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PRINCIPAL INVESTIGATOR: Michael J. Blend, Ph.D.
Steven M. Swanson, Ph.D.
Jeffrey Medin, Ph.D.

CONTRACTING ORGANIZATION: University of Illinois at Chicago
Chicago, Illinois 60612-7227

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6. AUTHOR(S)
Michael J. Blend, Ph.D.
Steven M. Swanson, Ph.D.
Jeffrey Medin, Ph.D

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Illinois at Chicago
Chicago, Illinois 60612-7227
E-Mail: **MJBlend@uic.edu**

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13. ABSTRACT *(Maximum 200 Words)*

The goal of this research is to develop stable radioimmunoconjugates (RICs) of *anti-HER2/neu* antibodies (MoAbs) for imaging and therapy in an animal model bearing human breast tumors. Pharmacy-grade Herceptin and an *anti-HER2/neu* antibody were conjugated with a DTPA linker. $^{111}InCl_3$ and $^{90}YCl_3$ were labeled to MoAb-CHX-A"–DTPA according to the procedure of Clarke et al. (*Cancer Res* 60: 4804, 2000). Binding capacity of RICs was tested with human cancer cell lines MCF-7, HCC-1954, BT-474 and SKBR-3. Results: The molar ratio of DTPA-CHX-A" to MoAbs was 1.4:1. Using a molar excess of 10:1 CHX-A"-DTPA to MoAb, a specific activity of 1.87 μCi ^{111}In/μg MoAb-RIC and 2.71 μCi ^{90}Y/μg MoAb-RIC was obtained. The purity of RIC was 96%+ for ^{111}In and 99%+ for ^{90}Y. Stability at 37° C for both RICs ranged from 98% at 24 hours in human plasma to 85% at 96 hours. ^{111}In- RIC bound to MCF-7 cells with a binding radio of 2.5:1 using a nonspecific IgG RIC as a control. A 98.3:1 binding ratio was observed when SKBR-3 cells were exposed. Conclusion: MoAbs can be labeled with ^{111}In and ^{90}Y using a CHX-A"-DTPA linker. Stable RICs are formed and significant cell binding of RICs occurs when *HER2/neu* over-expressing cells are used.

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INTRODUCTION

Many women continue to present to their physician with locally advanced or metastatic breast cancer. A portion of these patients may have a particularly aggressive form of this disease with little or no clinical signs of the disease. Biopsy evaluation as well as the measurement of serum tumor markers, hormone receptor status, and certain epidermal growth receptor proteins on the surface of tumor cells all help to determine the grade and aggressiveness of a particular cancer. Between 25 and 30% of all breast cancer patients have an aggressive form of breast cancer known as *HER2/neu* positive disease. The presence of *HER2/neu* over-expression signifies a poor prognosis for these patients. Innovative techniques and treatment modalities are needed to improve the outcomes of these particular breast cancer patients.

Recently the FDA approved an injectable monoclonal antibody (MoAb) that can slow the progress of *HER2/neu* over-expressing breast cancers in these patients. In clinical trials, the anti-*HER2/neu* MoAb Herceptin® (trastuzumab, Genentech) showed significant activity both as a single agent and in combination with traditional cytotoxic chemotherapy in the treatment of *HER2/neu* over-expressing metastatic breast cancer patients. Herceptin® is a recombinant anti-*HER2/neu* MoAb that targets the extracellular domain of HER-2 growth factor receptor, inhibiting signal transduction and cell proliferation. It also enhances antibody-dependent, cell-mediated cytotoxicity.

The long-term goal of this investigation is to determine if adding a diagnostic isotope known as Indium-111 (^{111}In) and alternately, a therapeutic isotope know as Yttrium-90 (^{90}Y) to Herceptin® can improve its effectiveness as an anticancer agent. Adding ^{111}In to Herceptin® may convert this MoAb into an effective diagnostic staging tool. Adding ^{90}Y to Herceptin® may improve its cytotoxic efficacy without adding significant risk to the patient.

The purpose of this midterm report is to describe the results of studies completed in our investigations to date. We began the investigation by labeling Herceptin®, anti-*HER2/neu*, and nonspecific IgG antibodies with ^{111}In and ^{90}Y using both a DOTA and DTPA linkers. Once a standard procedure for making these radioimmunoconjugates (RICs) was established, the new RICs were testing for chemical and in vitro stability. Results of completed studies are presented in the order in which they were proposed in the original approved grant. We are currently in the middle of a large imaging and biodistribution study using nude mice bearing normal and over-expression *HER2/neu* human breast cancer zenographs using ^{111}In-RICs.

BODY

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<u>OBJECTIVE 1:</u> To synthesize ^{111}In- and ^{90}Y-anti-*HER2/neu* antibody conjugates and perform <u>in vitro</u> analyses of stability, specificity, and affinity for normal expressing and over expressing HER2 human breast cancer cells.

The following two methods were used to label Herceptin® with ^{111}In.

<u>1. Labeling anti-HER2/neu and mouse IgG with ^{111}In using a DOTA Linker:</u>

The labeling procedure originally proposed for combining Herceptin® with radioisotopes was described by Li et al. (1,2) and Meares et al. (3). Multiple experiments using ^{111}In Chloride and the bifunctional chelating agent 1,4,7,10-tetraazacyclododecane-N', N'', N'''tetraacetic acid (DOTA) were performed. The procedure described in the literature was followed. Details of this labeling procedure can be found in the paper by Li et al.(2). Using the DOTA chelate, $^{111}InCl_3$ was successfully labeled to anti-*HER2/neu* monoclonal antibody obtained from Becton Dickinson (San Jose, CA) and nonspecific mouse IgG protein (BIO RAD, Richmond, CA). The results of this labeling experiment are presented in Table 1.

Table 1

Specific Activity, Purity, & Efficiency of ^{111}In Labeling of Anti-*HER2/neu* & IgG Using the p-SCN-Bz-DOTA Chelate

Protein	Specific Activity µCi/µg	Purity of RIC (%)	Efficiency of Labeling (%)
Anti-HER2/neu-DOTA	**1.55**	**97.7**	**14.3**
IgG-DOTA	**1.45**	**96.5**	**13.7**

As originally stated in our proposal, purified Herceptin® (to be obtained directly from the manufacturer) would be used for labeling purposes. However, the manufacturer, who had initially agreed to supply the antibody as a purified preparation (no preservatives added), stopped producing all purified preparations of Herceptin® for investigational purposes. Only pharmacy grade Herceptin® designed for direct infusion into patients could be obtained. **Labeling pharmacy grade Herceptin® with ^{111}In using the DOTA linker did not work.** The reason for this may be, in part, the presence of the following extraneous compounds (per manufacturer) in the pharmacy grade Herceptin®:

> "Herceptin® is a sterile, white to pale yellow, preservative-free lyophilized powder for intravenous (IV) administration. Each vial of Herceptin® contains 440 mg trastuzumab, 9.9 mg L-histidine HCl, 6.4 mg L-histidine, 400mg alpha, alpha-trehalose dihydrate, and 1.8 mg polysorbate 20."

The preparation is not designed for labeling using a DOTA chelator. We believe that some of the components present in the clinical preparation of Herceptin® may be interfering in the DOTA reaction. (The DOTA reaction worked well when purified anti-*HER2/neu* MoAb obtained from Becton Dickinson of San Jose, CA and control mouse IgG was used.)

Experiments were conducted to purify sufficient amounts of Herceptin® from the pharmacy grade preparation using ammonium sulfate precipitation techniques and Protein A column filtration (Sigma, St. Louis, MO). After collecting an adequate amount of pure Herceptin® we were successful in labeling it with ^{111}In using the DOTA chelator. However the new RIC failed to bind with tumor cells in vitro suggesting the process of purification destroyed to immunoreactivity of Herceptin®. The details of these experiments will be described in a paper in preparation for publication.

2. Labeling Pharmacy Grade Herceptin® with ^{111}In using a new DTPA Linker:

A. Synthesis of the ^{111}In RIC

The recently developed derivative of DTPA produced by investigators at the National Cancer Institute and the National Institutes of Health (NCI/NIH) was used. The new bifunctional chelator agent was obtained from Dr. Martin Brechbiel of the NCI/NIH. The details of labeling antibodies with this agent can be found in the article by Clarke et al. (4). We are now able to successfully label the pharmacy grade Herceptin® and the results of these experiments are described as follows:

We obtained Herceptin® from our hospital pharmacy (21 mg/ml, lot number 003419) as an IV preparation suitable for human use. The Herceptin® preparation was transferred to a dialysis system with a molecular cutoff of 10,000 Daltons and dialyzed overnight at room temperature against 3 L of 50-mM sodium bicarbonate buffer, pH 8.6. After dialysis, the total amount of protein was determined using the BIO-RAD Protein Assay Kit and an acidic solution of Coomassie Brilliant Blue G-250 dye. CHX-A''-DTPA was added to the dialyzed Herceptin® in molar excess of 10:1 (PREPARATION A) and 50:1 (PREPARATION B) and incubated overnight at room temperature in the dark. CHX-A''-DTPA-Herceptin® conjugate was mixed with an equal volume of 0.5 M Sodium Acetate buffer, pH 5.5. The mixture was added to ^{111}In-chloride obtained from Nycomed-Amersham of Chicago, Illinois (approx. 1 mCi per 100 μg Herceptin in the conjugate). The mixture was incubated for 20 minutes at room temperature and then the pH was raised to 7 with 2.0 M Sodium Acetate pH 9.5.

The radioimmunoconjugate (HERCEPTIN-CHX-A''-DTPA-^{111}In) was separated from CHX-A''-DTPA-^{111}In and free ^{111}In chloride by Sephadex G-25 PD-10 column (Supelco; Sigma, St. Louis, MO) as follows. The PD-10 column was first washed with 20 ml of 1% human serum albumin in 20 mM sodium acetate buffer, pH 6.3 in 0.15 M sodium chloride then with 25 ml of the same buffer without human albumin. The radiolabeled mixture was then loaded on the PD-10 column and fractions of 0.7 ml each were eluted with 20 mM sodium acetate buffer pH 6.3 in 0.15 M sodium chloride. The radioactivity of each fraction was

measured. The radioimmunoconjugate (HERCEPTIN-CHX-A''-DTPA-^{111}In) was eluted first (usually fraction #4). Purity was determined by ITLC using silica gel impregnated glass filter strips. To determine the efficiency of ^{111}In binding, ITLC strips were developed in a solvent system containing 10 mM EDTA (pH 4.5) and 0.9% saline in 10 mM NaOH as solvents. The yield, purity, and specific activity of the ^{111}In-labeled Herceptin® were estimated for the above two preparations. The results are presented in Table 2. The efficiency of labeling as well as the radiochemical purity was higher for the first preparation (PREPARATION A). However, Preparation A yielded lower specific activity than Preparation B.

Table 2

Specific Activity, Purity and Efficiency of Labeling of Herceptin® with CHA-A"-DTPA-^{111}In.

	PREPARATION A*	PREPARATION B*
Activity (μCi ^{111}In/μg Herceptin®)	1.87	3.10
Radiochemical Purity (% ^{111}In in RIC)**	96.8	92.7
Efficiency of Labeling (% ^{111}In bound to RIC)	31.8	19.4

* Molar excess of CHX-A"-DTPA: Herceptin®: PREPARATION A = 10:1, PREPARATION B = 50:1.
** Un-reacted chelate removed by dialysis.

The data clearly shows that ^{111}In labeling of Herceptin® using CHX-A''-DTPA is working in our laboratory. Because of the greater radiochemical purity and efficiency of labeling obtained with the molar excess of CHX-A"-DTPA: Herceptin® of 10:1, this molar concentration (PREPARATION A) will be used in the remainder of our studies. To our knowledge, this is the first time that Herceptin® has been labeled with ^{111}In using this new DTPA conjugate.

B. Stability of the new ^{111}In-RIC: The objective of this set of experiments was to test the stability of the RIC, produced in the second set of experiments, under control conditions. The RIC ^{111}In-CHX-A''-DTPA-Herceptin® at a molar excess of 10:1 (PREPARATION A) was incubated in a water bath at room temperature in a 50 mM sodium acetate solution (pH 6.3) and in human plasma (pH 7.4) at room temperature and 37^{0}C for 24, 48 and 96 hours post-labeling. Stability of the RIC was determined using instant thin layer chromatography (ITLC). The preparation was stable in both solutions and at both temperatures (Table 3). However, after 48 hr of incubation it appeared to be only slightly less stable in plasma than in the acetate buffer.

Table 3

Stability of newly formed Herceptin with CHA-A"-DTPA-^{111}In in sodium acetate buffer or human plasma under controlled conditions.

Time (h)	Temperature	% RIC Stability in: Buffer	Human Plasma
0	RT	97.9	---
24	RT	97.9	95.9
	37°C	98.1	95.5
48	RT	97.8	89.6
	37°C	97.7	89.3
96	RT	95.7	90.0
	37°C	94.8	85.0

C. In vitro binding affinity of RICs for human breast cancer cells: The binding capacity of the new RICs to defined human cancer cell lines MCF-7 and SKBR-3 was tested. MCF-7 human breast cancer cells express a normal amount of *HER2/neu* receptors whereas the SKBR-3 cells over-express *HER2/neu* receptors. Human breast carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 cells were cultivated in Eagle's minimum essential medium with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate and supplemented with 0.01 mg/ml bovine insulin and 10% fetal bovine serum. SKBR-3 cells were harvested by trypsinization, washed, counted and 2.5×10^5 cells were seeded in 12-well plates (6.25×10^4 cells/cm^2). The cells were incubated at 37° C for 18 hours and then the media was replaced with 2 ml of fresh McCoy's Medium with 10% fetal bovine serum containing one of the three RICs (1 μCi/ml). The cells were incubated in 5% CO_2 for 3 hours at 37° C. After the incubation period, the cells were washed 3 times with 2 ml of PBS and then solubilized with 1 ml of 1% SDS and the radioactivity bound to the cells counted in a gamma well counter. Table 4 shows the amount of radioactivity (expressed in counts per minute, CPM, per 100,000 cells) bound to both types of cells.

Table 4

Binding of ^{111}In labeled RICs to MCF-7 and SKBR-3 human breast cancer cells.

RIC	MCF-7 (*N*=3)	SKBR-3 (*N*=3)	Binding Ratio
Herceptin-CHX-A"-DTPA-^{111}In	620 ± 18	24,580 ± 1,405	40:1
HER-2-CHX-A"-DTPA-^{111}In*	1,540 ± 48	33,350 ± 2,800	22:1
IgG- CHX-A"-DTPA-^{111}In	250 ± 71	290 ± 25	1:1

Radioactivity is expressed and CPM per 100,000 cells ± standard deviation.
*Murine Antibody (Becton Dickinson, San Jose, CA) that reacts to cells over-expressing *HER2/neu*.

The in vitro binding studies were successful. The RIC ^{111}-In-CHX-A''-DTPA-Herceptin binds 40 times greater to human breast cancer cells that over-express *HER2/neu* (SKBR-3 cells) compared to normal *HER2/neu* expressing cells (MCF-7 cells). The RIC HER-2-CHX-A"-DTPA-^{111}In binds 22 times greater to the same *HER2/neu* over-expressing cells compared to the normal *HER2/neu* expressing cells. However, there is no difference in binding of the nonspecific RIC Mouse IgG- CHX-A"-DTPA-^{111}In in over-expressing and normal expressing breast cancer cells in vitro.

Additional binding studies with labeled Herceptin® [H-DTPA-^{111}In], labeled anti-*HER2/neu* MoAb [HER-DTPA-^{111}In], and labeled nonspecific IgG [IgG-DTPA-^{111}In] using new cells lines were performed. The new cell lines included HCC-1954 and BT-474 both of which over-express *HER2/neu* protein receptor. The same experimental conditions described above were used and the data is summarized in Table 5.

Table 5

Binding of ^{111}In-RICs to MCF-7, SKBR-3, HCC-1954 & BT-474 Human Breast Cancer Cells at 3 hours/37°C.*

RIC	MCF-7 (N=3)	SKBR-3 (N=3)	HCC-1954 (N=3)	BT-474 (N=3)
H-DTPA-^{111}In	**161 ± 14**	**6,310 ± 206**	**8,239 ± 598**	**4,920 ± 192**
HER-DTPA-^{111}In	**113 ± 53**	**2,206 ± 264**	**5,054 ± 336**	**2,875 ± 449**
IgG- DTPA-^{111}In	**50 ± 141**	**50 ± 8**	**50 ± 5**	**34 ± 5**

* Activity expressed in CPM per 100,000 cells ± SD. Specific Activity = 1.87 μCi/μg.

Again, in vitro binding studies were successful. The binding of RIC ^{111}-In-CHX-A''-DTPA-Herceptin (H) is significantly greater to human breast cancer cells that over-express *HER2/neu* (SKBR-3, HCC-1954, and BT-474 cells) than to normal *HER2/neu* expressing cells (MCF-7 cells). The RIC HER-CHX-A"-DTPA-^{111}In also binds greater to the same *HER2/neu* over-expressing cell lines compared to the normal *HER2/neu* expressing cells. However, there is no difference in the *in vitro* binding of the nonspecific RIC Mouse IgG- CHX-A"-DTPA-^{111}In in over-expressing and normal expressing breast cancer cells in vitro.

D. Labeling of Pharmacy Grade Herceptin® with ^{90}Y using a new DTPA Linker:

Pharmacy-grade Herceptin® was conjugated with a DTPA linker in preparation for labeling. $^{90}Ycl_3$ were labeled to H-CHX-A''–DTPA according to the procedure of Clarke *et al.* (4). The specific activity [μCi ^{90}Y/μg Herceptin®], radiochemical purity (% ^{90}Y in RIC), and efficiency of labeling (% of ^{90}Y bound to RIC) were measured. The data is presented in Table 6.

Table 6
Specific Activity, Radiochemical Purity & Efficiency of Labeling Herceptin-DTPA-^{90}Y

Specific Activity (µCi ^{90}Y/µg Herceptin)	**2.71**
Radiochemical Purity (% ^{90}Y in RIC)	**99.3**
Efficiency of Labeling (% OF ^{90}Y bound to RIC)	**27.1**

E. Stability of the ^{90}Y-DTPA Herceptin®: The objective of this set of experiments was to test the <u>in vitro</u> stability of ^{90}Y-DTPA Herceptin® under control conditions. The RIC ^{90}Y-CHX-A''-DTPA-Herceptin® at a molar excess of 10:1 was incubated in a water bath at room temperature and at 37°C in a phosphate buffered saline solution (PBS) at a pH of 7.4 and in human plasma for 24, 48 and 96 hours post-labeling. Stability of the RIC was determined using instant thin layer chromatography (ITLC). The preparation was stable in both solutions and at both temperatures (Table 7).

Table 7
STABILITY OF ^{90}Y-DTPA HERCEPTIN®

Time (hours) & Temperature		% RIC Stability in PBS Buffer at pH 7.4	% RIC Stability in Human Plasma
24	RT	97.7	97.6
	37°C	97.0	95.4
48	RT	97.0	93.1
	37°C	88.9	87.2
96	4°C	97.1	----
	RT	95.6	86.3
	37°C	81.5	80.5

Specific Activity of RIC = 2.71 µCi/µg.

F. Binding capacity of ^{90}Y-DTPA Herceptin® and ^{90}Y-DTPA-anti *HER2/neu* antibody:

The binding capacity of ^{90}Y-DTPA Herceptin® and ^{90}Y-DTPA-anti *HER2/neu* antibody was tested with the human cancer cell line BT-474 that expresses increased amounts of *HER2/neu* receptor. Cells were exposed to increasing amounts of ^{90}Y-RIC for 24 hours at 37°C. The specific activity of the ^{90}Y-DTPA Herceptin® was 1.3 µCi/µg and 1.2 µCi/µg for the ^{90}Y-DTPA-anti *HER2/neu* antibody. The radiochemical purity of the ^{90}Y-

DTPA'Herceptin® complex was 96.6% and 94.2% for ^{90}Y-DTPA Herceptin® and ^{90}Y-DTPA-anti *HER2/neu* antibody complexes respectively. The binding results are presented in Table 8.

Table 8
Binding of ^{90}Y labeled Herceptin® &
Anti-*HER2/neu* to BT-474 Cells for
24 hours at 37°C with Increasing amounts of ^{90}Y

µCi of ^{90}Y	^{90}Y-DTPA-Herceptin®	^{90}Y-DTPA-*HER2/neu*
0.2	2.16 ± 0.40*	2.39 ± 0.35
0.4	2.48 ± 0.32	4.08 ± 0.68
0.8	2.88 ± 0.15	6.83 ± 1.89

***Radioactivity expressed as cpm x 10^4. All measurements were made in triplicate (N=3).**

Because the ^{90}*Y* is a beta emitter and can be cytotoxic, the effect of increasing ^{90}Y concentration on the RIC was investigated by determining its binding to the cells in vitro over a period of time. There is a linear increase in binding of ^{90}Y when the ^{90}Y-DTPA-*HER2/neu* is exposed but this is not true for the ^{90}Y-DTPA-Herceptin® RIC suggesting that the Herceptin® is more sensitive to ^{90}Y activity. In the next experiment, ^{90}Y-DTPA-*HER2/neu* RIC was used to measure the binding to MCF-7 and BT-474 cell lines.

Table 9
Binding of ^{90}Y labeled anti-*HER2/neu* to
MCF-7 and BT-474 Cells for
3 hours at 37°C

MCF-7	2.46 ± 0.31*
BT-474	24.37 ± 1.42

***Radioactivity expressed as cpm x 10^3 per 100,000 cells ± SD. All measurements were made in triplicate (N=3).**

These data demonstrate that ^{90}Y labeled anti-*HER2/neu* RIC binds 10 times more avidly to BT-474 cells (HER2/neu over-expressing cells) than MCF-7 cells (normal expressing cells) in vitro.

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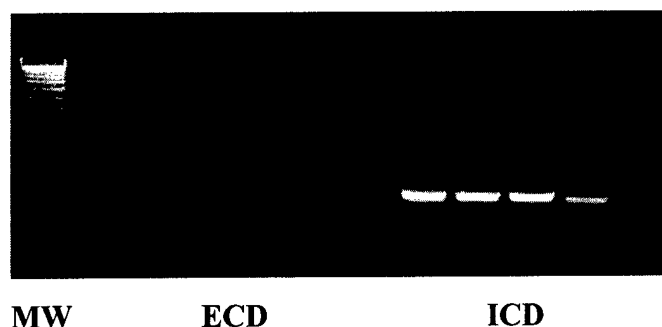
OBJECTIVE 2: Construction of a recombinant bicistronic onco-retrovirus for the stable genetic transfer of the HER2 cDNA and a fluorescent marker protein cDNA in tandem into breast cancer cell lines.

1. Isolation of full-length human *HER2/neu*.

DNA oligonucleotide primers were designed that correspond to the DNA sequence of the wild-type form of *HER2/neu*. Two sets of overlapping primers were designed in order to amplify the cDNA sequence in two manageable fragments for later manipulations. Sequences corresponding to the *HER2/neu* intracellular/transmembrane and extracellular/transmembrane regions were amplified from total RNA prepared

from SKBR3 cells (a human mammary epithelial cell line obtained from ATCC that overexpresses *HER2/neu*). Amplified products (ECD=2082 bp and ICD=1724 bp, respectively) were isolated by gel electrophoresis (Fig.1) and subcloned into plasmid PCR-Script SK+ (from Stratagene).

FIGURE 1



MW ECD ICD

Figure 1. Gel electrophoresis analysis of amplified *HER2/neu* extracellular domain (ECD) and intracellular domain (ICD) products. MW = molecular weight marker.

Resulting plasmid clones were analyzed by restriction digestions and sequenced by dideoxyflourescent-labeled sequencing methods to confirm fidelity of the clones. Some clones were found to have numerous mis-sensense mutations. A few clones, however, were found to have sequence that corresponded exactly to published cDNA sequences for *HER2/neu* intracellular/transmembrane and extracellular/transmembrane regions.

In order to join the ECD and ICD fragments into the full-length version of human *HER2/neu* for the eventual construction of the bicistronic retroviral vector (see below), a mutation was introduced into the ICD fragment plasmid to generate a unique restriction site called BspEI. This was done by the QuikChange mutagenesis kit (Stratagene) with appropriate primers using the manufacturer's protocol. Since BspEI is sensitive to *dam* methylation both the mutated ICD plasmid and the existing ECD plasmid were subpassaged through a *dam- e.coli* strain (SCS 110 from Stratagene). The ICD fragment was then released by digestion from its plasmid and ligated into corresponding restriction enzyme sites in the ECD plasmid. Diagnostic restriction enzyme digests and DNA sequencing revealed that the correct full-length human *HER2/neu* sequence was present and true in resulting clones. The resulting plasmid was called pCR-Script-*HER2/neu* FL. At this point the work was tabled briefly for transfer of the Medin laboratory to the Ontario Cancer Institute and the University of Toronto.

2. **Construction of a bicistronic recombinant retroviral vector for transfer of the *HER2/neu* and enYFP genes.**

Next the full-length *HER2/neu* sequence was subcloned into a shuttle vector (pSV-enYFP; Medin unpublished data) containing the internal ribosome entry site (IRES) from the encephalomyocarditis virus along

'with the sequence for the enhanced yellow fluorescence protein (enYFP; Stratagene). This shuttle vector allows rapid construction of bicistronic cDNA sequences that can be transferred into retroviral plasmids and later packaged, producing recombinant virions for infections of target cells. Retroviral gene transfer vector pUMFG/*HER2/neu*/IRES (10.6 kb) was then constructed by transferring a NcoI/ClaI fragment containing the full length human *HER2/neu* cDNA from the pCR-Script-*HER2/neu* plasmid to the pSV/IRES/enYFP shuttle vector. A NcoI/NotI fragment containing the sequence *HER2/neu*/IRES/enYFP was then transferred into the retroviral vector pUMFG (Takenaka et al 1999) to give pUMFG/*HER2/neu*/IRES/enYFP. *HER2/neu* was again sequenced to fully re-confirm its fidelity.

3. Transfection of retroviral producer cells to produce stable producer clones.

Amphotropic AM12 packaging cell line was grown to 90% confluency in 6 cm tissue culture plates in DMEM complete medium containing 10% FCS, 1% L-glutamine, and 1% Penicillin/Streptomycin (Bio-Whittaker). 0.7 mg of plasmid pGTN28, which confers resistance to neomycin, and 7 mg of plasmid pUMFG/*HER2/neu*/IRES/enYFP were mixed with ddH2O to reach a total volume of 438 ml, to which 62 ml of $CaCl_2$ were then added. Medium was aspirated from the AM12 packaging cell line plates and replaced with 4 ml of complete medium containing 25 mM chloroquine. 500ml of 2X HBS (Hepes Buffer Solution) were then added to the DNA/$CaCl_2$ mixture by "bubbling" and the final mixture transferred to each plate. Cells were incubated for 10 hrs at 37°C (5% CO_2). Medium was then replaced with fresh complete medium and left to incubate overnight (37°C, 5% CO_2). Cells were trypsinized with 0.25% Trypsin-1mM EDTA, split into two 10 cm plates, and placed under G418 (Bio-Whittaker) selection (800 mg/ml G418) for two weeks, then expanded in complete medium. Cells were then flow sorted using appropriate conditions and controls (see below) to obtain producer cells highly expressing (top 6%) both *HER2/neu* and enYFP. These pooled positive producer cells were then expanded and stored frozen or used in infection experiments.

4. Flow cytometry analyses for *HER2/neu* and enYFP expression.

Transfected AM12 producer cells expressing *HER2/neu* and enYFP (AM12/*HER2/neu*/enYFP) were trypsinized, washed in DPBS containing 0.1% (w/v) D-glucose and 0.02% (v/v) EDTA (final pH adjusted to 7.2), and stained with 20ml of IgG1 isotype conjugated to PE or mouse α-hu*HER2/neu* directly conjugated to PE (Becton Dickinson) according to the manufacturer's specifications. 10,000 cell events were acquired on a FACScan flow cytometer and analyzed using CellQuest software (Becton Dickinson). Results are shown in Figure 2.

Expression of huHer2/neu and enYFP in Packaging Cells Transfected with pUMFG/Her2/neu/IRES/enYFP

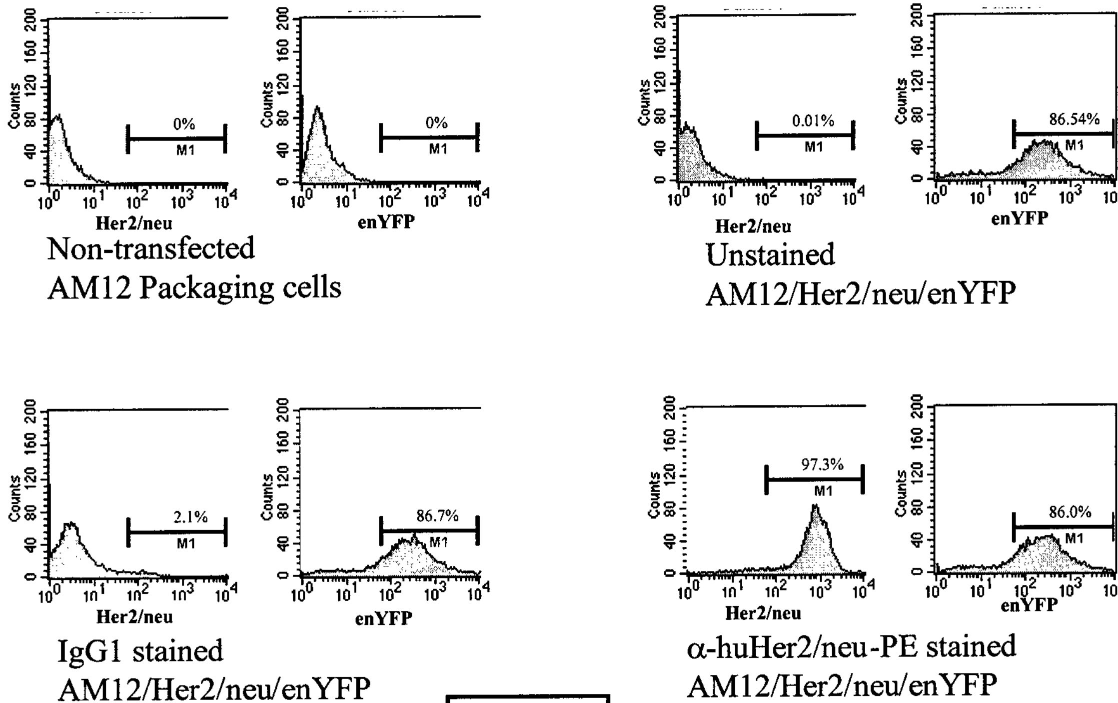


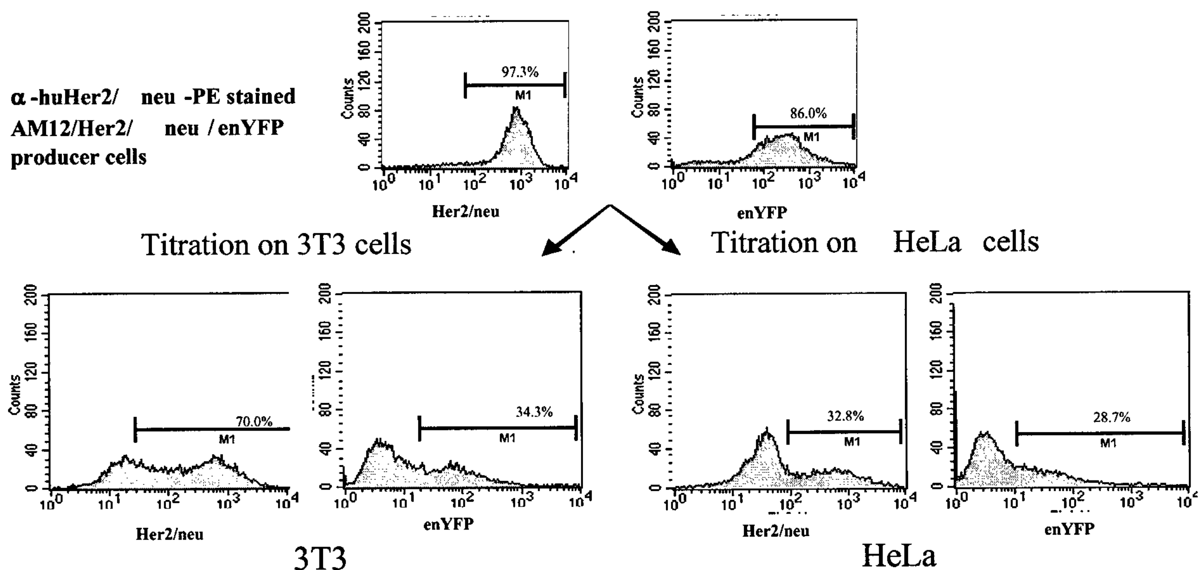
Figure 2

Figure 2. **AM12 packaging cells transfected with pUMFG/*HER2/neu*/enYFP plasmid were flow sorted, re-expanded, and stained with IgG1-PE and α-hu*HER2/neu*-PE antibodies. Analysis by flow cytometry for cell surface expression of hu*HER2/neu* and expression of enYFP showed that 87.8% of AM12/*HER2/neu*/enYFP producer cells were double positive for both hu*HER2/neu* and enYFP.**

5. Productive infection of test cells and titer determination.

Naïve, non-transfected AM12 and the pool AM12/*HER2/neu*/enYFP producer cell lines were grown in 10 cm tissue culture plates to 90% confluency in 7 ml of complete medium. Test recipient cell lines, NIH3T3 and HeLa, were grown to 20% confluency in 10 cm tissue culture plates and counted on a hemocytometer using Trypan Blue dye exclusion (Bio-Whittaker) prior to infection. Producer cell line supernatants were prepared by aliquoting 4 ml of filtered supernatant and 6 ml of medium in the presence of protamine sulfate (final concentration of 8 μg/ml). This medium was subsequently transferred to recipient cells and incubated overnight (37°C, 5% CO_2). Recipient cells were gently washed twice and incubated for 48 hrs in 10 ml of complete medium, before being stained and analyzed by flow cytometry as described above. Virus packaged by the AM12/*HER2/neu*/enYFP producer pool was shown to have a titer of $\sim 10^5$ productive infectious units/ml on NIH3T3 and HeLa cells, respectively (Fig. 3).

Figure 3. Determination of viral titer from amphotropic AM12/*HER2/neu*/enYFP pooled



packaging

cells on recipient NIH 3T3 and HeLa cells.

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OBJECTIVE 3: Measure the biodistribution of labeled Herceptin in mice bearing human breast cancers.

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Based on the in vitro binding affinity of RIC to normal and over-expressing *HER2/neu* receptor protein cells, it is hypothesized that the radiolabeled-Herceptin® will have a similar in vivo binding patterns. There should be significant RIC accumulation in HER2 over-expressing tumor cells and not as much in normally expressing HER2 tumor cells, or in normal mouse cells.

Cell lines that we are currently evaluating include the HER2 over-expressing lines BT-474 and the HER2 low expresser MCF-7. We are developing our own MCF-7 transfected cell line (Objective 2) for future studies. All of these lines are derived from different patients. Therefore, we will be able to evaluate the efficacy of the RICs in a broader genetic background than is possible using a single cell line. We are currently cultivating MCF-7 cells for the low HER2 expressers. For HER2 over expressers, we are growing HCC1954, SK-BR-3 and BT-474 cells. These cells are inoculated subcutaneously with Matrigel, since this maximizes take efficiency. We are also evaluating cell growth in bg/nu/xid mice since using a single mouse strain will be necessary for future biodistribution studies. MCF-7 cells were propagated in MEME (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum. Cells were harvested by trypsinization, washed and suspended in Matrigel. Ten million cells were inoculated subcutaneously in nude (nu/nu) mice. The mice were also implanted subcutaneously with a 0.72 mg pellet of 17β-estradiol (Innovative Research of America, Sarasota, FL). The mice were palpated weekly and were found to develop tumors within five weeks. A biodistribution experiment was performed using control and these tumor-bearing mice (implanted MCF-7 cells that expresses normal levels of the *HER2/neu* receptor). A second biodistribution and imaging experiment was performed on mice implanted with HCC-1954 cells that over-express levels of *HER2/neu* receptor.

Table 10 lists the radioactivity from selected tissues expressed as a ratio of that in the blood. These data indicated 4-time as much radioactivity in tumors than in blood of mice containing MCF-7 tumors. Levels of radioactivity in HCC-1954 tumors were about 10 times higher than in the blood. The data clearly shows that the ^{111}-In-RIC can accumulate in tumor cells, confirming the in vitro findings.

Table 10

Biodistribution of activity in tumor bearing mice 48 and 72 hours after intraperitoneal injection of radioimmunoconjugate HERCEPTIN-CHX-A'-DTPA-In^{111}. Five μCi of radiolabeled RIC was injected into 3 mice with MCF-7 tumors and sacrificed at 48 hours. One mouse containing an HCC-1954 tumor received 25 μCi of radiolabeled RIC and was imaged and sacrificed at 72 hours.

Tissue	MCF-7 Tissue/Blood Ratio	HCC-1954 Tissue/Blood Ratio
	Average ratio ± SD; N=3	N=1
Liver	0.323 ± 0.052	0.744
Lung	0.287 ± 0.065	0.562
HEART	0.195 ± 0.045	0.295
Spleen	0.179 ± 0.038	1.471
Kidney	0.247 ± 0.041	0.505
Stomach	0.064 ± 0.008	0.176
Tumor	0.718 ± 0.047	3.483

NOTE: We experienced considerable difficulty in growing SKBR-3 and HCC-1954 tumor cells in nude and SKID mice in our laboratory. For future experiments, the *HER2/neu* over-expressing cells lines BT-474 and our own transfected MCF-7 cell lines will be used for biodistribution studies.

KEY RESEARCH ACCOMPLISHMENTS

1. **Labeling Pharmacy Grade Herceptin®, Anti-*HER2/neu*, and IgG with ^{111}In using a DTPA linker.**

   As illustrated in Tables 2, and 3, we were able to label the above mentioned antibodies with ^{111}In resulting in complexes that have a high specific activity and radiochemical purity. The antibodies are stable over time and retain their ability to bind with several positive and negative tumor cell lines (see tables 3 and 4).

2. **Labeling Anti-*HER2/neu* antibody and IgG with ^{111}In using a DOTA linker.**

   The anti-*HER2/neu* and murine IgG antibodies are preparations of protein without an excess of cations or preservatives added and were labeled with ^{111}In using a DOTA linker successfully (Table 1). This was not true for pharmacy grade Herceptin®.

3. **Labeling Pharmacy Grade Herceptin®, Anti-*HER2/neu*, and IgG with ^{90}Y using a DTPA linker.**

   As illustrated in Tables 6, 7, 8 and 9 we were able to label the above mentioned antibodies with ^{90}Y resulting in RICs with high specific activity and radiochemical purity. ^{90}Y-labeled pharmacy grade Herceptin® is stable over time (Table 8). The ability of ^{90}Y-labeled pharmacy grade Herceptin® to bind to positive and negative tumor cell lines over time is demonstrated in Table 9.

4. **Standard labeling procedures have been established in our laboratory for labeling several antibodies using a DTPA linker. See attached procedure sheets.**

5. **Isolation of the cDNA for human HER2/neu.**

   As outlined above, the cDNA encoding human HER2/neu was isolated from a cDNA library as two fragments. These fragments were joined together and then the resulting full-length cDNA was fully sequenced to insure the fidelity of the target gene.

6. **Constructed a recombinant retrovirus that engineers over-expression of both human HER2/neu and enYFP in test recipient cells.**

REPORTABLE OUTCOMES

During the first year of our investigation we have accomplished the following:

1. Successful labeling of pharmacy-grade Herceptin® with ^{111}In using a new DTPA chelate method.
2. Successful labeling of nonspecific IgG protein and a purified monoclonal antibody directed against the *HER2/neu* receptor protein with ^{111}In using a DOTA chelate.
3. Successful and significant in vitro binding of ^{111}In labeled Herceptin® to human breast cancer cells that over-express the *HER2/neu* oncoprotein (receptor).
4. In vitro and in vivo stability of the new RIC (HERCEPTIN-CHX-A'-DTPA-In^{111}) has been demonstrated.
5. Isolation of the cDNA for human HER2/neu.
6. Constructed a recombinant retrovirus that engineers over-expression of both human HER2/neu and enYFP in test recipient cells.
7. Successful labeling of pharmacy-grade Herceptin® with ^{90}Y using a new DTPA chelate method.

NOTE: Five abstracts that have been submitted and accepted for publication and copies of these abstracts can be found in Appendix A.

CONCLUSIONS

We conclude that our work demonstrates the "proof-of-principle" evidence for Objectives 1 of our original proposal. Pharmacy grade Herceptin® can be labeled with ^{111}In using a new DTPA chelate and this labeled complex is stable in vitro. To our knowledge this information has not been previously published. This new radioimmunoconjugate of Herceptin® binds to breast tumor cells in vitro and in vivo (preliminary data not presented in this report). There is significantly more binding to breast tumor cells that over-express *HER2/neu* receptors than to those tumor cells which express normal concentrations of *HER2/neu* receptor. This is also true for anti-*HER2/neu* antibody but not for murine nonspecific IgG.

We also conclude that our work demonstrates "proof-of-principle" evidence for Objectives 1 and 3 of our original proposal. Pharmacy grade Herceptin® can be labeled with ^{90}Y using a DTPA chelate (to our knowledge this observation has not been published before) and that it binds to breast tumors cells in vitro and in vivo. There is significantly more binding to tumor cells that over-express *HER2/neu* receptor proteins than to those tumor cells which express normal concentrations of *HER2/neu* receptor protein. We are currently in the middle of a large imaging and biodistribution study using nude mice bearing normal and over-expression *HER2/neu* human breast cancer zenographs using ^{111}In-RICs.

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APPENDICES

Appendix A: Copy of accepted abstracts reporting the results of this investigation.

Appendix B: Procedures standardized in our laboratory for labeling MoAbs with ^{111}In and ^{90}Y using a DTPA chelating agent (see attached sheets).

APPENDIX A

Abstract presented at the 2002 Southern Ontario Gene Therapy meeting at Langdon Hall in Cambridge, Ontario, on April 21&22, 2002.

Immuno-gene therapy against prostate cancer using Her2/neu as a model antigen. **Miriam Mossoba, Christopher Siatskas and Jeffrey A. Medin. Department of Medical Biophysics. University of Toronto.**

Abstract

Prostate cancer is the second leading cause of cancer-related death in men in North America. Standard treatments for prostate cancer include chemotherapy, radiotherapy, androgen ablation, and prostatectomy. Better treatment modalities are needed to effectively eradicate/treat/contend with metastases. Efforts to harness the power of the immune system in recognizing and eliminating prostate tumors using antigen loading of dendritic cells have been met with limited success. Transferring tumor associated antigens genes directly into dendritic cells (DCs) overcomes some difficulties involved in antigen loading. In the present study, a prostate tumor associated antigen gene, *Her2/neu*, has been sub-cloned and incorporated into an MFG-based oncoretroviral vector. Her2/neu antigen is an EGF-R-related transmembrane tyrosine kinase that is an attractive target for immuno-gene therapy becuase it is selectively overexpressed in 20% of prostate tumors and 80% of its metastases in men. Vectors have been constructed that engineere expression of *Her2/neu* alone. Eventually, a cytokine, a co-stimulatory factor, or a fluorescent protein coding sequence will be added downstream of *Her2/neu* in a series of bicistronic vectors that may further potentiate effects or facilitate marking/detection. *Ex vivo* transduction of C57BL/6 mouse DCs will be performed. Transduced DCs will be injected into mice harboring Her2/neu-overexpressing prostate tumors. Tumor sizes as well as CTL and antibody responses against Her2/neu will be measured to determine the efficacy of this approach. Preliminary results show that the completed vector construct carrying the full length *Her2/neu* sequence alone yields cell surface expression in a producer cell line.

This abstract will be presented at the Annual Society of Nuclear Medicine Meetings on June 18, 2002 and was published in the Journal of Nuclear Medicine (43: May, 2002).

LABELING HERCEPTIN® WITH ^{111}In and ^{90}Y AND UPTAKE STUDIES IN MCF-7 AND SKBR-3 CELL LINES. **M. J. Blend, J. J. Stastny, S. M. Swanson, University of Illinois, Chicago, IL 60612 and M. W. Brechbiel, ROB, NCI, NIH, Bethesda, MD 20892.**

Objectives: **The goal of this research was to develop stable radioimmunoconjugates (RICs) of Herceptin® (H) for radioimmunoscintigraphy and radioimmunotherapy in an animal model bearing human breast tumors. H is used clinically for the treatment of breast cancer patients whose tumors over-express HER2/neu receptor.** Methods: **Pharmacy-grade H was conjugated with a DTPA linker in preparation for labeling. ^{111}In-Cl and ^{90}Y-Cl were labeled to H-CHX-A''–DTPA according to the procedure of Clarke *et al.* (*Cancer Res* 60: 4804, 2000). The binding capacity of ^{111}In-RIC was tested with human cancer cell line MCF-7 which expresses normal amounts of HER2/neu receptor and SKBR-3 cells which over-express HER2/neu receptors.** Results: **The molar ratio of DTPA-CHX-A'' to H in the tested immunoconjugate was 1.4:1. Using a molar excess of 10:1 CHX-A''-DTPA to H, a specific activity of 1.87 μCi ^{111}In/μg H-RIC and 2.71 μCi ^{90}Y/μg H-RIC was obtained. The purity of RIC was 96%+ for ^{111}In and 99%+ for ^{90}Y. Stability at 37° C for both RICs ranged from 98% at 24 hours in human plasma to 85% at 96 hours. ^{111}In- RIC bound to MCF-7 cells with a binding radio of 2.5:1 using a nonspecific IgG RIC as a control. A 98.3:1 binding ratio was observed when SKBR-3 cells were exposed.** Conclusion: **H can be labeled with ^{111}In and ^{90}Y using a CHX-A''-DTPA linker. Stable RICs are formed. To our knowledge, this is the first time that H has been labeled with ^{111}In or ^{90}Y using this DTPA conjugate. There is significant cell binding of ^{111}-In-RIC when HER2/neu over-expressing cells are used in culture. Animal *in vivo* and *in vitro* studies using ^{111}In and ^{90}Y-CHX-A''-DTPA-H are underway.**

Following 3 abstracts were presented at the Central Chapter Meetings of the Society of Nuclear Medicine in March 2002 and will be published in Clinical Nuclear Medicine in 2002.

LABELING HERCEPTIN® AND anti-HER2/neu ANTIBODY WITH IN-111 USING DTPA AND DOTA CONJUGATES. **M. J. Blend, J. J. Stastny, S. M. Swanson, University of Illinois, Chicago, IL.**

Objectives: The goal of this research was to develop stable radioimmunoconjugates (RICs) of Herceptin® (H) and an anti-HER2/neu antibody (anti-HER MoAb) for tumor localization in an animal model bearing human breast tumors. Methods: Pharmacy-grade H and pure anti-HER2/neu MoAb were conjugated with a DTPA and DOTA linker respectively. In-111-chloride was labeled to H-CHX-A"–DTPA (linker supplied by Dr. M. W. Brechbiel, NIH/NCI) according to the procedure of Clarke et al. (*Cancer Res* 60: 4804, 2000). In-111-chloride was labeled to *anti*-HER2/neu MoAb DOTA complex (Li *et al., Bioconjug Chem.* 4: 275, 1993). Results: The molar ratio of DTPA-CHX-A" to H in the tested immunoconjugate was 1.4:1. Using a molar excess of 10:1 CHX-A"-DTPA to H, a specific activity of 1.87 µCi In-111/µg H (RIC) was obtained. RIC purity was 96.8%. H-RIC in sodium acetate solution (SAS) and human plasma (HP) at room temperature (RT) and 37° C (body temperature) was quite stable. At 24 hr. the stability of H-RIC was 97.9% at RT and 98.1% at 37° C in SAS. The stability of H-RIC was 95.9% at RT and 95.5% at 37° C in HP. At 96 hr. the stability of H-RIC was 95.7% at RT and 94.8% at 37° C in SAS and in HP, 90% at RT and 85% at 37° C. HER2/neu MoAb DOTA complex was successfully labeled with a purity of 95.5% and a specific activity of 1.45 µCi In-111/µg RIC. Conclusions: H can be labeled with In-111 using a CHX-A"-DTPA linker to provide a stable RIC. To our knowledge, this is the first time that H has been labeled with In-111 using this DTPA conjugate. Animal in vivo and in vitro studies using In-111-CHX-A"DTPA-H are underway.

IN VITRO CELL BINDING AND KILLING OF HUMAN BREAST CANCER CELLS WITH IN-111/Y-90 LABELED HERCEPTIN®. **M. J. Blend, J. J. Stastny, S. M. Swanson, University of Illinois, Chicago, IL**

Objectives: **The goal of this research was to develop stable radioimmunoconjugates (RICs) of Herceptin® (H) for tumor imaging therapy in an animal model bearing human breast tumors. H is used clinically for the treatment of patients whose breast tumors over-express HER2/neu receptor.** Methods: **Pharmacy-grade H was conjugated with a DTPA linker (supplied by Brechbiel, NIH/NCI) in preparation for labeling. In-111 and Y-90 chloride were labeled to H-CHX-A''–DTPA according to the procedure of Clarke et al. (*Cancer Res* 60: 4804-4811, 2000). The binding capacity of In-111-RIC was tested with human cancer cell line MCF-7 which expresses normal amounts of HER2/neu receptor and SKBR-3 cells which over-express HER2/neu receptors. The killing capacity of Y-90-RIC was tested in MCF-7 and BT-474 cells (HER2/neu over-expressers).** Results: **The molar ratio of DTPA-CHX-A" to H in the tested immunoconjugate was 1.4:1. Using a molar excess of 10:1 CHX-A''-DTPA to H, a specific activity of 1.87 μCi In-111/μg H and 2.71 μCi Y-90/μg RIC was obtained. In-111-RIC bound to MCF-7 cells with a ratio of 2.5:1. There was a 98.3:1 binding ratio with SKBR-3 cells. At a concentration of 1.2 μCi Y-90-RIC/ml, 6.1 % of the MCF-7 cells and 66.7% of BT-474 cells were killed after 3 hours of incubation.** Conclusion: **H can be labeled with In-111 and Y-90 using a CHX-A''-DTPA linker. There is significant cell binding of In-111-RIC when HER2/neu over-expressing cells are used in culture. There is significant cell killing of BT-474 cells by Y-90-RIC in culture. Animal *in vivo* and *in vitro* studies using In-111/Y-90-CHX-A''-DTPA-H are underway.**

LABELING HERCEPTIN® WITH YTTRIUM (Y-90) USING A DTPA CONJUGATE. **M. J. Blend, J. J. Stastny, S. M. Swanson, University of Illinois, Chicago, IL**

Objectives:: **The goal of this research was to develop a stable radioimmunoconjugate (RIC) of Herceptin® (H) for radioimmunotherapy in an animal model bearing human breast tumors. H is used clinically for the treatment of patients whose breast tumors over-express HER2/neu receptor.** Methods: **Pharmacy-grade H was conjugated with a DTPA linker (linker supplied by Dr. M. W. Brechbiel, NIH/NCI) in preparation for labeling. Y-90-chloride was labeled to H-CHX-A"–DTPA according to the procedure of Clarke et al. (*Cancer Res* 60: 4804, 2000).** Results: **The molar ratio of DTPA-CHX-A" to H in the tested immunoconjugate was 1.4:1. Using a molar excess of 10:1 CHX-A"-DTPA to H, a specific activity of 2.71 μCi Y-90/μg H RIC was obtained. RIC purity was 99.3%. Y-90 labeling efficiency was 27.1%. Y-90-H-RIC in phosphate buffered saline (PBS) at pH 7.4 and in human plasma (HP) at room temperature (RT) and 37° C (body temperature) was quite stable. At 24 hr. the stability of Y-90-H-RIC was 97.7% at RT and 97.0% at 37° C in PBS. The stability of Y-90-H-RIC was 97.6% at RT and 95.4% at 37° C in HP. At 96 hr. the stability of Y-90-H-RIC was 95.6% at RT and 81.5% at 37° C in PBS and in HP, 86.3% at RT and 80.5% at 37° C.** Conclusion: **H can be efficiently labeled with Y-90 using a CHX-A"-DTPA linker to provide a stable RIC. To our knowledge, this is the first time that H has been labeled with Y-90 using this DTPA conjugate. Animal in vivo and in vitro studies using Y-90-CHX-A"-DTPA-H are underway.**

APPENDIX B

Procedures standardized in our laboratory for labeling proteins and monoclonal antibodies (MoAbs) with ^{111}In and ^{90}Y using a DTPA chelating agent (see attached sheets).

CURRENT METHOD FOR RADIOLABELING OF CONJUGATE:
CHX-A''-DTPA-IMMUNOGLOBULIN WITH In-111

A: PD-10 COLUMN PREPARATION:
1) Remove solution from PD-10 column.
2) Cut off bottom of the column.
3) Wash the column with 15 ml of Solution A
   (1% HSA in 20 mM Sodium Acetate, 0.15 M NaCl; pH 6.3)
4) Wash the column with 15 ml of Solution B
   (20 mM Sodium Acetate, 0.15 M NaCl; pH 6.3)

B: CONJUGATE RADIOLABELING WITH In-111:
1) Take immunoconjugate (IC):
   a) 100 μg HERCEPTIN in IC, or
   b) 100 μg mouse IgG in IC.
   Add 50 mM carbonate buffer pH 8.0 to get final volume 100 μl.
2) Add 50 μl dd water.
3) To the immunoconjugate add ~ 1.0 mCi of In-111 in 150 μl of 0.5 M Sodium Acetate pH 5.5.
4) Incubate at RT for 20 min.
5) After 20 min. add 40 μl of 2 M Sodium Acetate pH 9.5

C: RIC ISOLATION:
1) Transfer mixture from the tube on the PD-10 column.
2) Elute the column by Solution B (20 mM Sodium Acetate, 0.15 M NaCl; pH 6.3) and collect 7 fractions.
   Fraction #1 by 1 ml; Fraction #2 by 1 ml;
   Fractions #3 by 0.6 ml;
   Add 70 μl of 25% HSA to a tube #4 and elute Fraction # 4 by 0.8 ml of the Solution B. This fraction contains radioimmunoconjugate.
   Elute and collect Fractions # 5, 6 and 7 (0.7 ml each).
3) Measure radioactivity of each fraction including the column and calculate labeling efficiency and specific activity in μCi/μg IgG.

D: ITLC
1) Apply 5 μl of RIC to a TLC strip.
2) Separate RIC from free In-111 using EDTA (10 mM, pH 4.5).
3) Cut the TLC strip in ~2/3 length from start and measure radioactivity of "START" (RIC) and "FRONT" (FREE In-111)
4) Calculate RIC purity in %
5) Store the RIC at 4 oC until used.

April 08, 2002

Dr. J.J. STASTNY

CURRENT METHOD FOR RADIOLABELING OF CONJUGATE:
CHX-A''-DTPA-IMMUNOGLOBULIN WITH Y-90

A: PD-10 COLUMN PREPARATION:
1) Remove solution from PD-10 column.
2) Cut off bottom of the column.
3) Wash the column with 15 ml of Solution A
(1% HSA in 20 mM Sodium Acetate, 0.15 M NaCl; pH 6.3)
4) Wash the column with 15 ml of Solution B
(20 mM Sodium Acetate, 0.15 M NaCl; pH 6.3)

B: CONJUGATE RADIOLABELING WITH Y-90:
1) Take immunoconjugate (IC):
a) 50 μg HER2/new in IC, or
b) 50 ug mouse IgG in IC.
Add 50 mM of carbonate buffer pH 8.0 to get final volume 100 μl.
2) Add 50 μl dd water.
3) Add 3.3 mg of Ascorbic Acid (6.5 μl of CENOLATE: 500 mg Ascorbic Acid/ml).
4) To the immunoconjugate add ~ 0.5 mCi (500 μCi) of Y-90 in 150 μl of 0.5 M Sodium Acetate pH 5.5.
5) Incubate at RT for 20 min.
6) After 20 min. add 40 μl of 2 M Sodium Acetate pH 9.5

C: RIC ISOLATION:
1) Transfer mixture from the tube on the PD-10 column.
2) Elute the column by Solution B (20 mM Sodium Acetate, 0.15 M NaCl; pH 6.3) and collect 7 fractions.
Fraction #1 by 1 ml; Fraction #2 by 1 ml;
Fractions #3 by 0.6 ml;
Add 70 μl of 25% HSA to a tube #4 and elute Fraction # 4 by 0.8 ml of the Solution B. This fraction contains radioimmunoconjugate.
Elute and collect Fractions # 5, 6 and 7 (0.7 ml each).
3) Measure radioactivity of each fraction including the column and calculate labeling efficiency and specific activity in μCi/μg IgG.

D: ITLC
1) Apply 5 μl of RIC to a TLC strip.
2) Separate RIC from free In-111 using EDTA (10 mM, pH 4.5).
3) Cut the TLC strip in ~2/3 length from start and measure radioactivity of "START" (RIC) and "FRONT" (FREE Y-90)
4) Calculate RIC purity in %
5) Store the RIC at 4 oC until used.

April 08, 2002 Dr. J.J. STASTNY