uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG significantly decreased 1.6 fold in MDA-MB-468 xenografts (from 4.4 ± 0.9 %ID/g to 2.6 ± 0.5 %ID/g, p<0.05) when these mice were pre-administered an excess of HRG. This confirms that the tumour uptake of these bsRICs was specific. Tumour uptake was observed for $^{111}$In-DTPA-Fab in SK-OV-3 xenografts (7.9 ± 1.4 %ID/g), however low uptake was found for $^{111}$In-DTPA-HRG in MDA-MB-468 xenografts (2.1 ± 0.4 %ID/g) (Table 2-4).
Figure 2-6. Posterior whole-body small-animal SPECT/CT images of female CD1 athymic mice in HER2+/HER3+ tumour xenografts.

Mice were implanted subcutaneously in right hind flank with HER2+/HER3+ BT-474 human breast cancer xenografts. Imaging was performed at 4 (A), 24 (B), 48 (C), and 72 h (D) after injection of $^{111}\text{In}$-Fab-PEG$_{24}$-HRG. Tumour xenografts (arrows) were visualized in B–D but not in A. Also visualized are kidneys (large arrowhead) and bladder (small arrowhead).
Table 2-1. Tumour and Normal Tissue Uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG in CD1 Athymic Mice with HER2+/HER3+ BT474 Tumour Xenografts at Selected Times Post Intravenous (tail vein) Injection

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percent Injected dose/g (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
</tr>
<tr>
<td>Blood</td>
<td>7.4 ± 0.8</td>
</tr>
<tr>
<td>Heart</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Lung</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Liver</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Small intestines</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>Large intestines</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Tumour</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>Bladder</td>
<td>2.3 ± 1.2</td>
</tr>
<tr>
<td>Skin</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>Tail</td>
<td>2.0 ± 0.4</td>
</tr>
</tbody>
</table>

* Mice were administered 37 MBq/10 μg of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG (n=4)

$^\dagger$ Significantly higher than at other time points (p<0.05)
Table 2-2. Tumour:Normal Tissue Ratios of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG in BT474 Tumour Xenografts at Selected Times

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Tumour: Tissue Ratio</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
</tr>
<tr>
<td>Blood</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Heart</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Lung</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Small intestines</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Large intestines</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Muscle</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>Bladder</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Skin</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>Tail</td>
<td>1.1 ± 0.3</td>
</tr>
</tbody>
</table>

* CD1 athymic mice with HER2+/HER3+ BT-474 xenografts were administered 35-40 MBq/10 μg of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG (n=4) at selected time points post intravenous (tail vein) injection
Figure 2-7. Posterior whole-body small-animal SPECT/CT images of female CD1 athymic mice in HER2+ or HER3+ tumour xenografts.
Mice were implanted subcutaneously in right hind flank with SK-OV-3 (HER2+) human ovarian cancer xenografts (A) or MDA-MB-468 (HER3+) human breast cancer xenografts (arrows) (B) at 48 h after intravenous tail injection of $^{111}$In-Fab-PEG24-HRG.
Table 2-3. Tumour and Normal Tissue Uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG in CD1 Athymic Mice with SK-OV-3 (HER2+) Xenografts

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SK-OV-3 (HER2+)</th>
<th>MDA-MB-468 (HER3+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unblocked</td>
<td>Blocked †</td>
</tr>
<tr>
<td>Blood</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Heart</td>
<td>0.6 ± 0.1</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>Lung</td>
<td>0.6 ± 0.1</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Liver</td>
<td>1.8 ± 0.5</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.4 ± 1.0</td>
<td>6.5 ± 1.4</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.9 ± 0.3</td>
<td>1.9 ± 1.4</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.4 ± 0.1</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Small intestines</td>
<td>0.5 ± 0.1</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td>Large intestines</td>
<td>0.3 ± 0.1</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.2 ± 0.1</td>
<td>1.1 ± 0.9</td>
</tr>
<tr>
<td>Tumour</td>
<td>7.0 ± 1.2</td>
<td>2.6 ± 1.5 ‡</td>
</tr>
<tr>
<td>Bladder</td>
<td>0.6 ± 0.3</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>Skin</td>
<td>1.5 ± 0.4</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>Tail</td>
<td>0.4 ± 0.1</td>
<td>1.7 ± 0.6</td>
</tr>
</tbody>
</table>

* Mice were administered 37 MBq/10 µg of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG (n=4)
† To block HER2 or HER3 on tumours, mice were pre-administered an excess of unlabeled trastuzumab Fab (1 mg) or unlabeled HRG (1 mg), respectively by intraperitoneal injection 3 h prior to $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG injection
‡ Significantly different (p<0.001)
§ Significantly different (p<0.05)
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percent Injected ( \text{dose/g (mean ± SD)} )</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(^{111}\text{In-DTPA-HRG in MDA-MB-468 (HER3+)})</td>
</tr>
<tr>
<td>Blood</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Heart</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Lung</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Liver</td>
<td>1.1 ± 0.8</td>
</tr>
<tr>
<td>Kidney</td>
<td>7.1 ± 2.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Stomach</td>
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<tr>
<td>Small intestines</td>
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<tr>
<td>Large intestines</td>
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</tr>
<tr>
<td>Muscle</td>
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<tr>
<td>Tumour</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Tail</td>
<td>1.7 ± 1.1</td>
</tr>
</tbody>
</table>

* Mice were administered 37 MBq/10 µg of \(^{111}\text{In-DTPA-Fab-PEG}_{24}\)-HRG (n=3)
2.5 Discussion

Bispecific immunoconjugates (bsICs) provide a unique opportunity to specifically probe two antigens. For example bsICs have been formed from scFv fragments of antibodies for the enhancement of antigen specific immune recognition of cancer cells, which allowed for better targeted delivery of cytotoxic agents.\textsuperscript{219} Another bispecific antibody against HER2/HER3 inhibited HER3 signaling and demonstrated antitumour activity in preclinical models that are dependent on HER2 overexpression.\textsuperscript{220} In nuclear medicine they have been widely investigated as pretargeting agents, with the ability to bind a tumour associated antigen and a radionuclide carrier, allowing for faster imaging after injection and lower exposure of normal tissues to radioactivity.\textsuperscript{231,232} In this study we report for the first time the preparation, characterization, and receptor binding properties of \textsuperscript{\textit{111}}In-labeled bsICs capable of binding both HER2 and HER3. We further examined their tumour and normal tissue localization, and showed their specificity for these receptors through \textit{in vivo} blocking studies. We hypothesize that such probes may be able to differentiate heterodimers from monomeric receptors due to stronger bivalent binding and avidity for the dimers as opposed to monovalent binding to the individual HERs.\textsuperscript{233} In addition, heterodimerization of HER2 and HER3 results in a conformational change that produces a complex that binds HRG (the HER3 ligand) more strongly than the HER3 receptor alone.\textsuperscript{38} Since the HER3 binding domain of HRG was incorporated into the bsRICs, this may encourage high affinity binding to heterodimerized HER2-HER3 complexes. Finally, although the density of HER3 on cells is much lower than that of HER2 it has been shown to cluster with HER2 on lipid rafts, promoting HER2/HER3 heterodimerization and possibly increased binding of the bsRICs to heterodimers.\textsuperscript{234}

The bsICs described here were composed of a Fab fragment of trastuzumab crosslinked to the EGF-like domain of HRG, the endogenous ligand for HER3 (Figure 2-1). To examine the effects of steric hindrance on the ability to bind HER2 and HER3, various length PEG spacers (12 and 24 mer) were incorporated. Western blot analysis confirmed conjugation by probing with antibodies specific for the Fab or HRG components of the bsICs. In addition, the upward band shift corresponded to the increase in size expected with incorporation of longer PEG chains (Figure 2-2). These results were confirmed by SE-HPLC, using HRG labeled with \textsuperscript{\textit{111}}In in trace amounts incorporated into the reaction which only showed conjugation when both Fab and HRG were modified with crosslinkers (Figure 2-3).
The incorporation of the PEG spacers improved the receptor binding capabilities of each component (trastuzumab Fab or HRG; Figure 2-4). Incorporation of longer PEG chains improved overall receptor binding of the bsRICs, but also provided greater displacement of binding by excess unlabeled Fab or HRG in cells that expressed HER2 (SK-OV-3), HER3 (MDA-MB-468) or both receptors (BT-474), with the optimal properties displayed by $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG. Displacement by HRG was not obtained in SK-OV-3 cells that were HER2+/HER3-. Similarly, trastuzumab Fab did displace binding of these bsRICs to HER2-/HER3+ MDA-MB-48 cells. The apparently poor ability to completely displace binding of the bsRICs to MDA-MB-48 cells is due to the low HER3 density (about 35,000 receptors/cell) on these cells. In the case of BT-474 cells that express both HER2 and HER3 the total binding of the bsRICs was displaced to 32.7 ± 0.3% by unlabeled Fab, 51.2 ± 1.3% by unlabeled HRG and 15.0 ± 0.4% by Fab combined with HRG. When competition assays for $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG were performed over a range of Fab and HRG concentrations (Figure 2-5), the observed EC$_{50}$ values were found to be 9.1 and 6.7 nM with Fab on BT-474 and SK-OV-3 cells, and 41.1 and 32.7 nM with HRG on BT-474 and MDA-MB-468 cells, respectively. The overall higher displacement of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG by both ligands in cells that express both HER2 and HER3 as compared to displacement when treated with only Fab or HRG suggests that there may be some residual binding of the RICs as a result of interaction with heterodimerized receptors that cannot be displaced by a single competitor. This may also explain why higher concentrations of individual competitors were necessary to displace the binding of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG to BT-474 cells that express both HER2 and HER3 as opposed to SK-OV-3 or MDA-MB-468 cells that display only HER2 or HER3, respectively. Because of its favourable properties in vitro, $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG was selected for further study in vivo in tumour bearing mice. Optimal imaging conditions were first determined in mice implanted s.c. with BT-474 tumours, which are positive for both HER2 and HER3. Mice were imaged at 4, 24, 48 and 72 h after injection of bsRICs. Tumour uptake was not seen at 4 h but was observed at all time points after 24 h, with the highest uptake and T:B ratio found at 48 h (Table 2-1). These results are similar to those previously observed for $^{111}$In-labeled trastuzumab Fab fragments, which shows a tumour uptake of 8.4 ± 1.8%ID/g at 48 h p.i. in BT-474 tumour xenografts. Pharmacokinetic elimination of $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG from the blood was consistent with previous reports from our laboratory for $^{111}$In-DTPA-trastuzumab Fab (0.7 ± 0.1%ID/g vs 0.9 ± 0.3%ID/g at 48 h p.i.), and differed from $^{111}$In-DTPA-trastuzumab IgG, which
exhibited a blood concentration of 3.5 ± 1.0%ID/g at 72 h p.i. in mice with MDA-MB-361 tumour xenografts.\textsuperscript{235} Using this time point, the \textit{in vivo} properties of \textsuperscript{111}In-DTPA-Fab-PEG\textsubscript{24}-HRG, including its ability to bind HER2 and HER3 were further examined in mice bearing s.c. SK-OV-3 and MDA-MB-468 xenografts, respectively. To confirm specificity for these target receptors, blocking studies were performed by pre-administering an excess of Fab or HRG. In the case of SK-OV-3 xenografts, Fab blocking caused a significant 2.6 fold decrease in tumour uptake, while in MDA-MB-468 xenografts a significant 1.6 fold decrease was noted with HRG pre-administration (Table 2-3). Uptake in all other tissues remained unchanged with and without blocking. These results demonstrated that \textsuperscript{111}In-DTPA-Fab-PEG\textsubscript{24}-HRG bound specifically to both HER2 and HER3 \textit{in vivo}.

For comparison, the tumour and normal tissue uptake of \textsuperscript{111}In-DTPA-Fab in mice with SK-OV-3 xenografts, and \textsuperscript{111}In-DTPA-HRG in mice with MDA-MB-468 xenografts was examined at this same time point (Table 2-4). While the uptake of \textsuperscript{111}In-DTPA-Fab in SK-OV-3 xenografts was comparable to that of the bsRIC, much lower tumour uptake was observed with \textsuperscript{111}In-DTPA-HRG in MDA-MB-468 xenografts, possibly due to the rapid elimination of \textsuperscript{111}In-DTPA-HRG from the blood. To our knowledge this is the first time uptake of radiolabeled HRG into tumours expressing HER3 has been reported. As such the bsRIC may provide better imaging of HER3 expression in tumours than \textsuperscript{111}In-DTPA-HRG.

Here we have described the construction of a bsRIC that recognizes both HER2 and HER3, an important prerequisite for an imaging agent that could ultimately detect HER2-HER3 heterodimers. Our results show that these bsRICs bind in higher amounts and more strongly to cells that express both HER2 and HER3, as compared to cells that only express one of these receptors. We are now exploring methods to discriminate the binding of the bsRICs to heterodimerized receptors using inhibitors such as pertuzumab, which can diminish their binding,\textsuperscript{236} as well as assessing the extent of heterodimerization in the tumour cells using various techniques.\textsuperscript{38} Should these studies prove promising, the ability of the bsRICs to differentiate between tumours with low or high levels of heterodimerized HER2-HER3 receptors will be studied by imaging.
2.6 Conclusions

We conclude that $^{111}$In-labeled bsRICs composed of trastuzumab Fab fragments conjugated to the EGF-like domain of HRG through a PEG spacer of appropriate length bound specifically to both HER2 and HER3. Examination of these bsRICs in athymic mice bearing tumour xenografts expressing HER2 or HER3 or both receptors showed good tumour uptake and low normal tissue accumulation, with the exception of the kidneys. The optimal time point for microSPECT/CT imaging was 48 h p.i. Blocking studies in mice with tumours expressing only HER2 or HER3 demonstrated the specificity for both receptors in vivo. The design and approach for synthesis of these bsRICs recognizing HER2 or HER3 could be applied to the construction of other bsRICs that bind to more than one peptide growth factor receptor.
CHAPTER 3

MicroSPECT/CT Imaging of Co-expressed HER2 and EGFR on Subcutaneous Human Tumour Xenografts in Athymic Mice Using $^{111}$In-labeled Bispecific Radioimmunoconjugates

This chapter represents a reprint of Razumienko E, Dryden L, Scollard D, Reilly RM MicroSPECT/CT imaging of co-expressed HER2 and EGFR on subcutaneous human tumour xenografts in athymic mice using $^{111}$In-labeled bispecific radioimmunoconjugates. Breast Cancer Research and Treatment. 2013 Apr; 138(3):709-18.

All experiments and analyses of data were completed by Eva J. Razumienko in this chapter. L. Dryden assisted with the competition binding assays and biodistribution studies. SPECT/CT imaging was performed with the assistance of D. Scollard.
3.1 Abstract

Epidermal growth factor receptors (EGFR) form heterodimers with HER2 in breast cancer and increased EGFR expression has been found in HER2-positive tumours resistant to trastuzumab (Herceptin). Our objective was to synthesize bispecific radioimmunoconjugates (bsRICs) that recognize HER2 and EGFR and evaluate their ability to image tumours in athymic mice that express one or both receptors by microSPECT/CT. bsRICs were constructed by conjugating maleimide-derivatized trastuzumab Fab fragments that bind HER2 to a thiolated form of EGF with an intervening 24 mer polyethyleneglycol (PEG24) spacer. bsRICs were derivatized with diethylenetriaminepentaacetic acid (DTPA) for labeling with $^{111}$In. The ability of $^{111}$In-bsRICs to bind HER2 or EGFR was determined in competition assays using cells expressing one or both receptors. Tumour and normal tissue uptake were examined in CD1 athymic mice bearing subcutaneous tumour xenografts that expressed HER2, EGFR or both receptors, with or without pre-administration of Fab or EGF to determine specificity. Results: HER2 and EGFR binding and displacement of binding by competitors were found for $^{111}$In-bsICs. The highest uptake of $^{111}$In-bsRICs [7.3 ± 3.5 %ID/g] in 231-H2N human breast cancer xenografts (HER2+/EGFR+) occurred at 48 h post-injection (p.i.). Pre-administration of trastuzumab Fab decreased uptake in SK-OV-3 (HER2+/EGFR-) human ovarian cancer xenografts from 7.1 ± 1.2 %ID/g to 2.4 ± 1.5 %ID/g. Pre-administration of excess EGF decreased uptake in MDA-MB-231 (HER2-/EGFR+) human breast cancer xenografts from 5.9 ± 0.5 %ID/g to 2.0 ± 0.1 %ID/g. All tumours were imaged by microSPECT/CT. We conclude that $^{111}$In-bsRICs composed of trastuzumab Fab and EGF exhibited specific binding in vitro to tumour cells displaying HER2 or EGFR, and were taken up specifically in vivo in tumours expressing one or both receptors, permitting tumour visualization by microSPECT/CT. These agents may ultimately be useful for imaging heterodimerized HER2-EGFR complexes since their bivalent properties permit more avid binding to these complexes.
3.2 Introduction

Overexpression of the human epidermal growth factor receptor (HER) family of receptors, including HER2 and EGFR has been observed in many solid tumours, including breast cancer, and contributes to cellular transformation and proliferation. Both HER2 and EGFR homodimerize or heterodimerize which initiates mitogenic signalling, and cooperativity of EGFR and HER2 has been shown in mouse mammary tumourigenesis. EGFR binds its preferred ligand, EGF, which promotes recruitment of HER2 to the complex. There is no known ligand for HER2, but the receptor is locked in a “dimerization ready” conformation. Formation of the HER2/EGFR heterodimer results in autophosphorylation of the C-terminal intracellular kinase domains of these two receptors which activates signalling pathways including Ras/Raf/MEK/ERK and PI3K/Akt. These pathways propagate a cascade of intracellular signals that regulate proliferation and contribute to a more aggressive tumour phenotype.

New classes of therapeutic agents for breast cancer, known as HER dimerization inhibitors are intended to block HER2 heterodimerization with other HER family receptors, including HER3 and EGFR. The most well known example is the monoclonal antibody pertuzumab (Perjeta®; Roche). Recent clinical trial data has shown that pertuzumab, which binds and inhibits the dimerization domain of HER2, in combination with trastuzumab and docetaxel, significantly prolonged progression-free survival of HER2 positive metastatic breast cancer patients as compared to trastuzumab and docetaxel alone. Preclinically, novel agents that directly target HER2 heterodimers have been reported, including bispecific antibodies and bispecific scFvs that recognize HER2 and HER3 and which were developed as immunotherapeutics. There are currently no reports of agents targeting HER2/EGFR complexes.

Our objective was to develop bispecific radioimmunoconjugates (bsRICs) consisting of a domain that binds to HER2 covalently crosslinked to a domain that recognizes EGFR that would be capable of interaction with heterodimerized HER2/EGFR through bivalent receptor recognition. We propose that these bsRICs may ultimately be useful for probing HER2/EGFR complexes in tumours by single-photon emission computed tomography (SPECT). In the current study, our aim was to demonstrate specific binding of these bsRICs to HER2 and EGFR on tumours cells in vitro and specific localization in vivo in tumour xenografts in athymic mice. HER2 and EGFR are often co-expressed in breast cancer, and such coexpression has been associated with a poor
In addition, there is value in targeting HER2/EGFR, as EGFR has been found upregulated in HER2-positive breast cancer cells that exhibit trastuzumab resistance.\textsuperscript{112,242}

Previously we reported analogous bsRICs that recognize HER2 and HER3 which were composed of a binding moiety for each receptor and incorporated a flexible polyethyleneglycol (PEG) crosslinker between these two domains to provide greater mobility to the individual components which allowed better binding to both receptors.\textsuperscript{243} Here we show that bsRICs composed of trastuzumab Fab linked to EGF through this PEG spacer retained specificity for HER2 and EGFR \textit{in vitro} and these bsRICs showed good tumour uptake in mouse tumour xenografts models that express HER2, EGFR or both receptors. The bsRICs exhibited more attractive tumour imaging characteristics than the radiolabeled individual ligands. These properties render these bsRICs capable of interacting with HER2 and EGFR and with heterodimerized HER2/EGFR which may permit their application for probing these complexes in tumours by SPECT imaging.

### 3.3 Materials and Methods

#### 3.3.1 Tumour Cells

SK-OV-3 human ovarian carcinoma cells and MDA-MB-231 human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA). The 231-H2N cell line was derived from MDA-MB-231 cells that were transfected to stably overexpress \textit{c-erbB}-2 (HER2), and was kindly provided by Dr. Robert S. Kerbel (Sunnybrook Health Sciences Centre, Toronto, ON).\textsuperscript{244} SK-OV-3 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10\% fetal bovine serum (Invitrogen, Grand Island, NY). MDA-MB-231 and 231-H2N cells were cultured in Dulbecco's Modified Eagle medium supplemented with 10\% fetal bovine serum. All cells were cultured under a 5\% CO\textsubscript{2} atmosphere at 37\textdegree{}C. Cells were selected based on their expression levels of HER2 or EGFR as previously reported,\textsuperscript{245–247} but these receptor densities were confirmed by radioligand binding assays using \textsuperscript{111}In-labeled trastuzumab Fab fragments or \textsuperscript{111}In-labeled EGF. The EGFR expression of MDA-MB-231 and 231-H2N cells was $5.2 \times 10^5$ and $4.8 \times 10^5$ receptors/cell, respectively. The HER2 density of SK-OV-3 and 231-H2N was $9.6 \times 10^5$ and $4.5 \times 10^5$ receptors/cell, respectively.
3.3.2 Synthesis of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF

bsRICs were constructed as reported for analogous bsRICs recognizing HER2 and HER3. Briefly, trastuzumab (Herceptin) Fab fragments (50 kDa) were chemically crosslinked to human EGF (6.2 kDa, Peprotech, Rockey Hill, NJ) through the reaction scheme shown in Figure 3-1. Fab fragments were generated by proteolysis of trastuzumab IgG using immobilized papain. A maleimide functional group was introduced by reaction of the Fab fragments [1 mg/mL in phosphate-buffered saline (PBS), pH 7.2 with 5 mM ethylenediaminetetraacetic acid (EDTA)] with a 10-fold molar excess of succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (sulfoSMCC, Pierce, Rockford, IL) containing PEG chains with 24 repeating units (PEG$_{24}$) for 1 h at room temperature (RT). Primary amines on EGF were modified by reacting EGF with a 10-fold molar excess of 2-iminothiolane HCl (Pierce) in PBS (pH 8.0) containing 5 mM EDTA for 1 h at RT to insert a free thiol group. The number of thiols introduced into EGF was measured colorimetrically with Ellman’s Reagent (Pierce, Rockford IL). The assay showed that 1.3 ± 0.2 thiol groups were introduced into EGF. The number of maleimide groups introduced into trastuzumab Fab was not determined. Maleimide-modified Fab fragments were immediately reacted with a 2 fold molar excess of thiolated EGF for 1 h at RT. The bispecific immunoconjugates were purified and buffer-exchanged and re-concentrated to 1.0 mg/mL in 50 mM NaHCO$_3$ (pH 8.0) on a Microcon 30K device (Millipore), then derivatized with a 4-fold molar excess of diethylenetriaminepentaacetic acid (DTPA) dianhydride (Sigma-Aldrich) for 1 h at RT as previously described [17]. DTPA conjugation efficiency was measured by instant thin layer silica gel chromatography (ITLC-SG, Pall Life Sciences, Port Washington, NY) developed in 100 mM sodium citrate (pH 5.0) following radiolabeling of a sample of the unpurified reaction mixture with $^{111}$In [25]. The conjugation efficiency was multiplied by the 4-fold molar excess of DTPA in the reaction to determine the number of DTPA per molecule. Using this assay, there were 1.2 ± 0.3 DTPA groups per molecule of bispecific immunoconjugates. Excess DTPA was removed by purification on a Sephadex G25 (Sigma-Aldrich) mini-column eluted with 1 M sodium acetate (pH 6.0). Purified DTPA-Fab-PEG$_{24}$-EGF was labeled with $^{111}$InCl$_3$ (Nordion, Kanata, ON). The final radiochemical purity was >95% as measured by ITLC-SG.
bsRIs consisting of trastuzumab Fab fragments chemically crosslinked through a flexible 24-mer polyethylene glycol (PEG) spacer to epidermal growth factor (EGF). Fab fragments produced by proteolysis of trastuzumab IgG with papain were reacted with a tenfold molar excess of NHS-PEG<sub>24</sub>-maleimide cross-linker for 1 h at RT. Thiols were introduced into EGF by reaction with a tenfold molar excess of Traut’s reagent (2-iminothiolane) for 1 h at RT. Then a twofold molar excess of thiolated EGF was reacted with maleimide-modified trastuzumab Fab for 1 h at RT. Fab-PEG<sub>24</sub>-EGF immunoconjugates were purified and reacted with a fourfold molar excess of DTPA dianhydride for 1 h at RT. Following purification, DTPA-Fab-PEG<sub>24</sub>-EGF was labeled with ¹¹¹In by incubation with ¹¹¹InCl<sub>3</sub> in 1 M sodium acetate buffer for 1 h at RT.
3.3.3 Characterization of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF

The successful conjugation of Fab to EGF and purity of the bsRICs was assessed by size exclusion high performance liquid chromatography (SE-HPLC). To confirm conjugation to Fab, EGF was labeled with $^{111}$In$^{249}$ and used as a radiotracer in the conjugation reaction, which permitted analysis of the resulting bsRICs by SE-HPLC using radioactivity detector. $^{111}$In-DTPA-EGF was reacted with Traut’s reagent, as described above, to introduce a free sulfhydryl (SH) group. Combinations of $^{111}$In-DTPA-EGF (without SH) and Fab-SMCC or $^{111}$In-DTPA-EGF-SH and Fab were reacted for 1 h at room temperature (RT). SE-HPLC was performed on a BioSep SEC-S2000 column (Phenomenex, Torrance, CA) eluted with 100 mM NaH$_2$PO$_4$ buffer (pH 7.0) at a flow rate of 0.8 mL/min using a Series 200 pump (PerkinElmer, Waltham, MA, USA) interfaced with a UV diode array detector (PerkinElmer) set at 280 nm and a Radiomatic 610TR flow scintillation analyser radioactivity detector (FSA, PerkinElmer).

3.3.4 Competition receptor binding assays

The ability of the bsRICs to bind to HER2 and EGFR was measured by competition binding assays using SK-OV-3 (HER2+), MDA-MB-231 (EGFR+) and 231-H2N (HER2+/EGFR+) cells. Approximately $1.5 \times 10^5$ cells were seeded into wells in 24-well plates and cultured overnight. The cells were then exposed to 10 nM (5.0 MBq/µg) of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF in phosphate-buffered saline (PBS) in the presence of 1000 nM of unlabeled Fab, EGF or both for one point binding assays, or increasing concentrations (0-1200 nM) of unlabeled Fab, EGF or both for competition binding assays. After incubation for 3 h at 4°C, unbound radioactivity was removed and the cells were solubilised and transferred to $\gamma$-counting tubes and the cell bound radioactivity was measured in a $\gamma$-counter. For competition assays the cell bound $^{111}$In-labeled bsRICs in the presence of competitor was normalized against cell bound bsRICs in the absence of competitor (B/B$_0$) and plotted versus the logarithm of the competitor concentration (log C). The data was fitted to a one site competition model by Prism software version 4.0 (GraphPad, San Diego, CA) and the effective concentration that caused 50% displacement of the binding of the $^{111}$In-labeled bsRICs to the cells (EC$_{50}$) was calculated.
MicroSPECT/CT imaging

MicroSPECT/CT was performed on female athymic CD1 *nu/nu* mice (Charles River, Boston, MA, USA) bearing s.c. 231-H2N (HER2+/EGFR+) tumour xenografts at 4, 24, 48 and 72 h post injection (p.i.), and in mice bearing s.c. SK-OV-3 (HER2+/EGFR-) and MDA-MB-231 (HER2-/EGFR+) tumour xenografts at 48 h p.i. For 231-H2N xenografts, mice were inoculated s.c. in the thigh with $1 \times 10^7$ cells in 200 µL of a 1:1 mixture of Matrigel (BD Biosciences, Mississauga, ON) and serum free growth medium. For SK-OV-3 and MDA-MB-231 xenografts, mice were inoculated with $1 \times 10^7$ cells in 100 µL of serum free growth media. After 4-6 weeks, groups of 3 mice with $200 \pm 30$ mm$^3$ tumours (measured with external callipers and calculated using volume = length x width$^2$ x 0.5) were injected i.v. (tail vein) with $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF (10 µg, 35-40 MBq per mouse) in 100 µL of normal saline. For blocking studies 1 mg of unlabeled Fab or EGF was injected i.p. 3 h prior to $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF injection. Mice were anaesthetized by inhalation of 2% isoflurane in O$_2$. Imaging was performed on a NanoSPECT/CT tomograph (BioScan, Washington, DC) equipped with 4 NaI(Tl) detectors and fitted with 1.4-mm multipinhole collimators (full width at half maximum ≤ 1.2mm). A total of 24 projections were acquired in a 256 x 256 acquisition matrix with a minimum of 90,000 counts per projection. Images were reconstructed using an ordered-subset expectation maximization (OSEM) algorithm (9 iterations). Cone beam CT images were acquired (180 projections, 1s/projection, 45 kVp) before micro-SPECT images. MicroSPECT and CT images were co-registered using InVivoScope software (BioScan).

Tumour and normal tissue uptake

Biodistribution studies were performed immediately after microSPECT/CT imaging. Mice were sacrificed and the tumour and samples of blood and selected normal tissues collected, weighed and their radioactivity counted in a γ-counter. Tumour and normal tissue uptake were expressed as mean ± SD percentage injected dose per gram (%ID/g). All animal studies were conducted under a protocol (No. 989.13) approved by the Animal Care Committee at the University Health Network in accordance with Canadian Council on Animal Care (CCAC) guidelines. Statistical comparisons were made using a two-tailed Student’s t-test (p<0.05).
3.4 Results

3.4.1 Synthesis and characterization of bsRICs

Successful synthesis of Fab-PEG$_{24}$-EGF immunoconjugates was confirmed through use of $^{111}$In-DTPA-EGF as a radiotracer in the conjugation reaction with subsequent SE-HPLC analysis. A migration of the radioactivity peak from the EGF retention time ($t_R$) of 13.9 mins to closer to the Fab retention time of 10.1 mins was only observed when both components were modified with crosslinking agents. SE-HPLC analysis showed a major radioactivity peak with $t_R$ of 10.4 mins for Fab-PEG$_{24}$-EGF (Figure 3-2).

3.4.2 Competition receptor binding assays

The binding of $^{111}$In-labeled Fab-PEG$_{24}$-EGF to HER2 and EGFR was displaced by a 100 fold molar excess of unlabeled Fab, EGF or both ligands on cells that expressed only HER2 (SK-OV-3), EGFR (MDA-MB-231) or both HER2 and EGFR (231-H2N). In one point binding assays, an overall increase in total binding (in the absence of competitor) of the bsRICs was found for 231-H2N cells expressing both HER2 and EGFR when compared to the sum of the binding of $^{111}$In-DTPA-Fab or $^{111}$In-DTPA-EGF to these cells (Figure 3-3). There was no increased binding of the bsRICs to SK-OV-3 or MDA-MB-231 cells that express only HER2 or EGFR compared to the binding of $^{111}$In-DTPA-Fab or $^{111}$In-DTPA-EGF, respectively (Figure 3-3).

The bsRICs showed binding to HER2 and EGFR on MDA-MB-231, SK-OV-3 or 231-H2N cells and this binding was displaced in the presence of EGF or trastuzumab Fab (Figure 3-4). The binding to SK-OV-3 cells (HER2+/EGFR-) was reduced to 12.4 ± 2.6%, 105.0 ± 10.7% and 10.1 ± 1.6%, by trastuzumab Fab, EGF or both ligands (Figure 3-4b). The binding to MDA-MB-231 cells (HER2-/EGFR+) was reduced to 95.2 ± 7.5%, 20.3 ± 2.8% and 9.9 ± 2.9% by trastuzumab Fab, EGF or both ligands (Figure 3-4a). The binding to 231-H2N cells (HER2+/EGFR+) was reduced to 52.7 ± 2.8%, 65.6 ± 2.3% and 4.4 ± 0.9%, respectively by competition with trastuzumab Fab, EGF or both ligands compared to that without competitor (Figure 3-4c).

In order to examine HER2 or EGFR binding in more detail, competition binding curves were obtained by displacement of the binding of the bsRICs by increasing concentrations of trastuzumab Fab or EGF (Figure 3-5). Fitting of these curves to a 1-site competition binding
model estimated that the EC$_{50}$ values were 24.2 nM and 18.5 nM with Fab on 231-H2N and SK-OV-3 cells, and 24.9 nM and 36.3 nM with EGF on 231-H2N and MDA-MB-231 cells, respectively. These assays were also performed using $^{111}$In-DTPA-Fab and $^{111}$In-DTPA-EGF which were found to have EC$_{50}$ values of 8.32 nM and 17.14 nM, respectively, on 231-H2N cells.

Figure 3-2. SE-HPLC chromatograms of bsRIs.
bsRIs were analyzed on a BioSep-SEC-S2000 column eluted with 0.1 M NaH$_2$PO$_4$ (pH 7.0) at a flow rate of 0.8 mL/min with UV detection at 280 nm (blue line) and radioactivity detection using a flow scintillation analyzer (FSA; red line). $^{111}$In-EGF was used as a radiotracer to demonstrate that conjugation only occurred when both Fab and EGF were modified with the appropriate crosslinkers. The shift in the FSA signal only occurs when both moieties of the bsRIC are modified with the appropriate crosslinkers, confirming conjugation.
Figure 3-3. The binding of $^{111}$In-labeled Fab, EGF, or Fab-PEG$_{24}$-EGF to HER2 and EGFR on tumour cells in one-point competition binding assays.

Binding was evaluated using 10 nM radioligands in cell lines that are HER2+/EGFR− (SK-OV-3), HER2−/EGFR+ (MDA-MB-231), or HER2+/EGFR+ (231-H2N). Significantly greater binding to 231-H2N cells expressing both receptors was observed for $^{111}$In-Fab-PEG$_{24}$-EGF as compared to the sum of the individual binding for $^{111}$In-Fab or $^{111}$In-EGF (asterisks; $p < 0.0001$)
Figure 3-4. The binding of $^{111}$In-labeled bsRICs to HER2 and EGFR on tumour cells in one-point competition binding assays.

Binding was evaluated using 10 nM of $^{111}$In-Fab-PEG$_{24}$-EGF alone or competed with a 50 fold molar excess of unlabeled trastuzumab Fab, EGF, or both ligands in cells that are a HER2−/EGFR+ (MDA-MB-231), b HER2+/EGFR− (SK-OV-3), or c HER2+/EGFR+ (231-H2N)
bsRiCs were displaced by increasing concentrations of a trastuzumab Fab and b EGF on cells that are HER2+/EGFR+ (231-H2N), HER2+/EGFR− (SK-OV-3), or HER2−/EGFR+ (MDA-MB-231). B/B₀ represents the binding of the bsRiCs in the presence of competitors divided by the binding in the absence of competitors. Fitting of the curves to a 1-site competition binding model provided EC₅₀ values of 24.2 and 18.5 nM with Fab on 231-H2N and SK-OV-3 cells, and 24.9 and 36.3 nM with EGF on 231-H2N and MDA-MB-231 cells.

Figure 3-5. Displacement of the binding of 10 nM of ¹¹¹In-Fab-PEG₂₄-EGF bsRiCs to HER2 and EGFR.
3.4.3 MicroSPECT/CT imaging and tumour and normal tissue uptake

MicroSPECT/CT images of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF in mice bearing 231-H2N (HER2+/EGFR+) xenografts showed accumulation in the tumour and low normal tissue uptake at 24, and 48 h p.i., with the exception of the kidneys and liver (Figure 3-6).

Maximum tumour uptake was seen at 48 h p.i., and this was confirmed by biodistribution studies which showed 7.3 ± 3.5 %ID/g (Table 3-1), and a T/B ratio of 6.0 ± 1.8. The uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF was then examined in mice with SK-OV-3 (HER2+/EGFR-) and MDA-MB-231 (HER2-/EGFR+) tumours at 48 h p.i. Good uptake was seen in both tumour xenografts on microSPECT/CT images (Figure 3-7) and this was confirmed in biodistribution studies (Table 3-2). Moreover, when pre-administered an excess of Fab, $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF uptake in SK-OV-3 tumours was decreased by 2.9 fold (from 7.1 ± 1.2 %ID/g to 2.4 ± 1.5 %ID/g). Uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF decreased by 2.9 fold in MDA-MB-231 xenografts (from 5.9 ± 0.5% ID/g to 2.0 ± 0.1%ID/g) with pre-administration of an excess of EGF (Table 3-2). This confirmed that tumour uptake of these bsRICs was specifically mediated by binding to HER2 or EGFR.
Figure 3-6. Posterior whole body microSPECT/CT images of a female CD1 athymic mouse implanted s.c. in the right hind flank with a HER2+/EGFR+ 231-H2N human breast cancer xenograft.

Imaging was performed at a 4 h, b 24 h, c 48 h, and d 72 h after i.v. (tail vein) injection of $^{111}\text{In}$-Fab-PEG$_{24}$-EGF. The tumour xenograft (arrow) was visualized in panels b, c but not in panel a, d. Also visualized are the kidneys (large arrowhead) and liver (small arrowhead) illustrated in panel a only.
Table 3-1. Tumour and normal tissue uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF in CD1 athymic mice with 231-H2N tumour xenografts at selected times

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percent Injected dose/g (mean %ID/g ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
</tr>
<tr>
<td>Blood</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Heart</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Lung</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>8.5 ± 1.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>Small intestines</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Large intestines</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Tumour</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>Bladder</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>Skin</td>
<td>2.7 ± 2.0</td>
</tr>
</tbody>
</table>

* CD1 athymic mice with HER2+/EGFR+ 231-H2N xenografts were administered 35-40 MBq/10 $\mu$g of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF (n=4) and the tissue distribution was determined at the selected time points post i.v. (tail vein) injection.

$^+$Significantly different (p<0.05)
Table 3-2. Tumour and normal tissue uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF in CD1 athymic mice with SK-OV-3 and MDA-MB-231 xenografts at 48 h post-injection

<table>
<thead>
<tr>
<th>Tissue</th>
<th>TK-OV-3 (HER2+)</th>
<th>MDA-MB-231 (EGFR+)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unblocked</td>
<td>Blocked †</td>
<td>Unblocked</td>
<td>Blocked †</td>
</tr>
<tr>
<td>Blood</td>
<td>0.6 ± 0.1</td>
<td>1.5 ± 0.0</td>
<td>0.7 ± 1.3</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Heart</td>
<td>0.6 ± 0.1</td>
<td>1.0 ± 0.8</td>
<td>0.9 ± 0.3</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Lung</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.4</td>
<td>0.6 ± 0.8</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>3.8 ± 0.7</td>
<td>3.4 ± 0.3</td>
<td>4.3 ± 0.9</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.8 ± 1.3</td>
<td>5.5 ± 1.4</td>
<td>6.5 ± 2.6</td>
<td>5.9 ± 0.8</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 1.4</td>
<td>1.3 ± 0.8</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.3 ± 0.1</td>
<td>1.0 ± 0.4</td>
<td>0.4 ± 0.4</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Small intestines</td>
<td>0.6 ± 0.2</td>
<td>0.3 ± 0.9</td>
<td>0.7 ± 0.6</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Large intestines</td>
<td>0.3 ± 0.1</td>
<td>0.7 ± 0.5</td>
<td>0.8 ± 0.8</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.9</td>
<td>1.0 ± 0.4</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Tumour</td>
<td>7.1 ± 1.4</td>
<td>2.4 ± 1.5†</td>
<td>5.9 ± 0.5</td>
<td>2.0 ± 0.1§</td>
</tr>
<tr>
<td>Bladder</td>
<td>0.3 ± 0.1</td>
<td>0.7 ± 0.5</td>
<td>1.3 ± 0.8</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Skin</td>
<td>2.1 ± 0.4</td>
<td>1.7 ± 0.4</td>
<td>1.5 ± 0.4</td>
<td>1.7 ± 0.6</td>
</tr>
</tbody>
</table>

*CD1 athymic mice were administered 30-35 MBq/10 μg of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF (n=4) and sacrificed 48 h post i.v. (tail vein) injection.

† To block HER2 or EGFR on tumours, mice were pre-administered an excess of unlabeled trastuzumab Fab (1 mg) or unlabeled EGF (1 mg), respectively by intraperitoneal injection 3 h prior to $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF injection.

‡ Significantly different (p<0.003)

§ Significantly different (p<0.001)
Figure 3-7. Posterior whole body microSPECT/CT images of a female CD1 athymic mouse implanted s.c. in the right hind flank.

Mice were implanted with with a SK-OV-3 (HER2+/EGFR−) human ovarian cancer xenograft or b MDA-MB-231 (HER2−/EGFR+) human breast cancer xenograft (arrows) at 48 h after i.v. (tail vein) injection of $^{111}$In-Fab-PEG$_{24}$-EGF. Tumours were visualized in both cases (arrows).
3.5 Discussion

In this report we described for the first time the synthesis and evaluation of bsRICs capable of binding HER2 and EGFR \textit{in vitro} and \textit{in vivo}. The bsRICs were composed of a trastuzumab Fab fragment crosslinked through a flexible 24-mer PEG spacer to EGF. This design was intended to minimize any steric hindrance for binding to HER2 or EGFR, and was previously employed for cross-linking trastuzumab Fab fragments to the peptide heregulin (HRG) to synthesize bsRICs that recognized HER2 and HER3. Conjugation of Fab to EGF was confirmed by SE-HPLC, by incorporating EGF labeled with $^{111}$ In in tracer amounts into the conjugation reaction, which revealed that an immunoconjugate formed only when Fab and EGF were modified with the appropriate crosslinkers (Figure 3-1).

The ability of the bsRICs to bind to the target receptors was first evaluated \textit{in vitro} by competition receptor binding assays. The bsRICs retained their ability to bind specifically to HER2 and EGFR and binding was displaced by excess unlabeled Fab or EGF in cells that expressed HER2, EGFR or both HER2 and EGFR (Figure 3-4 and Figure 3-5). Displacement of bsRIC binding by EGF was not obtained in HER2+/EGFR- SK-OV-3 cells. Similarly, trastuzumab Fab did not displace the binding of bsRICs to HER2-/EGFR+ MDA-MB-231 cells. The greater total binding of $^{111}$ In-DTPA-Fab-PEG$_{24}$-EGF to 231-H2N cells that express both HER2 and EGFR, as compared to the sum of the binding for the individual $^{111}$ In-labeled Fab or EGF ligands to these cells (Figure 3-3) suggests that there may be more avid bivalent binding of the bsRICs to heterodimerized HER2-EGFR. This explanation is supported by the higher concentrations of competitors (EC$_{50}$ values) that were required to displace the binding of $^{111}$ In-DTPA-Fab-PEG$_{24}$-EGF to 231-H2N cells that express both HER2 and EGFR, than SK-OV-3 or MDA-MB-231 cells that display only HER2 or EGFR, respectively (Figure 3-5). The use of 231-H2N cells, a subclone of MDA-MB-231 cells that exhibited closely matched HER2 and EGFR densities ($4.5 \times 10^5$ and $4.8 \times 10^5$ receptors/cell, respectively) may be important for studying the binding of $^{111}$ In-DTPA-Fab-PEG$_{24}$-EGF to heterodimerized receptors. Ekerljung L et al. similarly concluded that receptor density was important in evaluating the binding of bispecific affibodies to HER2/EGFR.

Since $^{111}$ In-DTPA-Fab-PEG$_{24}$-EGF exhibited specific binding to HER2 and EGFR \textit{in vitro}, the bsRICs were then studied \textit{in vivo} for microSPECT/CT imaging of s.c. human breast cancer.
xenografts in athymic mice that express one or both receptors. The optimal time point for imaging was first determined in mice implanted s.c. with HER2+/EGFR+ 231-H2N tumours (Figure 3-6). Tumour uptake was not seen at 4 h or 72 h p.i. of the bsRICs but was noted at 24 h and 48 h, with the highest uptake at 48 h p.i. (7.3 ± 3.5%ID/g; Table 3-1). These results were similar to those previously reported for $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG bsRICs recognizing HER2 and HER3, which exhibited maximal tumour uptake of 7.8 ± 2.1 %ID/g at 48 h p.i. in BT-474 tumour xenografts. $^{243}$ 48 h p.i. $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF and $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG exhibited similar kidney uptake (7.8 ± 1.0 %ID/g vs. 6.4 ± 0.7 %ID/g), but $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF exhibited 2-fold higher liver uptake (3.4 ± 1.2 %ID/g vs. 1.8 ± 0.1 %ID/g), likely due to binding to EGFR on hepatocytes. $^{251}$ $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF exhibited higher uptake in MDA-MB-231 xenografts than previously reported for $^{111}$In-DTPA-EGF (7.3 ± 3.5 %ID/g at 48 h p.i. vs 1.5 ± 0.9 %ID/g at 72 h p.i.)$^{249}$. Following identification of the optimal imaging time point, the tumour uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF was examined in mice bearing s.c. SK-OV-3 and MDA-MB-231 xenografts that express only HER2 or EGFR, respectively (Figure 3-7). Pre-administration of an excess of Fab or EGF blocked tumour uptake of the bsRICs, demonstrating specific binding to HER2 or EGFR. In the case of SK-OV-3 xenografts, Fab blocking caused a 2.9 fold decreased tumour uptake, while in MDA-MB-231 xenografts a 2.9 fold decrease was found with EGF pre-administration (Table 3-2). This is similar to the 2.6-fold decreased uptake previously reported for $^{111}$In-DTPA-Fab-PEG$_{24}$-HRG in mice with HER2+ SK-OV-3 tumour xenografts with trastuzumab Fab pre-administration.$^{243}$ Interestingly, we noted that $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF appeared to exhibit more attractive properties for tumour imaging than either $^{111}$In-labeled trastuzumab Fab or EGF. Tumour uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF in SKOV-3 xenografts at 48 h p.i. (7.0 ± 1.2 %ID/g) was comparable to that reported for $^{111}$In-DOTA-trastuzumab Fab in SKOV-3 tumours in athymic mice at 24 h p.i. (5.4 ± 0.4 %ID/g)$^{178}$ or $^{111}$In-DTPA-trastuzumab Fab in BT-474 tumours at 48 h p.i. (5.2 ± 1.5 %ID/g)$^{177}$. However, kidney uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF (8.4 ± 1.8 %ID/g) at 48 h p.i. was 8-fold lower than $^{111}$In-DTPA-trastuzumab Fab (47.1 ± 5.7 %ID/g)$^{177}$. Liver uptake of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF at 48 h p.i. (3.4 ± 1.2 %ID/g) was 2-fold lower than for $^{111}$In-DTPA-EGF.$^{249}$ These results indicated that cross-linking trastuzumab
Fab to EGF through a 24-mer PEG spacer improved the pharmacokinetic properties of each of these ligands, yielding comparable tumour uptake but diminished normal tissue accumulation.

3.6 Conclusions

In conclusion, we have described the synthesis and characterization of novel bsRICs that recognize HER2 and EGFR. Our results show that these bsRICs bind in higher amounts to cells that express both HER2 and EGFR, as compared to cells that only express one of these receptors, suggesting there may be more avid binding of the bsRICs to cells expressing both receptors. We are now exploring methods to discriminate the binding of these bsRICs to heterodimerized receptors using inhibitors such as pertuzumab, which interferes with HER family heterodimerization, as well as assessing the extent of heterodimerization in tumour cells using biophysical techniques. Should these studies prove promising; the ability of the bsRICs to differentiate between tumours with low or high levels of heterodimerized HER2/EGFR receptors by SPECT imaging will be explored.
CHAPTER 4

Dual Receptor-Targeted Radioimmunotherapy of Human Breast Cancer Xenografts in Athymic Mice Co-Expressing HER2 and EGFR Using $^{177}$Lu- or $^{111}$In-Labeled Bispecific Radioimmunoconjugates

This chapter represents a reprint of Razumienko EJ, Chen JC, Cai Z, Chan C, Reilly RM. Dual Receptor-Targeted Radioimmunotherapy of Human Breast Cancer Xenografts in Athymic Mice Co-Expressing HER2 and EGFR using $^{177}$Lu- or $^{111}$In-Labeled Bispecific Radioimmunoconjugates. J Nucl Med. 2015 Oct 1. [Epub ahead of print]

All experiments and analyses of data were completed by Eva J. Razumienko in this chapter with the exception of the dosimetry analysis (Z. Cai). J. Chen assisted with the clonogenic assays, and C. Chan assisted with the radioimmunotherapy studies.
4.1 Abstract

Introduction: One mechanism of resistance to trastuzumab (Herceptin) in HER2-positive breast cancer (BC) is increased EGFR expression. We have developed $^{111}$In-labeled bispecific radioimmunoconjugates (bsRICs) that bind HER2 and EGFR on BC cells by linking trastuzumab Fab fragments through a polyethylene glycol (PEG$_{24}$) spacer to EGF. We hypothesized that tumours co-expressing HER2 and EGFR could be treated by dual-receptor targeted radioimmunotherapy with these bsRICs labeled with the $\beta$-particle-emitter, $^{177}$Lu or the Auger electron-emitter $^{111}$In. Methods: The binding of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF to tumour cells (MDA-MB-231, SK-OV-3, MDA-MB-231/H2N or TrR1) co-expressing HER2 and EGFR was assessed in competition assays. The clonogenic survival (CS) of these cells was measured after exposure to $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF or $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF or to monospecific $^{177}$Lu- or $^{111}$In-labeled trastuzumab Fab or EGF. The tumour and normal tissue biodistribution of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF was studied at 48 h post-injection in athymic mice bearing s.c. MDA-MB-231/H2N tumours. Radiation absorbed doses to tumours and normal tissues were estimated and compared for $^{111}$In and $^{177}$Lu-labeled bsRICs. The maximum injected amount of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF which caused no observable adverse effects (NOAEL) was identified in Balb/c mice. Athymic CD1 nu/nu mice bearing s.c. trastuzumab-sensitive MDA-MB-231/H2N or resistant TrR1 tumours were treated with $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF or $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF at the NOAEL, with unlabeled immunoconjugates or normal saline. Tumour growth was evaluated over a period of 49 days. Results: $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF bound specifically to HER2 and EGFR on tumour cells. Monospecific $^{177}$Lu- and $^{111}$In-labeled trastuzumab Fab or EGF killed tumour cells that predominantly expressed HER2 or EGFR, respectively, whereas bsRICs were cytotoxic to cells that displayed either HER2 or EGFR or both receptors. bsRICs were more effective than monospecific agents. $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF was more cytotoxic than $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF. The tumour uptake of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF was 2-fold greater than $^{177}$Lu-DOTA-trastuzumab Fab or $^{177}$Lu-DOTA-EGF. The NOAEL for $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF was 11.1 MBq (10 μg). Trastuzumab-sensitive MDA-MB-231/H2N and trastuzumab-resistant TrR1 tumours were growth-inhibited by $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF or $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF. Unlabeled immunoconjugates had no effect on tumour growth. $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF inhibited tumour growth more effectively than $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF due to a 9.3-fold higher
radiation absorbed dose (55.0 vs. 5.9 Gy, respectively). **Conclusion:** These results are encouraging for further development of these bsRICs for dual-receptor targeted radioimmunotherapy of BC co-expressing HER2 and EGFR, including trastuzumab-resistant tumours.

### 4.2 Introduction

Overexpression of the human epidermal growth factor receptor-2 (HER2) occurs in about 20-25% of breast cancers (BCs) and is the therapeutic target for trastuzumab (Herceptin; Roche Pharmaceuticals), pertuzumab (Perjeta®) and trastuzumab-emtansine (T-DM1; Kadcyla®). Although these HER2-targeted therapies combined with chemotherapy have improved the outcome for women with HER2-positive metastatic BC, not all patients respond and many patients with HER2-positive BC develop resistance within a year. Pertuzumab combined with trastuzumab and docetaxel has improved patient survival compared to trastuzumab and docetaxel alone. Trastuzumab combined with the HER2 tyrosine kinase inhibitor, lapatinib improved survival in patients with progressive HER2-positive BC. Recently, T-DM1 has been shown to be more effective than lapatinib combined with capecitabine for treatment of BC resistant to trastuzumab and taxanes. Despite these encouraging results, trastuzumab resistance remains a challenge. The reasons for tumour resistance are not completely understood but one mechanism is upregulation of other human epidermal growth factor receptor (EGFR) family members (e.g. EGFR and HER3). Our group has developed In-labeled bispecific radioimmunoconjugates (bsRICs) that recognize EGFR or HER3 expressed alone or co-expressed with HER2 on BC cells. These bsRICs may be useful for molecular imaging or radioimmunotherapy of trastuzumab-resistant tumours that co-express these receptors. These bsRICs were constructed by linking trastuzumab Fab fragments that bind HER2 through a 24-mer polyethylene glycol (PEG) spacer to human epidermal growth factor (EGF) or to heregulin-β (HRG), which are the natural ligands for EGFR and HER3, respectively. MicroSPECT/CT imaging demonstrated specific accumulation of these bsRICs in tumour xenografts in mice that displayed HER2 or EGFR or HER3 or HER2 co-expressed with EGFR or HER3.
Our hypothesis in the current study was that $^{177}$Lu- or $^{111}$In-labeled bsRICs that bind HER2 and EGFR would cause cytotoxicity \textit{in vitro} to BC cells expressing these receptors and tumour growth inhibition \textit{in vivo} in athymic mice bearing trastuzumab-sensitive or resistant BC xenografts co-expressing HER2 and EGFR. $^{177}$Lu ($t_{1/2} = 6.7$ d) emits moderate energy $\beta$-particles [$E_{\beta_{\text{max}}} = 0.50$ MeV (78.6%); 0.38 MeV (9.1%); 0.18 MeV (12.2%)] useful for radioimmunotherapy with a maximum range in tissues of 2 mm, as well as two low abundance $\gamma$-photons [$E_{\gamma} = 113$ keV (3%) and 210 keV (11%)] that can be exploited for single photon emission computed tomography (SPECT) imaging. $^{111}$In ($t_{1/2} = 2.8$ d) emits a cascade of 15 low energy (<25 keV) Auger electrons per decay, and two high abundance $\gamma$-photons [$E_{\gamma} = 171$ keV (90%) and $E_{\gamma} = 245$ keV (94%)] for SPECT imaging. Auger electrons have subcellular nanometer-micrometer range, but are lethally damaging to the DNA of cancer cells when emitted in close proximity to the cell nucleus.\textsuperscript{259} $^{111}$In-labeled trastuzumab modified with nuclear translocation sequence (NLS) peptides ($^{111}$In-NLS-trastuzumab) to promote its nuclear importation following internalization was highly effective for killing HER2-positive BC cells \textit{in vitro}\textsuperscript{260} and strongly inhibited tumour growth \textit{in vivo} in athymic mice bearing human HER2-overexpressing BC xenografts.\textsuperscript{209}

4.3 Materials and Methods

4.4 Cancer Cells

SK-OV-3 human ovarian cancer cells and MDA-MB-231 human breast cancer (BC) cells were purchased from the American Type Culture Collection (Manassas, VA). The MDA-MB-231/H2N cell line was derived from EGFR-positive MDA-MB-231 cells that were stably transfected to overexpress $c$-$erbB$-2 (HER2).\textsuperscript{244} TrR1 cells are a subclone of MDA-MB-231/H2N cells with acquired trastuzumab resistance but that continue to express HER2.\textsuperscript{244} Both MDA-MB-231/H2N and TrR1 cells were provided by Dr. Robert S. Kerbel (Sunnybrook Health Sciences Centre, Toronto, ON). SK-OV-3 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY). MDA-MB-231, MDA-MB-231/H2N and TrR1 cells were cultured in Dulbecco's Modified Eagle medium supplemented with 10% FBS. All cells were cultured in 5% CO$_2$ at 37°C. BC cells were selected based on their reported HER2 or EGFR expression\textsuperscript{235,258,261} but
these receptor densities were confirmed by direct (saturation) radioligand binding assays with $^{111}$In-labeled trastuzumab Fab fragments or $^{111}$In-labeled EGF. Since MDA-MB-231 cells exhibited low HER2 expression ($5.4 \times 10^4$ receptors/cell) and moderate EGFR density ($5.2 \times 10^5$ receptors/cell), these cells were designated as HER2$_{low}$/EGFR$_{mod}$. MDA-MB-231/H2N cells exhibited moderate HER2 and EGFR expression ($4.5 \times 10^5$ receptors/cell and $4.8 \times 10^5$ receptors/cell, respectively) and were designated as HER2$_{mod}$/EGFR$_{mod}$. TrR1 cells displayed $4.4 \times 10^5$ HER2/cell and $4.6 \times 10^5$ EGFR/cell and were designated as HER2$_{mod}$/EGFR$_{mod}$. SK-OV-3 cells exhibited high HER2 but low EGFR expression ($9.6 \times 10^5$ receptors/cell and $8 \times 10^4$ receptors/cell, respectively) and were designated as HER2$_{high}$/EGFR$_{low}$. The HER2 and EGFR density measured by radioligand binding assays in these cells were in agreement with the receptor expression assessed by Western blot.$^{244,246}$

4.4.1 Bispecific Radioimmunoconjugates (bsRICs)

Fab-PEG$_{24}$-EGF bispecific immunoconjugates (bsICs) recognizing HER2 and EGFR were constructed as reported by cross-linking trastuzumab Fab fragments (Mr = 50 kDa) produced by proteolytic digestion of trastuzumab IgG (Herceptin; Roche Pharmaceuticals, Mississauga, ON, Canada) to human EGF, Mr = 6.2 kDa, Peprotech, Rocky Hill, NJ) through a PEG$_{24}$ spacer.$^{258}$ Insertion of this PEG$_{24}$ spacer improved the binding of analogous $^{111}$In-diethylentriaminepentaacetic acid (DTPA)-trastuzumab Fab-heregulin (HRG) bispecific radioimmunoconjugates (bsRICs) to HER2 and HER3 on BC cells$^{243}$ and preserved the HER2 and EGFR binding of $^{111}$In-DTPA-trastuzumab-PEG$_{24}$-EGF bsRICs.$^{258}$ The bsICs were re-concentrated to 1.0 mg/mL in phosphate buffered saline (PBS, pH 7.0) on a Microcon centrifugal device (MWCO = 30 kDa; Millipore) then derivatized with a 10-fold mole excess of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono-N-hydroxysuccinimide ester (NHS-DOTA; Macroyclics Inc., Dallas, TX) for labeling with $^{177}$Lu. DOTA conjugation efficiency was measured by analysis of a sample of the unpurified reaction mixture trace-labeled with $^{177}$Lu by instant thin layer silica gel chromatography (ITLC-SG, Pall Life Sciences, Port Washington, NY) developed in 100 mM sodium citrate (pH 5.0). The conjugation efficiency was multiplied by the 10-fold mole excess of DOTA in the reaction to determine the number of DOTA chelators per molecule of bsICs. The bsICs were purified on a Sephadex G25 (Sigma-Aldrich) mini-column eluted with 400 mM ammonium acetate buffer, pH 5.0 to remove unconjugated DOTA.
Purified DOTA-Fab-PEG$_{24}$-EGF (200 µg; 100 µL) was labeled by incubation with 20 MBq (5 µL) of $^{177}$LuCl$_3$ (PerkinElmer, Waltham MA) for 90 mins at 42 °C. $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF bsRICs were synthesized as reported.$^{258}$ $^{111}$In-DTPA-trastuzumab Fab, $^{177}$Lu-DOTA-trastuzumab Fab, $^{111}$In-DTPA-EGF and $^{177}$Lu-DOTA-EGF were synthesized for comparison. There were 0.8 ± 0.3 DTPA or 0.7 ± 0.4 DOTA chelators per molecule of EGF, respectively. Fab fragments were derivatized with 2.3 ± 0.5 DOTA or 3.6 ± 0.7 DTPA per molecule. The final radiochemical purity of all radioimmunoconjugates (RICs) was >90% measured by ITLC.

The HER2 and EGFR binding of $^{177}$Lu-labeled bsRICs were evaluated by competition radioligand binding assays using SK-OV-3 (HER2$^{\text{high}}$/EGFR$^{\text{low}}$), MDA-MB-231 (HER2$^{\text{low}}$/EGFR$^{\text{mod}}$) or MDA-MB-231/H2N (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) cells. Approximately $1.5 \times 10^5$ cells were seeded into wells in 24-well plates and cultured overnight. The cells were then exposed to 10 nM (1.0 MBq/µg) of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF in phosphate-buffered saline (PBS) in the presence of 500 nM of unlabeled Fab, EGF or both competitors. After incubation for 3 h at 4°C, unbound radioactivity was removed and the cells were solubilised and transferred to γ-counting tubes and the cell bound radioactivity was measured in a γ-counter. The binding of $^{177}$Lu-DOTA-trastuzumab Fab or $^{177}$Lu-DOTA-EGF to MDA-MB-231 cells (HER2$^{\text{low}}$/EGFR$^{\text{mod}}$) or MDA-MB-231/H2N cells (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) was determined in the presence or absence of an excess of of trastuzumab Fab (69 nmols/L) or EGF (1,659 nmols/L). The HER2- and EGFR-binding properties of $^{111}$In-labeled bsRICs, $^{111}$In-DTPA-trastuzumab Fab or $^{111}$In-DTPA-EGF were previously reported.$^{258}$

4.4.2 Clonogenic Survival (CS) Assays

The clonogenic survival (CS) of SK-OV-3 (HER2$^{\text{high}}$/EGFR$^{\text{low}}$), MDA-MB-231 (HER2$^{\text{low}}$/EGFR$^{\text{mod}}$), MDA-MB-231/H2N (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) or trastuzumab-resistant TrR1 (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) cells exposed to $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF (350 ± 14.3 MBq/mg), $^{177}$Lu-DOTA-trastuzumab Fab (350 ± 9.4 MBq/mg) or $^{177}$Lu-DOTA-EGF (350 ± 13.5 MBq/mg) was determined. Approximately $1.5 \times 10^5$ cells were seeded into wells in 24-well plates and cultured overnight. The cells were then exposed for 24 h at 37°C to 500 µL of serum-free growth medium containing $2.5 \times 10^{-7}$ mols/L of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF, $^{177}$Lu-DOTA-trastuzumab Fab or $^{177}$Lu-DOTA-EGF. Controls consisted of cells exposed to growth medium
alone, or cells exposed to equivalent concentrations of unlabeled Fab-PEG$_{24}$-EGF, trastuzumab Fab or EGF. For comparison, the CS was evaluated in these cells exposed to the same concentrations of $^{111}$In-DTPA-trastuzumab Fab (350 ± 19.4 MBq/mg), $^{111}$In-DTPA-EGF (349 ± 15.2 MBq/mg) or $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF (350 ± 12.8 MBq/mg). After treatment, the cells were recovered by trypsinization, rinsed twice with medium and sufficient cells were seeded in triplicate into 6 well plates to obtain a measurable number of colonies after culturing for 5 days. Surviving colonies were stained with methylene blue and those with ≥30 cells were counted. The plating efficiency (PE) was determined by dividing the number of colonies formed by the number of cells seeded. The CS was calculated by dividing the PE for treated cells by that for untreated cells.

4.4.3 Biodistribution Studies

The tumour and normal tissue biodistribution of the $^{177}$Lu-DOTA-PEG$_{24}$-EGF bsRICs was compared to $^{177}$Lu-DOTA-trastuzumab Fab or $^{177}$Lu-DOTA-EGF in female athymic CD1 nu/nu mice (Charles River, Boston, MA) bearing s.c. MDA-MB-231/H2N (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) BC xenografts at 48 h post injection (p.i.). This time point was selected based on an earlier study which evaluated the biodistribution of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF from 4 to 72 h p.i. in mice with MDA-MB-231/H2N tumours and determined that maximum tumour uptake occurred at 48 h p.i. $^{258}$ Mice were inoculated s.c. in the thigh with $1 \times 10^7$ cells in 200 µL of a 1:1 mixture of Matrigel (BD Biosciences, Mississauga, ON) and serum free growth medium. After 4-6 weeks, groups of 3 tumour-bearing mice were injected i.v. (tail vein) with $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF, $^{177}$Lu-DOTA-trastuzumab Fab or $^{177}$Lu-DOTA-EGF (10 µg, 3-5 MBq per mouse) in 100 µL of normal saline. Mice were sacrificed and the tumour and samples of selected normal tissues including blood were collected, weighed and their radioactivity measured in a γ-counter. Tumour and normal tissue uptake were expressed as percent injected dose per gram (%ID/g). All animal studies were conducted under a protocol (No. 989.13) approved by the Animal Care Committee at the University Health Network in accordance with Canadian Council on Animal Care (CCAC) guidelines.
4.4.4 Radiation Dosimetry Estimates

The radiation absorbed doses to the tumour and normal tissues in CD1 athymic nu/nu mice with s.c. MDA-MB-231/H2N xenografts following injection of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF or $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF were estimated based on the reported biodistribution of $^{111}$In-DTPA-PEG$_{24}$-EGF in this same tumour xenograft mouse model, assuming that these two analogous bsRICs would exhibit comparable tumour and normal tissue uptake. The cumulative radioactivity in source organs was calculated and the absorbed doses estimated using OLINDA/EXT radiation dose assessment software, as described in the Supplementary Information.

4.4.5 Normal Tissue Toxicity Studies

Groups of 5 female non-tumour bearing Balb/c mice were injected i.v. (tail vein) with 3.7, 11.1 or 18.5 MBq of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF (10 µg, 100 µL). Control mice received injections of normal saline. Body weight was monitored every 2–4 d for 14 d. The mice were then sacrificed by cervical dislocation under anaesthesia and samples of blood collected into ethylenediaminetetraacetic acid (EDTA)–coated microtubes for biochemistry (serum alanine aminotransferase [ALT] and creatinine [Cr]) and hematology analyses. A complete blood cell (CBC) count as well as hematocrit and hemoglobin were measured on a HemaVet 950FS (Drew Scientific, Oxford, Connecticut) instrument.

4.4.6 Radioimmunotherapy Studies

The tumour growth–inhibitory properties of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF and $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF were compared in groups of 5 female athymic CD1 nu/nu mice implanted s.c. with trastuzumab-sensitive MDA-MB-231/H2N (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) or trastuzumab-resistant TrR1 (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) xenografts. Mice bearing 2–5 mm diameter tumours received a single intraperitoneal (i.p.) injection of 11.1 MBq (10 µg) of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF or $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF. Control mice received an i.p. injection of unlabeled DOTA-Fab-PEG$_{24}$-EGF (10 µg) or normal saline. We have previously found that $^{111}$In-labeled mAbs are rapidly absorbed after i.p. injection with an absorption half-life of 1.9 h that provides a bioavailability of
70% compared to i.v. injection. Others have similarly found equivalent blood concentrations at 24 h post i.p. or i.v. injection of radiolabeled antibodies. The tumour length and width were measured using calipers. The tumour volume was calculated as volume = (length \times width^2) \times 0.5, and the tumour growth index (TGI) was calculated by dividing the tumour volume at each time point by the initial tumour volume. The mean TGI was plotted vs. the time from the start of treatment to obtain the tumour growth curves. Treatment experiments were terminated when tumour size exceeded a mean diameter of 12 mm or at the planned end of the study (49 days).

### 4.4.7 Statistical Analysis

Statistical comparisons were made using a two-tailed Student’s t-test (p<0.05).

### 4.5 Results

#### 4.5.1 Bispecific Radioimmunoconjugates (bsRICs)

The synthesis and characterization of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF bsRICs were previously reported. $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF bsRICs were constructed by conjugating trastuzumab Fab fragments modified with NHS-PEG$_{24}$-maleimide to EGF functionalized with 2-iminothiolane (Traut’s reagent), then derivatizing these bsICs with NHS-DOTA for labeling with $^{177}$Lu (Figure 4-1). There were 3.2 ± 0.8 DOTA chelators substituted per molecule of bsICs. The binding of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF to SK-OV-3 cells (HER$_2^{\text{high}}$/EGFR$^{\text{low}}$) was reduced to 12.4 ± 2.6%, 105.0 ± 10.7% and 10.1 ± 1.6% by co-incubation with a 100-fold molar excess of trastuzumab Fab, EGF or both ligands, respectively compared to no competition (Figure 4-2A). The binding of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF to MDA-MB-231 cells (HER$_2^{\text{low}}$/EGFR$^{\text{mod}}$) was reduced to 95.2 ± 7.5%, 20.3 ± 2.8% and 9.9 ± 2.9% by trastuzumab Fab, EGF or both ligands, respectively (Figure 4-2B). The binding to MDA-MB-231/H2N cells (HER$_2^{\text{mod}}$/EGFR$^{\text{mod}}$) was reduced to 52.7 ± 2.8%, 65.6 ± 2.3% and 4.4 ± 0.9%, respectively by competition with trastuzumab Fab, EGF or both ligands (Figure 4-2C). An excess of unlabeled trastuzumab Fab inhibited the binding of $^{177}$Lu-DOTA-Fab to MDA-MB-231/H2N cells (HER$_2^{\text{mod}}$/EGFR$^{\text{mod}}$) but not to MDA-MB-231 cells (HER$_2^{\text{low}}$/EGFR$^{\text{mod}}$) while an excess of unlabeled EGF inhibited the binding of $^{177}$Lu-DOTA-EGF to both cell lines (Figure 4-3).
Figure 4-1. Synthesis of bispecific $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF radioimmunoconjugates.

Fab fragments of trastuzumab.

Fab fragments were produced by proteolytic digestion of the intact IgG were reacted with a 10-fold mole excess of NHS-PEG$_{24}$-maleimide for 1 hour at room temperature (RT). EGF was thiolated by reaction with a 10-fold mole excess of 2-iminothiolane (Traut’s reagent) for 1 hour at RT. Then a 2-fold mole excess of thiolated EGF was reacted with maleimide functionalized PEG$_{24}$-Fab for 1 hour at RT. Fab-PEG$_{24}$-EGF immunoconjugates were purified and reacted with a 2-fold mole excess of NHS-DOTA for 1 hour at RT. Following re-purification, DOTA-Fab-PEG$_{24}$-EGF was labeled with $^{177}$Lu by incubation with $^{177}$LuCl$_3$ in 1 M ammonium acetate buffer, pH 5.0 for 2 hours at 42°C.
Figure 4-2: Binding of $^{177}$Lu-DOTA-Fab-PEG24-EGF to MDA-MB-231, SKOV-3 or MDA-MB-231/H2N cells.

Binding of $^{177}$Lu-DOTA-Fab-PEG24-EGF to MDA-MB-231 (HER2$^{\text{low}}$/EGFR$^{\text{mod}}$), SKOV-3 (HER2$^{\text{high}}$/EGFR$^{\text{low}}$) or MDA-MB-231/H2N (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) cells in the absence or presence of competitors (EGF, trastuzumab Fab or a combination of EGF and trastuzumab Fab). Values shown represent the mean ± SD (n=3). Significant differences (P<0.05) are indicated by the asterisks.
Figure 4-3. Binding of bsRICs in the absence or presence of an excess of unlabeled trastuzumab Fab

Binding of (A) $^{177}$Lu-DOTA-trastuzumab Fab (1 nmol/L) or (B) $^{177}$Lu-DOTA-EGF (6.4 nmols/L) to MDA-MB-231 cells (HER2<sub>low</sub>/EGFR<sub>mod</sub>) and MDA-MB-231/H2N cells (HER2<sub>mod</sub>/EGFR<sub>mod</sub>) in the absence or presence of an excess of unlabeled trastuzumab Fab (69 nmols/L) or EGF (1,659 nmols/L). B/B₀: Binding in the presence of competitor divided by the binding in the absence of competitor. Values shown represent the mean ± SD (n=3).
4.5.2 Clonogenic Survival

There was no effect on the CS of MDA-MB-231 (HER2\textsuperscript{low}/EGFR\textsuperscript{mod}) cells exposed for 24 h to $2.5 \times 10^{-7}$ mols/L of unlabeled trastuzumab Fab, EGF or Fab-PEG\textsubscript{24}-EGF compared to untreated cells (Figure 4-4A). No significant decrease in survival was found for MDA-MB-231 cells treated with $^{111}$In-DTPA-trastuzumab Fab (CS = 94.5 ± 10.1%; $P$<0.01) compared to untreated cells. The survival of MDA-MB-231 cells treated with $^{111}$In-DTPA-Fab-PEG\textsubscript{24}-EGF was decreased by 1.4-fold compared to untreated cells but this difference was not significant (CS = 70.1 ± 15.7% vs. 100.0 ± 19.7%; $P$=0.09). The survival of MDA-MB-231 cells was significantly decreased by 2.6-3.3 fold by $^{177}$Lu-DOTA-EGF or $^{177}$Lu-DOTA-Fab-PEG\textsubscript{24}-EGF (CS = 38.4 ± 9.0%; $P$<0.0001 and 30.2 ± 4.8%; $p$<0.0001, respectively; Figure 4-4A). There was no significant effect of $^{177}$Lu-DOTA-trastuzumab Fab on the clonogenic survival of MDA-MB-231 cells (CS = 77.3 ± 19.2%; $P$=0.06).

The survival of SK-OV-3 (HER2\textsuperscript{high}/EGFR\textsuperscript{low}) cells exposed to unlabeled trastuzumab Fab or Fab-PEG\textsubscript{24}-EGF was significantly decreased compared to untreated cells (71.9 ± 9.0%; $p=0.02$ and 71.8 ± 2.2%; $P=0.01$, respectively vs. 100.0 ± 10.5%; Figure 4-4B). Unlabeled EGF had no effect on the CS of these cells (CS = 94.4 ± 4.5%). The survival of SK-OV-3 cells treated with $^{111}$In-DTPA-trastuzumab Fab or $^{111}$In-DTPA-Fab-PEG\textsubscript{24}-EGF was decreased compared to untreated cells (CS = 65.9 ± 7.0%; $P=0.01$ and 59.8 ± 4.3%; $p=0.003$, respectively). The survival of SK-OV-3 cells treated with $^{111}$In-DTPA-EGF was significantly decreased (CS = 75.5 ± 7.5%; $P=0.02$) compared to untreated cells. $^{177}$Lu-DOTA-trastuzumab Fab and $^{177}$Lu-DOTA-Fab-PEG\textsubscript{24}-EGF killed SK-OV-3 cells more efficiently than the corresponding $^{111}$In-labeled agents (CS = 35.5 ± 7.5% and 19.8 ± 4.2%, respectively; $P$<0.001). $^{177}$Lu-DOTA-EGF significantly reduced the CS of SK-OV-3 cells to 65.5 ± 7.5% ($P$<0.01).
Figure 4-4. Clonogenic Survival following exposure to $^{177}$Lu- or $^{111}$In-labeled bsRICs.

Clonogenic survival (CS) of (A) MDA-MB-231 (HER2$^{\text{low}}$/EGFR$^{\text{mod}}$), (B) SKOV-3 (HER2$^{\text{high}}$/EGFR$^{\text{low}}$), (C) MDA-MB-231/H2N (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) or (D) TrR1 (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) cells following exposure to $^{177}$Lu- or $^{111}$In-labeled trastuzumab Fab, EGF, Fab-PEG$_{24}$-EGF, unlabeled agents or no treatment. Values shown represent the mean ± SD (n=3). Significant differences ($P<0.05$) are indicated by the asterisks.
The survival of MDA-MB-231/H2N cells (HER2\textsuperscript{mod}/EGFR\textsuperscript{mod}) was significantly reduced to 71.6 ± 5.6% by treatment with unlabeled trastuzumab Fab for 24 h ($P$=0.01; Figure 4-4C) but not by unlabeled EGF (79.1 ± 8.5%; $P$=0.054) or Fab-PEG\textsubscript{24}-EGF (86.4 ± 8.0%; $P$=0.14). The survival of MDA-MB-231/H2N cells was significantly decreased by 2.3-3.2 fold to 31.6 ± 3.4% ($P$<0.001), 43.4 ± 5.9% ($P$=0.001) and 43.8 ± 0.03% ($P$<0.001) by exposure to \textsuperscript{111}In-DTPA-trastuzumab Fab, \textsuperscript{111}In-DTPA-EGF or \textsuperscript{111}In-DTPA-Fab-PEG\textsubscript{24}-EGF, respectively. The CS of MDA-MB-231/H2N cells was reduced to 37.6 ± 7.9%, 25.6 ± 17.6% and 12.2 ± 10.6% by \textsuperscript{177}Lu-DOTA-trastuzumab Fab, \textsuperscript{177}Lu-DOTA-EGF, or \textsuperscript{177}Lu-DOTA-Fab-PEG\textsubscript{24}-EGF, respectively. For MDA-MB-231/H2N cells, \textsuperscript{177}Lu-DOTA-trastuzumab Fab, \textsuperscript{177}Lu-DOTA-EGF or \textsuperscript{177}Lu-DOTA-Fab-PEG\textsubscript{24}-EGF were not more cytotoxic than the corresponding \textsuperscript{111}In-labeled agents.

There was no effect of unlabeled trastuzumab Fab, EGF or Fab-PEG\textsubscript{24}-EGF on the CS of TrR1 (HER2\textsuperscript{mod}/EGFR\textsuperscript{mod}) cells (Figure 4-4D). TrR1 cells treated with \textsuperscript{111}In-DTPA-trastuzumab Fab and \textsuperscript{111}In-DTPA-EGF also showed no significant decrease in survival (CS = 92.6 ± 17.6%; $P$=0.6 and 88.6 ± 0.6%; $P$=0.09, respectively). However, exposure to \textsuperscript{111}In-DTPA-Fab-PEG\textsubscript{24}-EGF decreased the CS of these cells to 67.4 ± 11.9% ($P$=0.01). Treatment of TrR1 cells with \textsuperscript{177}Lu-DOTA-trastuzumab Fab, \textsuperscript{177}Lu-DOTA-EGF, or \textsuperscript{177}Lu-DOTA-Fab-PEG\textsubscript{24}-EGF significantly reduced their CS to 51.7 ± 9.8% ($P$=0.003), 41.5 ± 8.3% ($P$=0.001) and 20.3 ± 5.6% ($P$<0.001), respectively. \textsuperscript{177}Lu-DOTA-trastuzumab Fab, \textsuperscript{177}Lu-DOTA-EGF and \textsuperscript{177}Lu-DOTA-Fab-PEG\textsubscript{24}-EGF were more cytotoxic than the corresponding \textsuperscript{111}In-labeled agents.

### 4.5.3 Biodistribution Studies

Tumour uptake at 48 h p.i. in mice bearing s.c. MDA-MB-231/H2N (HER2\textsuperscript{mod}/EGFR\textsuperscript{mod}) BC xenografts was 2.3-fold significantly greater for \textsuperscript{177}Lu-DOTA-Fab-PEG\textsubscript{24}-EGF (7.3 ± 1.5 %ID/g) than \textsuperscript{177}Lu-DOTA-trastuzumab Fab (3.2 ± 1.5 %ID/g; $P$=0.02) and 3.5-fold significantly higher than \textsuperscript{177}Lu-DOTA-EGF (2.1 ± 0.7 %ID/g; $P$<0.01; Figure 4-5). The blood concentration of radioactivity for \textsuperscript{177}Lu-DOTA-Fab-PEG\textsubscript{24}-EGF (1.2 ± 0.1 %ID/g) was 4-fold significantly higher than \textsuperscript{177}Lu-DOTA-EGF (0.3 ± 0.3 %ID/g; $P$<0.01) but not greater than \textsuperscript{177}Lu-DOTA-trastuzumab Fab (1.0 ± 0.1 %ID/g; $P$=0.07). Similar results were found for the heart. Spleen uptake of \textsuperscript{177}Lu-DOTA-Fab-PEG\textsubscript{24}-EGF (4.0 ± 1.8 %ID/g) was 2.8-fold significantly greater than \textsuperscript{177}Lu-DOTA-trastuzumab Fab (1.4 ± 0.1 %ID/g; $P$=0.01) and 2.2-fold higher than \textsuperscript{177}Lu-DOTA-EGF (1.8 ±
Liver uptake was 2.6-fold significantly lower for $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF (3.4 ± 1.2 %ID/g) than $^{177}$Lu-DOTA-EGF (8.9 ± 1.4 %ID/g; $P<0.01$) but was not different than $^{177}$Lu-DOTA-trastuzumab Fab (4.5 ± 1.4 %ID/g; $P=0.4$). There was lower intestinal uptake for $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF compared to $^{177}$Lu-DOTA-EGF, but not compared to $^{177}$Lu-DOTA-trastuzumab Fab.

### 4.5.4 Radiation Dosimetry Estimates

A comparison of radiation absorbed doses estimated for the tumour and normal organs in CD1 athymic mice with s.c. MDA-MB-231/H2N xenografts and injected with 11.1 (10 μg) of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF or $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF is shown in Table 4-1. The doses to normal organs were 1.5 to 8.2-fold higher for $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF than $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF. $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF deposited a 9.3-fold higher radiation dose in the tumour compared to $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF.
Figure 4-5. Tumour and normal tissue biodistribution of bsRICs.

$^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF, $^{177}$Lu-DOTA-trastuzumab Fab and $^{177}$Lu-DOTA-EGF at 48 h post i.v. injection in athymic mice bearing s.c. MDA-MB-231/H2N (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) BC xenografts. Values shown represent the mean ± SD (n=3). Significant differences ($P<0.05$) are indicated by the asterisks.
Table 4-1. Estimated Radiation Absorbed Doses in CD1 Athymic Mice with MDA-MB-231/H2N Human Breast Cancer Xenografts Injected with $^{111}$In or $^{177}$Lu-Labeled Bispecific Radioimmunoconjugates (bsRICs)

<table>
<thead>
<tr>
<th>Organ</th>
<th>$^{111}$In-DTPA-Fab-PEG$_{24}$-EGF</th>
<th>$^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>0.34 ± 0.06</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.29 ± 0.07</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Liver</td>
<td>0.92 ± 0.08</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td>Kidneys</td>
<td>1.8 ± 0.2</td>
<td>14.8 ± 1.7</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.79 ± 0.15</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.21 ± 0.03</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.24 ± 0.06</td>
<td>0.88 ± 0.35</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>0.395 ± 0.07</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>Large Intestine</td>
<td>0.30 ± 0.04</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Tumour</td>
<td>5.9 ± 2.1</td>
<td>55.0 ± 18.0</td>
</tr>
<tr>
<td>Total Body</td>
<td>0.043 ± 0.006</td>
<td>0.059 ± 0.008</td>
</tr>
</tbody>
</table>

CD1 athymic mice injected with 11.1 MBq (10 µg) of bsRICs assuming equivalent biodistribution as reported for $^{111}$In-DTPA-Fab-PEG24-EGF. Data shown are mean ± SD.
4.5.5 Normal Tissue Toxicity Studies

A dose-escalation acute toxicity study was performed to select the dose of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF for radioimmunotherapy studies. There was no significant change in body weight over 14 d for non-tumour bearing Balb/c mice injected with 3.7 to 18.5 MBq (10 µg each) of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF compared to normal saline treated mice, but there was a trend towards decreased body weight for mice receiving 18.5 MBq (Figure 4-6). There were significantly reduced leukocyte (WBC) counts in mice receiving 18.5 MBq of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF compared to saline treated mice ($P=0.015$; Figure 4-7A) but not for lower doses. Platelet (PLT) counts were not significantly decreased in mice receiving $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF compared to normal saline mice (Figure 4-7B). Erythrocyte (RBC) counts, hemoglobin (Hb) and hematocrit (Hct) were significantly lower in mice receiving 18.5 MBq of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF ($P=0.010$, $P=0.05$ and $P=0.013$, respectively; Figure 4-7C-E) than normal saline treated mice, but not at lower doses. There was no significant effect on serum alanine aminotransferase (ALT) or creatinine (Cr) at any dose of bsRICs (Figure 4-7F-G) but there was a trend towards higher Cr with increasing dose of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF.

Based on the overall normal tissue toxicity profile, 11.1 MBq (10 µg) was defined as the “no observable adverse effect level” (NOAEL) and was selected for radioimmunotherapy studies.
Figure 4-6. Body weight normalized to the initial body weight at different times post-injection of bsRICs

Body weight normalized to the initial body weight at different times post-injection of 3.7, 11.1 or 18.5 MBq (10 μg) of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF or for untreated mice. Values shown represent the mean ± SD (n=5). There were no significant differences ($P<0.05$) between the groups.
Figure 4-7. Blood cell counts, serum alanine aminotransferase (ALT) and creatinine (SCr) levels in non-tumour bearing Balb/c mice at 14 d post-injection of bsRCs.

Blood cell counts (WBC: leukocytes; PLT: platelets; RBC: erythrocytes), hemoglobin (Hb), hematocrit (HCT) Serum alanine aminotransferase (ALT) and creatinine (SCr) levels in non-tumour bearing Balb/c mice at 14 d post-injection of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF (3.7 to 11.1 MBq; 10 µg) or for untreated mice. Values shown are those for individual mice as well as the mean ± SD (n=5). Significant differences ($P<0.05$) are indicated by the asterisks.
4.5.6 Radioimmunotherapy Studies

Radioimmunotherapy studies in mice with MDA-MB-231/H2N (HER2\textsuperscript{mod}/EGFR\textsuperscript{mod}) BC xenografts demonstrated strong tumour growth inhibition after a single injection of 11.1 MBq (10 µg) of $^{177}$Lu-DOTA-Fab-PEG\textsubscript{24}-EGF (Figure 4-8A). The TGI at 49 d for $^{177}$Lu-DOTA-Fab-PEG\textsubscript{24}-EGF (2.3 ± 0.7) was 2.3-2.6-fold significantly lower than for mice treated with normal saline (6.2 ± 1.4; $P<0.001$) or unlabeled DOTA-Fab-PEG\textsubscript{24}-EGF (5.7 ± 1.2; $P<0.001$). $^{177}$Lu-DOTA-Fab-PEG\textsubscript{24}-EGF treatment was more effective than $^{111}$In-DTPA-Fab-PEG\textsubscript{24}-EGF (TGI at 49 d = 3.6 ± 1.0; $P=0.047$). Unlabeled DOTA-Fab-PEG\textsubscript{24}-EGF had no significant effect on the growth of MDA-MB-231/H2N tumours. $^{177}$Lu-DOTA-Fab-PEG\textsubscript{24}-EGF treatment moderately inhibited the growth of trastuzumab-resistant TrR1 (HER2\textsuperscript{mod}/EGFR\textsuperscript{mod}) BC xenografts in mice (Figure 4-8B). The TGI at 49 d post-treatment with $^{177}$Lu-DOTA-Fab-PEG\textsubscript{24}-EGF (3.5 ± 0.9) was 1.6-fold significantly lower than treatment with normal saline (5.6 ± 0.8; $P<0.001$) or unlabeled DOTA-Fab-PEG\textsubscript{24}-EGF (5.5 ± 0.9; $P<0.01$). $^{177}$Lu-DOTA-Fab-PEG\textsubscript{24}-EGF was 1.3-fold significantly more effective at inhibiting the growth of TrR1 tumours than $^{111}$In-DTPA-Fab-PEG\textsubscript{24}-EGF (TGI = 4.6 ± 0.5; $P=0.042$). Unlabeled DOTA-Fab-PEG\textsubscript{24}-EGF was not effective for inhibiting the growth of TrR1 tumours.
Figure 4-8. Tumour growth index (TGI) for athymic mice treated with bsRICs.

Mice bearing (A) trastuzumab-sensitive MDA-MB-231/H2N (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) tumour xenografts or (B) TrR1 (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) trastuzumab-resistant tumour xenografts were treated with unlabeled DOTA-Fab-PEG$_{24}$-EGF, $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF, $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF or received no treatment. Values shown represent the mean ± SD (n=5).
4.6 Discussion

We previously reported that $^{111}\text{In-DTPA-Fab-PEG}_{24}\text{-EGF}$ bsRICs imaged s.c. tumour xenografts in athymic mice that expressed EGFR or HER2 or both receptors. $^{258}$ We now extend these findings to radioimmunotherapy by complexing these bsRICs to the β-particle emitter, $^{177}\text{Lu}$ or by exploiting the Auger electron emissions of $^{111}\text{In}$. $^{177}\text{Lu-DOTA-Fab-PEG}_{24}\text{-EGF}$ bsRICs were constructed by linking trastuzumab Fab fragments to EGF, then derivatizing the bsICs with 3.2 ± 0.8 DOTA for labeling with $^{177}\text{Lu}$ (Figure 4-1). DOTA is likely substituted predominantly onto the Fab domain since trastuzumab Fab presents 25 ε-amino groups on lysines for reaction with NHS-DOTA, $^{265}$ whereas EGF contains only two lysines and one N-terminal amine for DOTA modification or thiolation with Traut’s reagent. $^{266}$ We previously reported that $^{111}\text{In-DOTA}$-trastuzumab Fab exhibited preserved HER2 binding affinity$^{178}$ and $^{68}\text{Ga-DOTA-EGF}$ was reported to bind with high affinity to EGFR. $^{267}$ The binding of $^{177}\text{Lu-DOTA}$-trastuzumab Fab or $^{177}\text{Lu-DOTA-EGF}$ to MDA-MB-231 cells (HER2$^{\text{low}}$/EGFR$^{\text{mod}}$) and MDA-MB-231/H2N cells (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) was inhibited by excess trastuzumab Fab or EGF in agreement with the receptor expression of these cells (Figure 4-3). Bivalent affibody molecules binding HER2 and EGFR have also been constructed. $^{250}$ However, these were not radiolabeled and not studied for tumour imaging or radioimmunotherapy. The $K_D$ for binding of these bispecific affibodies to EGFR or HER2 was 35-86 nM and 6-219 nM, respectively. In competition receptor binding assays with EGF or trastuzumab Fab, we previously measured an effective concentration-50% ($EC_{50}$ value) for displacement of the binding of $^{111}\text{In-DTPA-Fab-PEG}_{24}\text{-EGF}$ to EGFR of 25-36 nM and 18-24 nM for HER2. $^{258}$ These $EC_{50}$ values approximate the $K_D$. The specificity of binding of $^{177}\text{Lu-DOTA-Fab-PEG}_{24}\text{-EGF}$ to EGFR and HER2 was confirmed by competition with unlabeled EGF or trastuzumab Fab using MDA-MB-231 (HER2$^{\text{low}}$/EGFR$^{\text{mod}}$), SK-OV-3 (HER2$^{\text{high}}$/EGFR$^{\text{low}}$), MDA-MB-231/H2N (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) or TrR1 (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) cells (Figure 4-2). The inability of EGF to compete the binding of $^{177}\text{Lu-DOTA-Fab-PEG}_{24}\text{-EGF}$ to SK-OV-3 cells (HER2$^{\text{high}}$/EGFR$^{\text{low}}$) may be due to predominant HER2 binding on these cells due to the 12-fold greater density of HER2 than EGFR ($9.6 \times 10^5$ vs. $8 \times 10^4$ receptors/cell, respectively).

Exposure to $^{177}\text{Lu-DOTA-Fab-PEG}_{24}\text{-EGF}$ killed all four cell types: MDA-MB-231 (HER2$^{\text{low}}$/EGFR$^{\text{mod}}$), SK-OV-3 (HER2$^{\text{high}}$/EGFR$^{\text{low}}$), MDA-MB-231/H2N (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) and TrR1 (HER2$^{\text{mod}}$/EGFR$^{\text{mod}}$) (Figure 4-4). Monospecific $^{177}\text{Lu-DOTA-EGF}$ killed MDA-MB-
231 cells but was less effective for killing SK-OV-3 cells. Conversely, $^{177}$Lu-DOTA-trastuzumab Fab was more effective for killing SK-OV-3 cells than MDA-MB-231 cells. $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF bsRICs were less cytotoxic than $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF (Figure 4-4). Although HER2 contains a putative NLS, the receptor is slowly internalized which may limit the intracellular accumulation of the bsRICs. Moreover, the bsRICs were not modified with exogenous NLS peptides to promote more efficient nuclear importation following HER2-mediated internalization. Nuclear importation amplifies the DNA damage caused by the Auger electrons emitted by $^{111}$In. The longer range (2 mm) $\beta$-particles emitted by $^{177}$Lu do not require internalization or nuclear importation of the bsRICs for cytotoxicity. The bsRICs were more versatile than the monospecific agents since they were able to kill tumour cells displaying HER2 or EGFR or both receptors. This ability to target and kill tumour cells that are HER2- or EGFR-positive or which co-express these two receptors may overcome intratumoural HER2 heterogeneity. Intratumoural heterogeneity in HER2 expression has been found in 18% of HER2-positive BC, with HER2-amplified and HER2 non-amplified regions detected in the same specimen. HER2-negative cells may express EGFR. The bsRICs killed trastuzumab-resistant TrR1 cells that display both EGFR and HER2, whereas exposure to unlabeled Fab-PEG$_{24}$-EGF or trastuzumab Fab had no effect on the survival of these cells (Figure 4-4). These results confirm our previous report that Auger electron-emitting $^{111}$In-NLS-trastuzumab was cytotoxic to TrR1 cells, despite their resistance to trastuzumab.

Biodistribution studies at 48 h p.i. in CD1 athymic mice with s.c. MDA-MB-231/H2N xenografts (Figure 4-5) revealed 2-fold significantly greater tumour uptake of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF than $^{177}$Lu-DOTA-trastuzumab Fab or $^{177}$Lu-DOTA-EGF. These results indicate that cross-linking trastuzumab Fab through a PEG$_{24}$ spacer to EGF improved tumour localization compared to the monospecific agents. Tumour uptake of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF at 48 h p.i. (7.3 ± 3.5 %ID/g) was identical to $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF at this time point, indicating that substitution of DOTA did not affect tumour uptake of the bsRICs. A dose-escalation study was conducted in Balb/c mice to select a dose of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF for radioimmunotherapy studies. An administered dose of 11.1 MBq (10 $\mu$g) caused no significant decrease in body weight (Figure 4-6) or blood cell counts, Hb or Hct (Figure 4-7) and no increase in serum ALT or creatinine (Cr) over 14 d (Figure 4-7) and was therefore defined as the NOAEL. Interestingly, the hematological toxicity of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF was much
lower than for comparable amounts of $^{177}$Lu-DTPA-trastuzumab administered to mice, which may be due to its more rapid elimination from the blood (1.2 ± 0.1 %ID/g vs. 13.7 ± 0.8 %ID/g, respectively).

Radioimmunotherapy studies using a single NOAEL dose of 11.1 MBq (10 μg) of $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF in mice engrafted s.c. with trastuzumab-sensitive MDA-MB-231/H2N BC xenografts yielded strong tumour growth-inhibition (Figure 4-8) compared to mice treated with normal saline or unlabeled DOTA-Fab-PEG$_{24}$-EGF. In agreement with the results of in vitro cytotoxicity results, $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF was more effective for inhibiting tumour growth than $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF. Radiation dosimetry estimates (Table 4-1) revealed that $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF delivered a 9-fold higher dose to the tumour than $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF, which explains its greater potency. However, normal organ absorbed doses from $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF were 1.5- to 8-fold lower than $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF, which suggests that the administered amount of $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF could be increased to compensate for a lower potency. Radioimmunotherapeutic agents labeled with Auger electron-emitters have been found to be more effective for treating tumours in mice than the same agents labeled with β-emitters when administered at equitoxic doses. Trastuzumab-resistant TrR1 tumours also responded to treatment with the bsRICs, but were less sensitive than MDA-MB-231/H2N BC xenografts. $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF was the most effective for treating these tumours. $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF was not modified with NLS peptides to promote efficient nuclear translocation after HER2-mediated internalization and HER2 is slowly internalized, which may have limited the effectiveness of the subcellular range Auger electrons released by $^{111}$In for radioimmunotherapy. The resistance of TrR1 tumours to treatment with $^{111}$In- or $^{177}$Lu-labeled bsRICs compared to MDA-MB-231/H2N tumours may be due to their 3-fold higher expression of insulin growth factor-1 receptors (IGF-1R). Increased IGF-1R has been associated with radiation resistance. The resistance of TrR1 tumours could also be due to lower tumour accumulation of the bsRICs since this was not measured in biodistribution studies.

4.7 Conclusion

We conclude that $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF and $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF were effective for killing tumour cells in vitro that displayed HER2 or EGFR or both receptors and a
single dose of the bsRICs at the NOAEL yielded moderate to strong tumour growth inhibition  in vivo in mice bearing s.c. trastuzumab-sensitive or resistant human BC xenografts. $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF was more effective for inhibiting tumour growth than $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF, due to a 9-fold higher radiation absorbed dose in tumours. The lower normal organ absorbed doses deposited by $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF suggest that the administered amount of these bsRICs could be increased to compensate for a lower potency. These results are encouraging for further development of these bsRICs for dual-receptor targeted radioimmunotherapy of BC that co-expresses HER2 and EGFR, including trastuzumab-resistant tumours.
CHAPTER 5

Overall Summary and Conclusions
5.1 Summary of Research

In this thesis, the concept of targeting multiple receptors of the HER family with $^{111}$In and $^{177}$Lu labeled bsRICs was explored for molecular imaging and RIT of HER2 overexpressing breast cancer. The overall conclusions of the research described are:

I. $^{111}$In-labeled bsRICs composed of trastuzumab Fab fragments conjugated to the EGF-like domain of HRG through a PEG spacer of appropriate length bound specifically to both HER2 and HER3. In athymic mice bearing tumour xenografts expressing HER2 or HER3 or both receptors, bsRICs showed good tumour uptake and low normal tissue accumulation, with the exception of the kidneys. The optimal time point for microSPECT/CT imaging was 48 h p.i. Blocking studies in mice with tumours expressing only HER2 or HER3 demonstrated the specificity for both receptors in vivo.

II. $^{111}$In-labeled bsRICs composed of trastuzumab Fab fragments conjugated to EGF linked with a 24-mer PEG linker bound specifically to both EGFR and HER2, and bound in higher amounts to cells that expressed both HER2 and EGFR, as compared to cells that only expressed one of these receptors. SPECT/CT imaging of athymic mice bearing tumour xenografts showed that bsRICs improved the pharmacokinetic properties over the monospecific ligands, yielding comparable tumour uptake but diminished normal tissue accumulation.

III. $^{111}$In and $^{177}$Lu bsRICs were effective for killing tumour cells in vitro that displayed HER2 or EGFR or both receptors and a single dose of the bsRICs at the NOAEL yielded moderate to strong tumour growth inhibition in vivo in mice bearing s.c. trastuzumab-sensitive or resistant human BC xenografts. $^{177}$Lu-DOTA-Fab-PEG$_{24}$-EGF was more effective for inhibiting tumour growth than $^{111}$In-DTPA-Fab-PEG$_{24}$-EGF, due to a 9-fold higher radiation absorbed dose in tumours.
5.2 Discussion

5.2.1 The clinical role of imaging HER receptor status in breast cancer

HER2 overexpression occurs in approximately 20% of breast cancer cases, and HER2 is the target of multiple therapies. Because of the low incidence of HER2 positivity in the overall population of individuals with BC, nuclear medicine imaging of HER2 expression is not suitable for screening for BC. Currently mammography remains the imaging modality of choice for screening, with MRI is also used in high risk women. However, with the growing number of therapies that target HER2, there is value in using nuclear medicine imaging after diagnosis to better understand HER2 expression in lesions throughout the body and the particular phenotypic fingerprint of the disease in an individual patient. Currently, FISH and IHC are used to analyze HER2 gene amplification or protein expression, respectively, and are the basis for determining HER2 positivity in BC. While the accuracy of these methods is comparable, they have significant limitations, such as sampling errors due to tumour heterogeneity and discordance between primary tumours and metastases. As such, the ASCO and CAP expert panel reported that approximately 20% of current HER2 test results may be inaccurate. Nuclear medicine imaging can play an important role in addition to IHC and FISH in selecting patients for treatment with HER2 targeted therapies by visualizing HER2 expression in both the primary lesion and metastases, and doing so non-invasively without the need for tumour biopsy. This technique has already been shown to be able to detect early changes in HER2 expression levels after trastuzumab treatment, even before changes in tumour uptake of other probes such as \(^{18}\)F-FDG were visible.

However, HER2 is only one member of a larger network of cell surface signaling receptors that is responsible for aberrant progression and growth in BC. Recently, other members of the HER family, such as HER3 and EGFR, have been found to play an important role in oncogenic signaling, particularly in progression and treatment response to HER2 targeted therapies. EGFR and HER3 expression have both been shown to be correlated with significantly lower disease free survival. Furthermore, it has been shown that in HER2 overexpressing BC cells, loss of HER3 function has similar effects to loss of HER2 function on cell proliferation. Currently,
HER2 is the only approved biomarker for HER2-targeted therapy, and the only one routinely tested for in a clinical setting. However, biomarker analysis of patients enrolled in the CLEOPATRA study showed that low levels of ErbB3 (HER3) mRNA in patients treated with trastuzumab, pertuzumab and docetaxel were significantly associated with better prognosis. Furthermore, increased HER3 protein expression was significantly associated with poor prognosis in metastatic BC patients treated with trastuzumab. As described in Chapter 2, bsRICs targeted against HER2 and HER3 have been shown to bind to both receptors specifically and report on their expression using SPECT/CT imaging in preclinical BC xenograft mouse models, with good uptake in tumour tissues and low normal tissue accumulation. If translated into a clinical setting, these agents could be valuable tools in reporting on the status of multiple HER family receptors in a non-invasive manner. Prior to onset of treatment such images could provide clinicians vital information about the particular phenotype of a patient, helping guide treatment choices that are likely to be most efficacious for them. Furthermore, imaging could be performed during the treatment regimen to determine changes in receptor expression and potential response or resistance to therapy, allowing for more timely adjustments to the treatment regimen. Imaging with bsRICs will also prove useful in determining patient eligibility for HER3 targeted therapies, several of which are currently in Phase I clinical studies.

EGFR is another heterodimerization partner of HER2 that has been shown to play a key role in BC, as well as a number of other cancers. EGFR upregulation is also implicated as a potential mechanism for resistance to HER2 targeted therapies (see Section 1.4.1). In Chapter 3 the strategy for synthesizing bsRICs targeting two receptors was extended to create probes capable of binding both HER2 and EGFR. These bsRICs demonstrated more attractive properties for tumour imaging than either monospecific agent, with similar tumour uptake but lower normal tissue accumulation. These probes would be useful for reporting on receptor expression, not only in BC, but also in other cancers where EGFR is expressed such as colorectal cancer (CRC). Recently, HER2 gene amplification was identified in a minority of CRC patients, and further research is being done to determine the clinical implications of this finding. Furthermore, two antibodies that target EGFR are approved as therapy for CRC: cetuximab (Erbitux, Merck) and panitumumab ( Vectibix, Amgen), and bsRICs targeting HER2 and EGFR could provide information on which patients are best suited to these treatments. Trials are also currently ongoing to investigate the efficacy of these mAbs in other cancers, including BC.
5.2.2 Radioimmunotherapy of Breast Cancer in the Clinic

The idea of targeted therapy was first proposed in the early part of the 20th century, when Paul Ehrlich predicted that chemists would be able to produce “magic bullets” that would seek out specific disease causing agents, and deliver them specifically to affected areas of the body. This idea was first tested using a radionuclide conjugated to an antibody in the early 1950s, and has continued to be explored since. While the current state of RIT has shown clinical success in targeting CD20 with $^{90}\text{Y}$-ibritumomab tiuxetan (Zevalin) and $^{131}\text{I}$-tositumomab (Bexxar) for treating NHL, such success has yet to be achieved for solid tumours. The primary challenge so far has been achieving sufficiently high radiation absorbed doses to tumours, since only a small portion of the administered radiolabeled antibody localizes in tumour tissues. The most commonly used radionuclides in RIT to date are moderate-high energy β-particle emitters. Since β-particles have a relatively long path length in tissue (2-12 mm), these radionuclides possess the advantage of being able to deliver “crossfire” doses of radiation to neighboring tumour cells, addressing issues such as antigen heterogeneity and poor tumour penetration of large antibody molecules. However, hematopoietic toxicities often become dose-limiting before sufficiently high tumour doses can be reached. This is because circulating radiolabeled antibodies in the blood also irradiate and destroy surrounding normal stem cells in the bone marrow through the cross-fire effect. Auger electron-emitting radioisotopes such as $^{111}\text{In}$ have been explored as an alternative to β-particle emitters for targeted radiotherapy of cancer, as these short-ranged electrons may be better suited to the treatment of microscopic or small-volume disease if they can be selectively directed and internalized into the target cells. In Chapter 4 we compared the efficacy of $^{111}\text{In}$ vs. $^{177}\text{Lu}$ labeled bsRICs for cell killing and tumour growth inhibition, and found that at the NOAEL dose of 11.1 MBq, $^{177}\text{Lu}$-bsRICs were more effective at inhibiting tumour growth than $^{111}\text{In}$-bsRICs. Radiation dosimetry estimates revealed that $^{177}\text{Lu}$-bsRICs delivered a 9 fold higher dose to the tumour than $^{111}\text{In}$-bsRICs, explaining the increased potency. However, normal organ absorbed doses from $^{111}\text{In}$-bsRICs were 1.5- to 8-fold lower than $^{177}\text{Lu}$-bsRICs, which suggests that administered amount of $^{111}\text{In}$-bsRICs could be increased to compensate for a lower potency. RIT agents labeled with Auger electron-emitters have been found to be more effective for treating tumours in mice than the same agents labeled with β-emitters when administered at equitoxic doses. Costantini et. al have shown that using a
nuclear localizing sequence (NLS) peptide conjugated to an antibody labeled to an Auger emitting radionuclide improves internalization of the nuclide into the cell with routing to the radiation-sensitive cell nucleus, which improves cell killing, however without these sequences the localization of the radiolabeled antibody remained mostly on the membrane. As such, $^{111}\text{In}$-bsRICs could be conjugated to an NLS peptide to promote more efficient internalization and nuclear translocation and better efficacy for RIT.

The success of targeting HER2, as well as the growing interest in other members of the HER family as therapeutic targets, reinforces the feasibility of developing other radiopharmaceuticals that target these receptors. There has been success in targeting HER2 for RIT, however one of the major challenges remains inadequate delivery of the radiopharmaceutical to all cancer cells. HER2 heterogeneity, for example, has been observed in primary human breast cancer and metastases and these low antigen-expressing regions may not be eradicated with a radiolabeled antibody targeted to one particular tumour-associated antigen. Antibody mixtures ("cocktails") that combine two or more radiolabeled antibodies recognizing several antigens have been proposed as a useful strategy at circumventing the problems of non-uniform antibody delivery and antigen heterogeneity. It has been demonstrated that two non-competing $^{111}\text{In}$-labeled mAbs to HER2 used together resulted in increased binding of radioactivity on the surface of SK-BR-3 breast and SK-OV-3 ovarian cancer cells, and enhanced cytotoxicity compared to either mAb alone. The strategy described in this thesis of creating bispecific probes that can bind two distinct antigens (receptors) extends this strategy while also simplifying it. bsRICs can be tailored to bind to any two receptors, or even different epitopes on the same antigenic target. In Chapter 4 of this thesis I demonstrate that $^{177}\text{Lu}$ labeled bsRICs were able to specifically bind to both their target receptors. This allowed for killing of cells that expressed one or both receptors, as well as cells that were both trastuzumab resistant and responsive. Furthermore, the use of $^{177}\text{Lu}$ compared to $^{111}\text{In}$ was found to deliver a higher dose of radiation and resulted in more effective tumour growth inhibition in vivo. In the clinic, targeting of cells with different receptor phenotypes may help improve the effectiveness of RIT. By targeting cells that express one or both of the receptors recognized by the bsRICs, these probes may be able to overcome the challenge of phenotype heterogeneity within a single lesion, or discordance between receptor expression profiles in the primary lesion versus metastatic sites.
5.2.3 Overcoming resistance in HER2 breast cancer

One of the most challenging aspects of treating HER2+ BC is overcoming resistance to targeted therapies, particularly trastuzumab. Several mechanisms have been implicated in trastuzumab resistance (See Section 1.4.1), but of particular interest is the role that other members of the HER family play. While trastuzumab is believed to mediate HER2 signaling, it may not reduce signaling mediated through other receptors of the HER family, such as EGFR and HER3. Heterodimerization of HER2 with other HER (ErbB) family members can be induced by ligands of these receptors (eg. EGF for EGFR, and heregulin for HER3), and the resulting heterodimers initiate mitogenic signaling even in the presence of trastuzumab. An increase in expression of the ErbB family of ligands have been associated with increased signaling from HER2/EGFR and HER2/HER3 complexes, and blocking of trastuzumab mediated growth inhibition.

Clinically, EGFR overexpression has been correlated with disease progression in patients receiving trastuzumab. The promising results presented in this thesis demonstrate that imaging with bsRICs can be used to report on the potential mechanisms of trastuzumab resistance, particularly related to the role of other receptors of the HER family being coexpressed in these cells. In a clinical setting, patients who are not responding to HER2 therapy such as trastuzumab can be imaged with bsRICs against HER2/HER3 or HER2/EGFR to determine if multiple HER family members are coexpressed on the cell surface and are potentially influencing resistance. Information such as this can inform clinicians about alternate choices in therapy, such as if the patient should be treated with pertuzumab to block dimerization. In future, when HER3 and EGFR targeted therapies are available, reporting on the status of both receptors in resistant patients can also inform clinicians on which receptor may be a more favorable target for continued therapy. They will also be able to report on discordant receptor status between the primary lesion and any metastases.

The hypothesis of EGFR amplification as a mechanism for trastuzumab resistance is supported by data that shows that EGFR TKIs erlotinib and gefitinib, and the EGFR and HER2 TKI lapatinib were able to induce apoptosis in HER2 overexpressing cells that were resistant to trastuzumab. As such, targeting EGFR may be a viable tool for treating patients who have developed resistance to trastuzumab. In Chapter 5, Lu labeled bsRICs targeting HER2 and EGFR demonstrated promising efficacy for RIT of trastuzumab resistant BC. These bsRICs yielded moderate to strong tumour growth inhibition in vivo in mice bearing both trastuzumab-
sensitive or resistant human BC xenografts that were HER2+. Furthermore, in clonogenic survival assays they also demonstrated the ability to kill cells that expressed one or both of the target receptors at varying expression levels. If translated into the clinic, RIT of patients with HER2 BC who have developed trastuzumab resistance with these bsRICs could be a promising treatment strategy. This approach would not only allow for killing of tumour cells that are resistant to trastuzumab, but also target a wider population of tumour cells that overexpress one or both receptors, helping to eradicate the disease.

5.3 Future Research and Directions

While reaching interesting and promising conclusions on the role of bsRICs for imaging and treatment of HER2 BC, the research presented in this thesis also opens up many new avenues for investigation. In addition to the potential future applications of the bsRICs for imaging and RIT of HER2+ BC discussed in the previous sections, in the following section I provide some brief ideas for future research.

Firstly, Chapters 2 and 3 provide proof of concept that bsRICs can be labeled with $^{111}$In and effectively used to image cells expressing one or both receptors by SPECT/CT, with high tumour uptake and good tumour:blood ratios at 48 hours post injection. While gamma cameras and SPECT are still common, the effectiveness of $^{18}$F-FDG has vastly expanded the role that PET plays in the clinic, and represents the future of molecular imaging. As such there would be value in designing bsRICs labeled with positron emitting isotopes for PET imaging. Copper-$^{64}$Cu ($^{64}$Cu, $t_{1/2} \sim 12$ hrs) would be a suitable choice due to its half life, which is well matched to the pharmacokinetics of the bsRICs. Zirconium-$^{89}$Zr ($^{89}$Zr, $t_{1/2} \sim 78$ hours) would also be a reasonable choice, as it has a similar half life to $^{111}$In, and has already been studied in humans for PET imaging with radiolabeled antibodies.

Secondly, based on the results of Chapters 2 and 3, I propose that these bsRICs may be useful for monitoring response to treatment, particularly when using HER dimerization inhibitors such as pertuzumab. It would be interesting to confirm whether this is feasible by studying the effect pertuzumab treatment has on uptake of bsRICs. The aim of this study would be to determine if bsRICs are binding simultaneously to multiple receptors. If this were true, we would expect pertuzumab treatment to cause a decrease in the uptake of the bsRICs. In such a study, mice with
tumour xenografts that express HER2 as well as one of its dimerization partners (HER3 or EGFR) could be imaged with the appropriate bsRICs prior to initiating treatment, and during treatment of pertuzumab at therapeutic doses. $^{18}$F-FDG PET could also be performed as a positive control for tumour uptake. It would be interesting to see if bsRICs could report on changes in receptor expression more quickly than $^{18}$F-FDG shows slowing of tumour growth, as such a trend was previously observed with $^{111}$In-pertuzumab when used to monitor response to trastuzumab therapy. There would also be value in imaging mice with xenografts that are trastuzumab resistant being treated with pertuzumab, since pertuzumab is an alternative therapeutic option for patients that do not respond to trastuzumab.

In Chapter 4, I demonstrated the potential of $^{111}$In- and $^{177}$Lu-labeled bsRICs to be used for RIT. Future research could extend this by conjugating NLS peptides to $^{111}$In-bsRICs to improve the nuclear localization of $^{111}$In-bsRICs, where they would be capable of causing more DNA double strand breaks and cell killing. The internalization and nuclear localization of $^{111}$In-bsRIC-NLS would be measured and compared to that of $^{177}$Lu-bsRICs, and clonogenic assays would be performed to determine that the bsRICs are still able to specifically kill cells expressing one or both target receptors. Finally, tumour growth inhibition could be measured in cells that express either one or both receptors as well as those that are trastuzumab resistant, and compared to the efficacy observed with $^{111}$In and $^{177}$Lu-bsRICs that did not include NLS in Chapter 4.

The bsRICs developed here may play an important role in future clinical practice. Their ability to target more than one receptor simultaneously may prove advantageous in overcoming some of the challenges in treating HER2 BC. For example, bsRICs targeting multiple members of the HER family would be able to treat patients whose disease demonstrates receptor heterogeneity within a lesion, or patients who have metastases whose receptor expression differs from that of the primary lesion. While these probes show promise in a preclinical setting, the benefit that bsRICs may provide patients cannot be realized unless they are translated into the clinic. In order to test the bsRICs in a clinical trial, it would be necessary to create a pharmaceutical quality formulation manufactured under current Good Manufacturing Practices (GMP) and obtain regulatory approval from Health Canada with a Clinical Trial Application (CTA). This would allow enrollment of patients with HER2+ BC into Phase I clinical studies that would examine whether bsRICs could feasibly be used for imaging or RIT.
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