In-labeled nimotuzumab modified with nuclear localization sequences (NLS): An Auger electron-emitting radiotherapeutic agent for EGFR-overexpressing and trastuzumab-resistant breast cancer

By

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Abstract

Targeted Auger Electron Radiotherapy for Trastuzumab resistant Breast Cancer Cells

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Objective: The cytotoxic property of anti-EGFR-1 monoclonal-antibody nimotuzumab modified with nuclear localization sequence and radiolabeled with 111In was evaluated in trastuzumab-resistant breast cancer cells. Methods: $^{111}$In-nimotuzumab-NLS was constructed and its immunoreactivity was determined. Cellular and nuclear uptake was evaluated by cell fractionation. Finally, the cytotoxicity of conjugates ($^{111}$In-nimotuzumab/$^{111}$In-nimotuzumab-NLS) was studied by clonogenic assays. Results: The immunoreactivity of $^{111}$In-nimotuzumab-NLS was conserved. $^{111}$In-nimotuzumab-NLS exhibited 2-fold higher nuclear translocation as compared to $^{111}$In-nimotuzumab in MDA-MB-468 cells. Nuclear importation of $^{111}$In-nimotuzumab-NLS in MDA-MB-468 cells was 4-fold and 6-fold higher than moderate and low EGFR expressing cell lines, respectively. Clonogenic survival (CS) for MDA-MB-468 cells showed $^{111}$In-nimotuzumab-NLS to be 10-folds and 60-folds more potent than $^{111}$In-nimotuzumab and nimotuzumab, respectively. Moderate killing for TrR1 and MDA-MB-231 was observed. $^{111}$In-hEGF showed significantly higher cytotoxicity and 2-fold higher $\gamma$-H2AX foci integrated density/nuclear-area as compared to $^{111}$In-nimotuzumab-NLS. Preserved selectivity of $^{111}$In-nimotuzumab-NLS makes it an excellent tool for treating cancers.
“In the loving memory of my grandmother, who passed away fighting a battle against breast cancer”
Acknowledgments

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<tr>
<td>⁵¹I UdR 5</td>
<td>[¹²⁵I]-ido-2’-deoxyuridine</td>
</tr>
<tr>
<td>¹⁸FDG</td>
<td>[¹⁸F]-fluoro-2-deoxy-D-glucose</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-Dependent Cellular Cytotoxicity</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myeloid Leukemia</td>
</tr>
<tr>
<td>BCS</td>
<td>Breast Conserving Surgery</td>
</tr>
<tr>
<td>Bq</td>
<td>Becquerel</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDC</td>
<td>Complement-Dependent Cytotoxicity</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementary Determining Region</td>
</tr>
<tr>
<td>CS</td>
<td>Clonogenic survival</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal Carcinoma <em>In Situ</em></td>
</tr>
<tr>
<td>DFS</td>
<td>Disease Free Survival</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose Nucleic Acid</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriaminepentaacetic Acid</td>
</tr>
<tr>
<td>EC</td>
<td>Electron Capture</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>Effective Concentration 50%</td>
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<tr>
<td>ECD</td>
<td>Extracellular Domain</td>
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<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence <em>In Situ</em> Hybridization</td>
</tr>
<tr>
<td>γH2AX</td>
<td>Gamma Histone 2AX</td>
</tr>
<tr>
<td>GMP</td>
<td>Good Manufacturing Practices</td>
</tr>
<tr>
<td>HAMA</td>
<td>Human Anti-Mouse Antibody</td>
</tr>
<tr>
<td>HER</td>
<td>Human Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>hEGF</td>
<td>Human Epidermal Growth Factor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IGF-I</td>
<td>Insulin-like Growth Factor I</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>Insulin-like Growth Factor I Receptor</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>Insulin-like Growth Factor Binding Protein 3</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin Gamma</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ITLC-SG</td>
<td>Instant Thin-Layer Chromatography Silica-Gel</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Kd</td>
<td>Equilibrium Dissociation Constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LET</td>
<td>Linear Energy Transfer</td>
</tr>
<tr>
<td>mAb</td>
<td>Antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>NHL</td>
<td>Non-Hodgkin Lymphoma</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
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<tr>
<td>NLS</td>
<td>Nuclear Localizing Sequence</td>
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<tr>
<td>ORR</td>
<td>Objective Response Rate</td>
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<td>OS</td>
<td>Overall Survival</td>
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<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDSA</td>
<td>Pyridyl Disulfide Acrylate</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3’ kinase</td>
</tr>
<tr>
<td>PR</td>
<td>Partial Remission</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>RIT</td>
<td>Radioimmunotherapy</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SMCC</td>
<td>Succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate</td>
</tr>
<tr>
<td>SSTR</td>
<td>Somatostatin Receptor</td>
</tr>
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<td>SV-40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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Chapter 1

Introduction
1.1 Incidence, diagnosis and treatment of breast cancer

1.1.1 Epidemiology and etiology of breast cancer

Breast cancer (BC) is the most common epithelial cancer diagnosed in North American women and second largest cause of cancer-related death after lung cancer. In 2010, an estimated 207,090 new cases in U.S., [1] and 23,200 new cases in Canada [2] will be diagnosed with BC. Despite of the current treatment regimens, 39,840 and 5300 patient will die of this disease in U.S. and Canada, respectively [1, 2]. According to lifetime risk statistics approximately 12% of women born today will be diagnosed with cancer of the breast at some time during their lifetime [1]. There are several factors contributing towards BC, such as genetic mutation in the BRCA1 and BRCA2 genes, age, family history, ethnicity, radiation exposure, estrogen exposure, and lifestyle factors such as diet and lack of exercise. The most significant factors responsible for BC are, however, gender (being a woman) and age (being older) [3].

The functional structure of the female breast is the mammary gland. It is regulated by the endocrine system and it is derived from a modification of sweat glands. The mammary gland is made up of lobes which are subdivided into lobules. The lobules contain glandular alveoli that are composed of a unicellular layer of epithelial cells arranged in spheroid structure. The alveolar epithelial cells take up a variety of nutrients from the blood and secret them in the alveolar lumen as milk. The alveolar lumen drains into secondary tubules which converge to form mammary ducts. These ducts further converge to lactiferous ducts which end at the nipple. It has been found that two thirds of breast malignancies originate at the terminal ductal lobular unit (TDLU), which consists of lobes and ducts [3]. Malignancy can be induced by micro-environmental factors such as an abnormal expression level of circulating hormones or alterations in the local
extracellular matrix. This can lead to the evolution of a genetically unstable single cell or cluster of cells that spread through a single duct or involve the entire ductal system in malignant transformation [4]. Although the vast majority of BCs are sporadic in nature, the risk of developing cancer increases when atypical lobular hyperplasia, atypical ductal hyperplasia, lobular neoplasia, lobular or ductal carcinoma \textit{in situ} (LCIS or DCIS) are discovered [5].

1.1.2 Diagnosis and treatment of BC

After the diagnosis of BC is confirmed, the tumor is staged. The American Joint Committee on Cancer (AJCC) has designated staging by a tumor-node metastasis (TNM) classification to define BC. This system provides a strategy for grouping patients with respect to prognosis and helps in determining their therapeutic outcome. The staging category includes: i) tumor size (T), ii) lymph node status by assessing the number of axillary lymph nodes involved (N), and iii) presence or absence of distant metastasis (M). BC may be stage zero (T$_{is}$, N$_0$, M$_0$), the earliest stage, representing \textit{in situ} cancer. Stage IV (any T, any N, and M$_1$) represents the most advanced form of BC [6]. Furthermore, in order to assist with diagnosis, other factors such as estrogen-receptor and progesterone-receptor levels in the tumor tissue, human epidermal growth factor receptor 2 (\textit{HER2/neu}) status, menopausal status, and the general health of the patient are also used [6]. Finally histological analysis of tumors is done to assess the mitotic activity of the cell population, the level of differentiation of the cells, their pleomorphism, and their structural differentiation [7].

In BC multiple modalities such a diagnostic imaging, pathology, surgery, radiation therapy and medical oncology play a role in the management of the disease. In most cases patients are treated with surgery followed by single or a combination of multiple adjuvant local
and systemic therapy [3]. Treatment of a BC patient is often dependent on the stage of cancer, age and personal preference of the individual. Lumpectomy, i.e. breast conserving surgery (BCS) is considered a standard treatment option for ductal carcinoma in situ (DCIS) or invasive carcinomas of the breast, although in some cases women may elect for mastectomy. The incidence of local recurrence using this BCS therapeutic approach ranges from 6-19% [8]. In order to further reduce the recurrence of BC, draining nodes are either excised and/or radiation therapy is provided to the adjacent area to kill remaining cancerous cells [9]. Furthermore, the combination of hormonal therapy and chemotherapy in BC has been shown to reduce the risk of BC recurrence by 30-50% [3].

For patients who have resectable tumors, surgery is the first modality of treatment; however, primary (neoadjuvant) chemotherapy followed by surgery is also an available option. In the case of an unresectable tumor, primary radiation therapy following chemotherapy is an appropriate form of treatment. In terms of different chemotherapeutic drugs, resectable tumors are often treated with neoadjuvant doxorubicin-based or taxane-based regimens [10]. Patients with unresectable tumors in which disease has not metastasized, are treated with systemic therapy with or without tamoxifen (estrogen-receptor positive tumors) [11]. If the neoadjuvant treatment is successful in shrinking the tumor, the patient is eligible for BCS. If the metastasis has spread to other organs such as brain, liver, bones, and other visceral organs, at this stage, systemic therapy is the primary treatment using chemotherapy, endocrine therapy or both, depending upon the hormone receptor status of the tumor. Patients with high HER2 protein expression are treated with trastuzumab (Herceptin®) alone or in combination with anthracyclines and paclitaxel [12]. Trastuzumab has demonstrated delay in the progression of
disease and increased the survival of patients when used in combination with other endocrine and chemotherapy drugs [13].

1.2 Treatment of HER2-overexpressing BC with trastuzumab

1.2.1 Structure of HER2

The HER family comprises four genes encoding four homologous ErbB or type I tyrosine kinase receptors: epidermal growth factor receptor (ErbB1, EGFr or HER1), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4) [14]. Among these growth factor receptors, HER2 has been extensively studied in terms of its role in BC evolution [15]. The gene for HER2 is located on the long arm of chromosome 17. It encodes a protein that comprises 1255 amino acids (aa) with molecular weight of 185 kDa [16]. HER2 (also known as Neu) is a cell membrane, surface-bound receptor with an extracellular “ligand binding domain” (632 aa), a transmembrane region (22 aa) and an intracellular tyrosine kinase domain (580 aa) with a regulatory C-terminal segment [17]. There is no known ligand for HER2.

HER2 is expressed in many cell types including the nervous system, bone, muscle, skin, heart, lung, and intestinal tissue [18]. Expression of HER2 is higher in fetal tissues than adult tissues [19]. HER2 activation is dependent on the interaction of ligands with other HER receptors [20] [21]. HER2 is thought to be an orphan receptor, with none of the hEGF family of ligands able to activate it. Nevertheless, ErbB family receptors dimerize on ligand binding, and HER2 is the preferential dimerization partner of other members of the ErbB family [22].
Once HER2 is activated, it leads to a signaling cascade which promotes cell growth and activation of genes associated with cell division [15]. HER2 is a heterodimerization partner for other ErbB receptors which suggests that it has a critical role in the development of cancer [15]. Studies using immortalized cell lines have implicated the overexpression of HER2 in malignant transformation and tumorigenesis [23-25]. HER2 gene amplification and/or HER2 receptor overexpression is found in breast, ovarian and lung cancer. It was found that breast and ovarian tumor cells which contained increased numbers of copies of the HER2 gene similar to those seen in human tumors produced more aggressive growth characteristics compared to the parent cells. These cells also showed increased DNA synthesis, cell growth rates in vitro, tumorigenicity and metastatic potential [26]. HER2 gene amplification occurs in 25-30% of breast and 15-30% of ovarian carcinomas [27, 28]. Multiple studies have reproducibly identified that luminal subtypes of BC are characterized by HER2-positive/ER-negative. The basal-like subtype is characterized by low expression of ER- and HER2-related genes and clinically is ER/PR–negative and lack HER2 overexpression, thereby constituting the “triple-negative” phenotype [29]. However, in triple-negative breast tumors, HER1/EGFR is found to be expressed in approximately 60% of basal-like breast tumors [30].

Additional studies have also demonstrated that HER2 gene amplification occurs in the early development of BC. The frequency of HER2 expression levels was similar in DCIS, DCIS with invasion, and invasive BC, although some studies have found a higher incidence of HER2 overexpression in DCIS than in invasive tumors. Others have shown that HER2 amplification/overexpression is strongly associated with poorly differentiated DCIS, which is more likely to progress to invasive cancer [31, 32]. Thus, it is important to know the HER2 status in the patient at the time of diagnosis. This information is of great importance in the management
of patients with both primary and metastatic BC, particularly as HER2-targeted therapy is
introduced.

1.2.2 Antibodies and related molecules targeting HER2

Monoclonal antibodies (Abs) usually target cell surface antigens and can be used to treat
tpatients with different malignancies, including BC. Antibodies are immunoglobulins (Ig) that are
complex plasma proteins produced by plasma B-lymphocytes, and occur naturally as part of the
immune system of mammals. The recognition of an antigen by an antibody has been described as
a lock-and-key fitting model [33]. An intact IgG antibody has a molecular weight of about 150
kDa and contains 4 peptide chains. It contains 2 identical heavy chains of about 50 kDa which
may be denoted by α, β, ε, γ, or μ, and 2 identical light chains of about 25 kDa which may be
denoted by λ and κ, leading to a tetrameric quaternary structure. The two chains are linked to
each other and to the light chains by a disulphide bonds. The resulting tetramer has two identical
halves which together form a Y-like shape. Each heavy and light chain has similar structure, and
each contains a constant and variable region. The main differences between IgGs, i.e. antibodies
is observed in the variable region which is located in three small hyper-variable sequences that
are present in the N-terminal region of the heavy and light chains. This region at the end of the
fork contains an antigen binding site [34]. Monoclonal antibodies were first produced by
hybridoma technology in 1975, relying on the fusion of a myeloma cell line with B-cells from an
immunized animal. Later in the 1980’s, antibody technology was revolutionized when the genes
encoding monoclonal antibodies from murine hybridomas were efficiently cloned using the
polymerase chain reaction (PCR) [35].
For several decades and until recently, murine monoclonal antibodies were used extensively for treatment of cancer. The treatment was not as effective as anticipated because the human body would recognize these mouse antibodies as a foreign substance which would elicit an immune response resulting in the production of Human Anti-Mouse Antibodies (HAMA). The HAMA reaction was generally mild but limited the antibody efficacy and resulted in rapid clearance from the blood with the immune complexes deposited in the liver and kidneys [34]. A variety of humanization techniques were used to decrease the immune response. One such technique involved the replacement of murine constant regions with those of human IgG to form chimeras (chimeric mAbs). To further reduce the immunogenicity, the murine complementarity-determining region (CDR) region was transplanted to a human antibody framework (humanized mAbs). To improve the in vivo targeting and pharmokinetic properties, new antibody constructs have been produced using recombinant DNA techniques, e.g. scFv, minibodies, diabodies and affibodies [34]. However, the high renal uptake and low tumor accumulation of these molecules does not make them suitable for radioimmunotherapeutic applications [36]. Today there are several antibodies and molecules that target HER2 such as trastuzumab, pertuzumab, MDX-H210, 2B1, C6.5xscFv (NM3E2), and ertumaxomab that are either in clinical trial or already approved for use in patients [37].

1.2.3 Trastuzumab (Herceptin®) treatment for BC

Trastuzumab (type IgG\textsubscript{1}, rhuMAb-HER2, Herceptin\textsuperscript{®}, Genentech, San Francisco, CA) is a recombinant humanized monoclonal antibody that targets a cysteine-rich juxtamembrane region in domain IV on HER2. It is the only HER2-targeted immunotherapeutic agent that has
received marketing clearance from the U.S. Food and Drug Administration (FDA) and Health Canada for use in the treatment of patients with HER2-overexpressing BC [35].

1.2.4 Clinical efficacy of trastuzumab

In a Phase II clinical trial, the efficacy and safety of trastuzumab were evaluated in female metastatic BC patients with tumors overexpressing HER2, who progressed after chemotherapeutic regimens (i.e. doxorubicin) [38, 39]. In these trials, the patients were administered 4 mg/kg of trastuzumab as a loading dose, followed by a weekly dose of 2 mg/kg. The objective response rate (ORR) was defined in terms of percentage of complete and partial remissions (CR and PR, respectively). The median duration of response was 9 months in these trials and CR was obtained in 11.6% [36] and PR in 15% of patients [36, 37]. The most serious adverse side effect observed was cardiac dysfunction which occurred in 4.7% of patients who received doxorubicin. This finding suggested that trastuzumab may exacerbate myocardial damage caused by anthracycline treatment [36, 37].

The efficacy of trastuzumab was evaluated in another clinical trial that recruited patients with HER2 overexpressing metastatic disease who had never been treated with chemotherapy [40]. BC specimens were assessed for HER2 expression by immunohistochemical staining (IHC) and HER2 gene amplification by fluorescence in situ hybridization (FISH). A score of 3+ (strong staining) was assigned to a specimen when more than 10% of the tumor cells showed complete staining with 4D5 or CB11 anti-HER2 Abs. The patients, whose tumors were assigned a score of 3+ or HER2 gene amplification, had an ORR of 35% and 34%, respectively. The ORR results of these trials were double compared to the previously described clinical trial, possibly because in the previous study, the moderate HER2 expression in the tumors did not yield a good response to
trastuzumab [41]. Cardiac dysfunction was observed in 2% of the patients; however, these patients had a previous history of cardiac disease and did not require any intervention, such as providing patients with standard therapy for cardiac dysfunction, after discontinuing trastuzumab [38].

In a Phase III trial, Slamon et al. [12], showed that the addition of trastuzumab to chemotherapy (doxorubicin plus cyclophosphamide [AC] or single-agent paclitaxel) in metastatic BC, expressing moderate HER2 had a higher ORR, longer time to disease progression and a longer median overall survival (OS) compared to chemotherapy alone (OS: 25.1 months versus 20.3 months, respectively). Cardiotoxicity was however, more common in combined treatments that involved an doxorubicin, which led to a recommendation that concomitant anthracylines (AC) should be avoided while using trastuzumab. Consequently the use of non-anthracyline drugs (cisplatin, docetaxel, paclitaxel/carboplatin and vinorelbine) in combination with trastuzumab, showed promising results in various clinical trials [12].

The National Surgical Adjuvant Breast and Bowel Project (NSABP)-31 and the North American Intergroup randomized node-positive [42] have also studied the effects of adjuvant chemotherapy (AC plus paclitaxel) with or without trastuzumab in early stage HER2 positive BC patients. There was a greater percentage in patients with disease-free survival in those receiving the combination of chemotherapy and trastuzumab (87.1%) compared to patients receiving only chemotherapy (75.4%).

Finally, some studies have evaluated the role of neo-adjuvant trastuzumab therapy in patients with early-stage but inoperable HER2 positive BC. In one study, HER2 positive BC patients were pre-operatively assigned to chemotherapy (four cycles of paclitaxel followed by four cycles of 5-fluorouracil, epirubicin and cyclophosphamide) with or without trastuzumab.
Post surgery it was found that patients who received chemotherapy and trastuzumab had a higher pathologic complete response rate (pCR: 65%) compared with patients who only received chemotherapy (pCR: 25%) [43]. This trial was stopped after the superiority of trastuzumab was demonstrated [44]. Although no cardiotoxicity was observed, more long-term safety and efficacy data are required before trastuzumab can be applied widely as a neoadjuvant treatment for patients with early-stage disease [45].

1.3 Mechanism of action of trastuzumab

1.3.1 Immune mediated response (ADCC)

In addition to its cytostatic properties, trastuzumab is thought to activate antibody-dependent cellular cytotoxicity (ADCC). This immunological effect has been demonstrated in numerous BC cell lines. ADCC is mainly due to the activation of natural killer cells (NK) expressing the Fc gamma receptor which when bound to the Fc domain of trastuzumab, cause the lysis of cancer cells [46]. Athymic mice-bearing BT474 HER2-overexpressing xenografts demonstrated a tumor regression rate of 96% compared to 26% in mice lacking the Fc receptor (FcR-/-) after treatment with trastuzumab. This occurred, because although athymic mice lack T-cells, they retain natural killer cells which allowed trastuzumab to cause ADCC dependent cytotoxicity against BT474 tumor cells. ADCC response was also shown in patients [44]. In a study of 11 patients with HER2-positive BC, who received a standard dose of trastuzumab for 4 weeks, there was substantial infiltration of lymphocytes into the tumor tissue, suggesting an immune response which could have been mediated by trastuzumab binding to cancer cells. Furthermore patients with complete remission (CR) or partial remission (PR) were found to have
a higher in situ infiltration of leukocytes and a higher capability to mediate in vitro ADCC [45]. Thus, it seems that an active immune response contributes to the toxicity of trastuzumab.

1.3.2 Inhibition of tumor angiogenesis

Overexpression of HER2 in BC cells is closely associated with increased angiogenesis [36]. Mice-bearing HER2-overexpressing tumors, when treated with trastuzumab, showed regression and normalization of the vasculature associated with an anti-angiogenic effect. Similar studies found that mice bearing MDA-MB-435 HER2-expressing tumors, when treated with both paclitaxel and trastuzumab, showed the best tumor response as compared to trastuzumab given alone. Also in this study, a good concordance between tumor response and reduction of microvessels was observed [47]. Furthermore, another study found a reduction in cancer cell production of vascular endothelial growth factor (VEGF) and transforming growth factor-α (TGF-α) but this occurred only in vitro. Therefore a VEGF independent antiangiogenic mechanism might act in vivo, which may be caused by the modulation of different factors involved in the machinery of angiogenesis [48].

1.3.3 Inhibition of PI3K pathway resulting in attenuation of cell signaling

PI3K (Phosphatidylinositol 3-kinase) is activated by HER2 overexpression/activation. Activated PI3K generates phosphoinositide-dependent kinase (PDK) and protein kinase B (PKB) by phosphorylating phosphatidylinositol-4,5-bisphosphate (PIP2) to form phosphatidylinositol-3,4,5-trisphosphate (PIP3). The latter causes translocation of Akt to the plasma membrane where it is activated. Ultimately this whole process leads to protein synthesis, cell proliferation, survival, and motility [36]. Phosphatase and tensin homologue (PTEN) is a tumor suppressor protein which negatively regulates PI3K signaling by dephosphorylating PIP3. One proposed
mechanism of trastuzumab is to decrease the signaling from these pathways, thus promoting apoptosis and growth arrest [36]. An alternative mechanism proposed by Nagata et al, is that trastuzumab inhibits signaling by disrupting the interaction between HER2 and Src tyrosine kinase which subsequently leads to activation of PTEN. This may, in turn, lead to rapid Akt dephosphorylation and inhibition of cell proliferation [49].

1.3.4 Prevention of HER2 ECD shedding

When HER2 is overexpressed, it undergoes a proteolytic cleavage of the HER2 extracellular domain (ECD) and produces a truncated membrane bound-fragment (p95) [50]. Once cleaved, the ECD can be detected in the serum by an FDA-approved ELISA-based test to follow-up and monitor patients with metastatic BC. A study done by Molina et al. [51] found phosphorylated truncated HER2 receptor in 14 out of 20 BC patients. Furthermore, in HER2-overexpressing cell lines (BT474 and SKBR3), HER2 shedding can be blocked by trastuzumab which inhibits metalloproteinase activity. Several clinical studies have observed a decline in serum HER2 ECD during trastuzumab treatment which predicts tumor response and improved progression-free survival, indirectly supporting the hypothesis that trastuzumab may act by inhibiting HER2 cleavage.

1.3.5 Inducing G1 arrest by modulating the expression of p27kip1

Cylin-D dependent kinase (Cdk-4) and cylin-E dependent kinase (Cdk2) are regulated by a group of proteins known as Cdk inhibitors. The cyclins, cdk's and their inhibitors play a crucial role in regulating the progression of cells though the G1-S phase of the cell-cycle [52]. Lane et al. [53] demonstrated that in vitro treatment of SKBR3 and BT474 cell lines with trastuzumab
increased the expression of the Cdk inhibitor, p27\(^{kip1}\). This sequestration of Cdk by p27\(^{kip1}\) resulted in decreased activity of the kinase and cell cycle arrest. Trastuzumab has also been shown to inhibit the ubiquitin-mediated degradation of p27\(^{kip1}\), thus delaying progression through the G1-S phase of the cell cycle [54].

### 1.4 Mechanism of resistance to trastuzumab

The objective response rate for trastuzumab monotherapy ranges from 12-34% depending on prior therapy for metastatic disease, and the median duration of response is 9 months. Therefore, the majority of HER2-overexpressing tumors demonstrate primary resistance to single agent trastuzumab (Figure 1.4) [55]. In fact, it has been reported that the rate of primary resistance to single-agent trastuzumab for HER2-overexpressing metastatic BC is 66-88% [39, 40]. Furthermore, studies have shown that when trastuzumab is combined with chemotherapeutic agents, the median time to progression increased from 4.9 months to 7.4 months [47]. Although combining trastuzumab with chemotherapies prolongs the interval before disease progression, the majority of the patients who achieve an initial response to trastuzumab based regimens ultimately develop acquired resistance within one year.

#### 1.4.1 Loss of PTEN

PTEN deficiency is correlated with resistance to trastuzumab in clinical samples. Nagata and colleagues [33] showed reduction of PTEN when BT474 and SKBR3 cells were exposed to antisense oligodeoxynucleotides that inhibited translation of PTEN mRNA, thereby giving rise to cells that were resistant to trastuzumab. They further demonstrated that decreased levels of PTEN phosphatase resulted in increased PI3K/Akt phosphorylation signaling which inhibited the
growth arrest mediated by trastuzumab in HER2 overexpressing cells. They also found in PTEN deficient cells, that PI3K inhibitors overcame trastuzumab resistance *in vitro* and *in vivo* [33]. These results showed that PTEN can be a predictor of trastuzumab resistance. Accordingly, PI3K inhibitors could be further explored in patients with trastuzumab-resistant tumors expressing low levels of PTEN.

### 1.4.2 Increased signaling from insulin-like growth factor-I receptor (IGF-IR)

IGF-IR is a transmembrane tyrosine kinase receptor frequently overexpressed in HER2-overexpressing cancers. IGF-IR activation stimulates mitogenic signaling pathways involved in cell proliferation and cancer invasion [56]. Trastuzumab resistance is associated with increased signaling from IGF-IR.

A recent study showed that crosstalk occurs between IGF-IR and HER2, and that IGF-IR physically interacts with and phosphorylates HER2 in trastuzumab resistant cells, but not in trastuzumab-sensitive cells. It also showed that resistant cells exhibited more extensive IGF-IR stimulation of downstream PI3K/Akt and MAPK pathways relative to sensitive parental cells. Increased signaling from this receptor has been associated with reduction in growth arrest mediated by trastuzumab in HER2 overexpressing cells. Furthermore, this resistance was reversed when cells were treated with either IGF-IR blocking antibody or IGF-IR tyrosine kinase inhibitors [47]. Lu et al. [57] demonstrated that trastuzumab activity was significantly reduced in HER2-overexpressing SKBR3 BC cells transfected with the IGF-IR gene (SKBR3/IGF-IR) compared to non-transfected cells. The resistance of SKBR3/IGF-IR cells was reversed, however, in the presence of insulin growth factor binding protein-3 (IGFB-3) which is known to sequester IGF. Similar results were obtained for the MCF-7/HER2-18 cell line that intrinsically
overexpresses IGF-IR, but is transfected with HER2. In these cells, growth was inhibited by trastuzumab only in the presence of anti-IGF-IR (-IR3) mAbs or IGFBP-3. In another study, exposure of trastuzumab-resistant MCF-7/HER2-18 and SKBR3/IGF-IR cells to IGFBP-3 inhibited IGF-IR phosphorylation and enhanced killing by trastuzumab [58]. Furthermore, BT-474 cells, which acquired trastuzumab resistance through serial passage in the presence of trastuzumab, displayed elevated IGF-IR levels (3-5 fold) when compared with their trastuzumab-sensitive wild-type counterparts. Most importantly, this study revealed that inhibition of IGF-IR signaling in vivo by exposing cells to IGFBP-3 improved the anti-tumor effects of trastuzumab in athymic mice bearing MCF-7/HER-18 xenografts. When these mice-bearing MCF-7/HER-18 xenografts were treated with both IGFBP-3 and trastuzumab they exhibited 56% tumor regression as opposed to 6% when they were treated with trastuzumab alone [52].

Although these studies indicate a strong correlation between IGF-IR overexpression and resistance to trastuzumab, other studies have failed to find this correlation. For instance, one study found no relationship between tumor IGF-IR expression and patient outcome in trastuzumab-based therapies [59].

1.4.3 Receptor masking or epitope inaccessibility

A potential mechanism, by which resistance to trastuzumab develops, is via disruption of the interaction between the antibody and HER2. Expression of membrane-associated (MUC4) glycoprotein is associated with resistance to trastuzumab [60]. MUC4 has been shown to bind to HER2 and sterically hinder the binding of trastuzumab [60, 61]. MUC4 has also been shown to contribute to the malignant phenotype, promote tumor progression and metastasis, suppress apoptosis and activate HER2. Furthermore, MUC4 has been shown to directly interact with
HER2, this occurs due to the binding of the transmembrane subunit of MUC4, asialoglycoprotein subunit-2 (ASGP2) to HER2 intracellular domain. Through this interaction, it is proposed that MUC4 serves as a ligand for HER2 on residue Tyr1248, a major phosphorylating site contributing to the transforming ability of the HER2 oncoprotein [54].

Nagy et al. [55] used the JIMT-1 trastuzumab-resistant cell line with HER2 gene amplification to demonstrate the role of MUC-4 in trastuzumab resistance. They found that the level of MUC4 was inversely correlated with trastuzumab binding capacity, and that knockdown of MUC4 by interference RNA (RNAi) increased the sensitivity of JIMT-1 to trastuzumab. From these forementioned studies, it was proposed that elevated MUC4 expression masks the trastuzumab binding epitopes of HER2, resulting in steric hindrance of the interaction between the antibody and its epitope, thereby leading to drug resistance.

### 1.4.4 Alternative cell signaling mediated by ErbB1 family

Although trastuzumab is known to inhibit the downstream signaling pathway for HER2 it fails to prevent HER2 receptor heterodimerization with HER1/EGFR, HER3 and HER4 [55]. All these receptors, with the exception of HER3, contain a cytoplasmic tyrosine kinase domain. Upon activation by the binding of specific ligands to the extracellular domain, these receptors dimerize using HER2 as their preferred dimerization partner [62]. Heterodimerization induces intrinsic receptor tyrosine kinase activity and subsequent activation of downstream signaling components via the MAPK and PI3K pathways. Thus cells with EGFR/HER3 heterodimers or EGFR/EGFR homodimers may initiate mitogenic PI3K and MAPK signaling even in the presence of trastuzumab which blocks HER2. In addition, increased levels of the ErbB family ligands e.g. heregulin, betacellulin, and HEGF reversed trastuzumab-mediated growth inhibition.
and this inhibition was associated with increased signaling from HER2/HER3 and HER2/EGFR complexes in BT474 and SKBR3 cells [63].

Furthermore, decreased activity of endogenous inhibitors of HER2 signaling has also been proposed as a mechanism contributing to trastuzumab resistance. One example is mitogen-inducible gene 6 protein (MIG-6), a cytoplasmic protein initially identified as a serum inducible gene [64]. MIG-6 has been shown to interact with and inhibit activation of EGFR and HER2 [65]. Thus, molecular profiling of tumor cells to determine the relative levels of EGFR family members, their ligands and endogeneous inhibitors could be useful for predicting the onset of trastuzumab resistance.
Figure 1.4: This figure illustrates various mechanisms of trastuzumab resistance. These include loss of PTEN, up-regulation of IGF-1R, masking of HER2 receptor with MUC4 glycoprotein and, increased expression and signaling from the other members of epidermal growth factor receptor family (EGFR/HER1, HER3 and HER4).
1.5 Treatment of Herceptin-resistant cells overexpressing EGFR

1.5.1 Biology of EGFR

EGFR is a transmembrane glycoprotein of 170 kDa, encoded by the c-erbB1 proto-oncogene situated on chromosome 7 at 7q22. EGFR contains an extracellular domain, transmembrane region and intracellular tyrosine kinase domain with a regulatory carboxyl terminal segment. Known EGFR ligands are EGF, TGF-\(\alpha\), amphiregulin, heparin-binding EGF, betacellulin, epiregulin, and NRG2-alpha [66].

When EGFR is bound to its ligand, dimerization occurs with another EGFR molecule of another Type I growth factor receptor leading to subsequent tyrosine phosphorylation [67]. A signaling cascade begins at the intracellular level by phosphorylated EGFR activating important signaling molecules like phospholipase-C-\(\gamma\) (PLC-\(\gamma\)), Ras, and PI3K. In addition, activated EGFR phosphorylates signal transducer and activator of transcription-3 (STAT3) and activates STAT3 dimerization, nuclear localization and subsequent gene regulation. Together, the PLC-\(\gamma\)-Ca\(^{++}\) dependent calmodulin kinase (CaMK)/PKC, Ras-Raf-MAPK, PI3K-Akt-GSK and STAT pathways are considered the classical downstream signaling pathways of EGFR [Figure 1.5.1A] [68-71]. As a result of increased activation of EGFR, these downstream signaling pathways are often deregulated in tumors, leading to tumorigenesis, tumor proliferation and therapeutic resistance. Furthermore, in recent studies a novel EGFR signaling pathway was discovered which involves shuttling of the activated EGFR from the membrane to the nucleus [72, 73]. Nuclear EGFR functions as a transcription co-factor which interacts and activates the cyclin D1 gene and the iNOS gene. Both these genes are found to positively regulate proliferation [Figure 1.5.1A][72].
EGFR is frequently overexpressed (levels $\geq 10^6$ EGFR/cells) in many types of human malignancy such as head and neck squamous cell carcinoma (HNSCC), glioblastoma, non-small cell lung cancer (NSCLC), breast, prostate, colorectal, and ovarian carcinomas [74]. Overexpression of EGFR in cancer is often correlated with an unfavorable prognosis, mainly in HNSCC, ovarian, cervical and esophageal cancer and modestly correlated to gastric, breast, endometrial and colorectal cancers [72, 66].
Figure 1.5.1A: The EGFR signaling pathway in human cancers. EGFR signaling can be classified into two major pathways. (A) The traditional pathway consisted of several transduction cascades, namely those involve PLC-γ-CaMK/PKC, Ras-Raf-MAPK, PI3K-Akt-GSK, and STATs. Dysregulation of these pathways lead to malignant transformation, increased proliferation rate and tumor progression. (B) The novel direct EGFR pathway involves EGF activated nuclear translocation of cell-surface EGFR and transcriptional regulation of target genes. The accumulation of nuclear EGFR is associated with increased tumor proliferation.
1.5.2 Role of EGFR in trastuzumab resistance

An inverse relationship between HER2 and EGFR expression is present in most BC cell lines [75] and only 5% of patients have tumors with high densities of both these receptors [76]. Nevertheless, 10-36% of tumors co-display low levels of EGFR and high HER2 expression. EGFR has been shown to contribute to trastuzumab resistance [77]. Studies have observed higher activity and expression of EGFR in HER2 overexpressing BC cells that are resistant to trastuzumab. In one study Ritter et al. [78] developed a trastuzumab-resistant cell line (BT-474/HR) \textit{in vivo} by exposing BT-474 cells transplanted into nude mice to trastuzumab. They demonstrated that the resistant cells expressed higher levels of phosphorylated EGFR and EGFR/HER2 heterodimers than the parental BT-474 cells. Interestingly, they also found that phosphorylation of HER2 was reduced by EGFR tyrosine kinase inhibitors (TKI) such as erlotinib and gefitinib suggesting interaction between the two receptors. Furthermore, both these inhibitors increased apoptosis in BT-474/HR cells and inhibited their tumor growth \textit{in vivo}. They also found higher EGFR, TGF-\(\alpha\), heparin-binding EGF, and heregulin RNAs in trastuzumab resistant cell lines compared to parental trastuzumab sensitive cells [70].

In another study, the role of EGFR in acquired trastuzumab resistance in HER2 overexpressing SKBR3 cells was established [57]. In this study, SKBR3 clones expressing various level of EGFR were generated to examine the role of EGFR overexpression on trastuzumab sensitivity. A stable clone, SKBR3/EGFR (clone 4) expressing moderate levels of EGFR, remained sensitive to trastuzumab, whereas a stable clone, SKBR3/EGFR (clone 5) expressing high levels of EGFR, became resistant to trastuzumab. The clone 5 cell line, however, showed response to EGFR TKI. Similar to the previous study, this study also found high levels of EGFR phosphorylation, EGFR/EGFR homo-dimerization, and EGFR-HER2
heterodimerization in trastuzumab-resistant cell lines. Both these studies showed that EGFR overexpression can mediate trastuzumab-resistance in HER2-overexpressing cells [79].

In clinical settings, EGFR and its natural ligand have been associated with the progression of BC [36, 58]. Valabrega et al. [46] compared tumor tissue from patients with advanced HER2-positive BC before and after trastuzumab treatment, and observed a strong increase in TGF-α production upon disease progression, suggesting a possible role of EGFR signaling by TGF-α as mediator of acquired resistance to trastuzumab. Furthermore, EGFR overexpression has been correlated clinically with disease progression in patients receiving trastuzumab [80]. Finally, in addition to EGFR, HER3 and HER4 have also been found to dimerize with HER2 and induce mitogenic signaling which may contribute to trastuzumab resistance in BC cells. Thus, in order to reduce trastuzumab resistance, a combination of ErbB family inhibitors or TKI should be used in conjunction with trastuzumab to regain sensitivity.

1.5.3 Antibodies specific for EGFR

Different recombinant antibodies have been developed to target human EGFR and inhibit signal transduction pathways which lead to cell growth, proliferation and anti-apoptotic activity in tumor cells. Cetuximab (IgG1) (Erbitux/C225; ImClone), a chimeric mAb and panitumumab (IgG2) (Vectibix/ABX-EGF; Amgen), a human mAb are used for treating EGFR-overexpressing colorectal cancer and head and neck cancer with non-mutated KRAS (wild type). KRAS is a GTPase protein that when mutated, predicts response to antibody treatment. Once it is established that KRAS is not mutated, antibodies can be administered for treatment [81]. Currently, cetuximab is the only mAb for EGFR that is approved by Health Canada, however panitumumab is conditionally approved and still assessed in clinical trials [82].
Cetuximab and panitumumab reduce tumor cell growth both *in vitro* and *in vivo* [83, 84]. Furthermore, both these drugs have shown an increase in the overall survival when given alone or in conjunction with chemotherapy in colorectal cancer [85, 86]. Although to date, there are no anti-EGFR mAb-approved therapies for treating EGFR overexpressing BC, there are some preclinical studies currently examining the efficacy of cetuximab in triple negative (TN) BC cells [87]. One of these studies showed that cetuximab has similar ability to the DNA cross-linking agent, cisplatin (cis-diammedichloroplatinum; CDDP), to increase BRCA1 (DNA repair) protein in MDA-MB-468 cells. However, when cetuximab was combined with low doses of CDDP, the outcome was reversed. This was believed to be caused by unexpected ability of CDDP to promote a complete depletion of the cetuximab target EGFR. Although the mechanism by which it occurs is unclear, however the additive models of interactions reveal that antagonistic interactions occurred when cetuximab was concurrently combined with low doses of CDDP, whereas additive to synergistic effects took place in the presence of an optimal concentration of CDDP [83]. Thus, treatment schedules, cetuximab/CDDP doses and BRCA1 status should be carefully considered when combining anti-EGFR antibodies and platinum derivatives in TN/basal-like breast carcinomas. Another study found a significant decrease in skin metastasis in TN invasive ductal breast carcinoma by administering cetuximab with paclitaxel [88]. In addition to antibodies, there are also some small TKI molecules that have been approved for clinical use for targeting EGFR overexpression in various cancers, such as, gefitinib (TKI) (Iressa/ZD1839; AstraZeneca) for NSCLC and erlotinib (Tarceva/OS1774; Genentech) for metastatic NSCLC [72].
1.5.4 Nimotuzumab

Despite the clinical efficacy of cetuximab and panitumumab, these antibodies have caused severe acneiform rash (90-100%), which either leads to dose reduction or termination of cancer therapy [89]. This is believed to be due to the high affinity of cetuximab ($K_d=10^{-10}$) and panitumumab ($K_d=10^{-11}$) for EGFR, which causes them to bind equally well to cells that express normal/low EGFR, for example, normal skin cells. To eliminate this problem, nimotuzumab (h-R3/YM Biosciences Inc.), an IgG1 anti-EGFR antibody with low binding affinity ($K_d=10^{-9}$), has been developed [90]. This antibody has shown lack of or only mild skin toxicity (~3%) in numerous clinical trials [91]. The lower skin toxicity may be due to a requirement for bivalent binding by nimotuzumab, i.e. binding with both antibody arms to two epitopes on different EGFRs simultaneously, for stable attachment to cellular surfaces. This could result in selective binding to cells that express moderate to high EGFR levels ($10^5$ to $10^6$ EGFR/cell) compared to those with low EGFR density [92].

To date, nimotuzumab has mostly been effective against gliomas and squamous cell carcinoma of the head and neck (SCCHN), and is approved in some countries for such treatment. Recently, nimotuzumab’s efficacy was assessed in a Phase II clinical trial for SCCHN in combination with chemotherapy or radiotherapy [93]. The overall response rate (ORR) was measured as well as the percentage of progression-free survival (PFS), disease-free survival (DFS) and overall survival (OS). The PFS, DFS and OS were measured at 30 months after treatment. The nimotuzumab therapy regimen was 200 mg administered intravenously (I.V); the chemotherapy regimen was 50 mg of cisplatin, and radiation therapy dose was 60-66 Gy; all these regimens were given once a week for 6-6.5 weeks. It was found that the PFS, DFS and OS for the combined radiotherapy (RT), chemotherapy (CT) and nimotuzumab (N) treated group
was much higher (PFS/DFS=56.52% and OS=69.75%) than the group that received combined RT and CT only (PFS/DFS=12.66% and OS=21.74%). Furthermore, superior PFS, DFS and OS values were also observed with nimotuzumab combined with RT (PFS/DFS=34.78% and OS=39.13%) compared to RT alone (PFS/DFS=13.04% and OS=21.74%). Additionally, nimotuzumab was well tolerated, even after one year of treatment. Combining nimotuzumab with RT and CT therefore sensitized cancer cells and improved the treatment outcome without causing severe adverse side effects.

Nimotuzumab has also been studied for treatment of malignant gliomas. In a Phase II trial, 47 children and adolescents with refractory or relapsed high-grade gliomas were treated with nimotuzumab. The tolerability was good and the clinical activity was promising (partial response=9% and stable disease =22%) [94]. In another phase II study, the combination of RT and nimotuzumab was explored in 21 patients with malignant gliomas. There were 17% complete responses, 21% partial responses, and the median survival was 22 months [95]. Nimotuzumab is now being studied in combination with various chemotherapeutic agents (docetaxel, carboplatin and capecitabine) in 19 patients with malignant gliomas and squamous cell carcinoma of the head and neck [96].

Despite the efficacy of nimotuzumab in gliomas and SCCHN where EGFR overexpression is related to disease progression, there is a lack of data on the efficacy of this drug for treating BC patients. This is due to limited research that correlates EGFR overexpression to poor prognosis in BC patients. A preclinical study has shown promising results of nimotuzumab using a TN BC cell line. In this study [97], an aggressive metastatic variant of the MDA-MB-231 human BC cell line was selected through passage in vivo, resulting in a line termed 164/8-1B. This cell line is characterized as highly metastatic to many organs, including lymph nodes, lung,
and brain in mice. This variant also had increased EGFR expression compared to the parental cell line. Exposing this cell line to a combination of low-dose cyclophosphamide and nimotuzumab resulted in significant primary tumor growth delay in the metastatic variant compared to the parental cell line. In addition, the combination resulted in a significant survival advantage over cyclophosphamide alone in a metastatic model. However, nimotuzumab displayed single agent activity in primary tumor xenografts, but not in the treatment of metastatic disease. Therefore, combination therapy of continuous low-dose chemotherapy and nimotuzumab was an effective regimen in metastatic, triple negative, EGFR-overexpressing BC xenograft model [90]. Nevertheless, further pre-clinical and clinical trials need to be done to evaluate the efficacy of nimotuzumab, alone or in combination with radiotherapy and chemotherapy, in BC patients.

1.6 Targeted radiotherapeutics for malignancies

In molecular medicine, a promising strategy for treating malignancies that exploits their biological phenotype is targeted in situ radiotherapy. In this approach, mAbs that recognize tumor-associated antigens or peptide ligands that specifically bind to cell surface growth factor receptors, are used as targeting vehicles to deliver radionuclides selectively to cancer cells for radiation treatment. These two approaches are called radioimmunotherapy (RIT) or peptide directed radiotherapy (PDRT). To achieve specific uptake and minimize normal tissue accumulation, the antibody/peptide recognizing epitope/receptor must be expressed uniquely, or at least preferentially, on cancer cells compared with normal cells [98]. One benefit of using RIT is that it is targeted to cancer cells, although there can be significant normal tissue accumulation. In external beam radiation therapy, both normal and cancer cells in the path of the beam are exposed causing damage to non-cancerous cells [36]. Another potential benefit of using RIT is
that it employs an antibody which may have anti-tumor properties itself, such as activating CDC/ADCC or other effects; this causes further damage to cancer cells [34]. In RIT several factors are important to construct an effective treatment. These include the selection of an optimal radionuclide, identification of a promising tumor associated antigen/receptor and selection of an antibody with high specificity and low immunogenicity.

1.6.1 Radionuclides used for RIT

Radionuclides suitable for targeted in situ radiotherapy of tumors emit either α–particles (213Bi, 225Ac and 211At), β-particles (131I, 186Re, 188Re, 90Y, 64Cu, and 177Lu), or Auger and conversion electrons (125I, 123I, 111In, and 67Ga) [94]. α-Emitters are radionuclides having a proton-to-neutron ratio that exceeds that for stable elements of similar atomic number. Thus in order to reduce the repulsive forces between the positively charged protons, α–particles (consisting of two protons and two neutrons with a 2⁺ charge) are emitted, which brings the radionuclide to a more stable configuration. In β-particle decay, the radionuclide either carries excess protons or neutrons. In a neutron-excessive radionuclide, neutrons are converted into protons and a β⁻ particle and antineutrino are emitted. However, in a proton excessive radionuclide, protons are converted into neutrons and a β⁺ particle (positron) and neutrino are emitted. After the emission of beta particles the radionuclide becomes more stable. Generally, in targeted radiotherapy, we are most interested in β⁻ decay and not β⁺ (positron) decay, which is used mainly for PET imaging. For Auger electrons, the radionuclide decays by electron capture (EC). In EC, a proton in the nucleus captures an electron from an inner orbital shell, creating a vacancy in the shell. This vacancy is filled by the decay of an electron from a higher shell. The
excess energy released is transferred to an outer orbital electron, which is then ejected from the atom as an Auger electron, creating a 2\(^+\) charged nucleus.

The important difference between these different forms of radiation is their range in tissue and their linear energy transfer (LET) [94]. The \(\alpha\)-particles have the highest LET (100 keV/\(\mu\)m), are densely ionizing, and travel 50-100 \(\mu\)m (5-10 cell diameters) in tissue. \(\alpha\)-Emitters are best for eradicating small clusters of cancer cells or micrometastasis [94]. \(\beta\)-Particles have high energy and travel 2-12 mm in tissues (200-1200 cell diameters). In comparison with \(\alpha\)-emitters, the average LET of \(\beta\)-particles is quite low; for instance \(\beta\)-particles emitted by \(^{131}\)I (\(E_\beta=0.6\) MeV) have a LET of 0.3 keV/\(\mu\)m over a track length of 2 mm. Moreover, \(\beta\)-particles deposit most of their energy at the end of their track length (Bragg peak). Thus, due to the long range of the \(\beta\)-particle and its small LET, it is possible to kill distant non-targeted tumor or normal cells (cross-fire effect). If these cells are tumor cells, this is advantageous for large lesions i.e. 2-10 mm in diameter in which there is likely to be incomplete targeting of tumor cells by radiolabeled antibodies. However, the cross fire effect contributes to dose-limiting bone marrow toxicity in RIT, due to nonspecific irradiation of the hematopoietic stem cells by circulating radiolabeled antibodies perfusing the marrow [94]. In contrast to high energy \(\alpha\)-emitters (4-9 MeV) which directly deposit their energy over short distances in tissue (40-90 \(\mu\)m) resulting in high LET, Auger electrons have very low energy (<30 keV) [94]. As these very low energy Auger electrons travel an extremely short distance in tissue (nm-\(\mu\)m), their LET approaches that of \(\alpha\)-emitters (100 keV/\(\mu\)m) [94]. The advantage of the Auger electron emitting radionuclide when it is conjugated to a peptide or mAb is that the cells it kills are restricted to those in which binding and internalization occurs (due to the short range and the requirement to cause lethal DNA damage). Moreover, in contrast to \(\beta\)-emitters, Auger electrons lack the “cross-
fire effect”, thus sparing non-targeted cancer and normal cells. Nevertheless, a “bystander effect” and a more local “cross-dose” effect have been reported with Auger electrons [94]. Auger electrons are, therefore more useful for treating small tumor deposits or micrometastases for which delivery of radiolabeled antibodies or peptides is more homogeneous [94].

1.6.2 Current clinical status of RIT

Currently the only radiolabeled antibodies with FDA approval for the treatment of cancer are $^{90}$Y-ibritumomab tiuxetan (Zevalin®, IDEC Pharmaceuticals Corporation, San Diego, CA, USA) and $^{131}$I-tositumomab (Bexxar®, Corixa Corporation, Seattle, WA, USA) [94]. Ibritumomab is a murine anti-CD20 antibody which is conjugated to tiuxetan, an MX-DTPA linker-chelator for $^{90}$Y that forms Zevalin® [94]. $^{90}$Y-ibritumomab tiuxetan emits pure beta radiation. Tositumomab is an IgG$_{2a}$ murine mAb directed against CD20 and labeled with $^{131}$I, which is also a beta emitter [94]. Both these radiopharmaceuticals are used in the treatment of chemotherapy-refractory non-Hodgkin’s lymphoma (NHL) [94]. The target antigen (CD20) for both these antibodies is a 35 kDa transmembrane glycoprotein which functions as a calcium channel and is frequently expressed on both B-cells and B-cell tumors. CD20 appears only as the B-cell is maturing within the bone marrow before the cells are released into the circulation [99]. This provides an excellent specific target of opportunity as the earlier B-cell precursors and, subsequently, differentiated plasma cells are not targeted by anti-CD20 antibodies [95].

Encouraging results have been reported at both higher and lower doses of Bexxar and Zevalin. In several Phase III clinical trials, the efficacy of Bexxar for treatment of chemotherapy-refractory, low-grade or transformed NHL and Zevalin for treatment of relapsed/refractory low-grade, follicular or transformed NHL were evaluated [95]. In a Bexxar study, patients receiving
RIT (1.7-7.8 GBq) had both higher ORR and median duration of response (DR) compared to patients who received only chemotherapy (ORR: 65% and DR: 6.5 months v.s. ORR: 28% and DR: 3.4 months, respectively) [100]. In the case of Zevalin, the ORR was also significantly higher for patients receiving RIT (0.4 mCi/kg) compared to patients receiving only chemotherapy or rituximab (anti-CD20 antibody) (ORR: 80% v.s. 56%, respectively) [100]. Thus RIT for is a promising strategy designed to increase the efficacy of an already effective immunotherapeutic agent (i.e. rituximab) and minimize toxicity by only targeting specific cell types. Nevertheless, future research should explore more potent radionuclides, mAbs targeting alternative epitopes, novel pre-targeted methods for radionuclide delivery, and incorporate clinical trials comparing RIT to more standard approaches alone and in combination to make advances [94].

1.6.3 RIT targeting HER2/neu for BC

HER2/neu is an attractive target for RIT of BC because of the high expression of this receptor in a select group of patients and because promising results have already been reported from the use of unmodified mAbs directed toward this receptor [59]. Studies have found encouraging results for anti-HER2 mAbs radiolabeled with β-emitting radionuclides. In one study, De Santes and colleagues [101] examined the effects of radiolabeled 4D5 anti-HER2 mAb in beige/nude mice bearing NIH3T3 HER-2/neu (HER2 transfected NIH3T3 cells) xenografts. The 4D5 mAb was radiolabeled with $^{131}$I. After treating the mice systemically with 400 μCi they found a marked inhibition of tumor growth. Furthermore, $^{131}$I-4D5 was found to be 20 times more effective than $^{131}$I-labeled non-specific IgG mAb and 75 times more effective than un-labeled 4D5; however, rapid intracellular dehalogenation by deiodinases and the subsequent
export of radioiodine from the cells were observed [102]. In order to avoid *in vivo* limitations of radioiodinated antibodies, researchers have used the bifunctional chelate DOTA to conjugate anti-HER2 mAbs to radiometals such as $^{111}$In, $^{90}$Y and $^{212}$Pb. Thus, in a study by Tsai et al. [103], found that compared to radiohalogen-labeled conjugates (i.e. $^{131}$I), radiometal conjugates (i.e. $^{111}$In- or $^{90}$Y-4D5) have a longer intracellular retention. This may be due to the formation of charged radio-catabolites that have difficulty in penetrating the cell membrane and thus being released from the cells [103]. Furthermore, in biodistribution studies, $^{111}$In-labeled 4D5 had a higher tumor uptake compared to $^{131}$I-4D5 in mice-bearing MCF7/HER2 BC xenografts (30% ID/g versus 17% ID/g). Moreover, the radioactivity that was required to achieve maximum tumor growth inhibition was 4-fold lower for $^{90}$Y-4D5 compared to $^{131}$I-4D5 (100 μCi versus 400 μCi, respectively) [103].

The anti-HER2 antibodies have also been radiolabeled with α-emitting radionuclides. In one study, the effects of radiolabeled trastuzumab on carcinomatous meningitis (CM) (disease characterized by the dissemination of malignant tumor cells into the subarachnoid space along the brain and spine) were investigated [104]. To create a CM model, MCF-7/HER18 cells were injected intrathecally in athymic rats. These rats were then injected with 28 μCi $^{211}$At-labeled trastuzumab, 30 μCi $^{211}$At-labeled TPS3.2 control mAb, or saline. It was found the median survival increased from 20 days to 29 and 36 days for animals treated with saline, $^{211}$At-labeled TPS3.2 and $^{211}$At-labeled trastuzumab, respectively [104]. Furthermore, the median survival was also found to be significantly higher after increasing the dose to 96 μCi $^{211}$At-trastuzumab (96 days) compared to control or trastuzumab (21 days) treated groups [104]. Consequently, intrathecal $^{211}$At-labeled trastuzumab shows promise as a treatment for patients with HER2-positive breast CM.
Finally, HER2 antibodies have been radiolabeled with radionuclides that emit both $\alpha$ and $\beta$ particles. Horak et al. [105] studied the effects of radiolabeled AEI (anti-HER2 mAb). AEI was radiolabeled with $^{212}$Pb. $^{212}$Bi, the $\beta^-$ decay product of $^{212}$Pb, was subsequently the source of both $\alpha$ and $\beta^-$ emission. After radiolabeling AEI with $^{212}$Pb by using DOTA as a chelator, 10-20 $\mu$Ci of $^{212}$Pb/$^{212}$Bi-AEI was injected intravenously in mice bearing SK-OV3 tumor cells. It was demonstrated that $^{212}$Pb/$^{212}$Bi-AEI prevented tumor development in all animals and inhibited the growth of small tumors. Dose-limiting hematopoietic toxicities associated with circulating large amounts of $^{212}$Pb-AE1 mAbs in the blood limited the effectiveness of this agent in the therapy of larger tumors, suggesting that it may be most useful in the adjuvant setting for eradicating micrometastatic disease. Thus, these preclinical studies of HER2 targeted RIT are promising and justify further investigation, so that it can ultimately be employed for treating patients with HER2-positive BC.

1.7 Auger-electron radiotherapy

Auger electron emitting radionuclides such as $^{125}$I and $^{111}$In, represent an appealing alternative to $\alpha$ and $\beta^-$ emitters for targeted radiotherapy of cancer [106]. Most Auger electrons have an energy of <30 keV and a very short subcellular path (2-12 $\mu$m) in tissues. Hence, Auger electron emitters can exert their radiotoxic effects on cells only when internalized into the cytoplasm and, particularly when they are imported into the cell nucleus where they can cause DNA fragmentation and cell death [107, 108]. The decay of an Auger-emitting radionuclide outside the cell or at the cell surface usually delivers an insufficient dose of radiation to cause radiotoxicity [109]. The selective toxicity of Auger electron-emitters only for cells that bind and internalize the agents could, in theory, minimize or even eliminate the non-specific radiotoxicity.
(i.e. cross-fire effect) against bone marrow stem cells that was previously observed with β-emitters in RIT [110].

1.7.1 Targets for Auger electron radiotherapy

Many different molecular targets overexpressed by cancer cells have been exploited for Auger electron radiotherapy using mAbs and peptides as well as small molecules labeled with $^{111}$In, $^{99m}$Tc, $^{123}$I or $^{125}$I.

1.7.2 DNA synthesis as a target

$^{125}$I or $^{123}$I-iododeoxyuridine ($^{125/123}$I UdR) are the most widely studied Auger electron-emitting radiopharmaceuticals [110-115]. IUdR is a thymidine analog that is transported into cells and incorporated directly into DNA during S phase [111]. Studies have found that after intraperitoneal (i.p.) injection of $^{125}$I-UdR [112] or $^{123}$I-UdR [113] into mice with ovarian cancer xenografts, there is 4-5 log reduction in tumor cell mass. Furthermore, administration of $^{125}$I-UdR intrathecally delayed paralysis in mice especially when the radiopharmaceutical was co-administered with methotrexate (MTX), an antimetabolite that increases the uptake of $^{125}$I-UdR in DNA-synthesizing cells. The combination of both these drugs leads to 5-6 logs tumor cell kill and cured 30% of the tumor-bearing mice [114, 115]. Subsequent to these promising results, Kassis et al. [116] administered MTX and $^{125}$I-UdR (1.85 GBq) to a patient with pancreatic cancer metastasis to the CNS who failed to respond to conventional therapy. A dramatic drop in spinal fluid CA19.9 levels was observed after a single treatment that was also accompanied by clinical improvement [116]. Despite the efficacy of this radiopharmaceutical, it has several limitations including lack of specificity to tumor cells and extensive deiodination in the liver.
Nevertheless, $^{125}$I-UdR is currently being investigated for the treatment of bladder cancer [118], gliomas [119], and hepatic metastases [120] in which normal tissue uptake can be minimized by local administration.

### 1.7.3 Estrogen receptor (ER)

The ER translocates from the cytoplasm to the nucleus when bound to estradiol [117-118]. Several studies have explored targeting ER-expressing BC cells with ER-specific ligands, i.e. estrogen, 17-β-estradiol, or tamoxifen labeled with $^{123}$I or $^{125}$I [121, 122]. Beckham et al. [121] showed that when ER-positive cells were treated with 16-α-$^{125}$I-iodo-17-β-estradiol ($^{125}$I-E2) they exhibited DNA strand breaks. Furthermore, the surviving fraction (SF) of ER-positive MCF-7 BC cells decreased 5-fold following exposure to $<0.2$ MBq/mL of $^{125}$I-E2, whereas the SF of an ER-negative MCF-7 subclone variant did not decrease. In another study, DeSombre et al. [122] showed that the estradiol analogue, 2-$^{123}$I-iodo-1,1-bis-(4-hydroxyphenyl)-2-phenylethylene ($^{123}$I-BHPE) (8.9 MBq/pmole) was cytotoxic in vitro to Chinese hamster ovarian (CHO) cells that were gene-transfected to express ER. Furthermore, in subsequent study, a 2-log decrease in the tumor size of MCF-7 xenografts was observed when cells were pre-treated with the estradiol analogue, E-17-α-$^{125}$I-iodovinyl-11-β-methoxyestradiol ($^{125}$I-VME2) [123]. Thus, these promising studies show that ER-positive cells can be successfully targeted using Auger electron therapy.

### 1.7.4 Somatostatin receptors (SSTR)

Somatostatin receptors (SSTRs) are a family of G-protein coupled receptors that specifically bind somatostatin (SMS), a naturally occurring peptide ligand [124, 125]. SMS has
many physiological actions, including inhibition of exocrine and endocrine secretion as well as antiproliferative effects [126]. SSTRs are increased in most neuroendocrine gastroenteropancreatic (GEP) tumors as well as other malignancies including BC, neuroblastoma, and lymphoma [127]. Pentetreotide (Octreoscan®, Mallinckrodt Medical) is a diethylenetriaminepentaacetic acid (DTPA)-derivatized form of octreotide, a SMS analogue that complexes $^{111}$In, either for single photon emission tomography (SPECT) imaging [128] or for targeted Auger electron radiotherapy of SSTR-expressing tumors. In BC, 21-46% of tumors are positive for sst2 (SSTR subtype II) and there is an inverse correlation with expression of EGFR [129]. Because the internalization of native SMS and $^{111}$In-pentreotide is due to receptor-mediated endocytosis, this mechanism has the potential to deliver cytotoxic radiation from $^{111}$In-pentreotide in the cytoplasm to the cell nucleus.

Capello et al. [130] found that the clonogenic survival of CA20948 rat pancreatic cancer cells was virtually eliminated \textit{in vitro} by exposing cells to 37 MBq ($10^{-7}$ mol/L) of $^{111}$In-pentreotide. Furthermore, when $^{111}$In-DTPA which does not bind or internalize into the cells was incubated at similar concentration with these cells, there was no significant effect on the SF. This means that receptor-mediated binding and internalization of $^{111}$In-pentreotide were required for the cytotoxicity of the radionuclide. Similarly, SSTR-mediated, tumor growth inhibition by $^{111}$In-pentreotide was observed \textit{in vivo} in a rat hepatic metastasis model. Administration of 370 MBq of $^{111}$In-pentreotide significantly decreased the number of metastases formed following the inoculation of $5 \times 10^5$ SSTR-positive CA20948 cells into the portal vein of rats. The effectiveness of $^{111}$In-pentreotide was diminished by co-administration of 1 mg of octreotide to block SSTR [131]. Complete remission (CR) was achieved in 50% of rats bearing small ($\leq 1$ cm$^2$) s.c. CA20498 tumors when administered 370 MBq of $^{111}$In-pentreotide in three fractionated
doses (total 1,110 MBq). However, only partial remission (PR) was observed in large (>8 cm²) tumors using this regimen [132].

Following these preclinical studies, Krenning et al. [133] reported treating patients with metastatic neuroendocrine tumors with 111In-pentreotide using doses ranging from 1,590 to 4,810 MBq (10-120 µg) over a period of 10 months. A decrease in the size of liver metastases was observed as well as transient decreases in tumor-associated glucagon and γ-glutamyltransferase levels associated with these tumors. In a much larger clinical trial, Valkema et al. [134] treated 50 patients with mainly carcinoid or medullary thyroid carcinomas with multiple doses of 6,000-11,000 MBq (40-50µg) of 111In-pentreotide separated by 2 weeks. Therapeutic responses were observed in 21 out of 40 evaluable patients, including one PR, 6 minor remissions (MR), and 14 stabilization of previously progressive disease (PD). Similarly, Anothony et al. [135] treated 27 patients with two doses of 111In-pentreotide (6,660 MBq) who had rapidly progressing GEP tumors. It was found that 8% of patients achieved radiological PR and 62% showed significant clinical improvement. Furthermore, Nguyen et al. [136] reported significantly prolonged survival in 20 patients with advanced metastatic tumors treated with 3 monthly doses of 111In-pentreotide (7,000 MBq). Thus, based on the clinical trials described above, it can be concluded that 111In-pentreotide provides stabilization of disease and some PR in previously progressive metastatic SSTR-positive tumors.

1.7.5 Epidermal growth factor receptors (EGFR)

The epidermal growth factor receptor (EGFR) is the first member of the Type I growth factor receptor family [137]. When EGF, a 53-amino acid ligand binds to the EGFR, it causes dimerization and autophosphorylation with subsequent activation of mitogenic signaling.
cascades [138]. Overexpression of EGFR has been detected in many malignancies including cancer of the breast, head and neck, colon, bladder, and lung [134]. In BC, overexpression of EGFR is directly correlated with low ER expression, insensitivity to hormonal therapy and poor long-term survival [139-141]. Thus, EGFR is an attractive target for Auger electron radiotherapy to treat EGFR-positive malignancies, including BC. Our group has shown that exposure to $^{111}$In-DTPA-hEGF (111-112 mBq/cell), an analogue of hEGF labeled with the Auger electron emitter, $^{111}$In, reduced the survival of MDA-MB-468 cells to <3% in vitro [110]. Furthermore a comparison of the growth inhibitory properties in vitro of high specific activity (SA) $^{111}$In-DTPA-hEGF (30 MBq/ug) on MDA-MB-468 cells and commonly used chemotherapeutic agents for BC revealed that the radiopharmaceutical was 85-300 fold more potent on a molar concentration basis. The IC$_{50}$ for $^{111}$In-DTPA-hEGF was <70 pmol/L; in contrast, the IC$_{50}$ values for paclitaxel, methotrexate, and doxorubicin were 6, 15 and 20 nmol/L respectively [142]. Moreover, the cytotoxic affect of $^{111}$In-DTPA-hEGF was also observed in vivo when 5 subcutaneous (s.c.) injections of $^{111}$In-DTPA-hEGF (18.5 MBq, 3.4 ug each) were administered to athymic mice bearing s.c. MDA-MB-468 tumors, which resulted in 3-fold decrease in tumor growth rate compared to untreated mice [143]. However, in a Phase I clinical trial, a relatively low single dose of $^{111}$In-DTPA-hEGF (<2,290 MBq) administered to 16 chemotherapy refractory BC patients did not show any objective tumor response, although there was stabilization of disease in several patients as well as tumor uptake documented by SPECT imaging [144]. Thus, our group is planning to extend this Phase I clinical trial where patients will receive multiple treatment doses as previously studied in mice.

Monoclonal antibodies specific for EGFR have also been studied in vitro using tumor cell cultures and in vivo in tumor xenograft models [145-147]. Michel et al. showed that anti-EGFR
mAb 528 labeled with $^{111}$In or $^{125}$I (1.5 MBq/mL) dramatically reduced the SF of A431 squamous cell carcinoma cells overexpressing EGFR ($2-3 \times 10^6$ receptor/cell) \textit{in vitro} by 97-100\% [148]. Moreover, Mattes and Goldenberg reported that in mice-bearing A431 tumor xenografts, treatment with $^{111}$In-anti-EGFR mAb 525 significantly prolonged survival compared to $^{111}$In-labeled control mAb or no treatment [149]. Targeted Auger electron RIT for EGFR has also been investigated in a clinical trial. Quang et al. administered multiple doses (1,295-3,330 MBq; total 1,480-8,288 MBq) of $^{125}$I-528 mAb to 180 patients with glioblastoma multiforme (GBM) and astrocytomas as adjuvant therapy. A significant increase in the expected survival of these patients was noted with median survival of 4-150 months for GBM and 4-270 months for those with astrocytoma [143]. Taken together, the study described above shows that anti EGFR Auger electron RIT is cytotoxic to EGFR-overexpressing cells \textit{in vitro} and \textit{in vivo} and may be useful for treatment of patients with EGFR-positive tumors to increase their survival.

1.7.6 \textbf{Epidermal growth factor receptor-2 (HER2)}

HER2 is the second member of the Type I transmembrane epidermal growth factor receptor family. As previously discussed, HER2 overexpression is present in about 15-20\% of BC patients as a consequence of gene amplification [19]. Several studies have used HER2 as a target for RIT. Michel et al. [148] demonstrated 100\% killing of HER2-overexpressing SKBR-3 cells \textit{in vitro} by incubation with increasing concentrations (up to 7.4 MBq/mL) of a mixture $^{111}$In-labeled anti-HER2 mAbs 21.1 and 4D5 (murine analogue of trastuzumab) and mAbs against epithelial glycoprotein-2 (EGP-2). The cytotoxicity of these $^{111}$In-labeled EGP-2 and HER2 mAbs was more than 4 logs greater than for $^{111}$In-labeled non-reactive mAbs. In a follow up \textit{in vivo} study, Mattes and Goldenberg reported significantly prolonged survival in mice.
bearing HER2 positive s.c. SK-OV-3 tumor xenografts treated with 59 MBq of $^{111}$In-4D5 compared to mice receiving the same dose of an irrelevant $^{111}$In-labeled control mAb [149].

Recently our group has also studied Auger electron RIT of HER2-amplified BC using $^{111}$In-trastuzumab modified with 13-mer peptides [CGYGPKKKRKVVGG] that harbor the nuclear translocation sequence (NLS; underlined) of SV-40 large T antigen which promotes its nuclear importation following HER2-mediated internalization into BC cells [150, 151]. In this study, it was verified through confocal fluorescence microscopy that trastuzumab-NLS localized in the nucleus following 24 hr incubation, whereas trastuzumab was mostly membrane bound. Also, greater DNA damage and cell killing was observed in BC cells exposed to $^{111}$In-trastuzumab-NLS than $^{111}$In-trastuzumab or trastuzumab. Furthermore, $^{111}$In-trastuzumab-NLS was 2-5 fold more potent at killing cell lines such SKBR3 (HER 3+) and MDA-MB-361 (HER 2+) cells than $^{111}$In-trastuzumab. More importantly, $^{111}$In-trastuzumab-NLS was 6-fold and 3-fold significantly more potent at killing SKBR3 and MDA-MB-361 cells, respectively than unlabeled trastuzumab. More intriguingly, cell lines that were resistant to trastuzumab were sensitive to $^{111}$In-trastuzumab-NLS. It was found that trastuzumab-resistant, TrR1 cells were efficiently killed by $^{111}$In-trastuzumab-NLS whereas trastuzumab had very little effect on these cells [150, 152]. These findings show that the efficacy of an antibody on resistant cells can be increased by radiolabeling it with an Auger electron emitter and conjugating with an NLS sequence. This may similarly be a useful method for targeting trastuzumab-resistant cell lines that gain resistance by overexpressing other members of the EGFR family such as the EGFR itself.

1.8 Nuclear localizing sequences (NLS)

Several antibodies and other macromolecules that are targeted to kill cancer cells are unable to translocate into the nucleus readily, therefore, nuclear importation can be encouraged by
conjugation to a peptide containing an NLS [153-155]. This is an especially useful feature for Auger electron RIT because it enables the radionuclide to be in close proximity to the DNA so that the nanometer-range electrons can cause DNA damage [156]. Typical NLS peptide motifs possess a cluster of four or more cationic residues [i.e. lysine (K), or arginine (R)] and are categorized as either monopartite containing a single cluster of basic amino acids, or bipartite containing two clusters of basic amino acids separated by a linking sequence of 10-12 unconserved amino acids. The prototypical monopartite NLS is demonstrated by the SV-40 large-T antigen NLS (\textsuperscript{126}PKKKRKV\textsuperscript{132}), whereas the NLS of the xenopus phosphoprotein nucleoplasmin (KRPAATKKAGQAKKKKL\textsuperscript{170}) is defined as bipartite NLS. Mutagenesis studies have shown that substitution in the key cationic peptide sequence compromises the nuclear importation ability of the peptide [156].

Carrier proteins belonging to the karyopherin/importin family recognize NLS. Importin-\(\alpha\) directly binds to the NLS of the cargo protein, and forms a heterotrimeric complex when further recognized by importin-\(\beta\). This complex interacts with the hydrophobic repeat domains of nucleoporins that constitute the nuclear pore complexes (NPCs). Translocation of the importin-cargo complex across the nuclear pore is regulated by a small GTPase, Ran, which binds importin-\(\beta\) and induces the release of the cargo protein into the nucleoplasm. Importin-RanGTP complexes are then exported back to the cytoplasm where they are dissociated [156].

1.8.1 Targeting nuclear DNA with Auger electron-emitting radiopharmaceuticals

Introduction of NLS to peptides, small molecules, mAbs and oligonucleotides can direct Auger-electron emitting radionuclides into the nucleus of cancer cells. For example, Chen et al. employed this approach for the treatment of acute myelogeneous leukemia (AML) using the anti-
CD33 mAb HuM195. Introduction of as many as 12 SV-40 peptides (CGYGPKKKRKVEG) harboring the NLS of the SV-40 large antigen (underlined) into the HuM195 mAb, promoted the nuclear translocation of the radiolabeled mAb into the nucleus. Thus, nuclear uptake of $^{111}$In-HuM195-NS greatly enhanced the toxicity of the emitted Auger electrons in leukemic cells [157].

Similarly Ginj and colleagues [158] found that an $^{111}$In-labeled somatostatin analog Tyr$^3$-octreotide (TOC) ($^{111}$In-NLS-DOTA-TOC) exhibited an enhanced cellular uptake and a 6-fold increase in the cellular retention compared to its parent compound in SSTR-positive rat AR4-2J pancreatic tumor cells, as well as in human embryonic kidney cells transfected with SSTR subtype 2A (HEK-SSTR2A). Furthermore, the nuclear uptake of $^{111}$In-NLS-DOTA-TOC was increased 45-fold, while the peptide conjugate with no NLS ($^{111}$In-DOTA-ahx-PKKKRKV) had no uptake in both rat (AR4-2J) and human (HEK-SSTR2A) cell lines, thereby indicating the prerequisite of specific binding to the SSTR for nuclear importation [158].

Recently, our group also conjugated $^{111}$In-labeled trastuzumab with NLS and found it to be 6-fold and 3-fold significantly more potent at killing SKBR3 (HER2 3+) and MDA-MB-361(HER2 2+) cells, respectively, than unlabeled trastuzumab. Furthermore, $^{111}$In-trastuzumab-NLS demonstrated significantly higher γ-H2AX foci compared to $^{111}$In-trastuzumab or trastuzumab-treated groups. This means that the NLS sequence facilitated the nuclear transportation of $^{111}$In-trastuzumab and increased the cytotoxicity of the Auger electrons in HER2-positive BC cells [150]. In another study, a 12-mer peptide (PKKKRKVEDPYC), harboring the NLS of SV-40 large-T antigen, was conjugated to an $^{125}$I-labeled TFO (Triplex-Forming Oligodeoxynucleotide) specific for the mdr-1 gene to promote its translocation to the
nucleus of KB-V1 cells [159]. NLS conjugation markedly enhanced NLS nuclear uptake and gene-specific strand breaks within intranuclear DNA.

It is possible, however, that the non-specific cell-membrane penetrating potential of cationic NLS-peptides could increase nonspecific toxicity. Therefore, further evaluation is required to determine the extent to which this approach will improve the clinical efficacy of Auger-electron radiotherapy. Nevertheless, NLS-conjugation represents a novel strategy that can i) overcome the challenge of delivering radiolabeled antibodies and peptides into the nucleus of cancer cells, and ii) dramatically enhance the effectiveness of targeted Auger electron-emitting radiotherapeutics for malignancies [156].

1.9 Hypothesis of the thesis

In BC patients, the rate of primary resistance to single-agent trastuzumab for HER2-overexpressing metastatic BC is 66-88% [39, 40] with a median survival of 9 months [57]. In some instances, this resistance is related to overexpression and increased signaling from other epidermal growth family receptors such as EGFR (HER1), HER3 and HER4 [53,57,74,75]. In order to overcome this resistance, these growth factor receptors can be targeted. Therefore, the hypothesis of this thesis was that radiotherapeutically targeting EGFR with $^{111}$In-labeled anti-EGFR nimotuzumab mAbs modified with NLS peptides could overcome resistance to trastuzumab in BC cells that are intrinsically resistant due to minimal or low HER2 expression or that acquire such resistance through increasing their EGFR expression.
1.10 Specific Aims

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

**Aim 1:** To construct and characterize $^{111}$In-nimotuzumab-NLS *in vitro* by analytical techniques that measures its purity and homogeneity and immunoreactivity.

**Aim 2:** To measure the internalization and nuclear importation of $^{111}$In-nimotuzumab-NLS in a panel of trastuzumab-resistant BC cells and compare this with that of $^{111}$In-DTPA-hEGF.

**Aim 3:** To quantify the *in vitro* cytotoxicity of $^{111}$In-nimotuzumab-NLS in a panel of trastuzumab-resistant BC cells that display EGFR and compare this to that of $^{111}$In-DTPA-hEGF.

The experimental approach, results and discussion for these aims is described in Chapter 2, while directions for future research are described in Chapter 3.
Chapter 2

$^{111}$In-labeled nimotuzumab modified with nuclear localization sequences (NLS): An Auger electron-emitting radiotherapeutic agent for EGFR-overexpressing and trastuzumab-resistant BC.

All experiments and analyses of data were carried out by Aisha Fasih, except for the analysis of $\gamma$H2AX assay and radiation absorbed dose (Cai Z).
2.1 Introduction

The Canadian Cancer Society predicted that there will be over 22,400 new cases of breast cancer (BC) diagnosed in 2009, and more than 5,300 deaths due to this disease [1]. Current treatment regimens, including hormonal, radiation and chemotherapy, can increase survival. However the doses of these agents are limited by their potential to cause serious side-effects. Therefore, there is a need to develop more selective agents for targeted therapy which are more specific for cancer cells. Targeted therapy can be achieved by using recombinant monoclonal antibodies (mAbs) that bind to cancer cells overexpressing tumor-associated antigens. Trastuzumab (Herceptin®, Genentech, CA), is a humanized IgG1 anti-HER2 monoclonal antibody (mAb) approved for immunotherapy of HER2-amplified BC [51]. Despite its effectiveness, patients who respond to this drug initially, become refractory within a year due to the emergence of resistance [40]. One of the proposed mechanisms of resistance is an increased signaling from other EGFR Type I growth factor receptor tyrosine kinases including the EGFR itself [12]. Furthermore, it has also been found that EGFR expression is positively correlated with disease progression in patients receiving trastuzumab [80]. Therefore, our aim was to target EGFR to overcome resistance to trastuzumab in BC cells.

In the past our laboratory has developed a novel radiotherapeutic agent, $^{111}$In-labeled human EGF ($^{111}$In-hEGF) which exploits the overexpression of EGFR found in 30-50% of cases of ER-negative, hormone resistant and poor prognosis BC [140, 160]. $^{111}$In-hEGF was internalized in the nucleus of EGFR-positive MDA-MB-468 BC cells and exhibited potent cytotoxic effects in vitro alone/or in combination with chemotherapeutic drugs as well as the EGFR tyrosine kinase inhibitor, gefitinib [142, 161]. Furthermore, $^{111}$In-hEGF significantly inhibited the growth of established s.c. MDA-MB-468 tumours in athymic mice [161]. Despite
its effectiveness, $^{111}$In-hEGF showed rapid blood clearance which minimized the amount accumulating in tumors compared to an $^{111}$In-labeled anti-EGFR antibody[162]. Thus, to prevent rapid elimination and provide greater tumor uptake, it would be advantageous to use a larger molecule that is specific to EGFR.

Nimotuzumab is a humanized IgG1 mAb (h-R3) (YM BioSciences Inc., Mississauga) that targets EGFR and is currently in clinical trials for treatment of EGFR-overexpressing tumors. Competition assays show that nimotuzumab binds to domain III of the extracellular region of the EGFR, within an area that overlaps with both the surface patch recognized by cetuximab (another anti-EGFR antibody) and the binding site for EGF, thus blocking ligand binding sites [163], and therefore inhibits mitogenic signaling [90]. More importantly, it rarely causes Grade 4 radiation dermatitis or Grade 3-4 acneform rash, which is a severe and dose limiting side-effect observed for many other antibodies and small molecules (gefitinib) targeting the EGFR tyrosine kinase signaling pathway [91]. Thus the proven efficacy of nimotuzumab in an unlabeled form in clinical trials makes it a promising candidate to develop a novel radioimmunotherapeutic (RIT) agent that targets cells overexpressing EGFR.

Recently, our laboratory reported $^{111}$In-labeled trastuzumab modified with peptides containing the NLS of SV-40 large T antigen for Auger electron RIT of HER2-amplified BC. It was verified through confocal fluorescence microscopy that trastuzumab-NLS localized in the nucleus of HER2-positive BC cells following 24 hours incubation, whereas trastuzumab was mostly membrane bound. There was greater DNA damage and cell killing observed in BC cells exposed to $^{111}$In-trastuzumab-NLS compared to $^{111}$In-trastuzumab or trastuzumab. Furthermore, $^{111}$In-trastuzumab-NLS was 6-fold and 3-fold significantly more potent at killing HER2-positive SKBR3 and MDA-MB-361 human BC cells, respectively than unlabeled trastuzumab [151, 152].
More intriguingly, cell lines that were resistant to trastuzumab such as TrR1 were responsive to $^{111}$In-trastuzumab-NLS. However, a second trastuzumab resistant cell line (TrR2) was less sensitive to $^{111}$In-trastuzumab-NLS due to 10-fold lower HER2 density [164]. It was speculated that the reason $^{111}$In-trastuzumab-NLS can kill trastuzumab resistant cells is that it’s binding, internalization and nuclear translocation properties are preserved, thus causing lethal radiation damage, despite the existence of resistance pathways that block cell growth-inhibition by the drug.

Thus, these findings suggest that it is possible to increase the potency of effective immunotherapeutic agents by radiolabeling them with an Auger electron emitter such as $^{111}$In and conjugating with a peptide harbouring an NLS sequence. These RIT agents can be used to target HER2 on cells that are either intrinsically resistant or acquire resistance to trastuzumab. In this study, our aim was to take an alternative approach aimed at targeting EGFR on BC cells. In BC cells an inverse relationship is present between HER2 and EGFR expression [75] however, 5% of patients have tumors with high densities of both these receptors [76]. EGFR has been shown to contribute to trastuzumab resistance [77]. Studies have observed higher activity and expression of EGFR in HER2 overexpressing BC cells that are resistant to trastuzumab [70, 75]. Furthermore, EGFR overexpression has been correlated clinically with disease progression in patients receiving trastuzumab [80]. Thus, BC cells that are intrinsically resistant to trastuzumab due to low HER2 density or those that have acquired trastuzumab resistance through upregulation of EGFR expression can be targeted using nimotuzumab labeled with $^{111}$In and modified with NLS peptides. Due to its higher molecular weight, this agent is expected to have a longer half-life in the plasma and greater tumor uptake than $^{111}$In-hEGF, making it potentially more potent and lethal towards EGFR positive BC tumors that show resistance to trastuzumab.
2.2 Materials and Methods

2.2.1 Cell culture

MDA-MB-468, MDA-MB-231 and MCF-7 human BC cell lines were obtained from the American Type Culture Collection (ATCC). Trastuzumab-resistant 1 and 2 (TrR1 and TrR2) cells were isolated from 231-H2N tumors in athymic mice with acquired trastuzumab resistance [165]. 231-H2N cells are MDA-MB-231 cells transfected with HER2 receptors and exhibit sensitivity towards trastuzumab. TrR1 and TrR2 cell lines were kindly provided by Dr. Robert S. Kerbel (Sunnybrook Health Sciences Centre). MDA-MB-231, MCF-7, TrR1, and TrR2 were grown in Dulbecco’s modified Eagle’s medium with 10% FBS and 1% Penstrep (Invitrogen, California), whereas MDA-MB-468 were maintained in RPMI 1640 with 10% FBS and 1% Penstrep. MDA-MB-468 cells have the highest number of EGFR (10^6 receptors/cell), MDA-MB-231 and TrR1 have a moderate number of EGFR (10^5 receptors/cell) and MCF-7 has the lowest number of EGFR (10^4 receptors/cells). The number of EGFR on TrR2 cell line is unknown.

2.2.2 111In-nimotuzumab-NLS

Nimotuzumab (h-R3), a humanized IgG1 mAb (YM-Biosciences, Mississauga, ON) was derivatized with benzyl-isothiocyanate diethylenetriaminepentaacetic acid (SCN-Bn-DTPA) (Macrocylics, Dallas, TX) for complexing with 111In. Briefly, nimotuzumab (1 mg, 7-12 mg/mL) was reacted with 15-fold molar excess of SCN-Bn-DTPA (for 1 h at room temperature (RT) in sodium bicarbonate buffer (0.05 M), pH 8.4 and then purified on a Sephadex-G50 minicolumn (Sigma-Aldrich, St Louis, MO) eluted with phosphate-buffered saline (PBS), pH 7.6. Fractions 8-15 containing 800ul of the purified nimotuzumab-Bn-DTPA was collected. The purified
nimotuzumab-Bn-DTPA was then conjugated to synthetic 13-mer NLS-peptides (CGYGPKKKRKVG) synthesized by the Advanced Protein Technology Centre (Hospital for Sick Children, Toronto, ON) using a heterobifunctional cross-linking agent, sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC; Pierce, Waltham, MA). Briefly, Bn-DTPA-nimotuzumab (0.5-5 mg, 7-12 mg/mL in PBS, pH 7.6) was reacted with a 20-fold molar excess of SMCC (3-5 mmol/L) at RT for 1 h, then purified on a Sephadex-G50 minicolumn eluted with PBS pH 7.0. The maleimide-derivatized bn-DTPA was concentrated on a YM-50 Microcon ultrafiltration device (Millipore, Billerica, MA). The purified Bn-DTPA-nimotuzumab-SMCC (3-5 mg, 7-12 mg/ml) was then reacted with a 60-fold molar excess of NLS-peptides in PBS, pH 7.0 overnight at 4ºC. Bn-DTPA-nimotuzumab-NLS was purified on a Sephadex-G50 minicolumn eluted with 1 M sodium acetate pH 6.0. Bn-DTPA-nimotuzumab-NLS and Bn-DTPA-nimotuzumab (50 -100 µg) was labeled with 111In-acetate (50-100 MBq) for 1 h at RT. 111In-acetate was prepared by mixing 111In-chloride (Nordion, Inc, Vancouver, B.C) with 1.0 M sodium acetate, pH 6.0. After incubation, the radioimmunoconjugate was purified on a Sephadex-G50 minicolumn. The radiochemical purity was routinely >98% as determined by instant thin layer silica-gel chromatography (ITLC-SG; Pall Corp. Port Washington, NY) developed in 100 mmol/L sodium citrate, pH 5.0. Radioactivity on the ITLC strip was measured by cutting the strip in two sections at Rf = 0.5 and placing these in using an automatic γ-well-counter to assess radiochemical purity (RCP) (Rf 111In-nimotuzumab-NLS/111In-nimotuzumab: 0.0; free 111In /111In-Bn-DTPA: 1.0) (Wizard 3, PerkinElmer, Waltham, MA). For subsequent descriptions, 111In-Bn-DTPA-nimotuzumab will be represented as 111In-nimotuzumab, and 111In-Bn-DTPA-nimotuzumab-NLS as 111In-nimotuzumab-NLS.
2.2.2 Characterization of $^{111}$In-nimotuzumab-NLS

The purity and homogeneity of nimotuzumab-NLS immunoconjugates were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 6% gel. NLS conjugation to the antibody was confirmed by the shift in the band for nimotuzumab-NLS relative to nimotuzumab on the SDS PAGE gel. The migration distance of the bands relative to the front ($R_f$) was measured and was compared to a plot of the molecular weight ($M_r$) versus $R_f$ for standard $M_r$ reference markers (10-250 kDa; Fermentas, Burlington, ON). Alternatively, the number of NLS-peptides incorporated into nimotuzumab was quantified by incorporating a trace amount of $^{123}$I-labeled NLS-peptides into the conjugation reaction, and then measuring the proportion of the bound radioactivity following purification, and multiplying by the peptides-to-nimotuzumab molar ratio used in the reaction. NLS peptides were radiolabeled to a specific activity of 3-4 MBq/mg with $^{123}$I-sodium iodide (Nordion, Inc, Vancouver, B.C) using the Iodogen® method [157]. The radiochemical purity of $^{123}$I-labeled NLS-peptides was >95% as determined by paper chromatography developed in 85% methanol ($R_f$ $^{123}$I-NLS-peptides: 0.0; $^{123}$I iodide: 1.0). The number of Bn-DTPA was quantified by running an ITLC on an unpurified $^{111}$In-nimotuzumab sample. After running the ITLC the percentage of the radioactivity achieved by $^{111}$In-nimotuzumab is divided by the molar ratio of Bn-DTPA, which was used in reaction with nimotuzumab (i.e 15-fold molar excess) ($R_f$ $^{111}$In -nimotuzumab: 0.0; free $^{111}$In/$^{111}$In-Bn-DTPA: 1.0).

The immunoreactivity of $^{111}$In-nimotuzumab-NLS was evaluated in a competition receptor-binding assay as well as in a direct receptor-binding assay. For the competition assay, approximately $1 \times 10^5$ MDA-MB-468 cells were grown in 12 well plates for 24 h in culture medium. After a gentle rinse with normal saline, the cells were incubated with $^{111}$In-
nimotuzumab-NLS (125 nM) in the presence of unlabeled nimotuzumab (0-7,500 nM) in 400 µL of PBS containing 0.1% bovine serum albumin (BSA) at 4ºC for 3 h. The cells were rinsed to remove unbound $^{111}\text{In}$ then dissolved in 0.1 M NaOH to measure the cell-bound radioactivity. The dissolved cell suspensions were collected and the radioactivity was measured in a gamma-counter. The proportion of $^{111}\text{In}$-nimotuzumab-NLS bound to MDA-MB-468 cells displaced vs. increasing concentrations of nimotuzumab was plotted by Origin® v.6.0 (Microcal Software, Inc., Northampton, MA) and the curves fitted to a 1-site competition model to determine the dissociation constant ($K_d$). The equation used to model this curve is $Y=\text{binding and } X=\log(\text{concentration})$ and $Y= \text{Bottom } + \left(\frac{\text{Top} - \text{bottom}}{1 + 10^{(x-\text{LogEC50})}}\right)$.

A direct (saturation) receptor-binding assay was also used to evaluate immunoreactivity as well as to measure the number of EGFR on MDA-MB-468 and the other cell lines. Approximately $10^4$ cells were incubated at 4ºC with $^{111}\text{In}$-nimotuzumab-NLS or $^{111}\text{In}$-nimotuzumab in concentrations ranging from 0 to 125 nM in 400 µL of normal saline for 3 h. After incubating, the cells were gently rinsed with cold normal saline to remove unbound radioactivity. The cells were dissolved using 0.1 M NaOH and collected to measure the cellular-bound radioactivity in the gamma-counter. The assay was performed in the absence (total binding) or in the presence of unlabeled nimotuzumab (nonspecific binding) at a 60-fold molar excess compared to the radiolabeled immunonoconjugate (0-7,500 nM). Subtraction of non-specific binding from total binding yielded specific binding (nmol/L), which at saturation represented the maximum number of binding sites on the cells ($B_{\text{max}}$) assuming 1:1 binding of nimotuzumab-to-receptor. Similarly, the number of EGFR was determined for MDA-MB-231 and TrR1 cell lines, however, in these cases, $^{111}\text{In}$-nimotuzumab was used to perform the assay. EGFR on the MCF-7 cell line were quantified by using $^{111}\text{In}$-hEGF as described above. The receptor binding curves
for all cell lines were fitted by using Prism Version 4 (GraphPad, SanDeigo, CA) to a 1-site saturation binding model to estimate the dissociation constant ($K_d$) and $B_{\text{max}}$. The equation used to model the saturation binding curve was specific binding = $(X \times B_{\text{max}} / K_d + X)$, non-specific = $NS \times X$ and total binding ($Y$) = specific + non-specific. Finally, a one point binding assay was performed on TrR2 cells with $^{111}\text{In}$-nimotuzumab (150 nM) in the presence, and absence of unlabeled nimotuzumab (7,500 nM) to evaluate the specific binding to these cells and quantify the number of EGFR.

2.2.3 Internalization and Nuclear Importation Assays

The internalization and nuclear importation of $^{111}\text{In}$-nimotuzumab-NLS and $^{111}\text{In}$-nimotuzumab in BC cells was evaluated and compared to that of $^{111}\text{In}$-hEGF. Approximately $0.5 \times 10^6$ cells were incubated at 37°C with agitation in Eppendorf tubes containing $^{111}\text{In}$-nimotuzumab or $^{111}\text{In}$-nimotuzumab-NLS in 500 µL of PBS. After 4, 6 and 24-h time points, the cells were centrifuged and the medium was removed. Cells were rinsed with PBS, pH 7.5 to remove unbound radioactivity, then re-suspended in a 500 µL mixture of 200 mmol/L sodium acetate and 500 mmol/L of sodium chloride, pH 2.5 (stripping buffer) for 10 min at room temperature. Cells were then re-centrifuged and the supernatant (membrane bound radioactivity) was collected. Cells were re-suspended in 1 mL of Nuclei Ez-lysis buffer (Sigma-Aldrich) and incubated for 22 min at room temperature to lyse the cells. After incubation, nuclei (pellets) were separated from the cytoplasmic fraction (supernatant) by centrifugation for 5 min at 3,000 $\times g$, and the radioactivity in each fraction was measured in a gamma-counter. We previously determined that this cell fractionation procedure yields a pure nuclear fraction[166]. Cell fractionation procedures were carried out at both 4°C and in the presence of unlabeled
nimotuzumab to determine if the process was receptor-mediated. Assays were performed on MDA-MB-468, MDA-MB-231, TrR1, TrR2, and MCF-7 cell lines. The cell fractionation results were expressed as nanomoles of antibody localized in each cellular fraction. Analogous studies were also carried out for $^{111}$In-hEGF on MDA-MB-468 cells at 4 h incubation at 37 °C for comparison with $^{111}$In-nimotuzumab-NLS.

2.2.4 Clonogenic survival assays

The effect of $^{111}$In-nimotuzumab-NLS or $^{111}$In-nimotuzumab on the clonogenic survival of BC cells was studied and compared to that for $^{111}$In-hEGF treatment. Approximately $10^6$ cells were plated in T-25 flasks and cultured overnight. The cells were then rinsed with normal saline and incubated with 2 nM, 9.5 nM, 20 nM, or 35 nM of $^{111}$In-nimotuzumab-NLS (350 ± 9.50 MBq/mg) and $^{111}$In-nimotuzumab (320 ± 14.0 MBq/mg) in 5 mL of growth medium for 3 days at 37°C. Controls consisted of cells cultured with medium alone or medium containing unlabeled nimotuzumab. Cell were then trypsinized and rinsed twice with medium. Sufficient cells were plated in triplicate in T-25 flasks to obtain a measureable number of colonies after culturing in medium at 37°C for 7-14 days. Colonies were stained with methylene blue and colonies with $\geq 50$ cells (or $\geq 0.10$ mm for MDA-MB-468 cells) were counted. The clonogenic survival (CS) was calculated by dividing the plating efficiency (PE) for treated cells by that for untreated cells. The PE was determined by dividing the number of colonies formed by the number of cells seeded into each dish. Survival graphs were obtained by plotting the CS values vs. the concentration (nM) of radioimmunoconjugates or unlabeled antibodies used. Clonogenic survival assays were also performed for MDA-MB-231, TrR1, TrR2, and MCF-7 cell lines using similar conditions. Finally, clonogenic survival assays were performed with $^{111}$In-hEGF on MDA-MB-468 cells to
compare with $^{111}$In-nimotuzumab-NLS at similar concentrations and radioactivities (2 nM /0.45 MBq and 10 nM /0.75 MBq).

### 2.2.5 Evaluation of DNA Damage by confocal immunofluorescence microscopy

An initial response of cells to ionizing radiation (including Auger electrons) is the accumulation and phosphorylation of histone-H2AX at serine-139 (γH2AX) at sites of DNA-double strand breaks (DSBs). This damage can be identified as discrete foci in the nucleus by using γH2AX-specific antibodies [167]. To assess this DNA damage mechanism of cytotoxicity 10 nM / 0.75 MBq of $^{111}$In-nimotuzumab-NLS and $^{111}$In-hEGF were used to treat MDA-MB-468 cells. For controls, cells were either incubated with medium only or medium containing unlabeled hEGF or nimotuzumab-NLS. In order to examine effects of the conjugate, cells were seeded on chamber slides (Nunc, Life Technologies) overnight at 37°C in growth medium. The next day, the medium was removed and cells were incubated for 4 h suspended in 400 µL of medium containing various treatments ($^{111}$In-nimotuzumab-NLS, $^{111}$In-hEGF, nimotuzumab-NLS, hEGF and medium only) at 37°C. After incubation, the medium was removed and cells were rinsed twice with normal saline and fixed with 2% paraformaldehyde containing 0.5% Triton X-100 in PBS, pH 8.2 for 15 min. After three 10 minute washes with PBS, pH 7.5 containing 0.5% BSA and 0.2% Tween-20, the cells were permeabilized for 15 mins with PBS, pH 8.2 containing 0.5% Nonidet P-40, and blocked for 1 h in 2% BSA and 1% donkey serum. The chamber slides were then incubated with anti-phospho-γH2AX antibodies (1:800, Upstate Biotechnology) in 3% BSA for 1 h at 37°C. After three 10-minute rinses with PBS, pH 7.5 containing 0.5% BSA and 0.2% Tween-20, the cells were probed with AlexaFluor-488 anti-mouse IgG (Molecular Probes, Invittrogen) for 45 mins at room temperature. The chamber
slides were mounted with Vectashield® mounting media containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) to counterstain the cell nucleus. Images were taken with an inverted LSM510 confocal microscope (Carl Zeiss) at the Advanced Optical Microscopy Facility (Princess Margaret Hospital, Toronto, ON). Excitation at 364 nm and 488 nm was used for visualization of DAPI and AlexaFluor-488 using 385-470 nm and 505-550 filters, respectively. For imaging of γH2AX foci, a 1.2 µm slice was acquired and saved into LSM-Viewer software (v.3.5.0.0376, Zeiss). The γH2AX foci were expressed in terms of integrated density of the foci (area of all γH2AX foci × mean density pixel)/unit area of the nucleus [168].

2.2.6 Statistical Methods

Data were presented as mean ± standard error of the mean (SEM). Statistical comparisons were made using Student’s t-test. P < 0.05 was considered significant.

2.3 Results

2.3.1 Characterization of 111In-nimotuzumab-NLS

There were 4.2 ± 0.09 Bn-DTPA groups per nimotuzumab molecule when reacted with SCN-Bn-DTPA at a molar ratio of 15 and protein concentration of 12 µg/µL (Table 2.3.1). SDS-PAGE revealed an increase in the apparent molecular weight of nimotuzumab from 196 ± 5 kDa to 230 ± 7 kDa, when nimotuzumab was reacted with a 15-fold excess of SMCC then reacted with a 60-fold excess of NLS peptides (Figure 2.3.1A). The shift in the SDS PAGE reveals NLS-peptide conjugation to nimotuzumab, but the small shift in the band was not considered accurate to quantify the number of peptides introduced. Therefore we used the 123I-NLS peptide
substitution method to quantify number of NLS peptides on nimotuzumab. Using this method, there were $5.0 \pm 0.4$ NLS peptides per molecule of nimotuzumab (Table 2.3.1A). When radiolabeled with $^{111}\text{In}$, the specific activity was $350 \pm 9.50 \text{ MBq/mg}$ and the radiochemical purity was $99.23 \pm 0.12\%$.

**Table 2.3.1A:** The following table shows the characteristics of $^{111}\text{In}$-nimotuzumab-NLS.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>$^{111}\text{In}$-nimotuzumab-NLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN-Bn-DTPA/mAb</td>
<td>$4.2 \pm 0.1$</td>
</tr>
<tr>
<td>NLS peptides/mAb</td>
<td>$5.0 \pm 0.4$</td>
</tr>
<tr>
<td>Specific activity</td>
<td>$350 \pm 9.50 \text{ MBq/mg}$</td>
</tr>
<tr>
<td>Radiochemical purity</td>
<td>$99.23 \pm 0.12%$</td>
</tr>
</tbody>
</table>
**Figure 2.3.1A**

**Figure 2.3.1B**

**Figure 2.3.1:** (A) SDS PAGE (6%) gel with coomassie staining (BioRad, Biosciences, Hercules, CA) to visualize the bands. The SDS PAGE gel shows a band shift associated with the conjugation of nimotuzumab to NLS peptides. Lane 1 shows the protein ladder (Page ruler™ stained, Fermentas Life Sciences). Lane 2 shows unconjugated nimotuzumab (molecular weight: 196 kDa), and Lane 3 shows NLS-nimotuzumab (apparent molecular weight: 230 kDa). (B) The graph shows the plot of log molecular weight ($M_r$) for standard reference markers (10-250 kDa; Fermentas, Burlington, ON) versus the migration distance of the bands relative to the front ($R_f$). The graphs was plotted to obtain the standard equation of the line. The $R_f$ values of nimotuzumab and nimotuzumab-NLS were used in this standard equation to evaluate the apparent molecular weight of the conjugate (nimotuzumab and nimotuzumab-NLS).
2.3.2 Immunoreactivity of the conjugates

In competition receptor-binding assays using MDA-MB-468 cells, $^{111}$In-nimotuzumab-NLS was displaced by increasing concentrations of unlabeled nimotuzumab (Figure 2.3.2C). The dissociation constant measured in this competition binding assay was $17.0 \pm 0.02$ nM. This value was close to the previously reported $K_d$ value for nimotuzumab ($K_d=1 \times 10^{-9}$ M) [90] and to that determined by a direct receptor-binding assay ($K_d = (14.0 \pm 0.64) \times 10^{-9}$ M). These results indicate that the immunoreactivity of $^{111}$In-nimotuzumab-NLS for EGFR was conserved.

The direct receptor-binding assay showed saturation of binding sites by both $^{111}$In-nimotuzumab and $^{111}$In-nimotuzumab-NLS (Figure 2.3.2A and 2.2B). The dissociation constant ($K_d$) of $^{111}$In-nimotuzumab-NLS and $^{111}$In-nimotuzumab in MDA-MB-468 cells ($13.0 \pm 0.58$ nM vs. $14.0 \pm 0.64$ nM). There was no significant difference between the $B_{\text{max}}$ of $^{111}$In-nimotuzumab-NLS and $^{111}$In-nimotuzumab for approximately $3 \times 10^5$ MDA-MB-468 cells ((1.2 $\pm$ 0.04) $\times$ 10$^{-3}$ nmol and (1.3 $\pm$ 0.06) $\times$ 10$^{-3}$), respectively ($P > 0.05$). The binding site density on MDA-MB-468 cells assessed by $^{111}$In-nimotuzumab-NLS was (2.4 $\pm$ 1.7) $\times$ 10$^6$ receptors/cell and by $^{111}$In-nimotuzumab was (2.5 $\pm$ 1.3) $\times$ 10$^6$ receptors/cell. The direct receptor-binding assay showed that the binding of nimotuzumab to EGFR was conserved after conjugation with NLS and Bn-DTPA and radiolabeling with $^{111}$In. The number of EGFR, $K_d$ and $B_{\text{max}}$ for MDA-MB-231, TrR1 and MCF-7 cells were also determined (Table 2.2). The number of EGFR on TrR2 cells could not be determined using a saturation curve. Therefore, a one-point binding assay at an excess of $^{111}$In-nimotuzumab was performed to assess specific binding (Figure 2.3.2D). This one point binding assay revealed that the specific binding of $^{111}$In-nimotuzumab to TrR2 cells was very low.
Figure 2.3.2: Direct receptor-binding assay with increasing concentration of $^{111}$In-nimotuzumab (0-150 nM) in MDA-MB-468 cells (A) or $^{111}$In-nimotuzumab-NLS (0-150 nM) in MDA-MB-468 cells (B). The total binding curve is generated by adding increasing concentration of the labeled conjugate (i.e. $^{111}$In-nimotuzumab-NLS or $^{111}$In-nimotuzumab). The non-specific binding curve is obtained by adding increasing concentrations of the radioimmunoconjugate in the presence of 60-fold increasing concentration of unlabeled nimotuzumab (0-9000 nM), and
finally, the specific binding curve is obtained by subtracting non-specific binding from total binding (i.e. Specific binding = Total binding – non-specific binding) (C) Competition binding curve for $^{111}$In-nimotuzumab-NLS in the presence of increasing concentrations of nimotuzumab for MDA-MB-468 cells. The y-axis is plotted as the fraction of radioligand bound in the presence of competitors (B) divided by the initial fraction of radioligand bound (in the absence of competitors) ($B_0$). The x-axis is plotted as logarithmic concentration of nimotuzumab. The dissociation constant ($K_d$) for $^{111}$In-nimotuzumab-NLS measured in this assay was $17.0 \pm 0.02$ nM. Each point represents the mean ± SEM of 3 assays performed in triplicate. (D) One point binding assay with $^{111}$In-nimotuzumab (150 nM) on MDA-MB-468 and TrR2 cell line in the presence or absence of unlabeled nimotuzumab (6,250 nM).
**Table 2.3.2A:** The dissociation constant ($K_d$) and number of receptors ($B_{max}$) for various cell lines measured in a direct receptor-binding assay using radioimmunoconjugates.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Radioimmunoconjugate</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (nmol)</th>
<th>Number of EGFR/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-468</td>
<td>$^{111}$In-nimotuzumab</td>
<td>14.0 ± 0.64*</td>
<td>$(1.3 \pm 0.06) \times 10^{-3}^*$</td>
<td>$(2.5 \pm 1.3) \times 10^6$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>/ $3 \times 10^5$ cells</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>$^{111}$In-nimotuzumab</td>
<td>85.6 ± 12.8*</td>
<td>$(4.2 \pm 1.3) \times 10^{-4}^*$</td>
<td>$(5.4 \pm 0.8) \times 10^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>/ $4.5 \times 10^5$ cells</td>
<td></td>
</tr>
<tr>
<td>TrR-1</td>
<td>$^{111}$In-nimotuzumab</td>
<td>78.0 ± 23.0*</td>
<td>$(1.9 \pm 0.8) \times 10^{-4}^*$</td>
<td>$(4.2 \pm 1.2) \times 10^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>/ $2.7 \times 10^5$ cells</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>$^{111}$In-hEGF</td>
<td>0.08 ± 0.1</td>
<td>$(1.0 \pm 0.5) \times 10^{-5}$</td>
<td>$(5.2 \pm 1.6) \times 10^4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>/ $2.2 \times 10^5$ cells</td>
<td></td>
</tr>
</tbody>
</table>

*The $K_d$ of $^{111}$In-nimotuzumab in MDA-MB-468 is significantly different than the $K_d$ of MDA-MB-231 ($P < 0.01$) and TrR-1 ($P < 0.01$) cells.

The $B_{max}$ of $^{111}$In-nimotuzumab in MDA-MB-468 is significantly different than the $B_{max}$ of MDA-MB-231 ($P < 0.05$), however there is no significant difference in the $B_{max}$ of $^{111}$In-nimotuzumab in MDA-MB-468 and $B_{max}$ of TrR-1 cells ($P > 0.05$).

The $K_d$ and $B_{max}$ of $^{111}$In-hEGF is significantly different than $K_d$ and $B_{max}$ of $^{111}$In-nimotuzumab assessed in all the other cell lines (MDA-MB-468, MDA-MB-231 and MCF-7) ($P < 0.001$).
2.3.3 Internalization and Nuclear Importation Assays

The internalization and nuclear importation of $^{111}$In-nimotuzumab and $^{111}$In-nimotuzumab-NLS were compared in MDA-MB-468 cells expressing a high number of EGFR. This was done to assess how much radioactivity was deposited in different cell compartments especially the nucleus where it can cause lethal damage to the DNA. The fraction of cell-associated radioactivity that was internalized in the nucleus of MDA-MB-468 cells was $31.6 \pm 3.7\%$ at 4 h, $28.2 \pm 1.5$ at 6 h, and $17.4 \pm 0.2\%$ at 24 h for $^{111}$In-nimotuzumab-NLS (Figure 2.3.3A). The corresponding nuclear internalized fraction values for $^{111}$In-nimotuzumab were $16.1 \pm 1.5\%$ at 4 h, $14.5 \pm 2.2$ at 6 h and $15.2 \pm 2.0\%$ after 24 h (Figure 2.3.3A). These results showed that the nuclear internalization of cell associated radioactivity was 2-fold greater for $^{111}$In-nimotuzumab-NLS, compared to $^{111}$In-nimotuzumab at 4 h and 6 h indicating the NLS sequence promoted the internalization of the radioimmunoconjugate (t-test p=0.0006), however, there is no significant difference in nuclear internalization observed at 24 h between $^{111}$In-nimotuzumab and $^{111}$In-nimotuzumab-NLS (p>0.05). The uptake of radioactivity was blocked in the presence of excess unlabeled nimotuzumab (Figure 2.3.3B), demonstrating EGFR-mediated uptake. Furthermore, the inhibition of $^{111}$In-nimotuzumab-NLS uptake observed at 4°C also supported the finding that the uptake was receptor-mediated and only functional at 37°C. The nuclear uptake was significantly lower in MCF-7 cells which express low levels of EGFR ($10^4$ receptors/cell) that are similar to EGFR levels on normal epithelial tissues (Figure 2.3.3D). Thus, $^{111}$In-nimotuzumab-NLS is specific for EGFR and is internalized to a much greater extent in cells that overexpress EGFR compared to cells which express low EGFR density. Interestingly, in MCF-7 cells $^{111}$In-nimotuzumab-NLS has promoted significant internalization in the cytoplasm ($P < 0.05$) and nucleus ($P < 0.001$) when compared to $^{111}$In-nimotuzumab (2.3D). Furthermore,
the nuclear importation in MDA-MB-468 cells (10^6 receptors/cell) was found to be 4-fold higher compared to moderate EGFR expressing cell lines (i.e MDA-MB-231, TrR1 expressing 10^5 receptor/cell) and 6-fold higher compared to low EGFR expressing cell line (i.e MCF-7 cells expressing 10^4 receptor/cell) \( (P < 0.05) \) (Figure 2.3.3E). For comparison, the cell-associated nuclear radioactivity for \(^{111}\text{In-hEGF}\) was found to be 3.6 ± 0.2% as compared to 31.4 ± 3.1% in the case of \(^{111}\text{In-nimotuzumab-NLS}\) (Figure 2.3.3F). These results confirmed that \(^{111}\text{In-nimotuzumab-NLS}\) localized in the nucleus of EGFR-expressing BC cells and thus has the potential to damage DNA and be lethal to the cells. Finally the accuracy of the cell fractionation studies, which was used evaluate internalization and nuclear translocation, were verified by calpain I assay (Santa cruz biotechnology inc, CA). Calpain (molecular weight: 81.8kDa) is an intracellular \(\text{Ca}^{2+}\)-dependent protease that is only found in the cytoplasm [169]. Western blot analysis revealed that calpain was only present in the whole cell lysate and cytoplasmic fractions and not in the nuclear fraction (Supplementary Figure 1). This revealed there was very little or no cross contamination between the cellular fractions.
Figure 2.3.3A: Comparison of the cell associated radioactivity in the nucleus from $^{111}$In-nimotuzumab-NLS and $^{111}$In-nimotuzumab in MDA-MB-468 cells at different time points. Internalization is expressed in terms of cell-associated radioactivity internalized in the nucleus at 4, 6 or 24 h. Internalization assay was performed at 37 °C Values shown are the mean ± SEM of triplicate determinations. * Significantly different; $P < 0.05$. 
Figure 2.3.3B: Comparison of percentage of cell associated radioactivity in the nucleus from $^{111}$In-nimotuzumab-NLS (150 nM) or $^{111}$In-nimotuzumab (150 nM) with or without blocking in the MDA-MB-468 cells. Nuclear internalization was measured in the presence (blocked) or absence (no blocking) of an excess of unlabeled nimotuzumab (7,500 nM) contained in the medium to block EGFR. The nuclear internalization of radioactivity in the absence of unlabeled nimotuzumab (un-blocked) is significantly higher than the nuclear internalization of radioactivity in the presence of unlabeled nimotuzumab (blocked) for both $^{111}$In-nimotuzumab-NLS and $^{111}$In-nimotuzumab. Internalization assay was performed for 4 h incubation at 37 °C. Values shown are the mean ± SEM of triplicate determinations. * Significantly different; P < 0.05.
**Fig. 2.3.3C:** Comparison of cell associated radioactivity of $^{111}$In-nimotuzumab-NLS (150nM) associated at 4°C and 37°C in different cellular fractions of MDA-MB-468 cells. The radioactivity associated with cytoplasmic and nuclear fraction is significantly greater at 37°C compared to 4°C, however the radioactivity associated with the membrane fraction at 4°C is significantly greater than the radioactivity at 37°C. Values shown are the mean ± SEM of triplicate determinations. *Significantly different; $P < 0.05$. 
Figure 2.3.3D: Comparison of percentage of the total radioactivity of $^{111}\text{In}\text{-nimotuzumab-NLS}$ (150 nM) and $^{111}\text{In}\text{-nimotuzumab}$ (150 nM) in different cellular fractions of MCF-7 cells. Internalization assay was performed for 4 h incubation at 37 °C. The radioactivity associated with the cytoplasmic ($p < 0.0012$) and nuclear fraction ($p < 0.0001$) in MCF-7 cells is significantly greater for $^{111}\text{In}\text{-nimotuzumab-NLS}$ than $^{111}\text{In}\text{-nimotuzumab}$. Values shown are the mean ± SEM of triplicate determinations. * Significantly different, $P < 0.05$. 
**Figure 2.3.3E:** Comparison of $^{111}\text{In}$-nimotuzumab-NLS associated with different cellular fractions in trastuzumab resistant cell lines (MDA-MB-468, MBA-MD-231, TrR1, TrR2, and MCF-7. Internalization assay was conducted after incubation with $^{111}\text{In}$-nimotuzumab-NLS (125 nM/6.5 MBq) at 37°C for 4 h. Internalization is expressed as the amount of radioimmunoconjugates (nmol) localized in different compartments of the cells. Radioactivity associated with the cellular fractions of MDA-MB-468 cell line is significantly greater than the cellular fraction radioactivity of both moderate EGFR expressing cell lines (MDA-MB-231 and TrR1) and low EGFR expressing cell line (MCF-7). The radioactivity associated with the membrane and nuclear fraction of moderate EGFR expressing cell line is significantly greater than low EGFR expressing cell line. Values shown are the mean ± SEM of triplicate determinations. * Significantly different, P < 0.05.
Figure 2.3.3F: Comparison of cell associated radiactivity of $^{111}$In-nimotuzumab-NLS and $^{111}$In-hEGF associated with different cellular fractions in MDA-MB-468 cells. Internalization assay of $^{111}$In-nimotuzumab-NLS (125 nM/6.5 MBq) and $^{111}$In-hEGF (125 nM/6.5 MBq) was performed after 4 h incubation at 37 °C. Internalization and nuclear uptake are expressed as the percentage of cell associated radioactivity. Radioactivity associated with nuclear fraction is significantly greater for $^{111}$In-nimotuzumab-NLS than $^{111}$In-EGF, however the radioactivity associated with membrane fraction is significantly greater $^{111}$In-EGF than for $^{111}$In-nimotuzumab-NLS. Values shown are the mean ± SEM of triplicate determinations. * Significantly different, P < 0.05.
2.3.4 Clonogenic survival assays

The fraction of clonogenic survival (CS) for MDA-MB-468 cells was determined after exposing cells to various concentrations of the radioimmunoconjugates. The survival of MDA-MB-468 cells exposed to 20 nM of $^{111}$In–nimotuzumab-NLS was significantly lower (2.8 ± 0.2% vs. 21.0 ± 5.3%; $P=0.004$) compared to $^{111}$In-nimotuzumab, confirming that nuclear localization enhanced the lethal properties of the Auger electrons (Figure 2.3.4A). The CS of MDA-MB-468 cells exposed to 20 nM unlabeled nimotuzumab was 60.0 ± 7.0% (Figure 2.3.4A). The CS of MDA-MB-468 cells decreased as the concentration and radioactivity of the radioimmunoconjugates increased. At low concentrations of unlabeled nimotuzumab (2 nM and 9.5 nM) there was, however, an apparent growth stimulatory effect observed in MDA-MB-468 cells. The CS of MDA-MB-231 cells exposed to 40 nM of $^{111}$In-nimotuzumab-NLS, $^{111}$In-nimotuzumab and nimotuzumab was 55.4 ± 5.5%, 56.7 ± 4.8%, and 103.9 ± 13.6%, respectively. In the case of MCF-7 cells the CS after treatment with $^{111}$In-nimotuzumab-NLS, $^{111}$In-nimotuzumab or nimotuzumab was 82.6 ± 6.4%, 80.0 ± 1.6%, and 95.0 ± 19.4%, respectively (Figure 2.3.4B). These results demonstrated that the cytotoxicity of $^{111}$In-nimotuzumab-NLS was dependent on EGFR density. The survival of TrR1 cells exposed to 40 nM of $^{111}$In-nimotuzumab-NLS, $^{111}$In-nimotuzumab or nimotuzumab was 52.3 ± 18.2%, 69.2 ± 4.9% and 91.2 ± 3.8%, respectively (Figure 2.3.4B). The CS of TrR2 cells exposed to 40nM $^{111}$In-nimotuzumab-NLS, $^{111}$In-nimotuzumab and nimotuzumab was 96.2 ± 8.2%, 94.0 ± 18.0%, and 98.9 ± 5.8%, respectively (Figure 2.3.4B). It was concluded that BC cells with negligible HER2 that are intrinsically resistant to trastuzumab, but with a high EGFR expression (e.g. MDA-MB-468), were efficiently killed by $^{111}$In-nimotuzumab-NLS. The survival of BC cells with intermediate levels of EGFR and very low HER2 that exhibit resistance to trastuzumab (e.g.
MDA-MB-231 or TrR2) was significantly diminished with $^{111}$In-nimotuzumab-NLS, but these effects were less dramatic than against cells with high EGFR density. Finally, for comparison, the CS of MDA-MB-468 cells with $^{111}$In-hEGF revealed significantly higher toxicity of this agent compared with $^{111}$In-nimotuzumab-NLS under the condition used. The CS of $^{111}$In-nimotuzumab-NLS was significantly higher than $^{111}$In-hEGF at both 2nM/0.45MBq (87.2 ± 3.4 vs. 11.7 ± 2.2) ($P<0.0001$) and 10nM/0.75MBq (81.2 ± 7.6 vs. 1.0 ± 0.2) ($P<0.0001$) concentrations, respectively (Figure 2.3.4C). The reason may be due to a higher proportion of $^{111}$In-hEGF which is imported into the nucleus being closer to the DNA to cause more damage compared to $^{111}$In-nimotuzumab-NLS. This was not evaluated in the thesis research.
Figure 2.3.4A

Figure 2.3.4B
**Figure 2.3.4:** Clonogenic survival of MDA-MB-468 cells at various concentrations and amounts of radioactivity of nimotuzumab, $^{111}$In-nimotuzumab and $^{111}$In-nimotuzumab-NLS after a three-day culture period. There is a significant difference in CS between nimtuzumab and the radiolabeled conjugates ($^{111}$In-nimotuzumab or $^{111}$In-nimotuzumab-NLS) above 9.3nM/2.47MBq concentration. Also there is a significant difference in killing between $^{111}$In-nimotuzumab-NLS and $^{111}$In-nimotuzumab above 20nM/4.5MBq concentration (A). Clonogenic survival of MDA-MB-231, TrR1, TrR2 and MCF-7 cells treated with 40 nM/10.5 MBq of $^{111}$In-nimotuzumab or $^{111}$In-nimotuzumab-NLS or 40 nM of nimotuzumab after a three-day treatment period. There is a significant difference in the CS of unlabeled nimotuzumab and radiolabeled conjugates for MDA-MB-231 and MCF-7 cells (B). Values shown are the mean ± SEM of triplicate determinations. *Significantly different; P < 0.05.*
Figure 2.3.4C: Comparing clonogenic survival of MDA-MB-468 cells treated with 2 nM/0.45 MBq or 10 nM/0.75 MBq of $^{111}$In-nimotuzumab-NLS and $^{111}$In-hEGF after a three-day culture period. $^{111}$In-hEGF shows significantly higher toxicity than $^{111}$In-nimotuzumab-NLS under the conditions used. Values shown are the mean ± SEM of triplicate determinations. *, $P < 0.05$. 
2.3.5 Evaluation of DNA damage by confocal Immunofluorescence Microscopy

Exposure to radioactivity from $^{111}$In-hEGF resulted in significantly greater numbers of $\gamma$-H2AX foci expressed as integrated density/nuclear area as compared to $^{111}$In-nimotuzumab-NLS (6.7 ± 0.72 vs. 3.2 ± 0.75; $(P < 0.05)$ Figure 2.3.5A). Furthermore, both $^{111}$In-hEGF and $^{111}$In-nimotuzumab-NLS produced significantly higher $\gamma$-H2AX foci integrated density/nuclear area than the medium control or unlabeled hEGF or nimotuzumab-NLS (Figure 2.3.5A). Although the nuclear uptake of radioactivity associated with $^{111}$In-hEGF was less than $^{111}$In-nimotuzumab-NLS (see section 2.3.3), there were more double strand breaks (DSB) with $^{111}$In-hEGF detected using the $\gamma$-H2AX assay, which also corresponded to its greater potency in the clonogenic survival assays against MDA-MB-468 cells (see section 2.3.4).
Fig. 2.3.5: Induction of γ-H2AX foci by $^{111}$In-hEGF or $^{111}$In-nimotuzumab-NLS in MDA-MB-468 cells (A). Cells were incubated for 4 h with 10 nM/0.75 MBq of $^{111}$In-hEGF or $^{111}$In-nimotuzumab-NLS, 10 nM of unlabeled hEGF or nimotuzumab-NLS, or with growth medium alone as a control. Confocal microscopy of MDA-MB-468 cells exposed to $^{111}$In-hEGF, $^{111}$In-nimotuzumab-NLS and medium only (B). Cells were immunostained for γ-H2AX (bright foci) and 4, 9, 6-diamidino-2-phenylindole (DAPI) was used to counter stain the cell nuclei blue. Exposure to $^{111}$In-hEGF caused significantly greater numbers of γ-H2AX foci corresponding to DSB than $^{111}$In-nimotuzumab-NLS.
2.4 Discussion

The results of this study showed that synthetic 13-mer peptides (CGYGPKKKRKVGG) harboring the NLS of SV-40 large T-antigen facilitated the translocation of $^{111}$In-nimotuzumab into the nucleus of EGFR-overexpressing and trastuzumab-resistant BC cells, where the cytotoxicity of the emitted Auger electrons was enhanced. Furthermore, the dose-dependent cytotoxicity of $^{111}$In-nimotuzumab-NLS was shown to directly correlate with the EGFR expression on the BC cells. In clinical settings, trastuzumab is administered to patients who have tumors that exhibit moderate to high expression of HER2 on their cell surface [40]; however, those patients who initially show response to trastuzumab acquire resistance to the drug in less than a year [55]. This resistance to trastuzumab in some cases is followed by an increase in the expression of EGFR on the BC cells [38, 46, 60], leading to disease progression in these patients [80]. Thus, a rational targeted therapy for trastuzumab-resistant BC cells would be to target EGFR expression. If the significantly enhanced cytotoxic effects of $^{111}$In-nimotuzumab-NLS compared to unlabeled nimotuzumab in this study are also found clinically, then this radiotherapeutic agent could potentially offer an effective treatment for trastuzumab-resistant metastatic BC that displays EGFR.

Recently our group found that trastuzumab labeled with $^{111}$In and conjugated to NLS ($^{111}$In-trastuzumab-NLS) was effective for killing SKBR3 (HER2 3+) and MDA-MB-361 (HER2 2+) either alone or in combination with methotrexate [151, 152]. Thus, we anticipated that labeling nimotuzumab, an anti-EGFR mAb with $^{111}$In and conjugating it to a NLS would have similar cytotoxic effects on cell lines having moderate to high EGFR expression. At the specific activities used in this study (350 ± 9.50 MBq/mg), $^{111}$In-nimotuzumab only demonstrated a 3-fold increase in toxicity compared to unlabeled nimotuzumab (Figure 2.3.4A). Almost 99% of
the low-energy Auger electrons emitted by $^{111}$In have a range of less than 1 µm in tissues [170], and the radiation absorbed dose to the nucleus is 2-fold and 35-fold greater when $^{111}$In decays in the nucleus compared to when the decay occurs in the cytoplasm or on the cell surface, respectively [110]. Therefore, we further modified $^{111}$In-nimotuzumab with NLS peptides in order to enhance its radiotoxicity by promoting its nuclear uptake following receptor-mediated endocytosis. Indeed, $^{111}$In-nimotuzumab-NLS was 10-fold more potent at killing MDA-MB-468 cells overexpressing EGFR ($10^6$ receptors/cell) than $^{111}$In-nimotuzumab and nearly 30-fold more effective than unlabeled nimotuzumab. This toxicity was correlated with 2-fold higher nuclear importation of $^{111}$In-nimotuzumab-NLS (31.2 ± 2.6%) compared to $^{111}$In-nimotuzumab (16.0 ± 0.6%) (Figure 2.3.3A), indicating that the NLS peptides delivered more radioactivity into the nucleus which was lethal to the cells. These findings are in agreement with our previous observations where the NLS of SV-40 large T-antigen was able to mediate the translocation of covalently linked $^{111}$In-trastuzumab molecules into the nuclei of HER2-overexpressing BC cells following their receptor-mediated internalization [152]. Furthermore our group also reported that NLS-peptide modification of $^{111}$In-HuM195 mAbs, specific for CD33-epitopes, promoted their nuclear translocation in HL-60 leukemic cells, rendering them 12-fold more radiotoxic than unmodified $^{111}$In- HuM195 [157]. Similarly, NLS-peptides have also been shown to promote the translocation of a $[^{99m}\text{Tc(OH}_2)_3\text{(CO)}_3]^+\text{-labeled DNA-intercalating pyrene moiety into the nuclei of B16F1 mouse melanoma cells where the Auger electrons from }^{99m}\text{Tc were lethal}[171]$. Taken together, these studies demonstrate that NLS-containing peptides can efficiently route radiolabeled molecules internalized into tumor cells through receptor-mediated processes into the nucleus.
In the case of BC cell lines with moderate EGFR expression (10^5 EGFR/cell), the internalization and nuclear uptake of ^{111}\text{In}-\text{nimotuzumab-NLS} was lower (Figure 2.3.3E) and, hence, the agent exhibited lower cytotoxicity towards these cells (Figure 2.3.4B). The implications of this finding are that ^{111}\text{In}-\text{nimotuzumab-NLS} is most effective against cells having high or moderate EGFR expression (>10^5 receptors/cell). It was further observed that cell lines with a low number of EGFR (i.e. MCF-7; 10^4 receptors/cell) showed very little internalization and nuclear uptake or cytotoxicity from ^{111}\text{In}-\text{nimotuzumab-NLS}. This suggests that ^{111}\text{In}-\text{nimotuzumab-NLS} will not likely harm cells that have low levels of EGFR, i.e. healthy normal epithelial cells. Interestingly, we noted no significant increase in the cytotoxicity of ^{111}\text{In}-\text{nimotuzumab-NLS} compared to ^{111}\text{In}-\text{nimotuzumab} in MDA-MB-231 and TrR1 cells (Figure 2.3.4B), implying that NLS-peptide enhanced cytotoxicity appears to occur only in cells with EGFR overexpression (>10^6 receptors/cell). Nonetheless, both ^{111}\text{In}-\text{nimotuzumab-NLS} and ^{111}\text{In}-\text{nimotuzumab} were moderately cytotoxic to MDA-MB-231 and TrR1 cells. To increase the amount of ^{111}\text{In} delivered to these cells per EGFR recognition event and increase the cytotoxicity of ^{111}\text{In}-\text{nimotuzumab-NLS} will require strategies to increase the specific activity of the radioimmunoconjugates. These strategies could include the use of G4-dendrimers, a polymer/chelator that can be conjugated to as many as 64 DTPA chelators which, in turn can complex 64 ^{111}\text{In} atoms [172].

There was no cytotoxicity against TrR2 cells; this may be due to the apparent lack of binding sites for nimotuzumab on the cell membrane, which was shown by a one point binding assay (Figure 2.3.2D). Interestingly, a study by Du Manoir et al. [165] who developed the TrR2 cell line found that it was EGFR-positive by Western blot on whole cell lysate. Thus, the presence of EGFR in the whole cell lysate but inefficient binding of nimotuzumab to the intact
TrR2 cells could be related to either receptor down-regulation, mutations [extracellular domain (ECD) cleavage], or hindered binding, i.e. by glycoprotein MUC4, as shown previously for inhibition of binding of trastuzumab to HER2-positive but resistant BC cells [61]. Our group has recently verified that the HER2 ECD in TrR2 is not present, blocking trastuzumab binding and rendering the cells insensitive to trastuzumab. Alternatively, the inefficient binding of nimotuzumab may be related to lack of expression of EGFR on the cell surface, implying that all EGFR are retained within the cytoplasm or nucleus. Thus, further analysis is required to study the mechanism of inefficient binding and resistance of TrR2 cells towards $^{111}$In-nimotuzumab-NLS; new treatments are required to overcome this resistance.

The internalization of $^{111}$In-nimotuzumab-NLS was correlated with the number of EGFR on the different cell lines (Figure 2.3.3E). It should be noted, however, that the internalization values are possibly underestimates since it has been shown that exposure of cells to low pH buffers to remove surface-bound radioligands may also remove some internalized radioactivity [173]. Nevertheless, the internalization and nuclear importation of $^{111}$In-nimotuzumab-NLS was significantly higher in MDA-MB-468 cells than the uptake of $^{111}$In-nimotuzumab without NLS peptides. Additionally, despite the presence of an endogenous NLS peptide associated with the transmembrane region of the EGFR, the additional NLS peptide modification of nimotuzumab enhanced the uptake of radioactivity in the nucleus. It has also been suggested that cationic charges on the NLS peptide promote endocytosis of macromolecules via an absorptive-mediated process through binding to negatively charged cell-surface proteins [174]. Although the SV-40 NLS-peptides employed in our study possess several cationic lysine residues, the uptake of radioactivity by MDA-MB-468 cells exposed to $^{111}$In-nimotuzumab-NLS was blocked by unlabeled nimotuzumab, demonstrating that internalization was EGFR-mediated. Ginj et al.
showed that unconjugated $^{111}$In-labeled NLS peptides (PKKKRKV) harboring the same SV-40 large-T antigen NLS as that in the NLS-peptides employed in this study were not bound or internalized by tumor cells. Nevertheless, adding NLS peptides (in our case 5 NLS/mAb) to the antibody increases the overall positive charge of the antibody which can facilitate cell internalization and nuclear translocation of the radioimmunoconjugate into the cell.

Finally, we also compared the efficacy of $^{111}$In-labeled hEGF, an analogue of the natural ligand for EGFR with $^{111}$In-nimotuzumab-NLS. It was found that $^{111}$In-hEGF was 8 to 80 fold more cytotoxic than $^{111}$In-nimotuzumab-NLS ($p < 0.0001$) for MDA-MB-468 cells (2.3.4C) under the conditions used. Moreover, the increased sensitivity of MDA-MB-468 cells to $^{111}$In-hEGF correlated with an increase in γH2AX-foci integrated density/nuclear area compared to that for $^{111}$In-nimotuzumab-NLS (6.7 ± 0.23 vs. 3.2 ± 0.28, respectively) (Figure 2.3.5A), suggesting that $^{111}$In-hEGF caused more DNA double strand breaks leading to increased cell killing. In spite of the observed potent cytotoxicity, it was found that the percentage of the total radioactivity added to the cells that was internalized and the cell-associated radioactivity taken up into the nucleus for $^{111}$In-hEGF was significantly lower compared to $^{111}$In-nimotuzumab-NLS (0.3 ± 0.02 and 2.5 ± 0.1 vs. 1.2 ± 0.1 and 32 ± 4.0, respectively) (2.3F). This may seem contradictory, however, hEGF itself may also play a role in killing MDA-MB-468 cells despite relatively low uptake of radioactivity in the nucleus for $^{111}$In-hEGF. Previous research has shown that in cell lines which express EGFR above a critical threshold ($>10^5$ EGFR/cell), hEGF $> 1\text{nM}$ has cytotoxic effects on these cells [175]. Other studies have shown that hEGF greater than 1 nM increases glucose uptake which leads to increased formation of lactate in cells and eventually causes cell death [176]. Nevertheless, this effect was only reported in _in vitro_ experiments because, unlike plasma, the medium has a constant amount of glucose which is not replenished
over time. Furthermore, there is speculation that hEGF binds strongly to DNA enabling it to deliver more Auger electrons in closer proximity and cause more DSB than $^{111}$In-nimotuzumab-NLS despite lower overall nuclear uptake. Previously our group has found that two thirds of the internalized $^{111}$In-hEGF is bound to the chromatin region [110]. Thus binding of $^{111}$In-hEGF to the DNA allows closer delivery of Auger electrons which cause more damage to the DNA thereby leading to cell death. Further experiments are required to quantify how much internalized radioactivity is associated with the DNA in the case of $^{111}$In-nimotuzumab-NLS for comparison with $^{111}$In-hEGF to verify the cause of greater toxicity by $^{111}$In-hEGF. Nevertheless, these studies should be repeated under comparable EC$_{50}$ concentrations to conclude that $^{111}$In-hEGF was more potent at comparable receptor occupancy.

Radiation absorbed dose estimates to the nucleus of a single MDA-MB-468 cell from exposure to $^{111}$In-nimotuzumab-NLS and $^{111}$In-nimotuzumab (specific activity 350 MBq/mg) at receptor-saturation conditions were calculated using the method of Monte Carlo N-particle computer code (MCNP) [177] (Table 2.4A and 2.4B). The calculation was based on the compartmental distribution in the cells, for $^{111}$In-nimotuzumab (72% bound to the membrane, 12% in the cytoplasm and 16% in the nucleus) or for $^{111}$In-nimotuzumab-NLS (58% bound to the membrane, 10% in the cytoplasm and 32% in the nucleus) and it was assumed that the i) elimination from these subcellular compartments was only by radioactive decay, and ii) the cells double every 24 hrs (to incorporate elimination of radioactivity by mitotic division in which the radioactivity in each cell compartment would decrease by half). The absorbed dose estimate revealed that $^{111}$In-nimotuzumab-NLS delivered as much as 15.9 Gy while $^{111}$In-nimotuzumab delivered about 3.0 Gy, to the nucleus of a MDA-MB-468 cell. These estimates show that the NLS sequence increases the exposure of radiation in the nucleus where it is also more lethal.
Table 2.4A

Radiation absorbed dose estimates to the cell nucleus by $^{111}$In-nimotuzumab localized in compartments of a MDA-MB-468 human BC cell*

<table>
<thead>
<tr>
<th>Cell Compartment</th>
<th>$\bar{A}$ † (Bq × secs)</th>
<th>$S$ ([Gy/Bq × secs] × 10$^{-4}$)</th>
<th>Radiation dose to cell nucleus $D$ ‡ (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell membrane</td>
<td>10688.1</td>
<td>0.18</td>
<td>1.9</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>3469.2</td>
<td>0.26</td>
<td>0.6</td>
</tr>
<tr>
<td>Nucleus</td>
<td>2557.6</td>
<td>1.40</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>3.0</td>
</tr>
</tbody>
</table>

Table 2.4B

Radiation absorbed dose estimates to the cell nucleus by $^{111}$In-nimotuzumab-NLS localized in compartments of a MDA-MB-468 human BC cell*

<table>
<thead>
<tr>
<th>Cell Compartment</th>
<th>$\bar{A}$ † (Bq × secs)</th>
<th>$S$ ([Gy/Bq × secs] × 10$^{-4}$)</th>
<th>Radiation dose to cell nucleus $D$ ‡ (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell membrane</td>
<td>15817.1</td>
<td>0.18</td>
<td>2.84</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>10614.8</td>
<td>0.26</td>
<td>2.75</td>
</tr>
<tr>
<td>Nucleus</td>
<td>7367.0</td>
<td>1.40</td>
<td>10.31</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>15.9</td>
</tr>
</tbody>
</table>

*Cellular radiation dosimetry model of MCNP was used to estimate the radiation absorbed dose (D) to cell nucleus: $D = \bar{A} \times S$, where S is radiation absorbed dose to the nucleus (Gy) per unit of cumulated radioactivity in the source compartment, $\bar{A}$ (Bq × secs).
† Å (Bq × secs) value was calculated from the area under the curve of activity in each cell compartment vs. time (Figure 2.4A and 2.4B).

**Figure 2.4A**

**Figure 2.4B**

![Graphs showing cellular fractionation data](image)

**Figure 2.4:** (A) Cellular fractionation data (membrane, cytoplasm and nucleus) at 4, 6 and 24 hrs expressed of $^{111}$In-nimotuzumab expressed as radioactivity (Bq/cells) in MDA-MB-468 cells. (B) Cellular fractionation data (membrane, cytoplasm and nucleus) at 4, 6 and 24 hrs expressed of $^{111}$In-nimotuzumab-NLS expressed as radioactivity (Bq/cells) in MDA-MB-468 cells. The graphs were plotted to evaluate the area under the curve for calculating the absorbed dose.

### 2.5 Conclusion

It is concluded that NLS-peptides efficiently routed $^{111}$In-nimotuzumab to the nucleus of EGFR-positive and trastuzumab-resistant human BC cells, where the nanometer-to-micrometer range Auger electrons rendered the radiotherapeutic agent damaging to DNA and lethal to the cells. The efficacy of $^{111}$In-nimotuzumab-NLS/$^{111}$In-nimotuzumab for eradicating EGFR-positive...
BC cells *in vitro* was directly correlated with the level of EGFR expression on the cell surface. Furthermore, $^{111}\text{In}$-hEGF was found to be significantly more cytotoxic than $^{111}\text{In}$-nimotuzumab-NLS for killing EGFR-positive BC cells under the conditions used. However, potentially more favorable tumor targeting properties of $^{111}\text{In}$-nimotuzumab-NLS warrant future RIT studies in mice to evaluate its anti-tumor properties and normal tissue toxicities. These studies are currently in progress by our group.
Chapter 3: Overall thesis discussion and future directions
3. Thesis discussion

3.1 Development of Radioimmunotherapy

Around 1908, Dr. Paul Erlich changed medical practice by focusing on drug delivery systems for diseases, which he called "magic bullets". Ehrlich reasoned that if a compound could be made that selectively targeted disease-causing cells, then this could represent a drug for these cells which could be delivered selectively to disease-causing cells while sparing normal cells [178]. The concept of a "magic bullet" was fully realized with the invention of monoclonal antibodies (mAbs) in 1975 by Kohler and Milstein [179]. The properties of these magic bullets (mAbs) were further enhanced by radiolabeling them to direct radiation to tumor cells (RIT) [180]. Despite the demonstrated efficacy of these radiolabeled mAbs in preclinical mouse xenograft models of solid tumors, RIT has not been effective in curing tumors in humans due to the low proportions of the administered radiolabeled mAbs localizing in the tumor [181]. The most common radionuclides used in RIT have been beta emitters such as $^{131}$I or $^{90}$Y. Beta-particles from these radionuclides have high energy and travel 2-12 mm in tissues [98]. Thus, due to the long range of beta particles, they cause a cross-fire effect and due to their low linear energy transfer (LET), they are not densely ionizing to DNA of tumor cells. More distant non-targeted normal cells may be killed from the cross-fire effect, potentially resulting in normal tissue toxicities including hematopoietic toxicity [98]. This property becomes a dose-limiting factor before a tumor-killing dose can be reached.

As previously described in Chapter 2, Auger electron-emitting radioisotopes represent an intriguing alternative to beta-particle emitters for targeted radiotherapy for cancer. Auger electrons also have a very short, subcellular path length in tissue (< 0.5 µm) which results in high LET causing DNA damage and cell death [98]. In particular, studies have shown that these
electrons internalized in the cytoplasm of the cells or particularly localized in the nucleus cause cytotoxicity comparable to alpha-emitters [98]. Therefore, not only is the presence of an Auger electron-emitting radionuclide important, but also a mechanism to translocate the radionuclide into the nucleus of the cancer cells can increase the efficacy of such a targeted RIT agent.

A recent RIT agent investigated by our laboratory is $^{111}$In-labeled trastuzumab modified with peptides containing the NLS of SV-40 large T antigen for treatment of HER2-amplified BC. It was verified through confocal fluorescence microscopy that $^{111}$In-trastuzumab-NLS localized in the nucleus following a 24 h incubation, whereas trastuzumab was mostly membrane bound. There was greater DNA damage and cell killing observed in BC cells exposed to $^{111}$In-trastuzumab-NLS compared to $^{111}$In-trastuzumab and trastuzumab [164]. Furthermore, NLS conjugation also enhanced the nuclear uptake and cytotoxicity of anti CD33 mAbs $^{111}$In-HuM195 and $^{111}$In-M195 toward HL-60 myeloid leukemia cells and primary AML specimens [157]. Thus, these findings show that we can increase the efficacy of mAbs by radiolabeling them with an Auger electron emitter and conjugating them with an NLS sequence. As discussed in Chapter 1, this proved to be a useful method for targeting and killing HER2-positive but trastuzumab resistant BC cell lines with the Auger electron-emitter, $^{111}$In.

Studies in the past have found an inverse relationship between HER2 and EGFR expression in BC cell lines [75]. It is proposed that increased signaling from other EGFR type I growth factor tyrosine kinase family members (EGFR, HER-3 and HER-4) is one of the routes by which HER-2-positive cells gain trastuzumab resistance [55]. Since trastuzumab is only capable of blocking HER-2 mediated signaling, it fails to reduce signaling mediated from other HER receptors which maintains mitogenic signaling from these receptors. Moreover, EGFR positivity has been correlated clinically with disease progression in patients receiving
trastuzumab, despite HER2 amplification [80]. In the light of the evidence that EGFR may play a major role in trastuzumab resistance, I utilized a strategy to insert radiolabeled nimotuzumab (anti-EGFR mAb) with $^{111}$In directly into the nucleus of EGFR-amplified but trastuzumab-resistant BC cells. This was done by conjugating $^{111}$In-nimotuzumab to 13-mer synthetic peptides (CGYGPKKKRKVGG) harboring the NLS of SV-40 large T-antigen, so that low doses of Auger electron radiation in the nucleus could cause lethality. Indeed $^{111}$In-nimotuzumab-NLS was able to internalize into the nucleus and cause cytotoxic damage due to DNA double-strand breaks in BC cells overexpressing EGFR that are insensitive to trastuzumab.

### 3.2 Transport of biomolecules into the nucleus

Biomolecules such as peptides and antibodies that target overexpressed receptors on tumor cells are normally excluded from the nucleus [156]. In order to localize these molecules inside the nucleus, an NLS peptide is required. NLS are short, positively-charged, basic peptides that actively transport large proteins across the nuclear membrane [182]. These NLS peptides are of great significance as they enable the delivery of biomolecules labeled with Auger electron-emitting radionuclides into the nucleus where they can cause lethal damage to the DNA of the tumor cell. In some studies, the SV-40 NLS-motif (PKKKRKV) has been synthetically introduced into antibodies and also oligodeoxynucleotides and this has demonstrated significant radiotherapeutic efficacy [156]. Small molecules and chemotherapeutic agents such as carboplatin have also been modified with an NLS and labeled with other Auger electron-emitting radionuclides such as $^{191}$Pt, $^{193m}$Pt or $^{195m}$Pt [156] and these demonstrated efficient nuclear translocation following NLS conjugation. Naturally occurring NLS are also found in some peptide growth factors or their receptors, where they function to deliver internalized ligands to
the nucleus [156]. Previously, it has been proposed by our group that $^{111}$In-hEGF is transported to the nucleus by the NLS in the transmembrane domain of the EGFR [156]. It is likely that the NLS-peptide in $^{111}$In-nimotuzumab-NLS facilitated nuclear importation of this agent, but it is also possible that the NLS in the EGFR itself contributed to uptake in the nucleus following the pathway proposed by Lin et al, [73] in which internalized EGF/EGFR complexes were transported across the nuclear pore complex by interaction with importin $\beta_1$ through a putative NLS (NLS:RRRHIVRKRTLLR) found in the transmembrane domain of the receptor. Importin-$\beta_1$ complexes with importin-$\alpha$ to transport macromolecules across the nuclear pore in an energy dependent process powered by GTPase. Nonetheless, despite the endogeneous NLS in the EGFR, it was observed that the synthetic NLS peptide conjugated to $^{111}$In-nimotuzumab enhanced the nuclear uptake of the radiolabeled mAbs. Interestingly, it was also observed that internalization of $^{111}$In-nimotuzumab-NLS was significantly greater than $^{111}$In-EGF. This may be caused by additive effect of the NLS-peptide attached to $^{111}$In-nimotuzumab combined with the effect of the endogenous NLS in the EGFR. Furthermore, there was internalization of $^{111}$In-nimotuzumab-NLS observed in MCF-7 cells with very low EGFR density. This internalization may have been facilitated by the inherent positive charges of the NLS peptides that makes them bind to the cell surface, internalize and eventually translocate into the nucleus.

The efficacy of NLS-peptide to deliver Auger electron emitting bioconjugates to the nucleus could be further increased. One such approach is to increase the endosomal compartment escape, thereby allowing more radioactivity to be delivered to the nucleus. Bulmus et al. [183], described a novel pH-responsive polymer (pyridyl disulfide acrylate [PDSA]) containing a mixture of hydrophobic and acidic amino acids that are in a hydrated random coil conformation at physiological pH. At low pH such as those encountered in the endosome or lysosome, these
amino acids are protonated and form hydrophobic α-helices that become membrane-disruptive. Thus, modifying $^{111}$In-nimotuzumab-NLS with an endosomal membrane disruption polymer such as PDSA would increase its delivery to the nucleus by avoiding endosomal routing and subsequent proteolytic degradation [184]. Another approach to enhance nuclear uptake by NLS peptide is to employ bipartite instead of monopartite NLS, which may improve interaction with importins [182], thereby potentially increasing the efficiency of nuclear transport of $^{111}$In-nimotuzumab-NLS and further amplifying the cytotoxicity of the Auger electrons.

3.3 Radiation damage to tumor cells

Radiation causes several types of damage in cells including, production of free radicals, breaking of chemical bonds, production of new chemical bonds, and cross-linkage between macromolecules and damaged molecules that regulate vital cell processes (e.g. DNA, RNA, proteins) [185]. The main mechanism by which radiation causes damage to the cell is through DNA double-strand breaks (DSB) the effects of which are measured by clonogenic assays [185]. Although most DSBs are repaired, the residual breaks or incorrectly repaired DNA leads to increase mutational frequency and chromosomal aberrations that may lead to cell death [185]. Auger electrons with energy less than 30 keV, are able to cause DNA DSB by disrupting both DNA helices over distances of several nucleotides [98].

Phosphorylation of histone 2AX ($\gamma$-H2AX) occurs in proximity to DSBs and allows the recruitment of DNA repair and signaling factors at the site of the damage. The gamma-H2AX assay ($\gamma$-H2AX) is a immunocytochemical technique which identifies $\gamma$-H2AX foci and is an extremely sensitive and specific indicator of the existence of unrepaired DSBs induced by radiation [168]. In this study, there were high levels of $\gamma$-H2AX foci after incubating MDA-MB-
468 cells with $^{111}$In-EGF or $^{111}$In-nimotuzumab-NLS. Thus, DNA damage is likely the cause of diminished survival of these cells treated with these agents. Another contributing factor however may be the “bystander effect”, in which radiobiologically damaged cells induce cell death in non-irradiated cells through the release of cytokines and free radicals [186]. Xue et al.[187] demonstrated that pre-loaded Auger electron-emitting $^{125}$I-IUdR cells mixed with unlabeled cells exerted a cytotoxic effect on neighboring unlabeled tumor cells growing subcutaneously in athymic mice. This shows that Auger electrons can exhibit a bystander effect in vivo although it is not certain that this effect would be present in vitro, especially at the low cell densities used in clonogenic assays. Nonetheless, this effect would be useful to amplify the cytotoxicity of Auger electron-emitting agents by killing neighboring non-targeted tumor cells.

### 3.4 Future Directions

In the light of the encouraging in vitro results for $^{111}$In-nimotuzumab-NLS, this agent has great potential for further evaluation in preclinical in vivo studies. Currently our group is evaluating the pharmacokinetic and tumor and normal tissue distribution properties of $^{111}$In-nimotuzumab-NLS in vivo in athymic mice with s.c. EGFR overexpressing tumors. Once these properties are known, $^{111}$In-nimotuzumab-NLS can be further evaluated preclinically for its antitumor effects and normal tissue toxicities, and eventually in patients in a Phase I clinical trial. A pharmaceutical quality formulation manufactured under current Good Manufacturing Practices (cGMP) will need to be developed and regulatory approval obtained from Health Canada in the form of a Clinical Trial Application (CTA) [188]. The formulation, quality control specifications and analytical tests would be analogous to those that have previously been reported for similar
kits already approved by Health Canada for preparation of $^{111}$In-DTPA-hEGF, a radiotherapeutic agent for EGFR-overexpressing BC [188].

Another area of future research is to study chemotherapy-induced radiosensitization of EGFR-positive BC cells to $^{111}$In-nimotuzumab-NLS. Methotrexate has also been shown to radiosensitize normal and malignant cells to $\gamma$- radiation by causing cell cycle arrest in G1-S transition, which is a relatively radiosensitive phase of the cell cycle [151]. Previously our group has found that methotrexate radiosensitized HER2 expressing tumor cells to $^{111}$In-trastuzumab-NLS, thereby enhancing radiation-induced apoptosis in BC cells [164]. Thus, the combination of treatment using $^{111}$In-nimotuzumab-NLS and a chemotherapeutic drug may induce apoptosis in EGFR-expressing cells; this could be evaluated by probing with annexin V which has high affinity for phosphatidylserine which is externalized on the membrane of apoptotic cells [189]. This approach may be extremely useful because it could enhance killing of cells that express moderate EGFR, for instance MDA-MB-231, TrR1 cell lines that were not completely eradicated by exposure to $^{111}$In-nimotuzumab-NLS or $^{111}$In-nimotuzumab.

Finally, $^{111}$In-nimotuzumab-NLS is useful for single photon emission computed tomography (SPECT). SPECT imaging is performed using a gamma-camera that acquires images by capturing gamma photons released by the radionuclide to form 3-dimensional images [190]. SPECT imaging by $^{111}$In-nimotuzumab-NLS could be useful as it will allow the clinician to detect tumors that overexpress EGFR, that could then be treated with the same agent, $^{111}$In-nimotuzumab-NLS. Currently, Dr. Fonge is studying the imaging properties of $^{111}$In-nimotuzumab-NLS by using a MDA-MB-468 and MCF-7 tumor xenograft mouse model. He has shown specific localization of the radioimmunoconjugate in the MDA-MB-468 tumors (Fig 3.1). Finally, positron emission tomography (PET) imaging studies using $^{18}$F-2-fluorodeoxyglucose
(\(^{18}\text{F-FDG}\)) could be employed to assess early tumor response to \(^{111}\text{In-nimotuzumab-NLS}\) therapy in mice bearing BC xenografts. In PET imaging, annihilation photons produced by the decay of positron-emitting radioisotopes are detected by a ring of scintillation crystals in the tomograph [191]. A dedicated PET scanner designed for high resolution imaging of small laboratory animals is available in the STTARR facility for these studies. \(^{18}\text{F-FDG}\) is the most commonly used PET radiotracer and is avidly taken up by cancer cells [191]. \(^{18}\text{F-FDG-PET}\) is used in clinical oncology for cancer staging and monitoring of response to treatment with the goal of better managing patient therapy. [191]. Until recently, PET has been primarily used to detect response to chemotherapy in patients, but there has been a single report examining the role of \(^{18}\text{F-FDG-PET}\) in evaluating a response to targeted radiotherapeutics [192]. Therefore, PET studies in mice will provide insight into implementing PET into future clinical trials to monitor patient response to HER2-targeted Auger electron RIT.
**Figure 3.1:** *In vivo* SPECT imaging with $^{111}$In-nimotuzumab-NLS 48 h post-injection in an athymic mouse with MDA-MB-468 xenograft on the left flank and MCF-7 xenograft on the right. This study was performed by Dr. Humphrey Fonge.
Supplementary Figure 1: Western blot for calpain I assay done on whole cell lysate, cytoplasmic and nuclear fractions to evaluate the accuracy of cellular fractions. Calpain I is only found in cytoplasmic and whole cell lysate fraction, while it is absent in the nuclear fraction.
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