Targeted Delivery of Radioisotope or chemotherapeutic Loaded Prodrug-Polymers after Pretargeting Tumors with Bispecific Antibody Complex

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Abstract:

Although significant progress has been achieved in cancer therapy, in the last few decades, there still remains much that is incurable. Treatment usually results in temporary effects, such as improvement in the quality of life, not complete remission. The lack of complete understanding of early stage detection of small lesions, tumor progression and metastases is a major impediment to effective therapy that could lead to complete remission. Effective cancer therapy should be one with limited non-specific activity and show less normal cellular cytotoxicity. Cancer chemotherapeutic agents used to date are cytotoxic to tumors but also have other serious toxicity towards normal tissue. Doxorubicin which is used as a potent chemotherapeutic agent has a wide range of toxicity such as cardiotoxicity at optimal therapeutic dosing. Many other complications are associated with chemotherapy at normal chemotherapeutic dosages. To increase the specificity of chemotherapeutic agents and also reduce other unwanted cytotoxicities one needs to enhance the targeting of tumor as well as reduce non-targeted toxicities.

Bispecific antibodies complexes have been developed as a pretargeting tool to reduce background activities thereby increasing Target to Background (T: B) ratios. To enhance visualization of small lesions in vivo, we have engaged the pretargeting approach of bispecific monoclonal antibody (bsMAb) or bispecific affibody-antibody complexes (BAAC) and the use of negatively charged polymers radiolabeled with high specific radioactivity. Imaging of metastatic melanoma lesions localized in lung tissue or breast tumor lesions pretargeted with bispecific antibody complexes and targeted with high specific radioactivity polymers was undertaken. The bispecific antibody complexes were prepared by covalent conjugation via the thioether linkages. Pan cancer anti-nucleosome monoclonal antibody
(MAb) 2C5 crosslinked to anti-DTPA MAb (bsMAb) or anti-HER/neu affibody crosslinked to anti-DTPA Fab (BAAC) were prepared. Gamma scintigraphic visualization studies were performed to demonstrate tumor imaging in vivo. Then in vivo and ex vivo target (T) to background (B) activity ratios were obtained by computer planimetry and biodistribution studies.

In the lung metastatic melanoma murine model, in vivo gamma-images showed that lesions were observed unequivocally by 3 hr. Ex vivo gamma-scintillation counting corrected for the lesion mass showed that the mean lesion activity was 24.85 ± 13.53 %ID/g (percent injected dose per gram) when pretargeted with bsMAb, whereas control group injected only with radioactive polymers also corrected similarly was 0.977 ± 0.465 %ID/g ($P < 0.001$).

In the breast cancer tumor model, in vivo gamma-images showed specific targeting of HER2/neu overexpressing human breast carcinoma (BT-474) xenografts by 24 hrs when pretargeted with BAAC, while none of the images of mice showed targeting to HER2/neu negative human mammary adenocarcinoma (BT-20) tumors. Ex vivo gamma-scintillation counting for the BT-474 tumors pretargeted with BAAC showed that the mean lesion activity was $5.274 \pm 1.32$ %ID/g, whereas control group pretargeted with anti-DTPA Fab was $0.344 \pm 0.161$ ID/g ($p \leq 0.001$). Radiotracer accumulation in BT-20 tumor of the same mice pretargeted with BAAC was $0.315 \pm 0.109$ % ID/g and for the mice pretreated with anti-DTPA Fab was $0.303 \pm 0.087$ % ID/g ($p = \text{Not Significant}$).

Whether there are therapeutic benefits when the high specific radioactivity loaded polymers were replaced with polymers loaded with doxorubicin (D-Dox-PGA) in targeted therapy are also determined. Toxicity to embryonic cardiocytes and human mammary carcinomas were investigated. IC$_{50}$ of D-Dox-PGA and free Dox in embryonic cardiocytes was 1.20 and
15.75 µg/ml respectively. When BT-474 and BT-20 cells were pretargeted with BAAC followed by targeting with D-Dox-PGA, higher tumor cell-killing was observed only in BT-474 as compared with free-Dox. No therapeutic effect of pretargeting on HER2/neu negative BT-20 cells was obtained.

In vivo therapeutic efficacy was assessed in xenografted SCID mice at the maximum tolerated dose (MTD) of doxorubicin at 7.5mg/kg body weight. Three doses of either pretargeting with BAAC followed by specific targeting with D-Dox-PGA in BT-474 and BT-20 tumors xenografted mice showed tumor regression similar to treatment with free Dox. Both pretargeted-D-Dox-PGA and free Dox therapy showed significantly greater tumor regression than mice treated with either D-Dox-PGA alone or pretargeted with anti-HER2/neu affibody alone followed by targeting with D-Dox-PGA. No change in body weight was observed in all groups of mice receiving D-Dox-PGA whether pretargeted or not-pretargeted. In double xenografted mice (BT-474 and BT-20) treated with free Dox tumor regression appears to be slightly better than BAAC pretargeted mice, however all the mice treated with free Dox died after the 2nd dose (cumulative doxorubicin dose of 15 mg/kg body weight per mouse) with approximately 15-20 % decrease in total body weight. Evidence for cardiotoxicity and other organ toxicity will be determined from tissue samples collected from all animals in this study.

Use of bsMAb or BAAC and In-111 or Tc-99m-DSPL enabled early in vivo visualization of small metastatic melanoma lesions localized in lungs or human breast cancer lesions (3-4 mm). The therapeutic efficacy of pretargeted polymer drug conjugate therapy was highly target specific with lower non-specific non-target toxicities.
This pretargeting approach for targeting of polymer drug conjugates may lead to diagnostic imaging of very small cancerous lesions and potential targeted therapy with minimal cardiotoxicity and normal tissue toxicity in patients.
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<tr>
<td>BsMAb:</td>
<td>Bispecific Antibodies.</td>
</tr>
<tr>
<td>BAAC:</td>
<td>Bispecific Affibody-Antibody Complex.</td>
</tr>
<tr>
<td>BSA:</td>
<td>Bovine Serum Albumin.</td>
</tr>
<tr>
<td>CT:</td>
<td>Computed Tomography.</td>
</tr>
<tr>
<td>SPECT:</td>
<td>Single-photon emission computed tomography.</td>
</tr>
<tr>
<td>Dox:</td>
<td>Doxorubicin.</td>
</tr>
<tr>
<td>EDC:</td>
<td>1-ethyl -3-(3-dimethylaminopropyl) carbodiimide-HCL</td>
</tr>
<tr>
<td>D-Dox-PGA:</td>
<td>Doxorubicin-loaded polyglutamic acid polymer.</td>
</tr>
<tr>
<td>PGA:</td>
<td>Polyglutamic acid polymer.</td>
</tr>
<tr>
<td>DPL:</td>
<td>DTPA-conjugated PolyLysine polymer.</td>
</tr>
<tr>
<td>DSPL:</td>
<td>DTPA-conjugated, Succinylated-PolyLysine polymer.</td>
</tr>
<tr>
<td>DTPA:</td>
<td>Diethylene TetraPentaacetic Acid.</td>
</tr>
<tr>
<td>FITC:</td>
<td>Fluorescein Isothiocyanate.</td>
</tr>
<tr>
<td>HRP:</td>
<td>HorseRadish Peroxidase.</td>
</tr>
<tr>
<td>H9C2:</td>
<td>Rat embryonic cardiocyte H9C2 cell line.</td>
</tr>
<tr>
<td>MAb:</td>
<td>Monoclonal antibody.</td>
</tr>
<tr>
<td>B16F10:</td>
<td>Murine melanoma cells.</td>
</tr>
<tr>
<td>BT-20:</td>
<td>Human mammary adenocarcinoma cell line.</td>
</tr>
<tr>
<td>BT-474:</td>
<td>Human breast cancer cell line.</td>
</tr>
<tr>
<td>RES:</td>
<td>Reticuloendothelial system.</td>
</tr>
<tr>
<td>6C31H3:</td>
<td>Anti-DTPA MAb.</td>
</tr>
<tr>
<td>2C5:</td>
<td>Anti-Nucleosomal MAb.</td>
</tr>
<tr>
<td>EDTA:</td>
<td>Ethylenediaminetetraacetic acid.</td>
</tr>
<tr>
<td>PBS:</td>
<td>Phosphate buffer saline.</td>
</tr>
<tr>
<td>OD:</td>
<td>Optical density.</td>
</tr>
<tr>
<td>NaHCO$_3$:</td>
<td>Sodium bicarbonate.</td>
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</table>
1.0 Introduction:

1.1 Statement of Problem:

Although significant progress has been achieved in cancer therapy, in the last few decades, there still remains much that is incurable. Treatment usually results in temporary effects, such as improvement in the quality of life, usually not complete remission. About 12.7 million cancer cases and 7.6 million cancer deaths were estimated to have occurred in 2008 worldwide, with 56% of the cases and 64% of the deaths in the economically developing world (1). Breast cancer in females and lung cancer in males are the most frequently diagnosed cancers and the leading cause of cancer death for each sex in both economically developed and developing countries, except lung cancer is preceded by prostate cancer as the most frequent cancer among males in economically developed countries (1).

The lack of complete understanding of tumor progression and metastases is a major impediment to effect therapy that could lead to complete remission (2). Classical treatment consists primarily of surgery, with additional chemotherapy and radiation therapy. Another limitation is non-target toxicity of the therapeutic agents despite major improvements in the development of the therapeutic approaches (3).

Effective cancer therapy should be one with limited non specific activity and show less normal cellular cytotoxicity. Cancer chemotherapeutic agents used to date are cytotoxic to tumors but also have other serious toxicity towards normal tissues (3).

Problems associated with cancer diagnosis and treatment:

High background activity which limits the efficacy for both the detection and treatment of tumors was observed with intact immunoglobulin’s directly labeled with a radioisotope as a result of their very slow clearance from the circulation (4). When compared
with the entire IgG, the use of antibody fragments, such as Fab or F(ab’)_2, improves target: background ratios, as these fragments clear from the blood more rapidly than whole IgG (4). Consequently, lower uptake of the radiolabeled antibody in the tumor is seen. Still, unequivocal visualization of small lesions or metastatic lesions in deep tissues remains a challenge.

Apart from early stage detection there are several challenges associated with side effects of radiotherapy and chemotherapy. With the use of radiotherapy there is always a danger of toxicity to the nearby tissues which can lead to fatal outcomes (5). Chemotherapy is among the most preferred ways of treating the menace of cancer. It is also used in combination with other therapies with varying degrees of success (5). However, the negative side of chemotherapeutic drugs is that they are toxic by nature and therefore impact the human body in adverse ways. Chemotherapeutic toxicity is a phenomenon that is seen more in the elderly population, particularly in people above sixty years of age. Side effects are lesser in younger cancer patients (5). Chemotherapeutic toxicity resulting in cardiodepression, central neurotoxicity and myelosuppression can complicate therapy (6, 7). Certain age specific physiological factors such as loss of body fat and protein, and reduced capacity to regenerate cells can increase the undesired toxic effects of chemotherapy (6, 7). Chemotherapeutic drug induced anemia may also decrease the efficacy of chemotherapy.

The variability in tumor microenvironments of various cancers is a challenge for developing an ideal therapeutic regimen. Tumor vasculature, tumor oxygen supply, and high pressure within the core of the tumor make single drug therapy less optimal for therapy of all cancers (2). Considerable heterogeneity in cellular morphology, pigmentation, and marker expression in individual melanomas (8) is another example of this variability. Furthermore,
the clonal evolution model of tumor heterogeneity predicts that there should be genetic heterogeneity among cancer cells leading to heterogeneity in phenotype, function, and response to therapy (8). Epigenetic differences are also presumed to compound these genetic differences.
1.2 Review of Literature:

The concept of the magic bullet was first introduced by the bacteriologist Paul Ehrlich in the late 1800’s to describe a chemical with the ability to specifically target microorganisms while sparing normal host cells (9). His concept of the “magic bullet” was later expanded to include treatments for cancer with antibodies. In 1970’s revolutionary monoclonal antibody (MAb) technology was developed (9). MAb’s have found use in both diagnostic and therapeutic applications (10, 11). The development of tumor selective MAb’s or antibody fragments is a promising avenue for targeting and delivery of the therapeutic agents to tumors that over express tumor associated or specific antigens (10, 11).

1.2.1 Antibody for targeting therapeutic drugs:

MAb’s have been used for targeting therapeutic agents. Such approaches utilize directly linked therapeutic molecules on antibodies for delivery. Examples of drug molecules directly linked to antibodies include:

1) Toxins such as ricin which are delivered to the target cells that lead to disruption of protein synthesis and cell death (12),
2) Radioactive compound for radio diagnosis and therapy (12),
3) Signaling molecules such as cytokines, to locally activate the immune system and mark the target cells as "dangerous" (12),

Although antibodies with directly linked therapeutic agents have shown some efficacy, only limited concentration of the drug can be loaded on individual antibody molecule leading to sub-optimal drug delivery.
1.2.2 Bispecific Antibodies:

Bispecific antibodies (bsMAb) are unique macromolecular heterobifunctional crosslinked antibodies with two different binding specificities in a single molecule (13). They form ideal bioconjugates of two different antibody molecules joined together with or without chemical conjugation. The technology for the development and application of bispecific antibodies for biological research has advanced steadily since the concept of creating hybrid reagents with dual specificity was first proposed by Nisonoff and Rivers (14). Bispecific antibodies have found applications in immunohistochemistry, immunoassays, radioimmunodiagnosis, radioimmunotherapy and immunotherapy (14).

BsMAb have for several decades being developed to enhance site specific concentration of cytotoxic T cells (15) or other effector cells to promote immunotherapeutic tumor cells killing (15). This therapeutic effect is due to binding of target cells via one arm of the bispecific antibody while the other arm binds to either the effector cells or therapeutic drugs. Bispecific antibodies also provide means for selectively delivering biologically active agents to the surface of target cells that could ultimately lead to internalization of the therapeutic drug (16). Cytotoxic drugs and toxins loaded on carriers have been delivered to target cells via bispecific reagents because the entry of these potent molecules into the target cells is signaled by an easily measured intracellular activity (16, 17).

There are other approaches to target therapeutic agents or effector functions using antibodies apart from bispecific antibody targeting. Such an approach utilizes directly linked effector molecules to the antibody for delivery (17). Recent breakthroughs in recombinant DNA technology, the increased number of identified disease targets as the result of the completion of human genomic map project, and a better understanding of the mechanism of
the human immune system has all contributed to enhancement of the bispecific antibody technology.

1.2.3 HER2/neu as a potential target:

A potential cancer target is HER2/neu (also called ErbB2), a transmembrane protein belonging to the human epidermal growth factor tyrosine kinase receptor family (18). Increased HER2/neu activity is associated with increased proliferation and decreased apoptotic capacity. HER2/neu is often overexpressed in different cancers, including breast and ovarian (18), but is expressed only to a small extent or not at all in many normal adult tissues (18). In patients, overexpression of HER2/neu is associated with short disease-free time and decreased overall survival and occurrence of metastases (18). Together, these factors provide strong arguments for the development of targeting strategies directed against HER2/neu.

1.2.4 Nucleosomes on tumor cell surface as targets:

Tumor cell surface-bound nucleosomes (NS) (supramolecular constituents of nuclear material consisting of well-characterized individual monoNS composed of DNA and four pairs of histones arranged in a characteristic pattern) are found on all tumor cells tested (19). Binding of extracellular NS’s to tumor cell surface might be mediated by specific NS receptors that have been reported by several investigators to be present on the surface of tumor cells (19). The tumor cell-bound NS’s which are also found as extracellular NS’s in tumor cell cultures and in patients with tumors (19), are hypothesized to have originated from apoptotic tumor cells that exist in every in vivo developing tumors (19). Elevated free extracellular nucleochromatin has also been observed under non-cancerous conditions accompanied by massive apoptotic cell death, such as in lupus erythematosus and AIDS (19).
The monoclonal antibody 2C5 (MAb 2C5) with nucleosome (NS) restricted activity, that can recognize the surface of various tumors via the surface-bound nucleosomes released from apoptotically dying neighboring tumor cells but not normal cells has been demonstrated to be able to target pharmaceuticals such as chemotherapeutic and radioactive agents to tumor cells in vivo and in vitro (20).

1.2.5 Rationale for the use of Antibody Fragments and Affibody molecules:

Although monoclonal antibodies offer many attractive features for tumor targeting, they are associated with various side effects and limited efficacy. Intact antibodies are more immunogenic than antibody fragments and their large size leads to slow blood clearance, poor tissue penetration, high background activity and ultimate clearance into the liver. Therefore, smaller antibody fragments such as Fab, diabody, scFv, and various single domain antibody mimetics with increased blood clearance via enhanced renal excretion have been developed (21).

Additionally, antibody modification approaches have recently been developed based on grafting antigen binding regions on different protein scaffolds (21), e.g. ‘affibody’ ligands, ‘trinectins’ (small 94 residue structure developed from type III domain of fibronectin with similar binding properties as intact IgG), ‘anticalins’ (20kDa antibody mimetics with a barrel structure formed by eight antiparallel β-strands pairwise connected by loops and an attached α-helix) (21).

Affibodies are based on a 58 amino acid residue protein domain, derived from one of the IgG-binding domains of staphylococcal protein A. This cysteine-free three-helix bundle domain is designated the Z domain (22). Using the combinatorial protein engineering techniques, the surface residues of an α-helical receptor domain from staphylococcal protein
A are randomized. The mutant sequences were inserted into phagemids (plasmids used for protein expression by bacterial viruses or phages). The phages are then used to “pan” for proteins of interest. The simple, robust structure of the affibody molecules, together with their low molecular weight (Approx 10 kDa); makes affibody suitable for a wide variety of applications. Efficacy has been shown in bioprocess and laboratory-scale bioseparations, and promising results have been obtained when evaluating affibody ligands as detection reagents, to engineer adenoviral tropism and to inhibit receptor interactions (22). Thus, affibody might exhibit targeting and therapeutic potential.

1.2.6 Concept of Pretargeting:

Pretargeting is a technique which greatly improves tumor to blood activity ratios in vivo also known as Target: Background ratios (23). Pretargeting with bispecific antibodies allow targeting to specific antigens. Bispecific antibody circulates throughout the body and binds to cells expressing the target antigens. Clearance of the excess bsMAb from the circulation is achieved by waiting for reticuloendothelial (RES) or renal elimination (24). Since the bsMAb is not radiolabeled, sequestration by the RES should not result in increased non-target background activity which is associated with radioimmunodiagnosis and radioimmunotherapy (24). However, targeted bsMAb should allow capture of the signal molecule such as radiolabeled monovalent or bivalent hapten or in our case, polymer loaded with radiolabel or therapeutic drugs (23, 24). The condition for successful application of pretargeting with bsMAb is that the bispecific antibody should not be totally internalized upon binding to tumor-surface antigens. Internalization of bsMAbs may be minimized by minimizing cross-linking of the surface tumor antigens by the bispecific antibodies. The radiolabeled polymers utilized in our approach will localize at sites where bispecific
antibodies have accumulated in sufficient concentration. If the radiolabeled component has high tissue permeability, clearance and diffusion, the radionuclide will localize more rapidly at the targets, and clear from the in vivo environment quickly (24). Therefore, higher tumor selectivity may be achieved. Use of negatively charged polymer loaded with radionuclide is preferable since the negative charge of the polymers and the negative charge of the cell membranes and ground substances will lead to ionic repulsion keeping non-specific uptake of the polymers at a minimum. This should lead to further increase in the target to background ratio (T: B). This approach should minimize non-target toxicity in therapy and maximize tumor targeting in diagnostic imaging.

1.2.7 Development of polymer loaded chemotherapeutic agents:

**Doxorubicin associated Cardiotoxicity:**

Although doxorubicin or its modified carriers is the first-line chemotherapeutic drug for cancer therapy, the dose limiting factor is cardiotoxicity. Doxorubicin produces a) early transient electrocardiographic changes and b) a delayed progressive cardiomyopathy. Acute changes in the electrocardiogram, including tachycardia and arrhythmias generally reverse within a few hours. Drug induced cardiomyopathy however is severe and leads to progressive congestive heart failure with symptoms such as tachycardia, hepatomegaly, cardiomegaly, pulmonary edema and pleural effusion. This type of cardiomyopathy is cumulative, irreversible and is dose dependent (25).

Several hypotheses have been proposed to explain doxorubicin cardiotoxicity including free radical damage, metabolite and calcium overload hypothesis. However, none of them is adequate to explain the mechanism of irreversible cardiomyopathy (25).
**Polymer based drug delivery system:**

Ringsdorf in 1970 proposed that polymer-drug conjugates could enhance the delivery of anti-tumor drugs to the tumor. Hydrophilic polymer-drug conjugates administered intravenously have been designed to remain longer in the circulation. The clearance rate depends on the molecular weight of the conjugated drug. Drugs that are covalently bound by stable linkers are largely prevented from accessing normal tissues. Biodistribution is initially limited to the blood pool (26). Blood concentration of the drug conjugates drives tumor targeting due to increased permeability of angiogenic tumor vasculature relative to normal vasculature. This type of passive targeting is due to the enhanced permeability and retention (EPR) effect (27). Addition of cell-specific recognition ligands would allow further targeting of tumor cells. If the polymer-drug linker is stable in the circulation, for example, N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-Gly-Phe-Leu-Gly–doxorubicin, the relatively high level of renal elimination (whole body $t_{1/2}$ clearance $> 50\%$ in 24 h) compared with free drug ($t_{1/2}$ clearance $\sim 50\%$ in 4 days), decrease in non-target toxicity can be achieved as compared to free drug (28, 29). At the tumor interstitium, polymer-drug conjugates are internalized by tumor cells through either fluid-phase or receptor-mediated pinocytosis following non-specific membrane binding or ligand-receptor docking. Depending on the linkers used, the drug will usually be released intracellularly on exposure to lysozymes. Polymers such as Gly-Phe-Leu-Gly and polyglutamic acid (PGA) are cleaved by cathepsin B or hydrazone linkers are degraded in endosomes and lysosomes due to the acidic pH (pH 6.5 - <4.0) (30). The transport of drugs such as doxorubicin and paclitaxel out of these vesicular compartments ensures delivery to the pharmacological targets. Additionally intracellular delivery of pro-drugs may bypass mechanisms of resistance associated with membrane efflux.
pumps such as p-glycoprotein (31). Therefore the development of polymers as targeted novel
delivery systems would enhance therapy and diagnosis.
Specific Aims:

The overall objective of this thesis is to develop different bispecific antibody complexes and polymers loaded with either radioligands or chemotherapeutic agents to detect small cancer lesions and affect therapy by increasing the targeted payload to tumors. The specific aims are as follows:

Aim 1: Generate polymer-prodrug complexes.

Two kinds of polymer-prodrug complexes will be prepared a) Polymers as carriers for radioisotopes and b) polymers as carriers for chemotherapeutic agents.

Aim 2: Prepare and characterize bispecific antibodies targeted to nucleosomal tumor associated antigen (2C5-6C31H3).

Monoclonal antibodies 2C5 and 6C31H3 will be crosslinked via the thioether bonds. MAb 2C5 crosslinked MAb 6C31H3 bispecific antibody complex will be characterized for molecular weight and immunoreactivity by ELISA. Binding assay will be undertaken using FACS analysis on murine melanoma cells (B16F10 cells).

Aim 3: Isolate and characterize recombinant anti-HER2/neu affibodies.

The pDNA encoding affibody gene against HER2/neu antigen was a gift from Dr. Arash Hatefi. It will be expressed in E.coli. Pure anti-HER2/neu affibody will be characterized for molecular weight, affinity and binding to HER2/neu positive and negative human breast cancer cell lines.
Aim 4: Prepare and characterize anti-HER2/neu affibody crosslinked anti-DTPA Fab bispecific complex.

Bispecific complex will be prepared by chemical conjugation. Fab of anti-DTPA antibody crosslinked to anti-HER2/neu affibody will be characterized for immunoreactivity, molecular weight, and cell binding by ELISA, SDS-PAGE and FACS analyses respectively.

Aim 5: Demonstration of reduction of cardiotoxicity of Polymer conjugated Dox.

Equivalent concentrations of doxorubicin on polymers and free Dox will be used in cell culture of embryonic cardiocytes to demonstrate cytotoxicity and IC$_{50}$ will be determined in various cell lines relative to free Dox.

Aim 6: Demonstrate in vitro cytotoxicity in tumor cells after pretargeting with the bispecific antibodies.

Human mammary adenocarcinoma cells (BT-474) will be used to determine tumor toxicity. In studies using anti-HER2/neu bispecific antibody complexes, toxicity in HER2/neu positive human breast cancer cells will be assessed.

Aim 7: Demonstrate in vivo targeting and biodistribution in tumor models:

Murine metastatic lung melanoma model and xenografted tumor model of human breast cancer will be used to evaluate pretargeting effectiveness by in vivo imaging and biodistribution.

Aim 8: In vivo therapeutic efficacy studies in HER2/neu positive and negative xenografts to demonstrate targeted therapy and imaging to assess therapeutic efficacy.

Dual Xenografted tumor model of human breast cancers (BT-474 and BT-20) will be used to evaluate the therapeutic efficacy of pretargeting with anti-HER2/neu affibody-anti-DTPA Fab bispecific complexes and specific targeting of D-Dox-PGA for therapeutic efficacy.
2.0 Materials and Methods:

Monoclonal antibody 2C5 (anti-nucleosomal) was a generous gift from Dr. Vladimir Torchilin of Northeastern University. Anti-DTPA antibody (6C31H3) was developed in our laboratory. 2-Iminothiolane, N-hydroxy Succinimide ester of bromoacetic acid, N, N Dimethyl sulfoxide (DMSO) and bicyclic anhydride of diethylene-triaminepentaacetic acid (DTPA) were purchased from Sigma Chemicals Co. Zorbax GF-250 column was purchased from Agilent Technologies. C57/BL6 and SCID-SHO mice were purchased from Charles River Labs. Bispecific antibody was generated using chemical conjugation method via thioether linkages. The targeting arm consisting of murine anti-nucleosomal (2C5) antibody or anti-HER2/neu affibody were cross-linked to the capturing arm antibody, intact or Fab of murine anti-DTPA monoclonal antibody (6C31H3).

2.1 Production of anti-HER2/neu affibodies:

pDNA for anti-HER2/neu affibody:

DNA fragment encoding HER2/neu affibody gene was subcloned into the expression vector pET28b (kanamycin resistant) between NcoI and HindIII restriction sites (32). The ligation resulted in the expression vector denoted pET28b-HER2/neu encoding the affibody ligand fused to an N-terminal 6 Histidine tag, allowing purification by immobilized metal ion affinity chromatography (IMAC) (32, 33).

Affibody production and purification:

After confirmation of transfection of pDNA of anti-HER2/neu affibody in E coli strain BL21(DE3), the bacteria were inoculated in 15 ml of Lucia-broth (LB) media, containing 30 µg/ml kanamycin, and grown in 1 liter Erlenmeyer flasks overnight at 37° C. Fresh Lucia-broth (LB) (100 ml), supplemented with 5 g/l yeast extract and 30 µg/l
kanamycin, were inoculated with 1 ml of the overnight cultures and bacteria were grown at 37°C until absorbance at 600 nm of 0.6-1.0 was achieved. At this bacteria concentration gene expression was induced by addition of isopropyl b-D-thiogalactoside (IPTG; Sigma) to a final concentration of 1 mM (32, 33, 34). After overnight cultivation at 37°C, the cell cultures were harvested by centrifugation (4000 g, 25 min, 4°C). The cell pellets were subsequently resuspended in 25 ml of binding buffer (0.5M NaCl, 5mM imidazole, 20 mM Tris–HCl, pH 7.9) and disrupted by sonication (32, 34).

Anti-HER2/neu affibody proteins were recovered by IMAC purification on His-Mag Agarose beads affinity columns pre-charged with Ni$^{2+}$ (EMD serono, CA). The IMAC columns were equilibrated with 20 ml of binding buffer. Supernatants containing the dissolved anti-HER2/neu affibody proteins were diluted with 25 ml of binding buffer. Equilibrated His-Mag Agarose beads pre-charged with Ni$^{2+}$ were then combined with the affibody proteins in a centrifuge tube followed by mixing thoroughly by inverting the tube several times and incubation for 10mins with occasional mixing. The tube was then placed in a magnetic rack to recollect the beads bound with anti-HER2/neu affibody and the supernatant was discarded. His-Mag Agarose beads were then washed with 10 ml of wash buffer (0.5M NaCl, 60mM imidazole, 20 mM Tris–HCl, pH 7.9) several times. After thorough washing, affibody was eluted with 2 ml of elution buffer (0.5M NaCl, 1 M imidazole, 20 mM Tris-HCl, pH 7.9). Eluted anti-HER2/neu affibody fraction was assessed for purity (32, 34). Protein concentration was calculated from absorbance at 280 nm, using the standard protein assay with bovine serum albumin (BSA) as a standard.
2.2 Characterization of anti-HER2/neu affibodies using ELISA and SDS-PAGE:

2.2.1 ELISA for anti-HER2/neu affibody:

A 96 well micro-titer plate was coated with approximately 100µl/well of anti-HER2/neu affibody at a concentration of 1µg/ml in 0.1 M Tris buffer saline (TBS). Then the plate was incubated for 1 hr at 37°C followed by washing 3X with 0.1M Tris buffer saline with tween (TBS-T). The aliquots of 3% bovine serum albumin (200µl) was used as blocking agent and was incubated for 1 hr at 37°C. The wells were washed in 0.1 M TBS-T (3X). The wells were then loaded with 100 µl/well of serial dilutions of anti 6-His tag MAb (Abcam) with starting concentration at 1µg/ml. The micro-titer plate was then incubated for 1 hr at 37°C and washed 3X again with 0.1 M TBS-T. Aliquots of 50 µl of GAM-HRP (1/1000 diluted) were added. Incubation and washing were as described. K-Blue substrate was then added (50µl/well) as a chromogen for the assessment of antibody binding. OD at 405 nm was obtained using a BioTek ELISA reader (Model: EL 800, VT, USA). Assays were performed in quadruplicates and analyzed using GEN 5.0 software for mean and Standard deviation (Figure 1A).

2.2.2 SDS-PAGE for anti-HER2/neu affibody:

The purified anti-HER2/neu affibody was characterized by SDS-PAGE using a BIORAD Mini-PROTEAN Tetra cell kit. Handcast gels were made using Acrylamide/Bis-acrylamide. A 6% resolving gel and 4% stacking gel (about 2cm) combination was used. The aliquots of 10 µg protein samples in bromophenol blue tracking dye in sample buffer were prepared. The samples were heated at 70°C for about 10 minutes prior to gel electrophoresis. The MOPS-SDS running buffer [100mM 3-(N-morpholino) propane sulfonic acid (MOPS), 6.93mM Sodium dodecyl sulfate (SDS), 100mM Tris Base, 2.05mM EDTA] was used for
electrophoresis at 200 V and a run time of about 35 minutes.

After electrophoresis, the gel was carefully removed from the gel cassette and rinsed three times in deionized water. The gel was stained in 0.0025% comassie blue (staining solution) for about three hours. The gel was then de-stained with three changes of destaining solution (40 % methanol, 10 % glacial acetic acid). Upon de-staining, the gel was rehydrated with deionized water and transferred to a wet chromatographic filter paper followed by overlaying with a plastic sheet. The gel assembly was then transferred to the Bio-Rad gel dryer (model # 583) for two hours under vacuum.

Affibody molecule has a C-terminal cysteine residue with free sulphdryl group which has a tendency to oxidize and form dimmers. Both reduced and unreduced samples of anti-HER2/neu affibody were applied to the same gel for analysis. (Figure 1B).

2.3 Anti-HER2/neu affibody labeling with FITC:

The affibody molecules contain a unique C-terminal cysteine residue that allows for site-specific labeling (32, 34). This cysteine was used to label the affibody with thiol-reactive Fluorescein-maleimide dyes (Sigma Co). To reduce oxidized cysteines before conjugation, the affibody was incubated with 20 mmol/L of dithiothreitol (DTT) at pH 7.4 for 2 hrs at room temperature. After reduction of the affibody dimmer, the solution was dialysed against 0.1M PBS buffer with 10 mM EDTA for 24 hrs at 4ºC. Fluorescein-maleimide dyes dissolved in DMSO were added and conjugation was completed according to the manufacturer's protocol. Unreacted Fluorescein-maleimide dyes were removed by Sephadex G-10 desalting column chromatography.
2.4 Characterization of anti-HER2/neu affibody using flow cytometry:

Cultured cells (BT-474, BT-20 cells) were incubated with fluorescein labeled conjugates at different concentrations for 30 min at 37°C. Cells were then trypsinized and detection of the labeled cells was verified by flow cytometry. Flow cytometry using a FACS Calibur instrument (BD Biosciences) equipped with an argon-ion laser and an optional second red diode laser (source energy, 15 mW; detection time, 500 counts per second) was used. Cell Quest software was used for data acquisition and analyses. Ten thousand cells were gated. Data were live gated by FL1 (blue laser, 488 nm) (Figure 2).

2.5 Preparation of Fab fragment of anti-DTPA antibody (6C31H3):

Monoclonal anti-DTPA (6C31H3) Fab were prepared by enzymatic digestion of intact monoclonal anti-DTPA antibody with immobilized papain beads (Pierce) (35). 5mg/ml of intact anti-DTPA antibody was dialyzed overnight against sample buffer (20mM sodium phosphate, 10mM EDTA, pH 7.0). The papain beads (125 µg papain/ 500 µl of gel) were then equilibrated in the digestion buffer (sodium phosphate, 10mM EDTA, 20 mM cysteine hydrochloride, pH 7.0). After equilibration, the papain beads were added to the dialyzed sample followed by incubation for 20 hours at 37°C in a shaking water bath. The immobilized papain beads were removed after incubation and the crude digest (a mixture of anti-DTPA Fab and Fc fragments) was mixed with 1 ml of 1.5M Tris-HCl, pH 7.5. The anti-DTPA Fab were separated from the Fc and undigested antibody by Protein-A affinity chromatography. The digestion mixture was dialyzed overnight using a 5kDa MWCO dialysis membrane in binding buffer (20 mM Sodium phosphate dibasic, 0.15 M Sodium chloride, pH 8.0). Then dialyzed crude digest was added to an equal volume of binding buffer and applied to the Protein-A column. Anti-DTPA Fab was recovered in the fall
through wash whereas undigested antibody and Fc fragments were bound to the Protein-A column. Characterization of the anti-DTPA Fab was by ELISA and SDS-PAGE as described below.

2.6 Characterization of anti-DTPA Fab:

2.6.1 Immunoreactivity of anti-DTPA Fab:

100µl/well of DTPA-BSA (1µg/ml) was added to a 96-well micro titer plate (BD Biosciences) for the assessment of immunoreactivity of anti-DTPA Fab fragment (36). The plate was incubated at 37°C for 1 hour followed by washing with 0.1M PBS-T (5X). The plate was then blocked with 200µl/well of 3% non-hybridoma supportive fetal bovine serum and blocking was carried out for one hour at 37°C. After blocking, the plate was washed with 0.1M PBS-T (5X). Then 100µl aliquots of serial dilutions of purified anti-DTPA Fab starting at 1 µg antibody were added to the wells. Negative control of anti-myosin MAb was included. The plate was incubated for 1 hour at 37°C. Washing was as described. Aliquots (50µl/well) of Goat anti-Mouse antibody conjugated with horseradish peroxidase (1:1000 dilution) were added to the wells. Incubation (1 hour at 37°C) followed by washing was carried out with 0.1M PBS-T (5X). To assess the immunoreactivity of anti-DTPA Fab, K-Blue substrate (chromogen) was added (50µl/well). The micro-titer plate was then kept in dark for about 15 minutes. After 15 minutes, the plate was read at 405 nm in an ELISA reader. The assay was performed in quadruplicates and the results were analyzed using the GEN 5.0 software (Figure 3A).

2.6.2 SDS-PAGE analysis of anti-DTPA Fab:

Purity of the antibody fragments was characterized by SDS-PAGE as described previously (Figure 3B).
2.7 Preparation and characterization of 2C5-6C31H3 Bispecific antibody (bsMAb):

2.7.1 Preparation of bsMAb:

2C5 MAb, specific for the auto-antigen nucleosomes and anti-DTPA MAb (6C31H3) antibody are used for covalent coupling via thioether linkages to generate the bsMAb. One mg aliquot of anti-DTPA antibody in 50mM NaCl and 1mM EDTA (pH 8.0) was modified using 100 x molar excess of 2-iminothiolane in dimethyl sulfoxide (DMSO). An aliquot of 1 mg of 2C5 anti-pan cancer monoclonal antibody in 0.1 M PBS was modified with 100 x molar excess N-hydroxy succinimide ester of bromoacetic acid in DMSO at 25ºC for 1 hour and incubated additionally for 6 hours at 4ºC (37). Thiolated 6C31H3 and bromoacetylated 2C5 were separated from free iminothiolane or unreacted N-hydroxysuccinimide ester of bromoacetic acid respectively by Sephadex G-10 column centrifugation using 0.1M PBS as the elution buffer. The extent of modification of thiolated anti-DTPA Ab and bromoacetylated 2C5 Ab was assessed by Tri-nitro benzene sulfonic acid assay (38). Aliquots of the modified antibodies were saved to assess the immunoreactivity. Addition of equi-molar concentrations of each activated antibody resulted in conjugation of 2C5 to 6C31H3 via the thioether bonds. The reaction mixture was incubated at 4ºC overnight in the dark.

2.7.2 TNBS Assay for Modified Antibodies:

After recovering the modified antibodies (2C5 and 6C31H3) from the sephadex G-25 spin column chromatography, percent modification was calculated using tri-nitro benzene sulfonic acid assay. An aliquot of 10 µg of the modified antibody was compared to unmodified antibody (10 µg). Briefly, 375 µl of distilled water and 375 µL of 0.1M sodium tetraborate solution (pH 7.4) were added to 10 µg of each MAb. Then, 187.5 µL of 2mg/ml
solution of trinitro benzene sulfonic acid solution (Sigma Co.) were added. The reaction mixture was then incubated for 30 mins at 37°C. After incubation, 375 µl of freshly prepared Sodium sulphite solution (1.5mg/ml made in 0.2 M Sodium phosphate dibasic solution) were added to the above reaction mixtures. After thoroughly mixing the reaction mixtures, each sample was read at OD 420nm (38). Percent modification was calculated relative to the reading of the unmodified MAb. The assays were performed in triplicates to ensure reproducibility (Table 1 and 2). 

**2.7.3 Assessment of the Immunoreactivity of the Modified Antibodies:**

**Anti-DTPA Antibody Immunoassay:**

Micro-titer plates (96 well) were coated with 100 µL aliquots of DTPA modified bovine serum albumin (BSA) (1 µg/ml). The DTPA-BSA coated wells were used to assess anti-DTPA activity by the standard ELISA protocol (36). Aliquots of the thiolated antibody (1 µg/ml) and the standard unmodified anti-DTPA antibody were used to assess the immunoreactivity. Detection was achieved using a secondary antibody (goat anti-mouse IgG coupled horseradish peroxidase, 1:1000 dilution) followed by the addition of 50 µl aliquots of the enzyme substrate K-blue (Neogen Corp. MA, USA). The micro-titer plate was then read at OD 405 nm in an automated ELISA plate reader. The assay was carried out in quadruplicates and analyzed using GEN 5.0 software for mean and standard deviation (Figure 4A).

**Anti-Nucleosomal (2C5) Antibody Immunoassay:**

Briefly, Poly-lysine precoated micro-titer plate (96 well) were coated with 100 µl aliquots of nucleosomal preparation (40 µg/ml) (19). The nucleosomal preparation coated wells were used to assess 2C5 Ab activity by the standard ELISA protocol as described (19).
Aliquots of the bromoacetylated (1 µg/ml) and the unmodified 2C5 antibody were used to assess the immunoreactivity. The application of the secondary antibody followed by the enzyme substrate K-blue and the use of the ELISA plate reader were as described above (Figure 4B).

2.7.4 Isolation of bsMAb (2C5-6C31H3):

BsMAb was purified from unconjugated MAb by HPLC chromatography using Zorbax-GF 250 size exclusion column (9.4 D x 250 L mm) (Figure 5A). Briefly the reaction mixture of modified 2C5 and modified anti-DTPA antibody was first dialyzed against 0.2M sodium phosphate buffer (PBS, pH 7.0) and then an aliquot of 350 µl of the sample was applied to the Zorbax GF-250 size exclusion column pre-equilibrated and calibrated with proteins of known molecular weights such as IgG and BSA. The column was eluted with 0.2 M PBS. The elution was monitored at OD 214 nm. The flow rate of the column was optimized to 500µl/min and the pressure was maintained at around 400 psi. Aliquots of 250 µl fractions were collected.

2.7.5 Assessment of the Bispecificity:

A 96 well micro-titer plate (Falcon) was coated with aliquots of 100µl of 40 µg/ml polylysine (15 kDa) solution in 0.1M PBS and incubated for 24 hr at 4ºC in the dark (36, 39). The micro-titer plate was then washed with 200 µl/well of 0.1M PBS-Tween (3 X) followed by addition of 100µl/well of 40 µg/ml nucleosomal preparation in 0.1M PBS and incubated for 1 hr at 37ºC (19). Then the micro-titer plate was washed again with 0.1M PBS-Tween x 3 followed by addition of 5% non-hybridoma growth supportive fetal Calf serum (200µl) to each well as blocking agent and incubated at 37º C for 60 minutes. The wells were then washed with 0.1 M PBS-Tween x 3 and an aliquot of 50 µl of 1/10 dilution of each fraction
of the HPLC elution of the bsMAb from the Zorbax-GF 250 column chromatography was added to each well. The micro-titer plate was incubated for 1 hr at 37°C and then was washed 3 times with 0.1 M PBS-T. After washing, aliquots of 50 µl of 1/500 dilution of DTPA conjugated horseradish peroxidase (DTPA-HRP) were added to each well and incubated at RT for 1 hr followed by washing in PBS-T. DTPA-HRP used for analysis was prepared and characterized by described Khaw et al. (36). Then 50 µl aliquots of K- blue substrate (Neogen Corp. MA, USA) were added and incubated at room temperature (RT) in the dark for 30 minutes. The micro-titer plate was then read at O.D. 405 nm in an automated ELISA plate reader. The assay was performed and analyzed as described previously (Figure 5B).

2.7.6 SDS-PAGE Analysis:

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the bsMAb reaction mixture was compared to that of monomeric MAb before HPLC chromatography (Figure 6A). Then different fractions of the antibodies eluted from the HPLC Zorbax-GF 250 column were subjected to SDS-PAGE under nonreducing condition and compared with the molecular weight standards (Sigma Chemical Co.) (Figure 6B).

2.8 Preparation of anti-HER2/neu affibody crosslinked anti-DTPA Fab Bispecific antibody (BAAC):

2.8.1 Preparation of BAAC:

Anti-HER2/neu affibody is specific for the HER2/neu antigen highly over expressed in some breast and ovarian cancers and the anti-DTPA Fab fragment (6C31H3) are used for covalent coupling via the thioether linkage to generate the bispecific antibody complex. An aliquot of 1 mg of anti-DTPA Fab in 0.1 M PBS was modified with 100 molar excess N-hydroxy succinimde ester of bromoacetic acid in DMSO at 25°C for 1 hour and then 6 hours
at 4°C (37). Bromoacetylated anti-DTPA Fab was separated from unreacted N-hydroxysuccinimide ester of bromoacetic acid by Sephadex G-10 column centrifugation using 0.1M PBS as the elution buffer. Modification of bromoacetylated anti-DTPA Fab was assessed by Tri-nitro benzene sulfonic acid assay (38). Anti-HER2/neu affibody exists as dimers because of oxidized cysteine residue (32, 34). Treatment of the dimeric affibody with 20 mM DTT for 2 hrs at room temperature followed by dialysis against 4 L of 0.1 M PBS and 10mM EDTA at pH 7.4 produce reduced monomers of affibody ligands. Addition of equi-molar concentrations of bromoacetylated anti-DTPA Fab and reduced anti-HER2/neu affibody with free sulphydryl group resulted in conjugation of reduced anti-HER2/neu affibody and anti-DTPA Fab via thioether bonds. The reaction mixture was incubated at 4°C overnight in dark.

2.8.2 TNBS Assay for modified anti-DTPA Fab:

After recovering the modified anti-DTPA Fab from the sephadex G-25 spin chromatography, percent modification was calculated using tri-nitro benzene sulfonic acid assay. 10 µg of the modified anti-DTPA Fab was compared to unmodified antibody (10µg) as previously described. Percent modification was calculated relative to unmodified anti-DTPA Fab. Assay was carried out in triplicates to ensure the reproducibility (Table 3).

2.8.3 Immunoreactivity of modified anti-DTPA Fab:

Micro-titer plates (96 well) were coated with 100 µl aliquots of DTPA modified bovine serum albumin (BSA) (1µg/ml). The DTPA-BSA coated wells were used to assess anti-DTPA Fab activity by the standard ELISA protocol (36). Aliquot of the bromoacetylated antibody (1µg/ml) and the unmodified anti-DTPA Fab were used for comparison of the immunoreactivities as previously described (Figure 8).
2.8.4 SDS-PAGE Analysis for the formation of BAAC:

BAAC and the unconjugated proteins from the reaction mixture of modified anti-DTPA Fab and the reduced affibody molecules were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under nonreducing condition as previously described (Figure 9).

2.8.5 Purification of Bispecific affibody antibody complex (BAAC):

Bispecific antibody complex was purified from unconjugated affibody or anti-DTPA Fab by HPLC chromatography using Zorbax-GF 250 size exclusion column (9.4 D x 250 L mm) (Figure 10A). Briefly the reaction mixture of anti-HER2/neu affibody and modified anti-DTPA Fab was first dialyzed against 0.2M PBS (pH 7.0) and then 350 µl of sample was applied to the Zorbax GF-250 size exclusion column pre-equilibrated and calibrated with molecular weight standards such as IgG, BSA and anti-DTPA Fab. The column was eluted with 0.2M PBS. The elution was monitored at OD of 214 nm. Flow rate and pressure of the column were as described previously. Fractions (250 µl) were collected (Figure 10 A).

2.8.6 Bispecificity ELISA:

A 96 well micro-titer plate (Falcon) was coated with 100µl of 1µg/ml of anti-HIS monoclonal antibody (Abcam Inc.) solution in 0.1M PBS and incubated for 1 hr at 37°C. The micro-titer plate was then washed with 0.1M PBS-Tween (3X) followed by blocking with 5% fetal calf serum (200µl). After washing 50 µl of 1/10 diluted each fraction of elution of BAAC and unconjugated proteins from the Zorbax-GF column were added to each well. The micro-titer plate was incubated for 1 hr at 37°C, washed then aliquots of 50 µl of 1:500 dilution DTPA-HRP were added to each well. Incubation and washing were as described. Then 50 µl aliquots of K- blue substrate (Neogen Corp.) were added and incubated at room
temperature in the dark for 30 minutes. The micro-titer plate was then read in an automated ELISA plate reader. Assay was carried and analyzed as described previously (Figure 10B).

2.9 Preparation of DTPA-Succinyl Fluorescein Labeled Polyllysine polymers (DSPL-FITC):

DTPA-polylysine polymers (DPL) were prepared as described by Khaw et al. (24). Briefly, 50 mg of polylysine (PL, MW 14.6 kDa) (Sigma Chemical Co.) were dissolved in 0.1 M Sodium Carbonate, pH 8.3, and 50 molar excess of bicyclic anhydride of DTPA in 0.1-0.5 ml of anhydrous DMSO was added to the solution. The mixture was vigorously stirred for approximately 5 minutes followed by incubation at room temperature for 2 hrs while stirring. The percent modification of lysyl residues was assessed by the TNBS assay relative to unmodified polymer as described above.

Residual free lysyl residues were modified by addition of 24 molar excess of fluorescein isothiocynate (Sigma Chemical Co.). The fluorescein conjugated DTPA-PL was then subjected to succinylation with 100 molar excess of succinic anhydride (Sigma Chemical Co.) to provide DTPA-succinylated-Fluorescein labeled -polylysine (DSPL-FITC) (24, 40).

2.10 Preparation and characterization of D-Dox-PGA complex:

2.10.1 Preparation of D-Dox-PGA complex:

Aliquots of 50 mg of (10mg/ml) polyglutamic acid (PGA, MW 13.3 kDa) (Sigma) in 0.1 M NaHCO₃, pH 8.6 was reacted with 3X molar excess of anhydride of DTPA (sigma) which generated the D-PGA polymers. DTPA was linked to the N-terminal amino group of polyglutamic acid. The mixture was subjected to vigorous mixing and was then incubated at room temperature for 2 hrs. DTPA conjugated PGA (D-PGA) was dialyzed in 0.1 M
Phosphate buffered saline (PBS) (pH 7.4) using a dialysis membrane with a cut-off of 3000 Daltons. DTPA incorporation was demonstrated by ELISA using anti-DTPA antibody. Then 4.5 mg of doxorubicin were dissolved a minimal amount of DMSO and water (1:1), and were covalently linked via peptide bonds to the carboxylic acid of D-PGA using 9 mg water soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCL (EDC) (41). The free amino group of doxorubicin was linked to the carboxylic groups of polyglutamic acid. The reaction was undertaken at 4°C for 1 hr in the dark and then incubated overnight at room temperature. Higher amounts of doxorubicin (9mg) were also coupled to D-PGA complex in order to increase the number of moles doxorubicin per mole polyglutamic acid.

2.10.2 Isolation of Free and Polymer-linked doxorubicin using Sephadex G-25 Column:

The D-Dox-PGA complex was isolated from free doxorubicin by Sephadex G-25 (10cm x 0.5 cm) size exclusion chromatography. Fractions (0.8ml/16 drops) were collected using 0.1M PBS (pH 7.4) as the elution buffer (Figure 12). OD 490 nm was read using a spectrophotometer to estimate the concentration of doxorubicin (42).

2.10.3 Demonstration of incorporation of DTPA on polymers by ELISA:

A 96 well micro-titer plate was coated with 100µl of DTPA-bovine serum albumin (BSA)/well as a positive control standard or D-Dox-PGA at concentration of 1 µg/ml equivalent and then the plate was incubated for 1 hr at 37°C. The micro-titer plate was washed with 0.1M PBS-T (3 X) followed by addition of 5% blocking FCS and incubated for 1 hr at 37°C. The micro-titer plate was then washed with 0.1 M PBS-T (3X) and the wells were loaded with 100µl aliquots of serial dilutions of anti-DTPA antibody starting with 1µg/ml of the antibody. The micro-titer plate was incubated for 1 hr at 37°C, washed 3 X with 0.1 M PBS-T and 50µl aliquots of goat anti-mouse conjugated with HRP (1/1000
dilution) were added to each well. Incubation and washing was again carried out. K-blue substrate was added (50µl each well) as a chromogen. OD was read at 405 nm. The assay was carried out in quadruplicates and analyzed using GEN 5.0 as described (Figure 14).

2.11 Tissue Cultures:

Rat embryonic cardiocytes (H9C2), B16-F10 murine melanoma cells, BT-20 and BT-474 breast carcinoma cells were purchased from American Type Culture Collection (USA). All cells were cultured in Dulbecco Minimum Essential Medium (Cassion Labs, UT, USA) with 10% Fetal clone (Thermo Fisher, USA), penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin (0.25 µg/ml) at 37°C in an atmosphere of 95% air and 5% CO2. At about 80% confluence, the cells were harvested and used for induction of tumor growth.

2.12 Demonstration of specificity of bsMAb for B16-F10 murine melanoma cells by flow cytometry:

B16F10 murine melanoma cells were grown in 6 well plates. Cultured cells (B16F10) were incubated with serial dilutions of 2C5-Oregon green (1, 0.1, 0.01, and 0.001 µg/ml) for 30 min at 4°C. Another set of B16-F10 cells were incubated with serial dilutions of bsMAb (1, 0.1, 0.01, and 0.001 µg/ml) for 30 min at 4°C (43). Cells were then washed with cold 0.1 M PBS (3X). After washing, cells pretargeted with bsMAb were incubated with DSPL-FITC (2.5 µg/ml equivalent polymer concentration) for 1 hr at 37°C. The cells were then trypsinized and detection of labeled cells was verified using flow cytometry. Flow cytometry was performed using a FACS Calibur instrument (BD Biosciences) equipped with an argon-ion laser and an optional second red diode laser (source energy, 15 mW; detection time, 500 counts per second). Cell Quest software was used for data acquisition and analysis. A
minimum of ten thousand cells were gated. Data were live gated by FL1 (blue laser, 488 nm) (Figure 7).

2.13 Demonstration of specificity of BAAC for BT-474 human breast cancer cells by flow cytometry:

Human breast cancer (BT-474) cells were grown in 6 well plate. Cultured cells (BT-474) were incubated with serial dilutions of anti-HER2/neu labeled FITC (1, 0.1, 0.01, and 0.001 µg/ml) for 30 min at 4°C (32, 34). Another set of BT-474 were incubated with serial dilutions of BAAC (1, 0.1, 0.01, and 0.001 µg/ml) for 30 min at 4°C. Cells were then washed with 0.1 M PBS (Cold, 3X). After washing, cells were incubated with DSPL-FITC for 1 hr at 37°C. Cells were washed again with 0.1 M PBS. Cells were trypsinized and analyzed by flow cytometry as described previously (Figure 11A).

2.14 Demonstration of specificity of BAAC for BT-474 human breast cancer cells by epi-fluorescent microscopy:

Human breast cancer (BT-474) cells were grown in 6 well plate. Cultured cells (BT-474) were incubated with serial dilutions of anti-HER2/neu affibody labeled FITC (1 µg/ml) for 30 min at 4°C (32, 34). Another set of BT-474 were incubated with serial dilutions of BAAC (1, 0.1, 0.01, and 0.001 µg/ml) for 30 min at 4°C. The cells were then washed 3X with cold 0.1M PBS and incubated with DSPL-FITC at 37°C for 1 hr. Fluorescent intensity of treated cells was measured by obtaining digital micrographs of the samples using Olympus DP70 and X-cite 120 Fluorescence illumination system. Fluorescent intensity data were analyzed using Adobe Photo Shop 7. Control set of cells were treated with DSPL-FITC alone in order to check the non-specific binding (Figure 11B).
2.15 In-vitro cardiotoxicity assay on rat embryonic cardiocytes:

Rat embryonic cardiocytes (H9C2) were used to determine the inhibitory concentration at 50% cell survival. Aliquots of 40,000 cells were grown in 6 well plate to 90% confluence and serial dilutions of 2.5, 5, 10, 15 and 30 µg /ml of free Dox or Dox equivalent concentrations of D-Dox-PGA were added to the culture media and cells were incubated for 24 h at 37 °C. Each set were prepared in triplicate. Cell death and survival was assessed by Trypan Blue exclusion test (44) (Figure 15).

2.16 In Vitro tumorotoxicity assay on BT-20 and BT-474 cancer cells:

Human breast cancer cells (BT-474) were grown in 6 well plates. The cell culture studies were performed in triplicates. Medium of each well was replaced with medium containing 1µg/ml BAAC, 1µg/ml free anti-HER2/neu affibody, 1µg/ml anti-DTPA Fab antibody or culture medium alone respectively. After 30 min of incubation at 4ºC, the medium was removed and washed with 3X medium alone. Aliquots (1000 µl) of media containing serial dilutions of 5, 10, 15, and 30 µg/ml of Dox or Dox equivalent concentrations of D-Dox-PGA were added to the wells. After 24 hr incubation 37ºC, viability was assessed by Trypan Blue exclusion test (44). Data were expressed in % cell death and were normalized to control group with no treatment at the end of the study (Figure 16A).

2.17 Demonstration of specificity of BAAC and D-Dox-PGA for BT-474 human breast cancer cells using epi-fluorescent microscopy:

Human breast cancer (BT-474) cells were grown in 6 well plate. Cultured cells (BT-474) were incubated with either BAAC (1 µg/ml) or control treatment of culture media alone for 30 min at 4ºC. The cells were then washed 3X with cold 0.1M PBS and sets of cells were incubated with either D-Dox-PGA pretargeted with BAAC, D-Dox-PGA alone (Dox
equivalent concentration of 10µg/ml), or Dox alone at 37°C for 24 hrs. Fluoro-micrographs for Dox fluorescence were obtained using 475 nm excitation and 580 nm emission wavelength by epifluorescent microscopy (20). Dox fluorescent intensity of the treated cells was quantitated from the digital micrographs obtained with Olympus DP70 and X-cite 120 Fluorescence illumination system. Fluorescent intensity data were analyzed using Image J software from NIH. All the images were acquired at 245 msec exposure (Figure 16B and 16C).

2.18 Animal model:

2.18.1 Lung metastatic model:

All experiments were performed in 6-8 week old male C57/BL6 mice (Charles River Laboratories, Wilmington, MA) following protocol # 0309R approved by the Institutional Animal Care and Use Committee in accordance with the NIH guidelines. Food and water were available ad libitum. Approximately 2x10^5 B16F10 cells in 0.2 ml of serum free DMEM were injected intravenously via the tail vein to induce metastatic lesions in lungs (45, 46). After 10-14 days, the animals were used for in vivo studies.

2.18.2 Breast cancer model:

Imaging of pretargeted BAAC in breast tumor model was performed in 6-8 week old male SCID-SHO mice (Charles River Laboratories, Wilmington, MA) following protocol # 0309R approved by the Institutional Animal Care and Use Committee in accordance with the NIH guidelines. Food and water were available ad libitum. Approximately 4 x10^6 BT-474 and BT-20 cells in 0.2 ml of serum free DMEM were injected subcutaneous in the right and the left shoulder regions of the arm pits of the mice respectively (47). After about 6 weeks, the animals were used for in vivo studies. Similar animal model was used to perform the
therapeutic efficacy studies pretargeted with BAAC and targeted with D-Dox-PGA or controls treated with Dox alone, and D-Dox-PGA alone.

2.19 Preparation of $^{99m}$Tc-Labeled DTPA-Succinyl-PL (14.6 kDa):

An aliquot of 50 mg of polylysine (PL) (14.6 kDa; Sigma Chemical Co.) was dissolved in 5 ml of 0.1 M NaHCO$_3$, pH 8.7. Ten times molar excess (relative to lysine residues) of bicyclic anhydride of DTPA (Sigma Chemical Co.) in 1 ml of dimethyl sulfoxide (DMSO) was added slowly to the above solution while stirring vigorously (24). The number of lysyl residues modified was assessed by the Tri-nitro benzene sulfonic acid (TNBS) assay relative to the standard unmodified PL solution (38). The reaction mixture was dialyzed against excess (4 L) 0.1 M carbonate buffer, pH 9.6, at 4°C overnight. Then the DTPA-PL solution was succinylated with 100 times molar excess of succinic anhydride to succinylate any residual unmodified lysyl residues. The DTPA–Suc-PL (14.6 kDa) was dialyzed in 0.1 mol/L carbonate buffer, pH 9.6, and stored at 4°C until used. Approximately 100 µg aliquot of DTPA-PL (14.6 kDa) in 0.1 M carbonate buffer was reacted with 740 MBq of $^{99m}$Tc O$_4^-$ in 50 µg of SnCl$_2$ in 100 µl of 0.1N HCl that has been flushed with N$_2$ for 15 min. After 30 min of incubation, the Tc-$^{99m}$DSPL (14.6 kDa) was separated from free $^{99m}$Tc by Sephadex-G25 (10 mL) column chromatography (24) (Figure 17).

2.20 Clearance studies for BAAC:

Approximately 100µg of BAAC dialyzed in saline was used to labeled directly with Tc-$^{99m}$DTPA. Pre-incubation of BAAC with Tc-$^{99m}$DTPA was carried out for about 1 hr. After incubation, Tc-$^{99m}$DTPA was separated from BAAC labeled Tc-$^{99m}$DTPA using Sephadex G-25 (10 ml) column chromatography (24). Approximately 200 µci of Tc-$^{99m}$DTPA-BAAC were injected in each SCID mice via the tail vein and in vivo gamma imaging
was initiated. Images were acquired at 0, 1, 3, and 8 hr post injection. Image acquisition was for 300 seconds at each time point (1x10^6 - 6x10^5 total counts) (Figure 18). At the end of 8 hrs biodistribution studies were performed on the same animals (Figure 19).

2.21 In vivo imaging experimental protocol for the lung metastatic model:

Aliquot of 10 µg of bsMAb was injected i.v. 24 hours before the imaging schedule. Approximately 37 MBq of ^{99m}Tc-DSPL (14.6 kDa) (2 µg) was injected on the day of imaging (24). Gamma imaging was initiated at 0 hr and was followed for 1, 3, 8 and 24 hrs. A total of 9 C57/BL6 mice (5 pretargeted and 4 non-pretargeted) were used in this study. Two tumor mice pretargeted with bsMAb and 2 non-pretargeted mice were imaged in the anteroposterior view using a planar SPECT micro-camera provided by the Jefferson National Laboratory (48). Image acquisition was for 300 seconds at each time point (1x10^6 - 6x10^5 total counts), except at 24 hr when 30 min data acquisition time (10,000 counts) was employed. Data acquisition time of 2700 seconds was used for imaging the excised lungs (Figure 20A). Mice (n=2) after excision of the lungs were re-imaged for 2400 seconds (Figure 20B). Additionally 2 pretargeted and 2 non-pretargeted mice were imaged with the BIOSCAN SPECT/CT camera equipped with 3 X 9 pinhole collimators (Center for Translational Neuroimaging (CTNI) at Northeastern University, Figure 21A and 21B) for up to 3 hours. The imaging time was 1200 seconds for each SPECT data acquisition. One additional mouse pretargeted with bsMAb was not imaged but the data were included in the total biodistribution data. After 24 hours, the mice were sacrificed for biodistribution or in the case of two mice (Figure 20B) the lungs were excised after the 20 hours images and mice were reimaged. Additional 3 normal C57/BL6 (non-tumor) mice were pretargeted with bispecific antibody and targeted with Tc-99m-DSPL. Three more SCID mice were pretargeted with anti-DTPA antibody
followed by targeting with either Tc-99m-DSPL (n=1) or In-111-DSPL (n=2) to determine non-specific localization in the lungs.

2.22 Biodistribution studies for lung metastatic model:

After euthanasia of mice (5 pretargeted and 4 non-pretargeted allografted tumor mice, 3 non-tumor SCID mice pre-targeted with anti-DTPA antibody and 3 normal mice pretargeted with bsMAb) with IP overdose injection of ketamine/xylazine (100 mg/kg and 10 mg/kg respectively) total lungs with or without tumors, blood, heart, liver, spleen, kidneys, stomach, intestines, salivary glands, thigh muscle and chest skeletal muscle were excised, weighed, and then counted in a gamma scintillation counter (Compugamma model 1282; LKB Instruments, Inc.). Aliquots (5 µl) of the total injection volume (100 µl/37 MBq) of the radiolabeled polymer were saved and counted together with the tissue samples. Total injection dose was calculated by multiplying the counts per minute (cpm) of the 5 µl aliquot of the injected dose by 20. All counts were corrected for isotope decay (49). Percent Injected Dose/g (% ID/g) in tissues was estimated relative to the total injected dose. Since whole lungs were counted for radioactivity but only portions contained tumors, activity in tumor mass was assessed after correction for normal lung tissue. This correction was undertaken by determination of the area of the total lungs and tumors by computer planimetry of the digital photographs using ImageJ software (NIH). Each set of lungs is assumed to have uniform thickness for our calculations. The total lung and lesion masses were assessed for each set of lungs as pixels and % tumor mass relative to total lung mass was calculated. The % lesion relative to total lung mass was used to correct the %ID/g of the tumor (Figure 22).
2.23 In Vivo imaging protocol for breast cancer studies:

A total of 8 SCID-SHO mice with human breast cancer xenograft (4 BAAC pretargeted and 4 control mice pre-targeted with anti-DTPA Fab) were used in this study. The pretargeting dose of 10 µg of BAAC was injected i.v. 8 hours prior to the imaging schedule as previously reported. Approximately 37 MBq of Tc-99m-DSPL (1.77 µg) were injected. Gamma imaging was initiated at time of injection and repeated at 1, 3, and 24 hrs. Two tumor mice pretargeted with BAAC and 2 anti-DTPA Fab pretargeted mice were imaged in the posterior-anterior view using a planar SPECT micro-cameras provided by Jefferson National Laboratory (48). Image acquisition was for 300 seconds at each time point (1x10⁶ - 6x10⁵ total counts), except at 24 hr when 30 min data acquisition time (10,000 counts) was employed. At 24 hours, the mice were sacrificed for biodistribution.

2.24 Biodistribution studies for breast cancer model:

After euthanasia of mice with IP overdose injection of ketamine/xylazine (100 mg/kg and 10 mg/kg respectively), BT-20 and BT-474 tumors, the lungs, blood, heart, liver, spleen, kidneys, stomach, intestines, salivary glands, thigh muscle and chest skeletal muscle were excised, weighed, and counted in a gamma scintillation counter (Compugamma model 1282; LKB Instruments, Inc.). Total injection dose was calculated as described above. Radioisotope T_1/2 correction was performed as above. Percent Injected Dose/g (% ID/g) of each tissue was estimated relative to the total injected dose (Figure 24).

2.25 In vivo breast cancer therapy protocol:

Therapy studies were conducted on SCID mice using same tumor model as used for the imaging studies. A total of 18 mice were used for this study. Five SCID mice were pretargeted with 20 µg of BAAC followed by treatment with D-Dox-PGA complex
(7.5mg/kg Doxorubicin equivalent dose), 5 SCID mice were treated with Doxorubicin alone (7.5mg/kg Doxorubicin dose), 4 mice were treated with D-Dox-PGA complex alone (7.5mg/kg Doxorubicin equivalent dose), and 4 SCID mice were pretargeted with 20 µg of anti-HER2/neu affibody followed by D-Dox-PGA complex (7.5mg/kg Doxorubicin equivalent dose) (50). Respective doses were again administered on Day 14 and Day 28 of the study. Tumor sizes were monitored by vernier caliper measurements of the length (L) and width (W) of the tumors. Tumor mass was calculated using the formula: $L \times W^2 / 2$ (51). Additionally mice were also monitored for change in body weight. Data for curve fitting and analyses were generated by using Microsoft Excel XP.

2.26 Statistics:

Data are expressed as mean +/- SD. ANOVA (single factor) was used to determine statistical significance using the statistical package of Microsoft Excel XP. P value of ≤ 0.05 was considered significant.
3.0 Results:

Characterization of anti-HER2/neu affibody using ELISA, SDS-PAGE and Flow cytometry:

Anti-HER2/neu affibody possess a unique carboxy terminal end cysteine residue which have a tendency to dimerize. Affinity purification was used as the pDNA sequence had 6 histidine residues for metal ion affinity chromatography for purification using HIS-MAG agarose beads. Figure 1A shows ELISA for identification of isolation of anti-HER2/neu affibody from the bacterial culture. The ELISA shown below does not give direct correlation between HER2/neu antigen and indicates only the isolation of affibody molecule. Figure 1B shows both the reduced and unreduced SDS-PAGE for the anti-HER2/neu affibody. Molecular weight estimation for affibody monomer and dimmers were done based on Rf value. Anti-HER2/neu affibody has a molecular weight of around 9 kDa and affibody dimers had a molecular weight if approximately 18 kDa. SDS-PAGE also indicated the isolated protein of purity greater than 99%.

![Image](image-url)

**Figure 1.** A. ELISA for anti-HER2/neu affibody after HIS-MAG agarose protein purification
B. SDS-PAGE for the anti-HER2/neu affibody after HIS-MAG agarose protein purification showing 99% purity.
The isolated pure anti-HER2/neu affibody was tested against human breast cancer cell line (BT-474) overexpressing HER2/neu and human mammary adenocarcinoma cells (BT-20) which does not express or have minimal basal levels of HER2/neu antigen. Flow cytometry data showed complete shift in signal when BT-474 cells were treated with anti-HER2/neu affibody-FITC, while there was no shift in signal when BT-20 cells were treated with anti-HER2/neu affibody FITC. This data confirms that isolation of anti-HER2/neu affibody was achieved (Figure 2).

![Figure 2](image.png)

**Figure 2.** Binding of anti-HER2/neu affibody to human breast cancer cells BT-474 and BT-20 using flow cytometry. Positive shift was observed in BT-474 cells over-expressing HER2/neu antigen.

**Characterization of anti-DTPA Fab (SDS-PAGE and ELISA)**

Anti-DTPA Fab was prepared using papain digestion of pure anti-DTPA MAb. Starting concentration of 5mg/ml pure anti-DTPA MAb was used for digestion. The yield of anti-DTPA Fab after papain digestion was approximately 35% after protein A purification. Figure 3 shows immunoreactivity of pure anti-DTPA Fab. Nano-molar affinity was achieved after protein A purification of the papain digest against DTPA-BSA as the antigen at 1µg/ml (Figure 3A). SDS-PAGE showed anti-DTPA Fab to be highly homogenous (Figure 3B).
Figure 3. A. Immunoreactivity of the anti-DTPA Fab after protein A purification. B. SDS-PAGE for the anti-DTPA Fab after protein A purification.

**TNBS assay for modified anti-DTPA and 2C5 MAb:**

Modification of anti-DTPA MAb (6C31H3) with 100 molar excess of 2-Iminothiolane (Table 1) and anti-nucleosomal MAb(2C5) (Table 2) with 100 molar excess of N-hydroxy succinimide ester of bromoacetic acid was assessed using Tri-Nitro benzene sulfonic acid assay comparing to their respective unmodified MAbs. Modification of the epsilon amino groups of the lysine residues of anti-DTPA MAb was approximately 80%, while modification of 2C5 MAb was approximately 40%. This lower modification was expected using N-hydroxy succinimide ester of bromoacetic acid as it is prone to hydrolysis.
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**Table 1:** TNBS assay for anti-DTPA Ab modified with 2-iminothiolane.
Table 2: Estimation of modification of intact 2C5 MAb with N-hydroxy succinimide ester of bromoacetic acid using TNBS assay.

**Immunoreactivity of Modified Antibodies:**

The immunoreactivity of anti-DTPA antibody modified with 2-Iminothiolane and 2C5 antibody modified with N-hydroxy succinimide ester of Bromoacetic acid as analyzed by ELISAs is shown in Figure 4A and 4B respectively. Neither antibody showed loss of immunoreactivity after chemical modification relative to the native antibodies.
Figure 4. A. ELISA for determination of the immunoreactivity of modified anti-DTPA Ab relative to unmodified anti-DTPA antibody. B. ELISA for determination of the immunoreactivity of modified 2C5 antibody relative to unmodified 2C5 antibody. OD = Optical Density. Vertical bars denote ± 1 standard deviation (95% confidence interval) in all figures.

Purification of Bispecific 2C5-6C31H3 antibody (bsMAb):

The elution profile of the bispecific antibody reaction mixture by HPLC chromatography showed the presence of polymeric bsMAb, dimeric bsMAb and free unconjugated MAb peaks (Figure 5A). The polymeric bsMAb peak was at 15 min, that of the dimeric bsMAb was at 16 min and that of the free unconjugated antibody was at 17-17.5 min. The bispecificity of the HPLC fractions is shown in Figure 5B using the bispecific ELISA. Both the polymeric and dimeric bsMAbs (vertical dashed lines) bound the nucleosomal antigens coated on the micro-titer wells, as well as to the DTPA-HRP via anti-DTPA of the bispecific antibody complexes. The monomeric MAb peak showed minimal bsMAb activity.
(17-17.5 min elution fractions). SDS-PAGE analyses of the bsMAb mixture before HPLC chromatography showed presence of polymeric and dimeric bsMAb, and unreacted monomeric MAb (Figure 6A). After HPLC chromatography polymeric and dimeric bsMAb and the unreacted monomeric MAb were separated into different fractions (Figure 6B). Fraction 16 showed greater than 95% dimeric bsMAb purity. Optical Density at 280 nm was used to determine the protein concentration using a molar extinction coefficient of 1.4 for antibodies. The yield of pure dimeric bsMAb was approximately 10%.
**Figure 5.** A. Zorbax-GF 250 column HPLC elution profile showing elution of polymeric bsMAb, dimeric bsMAb (2C5-6C31H3) and free unconjugated monomeric monoclonal antibody. B. Assessment of bispecificity of bsMAb (2C5-6C31H3) in fractions collected from the size exclusion HPLC. OD = Optical Density. The error bars indicate ± 1 standard deviation (95% confidence interval). Dashed vertical lines indicate peak bispecific antibody activity and the corresponding HPLC eluted peaks determined by OD$_{214}$ nm readings.

![Image of HPLC profiles](image)

**Figure 6.** SDS-PAGE of bsMAb (2C5-6C31H3). A. before HPLC Zorbax GF-250 size exclusion column chromatography and B. after chromatography showing polymeric bsMAb, dimeric bsMAb and free unconjugated antibodies. The numbers indicated at the bottom of the gel indicate time (min) of elution collected as fractions.

**Specificity of bsMAb for B16-F10 tumor cells by flow cytometry:**

Figure 7 shows enhanced targeting of B16F10 murine melanoma cells by bsMAb pretargeting and targeting with FITC-DSPL (open bars). Binding was compared to MAb 2C5 labeled with Oregon green (solid bars). Both sets of data were read at same excitation and emission wavelengths. Oregon green is more stable and less susceptible to photobleaching (52, 53), yet the signal intensity obtained with bsMAb pretargeting and targeting with FITC-DSPL was greater at 0.1 and 0.01 µg/ml antibody concentrations than Oregon-Green labeled 2C5 MAb. Signal amplification using bsMAb and FITC-DSPL was approximately 25 times at 0.01 µg/ml antibody equivalent concentrations.
Figure 7A. In-vitro binding of Oregon-green labeled 2C5 (Solid Bars) or pre-targeting with bsMAb followed by specific targeting with FITC-DSPL (Open Bars) in murine B16F10 melanoma.
Figure 7B. In-vitro binding of Oregon green labeled 2C5Ab (panel b, c, d, and e) or pre-targeting with bsMAb followed by specific targeting with FITC-DSPL (panel f, g, h, i, and j, k) in B16F10 melanoma cells. Panel a represents mean fluorescent intensity for cells alone and panel f represents mean fluorescent intensity of the FITC-DSPL alone. The panel represents the individual curves for the graphical version in figure 7A.
TNBS assay for modified anti-DTPA Fab:

Modification of anti-DTPA Fab with 100 molar excess of N-hydroxy succinimide ester of bromoacetic acid (Table 3) was assessed using Tri-nitro benzene sulfonic acid assay comparing to the unmodified anti-DTPA Fab. Modification of epsilon amino group of the lysine residue of anti-DTPA Fab was approximately 40%. This lower modification was expected using N-hydroxy succinimide ester of bromoacetic acid as it is prone to hydrolysis.

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<td>0</td>
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<td>0.085</td>
<td>0.079</td>
<td>0.051</td>
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<tr>
<td>Mean OD 420</td>
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<td>0</td>
<td>0</td>
<td>0.082</td>
<td>0.085</td>
<td>0.079</td>
<td>0.051</td>
<td>0.056</td>
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Table 3. Estimation of modification of anti-DTPA Fab using TNBS assay.

Immunoreactivity of the modified anti-DTPA Fab:

The immunoreactivity of anti-DTPA Fab modified with 100 molar excess of N-hydroxy succinimide ester of bromoacetic acid as analyzed by ELISA is shown in Figure 8.
After modification, no loss in immunoreactivity was observed relative to the standard unmodified anti-DTPA Fab.

![Figure 8](image)

**Figure 8.** ELISA for determination of the immunoreactivity of modified anti-DTPA Fab relative to unmodified anti-DTPA Fab.

**SDS-PAGE analysis for BAAC:**

The molecular sizes in the reaction mixture of the reduced anti-HER2/neu affibody and the modified anti-DTPA Fab before HPLC chromatography were demonstrated by SDS-PAGE analysis relative to known molecular weight standards. The presence of polymeric and dimeric BAAC as well as unreacted monomeric and dimeric anti-HER2/neu affibody is represented in Figure 9. Almost 95-100 % crosslinking of anti-DTPA Fab to anti-HER2/neu affibody was achieved as no free anti-DTPA Fab was seen in the SDS-PAGE of the reaction mixture.
**Figure 9.** SDS–PAGE of BAAC (anti-HER2/neu affibody crosslinked anti-DTPA Fab) for molecular weight estimation. Before HPLC Zorbax GF-250 size exclusion column chromatography.

**Purification of the BAAC:**

After overnight incubation of reduced anti-HER2/neu affibody and modified anti-DTPA Fab, the reaction mixture was subjected to HPLC chromatography. The elution profile showed two peaks, consisting of the polymeric and the dimeric BAAC (Figure 10A). The bispecific immunoreactivity of various fractions was confirmed by ELISA (Figure 10B). Both the polymeric and dimeric BAAC (vertical dashed lines) bound the anti-HIS MAb coated on the micro-titer wells as well as to the DTPA covalently linked to HRP. OD 280 nm readings of the fractions were used to determine protein concentrations using the molar extinction coefficient of 1.4 for antibodies. The yield of the dimeric BAAC recovered was approximately 70%.
Figure 10. A. Zorbax-GF 250 column HPLC elution profile showing elution of polymeric BAAC, dimeric BAAC (anti-HER2/neu affibody crosslinked anti-DTPA Fab) and free unconjugated monomeric and dimeric anti-HER2/neu affibody B. Assessment of Bispecificity of the (anti-HER2/neu affibody crosslinked anti-DTPA Fab) BAAC corresponding to the fractions eluted from the size exclusion column chromatography. OD = Optical Density. The bars indicate ± 1 standard deviation (95% confidence interval). Dotted vertical lines indicate the correlation between HPLC elution profile and the bispecific activity by ELISA.

Specificity of BAAC for BT-474 tumor cells by flow cytometry and epi-fluorescent microscopy:

Figure 11A and 11B shows enhanced targeting of BT-474 HER2/neu positive breast cancer cells by BAAC pre-targeting and specific targeting with FITC-DSPL. Signal
amplification using BAAC and FITC-DSPL was approximately 10 times at 0.01 µg/ml antibody equivalent concentration. All the flow cytometry data with BAAC were adjusted for the background fluorescence with FITC-DSPL alone. Flow cytometry data of BT-474 HER2/neu overexpressing breast cancer cells also shows that BAAC (Open bars) bound specifically with higher signal intensity than with FITC labeled anti-HER2/neu affibody (Closed bars) (Figure 11A). Figure 11B (panel A) shows bright field images, nuclei stained with DAPI and the fluorescence after staining with anti-HER2/neu affibody labeled FITC (1 µg/ml) in BT-474 cells. Figure 11B (panel B), shows BT-474 cells pretargeted with BAAC (1 µg/ml) followed by specific targeting with FITC-DSPL The BAAC pretargeted cells showed higher fluorescent intensity as compared to FITC-anti-HER2/neu affibody corresponding the flow cytometry data. Panel C showed that FITC-DSPL did not bind non-specifically to BT-474 cells.

![Image](image.png)

**Figure 11A.** In-vitro binding of Fluorescein labeled anti-HER2/neu affibody (closed bars) or pre-targeting with BAAC followed by specific targeting with FITC-DSPL (open bars) in human breast cancer cells.
Figure 11 B. In-vitro binding of Fluorescein labeled anti-HER2/neu affibody (panel b, c, d, e, and f) or pre-targeting with BAAC followed by specific targeting with FITC-DSPL (panel h, i, j, k, and l) in human breast cancer BT-474 cells. Panel a represents mean fluorescent intensity for cells alone and panel g represents mean fluorescent intensity of the FITC-DSPL alone. represents the individual curves for the graphical version in figure 11A.
Characterization of D-DOX-PGA:

Isolation of intact D-Dox-PGA complex:

The D-Dox-PGA complex was separated from free dox after conjugation using sephadex G-25 size exclusion chromatography. The polymer conjugated doxorubicin (D-Dox-PGA) was eluted in the void volume at around fraction no.7 and free doxorubicin was eluted in the inclusion volume at around fraction no.33 (Figure12) by size exclusion Sephadex G-25 column chromatography. Approximately 4 ml of polymer conjugated doxorubicin (D-Dox-PGA complex) were acquired and used for further studies. Both the
samples of D-Dox-PGA (24 Dox mole/polymer mole or 36 Dox mole/polymer mole) eluted at the same fraction.

Figure 12. Superimposition of the elution profile of D-Dox-PGA complex (24 mole Dox/mole polymer-blue, 36 moles of Dox/mole polymer – Brown) and free doxorubicin (Green) using Sephadex G-25 column (10 X 0.5 cm) chromatography.

Estimation of Doxorubicin concentration in D-Dox-PGA complex:

Serial dilutions of known concentration of free doxorubicin were prepared and the concentration was read at 490 nm (Table 4). Concentration of doxorubicin on polymer-prodrug complex (D-Dox-PGA) was then extrapolated from the standard values using the equation of line. Concentration of doxorubicin on polymer-prodrug complex (D-Dox-PGA) was extrapolated from the standard curve. The concentration of doxorubicin on polymer complex thus prepared was approximately 525µg/ml or 24 moles Dox/mole polymer. By increasing Dox concentration approximately 36 moles Dox/mole polymer conjugates were also prepared.
### Table 4.
Optical density 490 nm values at different doxorubicin concentration to generate standard curve for concentration analysis of doxorubicin on D-Dox-PGA complex.

<table>
<thead>
<tr>
<th>Dox Conc. (µg/ml)</th>
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<td>1000</td>
<td>2.239</td>
</tr>
<tr>
<td>D-Dox-PGA complex</td>
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</tr>
</tbody>
</table>

**Figure 13.** Standard curve for different known doxorubicin concentration for estimation of doxorubicin concentration on D-Dox-PGA- complex at OD 490 nm.
Incorporation of DTPA on polymers (PGA):

ELISA assay was carried out to determine the presence of capture antigen (DTPA) on polyglutamic acid. As seen in Figure 14 standard anti-DTPA antibody bound to the D-Dox-PGA complexes as assessed by ELISA indicating the presence of DTPA on the polymers. Aliquots of 24 moles Dox/mole polymer and 36 moles Dox/mole polymer both showed equivalent binding to the anti-DTPA Ab at the concentration of 1µg/ml because only 1 mole of DTPA per mole of polyglutamic acid is present as compared to the positive control (DTPA-BSA) which have multiple moles of DTPA per mole of BSA. Assay was performed in quadruplicates and analyzed using GEN 5.0 software for mean and standard deviation. The colored points indicate the average of quadruplicate samples while the bars indicate the standard deviation (95% confidence interval) (Fig. 14).

Figure 14. Superimposition of assessment of presence of capture antigen on D-Dox-PGA complex using ELISA assay. (24 mole Dox/mole polymer - Blue, 36 mole Dox/mole polymer – Brown, STD DTPA-BSA - Red).
In vitro reduction in cardiotoxicity:

Rat embryonic cardiocytes treatment with varying concentrations of D-Dox-PGA and Dox alone (Dox equivalent 2.5, 5, 10, 15, and 30 µg/ml) after 24 hrs at 37ºC showed higher cell toxicity with free dox at all concentrations. IC₅₀ of D-Dox-PGA (Solid line) (15.75 ± 0.27 µg/ml) was significantly higher than that of free Dox (Dashed line) (1.20 ± 0.19 µg/ml, p <0.00005) resulting in approximately 13.1 times less nonspecific cardiocyte toxicity (Figure 15).

**Figure 15.** IC₅₀ of D-Dox-PGA complex (1.20 µg/ml) and Dox (15.75 µg/ml) alone on embryonic rat cardiocytes (H9C2) cells. Cardiotoxicity associated with D-Dox-PGA (Solid line) was significantly less than that of Dox (Dashed line) resulting in about 13 times less non-specific toxicity (p < 0.00005).
Tumorotoxicity study:

When BT-474 cells were pretargeted with BAAC at (1 µg/ml) or cells not pretargeted were treated with serial dilutions of 5, 10, 15, and 30 µg/ml Dox equivalent concentrations of D-Dox-PGA or Dox alone for 24 hr at 37°C, IC$_{50}$ of Dox was 4.23 ± 0.19 µg/ml and that of D-Dox-PGA pretargeted with BAAC was 1.82 ± 0.15 µg/ml, p < 0.05 (Fig. 16A). However, the IC$_{50}$ of D-Dox-PGA in controls pretargeted with either anti-DTPA Fab, or anti-HER2/neu affibody showed the same cytotoxicity as D-Dox-PGA, indicating that the D-Dox-PGA was only minimally toxic for cancer cells.

Figure 16 A. In vitro tumorotoxicity IC$_{50}$ of cells pretargeted with BAAC (1 µg/ml) followed by treatment with Dox equivalent concentrations of D-Dox-PGA (Dashed line) is significantly less than that with free Dox (Solid line). * indicates p <0.05.

Epi-fluorescent microscopy for tumorotoxicity studies:

Figure 16B shows images of BT-474 cells pretargeted with BAAC and targeted with D-Dox-PGA, or treatment with Dox alone or D-Dox-PGA alone without pretargeting. DAPI staining of the nuclei and the corresponding Doxorubicin fluorescence after internalization of D-Dox-PGA in BT-474 cells pretargeted with BAAC are shown in Panel A, top and bottom
respectively. Panel B shows the corresponding DAPI and Doxorubicin fluorescence in BT-474 cells treated with free doxorubicin. Panel C shows the corresponding images of BT-474 treated with D-Dox-PGA alone. All the images were analyzed for mean pixel intensity using Image J software from NIH. Pixel intensity for atleast 25 cells (n =3) from each group were assessed and the data for mean fluorescent intensity was analyzed. For the cells treated with BAAC and D-Dox-PGA relative mean intensity was 9.233 ± 2.56 which was significantly higher than cells treated with Dox alone with relative mean intensity of 3.43 ± 1.08 (p <0.00005). Higher doxorubicin fluorescence was observed when targeted specifically via BAAC as compared to cells treated with Dox Alone (Figure 16C). Very minimal background fluorescence was seen when cells were treated with D-Dox-PGA alone (p < 00000.5) as compared to BAAC pretargeted cells.
Figure 16 B. Epi-fluorescent microscopy of BT-474 cells pre-targeted with BAAC (1μg/ml) (Panel A) and specifically targeted with D-Dox-PGA (Dox equivalent concentration of 10μg/ml) showed significantly higher fluorescent intensity and internalization of Dox than cells treated with the Dox alone (Dox conc. of 10μg/ml) (Panel A), and no background signal was observed when cells treated with D-Dox-PGA (Panel C) (Dox equivalent concentration of 10μg/ml) at 245 msec exposure to obtain digital micrographs. All the sets of cells where imaged for DAPI (blue stain for nucleus using DAPI) and corresponding red fluorescence for doxorubicin.
Figure 16C. Fluorescent intensity of doxorubicin on BT-474 cells pre-targeted with BAAC and specifically targeted with D-Dox-PGA was significantly greater than, when BT-474 cells treated with Dox Alone or D-Dox-PGA Alone, * indicates p < 0.00005.

Radiolabeling of DSPL:

Using 5 mg of DSPL in 1 ml for Sephadex-G25 column chromatography, 3.56 mg of DSPL were recovered as assessed by Biuret assay (71% recovery, Assay was repeated three times). Assuming, the same amount of DSPL is lost when Tc-99m-DSPL (14.6 kDa) is used, 71 µg of the polymer were assumed to be recovered (Figure 17). Furthermore, since only the peak tube was used for in vivo studies, only 14.2 µg of the polymer were estimated from the area under the curve calculations from the elution profile to be in the peak tube. The radioactivity in the peak tube was 296 ± 18.5 MBq of $^{99m}$Tc. Each mouse was injected intravenously via the tail vein with 37 MBq of Tc-99m-DSPL which was equivalent to 1.77 µg of the polymer.
Figure 17. Elution profile for Tc-99m-DSPL using sephadex G-25 size exclusion column chromatography.

Clearance of BAAC and biodistribution of BAAC:

Anti-HER2/neu affibody crosslinked anti-DTPA Fab bispecific complex (BAAC) was labeled directly with Tc-99m-DTPA complex. Molecular weight of the dimeric BAAC was approximately around 60 kDa. Approximately 200 µCi of the labeled BAAC were injected in normal 6-8 weeks old SCID-SHO mice. Planar gamma imaging was initiated post-injection of Tc-99m-DTPA-BAAC. Images were acquired at 5 min, 3 hr and 8 hr post injection. BAAC was mostly cleared via kidneys and liver as seen in Figure 18. By the end of 8 hrs post injection no heart activity was observed when planar SPECT images were analyzed using ImageJ software from NIH. Mice were then euthanized for biodistribution studies.
Figure 18. Planar in vivo gamma images of SCID-SHO mice injected with Tc-99m-DTPA-BAAC alone at 5 min, 3 hr, and 8 hrs post injection.

Biodistribution data at the end of 8 hr post injection of Tc-99m-DTPA-BAAC complex showed around 22.02 ± 2.53 % ID/g and 30.14 ± 0.58 % ID/g uptake in liver and spleen respectively. 7.90 ± 1.67 % ID/g of the BAAC was associated with kidneys. Very minimal association was seen with blood (0.62 ± 0.25 % ID/g), and heart (0.80 ± 0.21 % ID/g) at the end of 8 hr post injection suggesting almost complete clearance from blood pool (Figure 19). Ideal pretargeting time for BAAC was therefore calculated around 8 hrs to minimize the background activity while imaging small tumor lesions.
In vivo Imaging of metastatic melanoma lesion in lungs:

In vivo gamma imaging studies were carried out with two different cameras. The same set of imaging experiment was repeated on different occasions using the same cameras to confirm reproducibility of tumor detection (Figure 20A and 20B). Planar images were obtained using a high resolution planar SPECT gamma camera provided by the Jefferson National Laboratory. Planar images were acquired approximately at 0, 1, 3, 8 and 24 hrs. Figure 20A shows images acquired at the time of injection (5 min), at about 1 hr (45 min) and at 24 hours. Figure 20B shows images obtained at 8 hrs and about 21 hrs as well as after surgical removal of the lungs to confirm that the hot spots seen in the region of the lungs were indeed associated with metastatic lesions in the lungs. Planar images showed presence of lesions of less than 2 mm as early as 1 hr (Figure 20A). While the control group injected
with Tc-99m-DSPL alone did not show any accumulation of activity in the lungs. Tc-99m-DSPL alone cleared rapidly via renal excretion. Minimal accumulation of the radioactivity was found in the liver. Both Figures 20A and 20B indicated that pretargeting with bsMAb had higher tumor accumulation (white arrows) as compared to very little or no activity in the control mice. Melanoma lesions were clearly visible in the necropsy pictures of the excised lungs from the mice.

Images were also obtained on a dual modality BIOSCAN SPECT/CT camera (Figure 21A and 21B). Figure 21A shows CT (top panels) and SPECT (bottom panels), 1mm thick computer reconstructed transverse images at 1 hr and 3hr post-radiotracer injection. Figure 21A.I shows the transverse CT and SPECT images of a mouse pretargeted with bsMAb followed by Tc-99m-DSPL at 1 (a and c) and 3 hours (b and d). Figure 21A.II shows the corresponding transverse CT and SPECT images of a control mouse injected with Tc-99m-DSPL alone. Accumulation of radioactivity in metastatic lesions is seen in the mouse pretargeted with bsMAb that corresponded to the CT lesions, whereas minimal radioactivity observed in the control animal did not correspond to the CT lesions. In the 1 hour image (Figure 21A. I), blood pool activity can be seen between 7 and 8 o’clock region (c) which corresponded to the heart regions apparent in the corresponding CT image (a). By 3 hours the blood pool activity has cleared (b and d).

Figure 21B represents the sagittal views of CT and SPECT images and the digital pictures of the excised lungs of mice pretargeted with bsMAb followed by Tc-99m-DSPL (a, b and c) and the control mice with Tc-99m-DSPL alone (d, e and f) respectively. Accumulation of radioactivity in the pulmonary lesions (arrows, b) is clearly evident in the mouse pretargeted with bsMAb corresponding to the lesions seen in the CT image (a) and
almost no activity was seen in the mouse injected with Tc-99m-DSPL alone (e). The CT image (Figure 21 b, and d) showed that the lungs of the control mouse were completely filled with the melanoma lesions (f). The lungs of the pretargeted mouse were less extensively infiltrated with melanoma lesions (Figure 21B, a, and c) yet there was significantly greater radiotracer accumulation in the lesions (b).

**Figure 20.** Planar gamma images from studies showing anteroposterior images of mice with B16F10 melanoma metastasized to the lungs, pretargeted with bsMAb and control mouse not pretargeted followed by i.v. injection of Tc-99m-DSPL. A. Images acquired at 5min, 45 min and 24 hr after polymer injection. Images a, b, and c are of a mouse pretargeted with bsMAb, while images e, f, and g are of the control mouse. Panels d and h are the gamma images and the corresponding digital photographs of the excised lungs for the experimental and control mouse respectively.
Figure 20B. Anteroposterior gamma images of different mice pretargeted with bsMAb (a, b, c and d) and control mouse (e, f, g, and h) at 8 and 21 hr. Images of the same mice after excision of the lungs are shown in c and g. Image d and h are the digital photographs of the excised lungs respectively. White arrows point to metastatic melanoma lesion activities; green arrows point to the liver activity and blue arrows point to the kidney activity.
Figure 21.A. Transverse CT and SPECT images at 1 and 3 hr post injection of Tc-99m-DSPL of a mouse pretargeted with bsMAb followed by targeting with Tc-99m-DSPL (A-I) and images of the control mouse injected with Tc-99m-DSPL alone (A-II).

Figure 21. B. Sagittal CT and SPECT images at 3 hr post injection and the digital pictures of the excised lungs at 24 hours. a, b, and c are of a mouse pretargeted with bsMAb followed by targeting with Tc-99m-DSPL and d, e and f are of a mouse injected with Tc-99m-DSPL alone. White arrows indicate metastatic melanoma lesions.
Biodistribution Studies of Lung metastatic model:

Biodistribution of Tc-99m-DSPL in all mice with melanoma metastasized to the lungs determined at 24 h after radiotracer administration in bsMAb pre-treatment group (n=5, horizontal lined bars) or Tc-99m-DSPL control group (n=4, solid bars) and mice with no tumors but pretargeted with bsMAb or anti-DTPA antibody followed by administration of radiolabeled DSPL (n=3 each, vertical lined and dotted bars respectively) is shown in Figure 22. The mean percent injected dose/g (%ID/g) of the whole lungs for the pretargeted group was 3.20 ± 2.06. The %ID/g after normalization for the tumor mass in this group was 24.85 ± 13.53. In the control group with melanoma metastasized to the lungs, receiving only Tc-99m-DSPL, %ID/g in the lungs was 0.40 ± 0.15 or 0.98 ± 0.47 after normalization for tumor mass. In the control groups with no tumors, pretargeted with bsMAb or anti-DTPA MAb, radiotracer accumulation in the lungs was 0.15 ± 0.01 and 0.066 ± 0.089 % ID/g respectively. Radiotracer accumulation in the tumors of bsMAb pretargeted group was significantly greater than all the control groups (p = 0.01, ≤ 0.001 or = 0.032) prior to or after normalization for the tumor mass in the lungs respectively. The control groups without tumors showed little or no accumulation of circulating bsMAb or radiotracer in the heart or the liver at 24 hours. There was no statistical difference in other organs in all the groups. Target to background ratio (T: B) for the bsMAb pretreatment group was 5.25:1 for the Lung to Liver ratio (not normalized), or 41.30:1 after normalization for the tumor mass.
Figure 22. Biodistribution of Tc-99m-DSPL in mice with melanoma metastasized to the lungs pretargeted with bsMAb followed by targeting with Tc-99m-DSPL (horizontal lined bars, n=5), injected with Tc-99m-DSPL alone (solid bars, n=4), normal C57BL6 mice pretargeted with bsMAb followed by Tc-99m-DSPL (vertical lined bars, n=3) and normal SCID mice pretargeted with anti-DTPA MAb followed by Tc-99m or In-111-DSPL (Dotted Bar, n=3). * and † indicate the p values equal to 0.01 and equal to 0.001 respectively.

In vivo imaging of human mammary carcinoma xenografts:

Mice carrying both HER2/neu positive human breast tumor (BT-474, right arm pit) and HER2/neu negative human mammary adenocarcinoma (BT-20, left arm pit) were used for the study. Approximately 37 MBq of Tc-99m-DSPL was injected via tail vein in all mice 8 hrs post administration of 10 µg of BAAC or 10µg of anti-DTPA Fab. At least 2 mice in each group were imaged. Planar gamma images of mice in the postero-anterior views were
acquired at 0 hr, 1 hr, 3 hr, and 24 hr post injection of Tc-99m-DSPL. Figure 23B is the 24 hr planar gamma image of a mouse pretargeted with BAAC followed by targeting with Tc99m-DSPL. The corresponding necropsy photograph showing the locations of the tumors in shown in Figure 23A. Figure 23C is the 24 hr planar gamma image of another mouse injected with Tc-99m-DSPL alone after pretargeting with anti-DTPA Fab. Figure 23D shows the necropsy digital photograph of the mouse control mouse. Unequivocal visualization of HER2/neu positive BT-474 tumor was seen by 24 hrs in the mouse pretargeted with BAAC, while no accumulation of Tc-99m-DSPL was seen in control group or in HER2/neu negative BT-20 tumors in all mice whether pretargeted with BAAC or not.

Figure 23. Planar gamma images from studies showing antero-posterior images of a mouse with BT-474 tumor (right arm pit) and BT-20 tumor (left arm pit) pretargeted with BAAC (B) and control mouse pretargeted with anti-DTPA Fab (C) followed by i.v. injection of Tc-99m-DSPL. Images 23A and 23D are necropsy photographs showing the location of the tumors for mice pretargeted with BAAC and anti-DTPA Fab respectively.

Biodistribution in xenografted SCID mice:

Biodistribution of Tc-99m-DSPL in all mice with human breast carcinomas (BT-474 and BT-20) determined at 24 h after radiotracer administration in BAAC pre-treatment group (n = 4, closed bars) or anti-DTPA Fab pretreatment control group (n = 4, open bars) is shown in Figure 24. The mean percent injected dose/g (%ID/g) of the BT-474 tumor for the BAAC
pretargeted group was 5.274 ± 1.32. In the control group pretargeted with anti-DTPA Fab, %ID/g in the BT-474 tumor was 0.344 ± 0.161. Radiotracer accumulation in the BT-474 tumors of BAAC pretargeted group was significantly greater than that of the control group pretreated with anti-DTPA Fab (p ≤ 0.001). Radiotracer accumulation in BT-20 tumor for mice pretargeted with BAAC was 0.315 ± 0.109 % ID/g and the control mice pretreated with anti-DTPA Fab was 0.303 ± 0.087 % ID/g. There was no significant difference of radiotracer accumulation in the BT-20 tumors between both the groups (p = NS).

**Figure 24:** Biodistribution of Tc-99m-DSPL in mice with BT-474 and BT-20 xenograft tumors pretargeted with BAAC followed by targeting with Tc-99m-DSPL (Closed bars, n = 4), pretargeted with anti-DTPA Fab followed by targeting with Tc-99m-DSPL (Open bars, n = 4). * indicates p values less than 0.001.
Therapeutic efficacy in human breast cancer xenografts:

In vitro data showed higher tumor toxicity in BT-474 cells when the cells were pretargeted with BAAC and targeted with D-Dox-PGA. Similarly, the breast cancer xenograft model described for the imaging protocol was also used for in vivo therapy efficacy assessment. Breast cancer xenografted mice were pretargeted with 20 µg of BAAC per mice and targeted with 7.5 mg/kg body weight of D-Dox-PGA (Dox Equivalent) (n =4). Control groups included treatment with either Dox Alone (7.5 mg/kg, n =4), D-Dox-PGA (7.5 mg/kg body weight, Dox Equivalent n =3) and tumors pretargeted with anti-HER2/neu affibody only (20 µg) followed by treatment with D-Dox-PGA (7.5 mg/kg body weight of Dox Equivalent n =3). Higher therapeutic dose of BAAC for pretargeting than the imaging dose was used in order to determine if better tumor regression can be achieved. Also, D-Dox-PGA has only one DTPA molecule per polymer as compared to Tc99m-DSPL with at least 50 DTPA molecules per polymer. Pretargeting time of 8 hrs which was used for imaging studies was also used for therapeutic protocol. A therapeutic dose of 7.5 mg/kg of doxorubicin which is the maximum tolerated dose (MTD) in SCID mice was used. Changes in tumor volume and body weight were monitored for the entire length of the study. At Day 0, mice were pretargeted with BAAC or anti-HER/neu affibody and 8 hrs later D-Dox-PGA was administered or in case of control groups Dox alone. At Day 14 and Day 28, a second and a third dose of either BAAC or anti-HER/neu affibody were injected followed by treatment with D-Dox-PGA or Dox alone. Mice with tumor volumes of approximately 40-50 mg were used for the studies.

Figure 25 shows therapeutic efficacy in HER2/neu overexpressing human breast cancer (BT-474) tumors, while Figure 26 shows therapeutic efficacy in HER2/neu non-
expressing human mammary adenocarcinoma (BT-20) tumors in the same mice. Mice treated with D-Dox-PGA alone or anti-HER2/neu alone pretargeted followed by treatment with D-Dox-PGA showed exponential increase in tumor volumes of both BT-474 tumor (Figure 25) and BT-20 tumor (Figure 26). In mice pretargeted with BAAC followed by targeting with D-Dox-PGA administration or Dox alone treatment showed decrease in the tumor volumes after 3 days of injection (Figure 25). At about Day 12 there as a slight increase tumor volume in both BAAC pretargeted group and Dox treatment group. Similar pattern was seen when second dose was injected at Day 14 of the study. However, in human mammary adenocarcinoma BT-20, increase in tumor volumes was seen in mice pretargeted with BAAC. Mice receiving Dox alone showed decrease in tumor volume of BT-20 tumors following a similar pattern to BT-474 tumor response (Figure 26).
Figure 25. Therapeutic efficacy in SCID-SHO mice with HER2/neu overexpressing (BT-474) tumor either pretargeted with BAAC followed by specific targeting with D-Dox-PGA (n = 4), control group pretargeted anti-HER2/neu affibody followed by administration of D-Dox-PGA (n = 3), control group administered with D-Dox-PGA alone (n = 3), and Dox alone treatment group (n = 4). Arrows indicate days at which dose were injected in all the groups. Data is expressed in fraction change of tumor volume (Mean tumor volume from each group with ± SD) in order to normalize mice with different tumor mass.
Figure 26. Therapeutic efficacy in SCID-SHO mice with non-expressing HER2/neu human mammary adenocarcinoma (BT-20) tumor either pretargeted with BAAC followed by specific targeting with D-Dox-PGA (n = 4), control group pretargeted anti-HER2/neu affibody followed by administration of D-Dox-PGA (n = 3), control group administered with D-Dox-PGA alone (n = 3), and Dox alone treatment group (n = 4). Arrows indicate days at which dose were injected in all the groups. Data is expressed in fraction change of tumor volume (Mean tumor volume from each group with ± SD) in order to normalize mice with different tumor mass.

Figure 27 shows the survival rate of different groups of breast cancer xenografted mice after receiving treatment. All mice receiving Dox alone treatment died by day 21 of the
study. Dox treated mice received a total dose of 15mg/kg/mouse by Day 14. The death in this group is most probably associated with cardiotoxicity. Furthermore, this group of mice treated with Dox showed approximately 15-20% decrease in total body weight by the time of death (Figure 28). In mice pretargeted with BAAC and targeted with D-Dox-PGA only one mouse died at Day 31 of the study after receiving the 3rd dose of D-Dox-PGA. This may be associated with anesthesia used to enable i.v. injection of BAAC and D-Dox-PGA and not due to cardiotoxicity since there was no loss of total body weights of mice in this therapy group. No mice died in the group pretargeted with anti-HER2/neu affibody and targeted with D-Dox-PGA. In the group of mice treated with D-Dox-PGA alone, one mouse died at Day 12 of the study. Since there was also almost no change in the body weight, death of this mice may have been also due to over-anesthesia administration. Mice treated with BAAC and D-Dox-PGA, D-Dox-PGA alone or anti-HER2/neu and-Dox-PGA maintained the body weight for the duration of the study unlike the Dox treated group which loss about 15% of the total body weight in 12 days (Figure 28). The data indicate that D-Dox-PGA is non-toxic in vivo even at a cumulative Dox equivalent dose of 22.5 mg/kg.
Figure 27: Survival rate curve of SCID-SHO mice with BT-474 and BT-20 tumors either pretargeted with BAAC followed by specific targeting with D-Dox-PGA (n = 4), control group pretargeted anti-HER2/neu affibody followed by administration of D-Dox-PGA (n = 3), control group administered with D-Dox-PGA alone (n = 3), and Dox alone treatment group (n = 4). Arrows indicate days at which dose were injected in all the groups. Arrows indicate days at which dose were injected in all the groups. Data is expressed in % Survival Rate.
Figure 28: Change in body weight of SCID-SHO mice with BT-474 and BT-20 tumors either pretargeted with BAAC followed by specific targeting with D-Dox-PGA (blue diamonds n=5), control group pretargeted anti-HER2/neu affibody followed by administration of D-Dox-PGA (purple squares, n =3), control group administered with D-Dox-PGA alone (green triangles, n = 3), and Dox alone treatment group (red circles, n = 5). Arrows indicate days at which therapeutic doses were injected in all the groups. Data is expressed as fraction of the body weight at the start of therapy (Mean body weight in gms from each group with ± SD).
Discussion:

After intravenous administration of monoclonal antibodies in patients, only a very small fraction of the injected dose is localized at the tumor sites (54). The small amount of antibodies is not quite sufficient to allow successful imaging of small lesions by the existing conventional radiological techniques and is certainly not sufficient to deliver tumoricidal concentrations of cytotoxic agents to tumors without causing damage to normal tissues and organs such as the bone marrow, liver, and kidneys (54).

When tumor uptake is limiting, the therapeutic dose required to achieve unequivocal therapeutic benefit cannot be administered because of toxicity to normal tissues (54). In radiation therapy, the slow plasma clearance of radiolabeled antibodies result in prolonged exposure of highly radiosensitive bone marrow to ionizing radiation. Despite the critical clinical need and a large potential market for efficient therapeutic drugs to treat small metastatic lesions and solid tumors, the goal remained elusive.

This challenge has led to a number of approaches to improving tumor to non-tumor radiation dose ratios to a level that will allow effective treatment of the tumor while sparing normal tissues. The tumor-to-nontumor dose ratio can be improved by a two-step targeting approach of pretargeting, which essentially separates the delivery of the radioactive isotope from that of the antibody (55). In this approach, a non-radiolabeled antibody (bsMAb) modified with a tag (MAb, Fab, or targeting ligands) is injected and allowed to reach maximum tumor uptake and sufficient clearance from blood. The clearance from the blood may be modified by using smaller molecular weight bsMAb complexes. Then, a relatively small and fast-clearing molecule carrying a radionuclide may be administered that quickly binds to the bsMAb localized at the tumor at the same time clear rapidly from the body.
Pretargeting approach combines the excellent tumor seeking properties of antibodies with the fast distribution and clearance of low molecular weight probe. This approach has allowed substantially higher tumor-to non-tumor ratios of radioactivity than those achievable employing the standard one-step radio-immunotherapeutic approach (55).

Bispecific antibody complexes are ideal for the pre-targeting approach. Bispecific antibodies can be made either by chemical cross-linking, quadroma technology (fusion of hybridoma cells with two different specificites) or via a recombinant technology (56). The use of appropriate technology for the production of bispecific antibodies depends on one’s needs. For a commercial scale production quadroma (fusion of hybridoma cells) or recombinant technology would be ideal. For research use applications, chemical conjugation approach may suffice. We have employed the chemical cross-linking method to prepare or bispecific antibodies as it is a fast approach and avoids several challenges of post bispecific antibody complex purification strategies. Quadroma technology produces various combinations of the light and heavy chains of the two hybridomas. The isolation and purification of the bispecific antibody from quadromas thus become highly complicated and inefficient. Genetic engineering approach may be the way of the future for bispecific antibody or antibody complex production.

Our bispecific antibody complexes (bsMAb or BAAC) are made via the use of thioether bonds that has the highest stability relative to the amide or disulfide linkages (57). Modification of individual MAb’s or recombinant proteins with heterobifunctional cross-linkers could possibly lead to reduction in immunoreactivity. However, with care, immunoreactivity may be maintained within nano molar affinity range after modification with hetero bifunctional cross-linkers such as 2-Iminothiolane or N-hydroxy succinimide
ester of bromoacetic acid (Figure 4). Purification of BsMAb or BAAC was achieved using size exclusion chromatography. Size exclusion chromatography of bsMAb (2C5 MAb crosslinked anti-DTPA MAb, 300 kDa) yielded 10-15 % dimeric bsMAb, and 20-30% polymeric bsMAb. Close to 50% of the total MAb used in the reaction was recovered as unconjugated monomeric antibodies (Figure 10A). The lower yield of bsMAb may be due to several reasons such as the availability of lysine residues of the MAbs for modification, the concentration of the reaction mixtures for crosslinking (1 mg/ml) as well as the size of the reactants. Cross-linking of BAAC where smaller anti-HER2/neu affibody was crosslinked to anti-DTPA Fab, (60 kDa) resulted in higher yields than preparation of bsMAb where intact antibody of one MAb is linked to the intact antibody of another. Using smaller molecular components, almost 100% crosslinking of the affibody to the Fab was achieved. No free anti-DTPA Fab was evident in the SDS-PAGE analysis (Figure 9). In addition the free sulphhydryl group on anti-HER2/neu affibody allows efficient site specific cross-linking since minimal steric hindrance at the sulphhydryl group is expected.

**Secondary polymer based probes to capture bispecific antibody:**

Higher affinity of the antibody for the hapten is an important requirement in the pretargeting approach because it would enhance noncovalent association between the antibody and hapten. The higher affinity of the antibody for the hapten would reduce the amount of antibody required to capture the hapten at the target site (4). Since the binding constants of antibodies are in the range of $10^{-10}$ M to $10^{-9}$ M (4), additional improvements coming from binding constants would have to rely on other existing noncovalent systems that display affinities much higher than $10^{-10}$ M (4). The amount of macromolecule available for capturing hapten at the target site is usually very small (in the order of nmol/gram tumor).
Consequently, the amount of hapten deliverable to the target is quite limited (4). With a hapten labeled at a higher specific activity, increased radiation dose can be imparted per unit mass to effect easier detection of the target, thereby improving the pretargeting efficacy. The availability of a suitable hapten-chelate for efficient labeling and the resultant formation of a stable in vivo complex that exhibits rapid diffusion, little uptake in nontarget tissues, and fast clearance from the circulation would be ideal for detection of small lesions in vivo (4, 24, 40). In such a case biodegradable polymers would best suit the requirement. We have designed such a polymer based system with very high specific activity and stability. The polymer that would be captured by the bsMAb or BAAC has repetitive lysine units and were easily modified with multiple DTPA molecules per mole of the polymer. Having multiple DTPA molecules per mole of the polymer permits labeling with radionuclide at very high specific radioactivity when using Tc-99m or In-111. In addition, the polymers were modified to be completely negative in charge so that ionic repulsion between the negatively-charged radiolabeled polymer and the negatively-charged cell surfaces and ground substances would keep non-specific interaction at a minimum. The molecular weight of the polymer used for our investigation is also small (14.6 kDa). The small size enabled fast clearance from the circulation by renal excretion. Another aspect of designing such as polymer based hapten system is that radionuclides may be replaced with fluorescein, rhodamine dyes or other fluorophores for in vitro cell binding assays as well as in vivo fluorescent imaging.

The fluorescein based polymers (FITC-DSPL) were used to screen bsMAb or BAAC pretargeted on tumor cells in vitro via flow cytometry or epi-fluorescence microscopy. Cultured B16F10 cells were pretargeted with several concentration of bsMAb followed by
specific targeting with FITC-DSPL and was compared to directly labeled Oregon green-2C5 MAb in B16-F10 cells. Flow cytometry analyses showed greater signal in bsMAb pretargeted B16F10 cells than those cells treated with Oregon green labeled MAb (Figure 7). Similar, signal amplification was obtained with HER2/neu overexpressing BT-474 cells pretargeted with BAAC, and targeted with FITC-DSPL (Figure 11). The in vitro binding assays show immense potential for tumor biomarker detection at the picogram levels.

For in vivo application, two important parameters are needed for unequivocal visualization of small lesions. First; high specific activity per mole polymer is needed. The specific radioactivity of the radiolabeled polymer used in our investigation is 20.90 MBq/µg of polymer or 0.0304 mol of $^{99m}\text{Tc}$ per mol of polymer. The radiospecific activity of the polymer used is 7.5 times greater than that of mono or divalent haptens (0.00367 $^{99m}\text{Tc}$ per mol of bivalent hapten IMP-192) (58, 59). Also, the specific radioactivity of our polymer is approximately 100,000 times higher than that obtained with $^{99m}\text{Tc}$-Glucarate Kit (9.7E-07 mol $^{99m}\text{Tc}$ per mol) or $^{99m}\text{Tc}$-Sestamibi Kit (5.9E-04 $^{99m}\text{Tc}$ per mol) (60, 61).

Second; the rapid clearance from the circulation and the body is needed to best suit the pretargeting strategy. Polymers of approximately 14.6 kDa molecular weight are used. Even though the starting polymers were polylysine, the final form of the polymers is rendered completely negatively charged by succinylation of residual free lysine residues. Polymer clearance ($\text{Tc-99m-DSPL}$) in vivo in C57/BL6 and SCID-SHO mice was characterized by Vishwesh Patil, PhD Candidate, Northeastern University. Briefly, after radiotracer injection in mice (C57/BL6 and SCID mice), planar images were acquired at 5 min, 45 min, 180 min, and 24hr time point. Each image was analyzed using ImageJ program from NIH. After 24 hours, the biodistribution studies showed maximum accumulation in the
kidneys (4.28 ± 0.33 % ID/gm). The heart (0.25 ± 0.02 %ID/gm) and blood (0.07±0.015 %ID/gm) showed minimum activity. Minimal accumulation was also seen in the liver. Most of the polymers cleared as early as 3 hours. By the end of 24 hrs no residual polymers were seen in the liver or kidneys. Some activity was still associated the bladder. Characterization of Tc-99m-DSPL in vivo helped in the development of a complete system for diagnostic imaging of small lesions in vivo.

Pretargeting as described above would be the best system for unequivocal visualization of small lesions in vivo, but further investigation is needed to estimate the appropriate wait period before radiotracer (Tc-99m-DSPL) administration. Estimation of the pretargeting wait period for bsMAb (300kDa) was done by analyzing 200 kDa bsMAb clearance as described by Khaw et al. (24). Clearance of the smaller bispecific antibody complexes (BAAC) was performed in SCID-SHO mice. Molecular weight of BAAC (60kDa) is at around the threshold of efficient clearance via renal elimination. Therefore hepatic clearance would facilitate total in vivo elimination of BAAC (62). Biodistribution at 8 hours after i.v. injection of BAAC directly labeled with Tc99m-DTPA showed maximum accumulation of radioactivity was seen in liver (22 ± 2.53 %ID/gm) and kidneys (7.90 ±1.675 % ID/g). The heart and blood showed minimum activity. Hence the 8 hours wait time was selected to ensure clearance of BAAC from the circulation (Figure 19).

Since early stage detection of small metastatic lesions would be crucial in cancer diagnosis and therapy, if higher specific radioactivity probes can be targeted, smaller lesions are expected to be detectable even if the resolution of the gamma cameras used are not optimal. PET and SPECT imaging of human colon cancer pretargeted with bsMAb and targeted with haptens radiolabeled with different radioisotopes have enabled visualization of
metastatic lesions in the lungs (63). The lesions were histological determined to be less than 0.3 mm in diameter, even though correlation of the lesions in the necropsied-lungs to the in vivo or ex vivo images was not reported (63). Unequivocal visualization of small cancer lesions still remains problematic due to low target radio-specific activity as well as the limitations of the camera technology. Current study with high resolution small animal gamma camera and the BIOSCAN SPECT/CT images confirmed that small metastatic lesions can be imaged as early as 1 hr post injection with the use of the pretargeting strategy (Figures 20A and 21A). Ex vivo gamma image of the excised lungs at the end of 24 hr in the pretargeted mouse showed that the radioactivity was localized to the lungs and was not due to residual blood pool activity (Figure 20B). Images taken with BIOSCAN SPECT/CT also showed localization of the radioactivity in the lesions in the pretargeted group after 1hr (Figure 21A). Transverse views of the SPECT images at 1 hr and 3 hr and the corresponding CT images confirmed that the activity was localized in the lesions. Furthermore, biodistribution data for the same animals correlated with the acquired gamma images. Radiotracer accumulation in the tumors of bsMAB pretargeted group was significantly greater than all the control groups (p = 0.01, ≤ 0.001 or = 0.032) prior to or after normalization for the tumor mass in the lungs respectively (Figure 22). The control groups without tumors showed little or no accumulation of circulating bsMAB or radiotracer in the heart or the liver at 24 hours. There was no statistical difference in other organs in all the groups. Target to background ratio (T: B) for the bsMAB pretreatment group was 5.25:1 for the Lung to Liver ratio (not normalized), or 41.30:1 after normalization for the tumor mass.

In addition we used 10µg bispecific antibody dose whereas in the mono and divalent haptens studies, the bispecific antibody dose was 15-16 µg/mouse (64, 65). Whether the
bsMAb pretargeting dose can be further decreased with the polymer targeting approach cannot be determined from the current studies. Although, optimal bsMAb pretargeting strategies such as dose of bsMAb, pretargeting time before the radiolabeled polymer can be injected were not optimized for this study as in (65), SPECT images (Figure 20, and 21) and biodistribution data (Figure 22) suggested that 24 hr pretargeting time was sufficient for the current study.

Detection of small lesions (3-4 mm) in vivo was further demonstrated in human breast cancer xenograft model. Dual xenograft model with HER2/neu overexpressing human breast (BT-474) tumor and HER2/neu negative human mammary adenocarcinoma (BT-20) on the same mouse was used in order to demonstrate specific targeting of small lesions. BAAC dose administered for pretargeting was again 10µg/mouse. Pretargeting wait period was 8 hrs as determined above. In vivo planar gamma images showed unequivocal visualization of tumor lesions by 24 hrs only in BT-474 human mammary xenografts (Figure 23). The 24 hr planar gamma images showed some billiary radiotracer activity but almost no activity was associated with blood or heart regions. Biodistribution data from the same animals correlated with the gamma images with almost 5.274 ± 1.32 % ID/g of Tc-99m-DSPL in BT-474 tumor pretreated with BAAC. A tumor: blood ratio of 14:1 was achieved. Radiotracer accumulation in the BT-474 tumors of BAAC pretargeted group was significantly greater than all the controls pretreated with anti-DTPA Fab (p ≤ 0.001). Radiotracer accumulation in BT-20 tumor for mice pretargeted with BAAC was 0.315 ± 0.109 % ID/g and for the mice pretreated with anti-DTPA Fab was 0.303 ± 0.087 % ID/g (Figure 24).
Additional investigation was undertaken to determine whether therapeutic benefits could be achieved by replacing radionuclides on the polymers with chemotherapeutic drugs. It is known that chemotherapeutic drugs modified on carriers led to the reduction of non-target cytotoxicity. Whether this toxicity reduced polymer-drug conjugates can impart enhanced toxicity to cancer lesions after pretargeting was investigated. Chemotherapeutic agents used currently in clinical practice are cytotoxic to all dividing cells. They are nevertheless used for cancer therapy because cancer cells have higher rate of replication resulting in differential cell attrition. Doxorubicin, the front-line chemotherapeutic agents for cancer therapy, is limited by its cardio-toxicity at the optimal therapeutic dose (25). Additional non-target toxicities include myelosuppression, nephrotoxicity and hepatotoxicity (6, 7). Doxorubicin was chosen for further evaluation as it has various non-target effects at its MTD. Several hypotheses have been proposed to explain doxorubicin cardiotoxicity. The hypotheses of the mechanisms of action of Dox include free radical formation, metabolite and calcium overload. However, none adequately explains the mechanism of irreversible cardiomyopathy.

Nevertheless, doxorubicin has been a favorite chemotherapeutic agent for developing strategies directed at changing the therapeutic index. Not only has its activity against various solid tumors been an attractive feature, many studies have pursued the mechanisms of action and differential effects on the heart relative to other tissues and tumors. Improvements in the therapeutic index of doxorubicin by liposomal encapsulation were developed about two decades ago (66). It has also been linked to monoclonal antibodies with highly impressive activity in animal tumors (66), but clinical experience remains unavailable. This drug has also been given by intra-arterial route and hepatic perfusion followed by extracorporeal
filtration. Such liposomal preparations have resulted in the treatment of Kaposi’s sarcoma (66), a tumor characterized by very abnormal vasculature and anthracycline sensitivity. Other methods of increasing the therapeutic index of anthracyclines are still under investigation.

Our goal was to specifically target cancer cells with polymer loaded doxorubicin (D-Dox-PGA) via the pretargeting strategy using bsMAb or BAAC and reduce the cardiotoxicity associated with doxorubicin. PGA was chosen as the polymer for conjugation because of biodegradable and hydrophilic properties. It has also been reported that hydrophobic or partially hydrophobic drugs attached to these polymers are rendered more soluble for drug delivery and increased bioavailability (67). PGA also possess a net negative charge which will be repelled by the negatively-charged cell surfaces and ground substances (24, 40) leading to decreased background activity and increased bioavailability for tumor targeting. Doxorubicin was loaded onto PGA via amide bong using EDC chemistry. Twenty-four to 36 moles doxorubicin per mole polymer polymer-drug conjugates have been made in our laboratory. Each PGA has an N-terminal modified DTPA permitting site-specific capture by bsMAb or BAAC. Poly glutamic acid of a mean molecular weight of 13.3 kDa, was used simulate in vivo properties of Tc99m-DSPL that would allow rapid clearance from the body via renal excretion. D-Dox-PGA complex formed was stable in 0.1 M PBS in vitro. Release of doxorubicin from D-Dox-PGA was obtained in an acidic (pH 4) or basic (pH 9.6) solutions (Savitri Mandapati, MS Thesis, Northeastern University). Only less than 10% dissociation of Dox was observed at pH 7.4 after 24 hours incubation at 37°C. However, for in vivo delivery, we propose that the internalized D-Dox-PGA would be digested by the
lysozymes and release free Dox intra-tumurally leading to highly targeted and localized tumor cell killing.

Rat embryonic cardiocytes when treated with several different concentration of either D-Dox-PGA complex or Dox alone in culture, very minimal cell killing was observed with D-Dox-PGA. IC$_{50}$ value for D-Dox-PGA was found to be 15.75 µg/ml while that of free doxorubicin was 1.20 µg/ml. D-Dox-PGA complex was found to be at least 13 fold less cardiotoxic in tissue cultures (Figure 15). Human breast cancer cells when pretargeted with BAAC followed by targeting of D-Dox-PGA resulted in significantly (p < 0.05) higher tumor cell killing relative to free doxorubicin treatment in vitro (Figure 16, 17).

Furthermore, in vivo therapeutic efficacy was assessed by the pretargeting approach in human breast carcinoma xenografts bearing SCID mice pretargeted with BAAC followed by targeting with D-Dox-PGA or Dox alone at doxorubicin equivalent concentration of 7.5 mg/kg body weight. When HER2/neu positive breast tumor (BT-474) were pretargeted with BAAC followed by targeting with D-Dox-PGA almost no tumor growth was observed compared to controls treated with D-Dox-PGA (Figure 25). In mice with free doxorubicin treatment tumor size reduction was similar to the pretargeted therapy. However, free Dox mice has a 15-20 % loss in total body weight and all mice died by the end of day 21 of the study after receiving a second 7.5 mg/kg dose at a total Dox dose of 15 mg/kg. Higher BAAC (20 µg) dose was used for therapy relative to the imaging dose of 10 µg to determine whether tumor regression can be achieved with D-Dox-PGA. No therapeutic effect was seen in the HER2/neu negative human mammary adenocarcinoma (BT-20) xenografts in BAAC/D-Dox-PGA treated mice. In mice treated with D-Dox-PGA alone or anti-HER2/neu affibody pretreated mice followed by treatment with D-Dox-PGA, no therapeutic effects
were seen and the tumor volume increased exponentially. The mice in these two groups did not show loss of total body weight. Loss in body weight was associated with free doxorubicin treatment. Deaths associated with free Dox therapy may be due to cardiotoxicity. The experiment with each mouse was terminated when the total tumor mass of 1-1.5 g were reached as per protocol restrictions. Data also suggest that the D-Dox-PGA used for therapeutic outcome (7.5 mg/kg body weight) is not at its MTD and also might not be enough for complete tumor remission because of faster clearance from the body. Further investigation is required to understand the role of cardiotoxicity and other systemic toxicity in the group of mice treated with doxorubicin alone as compared to mice treated with D-Dox-PGA.
Summary and Conclusions:

Highly specific targeting modality was developed for diagnostic imaging of small lesions in vivo. This pretargeting and targeting approach was modified for enhanced targeted therapy using xenograft murine model. Proof of concept for the ability to detect small metastatic lesions or solid tumors in vivo was demonstrated. Preliminary therapeutic efficacy studies in mice with xenografts also showed promise for future development of pretargeted polymer-drug-conjugate therapy that might result incomplete tumor regression. Additional evaluation is needed to ensure D-Dox-PGA to have no toxicity to normal tissues and organs especially reduced cardiotoxicity. Optimization of dose and treatment schedule needs to be investigated.

My thesis project has shown that small lesion can be images and that therapy with polymer-drug conjugates may have potential benefits towards development of translational therapeutic applications that may ultimately benefit cancer patients.
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Appendix 1: Radiation Safety Training.

**Refresher Radiation Training for SEALED sources (e.g. Co-57, Ru-106, PT-197):**

Northeastern University Office of Environmental Health & Safety

On March 12, 2010, Keyur Gada successfully completed 46: "Refresher Radiation Training for SEALED sources (e.g. Co-57, Ru-106, PT-197)" program on the web, answering 90% of the 10 questions correctly

(At least a 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note: Record Locator: tw0000000009730 (Session).

**Refresher Radiation Training for UNSEALED sources (e.g. H-3, C-14, P-32, S-35, IN-111):**

Northeastern University Office of Environmental Health & Safety

On March 12, 2010, Keyur Gada successfully completed 47: "Refresher Radiation Training for UNSEALED sources (e.g. H-3, C-14, P-32, S-35, IN-111)" program on the web, answering 90% of the 10 questions correctly

(At least a 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note: Record Locator: tw0000000009727 (Session).
Appendix 2: Occupational Health & Safety for Working with Animals in Research and Teaching.

In May, 2009, Keyur Gada completed the following requirements for working with Animals in Research and Teaching:

- Participated in the training program.
- Is added to the research protocol.
- Enrolled in the Occupational Health and Safety Program.
- Received Animal Facility Orientation from DLAM Staff.

For any questions, contact Sean Sullivan, Director of NU-IACUC & DLAM at (617)373-3958 or s.sullivan@neu.edu
Appendix 3: Laboratory Safety Training

Chemical Hygiene Training Part 1 - Right To Know.

Northeastern University Office of Environmental Health & Safety

On October 28, 2009, Keyur Gada successfully completed 01: "Chemical Hygiene Training Part 1 - Right To Know" program on the web, answering 100% of the 15 questions correctly (At least a 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note: To complete this training you must also take Part 2

Record Locator: tw0000000008094 (Session).

Chemical Hygiene Training Part 2 – Lab Safety and Hazardous Waste Management.

Northeastern University Office of Environmental Health & Safety

On October 28, 2009, Keyur Gada successfully completed 02: "Chemical Hygiene Training Part 2 – Lab Safety and Hazardous Waste Management" program on the web, answering 90% of the 10 questions correctly (At least a 80% score is required to pass).

For any questions, contact EHS at (617)373-2769 or ehs@neu.edu

Note: Record Locator: tw0000000008096 (Session).