Targeted *In Vitro* and *In Vivo* Delivery of Diagnostic and Chemotherapeutic Agents to Prostate Cancer Cells Pre-Targeted with Bispecific Antibody-Ligand Complexes.

Thesis Presented

By

Vishwesh Patil

To

The Bouvé Graduate School of Health Sciences in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Pharmaceutical Sciences with Specialization in Pharmaceutics and Drug Delivery Systems.

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ABSTRACT

Current standard cancer therapies include surgery, chemotherapy and radiation therapy. Although surgery is generally effective in the treatment of primary cancerous lesions, surgical resection of malignant tumors may not result in complete cure. By the time a tumor is detected by the existing diagnostic methods, it can be assumed that metastases may already have occurred and spread. These metastatic sites are not identified at such early stages by existing methods such as X-ray, ultrasound, Computed Tomography (CT) scans or even Nuclear Magnetic Resonance Imaging (MRI) because of poor limit of detection. Small cancerous lesions are difficult to detect in vivo due to high background activity, low target activity and contrast, as well as limited specificity of the targeting agents. Furthermore, chemotherapeutic agents lack specificity since normal cells are often also targeted by most of the chemotherapeutic agents. Radiation therapy has also been used to target cancer cells because cancer cells are less able to repair themselves after treatment with radiation. However, radiation cannot be used to treat many cancers because of the damage done to normal cells that surround the cancerous tissue. Thus, the development of combined and improved methods for the diagnosis and treatment of small cancerous lesions is needed.

To overcome the above problems, our objective is to develop diagnostic reagents that have high specific radioactivity, targeting ability and decreased non-target organ activity. This should allow the diagnosis of even very small lesions by delivering high specific radioactivity while keeping the background activity low. In addition, these targeted diagnostic agents may be modified to carry chemotherapeutic agents instead of radioisotopes.
When chemotherapeutic drugs are covalently conjugated to the carriers, the resulting drug-conjugates become pro-drug carriers that are expected to be less toxic to normal cells. To enable specific targeting of these pro-drug carriers, we proposed a pre-targeting approach with bispecific antibodies. A Bispecific Antibody-Ligand Complex (BALC) was constructed via conjugation of a peptide or peptide analog specific for a receptor to a monoclonal antibody or antibody fragment that can specifically capture an effector compound. We used a two-step approach using: i) Bombesin-Anti-DTPA antibody bispecific complex (Bom-bsCx) or Bombesin-Anti-DTPA antibody Fab' bispecific complex (Bom-bsFCx) for pre-targeting in step 1; and ii) targeted delivery of either Tc-99m/In-111-loaded polylysine (PL) polymers for diagnosis or Doxorubicin (Dox)-loaded polyglutamic acid polymers (D-Dox-PGA) for therapy in step 2. For diagnosis, specific tumor targeting and reduction of non-specific background activity were demonstrated by an in vivo prostate cancer xenograft model. Additionally for therapy, multiple Dox molecules attached to polymers are demonstrated to be less cytotoxic to embryonic cardiocytes relative to free Dox. However, targeting D-Dox-PGA polymers via Bom-bsCx led to enhanced prostate cancer toxicity.
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<th>Definition</th>
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<tr>
<td>ADC</td>
<td>Antibody-Drug Conjugate.</td>
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<td>BALC</td>
<td>Bispecific Antibody-Ligand Complex.</td>
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<td>BB1</td>
<td>Neuromedin B receptor of the Bombesin receptor family.</td>
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<td>BB2</td>
<td>Gastrin Release Peptide receptor of the Bombesin receptor family.</td>
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<td>BB3</td>
<td>Orphan receptor of the Bombesin receptor family.</td>
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<td>BiMAb</td>
<td>Bispecific Monoclonal Antibody.</td>
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<td>Bn, Bom</td>
<td>Bombesin.</td>
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<td>Bom-bsCx</td>
<td>Bombesin-anti-DTPA antibody bispecific complex.</td>
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<td>Bom-bsFCx</td>
<td>Bombesin-anti-DTPA antibody Fab' bispecific complex.</td>
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<td>BSA</td>
<td>Bovine Serum Albumin.</td>
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<tr>
<td>CDR</td>
<td>Complementarity Determining Region.</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System.</td>
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<tr>
<td>CT</td>
<td>Computed Tomography.</td>
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<td>D-Dox-PGA</td>
<td>Doxorubicin-loaded polyglutamic acid polymer.</td>
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<tr>
<td>Dox</td>
<td>Doxorubicin.</td>
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<tr>
<td>DPL</td>
<td>DTPA-conjugated PolyLysine polymer.</td>
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<tr>
<td>DRS-PL</td>
<td>DTPA-Rhodamine-Succinylated PolyLysine polymer.</td>
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<tr>
<td>DSPL</td>
<td>DTPA-conjugated, Succinylated-PolyLysine polymer.</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylene TetraPentaacetic Acid.</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced Permeability and Retention.</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein IsoThioCyanate.</td>
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<tr>
<td>GI</td>
<td>GastroIntestinal.</td>
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<tr>
<td>GPCR</td>
<td>G Protein-Coupled Receptor.</td>
</tr>
<tr>
<td>GRP</td>
<td>Gastrin Releasing Peptide.</td>
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<tr>
<td>HRP</td>
<td>HorseRadish Peroxidase.</td>
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<tr>
<td>H9C2</td>
<td>Rat embryonic cardiocyte H9C2 cell line.</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody.</td>
</tr>
<tr>
<td>MDR</td>
<td>Multiple Drug Resistance.</td>
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<tr>
<td>MRI</td>
<td>Nuclear Magnetic Resonance Imaging.</td>
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NMB: Neuromedin B.
PC3: Human prostate cancer PC3 cell line.
PDC: Polymer-Drug Conjugate.
PET: Positron Emission Tomography.
PGA: Polyglutamic acid polymer.
SAR: Structure-Activity Relationships.
1. INTRODUCTION:

1.1. STATEMENT OF THE PROBLEM:

Traditional methods of diagnosis are unable to detect and diagnose very small lesions such as metastatic lesions. In addition, traditional methods of therapy are “a shot in the dark” and are unable to treat small metastatic sites.

Current standard cancer therapies include surgery, chemotherapy and radiation therapy. Although surgery may generally be effective in the early treatment of cancer, surgical resection of malignant tumors does not usually result in complete cure due to metastatic growths. By the time a tumor of reasonable size is detected, it may be assumed that metastasis has already occurred. This is due to the inability of current diagnostic methods such as X-ray, ultrasound, CT scans or even MRI to detect small metastatic lesions. The difficulty may be compounded by high background activity, low target activity, and limited specificity of diagnostic reagents currently in use (1).

Therefore, chemo- and radio-therapies are traditionally applied in combination with surgical resection of primary tumors. Furthermore, chemotherapeutic agents may not kill quiescent metastatic cancer cells. In addition, some lesions develop multi-drug resistance due to the expression of a toxin excreting glycoprotein called Glycoprotein P (P-permeability). Glycoprotein P is found in the plasma membrane of tumor cells and is responsible for excreting the toxic chemotherapeutic agents from the cell interior. Due to the expression of a considerable amount of these glycoproteins, also known as “efflux pumps”, tumor cells very quickly excrete the chemotherapeutic substances effectively, developing a phenomenon so called “multiple-drug resistance” (MDR) of cancer cells (1, 2). Another drawback of chemotherapeutic agents is that they lack specificity since normal cells are most often also attacked by chemotherapeutic agents. Radiation therapy is another approach to
target cancer cells since cancer cells are less able to undergo DNA repair after radiation treatment. However, radiation cannot be used to treat many forms of cancer because of the damage done to normal cells in the neighboring of cancerous tissues (1).

1.2. LITERATURE REVIEW:

1.2.1. Need for Improved Methods for the Diagnosis and Treatment of Cancer:

In the first decade of the 20th century, Paul Ehrlich proposed the idea of using biological/chemical agents that possess specific affinity for certain organs, termed “magic bullet” (3). In the early 1970s, the lack of specificity of cancer therapy was a major impediment (4). It was predicted that a major advancement for cancer therapy or diagnosis would occur when a new class of agents with significant specificity for tumor cells would be developed (4, 5). Antibodies provided the initial impetus for specific diagnosis and treatment of cancer but were hampered by residual non-specificity due to the polyclonal nature of the antibodies used. By 1975, advancements in monoclonal antibody (mAb) technology marked the beginning of a new era in cancer therapy (5, 6). It was not until the advent of monoclonal antibodies that the concept of “magic bullets” for the specific targeting of tumors came one step closer to reality (6). Although there was much enthusiasm, skepticism remained (7). The major drawback of monoclonal antibody therapy was the immunogenicity of the mouse proteins used and some non-specific activity as many of the antigens targeted by monoclonal antibodies were embryonic antigens or shared antigens found on cancer cells and normal cells referred to as “tumor-associated antigens” (7, 8). Subsequently, the development of Bispecific Monoclonal Antibody (BiMAb) technology in the 1990s demonstrated the enormous potential of this technology for a wide array of diagnostic and therapeutic uses (7, 9). Targeting with mAbs can be enhanced by a two-step targeting approach in which two
mAbs are linked into a single agent, called a bispecific antibody, or one mAb is linked with a peptide analog (a ligand) specific for a receptor or antigen on the target moiety, called a bispecific antibody-ligand complex. This allowed targeting with one arm and capturing with the other arm the effector compound for diagnosis or therapy (9).

1.2.2. Antibody-Mediated Targeting:

To date, the monoclonal antibody technology invented by Kohler and Milstein has nourished the hope for therapeutic advancements in diseases that cannot be satisfactorily achieved by conventional therapies (10). mAb technology has been useful in both diagnostic and therapeutic applications (4, 5). The development of tumor-selective mAbs became a promising avenue for the targeting and delivery of therapeutic agents to tumors that over-express tumor-associated or specific antigens (5, 6).

The characteristic property of monoclonal antibodies is their specific binding to a particular antigen, which enables them to find their target precisely in vivo while reducing undesirable non-specific activity. These antibodies can be used for the delivery of toxic payloads, act as agonists or antagonists of receptors, or neutralize ligands (10). For therapeutic uses in malignant diseases, reducing the immunogenicity of rodent monoclonal antibodies became essential. Methods to reduce the immunogenicity of murine antibodies for human use include: chimerization; humanization through grafting of Complementarity Determining Regions (CDRs); or using various technologies such as phage display libraries and transgenic mice expressing human antibody repertoires for the recovery of fully humanized antibodies (11-14).

The reduction of the immunogenicity of antibodies led to prolonged half life and enhanced treatment efficacy. Another important modification is to arm humanized
antibodies with cytotoxic agents, such as radioisotopes, bacterial toxins, inflammatory cytokines, chemotherapeutics or prodrugs, also known as Antibody-Drug Conjugates (ADCs). There is a growing number of monoclonal antibodies approved for cancer therapy such as Rituximab, a chimerized antibody (11), Herceptin and Campath-1H, a humanized antibody (12, 13), and of ADCs conjugated to chemotherapeutics (Mylotarg (14)) or radioisotopes (Zevalin (15) and Bexxar (16)). In spite of this progress, the efficacy of monoclonal antibodies for cancer treatment is still limited (17) and needs further improvement.

1.2.3. Receptor-Mediated Targeting:

The discovery of certain tumor types that over-express specific receptors and the discovery of the associated ligands that bind these cell-surface receptors occurred in the mid-1980s (18, 19). Targeting the over-expression of neuropeptide receptors in a variety of tumors such as breast or prostate with neuropeptides for imaging and therapy has since gained popularity (18).

The use of ligands as targeting molecules is desirable because of the ease and cost effectiveness of their synthesis. Ligands can also withstand the harsh chemical conditions necessary for their modification and radiolabeling (20, 21). Ligands are less immunogenic than mAbs and possess better pharmacokinetic profiles such as faster blood clearance, tissue penetration, higher tumor uptake and greater tumor-to-background ratio (21, 22).

Recent clinical and laboratory studies have shown that many endocrine tumors over-express or ectopically express somatostatin receptors. Radiolabeled somatostatin analogs have been developed for imaging as well as for inducing receptor-mediated cytotoxicity (23, 24). In-111-labeled somatostatin analogs are now widely used for imaging neuroendocrine
tumors due to their greater sensitivity relative to conventional non-targeted diagnostic imaging modalities such as CT, MRI or ultrasound (23-27). Despite the success of In-111-labeled somatostatin in imaging endocrine tumors, other tumors such as colon, prostate, pancreatic and lung cancers do not over-express somatostatin receptors. Nevertheless these tumors have been shown to over-express other receptors such as the Gastrin Releasing Peptide (GRP) receptor or Bombesin (Bn) receptors, particularly of the BB2 receptor isoform (27, 28, and 29).

The discovery of these GRP receptors led to the development of radiolabeled analogs of Bn that could be used for the localization of tumors containing Bn receptors or for the development of Bn analogs coupled to cytotoxic agents for bombesin receptor-mediated cytotoxic therapy (30-32). Experiments using Bn analogs with high affinity for receptors coupled to radiolabeled compounds or cytotoxic agents such as Camptothecin, a topoisomerase inhibitor, Doxorubicin analogs, and Paclitaxel have been described (29, 30, and 33). Binding of these cytotoxic analogs to their associated receptors resulted in their internalization within Bn receptor-bearing tumor tissues and led to receptor-mediated tumoral cytotoxicity (30).

1.2.4. Bombesin Analogs as Potential Tumor-Targeting Agents:

The nomenclature of the Bombesin receptor family was derived from the discovery of Bombesin by V. Erspaner and his colleagues. They isolated a tetradecapeptide from the skin of the European frog Bombina bombina (33, 34). The mammalian version of Bombesin receptors consists of three G Protein-Coupled Receptors (GPCRs): Neuromedin B (NMB) receptor (BB1), GRP receptor (BB2) and orphan receptor (BB3) (34). All three receptors are widely distributed in the body, including in the GI tract and CNS. These receptors have
significant effects on normal physiology as well as on pathophysiological conditions (33).

Bombesin peptides, GRP and NMB, show a variety of pharmacological responses (35). GRP stimulates smooth muscle contraction and GI motility. It also stimulates the release of various GI hormones from the stomach, colon and pancreas. It has significant growth effects on both normal tissues and tumors. Its CNS effects include regulation of cardiac rhythm and thermoregulation (36). NMB causes contraction of smooth muscles, has CNS effects, including thermoregulation, and regulates the thyroid, stimulating hormone release (35, 36).

GRP and to a lesser extent NMB affect growth and differentiation of various human tumors, including colon, prostate and breast (37).

Preliminary studies on biological effects on smooth muscle contractions of different Bombesin peptides extracted from frog skins showed significantly different potencies suggesting the existence of more than one subtype of Bn receptors (35). One class of receptors has high affinity for GRP and lower affinity for NMB. Therefore they are designated GRP receptors. Another class of receptors has higher affinity for NMB and lower affinity for GRP. Therefore these receptors are named NMB receptors (38). Initial Structure-Activity Relationships (SAR) studies showed that Trp$^8$ and His$^{12}$ in the -COOH terminus of Bombesin are essential for the biological activity of the peptide (39). Various substitutions with D-amino acids (D-Phe and D-Tyr) for His$^{12}$ in Bombesin analogs resulted in decreased activity. Substituted Bombesin analogs inhibited Bombesin-stimulated amylase release from pancreatic cells because of BB2 (GRP) receptor activation (38, 39). SAR studies also demonstrated that the heptapeptide at the -COOH terminal was the minimal length required for BB2 receptor activation and that the nonapeptide length was necessary for full BB2 receptor affinity (40).
BB2 receptor functions have been extensively studied in various normal and neoplastic tissues. Studies demonstrated that tumor cells not only over-express BB2 receptors but also secrete Bombesin-like peptides resulting in growth stimulation in large numbers of cells from various types of cancer such as neuroblastomas, pancreatic tumors, colon cancer and prostate cancer (21, 37 and 38). BB2 receptors are over-expressed in large numbers of tumors including 85 to 100% of small cell lung carcinomas, 38 to 72% of breast cancers, 62 to 100% of prostate cancers and 72 to 85 % of neuroblastomas (29). In some tumors such as prostate cancer, over-expression of receptor was correlated with neoplastic transformation (41). Over-expressed BB2 receptors by tumors can be used as potential targets for the diagnosis, treatment and management of these tumors (29, 41).

1.2.5. Pre-Targeting Using Bispecific Antibody-Ligand Complexes:

The drawbacks associated with the use of antibodies for therapy and diagnosis led to the need for the development of new modalities such as antibody conjugates to increase the efficiency of therapeutic or diagnostic monoclonal antibodies (42). These conjugates can be generated by attaching antibodies to various agents such as bacterial toxins, radionuclides, peptide analogs and cytotoxic drugs (42, 43). Direct coupling of antibodies to effector compounds has some major disadvantages. Chemical modifications can inactivate antibody binding sites as well as cause essential alterations to the effector function (44), thus decreasing the efficiency of the immunoconjugates. Problems may also arise if the covalent bonds between the conjugates need to be broken for full biological activity as these bonds may be difficult to break (45). Another major problem associated with immunoconjugates is the non-specific interactions of their Fc domains with their associated receptors on the cells of the reticuloendothelial system, resulting in undesired accumulation of antibody in organs.
such as liver and spleen. This affects the sensitivity of tumor imaging due to the high background noise of the radiolabeled antibody. Fab fragments can be used to overcome some of these problems but cannot resolve them completely (46).

There is evidence that antibodies that bind non-specifically via Fc regions are cleared faster as compared to those that bind specifically to their tumor targets (46, 47). This has led to the development of an alternative approach for tumor targeting in which the effector compounds are directly coupled to the antibody. Bispecific antibodies that have two different antigen-specific binding sites, one for the tumor-associated antigen (target binding arm) and the other for the effector compounds (effector binding arm), have been developed (48). A specifically designed bispecific monoclonal antibody is first targeted to the tumor site based on its tumor specificity. After allowing a suitable period of time for the free and non-specifically bound antibody fraction to be cleared, the effector compound, which is recognized by the second specificity of the pre-targeted bispecific antibody, is then injected, leading to its specific localization at the tumor (49).

This approach may minimize non-target toxicity in therapy and maximize tumor targeting in imaging. These bispecific antibodies have many potential uses ranging from immunodiagnosis to the targeted delivery of toxic substances to tumors. They have also been used to target effector cells or toxins to tumors.

Based on the above bispecific antibody concept, Bispecific Antibody-Ligand Complexes can be constructed via conjugation of a peptide or peptide analog specific for a receptor or antigen on the target cell, conjugated to a monoclonal antibody specific for the effector compound. Binding to the effector compound is achieved following pre-targeting with BALCs.
1.2.6. Drug-Conjugated Polymers as Anti-Cancer Agents:

Approximately 85% of the human cancers detected are solid tumors and the first step towards successful cancer therapy starts with the surgical removal of the solid tumor followed by treatment of the remaining cancer cells by radio- or chemo-therapy (50). Chemotherapy has better outcomes as compared to radiation therapy. Ideally a chemotherapeutic drug candidate should be such that it should cause complete killing of cancer cells (51). However, this is a difficult goal to achieve because by the time a cancer is diagnosed it may have already metastasized. Also, the clinical utility of front-line chemotherapeutic drugs such as Doxorubicin, Camptothecin and Paclitaxel is limited by their non-specific toxicity to normal cells and tissues (52). To compensate for the above problems, it is recommended to use higher doses of the chemotherapeutic drugs by intravenous infusion. Complete cure may be achieved at higher therapeutic doses but elevated toxicity levels of the drugs to normal organs can increase mortality rates (53).

Another problem associated with chemotherapy is that most of the chemotherapeutic drugs are water insoluble and hence the use of solubility-enhancing solvents to administer them is a common practice. These solvents can themselves be toxic to patients (54). All these factors contribute to an overall reduction in the quality of life and survival time of cancer patients (52, 54).

Increasing medical needs for improved cancer treatments led to the design of different drug delivery systems that can deliver high payloads of drug at the desired site while keeping non-specific toxicity low and achieving the required therapeutic efficacy (54).

In 1975, Ringdorf conceived the idea of Polymer-Drug Conjugates (PDCs) thus giving birth to a new field of drug therapy (55).
The clinical aims for the design of polymer-anticancer drug conjugates are to enhance drug targeting to tumors, to reduce non-specific drug toxicity to normal tissues, and to overcome mechanisms of drug resistance. To reduce non-specific toxicity and improve drug delivery to tumors, anticancer drugs have been conjugated to polymers (56). The advantages of conjugating these hydrophobic chemotherapeutic agents (e.g. Doxorubicin and Paclitaxel) to hydrophilic polymeric carriers included solubility improvements enabling easier formulation and administration.

Almost all the PDCs that have been clinically tested rely on the “Enhanced Permeability and Retention” effect (also known as the EPR effect) of the tumor vasculature for their delivery (57). Since PDC delivery is not targeted, the concentration of PDCs needed for therapeutic efficacy may be quite high. Although the treatment of large tumors with well-established vasculature would benefit optimally (55, 57), the delivery of PDCs to small or metastatic lesions is problematic.

1.2.7. Combined Pre-Targeting and Polymer-Drug Conjugate Approach:

In the past 2-3 decades, radiolabeled monoclonal antibodies have been used for the diagnosis of cancer by targeting tumor-associated antigens (58). However high background and low target activities have limited their optimal diagnostic potential. Similar limitations are also seen in radioimmunotherapy resulting in high by-stander tissue toxicity (58). These limitations, associated with the use of antibodies for therapy and diagnosis, led to the development of ADCs to increase the efficiency of therapeutic or diagnostic monoclonal antibodies (42). Such conjugates can be generated by attaching antibodies to various agents such as bacterial toxins, peptide analogs and cytotoxic drugs (42, 43). Direct coupling of antibodies to effector compounds is a difficult procedure that often results in the inactivation
of the antibody and in alterations to the effector function of the drug molecules (44).

Additional problems may arise if the covalent bonds between the antibody and the drug need to be broken for full biological activity (45). Another major problem associated with immunoconjugates is the non-specific interaction of the Fc domain of the antibody with the Fc receptors on cells of the reticuloendothelial system. These effector functions may be circumvented by using antibody fragments (59). Nevertheless, drug-conjugated antibody fragments also accumulate albeit to a lesser extent in organs such as liver, spleen and bone marrow. Thus, in diagnostic imaging and radioimmunotherapy, the sensitivity for tumor imaging with radiolabeled antibodies may be compromised by high background noise or increased collateral injury to normal cells and tissues respectively.

An alternate “pre-targeting approach” using bispecific monoclonal antibodies was developed to reduce \textit{in vivo} background activity (59). These bsMAbs were designed to enable the targeting of tumors via their tumor recognition arm, after which the effector compounds may be delivered to their other arm (60). Such pre-targeting approach was applied to immunodiagnosis (61), the targeted delivery of toxic substances to tumors (62), or the activation of cytotoxic cells on tumors (63).

Based on the bsMAb concept, BALCs were constructed via conjugation of a peptide or peptide analog specific for a receptor to a monoclonal antibody that can specifically capture an effector compound. We propose a two-step approach using: a Bombesin-Anti-Diethylene TetraPentaacetic Acid (DTPA) antibody bispecific complex (Bom-bsCx) for pre-targeting in step 1; and a signal unit that is a negatively-charged chelating polymer of \( \sim 15 \) kDa that can be radiolabeled with In-111 or \( ^{99m}\text{Tc} \) at high specific radioactivity, or a therapeutic unit that is a Doxorubicin-loaded polyglutamic acid polymer (D-Dox-PGA) in step 2. This should allow specific tumor targeting to enable diagnosis of even very small
lesions by delivering high specific radioactivity while keeping background activity low. In addition, chemotherapeutic drugs are covalently conjugated to the carriers. Multiple Dox molecules attached to polymers were demonstrated to be less cytotoxic \textit{in vitro} to embryonic cardiocytes relative to free Dox. However targeting D-Dox-PGA polymers via Bom-bsCx led to enhanced prostate cancer toxicity \textit{in vitro}.
1.3. SPECIFIC AIMS:

The overall objective of the project is to design a Bispecific Antibody-Ligand Complex to enhance the delivery of diagnostic and chemotherapeutic agents to cancer lesions.

1.3.1. Aim 1:

To demonstrate selective in vitro targeting of prostate cancer cells pre-targeted with Bispecific Bombesin-Antibody Complexes (Bom-bsCx).

The following experiments will be undertaken:

- ELISA for assessing the immunoreactivity of bispecific antibody complexes.
  - ELISA for detecting Bombesin on Bom-bsCx.
  - ELISA for assessing the bispecificity of Bom-bsCx.
- Competitive inhibition ELISA to demonstrate that the binding of Bom-bsCx to an anti-Bombesin antibody can be inhibited with serial dilutions of free unmodified Bombesin.
- In vitro cell-based binding assay to demonstrate the specific and selective binding of Bom-bsCx to Bombesin receptors on human prostate cancer (PC3) cells.
- In vitro fluorescence ligand binding assay.
- Epifluorescent microscopy to demonstrate the binding of Bom-bsCx to Bombesin receptors on PC3 cells.

1.3.2. Aim 2:

To demonstrate enhanced in vitro drug delivery of Doxorubicin-conjugated polymers pre-targeted with bispecific bombesin-antibody complexes.
The following experiments will be undertaken:

- To determine the IC$_{50}$ of a N-terminal DTPA-modified, Dox-loaded polyglutamic acid polymer (D-Dox-PGA) and free Dox on PC3 cells.
- *In vitro* assay for assessing tumortotoxicity on PC3 cells.

1.3.3. Aim 3:

To demonstrate specific *in vivo* imaging of prostate cancer using radiolabeled polymers pre-targeted with bispecific bombesin-antibody complexes.

The following experiments will be undertaken:

- Radiolabeled polymer clearance studies.
- Bom-bsCx clearance studies.
- *In vivo* targeting of PC3 xenograft tumors with Bom-bsCx.

1.3.4. Aim 4:

To demonstrate enhanced targeting of radiolabeled polymers pre-targeted with bispecific bombesin-Fab' complex for cancer diagnosis.

The following experiments will be undertaken:

- ELISA for assessing the immunoreactivity of bispecific antibody Fab' complexes.
  - ELISA for detecting Bombesin on Bom-bsFCx.
  - ELISA for assessing the bispecificity of Bom-bsFCx.
- Competitive inhibition ELISA to demonstrate that the binding of Bom-bsFCx to an anti-Bombesin antibody can be inhibited with serial dilutions of free unmodified Bombesin.
• Bispecific Bom-bsFCx clearance studies.

• Comparison of targeting efficiency of Bom-bsCx and Bom-bsFCx on PC3 xenograft tumors.
2. MATERIALS AND METHODS:

The Bombesin analog is a gift of Dr. Alexandra D. Varvarigou, National Center for Scientific Research “Demokritos,” Athens, Greece (64). The Anti-DTPA antibody (6C31H3) and antimyosin antibody (2G42D7) were developed in our lab (65). Embryonic rat cardiocytes (H9C2) and human prostate cancer cells (PC3) were purchased from American Type Culture Collection (USA).

Cell Culture:

Cells were grown in high glucose (4.5g/l) DMEM supplemented with 10% Fetal Clone II (Hyclone) with penicillin (100units/ml), streptomycin (100µg/ml) and Amphotericin (2.5µg/ml) (Sigma Company Co.) at 37º C in 5% CO₂.

2.1. Preparation of Bom-bsCx:

Intact 6C31H3 (anti-DTPA) antibody (1mg/ml) (65) in 50 mM NaCl/ 1mM EDTA; pH 8, was modified with 100 molar excess of iminothiollane (Sigma Chemical Co.) in 0.2 ml of anhydrous dimethyl sulfoxide (DMSO) (Sigma Chemical Co.). Bombesin (1mg/ml) in 0.1M Phosphate buffered saline (PBS), pH 7.4, was modified with 10 molar excess of N-hydroxy-succinimide ester of bromoacetic acid (Sigma Chemical Co.) in 0.2 ml of anhydrous DMSO (64, 66). The modified antibody and the modified Bombesin were separated from unreacted reagents by Sephadex G-10 column (GE Healthcare Co.) centrifugation in 0.1M Phosphate buffered saline (PBS), pH 7.4. The extent of modification of anti-DTPA Ab and bromoacetylation of Bombesin were assessed by Tri-nitro benzene sulfonic acid (TNBS) assay (67). Incubation of 100 moles excess of bromoacetylated Bombesin and the thiolated antibody at 4ºC overnight resulted in 1:1 Bombesin to anti-DTPA 6C31H3 bispecific complexes via the thioether bond. The conjugated Bom-bsCx was then separated from free
Bombesin by size exclusion dialysis using a 3.5 KDa MWCO (Molecular weight cut off) dialysis bags (Spectrum Labs).

2.1.1. TNBS Assay for Modified Bombesin and Anti-DTPA Antibody:

After recovering the modified anti-DTPA Ab or bombesin from the Sephadex G-25 column spin chromatographies, percent modification was calculated using tri-nitro benzene sulfonic acid assay. An aliquot of 10 µg of the modified anti-DTPA or bombesin was compared to standard unmodified anti-DTPA or unmodified bombesin (10 µg) respectively. Aliquots of 375 µl of distilled water and 375 µL of 0.1M sodium tetraborate solution were added to 10 ug of each sample, and then 187.5 µL of 2mg/ml solution of trinitro benzene sulfonic acid solution (Sigma Co.) were added. The reaction mixture was incubated for 30 mins at 37°C. After incubation, freshly prepared 375 µl of Sodium sulphite (1.5mg/ml made in 0.2 M Sodium phosphate dibasic solution) were added to the above reaction mixture. After mixing the reaction mixture thoroughly, it was read at OD 420nm. Percent modification was calculated by comparison of the OD of the respective unmodified proteins. The assay was performed in triplicates to ensure reproducibility.

2.1.2. Assessment of the Immunoreactivity of the Modified Antibodies:

Modified 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. Serial dilution of aliquots of the thiolated antibody starting at 1 µg/ml and the unmodified anti-DTPA antibody were used to assess the immunoreactivity. Detection was achieved using a secondary antibody (goat anti-
mouse IgG coupled horseradish peroxidase) 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 O.D. 405 nm in an automated ELISA plate reader (BioTek, VT, USA, Model: EL 800). The assay was performed in quadruplicates and analyzed using GEN 5.0 software for mean and standard deviation.

2.2. Preparation of Bom-bsFCx:

Fab’ was coupled with bromoaceylated bombesin to produce Bom-bsFCx as described above.

2.2.1. NBS Assay for Modified Bombesin:

Assay was performed as described above.

2.2.2. Preparation of Fab’ Fragment of Anti-DTPA Antibody:

Intact anti-DTPA antibody was subjected to enzymatic digestion with immobilized pepsin beads to generate F(ab’)2 (Sigma Chemical Co.) as described by Khaw et al. (69). Briefly, 5mg/ml of the intact anti-DTPA antibody were dialyzed overnight in 0.2 M Acetate buffer, pH 4.0. Pepsin beads (Sigma Co.) were then equilibrated in the same buffer. Antibody digestion was completed using an equal volume of the pepsin bead and providing a final w/w ratio of pepsin to antibody ranging from 1:20 to 1:100. The resultant enzyme/antibody mixture was incubated at 37ºC for 12 hours. At the designated time, 2 M Tris buffer, pH 9.6 was added to adjust the pH to neutral pH to stop further enzymatic digestion.
F(ab\textsuperscript{\prime})\textsubscript{2} was purified by protein A affinity chromatography (Sigma Chemical Co.) to remove Fc and undigested Ab. After dialysis in 0.1 M PBS, pH 7.4, using a dialysis membrane with a 3500 MWCO (Spectrum Labs), F(ab\textsuperscript{\prime})\textsubscript{2} was stored at 4 °C. Prior to the cross-linking procedure F(ab\textsuperscript{\prime})\textsubscript{2} was reduced with 100 µl of 10 mM DTT (Dithiothreitol) (Sigma Chemical Co.) to obtain reduced Fab\textsuperscript{\prime} (70). The sample was dialysed in 0.1 M PBS, 50 mM EDTA, pH 7.4 using a dialysis membrane with a 3500 MWCO (Spectrum Labs) to maintain the reduced state of the Fab\textsuperscript{\prime}. Immunoreactivity of Fab\textsuperscript{\prime} was assessed using DTPA-BSA ELISA.

2.2.3. Characterization of Fab\textsuperscript{\prime} Antibody Fragments:

2.2.3.a. SDS-PAGE:

Purity of Fab\textsuperscript{\prime} was characterized by SDS-PAGE. Briefly, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (X Cell Sure lock Mini-Cell, Nu PAGE 4-12% precast gels; Invitrogen Inc.) of Fab\textsuperscript{\prime} was compared to that of monomeric MAb. Samples were subjected to SDS-PAGE under nonreducing condition and compared with the molecular weight standards (Sigma Chemical Co.).

2.2.3.b. Immunoreactivity of Anti-DTPA Fab\textsuperscript{\prime} Fragment:

Aliquots of 100µl DTPA-BSA (1µg/ml) were added to each well of the 96-well micro titer plate (BD Biosciences) for assessment of the immunoreactivity of anti-DTPA Fab\textsuperscript{\prime}. The plate was incubated at 37°C for one hour followed by washing with 0.1M PBS-T (5X). The wells were then blocked with 200µl/well of 3% non-hybridoma supportive fetal bovine serum for one hour at 37°C followed by washing in 0.1M PBS-T (5X). Then 100µl aliquots of serial dilutions of purified anti-DTPA intact Ab and anti-DTPA Fab\textsuperscript{\prime} starting with 1 µg/ml
were added to the wells. The plate was incubated for one hour at 37°C, washed again in 0.1M PBS-T (5X) and 50µl/well aliquots of goat anti-mouse antibody conjugated with horseradish peroxidase (1:1000) were added to each well. The microtiter plate was incubated for 1 hr at 37°C followed by washing. To assess the immunoreactivity of anti-DTPA intact Ab and anti-DTPA Fab', K-Blue substrate (chromogen) was added (50µl/well). The microtiter plate was then kept in dark for about 15 minutes then the plate was read at 405 nm. Assay was undertaken in quadruplicates and the apparent affinity was analyzed using the GEN 5.0 software.

2.3. Preparation of DTPA-Horse Radish Peroxidase (DTPA-HRP):

2.3.1. Preparation of DTPA-HRP:

DTPA-HRP conjugates were prepared as described by Khaw et al. (71). Briefly, 5 mg/ml of HRP (Sigma Chemical Co.) in 0.1M NaHCO₃, pH 8.5 was reacted with 10 molar excess of bicyclic anhydride of DTPA (Sigma Chemical Co.) in 0.2 ml DMSO with constant vigorous stirring. The reaction mixture was incubated overnight in the dark at 4°C. The reaction mixture was then subjected to Sephadex-G25 (10 ml) (GE Healthcare Co.) column chromatography in 0.1 M PBS to separate free DTPA from DTPA-HRP conjugate. DTPA-HRP was eluted in the void volume. Approximately 1ml fractions were collected and the optical density at 402 nm, the absorption maxima of HRP, was obtained. Presence of DTPA on HRP was assessed by ELISA using anti-DTPA antibody.

2.3.2. Demonstration of Incorporation of DTPA on DTPA-HRP by ELISA:

A 96 well micro-titer plate (Falcon) was coated with 50µl of serial dilutions of anti-DTPA Ab or anti-myosin Ab as control starting at 1µg/ml. The antibody coated micro-titer
plate was incubated at 37 ºC for 1hr. Washing and blocking of the microtiter wells were as described previously. Each micro-titer well was then loaded with 50µl aliquots of 1:50 dilution of DTPA-HRP (concentration 76 µg/ml) and incubated at 37ºC for 1 hr. After washing, 50 µl aliquots of K-blue substrate (Neogen Corp.) were added and incubated for 30 min at room temperature in the dark. Color intensity is read at 405 nm using an ELISA reader as previously stated. The assay was performed in quadruplicates. Mean and standard deviation were calculated as described previously.

2.4. Preparation of N-Terminal DTPA-Modified Dox-Loaded Polyglutamic Acid Polymer (D-Dox-PGA):

2.4.1. Preparation of D-Dox-PGA:

An aliquot of 50 mg of (10mg/ml) polyglutamic acid (PGA, MW 13.3 kda) (Sigma Chemical Co.) in 0.1 M NaHCO3, pH 8.6 was reacted with 5X molar excess of bicyclic anhydride of DTPA in 0.2 ml DMSO. The mixture was vigorously stirred for approximately 5 minutes followed by incubation at room temperature for 2 hrs. DTPA linked to the N-terminal amino group of polyglutamic acid was assessed by ELISA using anti-DTPA antibody (10ug/ml). Binding of anti-DTPA antibody to DTPA-BSA (Bovine serum albumin) was used for comparison. Then 4.5-9 mg of Doxorubicin (Dox) dissolved in 0.2 ml of anhydrous DMSO was covalently linked via peptide bonds to the carboxylic acids of D-PGA using 9-18 mg water soluble 1-ethyl -3-(3-dimethylaminopropyl) carbodiimide.HCL (EDC) (Sigma Chemical Co.). The reaction mixture of Dox and D-PGA was incubated at 4ºC for 1 hr in the dark and then incubation was continued overnight at room temperature. The solution of D-Dox-PGA was subjected to Sephadex-G25 (10 ml) column chromatography to separate free Dox from D-Dox-PGA in 0.1 M PBS.
2.4.2. Isolation of Free and Polymer-Linked Doxorubicin Using Sephadex G-25 Column:

The D-PGA-Dox 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 0.1 M PBS pH 7.4 as the elution buffer. OD 490 was read using a spectrophotometer to estimate the concentration of Doxorubicin.

2.4.3. Demonstration of Incorporation of DTPA on Polymers by ELISA:

A 96 well micro-titer plate was coated with 100µl of DTPA-BSA/well as positive control standard or with D-Dox-PGA complex at a concentration of 1 µg/ml equivalent and then the plate was incubated for 1 hr at 37°C. The micro-titer plate was washed, blocked and washed again as previously described. The micro-titer plate was then loaded with 100µl aliquots of serial dilutions of the anti-DTPA antibody starting with 10 µg/ml of the antibody. The micro-titer plate was incubated for 1 hr at 37°C and washed again. Aliquots of 50µl of horseradish peroxidase conjugated goat anti-mouse antibody (GAM-HRP) diluted 1:500 was added to each well. Incubation and washing was again carried out. K-blue substrate was added (50µl each well) as a chromogen for the assessment of antibody binding. OD was read at 405 nm. The assay was carried out in quadruplicates and analyzed as before.

2.5. Preparation of DTPA-Rhodamine-Succinylated Polylysine Polymers (DRS-PL):

DTPA-polylysine polymers (DPL) were prepared as described by Khaw et al. (71). Briefly, 50 mg of poly lysine (PL, MW 14.6 kDa) (Sigma Chemical Co.) was 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 (67). DTPA linked to the amino group of polylysine was assessed by ELISA using anti-DTPA antibody (10µg/ml) as described above. Binding of anti-DTPA antibody to DTPA-BSA (Bovine serum albumin) was used for comparison.

Residual free lysyl residues were further modified by addition of 24 molar excess of rhodamine isothiocynate (Sigma Chemical Co.). The rhodamine conjugated DTPA-PL was then subjected to succinylation with 100 molar excess of succinic anhydride (Sigma Chemical Co.). The above solution was subjected to Sephadex-G25 (10 ml) column chromatography to separate free unreacted DTPA and rhodamine in 0.1 M PBS to provide DTPA-rhodamine-succinylated-polylysine (DRS-PL).

2.6. Assessment of Immunoreactivity and Bispecificity of the Bom-bsCx:

2.6.1. ELISA for Detection of Bombesin on Bom-bsCx:

A 96 well micro-titer plate (Falcon) was coated with aliquots of 50µl of serial dilutions of samples starting at 10µg/ml for standard Bombesin and 100 µg/ml of antibody concentration for Bom-bsCx. The plate was incubated at 37 ºC for 1hr. The wells were washed then blocked as described above. The wells loaded with 50µl aliquots of 1:1000 dilution of rabbit anti-Bombesin antibody (Abcam, USA) and incubated at 37ºC for 1 hr. The wells were washed followed by addition of 50 µl aliquots of 1:2000 diluted goat-anti-rabbit-HRP (Thermo Scientific Inc.). The micro-titer plate was incubated for 45 min at 37ºC followed by washing. K-blue substrate was then added (50µl each well) and incubated for 30
min at room temperature in the dark. The micro-titer plate was read and analyzed as described above. The standard Bombesin curve generated was used to estimate the concentration of Bombesin in the Bom-bsCx.

2.6.2. ELISA for Bispecificity of Bom-bsCx:

The 96 well micro-titer plate was coated with 50µl aliquots of 1:1000 dilution of rabbit anti-bombesin antibody and incubated at 37 ºC for 1hr. The wells were washed then blocked as above. After washing again, the wells were loaded with aliquots of 50µl serial dilutions of bombesin, Bom-bsCx or anti-DTPA antibody starting at 1 µg/ml each (bombesin concentration in Bom-bsCx was determined as described above). The micro-titer plate was incubated overnight at 4 ºC followed by washing. An aliquot of 50 µl of DTPA-HRP (1:500 dilutions) was added to each well and the micro-titer plate was incubated for 45 min at 37ºC. After washing, K-blue substrate was added (50µl) as described and incubated for 30 min at room temperature in the dark. The micro-titer plate was read and analyzed as described above.

2.6.3. Competitive Inhibition ELISA for Bom-bsCx:

Competitive inhibition ELISA was undertaken to demonstrate that binding of Bom-bsCx to anti-bombesin antibody can be inhibited with serial dilutions of free unmodified bombesin.

Each well of a 96 well micro-titer plate was coated with 50µl of 1:1000 dilution of anti-bombesin antibody. The plate was incubated at 37ºC for 1hr. The wells were then washed followed by addition of the blocking agent and incubated for 1 hr at 37ºC. After washing, 50 µl aliquots of serial dilutions of unmodified bombesin (1 through 1x10⁻⁶ µg/ml)
in equal volume of Bom-bsCx (1 µg/ml) were added and incubated at 4 °C overnight. The wells were washed. Then 50 µl aliquots of 1:500 dilutions of DTPA-HRP were added and incubated for 1hr at 37ºC. After washing, K-Blue was added and incubated for 30 mins at room temperature in dark. The micro-titer plate was read and analyzed as described above.

2.7. In Vitro Cell-Based Binding Assay for Bom-bsCx:

The cell lines H9C2 (rat embryonic cardiocytes), 2G42D7 (anti-myosin murine hybridomas), and PC3 (human prostate cancer) were used for the assay. The cells were grown in 96 well plates at 10,000-20,000 cells per well and were grown until 70% confluence. The cells were incubated with 10 µg/ml of Bom-bsCx at 37 ºC for 1 hr. After washing with 0.1M PBS (5 X), targeting on various cells was demonstrated with 100 µl of 1:500 diluted HRP-conjugated rabbit anti-murine IgG antibody (RAMIgG-HRP) (Thermo Scientific Inc.). K-blue chromogen was added as described above and presence of Bom-bsCx assessed with the ELISA reader. Controls consisted of cells treated with 6C31H3 (anti-DTPA) antibody, bovine serum albumin (BSA), or culture medium alone.

2.8. Fluorescence Ligand Binding for Bom-bsCx by Flow Cytometry and Epifluorescent Microscopy:

Fluorescence ligand binding assay using Bom-FITC was undertaken to demonstrate differential expression of Bombesin receptors by 2G42D7, H9C2 and PC3 cell lines.

H9C2, 2G42D7 and PC3 cells were incubated with serial dilutions of Bom-FITC (10, 5, 2.5, 1, 0.1 and 0.01 µg/ml). The micro-titer plate was incubated for 1 hr at 37 ºC. Fluorescent activated cell analysis [BD Biosciences, USA] was undertaken using non-treated cells as control.
Epifluorescent microscopy was performed on PC3 cells pre-targeted with Bom-bsCx, Bombesin, and anti-DTPA antibody (10µg/ml each), or culture medium for 1 hr at 4 ºC to minimize internalization and targeting with DRS-PL. PC3 cells were then washed 3X with cold 0.1M PBS and incubated with DRS-PL at 37 ºC 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. The fluorescent intensity of each cell was computer planimetered and mean pixel density was determined for a total of 50 cells from each well. Regions without cells were planimetered and the pixel density determined for background fluorescence. The background pixel density was subtracted from the mean cellular pixel densities.

2.9. Determination of IC_{50} of D-Dox-PGA and Free Dox on PC3 Cells:

Human prostate cancer cells (PC3) were used to determine the inhibitory concentration at 50% cell survival (IC50). Aliquots of 40,000 cells were grown in 6 well plates (Falcon) to 90% confluence and serial dilutions of 5, 10, 20 and 30 µg /ml of free Dox or Dox equivalent concentrations of D-Dox-PGA were added in the culture medium and the cells were incubated for 24 hr at 37 ºC. The study was performed in triplicates. Cell death and survival was assessed by Trypan Blue exclusion test (68).

2.10. In Vitro Reduction of Cardiocyte Toxicity:

IC 50 of D-Dox-PGA and free Dox were determined in rat embryonic cardiocytes (H9C2). Aliquots of 40,000 cells were grown in 6 well plates 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 medium and cells were incubated for 24 h at 37 °C. Each set was prepared in triplicate. Cell death and survival was assessed by Trypan Blue exclusion test (68).

2.11. In Vitro Assay for Tumor Toxicity Using Bom-bsCx in Human Prostate (PC3) Cancer Cells:

Human prostate cancer cells (PC3) were grown in 6 well plates and studies were performed in triplicate. Medium of each well was replaced with medium containing 15µg/ml of Bom-bsCx, Bombesin or anti-DTPA antibody, or culture medium alone. After 24 hrs of incubation at 37 °C, the media were removed and wells washed with 3X medium alone. Aliquots (1000 µl) of media containing serial dilutions of 5, 10, 20 and 30 µg/ml of Dox or Dox equivalent concentrations of D-Dox-PGA were added to the wells. After 24 hr incubation at 37 °C, viability was assessed by Trypan Blue exclusion test (68).

2.12. Assessment of Immunoreactivity and Bispecificity of Bom-bsFCx:

2.12.1. ELISA for Detection of Bombesin on Bom-bsFCx:

Micro-titer plates (Falcon) were coated as above with serial dilutions of Bombesin (10µg/ml) or Bom-bsFCx (100 µg/ml). The plates were incubated at 37 °C for 1 hr. The wells were washed then blocked as described above. The wells were washed and loaded with 50µl aliquots of 1:1000 dilution of rabbit anti-Bombesin antibody (Abcam, USA) and incubated at 37°C for 1 hr. The wells were washed followed by addition of 50 µl aliquots of 1:2000 diluted goat-anti-rabbit-HRP (Thermo Scientific Inc.). The micro-titer plate was incubated for 45 min at 37°C followed by washing. K-blue substrate was then added (50µl each well) and incubated for 30 min at room temperature in the dark. The micro-titer plate
was read and analyzed as described above. The standard Bombesin curve generated was used to estimate the concentration of Bombesin in the Bom-bsFCx.

2.12.2. ELISA for Bispecificity of Bom-bsFCx:

The bispecificity was assessed for Bom-bsFCx was as described above. However, Bom-bsFCx was substituted for Bom-bsCx.

2.12.3. Competitive Inhibition ELISA for Bom-bsFCx:

Competitive inhibition ELISA for Bom-bsFCx was as described for Bom-bsCx.

2.13. In Vivo Animal Model Studies:

All experiments were performed in 6-8 week old male C57/BL6 and 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. The animals were allowed food and water *ad libitum*. PC-3 human prostate cancer xenografts were induced in SCID mice by subcutaneous injection of $3 \times 10^6$ cells in 0.1 ml of serum free DMEM into the left, right or both shoulder regions with or without matrigel (0.1 ml of matrigel) (BD Biosciences).

2.13.1. Preparation of $^{99m}$Tc-Labeled DTPA–Succinylated Polylysine:

DTPA–Succinylated-polylysine (DSPL) (14.6 kDa) was prepared as previously described (71). Approximately 100 µg aliquot of DSPL in 0.1 M carbonate solution 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\textsubscript{2} for 15 min. After 30 min of incubation, \textsuperscript{99m}Tc-DSPL (14.6 KDa) was separated from free \textsuperscript{99m}Tc by Sephadex-G25 (10 mL) column chromatography (72).

2.13.2. Preparation of \textsuperscript{111}In-Labeled DSPL:

DSPL was labeled with In-111Cl\textsubscript{3} using sodium citrate as a transchelator at pH 5.5 - 6.0. Briefly, 3-4 mCi of indium-111 in 200 \mu l of 1 M sodium citrate, pH 5.5, was added to 100 \mu g of DSPL. The mixture was stirred vigorously, and then incubated at room temperature for 30 min. Bound and free In-111 was separated by Sephadex-G25 (10 mL) (GE Healthcare Co.) column chromatography. \textsuperscript{111}In-DSPL was eluted in the void volume (72).

2.13.3. Preparation of \textsuperscript{99m}Tc-Labeled DTPA- Bom-bsCx for \textit{In Vivo} Clearance:

Approximately 100 \mu g of DTPA in 0.5 ml 0.1 M carbonate solution were reacted with 740 MBq of \textsuperscript{99m}Tc O\textsubscript{4}\textsuperscript{-} for 30 mins as described above. After 30 min, \textsuperscript{99m}Tc-DTPA mixture was incubated with 100 \mu g of Bom-bsCx for another 30 mins. \textsuperscript{99m}Tc-labeled DTPA- Bom-bsCx was separated from free \textsuperscript{99m}Tc-DTPA by Sephadex-G25 (10 mL) column chromatography (72).

2.13.4. Preparation of \textsuperscript{99m}Tc-Labeled DTPA- Bom-bsFCx:

DTPA was labeled with \textsuperscript{99m}Tc as described above. \textsuperscript{99m}Tc-DTPA was incubated with 100\mu g of Bom-bsFCx for 30 mins and then \textsuperscript{99m}Tc-labeled DTPA- Bom-bsFCx was separated from free \textsuperscript{99m}Tc-DTPA as described above (72).
2.13.5. *In Vivo* Bom-bsCx Clearance Studies:

C57/BL6 normal mice (n=3) were injected with 14-16 MBq $^{99m}$Tc-DTPA-Bom-bsCx (5-10 µg/mouse). After radiotracer injection, planar images were acquired for one mouse at 30 min, 45 min, 450 min, and 24hr time point. Each image was analyzed using ImageJ program from NIH. Microsoft Excel 2007 was used to fit trend lines. At 24 hours the mice were euthanized by an overdose of IP injection of sodium pentobarbital (100 mg/kg) or ketamine/xylazine (100 mg/kg and 10 mg/kg respectively) and biodistribution of radioactivities in the blood, heart, lung, liver, spleen, kidney, stomach, intestines and skeletal muscle tissue were determined by gamma scintillation counting (model 1282 Compugamma; LKB Instruments, Inc.) of weighed tissue samples. Aliquots (5-10 µL) of the radiolabeled Bom-bsCx were included for calculation of percent injected dose as described by Gada et al (72). Student's t-test was used to determine statistical significance.

2.13.6. *In Vivo* Bom-bsFCx Clearance Studies:

Similarly, C57/BL6 normal mice (n=4) were injected with 13–15 MBq $^{99m}$Tc-DTPA-Bom-bsFCx (5-10µg/mouse). After radiotracer injection, planar images were acquired at 30 min, 45 min, and 450 min time point for one mouse. Upon completion of gamma imaging at 8 hours, biodistribution, image analyses and trend lines fitting were undertaken as described above.

2.13.7. *In Vivo* Polymer Clearance Studies:

SCID mice (n=3) were injected with 30–35 MBq $^{99m}$Tc-DSPL (2-3µg/mouse). After radiotracer injection, planar images were acquired at 5 min, 45 min, 180 min, and 24hr time
points for one mouse. Biodistribution, image analyses and fitting of trend lines were as described above.

2.13.8. *In Vivo* Targeting of Xenograft Tumors with Bom-bsCx:

SCID SHO mice (n=9) were injected subcutaneously in the shoulder region (Right, Left or both) with $3 \times 10^6$ PC3 Human Prostate cancer cells with or without matrigel. After 14 days, the SCID mice (approx. 20 g) with the xenograph tumors (3-5 mice in each group) were injected with 10 µg of Bom-bsCx (n = 4), anti-DTPA (n=3) or Bombesin alone(n=2). The next day, when Bom-bsCx has cleared from the circulation, $7.03 \pm 1.74$ MBq In-111–DSPL (2-3 µg/mouse) was administered intravenously under Ketamine and xylazine or isoflurane anesthesia. Serial gamma imaging was performed for 3 hrs using Picker SX300 gamma camera equipped with a 3 mm pinhole collimator. Postero-anterior gamma images of 500 sec acquisition time were obtained at 5 min, 15 min, 30 min, 60 min, 120 min, and 180 min. At 24 hrs, the mice were re-anesthetized and imaged for 1500 sec each. Three mice were also imaged with BIOSCAN/CT camera. The 3 X 9 pinhole collimators of BIOSCAN/CT were set according to the manufacturer’s instructions. Image analyses, trend line fitting and biodistribution were performed as already described.

2.13.9. *In Vivo* Targeting of Xenograft Tumors with Bom-bsFCx:

PC3 xenografted SCID mice were injected with 100 µg of Bom-bsFCx (n =5) or Bombesin (n=4) alone. After 8-12 hours, when Bom-bsFCx has cleared from the circulation, approximately 45-55 MBq $^{99m}$Tc–DSPL (2-3 µg/mouse) were administered intravenously. Serial gamma imaging was performed for 3 hrs as above. Gamma images were acquired at 5 min, 15 min, 30 min, 60 min, 120 min, and 180 min for 500 seconds each. One mouse from
each group died before 6 hours due to over-anesthesia administration. At 24 hrs, mice were imaged for 1500 sec acquisition time. A SPECT micro-gamma camera (73) provided by the National Jefferson Labs was used for postero-anterior planar gamma imaging. Images were analyzed, trend lines fitted and statistical analyses performed as described above. Biodistribution and tumor activities were also determined as above.

2.14. Statistics:

Data are expressed as mean +/- SD. Student’s-t test was used to determine statistical significance using the statistical package of Microsoft Excel XP. P value of $\leq 0.05$ was considered statistically significant.
3. RESULTS:

3.1. Preparation of Bom-bsCx:

Bombesin was cross linked to anti-DTPA Ab using the thioether bond. The schematic diagram below (figure 1) shows the modification of Bombesin and MAb with cross linking reagents followed by formation of Bom-bsCx via the thioether bond.

![Diagram showing the preparation of Bom-bsCx](image)

**Figure 1:** Schematic diagram for Bom-bsCx formation.

3.1.1. TNBS Assay for Modified Bombesin and Anti-DTPA Antibody:

Modification of anti-DTPA Ab with 2-Iminothiolane and bombesin with N-hydroxy succinimide ester of bromoacetic acid was assessed using Tri-Nitro benzene sulfonic acid assay comparing to their respective unmodified standards (Table 1). Modification of epsilon lysine residues of anti-DTPA mAb was approximately 65 %, while modification of epsilon amino group residue of bombesin was approximately 99%. Bombesin has only one epsilon amino group residue to modify so higher % modification for bombesin is expected.
<table>
<thead>
<tr>
<th></th>
<th>dH2O</th>
<th>0.1 M Sodium Tetraborate</th>
<th>TNBS (2mg/ml)</th>
<th>Incubate</th>
<th>Na2SO3 (1.5mg/ml) in 50 ml of 0.2M NaH2PO4</th>
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<th>Mean OD 420</th>
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<tr>
<td></td>
<td>3</td>
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<td>375 µL</td>
<td>for 30 min</td>
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<td>0</td>
</tr>
<tr>
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<td>DTPA</td>
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<td>for 30 min</td>
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<td>375 µL</td>
<td>Incubate</td>
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<td>0.096</td>
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<td></td>
<td>2</td>
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<td>375 µL</td>
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<td></td>
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Table 1: TNBS Assay for Modified Bombesin and anti-DTPA Ab. Modification of epsilon lysine residues of anti-DTPA Ab was approximately 65%, while modification of epsilon amino group residue of Bombesin was approximately 99%.
3.1.2. Assessment of the Immunoreactivity of the Modified Antibodies:

Modified Anti-DTPA Antibody Immunoassay:

The immunoreactivity of anti-DTPA antibody modified with 2-Iminothiolane as analyzed by ELISA is shown in Figure 2. Modified antibody showed no loss of immunoreactivity after chemical modification relative to the native antibodies.

Figure 2: ELISA for determination of the immunoreactivity of modified anti-DTPA Ab (Squares) relative to unmodified anti-DTPA antibody (Triangles). OD = Optical Density. Vertical bars denote ± 1 standard deviation (95% confidence interval) in all figures.
3.2. Preparation of Bom-bsFCx:

Bombesin was cross-linked to anti-DTPA Fab' using the thioether bond.

3.2.1. TNBS Assay for Modified Bombesin:

Modification of bombesin with N-hydroxy succinimide ester of bromoacetic acid was assessed using Tri-Nitro benzene sulfonic acid assay comparing to the unmodified bombesin (Table 2). Modification of the epsilon amino group residue of bombesin was approximately 99%.

<table>
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<th>TNBS (2mg/ml)</th>
<th>Incubate</th>
<th>Na2SO3 (1.5mg/ml in50 ml of 0.2M NaH2PO4)</th>
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<tr>
<td></td>
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<td>375 µL</td>
<td>187.5 µL</td>
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<td></td>
<td>3</td>
<td>375 µL</td>
<td>375 µL</td>
<td>187.5 µL</td>
<td>at 37°C</td>
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<tr>
<td>STD Bombesin 10 µg</td>
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<td>187.5 µL</td>
<td>at 37°C</td>
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Table 2: TNBS Assay for Modified Bombesin. Modification of epsilon amino group residue of Bombesin was approximately 99%.
3.2.2. Preparation of Fab’ Fragment for Anti-DTPA Antibody:

Characterization of Antibody Fab’ Fragments:

3.2.2.a. SDS PAGE for Fab’ Fragments:

SDS-PAGE analyses (Figure 3) demonstrated that the F(ab’)2 preparation was homogenous without residual undigested intact antibody. Reduction of anti-DTPA F(ab’)2 resulted in complete formation of Fab’ (c). Anti-DTPA Fab’ was then refrigerated at 4°C until further use.

**Figure 3**: SDS–PAGE of following: A. purified anti-DTPA Ab (150Kd), B. purified anti-DTPA F(ab’)2,(100Kd) C. purified anti-DTPA Fab’ (51Kd).
3.2.2.b. Immunoreactivity of Anti-DTPA Fab' Fragment:

Figure 4 shows the normalized immunoreactivity of purified Fab' as compared to controls. No loss of immunoreactivity was observed.

Figure 4: ELISA for determination of the immunoreactivity of purified Fab' (Squares) as compared to anti-DTPA antibody (Circles).
3.3. Preparation of DTPA-HRP:

3.3.1. Elution Profile for DTPA-HRP:

The DTPA-HRP was eluted in the void volume with fraction no.5 showing peak HRP activity (figure 5) by size exclusion Sephadex G-25 column chromatography. Approximately 2 ml of DTPA-HRP (Peak tube no.5 activity of 76 µg/ml) were acquired and used for further studies.

Figure 5: Elution profile of DTPA-HRP by Sephadex G-25 column chromatography.
3.3.2. Incorporation of DTPA on DTPA-HRP by ELISA:

ELISA was undertaken to determine the presence of the capture antigen (DTPA) on HRP. As seen in Figure 6, anti-DTPA antibody captured DTPA-HRP indicating the presence of DTPA on HRP. The control antibody anti-myosin showed no binding of DTPA-HRP.

**Figure 6**: anti-DTPA antibody captured DTPA-HRP indicating the presence of DTPA on HRP (Circles) as compared to anti-myosin Ab controls (Squares).
3.4. Preparation of N-terminal DTPA-Modified, Dox-Loaded Polyglutamic Acid Polymer (D-Dox-PGA):

DTPA was first covalently conjugated to PGA followed by conjugation of Dox to PGA via amide bond formation. The schematic diagram below (figure 7) shows the synthesis of D-Dox-PGA.

**Figure 7:** Schematic diagram of D-Dox-PGA.
3.4.1. Isolation of Free and Polymer-Linked Doxorubicin Using Sephadex G-25 Column:

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 (Figure 8) by size exclusion Sephadex G-25 column chromatography. Approximately 4 ml of polymer conjugated Doxorubicin (D-PGA-Dox complex) were acquired and used for further studies.

Figure 8: Superimposition of the elution profile of D-Dox-PGA (24 moles of Dox/mole polymer (triangles) and free Doxorubicin (diamonds) by Sephadex G-25 column (10 X 0.5 cm) chromatography.
Standard curve for free Doxorubicin was generated to estimate the concentration of Doxorubicin covalently linked to the polymer (figure 9). 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.

Figure 9: Standard curve for estimation of Doxorubicin concentration at OD 490 nm.
3.4.2. Incorporation of DTPA on PGA Polymers by ELISA:

The presence of the capture antigen (DTPA) on polyglutamic acid was shown by ELISA in Figure 10. Standard anti-DTPA antibody bound D-Dox-PGA indicating the presence of DTPA on the polymers. Aliquots of 24 moles Dox/mole polymer showed equivalent binding to anti-DTPA Ab at the concentration of 1 µg/ml because only 1 mole of DTPA per mole of polyglutamic acid is present as compared to DTPA-BSA which was used as a positive control with multiple moles of DTPA per mole of BSA.

![Figure 10](image-url)

**Figure 10:** Superimposition of assessment of presence of capture antigen on D-Dox-PGA using ELISA assay (D-Dox-PGA – Diamonds, STD DTPA-BSA- Circles).
3.5. Preparation of DTPA-Rhodamine-Succinylated Polylysine Polymers (DRS-PL):

3.5.1. TNBS Assay for DTPA-Modified Poly-L-Lysine Polymers (D-PL):

Modification of PL was assessed using Tri-Nitro benzene sulfonic acid assay comparing to the unmodified standard (Table 3). Modification of epsilon amino groups of lysines was approximately 31%.

<table>
<thead>
<tr>
<th></th>
<th>dH2O</th>
<th>0.1 M Sodium Tetraborate</th>
<th>TNBS (2mg/ml)</th>
<th>Incubate</th>
<th>Na2SO3 (1.5mg/ml) in 50 ml of 0.2M NaH2PO4</th>
<th>OD 420</th>
<th>Mean OD 420</th>
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<tr>
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<td>2</td>
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<td></td>
<td>3</td>
<td>375 µL</td>
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<td>at 37 * C</td>
<td>375 µL</td>
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<tr>
<td><strong>STD PL 10 ug</strong></td>
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<td>365 µL</td>
<td>375 µL</td>
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<td>1.222</td>
<td>1.155</td>
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<td></td>
<td>2</td>
<td>365 µL</td>
<td>375 µL</td>
<td>Incubate</td>
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<td>3</td>
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<td>at 37 * C</td>
<td>375 µL</td>
<td>1.125</td>
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<tr>
<td><strong>Modified PL 10 ug</strong></td>
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<td>375 µL</td>
<td>Incubate</td>
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<td>at 37 * C</td>
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</tr>
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</table>

**Table 3:** TNBS Assay for modified PL. Modification of epsilon amino groups of PL was approximately 31%.
3.5.2. Incorporation of DTPA on PL Polymers by ELISA:

ELISA to determine the presence of capture antigen (DTPA) on PL is shown in Figure 11. Anti-DTPA antibody bound D-PL indicating the presence of DTPA on the polymers. Aliquots of D-PL showed equivalent binding to the anti-DTPA Ab at the concentration of 1µg/ml relative to DTPA-BSA binding.

Figure 11: Superimposition of assessment of presence of capture antigen (DTPA) on D-PL using anti-DTPA ELISA assay. Squares represent DTPA-BSA and Circles represent D-PL.
3.6. Assessment of Immunoreactivity and Bispecificity of the Bom-bsCx:

3.6.1. ELISA for Detection of Bombesin on Bom-bsCx:

A standard curve for bombesin was generated and the concentration of bombesin in Bom-bsCx was estimated using the standard bombesin curve (Fig.12).

The amount of bombesin in Bom-bsCx, calculated using the equation of the line \( Y = 0.0737\ln(X) + 0.5712, R^2 = 0.9996 \) was about 0.9 to 1 mole of bombesin per mole of antibody.

![Figure 12: Normalized Standard Bombesin curve and serial dilutions of Bom-bsCx. The amount of Bombesin in the Bom-bsCx, as quantitated by ELISA, was about 0.9 to 1 mole of Bombesin per mole of antibody. The line represented by squares shows the concentration of Bombesin in Bom-bsCx.](image-url)
3.6.2. ELISA for Bispecificity of Bom-bsCx:

Bispecificity of Bom-bsCx is demonstrated by bispecific ELISA (Fig. 13). Bombesin of Bom-bsCx captured by anti-bombesin antibody allowed binding of DTPA-HRP by the anti-DTPA of the Bom-bsCx (O.D.405 nm = 0.272± 0.002, mean ± SD) which was significantly greater than non-specific background activity of bombesin alone (0.0001±0.0003) or anti-DTPA antibody alone (0.0001±0.0004) (p < 0.0001) at 1 µg/ml concentration. Bom-bsCx binding was still significantly higher at 10^{-6} µg/ml concentration relative to bombesin or anti-DTPA alone.

Figure 13: ELISA demonstrating bispecific activity using DTPA-HRP. Bom-bsCx starting at 1µg/ml Bombesin equivalent concentration, Anti-DTPA Ab (1µg/ml) and Bombesin alone (1 µg/ml) showed that only Bom-bsCx bound to anti-bombesin and DTPA-HRP simultaneously (p <0.0001 compared to bombesin or anti-DTPA Ab alone at respective concentrations). * indicates p <0.0001.
3.6.3. Competitive Inhibition ELISA for Bom-bsCx:

Competitive inhibition ELISA demonstrated that binding of Bom-bsCx to anti-bombesin antibody can be inhibited with free unmodified bombesin (Fig 14). Maximum inhibition was obtained at 1µg/ml of free bombesin and 90% inhibition was obtained between 0.1 and 0.01 µg/ml of free bombesin.

*Figure 14:* Competitive inhibition ELISA demonstrating inhibition of binding of Bom-bsCx to anti-Bombesin antibody at various concentrations of free Bombesin.
3.7. *In Vitro* Cell-Based Binding Assay for Bom-bsCx:

*In vitro* cell-based binding assay showed that PC3, 2G42D7 and H9C2 cells contained different concentrations of bombesin receptors expressed on the cell surfaces (Fig. 15). PC3 prostate cancer cells incubated with Bom-bsCx, Anti-DTPA (6C31H3) antibody and BSA (10µg/ml each) at 37°C for 1 hr showed Bom-bsCx binding (O.D. 490nm = 0.141±0.017) to be significantly greater than with free anti-DTPA (6C31H3) antibody (0.0202±0.012, p <0.0001) or BSA control (0.036±0.016, p <0.0001). Bom-bsCx also bound 6C31H3 hybridomas (0.154±0.02) similar to PC3 cells (p = NS) and significantly greater than binding to H9C2 cells (O.D. 490nm = 0.042± 0.010, p<0.0001). Cells of all 3 cell lines did not bind anti-DTPA mAb.

![Bar graph showing binding levels](image)

**Figure 15:** Binding of Bom-bsCx to H9C2, 2G42D7 and PC3 cells was significantly greater than binding of anti-DTPA (6C31H3) antibody or non-specific binding in BSA in the same cells (* indicates p <0.0001).
3.8. Fluorescence Ligand Binding of Bom-bsCx by Flow Cytometry and Epifluorescent Microscopy:

Fluorescence ligand binding assessed by Flow Cytometry showed PC3, 2G42D7 and H9C2 cells to contain varying concentrations of bombesin receptor expression on the cell surfaces (Fig. 16). PC3 and 2G42D7 hybridoma cells showed similar bombesin receptor expression. As in Fig 16, H9C2 showed minimal expression of bombesin receptors.

![Graph showing concentration vs. adjusted mean intensity](image)

**Figure 16:** Flow cytometry analyses of high expression of Bombesin receptors in PC3 and 2G42D7 hybridoma cells relative to the low expression of the same receptor in H9C2 embryonic cardiocytes.
Epifluorescent microscopy of PC3 cells pre-targeted with Bom-bsCx showed presence of bombesin receptors by capturing DRS-PL on PC3 cells. However, PC3 cells incubated with anti-DTPA (6C31H3) (10µg/ml), free Bombesin (10µg/ml) or untreated cells incubated for 1 hr at 40°C, followed by incubation of the washed cells with DRS-PL (25µg/ml) at 370°C for 1 hr showed no epifluorescence (Fig. 17).

**Figure 17:** PC3 cells pre-targeted with Bom-bsCx (10µg/ml) [top left] showed significantly higher fluorescent intensity than cells treated with free anti-DTPA antibody (10µg/ml) [top right], Bombesin (10µg/ml) [bottom left] or Untreated cells [bottom right] targeted with DRS-PL at 49.5 msec exposure to obtain digital micrographs. The corresponding DIC micrographs are shown on the right of each fluorescent micrograph.
Quantitation of the epifluorescent intensity by computer planimetry showed that PC3 cells pre-targeted with Bom-bsCx showed the highest rhodamine fluorescent intensity (relative mean fluorescent intensity = 52.48±3.14) which was significantly higher than those of cells pre-targeting with anti-DTPA antibody (4.8638±1.30), free Bombesin (3.652±0.974) or untreated cells (2.452±0.596) (P < 0.0001) (Fig. 18).

Figure 18: Fluorescent intensity of PC3 cells pre-targeted with Bom-bsCx was significantly greater than free anti-DTPA antibody (p <0.0001), free Bombesin (p <0.0001) or Untreated cells (p <0.0001) targeted with DRS-PL. * indicates p <0.0001 compared to Bom-bsCx.
3.9. *In Vitro* Reduction of Cardiocyte Toxicity:

IC$_{50}$ of H9C2 embryonic rat cardiocytes treated with serial dilutions of 2.5, 5, 10, 15 and 30 mg/ml Dox equivalent concentrations of D-Dox-PGA or free Dox and incubated for 24 hr at 37°C is shown in Figure 4A. IC$_{50}$ of D-Dox-PGA (22.22±0.27mg/ml) was significantly higher than that of free Dox (2.24±0.05 mg/ml, p<0.00005) resulting in approximately 10 times less non-specific cardiocyte toxicity (Fig. 19).

![Graph showing IC50 values](image)

**Figure 19:** IC$_{50}$ of H9C2 embryonic rat cardiocytes treated with D-Dox-PGA to be significantly less than that of Dox resulting in about 11 times less non-specific toxicity (p <0.00005).
3.10. *In Vitro* Assay for Tumor Toxicity Using Bom-bsCx in Human Prostate (PC3) Cancer Cells and Determination of IC\textsubscript{50} of D-Dox-PGA and Free Dox on PC3 Cells:

When PC3 cells were either pre-targeted or not pre-targeted and followed by targeting with serial dilutions of 5, 10, 20 and 30 \( \mu \)g/ml Dox equivalent concentrations of D-Dox-PGA or just free Dox, and the cells were incubated for 24 hr at 37\(^\circ\)C, IC\textsubscript{50} of free Dox was 26.09±1.04 \( \mu \)g/ml and that of pre-targeted PC3 and targeted with D-Dox-PGA was (11.87±1.14\( \mu \)g/ml, \( p < 0.00005 \), Fig. 20). However, the IC\textsubscript{50} of D-Dox-PGA in controls (Anti-DTPA or no treatment) determined by extrapolation of the equation of the curves were 1.28±0.034 and 1.34±0.04 gm/ml respectively, indicating that D-Dox-PGA was by itself not toxic even to cancer cells.

![Graph showing IC\textsubscript{50} of PC3 cells pre-targeted with Bom-bsCx (10\( \mu \)g/ml) followed by targeting with Dox equivalent concentrations of D-Dox-PGA to be significantly less than that with free Dox. IC\textsubscript{50} of anti-DTPA + D-Dox-PGA and D-Dox-PGA alone extrapolated from the curves was 1.28±0.034 gm/ml and 1.34±0.04 gm/ml respectively. * indicates \( p <0.0001 \).]
3.11. Assessment of Immunoreactivity and Bispecificity of the Bom-bsFCx:

3.11.1. ELISA for Detection of Bombesin on Bom-bsFCx:

The amount of bombesin in Bom-bsFCx, calculated using the equation \( Y = 0.0838 \ln(X) + 0.6516 \) \((R^2 = 0.992)\) of the curve for bombesin and anti-bombesin antibody (Figure 21), was about 1.2 to 2 mole of bombesin per mole of anti-DTPA Fab\(\prime\).

**Figure 21:** Normalized Standard Bombesin curve and serial dilutions of Bom-bsFCx. The amount of Bombesin in the Bom-bsFCx, as quantitated by ELISA, was about 1.2 to 2 mole of Bombesin per mole of Fab\(\prime\). The line represented by squares shows the concentration of Bombesin in Bom-bsFCx.
3.11.2. ELISA for Bispecificity of Bom-bsFCx:

Bispecificity of Bom-bsFCx is demonstrated in Figure 22. Binding of DTPA-HRP by Bom-bsFCx (O.D.405 nm of 0.132±0.001, mean ± 1 SD) was significantly greater than that of DTPA-HRP by bombesin (0.001±0.003) or anti-DTPA antibody alone (0.000±0.003) (p < 0.001) at 1 µg/ml concentrations. Bom-bsFCx still bound DTPA-HRP significantly higher at 10^{-5} µg/ml concentration relative to bombesin alone.

![Figure 22: ELISA for demonstration of bispecific activity using DTPA-HRP. Bom-bsFCx starting at 1µg/ml Bombesin equivalent concentration, Anti-DTPA Ab (1µg/ml) and Bombesin alone (1 µg/ml) showed that only Bom-bsFCx bound to anti-bombesin and DTPA-HRP simultaneously (p <0.001 compared to bombesin or anti-DTPA MAb alone at respective concentrations). * indicates p <0.001, NS indicates no significance.](image-url)
3.11.3. Competitive Inhibition ELISA for Bom-bsFCx:

Binding of Bom-bsFCx to anti-bombesin antibody can be inhibited by free unmodified bombesin is shown in Figure 23. Maximum inhibition was obtained at 10µg/ml free bombesin with 90% inhibition between 1 and 0.1 µg/ml of free bombesin.

**Figure 23:** Competitive inhibition ELISA demonstrating inhibition of binding of Bom-bsFCx to anti-Bombesin antibody by various concentrations of free Bombesin.
3.12. *In vivo* Animal Model Studies:


**Elution Profile for Tc-99m-DSPL:**

Tc-99m-DSPL was eluted in the void volume starting at around fraction no. 6 in Sephadex G25 column chromatography. Free $^{99m}$Tc was eluted in the inclusion volume at around fraction no. 17 (figure 24). Approximately 2 ml of Tc-99m-DSPL were acquired in the peak fractions and used for in vivo studies.

In our laboratory, 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. 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 $296 \pm 18.5$ MBq of $^{99m}$Tc ($\approx 3.0 \times 10^8$ GBq/mole DSPL). 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 (72).
**Figure 24:** Elution profile for Tc-99m-DSPL. Tc-99m-DSPL was eluted in the void volume starting at fraction no. 6. Free $^{99m}$Tc was eluted in the inclusion volume at around fraction no. 17.

### 3.12.2. Preparation of $^{111}$In-Labeled DSPL:

**Elution Profile for $^{111}$In-Labeled DSPL:**

In-111-DSPL was eluted in the void volume at around fraction no. 7 and free In-111 was eluted in the inclusion volume at around fraction no. 18 (Figure 25) by size exclusion Sephadex G-25 column chromatography. Approximately 1.5 ml of In-111-DSPL were acquired and used for further studies.
Assuming approximately 71% recovery of In-111-DSPL (14.6 kDa) after Sephadex-G25 column chromatography (Figure 25), 71 µg of the polymer were assumed to be recovered. 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 35 ± 3.5 MBq of In-111.

**Figure 25:** Elution profile for In-111-DSPL. In-111-DSPL was eluted in the void volume starting at fraction no. 7. Free $^{111}$In was eluted in the inclusion volume at around fraction no.18.
3.12.3. $^{99m}$Tc-Labeled DTPA- Bom-bsCx In Vivo Clearance:

Elution Profile for $^{99m}$Tc-Labeled DTPA-Bom-bsCx:

$^{99m}$Tc-Labeled DTPA-Bom-bsCx was eluted in the void volume at around fraction no. 5 and free $^{99m}$Tc-DTPA was eluted in the inclusion volume at around fraction no.19 (Figure 26) by size exclusion Sephadex G-25 column chromatography. Approximately 1.5 ml of $^{99m}$Tc-Labeled DTPA-Bom-bsCx (49 ± 2.5 MBq) were acquired and used for clearance study.

![Elution profile for $^{99m}$Tc-Labeled DTPA-Bom-bsCx](image)

**Figure 26:** Elution profile for $^{99m}$Tc-Labeled DTPA- Bom-bsCx. $^{99m}$Tc-Labeled DTPA-Bom-bsCx was eluted in the void volume starting at fraction no. 5. Free $^{99m}$Tc-Labeled DTPA was eluted in the inclusion volume at around fraction no.19.
3.12.4. Preparation of $^{99m}$Tc-Labeled DTPA-Bom-bsFCx:

**Elution Profile for $^{99m}$Tc-Labeled DTPA-Bom-bsFCx:**

The elution profile of $^{99m}$Tc-Labeled DTPA-Bom-bsFCx by Sephadex-G25 column chromatography was similar to that of $^{99m}$Tc-Bom-bsCx (Figure 27). Approximately 1.5 ml of $^{99m}$Tc-Labeled DTPA-Bom-bsFCx (47 ± 1.5 MBq) were acquired and used for further studies.

![Figure 27](image)

**Figure 27:** Elution profile for $^{99m}$Tc-Labeled DTPA-Bom-bsFCx. $^{99m}$Tc-Labeled DTPA-Bom-bsFCx was eluted in the void volume starting at fraction no. 5. Free $^{99m}$Tc-Labeled DTPA was eluted in the inclusion volume at around fraction no.19.
3.12.5. *In Vivo* Clearance of Bom-bsCx and Bom-bsFCx:

Planar images of a mouse at 30, 45, 450 min, and 24hr after $^{99m}$Tc-Bom-bsCx injection are shown in Figure 28. Computer planimetry of serial image analyses showed time activity curves of the heart, liver, kidneys, salivary glands, spleen and bladder [Fig 29]. Biodistribution at 24 hrs showed maximum accumulation of $^{99m}$Tc-Bom-bsCx activity in the liver (16.75±3.6 %ID/gm). The heart (0.25±0.038 %ID/gm) and blood (0.73±0.040 %ID/gm) showed minimum radioactivity [Fig 30]. Therefore, a pre-targeting waiting time of 20 to 24 hours was considered sufficient for Bom-bsCx to clear.

**Figure 28:** Anteroposterior planar gamma images of C57 BL/6 normal mice (n=3) injected with 14 – 16 MBq $^{99m}$Tc-Bom-bsCx (5-10 µg/mouse). After radiotracer injection, planar images were acquired at 30 min, 45 min, 450 min, and 24hr time point. S: Salivary gland, H: Heart, L: Liver, Sp: Spleen, B: Bladder, K: Kidney.
Figure 29: Each image (n=1) was analyzed using ImageJ program from NIH.

Figure 30: Biodistribution of $^{99m}$Tc-Bom-bsCx (5-10 µg/mouse) injected mice (n=3) at 24 hrs. Error bars = Mean ± 1 SD.
Similarly serial planar images of a mouse injected with $^{99m}$Tc-Bom-bsFCx, at the time of injection, 180 and 480 min are shown in Figure 31. Computer planimetered time activity curves are shown in Figure 32. Biodistribution after 8 hrs showed maximum accumulation in liver (13.52±2.33 %ID/gm) and spleen (27.45±1.62 %ID/gm) whereas minimal radioactivity remained in the heart (0.71±0.071 %ID/gm) and blood (0.66±0.15 %ID/gm [Fig 33]. Therefore for subsequent in vivo imaging studies with Bom-bsFCx, 8 to 12 hours pre-targeting wait time was used.

**Figure 31:** Anteroposterior planar gamma images of C57 BL/6 normal mice (n=5) injected with 13-15 MBq of $^{99m}$Tc-Bom-bsFCx (5-10 µg/mouse). After radiotracer injection, planar images were acquired at 5 min, 3hr, and 8hr time points.

Figure 32: Each image (n=1) was analyzed using ImageJ program from NIH.

Figure 33: Biodistribution of $^{99m}$Tc-Bom-bsFCx (5-10 µg/mouse) at 8 hrs in C57 BL/6 normal mice (n=5). Error bars = Mean ± 1 SD.
3.12.6. *In Vivo* Polymer Clearance Studies:

Serial planar images acquired at 5, 45, 180 min, and 24hr after intravenous injection of $^{99m}$Tc-DSPL are shown in Figure 34. Time activity curves from computer planimetry are shown in Figure 35. Biodistribution at 24 hrs showed maximum accumulation in the kidneys (4.28±0.33 %ID/gm) with minimal heart (0.25±0.02 %ID/gm) and blood (0.07±0.015 %ID/gm) radioactivities [Fig 36]. Most of the radioactivity associated with the polymers has cleared from the blood by 3 hours.

**Figure 34:** Anteroposterior planar gamma images SCID mice (n=3) were injected with 30 – 35 MBq $^{99m}$Tc-DSPL (2-3µg/mouse). After radiotracer injection, planar images were acquired at 5 min, 45 min, 180 min, and 24hr time point.

H: Heart, B: Bladder, K: Kidney.
Figure 35: Each image (n=1) was analyzed using ImageJ program from NIH.

Figure 36: Biodistribution of $^{99m}$Tc-DSPL (2-3µg/mouse) at 24 hrs in SCID mice (n=3). Error bars = Mean ± 1 SD.
3.12.7. *In Vivo* Targeting Xenograft Tumors Using Bom-bsCx:

In vivo planar gamma images acquired at 1 hr, 3 hr and 24 hrs after intravenous injection of In-111 DSPL in pre-targeted xenografted PC-3 tumor bearing mice are shown in Fig 37. PC-3 prostate xenograft lesions of less than 2 mm were visualized within 1-3 hr. Control mouse injected with In-111-DSPL alone did not show any accumulation of radioactivity in the tumor. In-111-DSPL cleared rapidly via renal excretion. Very minimal radioactivity accumulated in the liver and the heart. Xenografted mice were also imaged with BIOSCAN/CT. The coronal, saggital and transverse tomographic views at 3hr post-In-111 DSPL injection showed tumor uptake. Figure 38 shows the transverse images of a mouse pre-targeted with Bom-bsCx followed by In-111-DSPL and a control mouse injected only with In-111-DSPL also at 3hr post-injection of the radiotracer. After 24 hours, the biodistribution data showed that the tumor activity in the pre-targeted tumors (1.21±0.36 %ID/g) was 5.4 times that of In-111-DSPL activity in the control tumors (0.22±0.08, p = 0.001). Other organ activities were significantly less than tumor activity (p = 0.0016-0.0026) [Fig 39] except for the kidneys.
Figure 37: Posteroanterior planar gamma images of pre-targeted xenografted PC-3 tumor bearing SCID mouse 1 at 1 hr (i), 3 hr (ii) and 24 hrs (iii), SCID mouse 2 at 30 mins (iv) and 24 hrs (v) after intravenous injection of In-111 DSPL and SCID mouse (control) at 3 hr (vi) and 24 hrs (vii) after intravenous injection of In-111 DSPL.
**Figure 38:** Transverse images of a mouse pre-targeted with Bom-bsCx followed by In-111-DSPL (slides 42 and 92, white arrows) and a control mouse injected only with In-111-DSPL at 3hr post-injection (slides 39 and 83). Black arrows indicate tumor lesions.
Figure 39: Biodistribution of In-111-DSPL in PC-3 tumor bearing SCID mice pre-targeted with Bom-bsCx followed by targeting with In-111-DSPL (filled bars, n=4), injected with In-111-DSPL pre-targeted with anti-DTPA or Bombesin alone (open bars, n=5). * indicate the p values of 0.001.
3.12.8. *In Vivo* Targeting Using Bom-bsFCx:

*In vivo* planar gamma imaging studies were also undertaken using the planar SPECT micro-camera with $^{99m}$Tc-DSPL in Bom-bsFCx pre-targeted and non-pre-targeted control mice. The imaging studies were undertaken at two different times to confirm reproducibility. Planar posteroanterior images at 1hr and 3 hr are shown in Figure 40. These images showed radiotracer localization in PC-3 prostate cancer lesions of less than 2 mm in length as early as 1-3 hr after $^{99m}$Tc-DSPL i.v administration.

Mice were imaged again at 24 hr post-injection. Figure 41 shows postero-anterior images of a mouse pre-targeted with Bom-bsFCx and another with bombesin alone and targeting with $^{99m}$Tc-DSPL. The control mice injected with bombesin and $^{99m}$Tc-DSPL alone did not show any accumulation of activity in the tumors. $^{99m}$Tc-DSPL cleared rapidly via renal excretion and very little accumulation was observed in the liver. Figure 41 shows that pre-targeting with Bom-bsFCx had higher tumor accumulation (white arrows) as compared to very little or no activity in the control mice.

After 24 hours, tumors pre-targeted with Bom-bsFCx had tumor activity of $6.54 \pm 1.58$ %ID/g with minimal liver activity ($1.68 \pm 0.46$), whereas the tumor and liver activities of Bombesin pre-targeted controls were $0.44 \pm 0.17$ and $0.85 \pm 0.28$ respectively ($p = 0.002$ and 0.07 respectively) [Fig 42].
**Figure 40:** Posteroanterior planar gamma images of PC-3 tumor xerographs bearing SCID mouse 1 pre-targeted with Bom-bsFCx followed by $^{99m}$Tc-DSPL at 1 hr (i) and 3 hr (ii) post injection.

**Figure 41:** Posteroanterior planar gamma images of PC-3 tumor xerographs bearing SCID mouse pre-targeted with Bombesin alone (i) and Bom-bsFCx (ii) followed by $^{99m}$Tc-DSPL at 24 hr post injection.
Figure 42: Biodistribution of $^{99m}$Tc-DSPL in PC-3 tumor bearing SCID mice pre-targeted with Bom-bsFCx followed by targeting with $^{99m}$Tc-DSPL (filled bars, n=4), injected with $^{99m}$Tc-DSPL pre-targeted with Bombesin alone (open bars, n=3). ‡ and * indicate the p values of 0.02 and 0.002 respectively.
4. DISCUSSION:

Monoclonal antibodies have become near ideal “magic bullets” for targeting tumors. However there are limitations and, to overcome some of these limitations for therapy and diagnosis, new modalities such as antibody conjugates were developed to increase their efficiency (42). Antibody conjugates can be generated by attaching bacterial toxins, radionuclides, peptide analogs and cytotoxic drugs (42, 60). Direct coupling of the effector compounds to antibodies may cause alterations to the effector function (44) or its immunoreactivity. Problems may also arise if the covalent bonds of the conjugates need to be broken for full biological activity at the target-antibody interface (45). An additional problem of immunoconjugates is the non-specific interaction of its Fc domain with Fc receptors on the cells of the reticuloendothelial system. This would increase the Fc receptor-mediated accumulation of immunoconjugates in organs such as liver and spleen. If such immunoconjugates are used for imaging of tumors, high non-target organ activity and background noise will ensue (46). The non-specificity of intact antibodies may be reduced by using fragments of antibodies lacking the Fc region.

Additional limitations include: a) non-specific activity of these antibodies for targeting tumors since many mAbs were generated to embryonic antigens or tumor associated antigens that were also present but in lower concentrations on non-malignant cells (7, 8); and b) antibodies are positively charged while cells surfaces and ground substances are negatively charged, therefore ionic interactions between radiolabeled antibodies and cells and ground substances result in higher non-target background activity (9).

An alternative approach for tumor targeting using a multi-stage delivery system with bispecific monoclonal antibodies (bsMAb) technology was developed to reduce non-specific background activity (7, 9). Bispecific antibodies that have two different antigen-specific
binding sites, one for the target antigen (such as tumor associated antigen) and the other for the effector compounds (effector binding arm), have been developed (45). Bispecific antibodies first target the tumor via its tumor specific arm. After allowing clearance of the circulating bsMAb, the effector compound, which is recognized by the second arm of the bsMAb, is injected leading to its specific localization at the tumor (74). This approach minimizes non-target background activity and maximizes tumor targeting for \textit{in vivo} imaging. BsMAb consists of a targeting MAb linked covalently to a second MAb that can capture a signal carrying molecule for diagnostic applications (75) or activate a receptor on the effector cells for therapeutic applications (9). This technology has led to the development of two-step imaging or pre-targeted imaging technology where bsMAbs were injected first and after the excess bsMAb has cleared from the blood, mono- or di-valent radiolabeled haptens were injected (9, 75). Non-specifically bound bsMAbs do not raise the background activity because the bsMAbs were not radiolabeled and clear from the circulation by phagocytosis and/or endocytosis (59, 69). In recent years, Positron Emission Tomography (PET) imaging using I-124-labeled haptens after pre-targeting with bsMAbs has been achieved (75). Clinical trials have also been successful (76). However unequivocal visualization of very small malignant lesions remains problematic due to the low target activity of radiolabeled mono and divalent haptens used for the delivery of the radiotracers. To overcome this limitation, we have developed negatively charged polymers that can be radiolabeled with high specific radioactivity and that are sequestered at the target sites pre-targeted with a bsMAb (69, 76). Experimental atherosclerotic lesions of 2.5 mg mean mass were imaged in ApoE knockout mice (69) and 4 µg of surrogate antigen-coated polystyrene beads were imaged in a surrogate antigen mouse model pre-targeted with bispecific
F(ab')₂xF(ab')₂ antibodies (77). The results of the imaging of metastatic melanoma lesions in the lungs of mice using bsMAbs and $^{99m}$Tc-DSPL are now in press (72).

Bombesin has been used as a targeting ligand for the development of bispecific complexes. We have replaced the targeting antibody of our bsMAb with Bombesin, a 14 amino acid ligand specific for the Bombesin receptors that are over-expressed in various cancers such as prostate cancer (64). Bombesin was isolated from the skin of the European frog Bombina bombina by V. Erspaner and his colleagues and led to the establishment of the Bombesin receptor family (34, 35). Bombesin was chosen as the targeting ligand for the development of bispecific antibody complexes because Bombesin receptors, also known as GRP receptors, are over-expressed in many different types of cancer such as prostate, breast and hematological cancers (29). Bombesin also has an amino group residue at its 13th peptide position for chemical modification without affecting its binding to Bombesin receptors (64).

GRP receptors are growth receptors and therefore their ligands have pharmacological and physiological functions. Bombesin has been reported to stimulate B lymphocyte proliferation by activation of the Bombesin receptors (78). Determination of the presence of Bombesin receptors in hybridoma, PC3 and H9C2 cells demonstrated that Bombesin receptors are over-expressed in PC3 (26) and hybridoma cells but are expressed only minimally in cardiocytes. Since hybridomas are generated by fusion of spleen cells and myeloma cells, which are cancer cells of the B lymphocyte lineage, it is not unexpected to find over-expression of Bombesin receptors in hybridomas. Since H9C2 cells are not malignant cells, upregulation of Bombesin receptor expression is not expected.

Since pre-targeting with bispecific antibodies greatly improves tumor-to-blood ratios in in vivo targeted drug delivery (59), we have utilized this two-step pre-targeting approach
to demonstrate that the targeting of PDCs improves the therapeutic index in initial in vitro studies. In in vivo applications, if Bom-bsCx is used as the pre-targeting moiety not labeled directly with signal nor drug molecules, it will bind to cells expressing the target receptors and the excess will be cleared from the circulation after some time. The rate of clearance will depend on the size and affinity of Bom-bsCx for the target and/or the reticuloendothelial system. If sufficient pre-targeted Bom-bsCx is not internalized into the target cells, cell surface localized Bom-bsCx will permit capture of the polymer-drug conjugates delivered in the second step whether they are radiolabeled (61) or loaded with therapeutic drugs (62). Our study indicated that there was sufficient Bom-bsCx remaining on the surface of PC3 cells after 24 hours of incubation permitting subsequent targeting with D-Dox-PGA and leading to greater cancer cell toxicity by 2.5 times that of free Dox treatment at the IC$_{50}$ concentration of 11.87 µg/ml (equivalent Dox concentration).

Polyglutamic acid was chosen as the polymer for conjugation of Doxorubicin because of its biocompatibility and hydrophilicity. 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 (55). The availability of PGAs of different molecular sizes also allows the preparation of PDCs with increased or decreased in vivo $T_{1/2}$ (55). PDCs consisting of PGA will also possess a net negative zeta potential that will cause it to be repelled by the negatively-charged cell surfaces and ground substances (79) and lead to decreased background activity and increased bioavailability for targeting. If the size of the PDCs is large enough (or if modified with polyethylene glycol (80) to render them long circulating), passive targeting due to the EPR effect will provide additional drug localization (55, 80).
Doxorubicin is the front-line chemotherapeutic agent for cancer therapy but its optimal use is limited by its toxicity to normal cells and to the heart (81). The primary clinical limitation of Dox is its cardiotoxicity (81). Its versatility on the other hand is due to the presence of an amino group in the 3’ position of the aglycone that is free to react with other molecules to produce covalently conjugated pro-drug complexes. Similar to previous studies (82), the current results confirmed that covalent linkage of Dox to PGA via the peptide bond between the amino group at the 3’ position of the aglycone of Doxorubicin and the carboxyl groups of PGA reduced non-specific cell toxicity in rat embryonic cardiocytes. We assumed that H9C2 rat embryonic cardiocytes were similar to adult cardiocytes and that the toxicity due to Doxorubicin in these cells (IC_{50} = 2.24 µg/ml) will also be similar to in vivo cardiotoxicity induced by reactive oxygen species (83). Cardiocytes possess lower amounts of superoxide dismutase and large amounts of mitochondria relative to other cells and therefore are more susceptible to oxygen radical injury (83). However, when Doxorubicin is covalently linked to PGA, it functions as an inactive pro-drug with an IC_{50} of 22.22 µg/ml in these cardiocytes. In addition, the negatively charged polymers prevent or reduce the uptake of Doxorubicin-PDC by ionic repulsion. Due to this reduction of PDC uptake by cells, even PC3 cells were only minimally affected in vitro after 24 hr of incubation. IC_{50} of non-pre-targeted PC3 cells is about 1.34±0.04 gm/ml equivalent Dox concentration, which was outside the range of the study concentrations. However when PC3 cells were pre-targeted with Bom-bsCx, followed by treatment with D-Dox-PGA, IC_{50} was 11.87 µg/ml while that of free Dox was 26.09 µg/ml (p < 0.05). Therefore, Bom-bsCx pre-targeted-PDC targeted treatment of PC3 prostate cancer cells has greater than 21 times the tumor-specific killing capacity [(Cardiocyte IC_{50} of D-Dox-PGA ÷ Dox) x (PC3 IC_{50} of Dox/pre-targeted-D-Dox-PGA) = (22.2/2.24) x (26.09/11.87) = 21.8] of free Dox in our in vivo experiments.
vitro culture system. How this observation will translate to the in vivo therapeutic index must await additional studies. Nevertheless, in cell cultures, the maximum effect of free Dox is favored whereas that of pre-targeting is disadvantaged since Dox in culture is not subjected to clearance whereas excess Bom-bsCx is washed away in the washing step. On the other hand, in in vivo situations, Dox clearance from the circulation would extensively decrease the therapeutic availability of the drug.

For in vivo applications, bispecific antibodies utilizing tumor specific/associated antibodies linked to hapten capture antibody have been used for pre-targeting, followed by targeting with radiolabeled haptens (60, 62). We have replaced the targeting antibody with bombesin, a 14 amino acid ligand specific for the Bombesin receptors over-expressed in various cancers such as prostate cancer (64).

Radiolabeled bombesin has been used for imaging prostate cancer in patients (64). However small lesions are difficult to detect due to insufficient concentration of target molecules to allow low specific radioactivity targeting agents to accumulate in sufficient concentration for in vivo imaging. By the time diagnosis of prostate cancer is made by determination of the elevation of PSA levels or digital rectal examination of enlarged prostate, prostate cancer metastases may already have occurred (84). Therefore, there is a need for highly sensitive diagnostic methods that can detect very small lesions. Small lesions are also amenable to more efficient targeted therapy than larger lesions because high intra-tumoral pressure would counter the delivery of therapeutic drugs (88). The limitation of the current study is that the leaky tumor vasculature may not be well developed and therefore delivery of imaging or chemotherapeutic agents via enhanced permeability and retention effect (88) may not have a prominent role. Therefore, small pre-targeting and targeting reagents that can freely diffuse to small lesions would be optimal for this application.
By substituting the targeting antibody with Bombesin in the development of our bispecific ligand antibody complexes, we have made bispecific antibody complexes with slightly larger molecular size than intact antibody or Fab’. Bom-bsCx has a molecular weight of about 152 kDa and Bom-bsFCx has a molecular weight of 52-53 kDa. The wait time for the clearance of Bom-bsCx was approximately 24 hours whereas the clearance time of Bom-bsFCx was about 8-12 hours. The polymer size was selected so that clearance from the circulation via renal excretion is within a few hours while allowing sufficient accumulation at the pre-targeted sites. The polymers are radiolabeled at high specific radioactivity with either In-111 or $^{99m}$Tc. The specific activity of $^{99m}$Tc-labeled DSPL is 19-21 mBq/µg of polymer or 0.0277 mol of $^{99m}$Tc per mol of polymer (72), which is greater than that of radiolabeled mono or divalent haptens (85, 86).

*In vivo* planar gamma images acquired after intravenous injection of In-111-DSPL in xenografted PC-3 tumor bearing mice pre-targeted with Bom-bsCx showed lesions of less than 2-3 mm in diameter within 1-3 hr after intravenous radiotracer injection. Control mice injected with In-111-DSPL alone did not show any accumulation of radioactivity in the tumors. In the 3 hr transverse SPECT/CT co-registered images, no blood pool activity was evident in the pre-targeted or control non-pre-targeted mice (Figure 37, lower SPECT/CT co-registered images) even though the CT images showed the hearts clearly. After 24 hours, the biodistribution data showed that the tumor activity in the pre-targeted tumors ($1.21\pm0.36$ %ID/g) was 5.4 times that of In-111-DSPL activity in the control tumors ($0.22\pm0.08$, $p = 0.001$). Other organ activities were significantly less than those of the pre-targeted tumor activity ($p = 0.0016-0.0026$).

*In vivo* imaging was improved using Bom-bsFCx and $^{99m}$Tc-DSPL. Postero-anterior images of a mouse pre-targeted with Bom-bsFCx and another with Bombesin alone followed
8 hours later by targeting with $^{99m}$Tc-DSPL enabled the visualization of tumors less than 2-3 mm in size by planar imaging as early as 1-3 hours. After 24 hours, tumors pre-targeted with Bom-bsFCx had tumor activity of $6.54\pm1.58$ %ID/g with minimal liver activity ($1.68\pm0.46$), whereas the tumor and liver activities of Bombesin pre-targeted controls were $0.44\pm0.17$ and $0.85\pm0.28$ respectively ($p=0.002$ and 0.07 respectively). Minimal liver activity was also evident in the *in vivo* images at 24 hours (Figure 41). The mean $^{99m}$Tc activity ratio in pre-targeted and non-pre-targeted tumors was approximately 15:1 by gamma scintillation counting and by computer planimetry of Figure 40 and 41 was 10.3:1 and 19.3:1 respectively.

Using this pre-targeting approach with bispecific antibody complexes and targeting with high specific radioactivity labeled polymers that enabled the visualization of very small cancer lesions, one should also be able to deliver high specific activity chemotherapeutic agent-conjugated polymers for therapy (88). Additionally, if both radiolabeled and chemotherapeutic agents are simultaneously loaded onto the polymers, one should be able to visualize and confirm delivery of the chemotherapeutic drugs to the lesions.

**Limitations of our current studies:**

Proper selection of anesthesia for use in mice is a very important parameter and depends mainly on which type of experiments needs to be performed as well as the different routes of the delivery systems available. Important parameters to be considered are strain of the mice, duration of the procedure, and the physical limitations needed for the animals of the planned study. Anesthetic regimens are of two types: injectable and inhalable. The most commonly used anesthetics in mice include injectable agents avertin, pentobarbital, thiobutabarbital, chloralose-urethane, ketamine (usually combined with other agents such as
xylazine, and/or diazepam), and inhalable anesthetic agents such as isoflurane, methoxyflurane, and halothane (89).

The advantage of using ketamine-xylazine in a long procedure is that the ketamine-xylazine combination will produce a deeper anesthesia which will permit longer and invasive procedures (89). Then as the effect of shorter acting ketamine begins to wear off, the level of anesthesia can be monitored extensively. It should be recognized that xylazine does not have a very long duration of action and therefore must be supplemented regularly. Nonetheless, it is generally accepted that its use for recovery surgeries should be avoided (90). However, it is highly recommended to use 100% O2 to anesthetized mice to prevent hypoxia. It is also observed that anesthetized mice are more prone to hypothermia and it is important to monitor body temperature at regular basis and carefully regulate it. Providing supplemental heat using a heat lamp is highly recommended and will ensure uniform warming (90).

The use of inhaled anesthetics (isoflurane in our case) for prolonged analytical procedures is highly recommended. The advantages of isoflurane are that it maintains sympathetic vasomotor activity, has minimal cardiodepressor effects, and allows very careful, minute-to-minute control of the anesthetic plane (91). The disadvantages of isoflurane are that: it requires expensive equipment; can be physically challenging (especially when the procedure requires extensive instrumentation and multiple changes in animal posture); the depth of anesthesia can be volatile if its administration is not finely and carefully controlled; and a mechanical ventilator is usually needed (91).
Future Prospect of the studies:

Surgery, chemo- and radiation-therapy still represent the golden standards for current cancer therapy. Surgery is generally effective in the treatment of primary cancerous lesions. However surgical resection of malignant tumors may not result in complete cure.Chemotherapy is not a reliable treatment because cancer cells often become immune to chemotherapy. The patients show more toxic effects and their suffering from chemotherapy is more extensive while in most cases the cancer itself is unaffected. Radiation therapy has also been used to target cancer cells because cancer cells are less able to repair themselves after treatment with radiation. However, radiation cannot be used to treat many types of cancers because of the damage done to normal cells surrounding cancerous tissues.

Some of the most common chemotherapeutic drugs used and their mechanism of action are described below:

1. Doxorubicin:

Mechanism of action: Topoisomerase II inhibitor (92).

Doxorubicin binds to Topoisomerase II resulting in the inhibition of DNA replication and hence causing cell cycle arrest. Double strands break to induce apoptosis in cancer cells (92).

2. Melphalan:

Mechanism of action: Covalent DNA binding.

“The chloroethyl side chain present in melphalan cyclizes spontaneously in an aqueous solution to form an aziridinium ion that is capable of targeting the nucleophilic sites of DNA.
This monoadduct can form a second aziridinium ion that can add to other nucleophilic sites of DNA resulting in crosslinking of two DNA bases” (93). This causes a halt in DNA replication and causes cell cycle arrest.

3. Camptothecin (CPT):

Mecanism of action: Topoisomerase I inhibitor (94).

“CPT binds to the Topoisomerase I and DNA complex (covalent complex), resulting in a ternary complex, and thereby stabilizing it. This prevents DNA re-ligation and therefore causes DNA damage which results in apoptosis” (94).

4. Paclitaxel:

Mechanism of action: Mitotic inhibitor (95).

“Paclitaxel stabilizes the microtubule polymer and protects it from disassembly. The inability of the chromosomes to achieve a metaphase spindle configuration leads to a mitotic block with subsequent triggering of apoptosis” (95).

In most cases cancer cells become immune to chemotherapeutic drugs. This phenomenon is called drug resistance and many cancers quickly become resistant to many drugs earning the title of Multi-Drug Resistance (MDR) (96). Chemotherapeutic treatments trigger cancer cells to take evasive action. They build fast acting pumping stations that round up the drugs and pump them out through the cell membranes, lowering the concentration and effect of the chemotherapy within the cancer cells (96). Normal healthy cells do not react this way and therefore continue being hit by the chemotherapy. This pumping action is done
by a specific protein, called P-glycoprotein (Pgp). The more P-glycoprotein a cancer cell makes, the less it is affected by drugs like Doxorubicin, Vincristine, and Taxol (97).

Quite a few substances have been found which have an inhibitory effect on the P-glycoprotein and reverse Multi Drug Resistance. The drug Verapamil is often used as a standard for comparing the effect of other substances. Theanine and Curcumin, the extracts from green tea and turmeric respectively, fit into this category of inhibiting P-glycoprotein and reversing Multi Drug Resistance (99). In a mouse experiment with drug resistant ovarian cancer, Theanine significantly enhanced the inhibitory effect of Doxorubicin on tumor growth and increased the drug concentration in the tumors relative to the control group which was given doxorubicin alone (99). Progesterone is a frequently used steroid hormone to treat PMS, miscarriage and menopausal complaints. It appears to be useful when used with Vincristine for cancer therapy. Progesterone increases concentrations of chemotherapeutic drugs within the cancer cells, acting like other P-glycoprotein inhibitors, but does not affect healthy cells (100). The use of these MDR inhibitors can improve chemotherapy.

Another approach for delivering chemotherapeutic drugs and avoiding MDR would be to deliver these drugs as prodrugs to malignant cells. The targeting of tumor cells can be achieved by active targeting using antibodies or ligand/receptor interactions. Therefore, it would be beneficial to deliver a combination of therapeutic reagents such as cytotoxic drugs, MDR inhibitors and anti-angiogenic drugs via a single delivery system to the tumors. Such a combination therapy at the molecular level may be achieved by loading multiple drugs on polymers and targeting them to the tumors.

The polymer-drug conjugates used in our studies consisted of Doxorubicin. It would be ideal if multiple drugs such as Doxorubicin, Melphalan, Paclitaxel, Theanine and
Camphotecin could be conjugated to the same polymer. This would help to avoid MDR, deliver adequate dose to the tumor, and prevent non-specific toxicity. Simultaneously radiolabeling these drug-conjugated polymers would allow the monitoring and confirmation of the delivery of the chemotherapeutic prodrugs to the cancerous lesions.
5. SUMMARY AND CONCLUSIONS:

Pre-targeting with Bom-bsCx demonstrated over-expression of Bombesin receptors on PC3 and Hybridoma cells, but minimal expression of the receptors on H9C2 embryonic cardiocytes. This pre-targeting approach with Bom-bsCx enabled delivery of PDCs that resulted in higher therapeutic index in PC3 cancer cell therapy in our initial \textit{in vitro} studies. Additionally, covalent conjugation of Doxorubicin to PGA polymers provided pro-drug polymer conjugates with less cardiocyte toxicity but greater PC3 cancer cell toxicity when the pro-drug polymer conjugates were pre-targeted with Bom-bsCx. Therefore, pre-targeting with Bom-bsCx and targeted delivery of pro-drug-polymer conjugates may lead to prostate cancer therapy with greater therapeutic outcome.

Pre-targeting with bispecific antibody complexes to human prostate cancer PC3 lesions followed by high specific radioactivity-labeled polymers, designed to clear from the blood quickly, allowed the capture of PDCs at the bispecific antibody pre-targeted sites and provided sufficient tumor lesion activity to enable visualization of cancerous lesions as small as 1-2 mm in diameter. Smaller lesions were visualized using a BIOSCAN/ CT camera, indicating that gamma cameras with higher sensitivity and better resolution may allow the diagnostic imaging of even smaller lesions.
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APPENDIX

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, Vishwesh Patil 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: tw0000000009729 (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, Vishwesh Patil 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: tw0000000009726 (Session).
Appendix 2: Occupational Health & Safety for Working with Animals in Research and Teaching.

In May, 2009, Vishwesh Patil 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 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 March 31, 2010, Vishwesh Patil 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: tw0000000009824 (Session).

Chemical Hygiene Training Part 2 – Lab Safety and Hazardous Waste Management.
Northeastern University Office of Environmental Health & Safety
On March 31, 2010, Vishwesh Patil 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: tw0000000009825 (Session).