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**Title and Subtitle**
Novel Radiopharmaceuticals for Radioguided Surgery of Ductal Carcinoma In Situ of the Breast

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**Abstract (Maximum 200 Words)**
Ductal carcinoma in situ (DCIS) is an early stage of breast cancer in which cancer cells remain confined by the basement membrane of the ducts. DCIS is treated by surgery combined with local radiotherapy. The surgeon has difficulty however in accurately defining the disease margins which may lead to re-excision or recurrent DCIS which can in some cases advance to invasive breast cancer. Our objective is to develop novel radiopharmaceuticals for radioguided surgery (RGS) of DCIS to improve the surgical management. In the 1st year of the project Fab' fragments of the TAG72 monoclonal antibody CC49 and the HER-2/neu antibody, trastuzumab (Herceptin®) reactive immunohistochemically with DCIS were constructed and labeled with $^{111}$In or $^{99m}$Tc. The Fab' fragments were pure, homogenous and exhibited preserved immunoreactivity. In mouse tumor xenograft models, the $^{111}$In and $^{99m}$Tc Fab' fragments exhibited specific tumor localization and were rapidly cleared from most normal tissues allowing tumor imaging as early as 2 h post-injection. Tumor/blood ratios were 4:1 at 24 h and more than 10:1 at 48 h. In the 2nd year of the project, the Fab' fragments will be evaluated in phantoms of DCIS to project the sensitivity and resolution of the approach. In the 3rd year, a pilot Phase I clinical trial will be conducted.

**Subject Terms**
Breast Cancer, DCIS, radiopharmaceuticals, In-111, Tc-99m

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TABLE OF CONTENTS

Cover .................................................................................................................. 1
SF298 .................................................................................................................. 2
Table of Contents ............................................................................................... 3
Introduction .......................................................................................................... 4
Summary of Research Accomplished in Year 1 (2000-2001) ................................4

Task 1: Identification of monoclonal antibodies reactive with DCIS .................... 4
Task 2: Construct novel radiopharmaceuticals for radioguided surgery of DCIS ...... 6
Task 3: Conduct testing of radiopharmaceuticals in animal tumor xenograft models ... 11

Planned Research for Year 2 (2001-2002) .......................................................... 14

Task 1 (completion): Identification of combinations of monoclonal antibodies reactive with DCIS ................................................................. 14
Task 4: Conduct preclinical testing of radiopharmaceuticals in phantom models .... 15
Task 5 (commence): Design a Phase I clinical trial protocol for a pilot study of radioguided surgery in patients with DCIS and obtain regulatory approval ........ 15

Planned Research for Year 3 (2002-2003) .......................................................... 15

Task 5 (completion): Conduct a Phase I clinical trial of radioguided surgery of DCIS 15

Key Research Accomplishments ......................................................................... 15

Reportable Outcomes ......................................................................................... 16

Manuscripts ....................................................................................................... 16

Abstracts .......................................................................................................... 16

Presentations .................................................................................................... 16

Applications for Funding Based on Research .................................................... 16

Conclusions and Future Research .................................................................... 17

References ....................................................................................................... 17
INTRODUCTION

Ductal carcinoma *in situ* (DCIS) is an early non-invasive stage of breast cancer in which malignant cells remain confined by the basement membrane of the ducts (1). The incidence of DCIS has been increasing due to widespread mammographic screening to detect breast cancer at an early stage and DCIS now represents 15-30% of all new cases of breast cancer diagnosed mammographically (2,3). DCIS is treated in most cases by breast conservation surgery (BCS) followed by local radiotherapy, but re-excision is frequently required due to the difficulty in determining “clear” margins at operation. In addition, residual disease may be present in up to 25% of patients assumed to be treated successfully, resulting in recurrent disease, which in about half of cases presents as invasive breast cancer (4,5). There is a need to improve the surgical treatment of DCIS through more accurate delineation of the extent of disease in order to decrease re-excision rates with their attendant costs and patient morbidity, and also decrease the rate of recurrence. Radioguided surgery (RGS) is a technique which employs highly specific radiopharmaceuticals directed towards malignant cells administered to the patient prior to surgery (6). The surgeon uses a sensitive γ-detecting probe intraoperatively to more clearly define the margins for resection of disease through identifying cancer cells specifically targeted by the radiopharmaceuticals. Our objectives in this research project are therefore: 1) to develop novel monoclonal antibody Fab’ fragments labeled with $^{111}$In or $^{99m}$Tc as potential radiopharmaceuticals for radioguided surgery of DCIS and evaluate these preclinically in tumor xenograft models, 2) project the sensitivity and resolution of the approach using phantom models of DCIS containing breast cancer cells targeted *in vitro* with the radiopharmaceuticals and 3) design and conduct a pilot Phase I clinical trial of radioguided surgery in a limited group of patients with DCIS.

SUMMARY OF RESEARCH ACCOMPLISHED IN YEAR 1 (2000-2001)

The following represents a summary of the research accomplished in the 1st year of the project.

**Task 1: Identification of monoclonal antibodies reactive with DCIS by immunohistochemistry**

The tumor-associated glycoprotein-72 (TAG-72) cell surface mucin is reported to be expressed on more than 80% of DCIS (7) and the HER-2/neu transmembrane receptor tyrosine kinase has been detected in 60-80% of cases of DCIS (8). Monoclonal antibody CC49 is a murine IgG1 antibody directed against the TAG-72 cell-surface mucin available to our group through a Material Transfer Agreement with the NCI (U.S.). Trastuzumab (Herceptin®, Hoffman La Roche) is a commercially available anti-HER-2/neu antibody approved in the U.S. and Canada for the treatment of advanced HER-2/neu positive invasive breast cancer. Importantly, these antibodies are available in pharmaceutical quality, a requirement for rapid translation of our preclinical research to a pilot Phase I clinical trial of radioguided surgery of DCIS planned for the 3rd year of the project. We have therefore initially selected these two antibodies as promising candidates for radiopharmaceutical development for radioguided surgery of DCIS. The suitability of the antibodies for radioguided surgery of DCIS was screened by immunohistochemical staining of a panel of tissue sections including DCIS, invasive breast cancer, normal breast epithelium and benign breast disease. Briefly, sections were cut, deparaffinized and endogenous peroxidase blocked with 3% $\text{H}_2\text{O}_2$ for 15 mins. The sections
were stained using the Histostain-SP Mouse Kit™ or Histostain Rabbit Kit™ (Zymed) in the case of CC49 or trastuzumab respectively. The sections were incubated with the primary antibody (CC49 or trastuzumab) using a 1:16,000 to 1:32,000 dilution of a 1 mg/mL solution for 1 h, washed 3 times in Tris buffer and then incubated with biotinylated anti-mouse antibody (CC49) or anti-rabbit antibody (trastuzumab) for 15 mins. The sections were washed again and streptavidin conjugated to horseradish peroxidase (HRP) was incubated with the sections for 15 mins. The colour was developed by incubating with diaminobenzidine (DAB) solution for 10 mins. The stained slides were counterstained with hematoxylin and eosin and examined by a pathologist (Dr. Harriette Kahn). The results of the immunostaining experiments are shown in Table 1.

**Table 1.** Reactivity of monoclonal antibodies CC49 and trastuzumab (Herceptin®) with DCIS, invasive breast cancer, normal breast epithelium and benign breast disease.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>n</th>
<th>Reactivity by Immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Monoclonal antibody CC49</td>
</tr>
<tr>
<td>Benign breast disease</td>
<td>6</td>
<td>4†</td>
</tr>
<tr>
<td>Pure DCIS</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>Combined DCIS and invasive ductal carcinoma</td>
<td>14</td>
<td>4</td>
</tr>
</tbody>
</table>

† Minor staining of secretory surface epithelium.

An example of staining of DCIS by monoclonal antibody CC49 or trastuzumab (Herceptin®) is shown in **Fig. 1**. These represent preliminary immunohistochemical staining results and we are continuing to expand the number of cases tested with the individual antibodies. We are also planning to test combinations of the antibodies to determine if the heterogeneity of staining can be minimized by targeting more than one cell surface marker.

**Fig. 1.** Immunohistochemical staining of DCIS section with monoclonal antibody CC49 (left) or trastuzumab (Herceptin®) (right). There was high reactivity of the antibodies with DCIS but not with adjacent normal breast epithelium.
**Task 2: Construct novel radiopharmaceuticals for radioguided surgery of DCIS**

Novel radiopharmaceuticals consisting of monoclonal antibody Fab’ fragments of trastuzumab (Herceptin®) and anti-TAG72 mAb CC49 labeled with $^{111}$In or $^{99m}$Tc were constructed and evaluated in vitro for their purity, homogeneity and retention of immunoreactivity. Fab’ fragments offer the advantages of rapid tumor localization and clearance from the blood and normal tissues as well as low immunogenicity (9).

**Preparation of Fab’ fragments of trastuzumab (Herceptin®).** Herceptin® Fab’ fragments were prepared by enzymatic digestion of intact IgG using a 10-fold molar excess of immobilized papain (Pierce Chemical Co.) in a digestion buffer (20 mM phosphate + 10 mM EDTA-Na$_4$, pH 7.0) containing 80 mM cysteine for 24 h at 37 °C. Fab’ fragments were purified by affinity chromatography on a Protein-A column (Pierce) followed by additional purification and reconcentration by ultrafiltration on Centricon-50 and 100 devices (Amicon). The purity of the Fab’ fragments was assessed by SDS-PAGE on a 4-20% Tris-HCl gradient gel under nonreducing and reducing conditions and by Western blot. Herceptin® Fab’ migrated as a single band with the expected M$_r$ of ~50 kDa on SDS-PAGE. Western blot showed that this band was reactive with anti-human Fab’ (Fig. 2). The purity of Herceptin® Fab’ fragments was >90% as determined by densitometry of the SDS-PAGE gels.

![SDS-PAGE and Western Blot](image)

**Fig. 2.** SDS-PAGE analysis of purified Fab’ fragments of trastuzumab (Herceptin®) on a a 4-20% Tris-HCl gradient gel under non-reducing and reducing conditions. Fab’ exhibited a single band under non-reducing conditions with the expected M$_r$ (50 kDa). Under reducing conditions the Fab’ fragment migrated as a single band with M$_r$ of 25 kDa. Western blot using an anti-human Fab’ antibody confirmed the identity of the M$_r$ 50 kDa band on SDS-PAGE.

**Radiolabeling of Fab’ fragments of trastuzumab (Herceptin®) with $^{99m}$Tc.** Fab’ fragments of trastuzumab (Herceptin®) were radiolabeled with $^{99m}$Tc using methodology described by Ultee et al. (10). Briefly, Fab’ fragments (5 mg/mL) or intact IgG were modified with hydrazinonicotinamide (HYNIC) by reacting the ε-NH$_2$ groups on lysine residues with a 5-fold to 20-fold molar excess of HYNIC N-hydroxysuccinimide ester. HYNIC-Fab’ was purified from excess free HYNIC by size-exclusion chromatography on a Sephadex G-25 (Pharmacia) mini-column. Purified HYNIC-Fab’ was aliquoted and stored at 4 °C until required for labeling with
The HYNIC substitution of trastuzumab (Herceptin®) Fab' was assayed as described by King et al. (11). The assay is based on reaction of the introduced hydrazine moieties with p-nitrobenzaldehyde to form a colored complex which absorbs at 405 nm. A standard curve was constructed by assaying solutions containing increasing concentrations of HYNIC (0 to 100 μM). A 100 μl aliquot of standard HYNIC solution was mixed in a 96-well plate with 100 μl of 1.0 mM p-nitrobenzaldehyde (Sigma) solution prepared by dissolving p-nitrobenzaldehyde in acetonitrile (6.4 mg/mL) then diluting to 20 mM citrate buffer pH 5.2. After incubation for 30 mins at room temperature, the absorbance of the wells at 405 nm was recorded by a plate reader to generate the standard curve (absorbance vs. HYNIC concentration). To determine the concentration of HYNIC in HYNIC-IgG or Fab' conjugates, the conjugates were diluted to 1 mg/mL in 20 mM citrate buffer pH 5.2, then reacted with 1 mM p-nitrobenzaldehyde at room temperature for 30 mins. The absorbance at 405 nm was recorded, and the molar concentration of HYNIC in the HYNIC-IgG or Fab' conjugates calculated from the standard calibration curve. The concentration of proteins (IgG or Fab') in HYNIC-IgG or Fab' was determined from their UV absorbance at 280 nm using an extinction coefficient of $E_{1cm}^{1%}$ of 15. The substitution level of HYNIC-IgG or Fab' was calculated by dividing the molar concentration of HYNIC by the molar concentration of IgG or Fab'. The HYNIC substitution using different reaction conditions is shown in Table 2. The desired substitution level of 1-3 HYNIC molecules/molecule trastuzumab Fab' required to retain immunoreactivity was achieved at a molar ratio (HYNIC:Fab') of 10:1.

### Table 2. Derivatization of trastuzumab (Herceptin®) Fab' with hydrazinonicotinamide (HYNIC) for radiolabeling with $^{99m}$Tc.

<table>
<thead>
<tr>
<th>Molar Ratio (HYNIC: Fab')</th>
<th>n</th>
<th>HYNIC Substitution (Mols HYNIC/mol Fab')</th>
</tr>
</thead>
<tbody>
<tr>
<td>5:1</td>
<td>3</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>10:1</td>
<td>3</td>
<td>2.3 ± 0.9</td>
</tr>
<tr>
<td>15:1</td>
<td>2</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>20:1</td>
<td>3</td>
<td>11.6 ± 3.5</td>
</tr>
</tbody>
</table>

Radiolabeling of HYNIC-trastuzumab Fab' was performed by incubating the antibody with $^{99m}$Tc-glucosperonate for 1 h at room temperature. $^{99m}$Tc-glucosperonate was prepared by adding $^{99m}$Tc-sodium pertechnetate (150 mCi) freshly eluted from a $^{99m}$Tc/$^{99m}$Tc generator (Dupont Pharma) to an in-house stannous glucosperonate kit containing calcium glucosperonate (100 mg/mL) and stannous chloride (0.07 mg/mL) in Sterile Water for Injection USP. $^{99m}$Tc-glucosperonate forms a covalent linkage with the introduced hydrazine groups on the antibody. The radiochemical purity of $^{99m}$Tc-trastuzumab (Herceptin®) was >94% as measured by silica gel instant thin layer chromatography (ITLC-SG) developed in 150 mM sodium chloride. The specific activity achieved ranged from 10-10 μCi/μg.

Radiolabeling of Fab' fragments of trastuzumab (Herceptin®) with $^{111}$In. Fab' fragments of trastuzumab (Herceptin®) were radiolabeled with $^{111}$In by modification of the proteins with the metal chelator, diethylenetriaminepentaacetic acid (DTPA) using methodology previously reported by us (12). Fab' fragments (10 mg/mL in 50 mM sodium bicarbonate buffer pH 7.4) were reacted with a 10-fold molar excess of the bicyclic anhydride of DTPA (Sigma), then
purified from excess DTPA by size-exclusion chromatography on a Sephadex G-25 mini-column. Purified DTPA-trastuzumab Fab' (Herceptin®) was radiolabeled by incubation with $^{111}$In acetate for 30 mins at room temperature. $^{111}$In-acetate was prepared by mixing equal volumes of $^{111}$In chloride (MDS-Nordion) with 1 M acetate buffer pH 6.0. The radiochemical purity of $^{111}$In-trastuzumab Fab' (Herceptin®) was >90% as measured by silica gel-instant thin layer chromatography (ITLC-SG) developed in 100 mM sodium citrate pH 5.0. The specific activity achieved was approximately 1 μCi/μg.

**Radiolabeling of Fab' fragments of trastuzumab (Herceptin®) with $^{125}$I.** Fab' fragments of trastuzumab (Herceptin®) Fab' and intact IgG were radiolabeled with $^{125}$I using the Iodogen™ (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) method (13). Briefly, trastuzumab (Herceptin®) Fab' or intact IgG were incubated for 10 mins with $^{125}$I-sodium iodide (Amersham) in a glass tube precoated with 10 μg of Iodogen. The radioiodinated antibodies were purified by size exclusion chromatography on a Sephadex G-25 mini-column eluted with 1% bovine serum albumin (BSA) in phosphate buffered saline pH 7.4 (PBS). The radiochemical purity of $^{125}$I-trastuzumab (Herceptin®) Fab' and intact IgG was >99% as measured by paper chromatography (Whatman No. 1) developed in 85% methanol/water.

**Determination of the immunoreactivity of trastuzumab (Herceptin®) IgG and Fab'.** The immunoreactivity of trastuzumab (Herceptin®) Fab' fragments was first assessed by competition binding assays against HER-2/neu (+) SKBr-3 breast cancer cells. Briefly, $1 \times 10^6$ SKBr-3 cells (ATCC) were incubated with $^{125}$I-trastuzumab (Herceptin®) IgG and increasing concentrations of trastuzumab IgG, Fab', HYNIC-IgG or HYNIC-Fab' at 4 °C for 3.5 h. The cell suspensions were centrifuged and the cell pellet washed twice with ice-cold PBS containing 0.1% BSA. The radioactivity bound to the cell pellet was measured in a γ-counter using a window for $^{125}$I (15-80 keV). The binding curves (Fig. 3) were fitted to a one-site competition binding model using Graphpad Prism™ obtain the $K_d$ values. $K_a$ values were calculated as $K_a = 1/K_d$.

![Fig. 3. Competition assay for trastuzumab IgG and Fab’ and HYNIC-derivatized analogs against HER-2/neu (+) SKBr-3 breast cancer cells. The affinity of trastuzumab-Fab’ for HER-2/neu in this assay was reduced 4-fold compared to IgG. HYNIC derivatization decreased the binding affinity of the antibodies 4-5 fold.](image)

The $K_a$ values were: $2.7 \times 10^8$ and $6.6 \times 10^7$ L/mol for non-derivatized IgG and Fab’ respectively and $5.9 \times 10^7$ and $1.5 \times 10^7$ L/mol respectively for HYNIC-derivatized IgG and Fab’. These assays demonstrated approximately a 4-fold decrease in binding affinity for trastuzumab Fab’
compared to intact IgG and a further 4-fold decrease in affinity following derivatization with HYNIC for labeling with $^{99m}$Tc.

The immunoreactivity of trastuzumab (Herceptin®) IgG or Fab' fragments labeled with $^{125}$I was also evaluated in a direct radioligand binding assay using HER-2/neu (+) SKBr-3 breast cancer cells. The direct radioligand binding assay was performed by incubating increasing concentrations of $^{125}$I-labeled antibodies with $1 \times 10^6$ SKBr-3 cells at 4 °C for 3.5 h. The cell suspensions were centrifuged and washed twice with ice-cold PBS containing 0.1% BSA. The cell pellet and supernatant were collected and measured in a γ-counter using a window for $^{125}$I (15-80 keV). The assay was performed in the absence (total binding, TB) or presence (non-specific binding, NSB) of an excess (100 nM) of unlabeled IgG or Fab'. Specific binding (SB) was obtained by subtraction of NSB from TB. The cell binding data were fitted to a one-site binding model using least-square regression analysis by GraphPad Prism™ software to estimate the $K_a$ and $B_{max}$ values. The direct radioligand binding assays (Fig. 4) showed that the binding affinity of trastuzumab Fab' labeled with $^{125}$I was decreased 2-fold compared to the intact IgG ($K_a$ 7.7 × 10$^7$ vs. 1.4 × 10$^8$ L/mol respectively). The number of binding sites ($B_{max}$) were actually higher however for Fab' than that for IgG (2.9 × 10$^6$ vs. 1.8 × 10$^6$ sites/cell respectively).

Fig. 4. Direct binding assay for $^{125}$I-trastuzumab (Herceptin®) IgG (left) or Fab' (right) to HER-2/neu positive SKBr-3 human breast cancer cells. The $K_a$ value for $^{125}$I-trastuzumab Fab' was reduced 2-fold compared to intact IgG ($K_a$ 7.7 × 10$^7$ vs. 1.4 × 10$^8$ L/mol respectively).

Preparation of Fab' fragments of monoclonal antibody CC49. The TAG-72 monoclonal antibody CC49 was purified from mouse ascites (supplied by Dr. J. Schlom, NCI) by affinity chromatography on a Protein G column (Pierce). Fab' fragments of monoclonal antibody CC49 were prepared by enzymatic digestion of the intact IgG using immobilized papain and purified as described previously for trastuzumab (Herceptin®) Fab'. The purity of the Fab' fragments was determined by SDS-PAGE, Western blot and size-exclusion HPLC as described for trastuzumab (Herceptin®) Fab'. CC49 Fab' migrated as a single band on SDS-PAGE with the expected $M_r$ of 50 kDa which was positive on Western blot using an anti-mouse Fab antibody (Fig. 5). Size-exclusion HPLC (not shown) demonstrated that the purity of the Fab' fragments was >92%.
Fig. 5. SDS-PAGE analysis of purified Fab' fragments of monoclonal antibody CC49 on a a 4-20% Tris-HCl gradient gel under non-reducing and reducing conditions. Fab' exhibited a single band under non-reducing conditions with the expected M_{r} (50 kDa). Under reducing conditions the Fab' fragment migrated as a single band with M_{r} of 25 kDa. Western blot using an anti-mouse Fab' antibody confirmed the identity of the M_{r} 50 kDa band on SDS-PAGE.

**Determination of the immunoreactivity of monoclonal antibody CC49 IgG and Fab'**. The immunoreactivity of monoclonal antibody CC49 IgG and Fab' fragments were assessed by a solid phase competition immunobinding assay. Briefly, 10 ng of bovine submaxillary mucin (BSM), a source of the TAG-72 antigen, was coated onto wells in a 96-well microELISA plate at 4 °C overnight. Non-specific binding sites were blocked by incubating the wells in 5% BSA in PBS for 1 h at 37 °C. Serial dilutions of CC49 IgG or Fab' were then incubated in the wells with ^{125}I-labeled CC49 IgG at 4 °C for 16-18 h. The wells were washed with PBS and the supernatant discarded. ^{125}I radioactivity bound to the wells was measured in a γ-counter. The binding data were fitted to a one-site binding competition model using Graphpad Prism™ software to obtain estimates of K_{d} and B_{max} (Fig. 6).

Fig. 6. Competition binding assay for CC49 IgG and Fab' fragments against bovine submaxillary mucin immobilized on a microELISA plate. The affinity for CC49 Fab' was reduced about 100-fold compared to the intact IgG.
These assays showed that there was a significant decrease in binding affinity of CC49 Fab' compared to the intact IgG (K_a 1.1 × 10^6 vs. 1.2 × 10^6 L/mol respectively). This suggests that alternative methods of preparing Fab' fragments of monoclonal antibody CC49 may have to be considered to obtain a higher immunoreactivity product.

Task 3: Conduct preclinical testing of radiopharmaceuticals in animal tumor xenograft models

Establishment of a mouse xenograft model of DCIS. Our original plan was to establish a mouse xenograft model of DCIS similar to that reported by Holland et al. (14) by implanting surgical specimens into non-obese diabetic severe combined immunodeficiency (NOD-scid) mice. However repeated attempts to establish the tumors were unsuccessful and it was therefore decided instead to establish xenografts using cancer cell lines expressing the appropriate antigens/receptors for monoclonal antibody CC49 or trastuzumab (Herceptin®) implanted subcutaneously into athymic mice. This was the alternative plan to establish a xenograft model for evaluation of the radiopharmaceuticals which was outlined in our original research proposal.

Tumor imaging and biodistribution studies of trastuzumab (Herceptin®) IgG and Fab'. The tumor and normal tissue localization of trastuzumab (Herceptin®) labeled with ⁹⁹ᵐTc or ¹¹¹In was evaluated in athymic mice implanted subcutaneously with HER-2/neu positive BT-474 human breast cancer xenografts. SKBr-3 cells were not used to establish the tumor xenografts because they do not grow as tumors in athymic mice. Female athymic mice received an intradermal injection of a sustained release estradiol pellet (Innovative Research) required for growth of BT-474 xenografts. One day following implantation of the estradiol pellet, 1 × 10⁷ BT-474 cells in 100 µL of Matrigel® (Becton-Dickinson) were injected subcutaneously in the right hind leg. When the tumors reached a diameter of 0.2-0.5 cm, trastuzumab (Herceptin®) Fab' labeled with ⁹⁹ᵐTc or ¹¹¹In was injected intravenously in the tail vein of the animals (30-50 µCi/mouse). A control group of mice received an identical amount of Fab' fragments of the irrelevant CD33 monoclonal antibody HuM195 (Protein Design Labs). The mice were imaged at selected time points up to 24 hours post-injection for ⁹⁹ᵐTc-labeled antibodies or 48 hours post-injection for ¹¹¹In-labeled antibodies. The mice were then sacrificed and the tumor and normal tissue uptake of the radiopharmaceuticals determined. The biodistribution data was expressed as percent injected dose/g (% i.d./g) (Fig. 7) and tumor/normal tissue (T/NT) ratios (Fig. 8).

Fig. 7. Tumor and normal tissue uptake of ⁹⁹ᵐTc-trastuzumab Fab' at selected times p.i. in athymic mice bearing s.c. HER-2/neu (+) BT-474 breast cancer xenografts. Organs are B (blood), H (heart), Lu (lungs), L (liver), K (kidneys), Sp (spleen), St (stomach), I (intestine), T (tumor) and M (muscle).
The specificity of tumor uptake of trastuzumab (Herceptin®) Fab' was determined by comparison with that for irrelevant HuM195 Fab'. Tumor uptake at 24 h for $^{99m}$Tc-trastuzumab (Herceptin®) Fab' (10.7 ± 3.5 % i.d./g) was significantly higher (t-test, p<0.05) than that for HuM195 Fab' (2.6 ± 0.5 % i.d./g) demonstrating specific tumor uptake. The major normal tissue uptake of $^{99m}$Tc-trastuzumab (Herceptin®) Fab' was mainly in the kidneys but also in the liver, lungs and spleen.

**Fig. 8.** T/NT ratios for $^{99m}$Tc-trastuzumab (Herceptin®) Fab' at selected times post-injection in athymic mice bearing s.c. HER-2/neu (+) BT-474 breast cancer xenografts. Organs are B (blood), H (heart), Lu (lungs), L (liver), K (kidneys), Sp (spleen), St (stomach), I (intestine), T (tumor) and M (muscle).

The T/NT ratios at 24 h for $^{99m}$Tc-trastuzumab (Herceptin®) Fab' were significantly higher (t-test, p<0.05) than those for irrelevant HuM195 Fab' demonstrating that the antibody localized specifically in the tumors. The tumor/blood ratio for $^{99m}$Tc-trastuzumab (Herceptin®) Fab' at 24 h (3.2 ± 0.7) was significantly greater than that for HuM195 Fab' (0.9 ± 0.2).

The short half-life of $^{99m}$Tc (6 hours) does not permit sensitive detection beyond 24 h post-injection of the radiopharmaceutical. In order to determine if improvements in tumor localization may be achieved at later time points, trastuzumab (Herceptin®) was labeled with $^{111}$In (half-life of 67 hours). The tumor and normal tissue localization (% i.d./g) at 24 and 48 h after injection for $^{111}$In-trastuzumab (Herceptin®) are shown in **Fig. 9** and the T/NT ratios in **Fig. 10**.

**Fig. 9.** Tumor and normal tissue uptake of $^{111}$In-trastuzumab (Herceptin®) Fab' at 24 and 48 h after injection in athymic mice bearing s.c. HER-2/neu (+) BT-474 breast cancer xenografts. Organs are B (blood), H (heart), Lu (lungs), L (liver), K (kidneys), Sp (spleen), St (stomach), I (intestine), T (tumor) and M (muscle).
**Fig. 9.** T/NT ratios for $^{111}$In-trastuzumab (Herceptin®) Fab' at 24 and 48 h after injection in athymic mice bearing s.c. HER-2/neu (+) BT-474 breast cancer xenografts. Organs are B (blood), H (heart), Lu (lungs), L (liver), K (kidneys), Sp (spleen), St (stomach), I (intestine), T (tumor) and M (muscle).

The tumor/normal tissue ratios were increased for $^{111}$In-trastuzumab Fab' compared to $^{99m}$Tc-trastuzumab Fab'. For example the tumor/blood ratio for $^{111}$In-trastuzumab Fab' was 4.1 ± 0.6 and 10.3 ± 1.0 at 24 h and 48 h post-injection respectively compared to 3.2 ± 0.7 for $^{99m}$Tc-trastuzumab Fab' at 24 h post-injection. These results suggest that a longer time period (ie. 48 h) between injection of the radiopharmaceutical and performance of the radioguided surgery in patients may provide higher tumour/background ratios which could enhance the resolution of the technique. The use of a longer half-life radiolabel such as $^{111}$In would permit the time delay required.

HER-2/neu (+) BT-474 breast cancer xenografts were imaged as early as 2 h post-injection of $^{99m}$Tc-trastuzumab Fab' (**Fig. 11**).

**Fig. 11.** Successful imaging of subcutaneous HER-2/neu (+) BT-474 human breast cancer xenograft at 2 hours post-injection of $^{99m}$Tc-trastuzumab (Herceptin®) Fab'. Normal organs also visualized are the kidneys and tail (injection site).

**Tumor imaging and biodistribution studies of monoclonal antibody CC49 IgG and Fab'.** Tumor imaging and biodistribution studies were performed with monoclonal antibody CC49 Fab' labeled with $^{99m}$Tc in athymic mice bearing subcutaneous TAG72 (+) LS174T human colon cancer xenografts. Although the LS174T xenografts were not of breast cancer origin, they nevertheless provided a suitable animal tumor model expressing the target antigens to evaluate
the tumor and normal tissue localization of the radiopharmaceuticals in vivo. The tumor accumulation (% i.d./g) and tumor/normal tissue ratios are shown in Fig. 12 and Fig. 13 respectively.

**Fig. 12.** Tumor and normal tissue uptake of $^{99m}$Tc-monoclonal antibody CC49 Fab' at selected times post-injection in athymic mice bearing s.c. TAG72 (+) LS174T colon cancer xenografts. Organs are B (blood), H (heart), Lu (lungs), L (liver), K (kidneys), Sp (spleen), St (stomach), I (intestine), T (tumor) and M (muscle).

**Fig. 13.** T/NT ratios for $^{99m}$Tc-monoclonal antibody CC49 Fab' at selected times post-injection in athymic mice bearing s.c. TAG72 (+) LS174T colon cancer xenografts. Organs are B (blood), H (heart), Lu (lungs), L (liver), K (kidneys), Sp (spleen), St (stomach), I (intestine), T (tumor) and M (muscle).

These studies demonstrated that $^{99m}$Tc-monoclonal antibody CC49 Fab' localized effectively in TAG72 (+) LS174T tumor xenografts despite the decreased immunoreactivity observed in vitro and was rapidly eliminated from the blood and normal tissues producing tumor/blood ratios of >4:1 at 24 h post-injection. The rapid tumor uptake and clearance from normal tissues allowed successful tumor imaging as early as 2 h post-injection (not shown).

**PLANNED RESEARCH FOR YEAR 2 (2001-2002)**

**Task 1 (completion): Identification of combinations of monoclonal antibodies reactive with DCIS by immunohistochemistry**

In the 2nd year of the project, we complete our immunohistochemical evaluation of the candidate antibodies (CC49 and trastuzumab) by expanding the number of cases of DCIS, invasive breast cancer, benign breast and normal breast epithelium evaluated. We will also test combinations of
the two antibodies to determine if this could increase the immunoreactivity towards DCIS and minimize the effect of antigen/receptor heterogeneity. We may also test additional candidate monoclonal antibodies directed against targets on DCIS for radiopharmaceutical development if these can be identified.

**Task 4: Conduct preclinical testing of radiopharmaceuticals in phantom models**

In the 2nd year of the project, we will design, construct and evaluate experimentally phantom models of DCIS to project the sensitivity and resolution of radioguided surgery of DCIS in patients. The phantoms will consist of anatomically correct models of DCIS in which breast cancer cells targeted *in vitro* with the radiopharmaceutical can be dispensed to simulate a lesion. The phantoms will be surrounded by a background containing a radioactive solution to simulate background (or adjacent normal tissue radioactivity) and overlayed with water to simulate attenuation by overlying normal tissue. The phantoms will be used to evaluate the detection sensitivity and resolution of the γ-detecting probe (Neoprobe). The γ-detecting probe required for these studies will be purchased early in the 2nd year of the project.

**Task 5 (commence): Design a Phase I clinical trial protocol for a pilot study of radioguided surgery in patients with DCIS and obtain regulatory approval**

We have prepared an initial draft protocol for the pilot Phase I clinical trial planned for the 3rd year of the project. This draft protocol will continue to be reviewed and revised taking into account the results of our preclinical experiments in animal tumor xenograft models and in the phantom models. Our goal is to submit the final protocol to our institutional Research Ethics Board (REB), Health Canada and the U.S. Army Human Subjects Review Board by the end of the 2nd year.

**PLANNED RESEARCH FOR YEAR 3 (2002-2003)**

**Task 6 (completion): Conduct a Phase I clinical trial of radioguided surgery of DCIS**

The pilot Phase I clinical trial of radioguided surgery of DCIS is planned for the 3rd year of the project once the preclinical research phase is completed and we have obtained institutional REB, U.S. Army and Health Canada regulatory approvals.

**KEY RESEARCH ACCOMPLISHMENTS**

- Evaluated the reactivity of monoclonal antibodies CC49 and trastuzumab (Herceptin®) against DCIS, invasive breast cancer, benign breast disease and normal breast epithelium by immunohistochemical staining.

- Constructed Fab’ fragments of monoclonal antibody CC49 and trastuzumab (Herceptin®) and evaluated their purity, homogeneity and retention of immunoreactivity *in vitro* using breast cancer cell lines expressing the appropriate receptors or by ELISA using immobilized soluble antigens.
- Radiolabeled monoclonal antibody CC49 and trastuzumab (Herceptin®) IgG and Fab' fragments with $^{111}$In and $^{99m}$Tc and evaluated their tumor and normal tissue localization in athymic mice bearing human tumor xenografts expressing the appropriate antigens/receptors.

- Prepared a preliminary draft of a protocol for the pilot Phase I clinical trial of radioguided surgery of DCIS in anticipation of submission for Research Ethics Board (REB) and Health Canada approval.

REPORTABLE OUTCOMES

Manuscripts


Abstracts


* Ms. Ying Tang, who is completing her PhD in Pharmaceutical Sciences at the University of Toronto under my supervision and whose thesis is based on this work, received a prestigious AACR-Smithkline-Glaxo Scholar-in-Training Award for the above abstract presented at the annual meeting of the AACR in New Orleans, March 24-28, 2001.

Presentations

Reilly, R.M. Molecular imaging and targeted radiotherapy of cancer: Past, present and future. Presented at Department of Medical Biophysics seminar, Sunnybrook and Women’s College Health Sciences Centre, Toronto, ON, July 4, 2001.


Applications for Funding Based on Research


