Studies in Multifunctional Drug Development:
Preparation and Evaluation of 11β-Substituted Estradiol-Drug Conjugates, Cell Membrane Targeting Imaging Agents, and Target Multifunctional Nanoparticles

by

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ABSTRACT OF DISSERTATION
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Abstract

Cancer is the second leading cause of death after cardiovascular disease in the United State. Despite extensive research in development of antitumor drugs, most of these therapeutic entities often possess nonspecific toxicity, thus they can only be used to treat tumors in higher doses or more frequently. Because of the cytotoxicity and severe side effects, the therapeutic window for the drug normally is limited. Beside the toxicity issue, antitumor drugs are also not selectively taken up by tumor cells, thus the necessitating concentrations that would eradicate the tumor can often not be used. In addition, tumor cells tend to develop resistance against the anticancer drugs after prolonged treatment. Therefore, alleviating the systemic cytotoxicity and side effects, improving tumor selectivity, high potency, and therapeutic efficacy are still major obstacles in the area of anticancer drug development. A more promising approach for developing a selective agent for cancer is to conjugate a potent therapeutic drug, or an imaging agent with a targeting group, such as antibody or a high binding-specificity small molecule, that selectively recognize the overexpressed antigens or proteins on tumor cells. My thesis combines several approaches to describe this strategy via using different targeting molecules to different diseases, as well as different potent cytotoxic drugs for different therapies.

Chapter 1 describes the rationale and motivation for my research

Chapter 2 provides an extensive review of all the works that over the past 30 years regarding the use of steroids (antiestrogens) as targeting molecules to deliver anticancer drugs to breast cancer. In this context, a comprehensive discussion for selecting a synthetic estrogenic scaffold,
techniques for conjugation, optimization of linker, and some initial biological data will be presented.

**Chapter 3** describes the development of the 11β substituted antiestrogen and conjugation strategies to make hybrids with the DNA-intercalator Doxorubicin. This section will describe our specific synthetic work and optimization process for the 11β substituted scaffold such that it retains high binding affinity to estrogen receptors, not only as individual component but also as the hybrid. Series of different bifunctional linkers, such as tetraethylene glycol were chosen and evaluated for the conjugation, with special emphasis on optimizing the chemistry to trigger drug release. Finally, the doxorubicin derivatives were screened its effects. *In vitro* studies were conducted to evaluate the best combination of all three components- antiestrogen, linker and therapeutic drug as

**Chapter 4** discusses the use of the convergent methodology for attaching radiolabeled groups onto biological targeting molecules. Two specific examples of using 1,3- cycloadditon chemistry will be described. One approach used the 11β substituted antiestrogen scaffold functionalized with either $^{18}$F (F) ethynyl, 1-$^{18}$F (F) triethylene glycol, and 1-$^{18}$F (F) tetraethylene glycol. The other approach used the prostate specific membrane antigen (PSMA) inhibitors with prosthetic imaging groups on the lysine handle. These prosthetic imaging agents contain radionuclides, such as $^{99m}$Tc or $^{18}$F, and were appended to the PSMA via a “click” process. Also, in this chapter, the enhancement in binding-specificity, image contrast and pharmcokenetics of using multimeric (i.e. trivalency) versus monomeric target molecule, are examined, specifically for the trimeric PSMA - $^{18}$F complex. Initial *in vivo* studies were conducted to determine whether the
radioligand assembled from all three components- targeting, linker and radionuclide-was better than the monomeric form.

**Chapter 5** emphasizes the application of small targeting molecules, therapeutic pH-sensitive drug moiety and radiolabeling ligand to the assembly of nanomedicine. In this approach, gold nanoparticles are used as the nanoparticle platform. Monofunctionalized AuNP, such as PSMA-AuNPs, Dox-AuNPs, and radiolabeling-AuNP are prepared, characterized and evaluated to achieve the optimum formulation of each. Then multifunctional-AuNP was prepared via convergent and top-down approach using appropriate azidolipoamide derivatives. The product was evaluated in cell based assays to determine pharmacokinetics, drug release and targeting via adjusting ligand ratios, length of individual linker, and degree of the stealth coating.

**Chapter 6** summarizes the major achievement of my thesis work and suggests future directions for the continuation of these studies.
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LBD  Ligand Binding Domain
mAbs  monoclonal antibodies
MeOH  Methanol
MfAuNPs  Multifunctional Gold Nanoparticles
MRI  Magnetic Resonance Imaging
NPs  Nanoparticles
NMR  Nuclear Magnetic Resonance Spectroscopy
NR  Nuclear Hormone Receptor
PCa  Prostate Cancer
PET  Positron Emission Tomography
PSMA  Prostate Specific Membrane Antigen
PSMAi  Prostate Specific Membrane Antigen Inhibitor
PEG  Polyethylene Glycol
RBA  Relative Binding Affinity
RES  Reticuloendothelial System
SERM  Selective Estrogen Receptor Modulator
SPR  Surface Plasmon Resonance
TEG  Tetraethylene glycol
TEM  Transmission Electron Microscopy
THF  Tetrahydrofuran
TKI  Tyrosine Kinase Inhibitor
TLC  Thin Layer Chromatography
UV  Ultraviolet Spectroscopy
Chapter 1

Background, rationale and potential impact of my research
Cancer is the second leading cause of death after cardiovascular disease in the United State. At this time, cancer still remains as a largely incurable disease and often poses a death threat to patient.\textsuperscript{1} Despite extensive research in developing drugs for treatment of cancer, most of these therapeutic entities are still limited by their nonspecific toxicity, high dosage, poor solubility, and aggregation, poor properties \textit{in vivo} metabolism and short circulation half-lives. As a consequence, the current drug treatment is often a tradeoff between efficacy and severe side effects, and therefore the therapeutic window is limited.\textsuperscript{2-4} Because antitumor drugs are not selectively taken up by tumor cells, whether intravenously or orally administered, the concentrations that would eradicate the tumor are often not achieved. In addition, tumor cells tend to develop resistance against the anticancer drugs following prolonged exposure.\textsuperscript{5,6} As a result, new methods for reducing systemic toxicity and side effects, improving in tumor selectivity, high potency, and therapeutic efficacy represent major targets in the area of anticancer drug treatment.

One of the more promising approaches for developing selective agents for cancer is to combine a potent drug, therapeutic and/or an imaging agent with a specific targeting group, such as an antibody or a high-affinity small molecule that selectively recognizes the overexpressed antigens or proteins on tumor cells. In essence, such an entity would not only be able to selectively target the selective tumor lesion, but also deliver sufficient amounts of therapeutic agents to effectively kill the tumor.\textsuperscript{2,7-9}

Selective drug targeting and delivery in the treatment of cancer is a complicated process and is considered as an interdisciplinary focus that has progressively gained attention. Many innovative technologies, such as nanotechnology and biodegradable materials for effective drug
targeted delivery, have been recently developed, including the utilization of biodegradable polymeric implants, peptides and proteins encapsulation, and nanofabrication.\textsuperscript{10-12} Although the initiatives in this field began decades ago, translation into the clinic still encounters different challenges from many technologies. Within these advancing technologies, the development of nanotechnology in cancer treatment is considered one the main driving forces to attract the funding resources to forefront the fundamental research in nanoscience. Whether it is a rising innovation or a mature technology, nanotechnology-based cancer treatment is often faced with several unique issues before commercialization, including the suitable scientific development, government regulations, policies, specific approval financial support and market forces for the final products.\textsuperscript{12}

The integration of nanotechnology in medicine (nanomedicine) specifically for drug delivery and drug-targeting has emerged as a multidisciplinary research field with its primary objectives being the investigation and control of nanomaterials by integrating chemistry, molecular biology, pharmaceutics and engineering.\textsuperscript{13} The ultimate goal of nanomedicine is a personalized medication that could stop the progression of cancer or genetic disease in a patient. Because of the complexity of nanomedicine research and development, as described by many experts, it could lead to many changes in our current practice in medicinal and clinical research.\textsuperscript{2-4,6,7,11-14} Nanomedicine has the potential to fundamentally change of the development landscape of pharmaceutical and biotech industries. Nanomedicine also includes drug formulation that utilizes sub-micron materials with the aim of improving the biodistribution and the target site accumulation of systemically administered chemotherapeutic agents. Many different types of nanomedicines have been developed in the past decade, for example, liposomes, polymeric micelles, and inorganic nanoparticles. The literature shows that these nano-size drug systems are
able to improve the balance between the efficacy and the toxicity for many therapeutic interventions.\textsuperscript{2,7,11,13} Although nanomedicines could be developed in many different forms, they all share a common aim, i.e., to precisely, safely, and reproducibility deliver a potent chemical to a specific target or location. Nanomedicine creates its own unique research strategies by integrating diverse areas of expertise including drug delivery, tissue engineering, molecular imaging, medical implants and devices. For example, in the areas of drug delivery and imaging for cancer therapy, nanomedicine is now considered one of the most highly active and exciting fields with many opportunities. As evidence, growth of nanotechnology in medicine has annually increased and is estimated to exceed $1$ trillion by 2015, making it one of the fastest-growing industries.\textsuperscript{1}

Despite the prediction of potential benefits in the treatment of cancer and other genetic diseases, translation of nanomedicine into the clinic still faces with numerous challenges.\textsuperscript{2,9,15} For example, clinical validation of nanomaterials \textsuperscript{1}, concurrent development of more complex nanomedicines\textsuperscript{2}, unforeseen toxicity of NPs\textsuperscript{3}, and lack of scalability\textsuperscript{5} of these systems will undoubtedly hinder their development and commercialization. The clinical validation of nanomedicines may lower the risk of translational studies, however, the toxicity of these systems will depend on multiple, interrelated parameters that include the biophysicochemical properties of nanomaterials (size, shape, rigidity, surface charge) as well as the payload ligands (imaging, targeting, therapeutics) and the accelerated blood clearance pegylation coating could potentially induce side effects upon repeated dosing.\textsuperscript{3,5,16} The limitations of current animal models (e.g., species-specific differences, insufficient cancer and clinical response models), could also prevent the effective clinical translation of nanomedicine technologies. Therefore, the development of therapeutic and/or imaging nanomedicines needs to be optimized and evaluated on a case-by-
case basis.\textsuperscript{2,9,11,17} To date, each potential nanomedicine that has been developed, has utilized an established method (whether liposomes, micelles, polymeric or nanoparticulate forms) based on “ready-made” drugs and/or targeting moieties such as antibodies, peptides or aptamers. The limited availability of targeting ligands restricts the development of nanomedicines. Another major issue associated with targeted nanomedicines involves control over surface and/or encapsulation composition, (i.e., the number and type of targeting group). Most current approaches use “off-the-shelf” materials, making the products reliant on the availability of bioconjugation sites on the targeting molecules. As a consequence, there is variability in the ligation efficiency. The approach also depends upon appropriate functional groups on the targeting unit. The bioconjugation reaction itself may significantly modify the biological activity of the targeting unit. As a result, it becomes increasingly difficult to reliably (reproducibly) introduce more than two targeting units onto the nanoparticle surface. Diversity for each functional target is also limited by the number of molecules available.\textsuperscript{6,12,16,18-22}

Success in nanomedicine development requires diligent integration of different scientific disciplines such as in chemistry, biology, pharmacy, nanotechnology, medicine and imaging. There is still a need to develop “enabling” chemical technologies for preparing these small target molecules, “nanomedicinal” chemistry. This subdiscipline of chemistry focuses on making versatile compounds that can be used in a variety of platforms. One aspect of my research is to develop a modular, convergent and “tunable” technology for preparing precision-guided targeted molecules (such as antiestrogen, PSMA, pH-sensitive doxorubicin and series of imaging modalities) such that they can be used to assemble multi-functional nanoparticles. Several key features make this innovative approach better than current methods. First, modularity allows us to construct the components from commercially available materials or from compounds that we
can readily prepare ourselves. This “designer” approach is not dependent on “ready-made” components. This aspect is very important for incorporating unusual or specialized targeting units that require multi-step synthesis. These units are often potent receptor ligands, or enzyme inhibitors that possess particular structure features. Bio-conjugation of these compounds, whether peptides, peptidomimetics or small molecules, must take place at specific sites that do not adversely affect their binding/inhibitory properties. For some compounds, X-ray crystal structure-based drug design strategies or computational methods are valuable adjuncts. Second, our strategy is convergent, meaning that establishing the surface composition of the nanoparticle is independent of the functionalization of the targeting units. We can prepare the surface of the nanoparticle with the appropriate array of terminally modified linkers (e.g. functionalized AuNPs with alkyne, carboxylate and maleic amide), and subsequently ligate the correspondingly modified targeting units. Another important feature of our strategy is that the terminal functional groups of the nanoparticle possess chemo-orthogonality, meaning that they will not cross-react with each other. Therefore, we can sequentially and selectively ligate the corresponding targeting units using a variation of solid phase organic synthesis (SPOS). Depending upon the number of different functional groups used, we can append up to five different targeting molecules to a single population of nanoparticles. Third, our strategy is “tunable,” meaning that we can control the extent to which each of the targeting elements would be displayed. Because all of the surface interacting groups are essentially identical (dithiols, i.e. lipoamides with polyethylene-oligoethylene glycol linkers), we can control the ratio of each group in the solution by adjusting to composition of the solution in which the coated gold nanoparticles are prepared. Since the subsequent ligation reactions are chemo-orthogonal (as previously described), the external
presentation of the targeting units is regulated by the composition of the surface interacting groups.

Targeted drugs and targeted-drug delivery systems, such as nanomedicines, can alter the chemo reagents more effectively and conveniently than those in classical medicine. This new approach, if successful, would increase patient compliance, extend product life, provide product differentiation and possibly reduce overall healthcare costs in cancer treatment. In addition, improved targeted drugs and targeted-drug delivery system may exhibit improved pharmacokinetics in the body, thus enhancing their therapeutic value. Therefore, the primary aim of my research is to develop new synthetic approaches, such as modular, convergent, or bioorthogonal conjugation methods, that can convert therapeutic drugs into move versatile forms that can be used in preparing hybrid drugs, small therapeutic molecules, such as targeting and imaging ligands in assembly a targeting multifunctional drug delivery system. My research also primarily focuses on using small targeting molecules to deliver a specific drug at the right time to a specific target at the required dose.

A major component of my research is focused on developing multifunctional drug therapeutic systems for breast and prostate cancers. The studies related to the preparation and biological evaluation of new therapeutic agents, such as estradiol-drug hybrids, cell membrane targeted molecular imaging agents, and multifunctional NPs will be discussed. The preliminary results of these studies indicated that these new reagents achieved their initial objectives and can be further improved for optimized synthesis and/or in vivo experiments. The following paragraphs briefly describe the content of each chapter of my thesis, focusing on primary objectives, experimental design and significant results.
Chapter 2- This chapter reviews the research that has been published over the past 30 years regarding the use of steroidal estrogens and antiestrogens as targeting molecules to deliver anticancer drugs to breast cancer cells. I will also provide a discussion of the criteria and biological assays for selecting synthetic estrogenic scaffolds and review the synthetic chemistry methods for conjugating the toxins, and biological assays used to evaluate the conjugates.

Chapter 3- This chapter describes the rationale and strategy used for developing the 11β substituted antiestrogen-doxorubicin conjugates. This section describes the specific synthetic efforts and the optimization process that resulted in high binding affinity to estrogen receptors. It will describe different bifunctional linkers that were chosen and evaluated for the conjugation, leading to the derivative that contained a pH release mechanism. In vitro studies were conducted to evaluate all three components- antiestrogen, linker and therapeutic drug as bioconjugates to target (ER+) breast cancer cells. Finally, the chapter will interpret the results as the basis for future antiestrogen-drug conjugate development.

Chapter 4- This chapter discusses the use of the convergent methodology for attaching different imaging modalities, e.g., fluorescent tags, Raman probes, and radiolabeling nucleides for PET and SPECT imaging, onto a biologically targeted molecules. In this section, two specific examples that use 1,3- cycloaddition (“click”) chemistry will be explored. For attachment, one involves the 11β substituted antiestrogens functionalized with either 18F (F) ethenyl, 1-18F (F) triethylene glycol, and 1-18F (F) tetraethylene glycol. The other example looks at the prostate specific membrane antigen (PSMA) inhibitors with a prosthetic imaging group ligated to a lysine. These prosthetic groups incorporate radionuclides, such as 99mTc or 18F, and are appended to the PSMA via the “click” process. This chapter also explains the strategy of localization.
enhancement for increased binding-specificity, improved image contrast and optimized pharmacokinetics by use of multimeric (trivalency) strategy. Initial \textit{in vivo} studies were conducted to evaluate the radiotracers. The results will be interpreted to provide an assessment of the imaging strategies.

\textbf{Chapter 5}- This chapter describes the development of multifunctional nanoparticles that incorporate small targeting molecules, a PSMAi, a therapeutic pH-sensitive drug moiety, an imaging ligand group and a polyethylene glycol moiety. These components are developed using variations of our modular, convergent and bioorthogonal synthetic approaches. The components are specifically chosen for use in assembling a nano-drug delivery. Specific methods for preparing a targeted multifunctional gold nanoparticle (MfAuNPs) are also described in this chapter. Preliminary studies are described in which monofunctionalized AuNP, such as PSMA-AuNPs, Dox-AuNPs, and radiolabeling-AuNP were prepared, characterized, and evaluated to achieve the optimal formulations for each. Finally a targeted multifunctional-AuNP was assembled via convergent and top-down approach. The nanoparticulate drug delivery system was then evaluated in cell based assays. Optimization in terms of pharmacokinetics, drug release and targeting was undertaken by adjusting ligand ratios, length of individual linkers, and degree of the stealth coating. This chapter will summarize the results and speculate of future modifications.

\textbf{Chapter 6} – In this chapter, I will summarize the outcome and potential impacts of my research, efforts and provide suggestion regarding future directions.
1.1 References


Chapter 2

Evolution of Chemotherapeutic Estradiol-Drug Conjugates
This chapter is largely adapted from my initial literature research and background survey for steroidal-toxin conjugates. The information that I collected, led to a published review paper in *Bioconjugate Chemistry*. Some of the key discussions in the paper are also presented in the following chapter.

### 2.1 INTRODUCTION

One of the major challenges in cancer chemotherapy involves delivery of potent drugs selectively to the pathological cells without exposing the toxic effects of the drugs to the normal tissue. Because few cytotoxic drugs used today possess such inherent selectivity for the disease, an entire field of targeted drug delivery has developed. The aim of this discipline is to devise methods by which nonselective, but highly active, agents can be modified such that they will recognize cancer cells in preference to normal cells. Among the many strategies devised, one that has received significant attention is the preparation of targeting group-drug conjugates. In this approach, the drug is covalently attached (ligated) to another molecule that possesses the requisite cancer cell recognition properties. The criteria that the resultant conjugate must meet are several- the modification must not alter significantly the recognition properties of the targeting moiety; the modification does not modify the therapeutic properties of the drug; and the entire conjugate demonstrates the biological properties regarding efficacy, potency and selectivity. In addition, the compounds must be chemically and biologically stable, and be prepared in as few steps as possible.

Achieving this goal for most cancers remains to be demonstrated. However, a number of studies in this regard have been undertaken for hormone (estrogen) responsive breast cancer. This form of breast cancer provides an excellent example, as it possesses a well-characterized
targeting mechanism (estrogen receptor) as well as many potentially useful therapeutic agents. Although this biological target has been the subject of research for over 30 years, few reviews have evaluated the strategies for targeted drug delivery.\textsuperscript{1-3} In this Chapter, I provide the therapeutic context for targeted drug delivery, a rationale for targeting the estrogen receptor, a survey of past approaches to estrogen receptor targeted drug delivery conjugates using the steroidal derivatives, and our current efforts which have resulted in the identification a potential estrogen receptor-based drug delivery.

2.2 DIAGNOSIS AND THERAPY OF BREAST CANCER

2.2.1 Breast Cancer

Breast cancer is the most common cancer among women in the United State, with an estimate that 1 in 8 women will develop breast cancer in their lifetime.\textsuperscript{4} It is still the most common cause of death in women between 35 and 55 years of age, and worldwide it is considered as the most common cause of cancer death.\textsuperscript{4-6} Currently, the mortality rates due to breast cancer have decreased in many industrialized countries, including the United States. Two possible explanations for this decline in breast cancer mortality are (1) more effective early detection due to widespread screening efforts, and (2) advances in adjuvant systemic therapy. In early-stage breast cancer without distant metastases, cure rates of up to 70\% are seem possible. However, even with currently available therapeutics, recurrent or metastatic breast cancer is still considered incurable.\textsuperscript{5,7}
2.2.2 Diagnostic Imaging for Breast Cancer

Early detection is a major factor that improves the chances of survival in all forms of cancers, and breast cancer has one of the more mature screening and detection regimes. Mammography is still the most common method for early detection, often detecting cancer before physical symptoms appear.\textsuperscript{8,9} This technique uses x-rays to noninvasively visualize an abnormal anatomical structure, such as non-homogeneous tissue density within the breast. The diagnostic mammogram typically is more accurate in postmenopausal women when compared to premenopausal women.\textsuperscript{10} Ultrasonography is another diagnostic modality that is used to discriminate between the fluid-filled and solid tissue structures. Ultrasound is typically used to evaluate a limited region of the breast, but not as a screening test of the entire breast. The third imaging modality is breast magnetic resonance imaging (MRI), which uses a magnetic field to create a detailed anatomical image. In order to enhance resolution, this method requires injection of a spin contrast agent, such as a gadolinium complex, to improve the quality of the images, as heterogeneity in the density of the breast tissue can be problematic. MRI is not recommended for screening cancer in most women because it is not as reliable as mammography for certain breast conditions, such as ductal carcinoma \textit{in situ} (a type of noninvasive or early breast cancer). In addition, MRI testing is more likely to identify suspicious regions that turn out not to be cancerous. All of these methods are non-invasive, and in combination with clinical and breast self-exams, represent the basis for breast cancer detection. However, these procedures only provide anatomical information for tissue abnormalities which may or may not be cancerous and therefore a biopsy is required to confirm if the abnormal tissue is a carcinoma.\textsuperscript{11}
2.2.3 Treatment of Breast Cancer.

Breast cancer therapy can be “personalized”, based on a variety of factors, including the stages of the disease and biopsy (histological) results. Localized disease is treated primarily with surgery, either a lumpectomy or mastectomy, to remove the gross cancerous tissue. Radiation and chemotherapy may be used prior to surgery, neoadjuvant setting, to reduce the tumor mass. Surgery is usually followed, adjuvant setting, by radiation therapy to kill any remaining cancerous cells. Advanced stage breast cancer is considered a systemic disease and therefore, adjuvant systemic therapy, such as endocrine therapy, chemotherapy and/or trastuzumab, is administered in addition to loco-regional treatment. Adjuvant chemotherapy is usually given for 3 to 6 months after radiation and typically consists of a combination or “cocktail” of drugs. Such combinations are normally more effective than single drug therapy for breast cancer treatment as each component imparts its benefit through a different mechanism. Most chemotherapeutic approaches are prone to significant systemic side effects due to their high toxicity and lack of cancerous tissue specificity. As a result, significant efforts have been directed toward the development of new therapeutic strategies with greater specificity, enhanced therapeutic efficacy and/or bioavailability.
2.3 THE ESTROGEN RECEPTORS AS TARGETS OF STEROID-DRUG CONJUGATES

2.3.1 Role of Estrogen and Estrogen Receptor (ER) in Breast Cancer

The underlying molecular mechanism by which estrogens increase the risk of breast cancer is still not fully understood, though estrogen metabolites are known to bind to DNA and induce mutations. For example, estradiol (the endogenous parent estrogen) undergoes hydroxylation (oxidation) by the cytochrome P450 system to form catechol estrogens, which are further oxidized to the corresponding quinones and/or semi-quinones. The quinone derivative of catechol estrogen can subsequently bind to DNA, forming DNA adducts. Furthermore, the semi-quinones generate free radicals that can react with oxygen, forming superoxide radicals (reactive oxygen species-ROS), which can also attack and alter DNA. In addition, direct estrogen stimulation of the breast epithelial cell proliferation is another increased breast cancer risk factor. Enhanced cell replication resulting from estrogen exposure results in the propagation of genetic errors, and the mutagenesis increases the risk of genetic alteration. Although estrogens enhance the growth of certain types of breast cancer cells in cell culture, the detailed mechanisms by which the steroids promote growth \textit{in vivo} are still not completely clear.\textsuperscript{16-20}

A discussion of estrogen receptors (ER) and predominately the alpha (\(\alpha\))-subtype is required to appreciate the role of estrogens in breast cancer. In general, the alpha-subtype of estrogen receptors (ER\(_\alpha\)) is the more dominant form in ER-positve (ER+) breast cancer cells undergoing proliferation than in normal cells, suggesting that paracrine factors, such as secreted growth factors, proteases, and growth receptors mediate the mitotic effects of estrogen. In addition, the dissociation between cell proliferation and the presence of ER\(_\alpha\) in normal epithelial cells, proliferating breast cancer cells express ER\(_\alpha\), suggests that estrogen can directly stimulate
the growth of breast cancer cells. More recent data has indicated that ER\(_{\alpha}\) and ER\(_{\beta}\) may have different roles in the formation and/or progression of breast cancer. For breast (ER\(^+\)) tumor cells, estrogens can stimulate growth only if ER\(_{\alpha}\) is present. By comparison, estrogen interactions with ER\(_{\beta}\) inhibited cell proliferation and prevented tumor formation. Further studies have shown that ER\(_{\alpha}\) is the subtype that is generally up-regulated during the conversion from normal mammary epithelial into carcinoma, whereas the \(\beta\) form is usually decreased. For example, Rao \textit{et al}, used immunohistochemistry (IHC) to demonstrate that higher level of ER\(_{\beta}\)-positive cells in normal mammary gland than in abnormal, but non-proliferating benign breast tissue.\(^{21}\) In contrast ER\(_{\alpha}\) levels increased during progression from normal to abnormal tissue. Therefore, the ER\(_{\alpha}\):ER\(_{\beta}\) ratio appears to change during breast cancer carcinogenesis, however, whether this relationship is a cause for malignant transformation is still unclear.

### 2.3.2 Review of Structure and Function of Estrogen Receptors

The estrogen receptors (ER) are members of nuclear hormone (NR) receptor, ligand-controlled transcriptional factor superfamily. These proteins mainly function as activators or repressors of transcription but can also mediate nongenomic pathways, such as signaling cascades or ion channels.\(^{22,23}\) As mentioned in the previous section, there are two major isoforms of ER, ER\(_{\alpha}\) and ER\(_{\beta}\), that possess similar affinities for 17\(\beta\)-estradiol (E2). ER\(_{\alpha}\) is predominately located in breast and uterus tissue, while ER\(_{\beta}\) is mainly found in central nervous system, cardiovascular and bone tissues.\(^{21,24-28}\) The two isoforms have modest sequence identity but are structurally homologous, maintaining conserved structural and functional distinct domains.\(^{29-31}\)
The current understanding of the cellular uptake and distribution of 17β-estradiol, as well as the other endogenous estrogens, is more complex than previously described. In addition to passive diffusion through the outer cellular membrane, estrogens may also bind to cognate estrogen receptors imbedded within the cell membrane (mER). These membrane estrogen receptors arise from the same genes as the nuclear receptors and are chemically identical. Interaction of estrogens with mER results in endosome formation, leading to an alternate entry and signaling pathway. Once within the cell, via either route of entry, free estrogen binds to the ligand binding domain (LBD) of the ER as part of the ER-Hsp90 chaperone complex. Upon ligand binding, ER undergoes a conformational change, causing dissociation of Hsp90 and permitting subsequent estrogen receptor dimerization. The conformational changes induced by the ligand-ERα interaction subsequently drive the transcriptional responses. For the endogenous ligands, the formation of the ligand-ERα complexes promotes the recruitment of coactivator proteins and association with the corresponding estrogen response elements (ERE) within the DNA. Completion of transcriptional complex leads to the downstream RNA and protein responses typically associated with the steroidal estrogens.

For synthetic ligands that target the estrogen receptor, the process for uptake, binding, coregulator recruitment and transcriptional activity may be significantly altered. Depending upon physico-chemical properties or ER-binding affinity of the compound, the balance between passive diffusion and membrane ER-endosomal uptake may vary widely. Within the cell, affinity for the ER-LBD can affect the rate and efficacy of ER-HSP dissociation and resultant ER dimerization process. More importantly, the conformational changes induced with ER-LBD by the synthetic ligand give rise to complexes with ER that may not resemble those formed with the endogenous ligands. Ligand-ER complexes similar to those formed by estradiol will tend to
recruit a similar (but not necessarily identical) set of coregulatory proteins and generate similar, but not identical transcriptional responses. Such compounds are called estrogen agonists. At the opposite extreme are ligands that bind within the same site as estradiol but produce different conformational changes. Such complexes fail to recruit the same set of coregulatory proteins or recruit coregulatory proteins that block the transcriptional responses. These compounds are called antagonists or anti-estrogens (AE). Most synthetic ligands for the ER have properties that fall between these two extremes, and these variations in properties must be taken into consideration when using the ligands for targeting the ER. The specific structure-activity relationships (SAR) for ER ligands are well described and therefore will not be reviewed here.

2.3.3 Biological Downstream Consequences of Agonists/Antagonist with Estrogen Receptors

The agonist hormone 17β-estradiol (E2) is a key regulator of growth, differentiation, and function in a wide array of target tissues, including the male and female reproductive tracts, mammary gland, and skeletal and cardiovascular systems. The biological effects of estradiol (E2) are mediated through at least four different ER pathways as shown in Figure 2-1.44 The classical ligand-dependent pathway 1 is the pathway in which E2-ER complexes bind to estrogen responsive elements (EREs) in target promoters leading to an up- or down regulation of gene transcription and subsequent leading to tissue responses. In the ligand-independent pathway 2, the growth factors (GF) or cyclic adenosine monophosphate activate intracellular kinase pathways, leading to phosphorylation (P) and activation of ER at ERE-containing sequence. The pathway 3 is ERE-independent, where E2-ER complexes alter transcription of genes containing
alternative response elements, such as AP-1, through association with other DNA-bound transcription factors (Fos/Jun). These factors then tether the activated ER to DNA, resulting in an up-regulation of gene expression. And finally, the pathway is cell-surface (nongenomic) signaling, in which E2 activates a putative membrane associated binding site, possibly an isoform of ER, linked to intracellular signal transduction pathways that generate rapid tissue responses. As indicated previously, modification of the structure of the ER ligand would be expected to generate alterations in each of these pathways, leading to a different overall biological response.

**Figure 2-1**- The multifaceted mechanisms of estradiol and estrogen receptor signaling “Reprinted with permission from Dao,K-L and Hanson, R. N. Bioconj. Chem. 2012, 23, 2139-2158. Copyright 2012 American Chemical Society.”
### 2.3.4 Concepts of using steroidal estrogens to target ER

Steroidal estrogens, such as E2 and its substituted derivatives, are attractive vectors for targeted drug delivery because they have been shown to localize in tissues that overexpress ER.\textsuperscript{2,47-49} Although steroidal estrogens may freely penetrate non ER-expressing tissues, they typically are not retained within them. Such observations provide the basis for developing estrogen-conjugates for therapeutic drugs that lack inherent breast cancer cell selectivity. The core structure of E2 provides a scaffold for incorporating functional groups, large or small, that can impart additional biological activity. The challenge is how to transform E2 into a targeting molecule for drug delivery without significantly disrupting its inherent binding properties for the ER.\textsuperscript{50-52}

A review of this field can essentially be divided into two distinct eras. The first era was pre-1990 when such agents were primarily based on the known structure–activity relationships (SAR) for ER agonists and antagonists. The second era began after 1990 when E2 conjugates could be designed based on the crystal structures of agonist or antagonist ligands complexed with the ER-LBD.\textsuperscript{53-55} The pre-1990 SAR studies emphasized both the necessity of free 3,17β-OH groups on estradiol and for small substituents on the aromatic “A” ring, as well as at the 6, 7, 11, 16 and 17 positions. Large substituents at those sites as well as the other positions on E2-scaffold were known to be detrimental to ER-binding. The advent of the ligand-ER LBD crystal structures rationalized the observations and it was clear that the phenolic-3-OH was oriented toward the inner core of LBD, an interaction with Glu- and Arg residues as well as a water molecule. The 17β-OH formed hydrogen bonds with His-544 located in helix 12. The remainder of the E2 structure is bounded by a predominantly hydrophobic surface. The crystal structures of
complexes with simple ER ligands do not provide insights into adaptive responses of ER compared to complexes with more highly modified steroidal structures. 17α-substituted phenylvinyl E2 derivatives, for example, induce a pocket in ER-LBD not previously observed, however, the pocket can accommodate high affinity ligands which are potent agonist or low affinity compounds that are essentially inactive. Substituents at the equivalent of 11β-position of E2 can be accommodated within the ER-LBD, with larger groups (beyond 3-9 atoms) resulting in a conformational change in which the helix-12 of the ER assumes an antagonist pose. The dialkylaminoethoxyphenyl groups found in many nonsteroidal antiestrogens occupy this space. For 7α-substituted E2 derivatives having large groups (3-4 atoms), the steroidal scaffold appears to rotate around the 3-17β axis, and projects the 7α-substituent into the 11β-pocket. Therefore, E2-drug conjugate development must consider these observations in the overall design process.

2.3.5 Roles of Chemotherapeutic Agents

Most of the conventional chemotherapeutic agents that have been used in developing conjugates were selected for their ability to target one of the six hallmark features associated with human cancer cells. As formulated by Hanahan and Wienberg et al., these include- (1) limitless proliferative potential; (2) self-sufficiency in growth signaling; (3) insensitivity to anti-growth signals; (4) evasion of apoptosis; (5) sustained angiogenesis; and (6) tissue invasion and metastasis. The majority of the anticancer compounds used for conjugation with estradiol are endowed with intrinsic immunosuppressive properties because they preferentially target rapidly proliferating cells. Figure 2-2 illustrates the functional targeting of anticancer reagents that have been used in estradiol-anticancer drug conjugates. In general, the drugs were chosen
because they demonstrated chemotherapeutic effects against breast cancer, however, they were non-selective with respect to the breast cancer tissue, and therefore produced serious systemic toxic side effects. These side effects led many research groups to couple the chemotherapeutic drugs with a high putative affinity targeting agent, such as estradiol, to make a conjugate that would be more selective to the disease and yet retain the potency of the original drug, thereby alleviating the off-target toxicity.

**Figure 2-2:** Targets of chemotherapeutic agents

2.4 EVOLUTION OF ANTIESTROGEN-DRUG CONJUGATES

2.4.1 General Concepts of Antiestrogen-Drug Conjugates

Targeted therapeutic drug conjugates are designed to synergize the two independent functionalities. The aim is to make a single entity that has therapeutic efficacy of the therapeutic compound and the selectivity of the targeting compound. The conjugates should possess better pharmacokinetics and enhanced pharmacological properties such as specificity, cellular uptake and/or retention.\textsuperscript{57-59} Considering the many roles that ERs play in normal and abnormal physiology, the list of potential therapeutic groups that could be attached to ER-targeting steroids becomes quite extensive. Nevertheless, because the ultimate objective in each substance is essentially the same, i.e. delivering a therapeutic group intracellularly to an ER responsive cell, the overall criteria for each conjugate will remain the same. As with all drug-conjugates, the compound must be chemically and biologically stable during the \textit{in vivo} circulation period. The ER-targeting moiety should retain high affinity for ER, with a relatively high binding affinity (RBA \( \geq 10\% \)) to that of estradiol. The conjugate should be capable of achieving an intracellular concentration, either by active uptake or passive diffusion, sufficient to promote the therapeutic effect. The conjugate should provide a mechanism by which the therapeutic component is effectively accessible to its site of action. That effect should occur at levels below those at which toxicity is generated in normal cells, or non ER-selective cells. The targeting component should not interfere with the therapeutic responses.\textsuperscript{1,2,47,59-61}

In the following section we will review examples of efforts to develop steroid-based ER targeted drug delivery systems. Most of the therapeutic targets are associated with cancer,
however, some are directed toward the other hormone related disorders. In each example, we will evaluate the choice of estradiol derivative, the therapeutic moiety and the linker as at contributing to the observed biological effects. The first set of examples focuses on agonist-like estradiol derivatives, i.e., those with position 3, 6, 7, 16, and 17-substitution patterns. The subsequent examples will look at the steroidal antiestrogens, beginning with the 7α-substituted estradiols and ending with a summary of our work which resulted in a highly active 11β-substituted estradiol derivative.

The evolution of conjugating chemotherapeutic agents to a steroid E2 scaffold is represented in the schematic below (Figure 2-3). This review will discuss details with each case, where R could be a specific design of a functional linker/tether that is attached to a specific anticancer reagent.

\[ \text{R= specific linker with chemotherapeutic reagents, e.g. Cis-platin, biotin, geldanamycin, ellipticine, daunorubicin} \]

\textbf{Figure 2-3-} Generic E2-drug conjugates
2.4.2 E2-Ellipticine Conjugates

Figure 2-4 E2-Ellipticine conjugates: A) E2-Ellipticine short linker\textsuperscript{62}; B) E2-Ellipticine long linker\textsuperscript{63}

Ellipticine is a DNA intercalating agent that has been evaluated as a breast cancer chemotherapeutic agent, however, its lack of selectivity and severe side effects negated its
clinical utility. Two sets of 17α substituted estradiol-ellipticine conjugates were prepared with the objective of enhancing breast cancer cell selectivity and cytotoxicity (Figure 2-4A, B). Delbarre, et al., used estradiol modified at the 17α-position with a propionic acid residue to conjugate ellipticine as its quaternary salt (Figure 2-4A). Conjugation of the estradiol derivative had no significant effect on enhancing cytotoxicity in ER-positive (ER+) MCF-7 breast cancer cells when compared with untargeted ellipticine alone. This effect was explained by the low binding affinity of the conjugate to the ER receptors. As is now, appreciated from the crystal structure of 17α substituted estradiol-ER-LBD complexes, large substituents such as the ellipticine moiety cannot be well accommodated within the binding pocket and would dramatically reduce binding affinity. The interactions of the E2-ellipticine conjugate with DNA remained similar to those of ellipticine modified with triarylethylene (nonsteroidal antiestrogen) derivatives, suggesting the steroidal group did not significantly interfere with DNA intercalation. Cytotoxicity studies with MCF-7 cells in the presence and absence of 3H-estradiol, indicated that the E2-ellipticine conjugate exerted both cytostatic and cytotoxic effects, which were primarily due to the pyridocarbazole moiety. The cellular uptake of the conjugate appeared to be independent of interactions with the estrogen receptors; therefore this was an example in which steroidal targeting and therapeutic effects were not synergistic.

Katzenellenbogen, et al., synthesized a series of E2-ellipticine conjugates with the longer linkers and neutral ellipticine derivatives (Figure 2-4B). The purpose of these modifications was to position the ellipticine nucleus further from the 17β-hydroxyl group of E2, since SAR of the estrogens indicated that the most crucial units required for binding were the free 3-OH and 17β-OH groups. Besides varying the chain length of the linkers, the authors investigated the point of
linker attachment on different regions of the ellipticin
e ellipticine ring system. The summary of biological
results showed that linker chain length had a major effect as the E2-ellipticine conjugates, with a
longer linkers having better ER binding properties compared to the shorter linker. Those
derivatives assembled with longer linker in group B had RBA values of 0.001 to 0.003 %, while
the derivative with a shorter linker (Figure 2-4 A) had a RBA value= 0.005, (E2=100). The
studies also indicated that attaching E2 to the 2-position of ellipticine promoted the enhancement
of potency and DNA binding affinity when compared to the 6- and the 9-positional derivatives of
the ellipticine. However, the E2-2-ellipticine conjugate displayed no selectivity toward the ER+
breast cancer cells, suggesting that conjugation did not impart significant ER recognition
properties. Again, the most logical explanation for the observed effects is that the linker-
ellipticine moiety cannot be readily accommodated in the inducible 17-alpha binding pocket,
thereby compromising the ER-binding capacity. The uptake into the cell and distribution to the
nucleus would most likely be passive-diffusion, assisted perhaps, by the physicochemical
properties of the estradiol moiety.

2.4.3 Estradiol-Nitrosoureas Conjugates

![Chemical structure](image)

**Figure 2-5:** CNU-alanine-estradiol-3, 6α, or 17-ester (CNU-E2)
The 2-chloroethylnitrosourea (CNU) is highly reactive component found in many anti-neoplastic chemotherapeutic agents. The clinical use of such agents is often limited by the nonselective mechanism of cytotoxicity, and therefore there have been numerous efforts to develop of new compounds with greater target tissue selectivity and lower systemic toxicity.

Eisenbrand, et al., prepared a series of 2-chloroethylnitrosourea-alanyl (CNU-ala)-estradiol hybrids, in which the point of ligation was via a 3/17-O ether linkage, or at 6α-position (Figure 2-5). The results showed that compared with parent CNU-ala, E2-CNU conjugates had 3-fold longer terminal plasma half-life, probably due to the higher lipophilicity of conjugates. Moreover, it was found that the CNU-E2 amino acid esters were also inhibitors of serum esterase, which may have contributed to the longer half-life. The experimental evidence suggested that affinity of the conjugates of CNU-E2 for ER may have played a role in its anticancer activity in hormone dependent tumors, however, the reported RBA values were relative low for all CNU-E2 conjugates (E2-CNU-3-ester = 4.7; E2-CNU-6α-ester 0.28; E2-CNU-17α-ester 0.8 and E2-CNU-3 and 17 esters 0.05; comparing to E2=100). It was noted that E2-CNU-6α-ester had the lowest affinity, even though the 3-OH and 17-OH groups, relevant for ER interaction, were left free. This may be because the parent 6-hydroxyl estradiol has low RBA (6.3%). The RBA value of the 3-ester was the highest in the series, contrary to the greater importance of the 3-OH for ER binding affinity, however, the reported value probably resulted from rapid cleavage of phenolic ester bond under the incubation condition.

Although the E2-CNU-17 ester had the lowest RBA values (0.8%) compared to other ester conjugates, biological results indicated that it was the most active antineoplastic agent. The
E2-CNU-3-ester displayed lower potency and the 6-ester was essentially in active. The lack of antineoplastic activity of the latter two compounds (3, 6-esters) may correlate with their lower RBA values, however, no evidence was provided to show that the conjugates acted via ER. The results showed that E2-CNU-17-ester conjugate had enhanced cytotoxicity and antineoplastic potency compared to the parent CNU-alanine, however, the conjugate was not evaluated in ER(+) cancer cells. Therefore, an association with ER, either for selective uptake or cytotoxicity, was not conclusively demonstrated.65

2.4.4 E2-Geldanamycin Conjugates

![Geldanamycin-Estradiol](image)

**Figure 2-6:** Geldanamycin-Estradiol at 16α (GDM-E2) 69

Geldanamycin (GDA) is an ansamycin benzoquinone antibiotic that has a well-established role in cancer chemotherapy, including breast cancer.70 It has been shown that replacement of the 7-methoxy group with an allylamine moiety (7-allylaminogeldanamycin-AAG) improves the in vivo pharmacokinetic properties. Geldanamycin exerts its antineoplastic effect by binding to chaperone (heat shock) proteins of cancer cells, leading to degradation and therapeutic response. As with most cancer therapeutic agents, its efficacy is limited by lack of
target tissue selectivity and systemic toxicity.\textsuperscript{71-73} Danishefsky, \textit{et al}, prepared a series of the 7-N-geldanamycin derivatives, linked through the 16\textalpha position of estradiol, in an effort to impart ER-selectivity. The linkers were alkyl groups that varied in length and some of which contained an imbedded alkene group. The investigators reported that the 2-butenyl linked E2-GDA conjugate demonstrated the highest ER-binding affinity, but even this value was very low compared to E2. The investigators reasoned, based on SAR studies, that substitution at 16\textalpha would allow retention of activity for both components- estradiol and geldanamycin.\textsuperscript{69}

The initial biological results of the conjugate with the butanyl linkage demonstrated significant inhibition of proliferation in ER-overexpressed cancer cells. The E2-GDA conjugate showed no effect on IGF1R and reduced activity against Raf-1, suggesting some degree of selectivity for ER vs. non ER responses. Nevertheless, the overall cytotoxic activity of the conjugate was less than GDA alone, and the biological effects were not blocked by coadministration of E2. Therefore, although the conjugates could be readily prepared, they did not achieve their therapeutic objectives. Neither conjugate has a synergistic with ER-mediated effects.

\textbf{2.4.5 E2-Chlorambucil Conjugates}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2-7.png}
\caption{E2-Chlorambucil conjugates A) 7\textalpha (Chlor-E2)\textsuperscript{74} and B) 16\textalpha (Chlor-E2)\textsuperscript{75}}
\end{figure}
Chlorambucil is an orally active nitrogen mustard, typically used in the treatment of chronic lymphocytic leukemia. Its mechanism of reaction involves crosslinking DNA, leading ultimately to cell death. Although highly effective against certain cancers, the non-selectivity of its mechanism of action limits its use against breast cancer. As a results, several efforts have been made to conjugate this drug to estradiol in order to impart the requisite selectivity while retaining efficacy.\textsuperscript{76,77}

In the first approach, described by Essigmann, \textit{et al}, chlorambucil was attached to estradiol at 7α-position via a series of linkers (Figure 2-7A). Evaluation of the products indicated that the carbamylated and the amino conjugates retained the highest ER affinity, RBA= 46\% and 40\% respectively. The high ER affinity was attributed to having the conjugate adopt a binding mode similar to that observed for the antiestrogenic 7α-undecylamine estradiol derivative, ICI-164,384.\textsuperscript{78} For this derivative, the 7α side chain is extended through the 11β-binding pocket, at the surface of which the lipophilic chain undergoes 90° flexion. The conformational adaptation allows the linker to track closely with the surface contours of the LBD, and in the case of the conjugate would make the terminal chlorambucil group available for reaction with the DNA. The studies indicated that the most potent conjugate interacted initially with the DNA, with the recruitment of ER occurring later. The result was that the treated cells were less likely to repair the defects caused by alkylation with the chlorambucil moiety in the presence of ER, lending to enhanced cell death.\textsuperscript{74} In this case, the ER-conjugate interaction enhanced the alkylation responses rather than providing a true targeting effect.\textsuperscript{74,79} Conjugates with linkers containing other groups, such as amido-, amino- or guanidine-, between the hexanyl-substituted estradiol
and the carboxyl group chlorambucil had lower RBAs values.\textsuperscript{74} As would be expected, these E2-chlorambucil conjugates demonstrated lower cytotoxicity against ER(-)-breast cancer cells.\textsuperscript{74,78,79}

In the second approach, described by Gupta \textit{et al}, chlorambucil was attached at the 16\(\alpha\)-position of E2 with alkyl linker groups of varying lengths(Figure 2-7B).\textsuperscript{75} Theoretically this approach would allow the estradiol moiety to retain the free 3, 17\(\beta\)-OH group required for high ER binding. However, as previously noted substitution at the 16\(\alpha\)-position with sterically demanding group generally is detrimental to ER binding and tends to impart other biological properties. This effect had been observed previously such as with the 16\(\alpha\)-geldanamycin substitution. In this series, the conjugate with a four carbon spacer was found to be more active in hormone-independent ER(-) cell lines, e.g. MDA-MB-486, while the conjugate with a six carbon spacer was more active toward ER-dependent MCF-7 cancer cells. ER binding data were not provided and, because the studies were done without co-incubation with estradiol, an ER-targeting effect could not be demonstrated.

In these two series of E2-chlorambucil conjugates one can see the influence of experimental design. Although both series retain the capacity to alkylate the DNA, only the 7\(\alpha\)-substituted estradiol derivative retained significant ER binding affinity and therefore, demonstrable \textit{in vitro} ER-related selective cytotoxicity. The 16\(\alpha\)-substituted E2-conjugates were readily accessible in a few steps, whereas the more effective 7\(\alpha\)-compounds required a more extensive synthetic scheme.\textsuperscript{75}
2.4.6 E2-Nucleoside Conjugates

![Diagram of E2-Nucleoside Conjugates]

Figure 2-8: E2-Nucleoside conjugates\textsuperscript{80}

Nucleosides, their derivatives and analogs, constitute one of the most important therapeutic classes for the treatment of neoplastic disease. As is in the case with most anticancer drugs, they are most effective against highly proliferative cells and therefore display little inherent selectivity for hormone responsive tissue. Because the 5-substituted uracils constitute one of the major classes of anticancer nucleosides,\textsuperscript{81,82} it seemed logical to develop an E2-nucleoside conjugate to impart tissue selectivity. Van Lier, \textit{et al}, prepared a series of estradiol-nucleoside conjugates using a palladium catalyzed cross-coupling reaction to ligate the 17-alpha-terminally alkynylated-estrogen derivatives with 5-halo-nucleosides (Figure 2-8).\textsuperscript{83} The RBA values of these conjugates were extremely low. Changing the alkyl spacer from two to eight carbons resulted in a modest increase in RBA values from 0.07% to 2.9%. Substitution at the N-1-position of pyrimidine with either a ribose or deoxyribose residue substantially decreased RBA values. As previously described, the 17\textalpha binding pocket has a limited capacity for sterically demanding substituents and the addition of bulky, hydrophobic groups probably cannot be accommodated. Evaluation of these conjugates against ER(-)- and ER(+)- breast cancer cell lines indicated that the compounds showed neither significant enhancement of cytotoxicity nor ER-related selectivity.\textsuperscript{80,83,84}
2.4.7 Therapeutic E2-Daunorubicin Conjugates

![Chemical Structures](image)

**Figure 2-9:** E2-Daunorubicin A)17-amine\(^85\) B)17\(\alpha\)-aminopropynyl\(^86\)

Daunorubicin is a member of the anthracycline antibiotics family frequently used for the treatment of advanced breast cancer.\(^87\) In spite of cumulative dose-related cardiotoxicity, the anthracycline antibiotics are still the preferred chemotherapeutic agents for metastatic breast cancer.\(^88\) Because of their beneficial effects, many significant efforts have been undertaken to enhance the target tissue selectivity of these compounds and thereby reduce the undesired cardiotoxicity. Two examples of E2-anthacycline conjugates reflect this effort. In the first study by Hartman, *et al.*, the E2-anthacycline conjugate was linked via a 17-oxime (Figure 2-9A). This conjugate demonstrated negligible ER binding affinity and no ER-responsive cancer selectivity. As previously noted, loss of 17\(\beta\)-OH group and the additional presence of a bulky substituent at the 17-position are detrimental to ER binding affinity.\(^85\)

In the second approach the estradiol was conjugated through an 17\(\alpha\)-aminopropynyl group to the sugar moiety of the anthracycline.\(^86\) Although more rational than the previous approach, this ligation strategy resulted in a loss of ER binding affinity and reduced cytotoxicity.
The conjugate had an RBA value < 1% that of E2 and was significantly less active than daunorubicin in both ER(+) and ER(-) breast cancer cell lines. Although the conjugate could be easily synthesized, the approach generated neither ER-related selectivity nor enhanced cytotoxicity. In these examples, the choice of ligation sites on the estradiol component and the chemotherapeutic agent resulted in the loss of biological activity.

2.4.8 E2-Taxol Conjugates

Paclitaxel (Taxol) is a potent antimitotic cancer drug, and, because its mechanism of action is different from most agents, it has become a valuable component in combination therapy. In particular, it has established a major role in the treatment of recurrent, metastatic breast cancer. Unfortunately, its solubility characteristics and inherent toxicity compromise its usefulness in the treatment of cancer patients. One approach to improve paclitaxel selectivity and efficacy for breast cancer, as well as improving its formulation properties, involved its conjugation to estradiol. Kingston, et al., described the preparation a series of E2-paclitaxel conjugates in which the site of attachment occurred moiety either at the 11β or 16α positions of the estradiol, and at the 2’, 7’ or 10- hydroxyls of the paclitaxel group (Figure 10). SAR for steroidal estrogens would suggest that substitution at the 11-positions would be tolerated, providing certain conditions were met, but that the 16-position substitution would be detrimental to ER binding (previously discussed in the case of E2-geldanamycin and E2-chlorambucil). On the other hand, ester conjugation of paclitaxel at its 2’, 7’ or 10 positions should not affect the drug activity, providing the ester bond underwent hydrolysis at that site to release the free drug within the cell.

96,97
One of the objectives in the synthesis of the conjugates was not only to improve the formulation properties of paclitaxel because it had poor aqueous solubility, but also to enhance the drug target selectivity. The use of the 2’-hemisuccinate or 7-hemisuccinate ester group was intended to improve the conjugate solubility. The results indicated that the 2’-substituted paclitaxel conjugates with either the 11β- or 16α- substituted estradiol were less active than paclitaxel, and demonstrated no significant selectivity for ER(+) MCF-7 cells as compared to the ER(-)MDA-MB-231 cells. Similar results were observed for the conjugates in which paclitaxel was linked to the 11β- or 16α- positions of E2. All conjugates had low ER binding affinity and were less active in the cell assays than paclitaxel. The conjugate of 10-substituted paclitaxel, and the 11β-substituted estradiol was 3-fold more active against the ER(+)-MCF-7 cells than the

**Figure 2-10:** E2-Paclitaxel (Taxol) Conjugates A) 16α-E2 ether linkage at 7’-O-Taxol; B) derivatives of E2-Paclitaxel conjugates
ER(-)- MDA-MB-231 cells, but less potent than paclitaxel alone in either cell line. In summary, the investigators demonstrated the ability to prepare a variety of E2-paclitaxel conjugates, designed to explore the contributions of different attachment sites and linker properties, but were unsuccessful in achieving enhanced potency and/or ER-related cytotoxicity. 89,96,97

2.4.9 E2-Photodynamic therapy (PDT) Conjugates

![Diagram of E2-PDT Conjugates](image)

**Figure 2-11:** E2-Photodynamic therapy (PDT) Conjugates A) 17α, B) 11β
Photodynamic therapy (PDT) is a form of cancer treatment that relies upon the accumulation of a photosensitizer into cancerous tissues followed by localized irradiation. Irradiation excites the photosensitizer to generate radicals, leading to reactive oxygen species (ROS) that cause damage to cellular components, ultimately leading to cell death (apoptosis). This effect is typically achieved through the use of metalated porphyrin derivatives, or less frequently through chemically reactive species that, upon appropriate activation, lead to the generation of radicals. One of the first E2-PDT approaches was developed by van Lier, et al., in which estradiol was modified with an alkynyl linker at the 17α position and then conjugated with a photosensitizing porphyrin or phthalocyanine derivatives (Figure 2-11A). Although the synthesis of the conjugate using spacers of different lengths was successful, the RBA values of the conjugate were dependent on the specific structure of the spacer. The highest RBA values were reported by those lipophilic E2-PDT conjugates via using the aliphatic alkynyl and benzylic linkers. The RBA increased with increased spacer length, for example 17α-(2-propynyl) estradiol-PDT, compound 1 RBA=1.35%; 17α-(2-buta-1,7-diynyl)estradiol-PDT compound 2 has RBA=3.08%; and 17α-phenyl-(3-buta-1,7-diynyl)estradiol has RBA=8.54% (Figure 2-11 A). Although the RBA values were also slightly dependent on the attachment position at phthalocyanines, the overall ER binding of these derivatives were relatively poor (<0.2 %). As consequence, the biological evaluation of the conjugates indicated that there was no synergy between the steroidal group and PDT component.\(^{100,101}\)

In the second approach, Ray, et al., conjugated the PDT component to an established, high affinity 11β-substituted estradiol derivative.\(^{102}\) Evaluation of the binding properties of the E2-PDT conjugate indicated that most of the ER binding affinity was lost (RBA = 0.4%). The conjugate was evaluated in ER(+) -MCF-7 cells as well as in other cancer cells that overexpress
ER, but the results demonstrated no pattern of ER-dependency. An analysis of the results based upon the current knowledge of the ER ligand binding pocket suggests that the linker between the PDT component and the 11-beta position of estradiol was too short to be readily accommodated. As a result the properties of the PDT component dominated the biological responses.\textsuperscript{103,104}

An alternate approach to generating radicals and the reactant ROS \textit{in situ} is via the Bergman cyclization of an ene-diyne moiety. This mechanism is presumed to be the operant mode by which the ene-diyne-containing antibiotics, such as calicheamicin and neocarzinostatin, exert their cytotoxic effects. While extremely potent, lack of tissue activity severely limits their clinical utility. Conjugation to tissue-selective monoclonal antibodies, e.g., MyloTarg, has been one successful method for limiting off target toxicity.\textsuperscript{107} Jones, \textit{et al.}, explored simpler ene-diyne derivatives, as shown in Figure 2-12, as an approach to developing selective radical-generating therapeutics. Introduction of thermoreactive ene-diyne ester and ether derivatives at the 3- and 17α positions of estradiol was accomplished in good overall yields.\textsuperscript{105-108}
As anticipated, based upon the estrogen SAR, the observed RBA values were extremely low - the ether linked conjugate (Figure 12A) had RBA=0.003%, and the 17α-alkynyl conjugate (Figure 11B) RBA= 0.5%. Therefore, lack of selectivity for ER(+) breast cancer cells was not unexpected.\(^{105}\)

2.4.10 E2-Platin Conjugates

The discovery of cis-platin as an agent for the treatment neoplastic disease represents one of the remarkable developments in cancer chemotherapy. This compound and its derivatives now comprise a major component in the treatment regimens for many forms of cancer, including breast cancer. In spite of its inherent activity, its usefulness is limited, like many anticancer
drugs, by its lack of tissue selectivity and systemic toxicity. In the field of breast cancer research, a number of efforts have been undertaken to develop E2-platin conjugates that would retain the anticancer activity of cis-platin but demonstrate the ER -selectivity of estradiol. A recent review specifically described the metalated-estrogen conjugates, and therefore only the relevant findings will be summarized here.

As shown in Figure 2-13, a substantial number of E2-platin conjugates have been prepared and evaluated as potential therapeutic agents. Conjugates in which the substituent was ligated at the 3- or 17β-position typically demonstrated very low ER-binding affinity. Although significant cytotoxic activity was retained with these derivatives, such effects could not be attributed to specific ER targeting events. Even the novel Pt(IV) complex prepared by Lippard, et al., failed to demonstrate significant ER(+) selectivity. Substitution at the 16α-position, as reported by Berbube, et al., generated a novel series of conjugates. These compounds were active in several cancer cell lines, but demonstrated little ER-based selectivity. The absence of an ER-related response was not surprisingly given the low ER-binding affinity of the E2-platin conjugates.
The more interesting E2-platin-estradiol conjugates were those in which the metal-containing group was attached at either the 7α- or 17α- positions, as these substitution patterns are known to be more sterically tolerant. As with the mono-and terpyridyl ethynyl estradiols, the steric interference with the ER-binding pocket was much greater with the bulkier substituent,
thus the RBA of the mono-pyridyl E2-platin conjugate was 10-fold higher compared to the terpyridyl derivative. However, these conjugates displayed little cytotoxic activity or ER-based selectivity.

Essigmann, et al., described the synthesis of (E-en)Pt(II)Cl$_{2n}$, a bifunctional cisplatin conjugate consisting of ethylenediamine dichlororidoplatinum(II) tethered to the 7-position of estradiol (Figure 13B). The investigators suggested that following internalization of the conjugate, hydrolysis of the carbamate group in the linker would release the biologically active cis-platin moiety. This E2-platin conjugate retained significant ER-binding affinity (RBA = unreported) and demonstrated enhanced cytotoxicity in ER(+) breast cancer cell lines as compared to cis-platin alone. The study did not compare the efficacy of the conjugate in ER(-)-breast cancer cells and therefore target cell selectivity was indeterminate. Nevertheless, this constituted one of the first studies in which efforts to impart ER-selectivity to inherently nonselective anticancer agents were moderately successful. Unfortunately, there have been no subsequent published studies following these preliminary results.

2.4.11 Miscellaneous E2 Drug conjugates.

Most of the examples described so far in this review have employed a well-recognized cancer chemotherapeutic agents, e.g. antibiotic, DNA intercalators, antimitotics, as the conjugate partner for estradiol. Other recent examples have used therapeutic components with noncancer targets, in which bioactivity or selectivity may be enhanced through ER-based targeting. Kim, et al., evaluated the effects of E2-conjugates containing a proteosome degrading peptide. Peptides that contain an E2 regulator motif promote ubiquitination of proteins, leading to their
degradation. In this study an active pentapeptide was ligated directly to the 17β-OH using esterification, while introduction of the pentapeptide at the 16α- or 7α- positions of estradiol required a 10-12 steps synthesis to yield the target E2-pentapeptide conjugate. All of the derivatives demonstrated retention of proteosomal degradation activity indicating that the presence of the steroid did not interfere with the action of the peptide. However, only the 7α-substituted estradiol-based conjugate demonstrated significant ER-binding affinity and therefore was selected for subsequent optimization studies.\textsuperscript{126}

Katzenellenbogen, \textit{et al.}, reported the preparation and evaluation of an E2 conjugate in which the second component would recruit FKBP, an abundant intracellular protein. The strategy was based on the premise that the estradiol component would initially target the ER, whereupon, the second component would recruit FKBP rather than the usual coregulatory protein.\textsuperscript{127} The peptidomimetic SLF moiety was conjugated at the 17α-position of estradiol using both short and long linker groups. The RBA value for the conjugate with the short linker was 1.5% while no value was provided for the longer linker variant. Although initial studies indicated that the E2 conjugate was capable of simultaneous binding to both ER and FKBP, it was unable to disrupt the formation of ER-coactivator complexes \textit{in vitro}.\textsuperscript{127}
2.5 REFERENCES


(18) Miller, W. R. Molecular endocrinology and breast cancer - A reed before the wind lives on (a tribute to Professor Mike Reed). *Steroids 2011*, 76, 745-749.


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Chapter 3

This chapter is largely based on published work in *Bioconjugation Chemistry* 2012, 23, pp 785-795, but is presented here as an expanded revision that includes materials related to experimental design and execution.

### 3.1 **INTRODUCTION**

Having conducted the literature review of existing estradiol-drug conjugates (Chapter 2) and determining that none had met the criteria as potential therapeutic agents, we undertook our own approach to develop such an agent. The Hanson lab had developed extensive experience in preparing high affinity estrogens as potential imaging agents for breast cancer. Previous students’ work with 17α-(substituted vinyl) estradiols, both published and unpublished, demonstrated the limitations of substituents at that position and therefore we focused our current efforts in the 11β-position of estradiol, a site that had not received significant attention. Structure activity relationships for the steroidal estrogens indicated that small substituents at that site conferred agonist activity, usually with an increase of binding affinity. Alkyl or heteroalkyl groups beyond 3-4 atoms in length resulted in antagonist properties without a significant loss of affinity. Unsubstituted aromatic groups at the 11β-position also were agonists while more highly substituted derivatives expressed antagonist properties. Although several papers described the synthesis and favorable biological properties of these 11β-substituted estradiol derivatives (RU39411 and RU58668), they were not translated into clinical use. The more demanding chemical syntheses needed to prepare these compounds made them less competitive to ER binding and therefore nonsteroidal antiestrogens, such as tamoxifen and raloxifene, or the simpler steroidal antagonist faslodex gained established roles in endocrine therapy. 1-6
The proposed estradiol-drug conjugates were based on 11β-aryl estradiol antiestrogens, scaffold possessed several potential of advantages. The criteria that the final conjugate would need to have includes: (1) high ER affinity, (2) expression of ER antagonist properties, (3) enhanced anticancer activity compared to the parent drug, (4) selectivity for ER-expressing cancer cells, and (5) improved pharmaceutical properties. The choice of the 11β-aryl substituent permits the parent estradiol scaffold to occupy the default ligand binding pocket of ER. Previous SAR studies indicated that the 11β-aryl group occupies a secondary pocket that extends to the surface of the receptor. In the antagonist conformation the 4’-position of the aryl group would access the solvent exposed space and should permit further conjugation without loss of binding affinity or efficacy. We would then be able to prepare conjugates or derivatives of therapeutic agents that could be linked to the 11β-aryl estradiols through the 4’-position of the aryl group. Our actual synthetic strategy called for the modular assembly of the ultimate conjugate by proceeding from both ends. We would prepare specifically functionalized 11β-aryl estradiol derivatives and functionalized therapeutic agents and then ligate them, using “click” chemistry to form the final estradiol-drug conjugate.7,8
The synthetic strategy for the estradiol component of the conjugate, starting from the estra-4,9-diene-3,17-dione monoethylene glycol ketal precursor, was initially developed by previous graduate students – Edward Hua and J. Adam Hendricks. They demonstrated that 11β-(4-substituted phenyl)estradiols can be prepared in good overall yields. Evaluation of the intended products for ERα- and ERβ-LBD binding affinity indicated that the compounds demonstrate high relative binding affinity (RBA = 20-80%) compared to estradiol and that they were subtype nonselective. The studies also indicated that 4-substituents on the phenyl ring that
were larger than methoxy were ER antagonists. Ligation of a prototypical quinone derivative using “click” chemistry also proceeded in high yield to give a product that retained high affinity and antagonist properties. Therefore, as shown in Figure 3-1, our hypothesis that we could use the 11β-aryl estradiol scaffold as the basis for developing the proposed drug-estradiol conjugates appeared to be valid.9-13

Before describing the development of antiestrogen-doxorubicin (AE-Dox) conjugate, I would like to summarize some of the steroidal conjugation studies that were undertaken by previous students to provide a context for the current work. Although, the final results did not achieve all of the defined criteria for estradiol-drug conjugates, their findings was vital in generating the strategy of making AE-Dox conjugates. The following sections will briefly discuss the conjugation studies of Dr. Edward Hua (estrogen -mitomycin C) and Dr. Adam J. Hendricks (antiestrogen-geldanamycin).11

3.2 Therapeutic mitomycin C-Estrogen Conjugate from 11β prosthetic group

The first target was the preparation of a mitomycin C-estradiol as shown in Figure 3-2. The choice of mitomycin C as the bioactive component was based on its clinical use for the treatment of advanced breast cancer.14 Although estradiol-mitomycin C conjugates had been explored previously, lack of success was largely due to factors previously described.15,16 Mitomycin C belongs to the class of compounds that require metabolic activation, i.e, quinone reduction, prior to alkylation of the DNA.17 It also displays a degree of sequence selectivity based upon its molecular structure.12 It has been shown that structural modifications of the 7-amino group also anticancer and DNA alkylation activity, suggesting that incorporation of a pendant group at that position would be tolerated.13,14 As a result, the compound shown in
Figure 3-2, which would incorporate all of the structural features suggested, and would be essential in the hybrid agent - the 11β-(4-alkoxyaryl) estradiol for anti-estrogenic effects: the alkylamino mitomycin C for DNA binding, and the hydrophilic triethylene glycol linker to span the two functional groups.

![Diagram of hybrid compound AE-Mito](image)

**Figure 3-2: Mitomycin-Antiestrogen hybrid at the 11β position**

The hybrid compound AE-Mito competitively displaced estradiol from ERα-LBD with RBA value of 7±1 %. The azido-estradiol derivative, in the same assay system had an RBA = 26±9 %, indicating that the presence of the additional mitomycin-C group at the terminus of the linker did not have an adverse effect on ER-LBD binding. The hybrid agent did not stimulate the production of alkaline phosphatase at any dose level, however, the compound potently blocked the stimulation caused by 1 nM estradiol. Again, the results demonstrated that the presence of the mitomycin C moiety did not interfere with the receptor binding or transcriptional response. The N-propargyl-porifiromycin component (Figure 3-2), as expected, exhibited low binding to the ERα-LBD (RBA= 0.3±0.2 %), and low level of inhibition in the
alkaline phosphatase assay. Our assays evaluating the effect of the mitomycin moiety on cellular proliferation of MCF-7 (ER-positive) and MDA-231 (ER-negative) breast cancer cell lines indicated that the cytotoxicity activity was comparable to that of mytomicin C alone in both cell lines. Therefore, while the conjugate met the criteria of retaining high affinity and antiestrogen efficacy in cells, it did not enhance the cytotoxicity effect nor did it demonstrate selectivity for ER expressing cells.\textsuperscript{15}

3.3 Therapeutic Geldanamycin-Estrogen Conjugate from 11β prosthetic group

The second effort was a conjugate composed of our steroidal antiestrogen linked to the quinone containing drug geldanamycin, which was developed by Dr. Hendricks. The choice of geldanamycin was based on several factors. Although an estradiol-geldanamycin conjugate, previously reported by Kuduk, \textit{et al.}, had been unsuccessful, the failure was largely due to the position on estradiol to which geldanamycin had been appended. Geldanamycin is another quinone-containing antibiotic for which active derivatives have been extensively explored. Introduction of an allylamino group on the quinone moiety produced a clinically useful agent, (allylaminogeldanamycin AAG), and therefore, further modification at that site, such as linker group, should be well tolerated. The study was undertaken to examine a variety of linkers used to conjugate geldanamycin to the steroidal antiestrogen as ER-targeted therapeutic agents.
Figure 3-3: Geldanamycin-Antiestrogen hybrid at the 11β position

Three 11βE2-GDA hybrids were assembled with three different linkers as shown in Figure 3-3. Two variations of the steroidal antiestrogen were prepared. The first was the 11β-(4 azidoethoxyphenyl) group. For the second, the propargylaminoethoxyphenyl group was introduced in an effort to more closely mimic the side chain found in most antiestrogens. To complement these derivatives, variations of the amino geldanamycin were prepared. The first had a short chain propargylamino group, comparable to that found in AAG. The second variant used a diaminoalkane which was functionalized at the quinone ring of geldanamycin and also alkylated which an activated cyclooctyl. Such reagents undergo spontaneous cyclization reactions with azides in a copperless click reaction. The third variation employed a long hydrophilic oligoethylene glycol linker with a terminal azido group that would react with the
corresponding alkyanyl steroidal derivative. The appropriate ligation gave the targeted antiestrogen-geldanamycin products in good yields.\textsuperscript{7}

These hybrids were tested for cytotoxicity against two cell lines, ER(+)\textsuperscript{-}MCF-7 and ER(-)\textsuperscript{-}SKBr3, which also overexpresses HER-2. The preliminary results indicated no significant differences in cytotoxicity potency between the two cell lines. The IC\textsubscript{50} of the compound B in Figure 3-3 was $1.2 \pm 0.09\mu\text{M}$ in MCF-7 which was similar to that in SKBr3 cells, $0.71\pm0.16\mu\text{M}$. Although the AE-GDC conjugates did not show either synergistic or selectivity effect of the molecule and resulted slightly less potency than the parent GDC, the data demonstrated that the hybrids mostly retain activity and begin to illustrate a trend regarding the GDA hybrids. There seems to be a correlation between the spatial proximity of the two compounds and their activity by the tether linker. For example, in Figure 3-3, comparing compound B ($1.2 \pm 0.09\mu\text{M}$) and C ($0.102 \pm .005\mu\text{M}$) in MCF-7, one can see an order of magnitude improvement in its EC\textsubscript{50} for MCF-7 cells. This suggests that the longer tether, providing greater distance between the two compounds, is beneficial to their construction and efficacy, while the shorter chains can affect their activity, most likely due to spatial issues. Compound A is more difficult to compare because its tether, while an intermediate length, possesses different spatial, steric and physicochemical properties. However, additional data and studies are needed to confirm these trends and further our understanding of these hybrids.

My primary objective was to investigate a series of different AE-toxins and determine which combination would give us the desired conjugation criteria as previously mentioned. Based on the literature review and previous studies in the research group, I did not plan on using the same chemo toxins or linkers. However the substituted steroid remained as my choice for
targeting ER-expressing cells. Most of my conjugation strategy was designed based on the convergent modular synthetic approach, which is represented in the Scheme 3-1 below.

The strategy involves the targeting molecule conjugated with the bifunctional oligomer ethylene glycol (OEG) containing a terminal alkyne or azide, so it can be appropriately ligated with its partner, a complimentary module, using Huisgen [3+2] cycloaddition (“click”) chemistry. This synthetic approach allows the terminal/biomolecule-containing component and the functionalized targeted module to be prepared independently. The separation of each element from the other component can be modulated by varying the length of the linker units. Although the OEG is indicated as our linker, one can use other linker moieties, thereby generating further structural diversity.25-32

![Scheme 3-1 Convergent synthesis model of bioconjugate molecule](image)

To demonstrate that our convergent synthesis model approach would be useful for ligating two therapeutic molecules together as a single entity, I had prepared several tamoxifen (antitestrogen)-drug conjugates (Table 3-1). Because the parent compounds are potent estrogen/antiestrogen, they might lose potency once they are conjugated. To verify the potency of the compounds, I evaluated the final conjugates in ER(+) and ER(-) expressing breast cancer
cells. The results of some representative conjugates in Table 3-1, indicated that none of the compounds demonstrated different effects when compare with the parent compounds. The results in cytotoxicity studies of TAM-TKI (compound C) conjugates suggested no antiproliferative activity in both cell lines MCF-7 and MDA-MB-231 (Figure 3-6).

**Table 3-1**: Bioconjugate of Tamoxifen with antiestrogen (AE) and tyrosine kinase inhibitors

<table>
<thead>
<tr>
<th>Therapeutic 1</th>
<th>Bifunctional linker</th>
<th>Therapeutic 2</th>
<th>Bioconjugates</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Image A]</td>
<td>![Diagram A]</td>
<td>Tamoxifen (Tam)-17β estradiol derivative (AE-Tam)</td>
<td></td>
</tr>
<tr>
<td>![Image B]</td>
<td>![Diagram B]</td>
<td>Tamoxifen (Tam)-11β estradiol derivative (11βAE-Tam)</td>
<td></td>
</tr>
<tr>
<td>![Image C]</td>
<td>![Diagram C]</td>
<td>Tamoxifen- Tyrosine kinase inhibitor (TKI-Tam)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3-4: Cytotoxicity evaluation Tam-TKI (compound C) in (ER+)-MCF-7 and (ER-)-MDA-MB-231

The real motivation behind the building these conjugates was to look at the use of a high potency RU39411 (Table 3-2), as the parent scaffold for making an ER targeting compound. However, studies involving with RU39411 would first require the synthesis of the parent drug, then N-demethylation and linker ligation, which requires a multistep process. Therefore, I decided to do pilot studies using a readily accessible anti estrogen, tamoxifen, which has the same N,N-dimethyl-2-phenoxyethanamine group as RU 39411.
**Scheme 3-2**: “Click” ligation of tamoxifen derivatives with other therapeutic moieties

**Figure 3-5**: Cytoxicity evaluation of Tam-AE (compound B) in (ER+) MCF-7 and (ER-) MDA-MB-231
Synthesis of tamoxifen (Tam) hybrids, Scheme 3-2, began with N-demethylation to give desmethyl Tam. Alkylation of the N-methylaminoethoxy Tam derivative with either the azido or alkyne substituted TEG tosylate gave the corresponding conjugated terminal functionalized Tam component (75% yield). Ligation of the alkynyl Tam to the complementary 2-azidoethoxyphenyl 11β estradiol, using the copper assisted cyclization reaction, yielded the tethered anti-estrogen-Tamoxifen hybrid in good isolated yields. Using a similar route, the azido Tam was coupled to the alkylnyl TKI to give Tam–TKI (compound C) hybrid.

From the cell culture results, indicated in Figure 3-5, it is clear that Tam-AE (compound B) is less potent in both cell lines than tamoxifen alone or its derivatives (Tam attached to linkers). A similar result was observed for the Tam-TKI conjugate, in which the hybrid did not inhibit the cell proliferation, even at high doses. It was noted that the conjugate had lost the potency compared to the parent compound tamoxifen. One of the immediate observations observed in these assays was that while some conjugations may have a synergistic effect, the potency of the individual compounds may also be lost when each is conjugated with another compound. Therefore, as mentioned previously, the conjugate and its components must be carefully selected in order to generate the desired therapeutic efficacy.

3.4 Therapeutic Doxorubicin-Estrogen Conjugate from 11β prosthetic group

3.4.1 The 11β-Antiestrogen (AE) component

In designing the anti-estrogen – doxorubicin conjugate, we developed a model strategy, i.e. each component can be prepared independently and ultimately incorporated in a modular
fashion. The selection of the right 11β-substituted antiestrogen for developing the AE-Dox conjugate was carefully considered. The previous studies by Drs. Hua and Hendricks showed that the 11β-substituted phenyl estradiols retain a very good binding affinity to ER, however, none of AE components in their conjugates demonstrated synergy as antiproliferation agents. Therefore, I carefully examined the next “generation” of 11β-AE agents as listed in the Table 3-2. Of all the compounds, the AE with the aminoethoxy group extending from the phenyl had the best binding affinity to the ER and appear to exert the best potency against (ER+)-MCF-7 cells. ²

**Table 3-2: Similarity in development of antiestrogen (AE) generation**

<table>
<thead>
<tr>
<th>1&lt;sup&gt;st&lt;/sup&gt; gen. of 11β-AE</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; gen. of 11β-AE</th>
<th>RU39411</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; gen. of 11β-AE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Dr. Ed Hua thesis)</td>
<td>(Dr. Hendricks thesis)</td>
<td></td>
<td>(This work)</td>
</tr>
</tbody>
</table>

![Chemical structures](image)

Therefore, an analog of steroidal anti-estrogen similar to RU39411 was selected as the targeting component. Not only is the parent compound a pure antagonist, it possesses significantly higher ER binding affinity as compared to most non-steroidal anti-estrogens, such as tamoxifen and raloxifene. It is also less lipophilic. ²¹-²⁵ The deletion of the triarylethylene pharmacophore would also reduce interactions with the anti-estrogen binding sites not associated with the estrogen receptor. The analysis of its binding mode in comparison to tamoxifen-ERα-LBD complexes suggested that the ligation of linker groups via the tertiary amine should result in retention of ER affinity, as the linker would be external to the ligand-binding pocket. The modified anti-estrogen 9 in Scheme 3-3, is synthetically accessible via a multistep process
starting from the steroidal intermediate deltenone (as shown in the experimental section).\textsuperscript{10,11} The intermediate for compound 9 can also be made via a single step using demethylation from RU39411 compound to form the demethylated amino AE 9. However, RU39411 is no longer available from its source (Sanofi-initially Roussel Uclaf) which limits that route. We did have a small quantity (<200mg) obtained in the late 1980 with which to conduct initial studies.

**Synthesis of 11β-AE scheme**

**Scheme 3-3:** Synthesis of azido 11β-AE (the 3\textsuperscript{rd} generation)
The initial biological results have indicated that neither the 11β-substituted antiestrogen nor the modified azido alkynated-AE, compound 9, showed any significant differences in activity on breast cancer cells as compared to estradiol, Figure 3-6. Therefore, it was concluded that modifying 11β-AE with the linker should not produce any significant changes in the compound activity.

**Figure 3-6**: Cytoxicity evaluation 11β-AE (compound 9) in (ER+)-MCF-7 and (ER-) MDA-MB-231
3.4.2 The doxorubicin (Dox) hydrazone component

There are several reasons that for choosing doxorubicin as the therapeutic component for our AE-Dox bioconjugate development. As previously mentioned, doxorubicin is considered the most clinically effective anthracycline drug for estrogen-responsive breast cancer. While highly effective as a cytotoxic agent, its use is compromised by dose-limiting cardiotoxic side effects. Strategies to improve its clinical utility have focused on prodrug approaches to reduce side effects and targeted drug delivery to improve its efficacy. Some of the most promising doxorubicin derivatives involve hydrazone formation through the ketone or amide conjugation on the carbohydrate amino-group. In both approaches, intracellular processes, such as pH-dependent hydrolysis or enzymatic cleavage of the amide bond, lead to free doxorubicin that generates the observed therapeutic response. While such conjugation strategies may reduce cardiotoxic effects, selective or enhanced delivery of the agent to the tumor was not improved.²⁶-

Figure 3-7: Prodrug approaches in the development of doxorubicin conjugates

Enhanced tumor delivery of doxorubicin requires the incorporation of an appropriate targeting agent.¹⁶-¹⁷ As discussed in Chapter 2, a few attempts were made to conjugate
doxorubicin derivative (daunorubicin) through the 17 position of estradiol via amide or cyano bond. However, no synergistic or potency-enhancing effects were observed. Until recently, several examples of doxorubicin derivatives bearing tumor-selective groups, including nonestradiol breast cancer selective agents have been reported.\textsuperscript{18} For example, a non-steroidal anti-estrogen, such as, tamoxifen, (Figure 3-8) was used to target doxorubicin to ER(+) breast cancer, however, had significant problems. The parent compound, tamoxifen, has low ER affinity and exhibits substantial non-ER binding. Although the hydroxylated metabolite, 4-hydroxytamoxifen, has higher ER affinity, this compound exists as a mixture of E/Z-isomers and is chemically less stable.\textsuperscript{24,29} Nevertheless, preliminary studies suggested that an enhanced and selective cytotoxicity in breast cancer cells may be achieved using a better targeting group. The conjugation of amino-sugar component doxorubicin to the amino terminus of hydroxyl tamoxifen using a releasable linker led to an increase in anti-proliferative activity in a variety of breast cancer cell lines. The investigators suggested that the targeting in this case was due to a combination of ER and anti-estrogen binding site (AEBS) effects.\textsuperscript{23-25,29-31}

\textbf{Figure 3-8:} Tamoxifen-Doxorubicin Conjugate
3.4.3 The benzoyl hydrazone and TEG linkers components

Much of the (anti-)estrogen-doxorubicin conjugate research has focused on the targeting and therapeutic groups, however, the linking moiety is also important. For the hybrid to be effective in vitro or in vivo, the linker must be long enough to permit the ER-binding component to interact with the target protein while maintaining a stable bond with the doxorubicin. Likewise, the interaction with doxorubicin must be stable in the extracellular environment while permitting facile dissociation within the target breast cancer cells. The linker also needs to have physicochemical properties that do not compromise its formulation or its biological compatibility. Therefore, our strategy in this study considered all three components in the drug design process - the estrogen targeting component, the doxorubicin drug delivery component as well as the linker component that would tether the targeting, readily release mechanism and chemotherapeutic units.

In the process of evaluating the linkers, there were a variety of choices available, such as polyalkyl, polyamide or polyethylenene glycols. Based upon their prior use in pharmaceutical and other biological materials, oligoethylene glycols were chosen. The length can be anywhere from 3 to 9 repeating ethylene glycol units, however, the choice of selecting tetraethylene glycol (TEG) seemed most appealing to us for several reasons. First, the polydispersity of TEG is about 93%, a higher uniformity compared to other higher molecular weight OEG. Secondly, TEG is small enough to be soluble in most of organic solvents. It is commercially available at an affordable price for large quantity. The idea was to make each half linker from the same length of OEGs with different heterobifunctional head groups. Each half linker could be attached covalently to either the targeting (anti-estrogen) or chemotherapeutic (doxorubicin) moiety. The
other terminal group consists of a “click” partner that would be ligated in the final step to form the targeted conjugate. In this case, I selected TEG as for both half linkers to develop the tether for the conjugate. The important reason of selecting TEG for both sides of the conjugate molecule is that it would give the symmetrical orientation of the molecule in space. In addition, the polarity of two sides of the triazole (after click conjugation) would not be so much different, perhaps enhancing the stability and ease in separation of the final compound (Scheme 3-4).

Scheme 3-4: AE-Dox conjugates derived from one simple TEG
The preparation of doxorubicin hydrazones is well described, however, we needed to develop a specific linker to form the desired hydrazone. There were two reasons for selecting benzoyl hydrazones as the Dox linker as supposed to other hydrazones. First of all, the aroyl hydrazone intermediate is more stable than alkanoyl hydrazone because in the protonated form the charge could be resonated through aromatic ring. While the alkyl form of the hydrazone bond would be cleaved, therefore it is less hydrolytic instability comparing to the aryl form. The second reason for incorporating aromatic ring into the linker was that it not only improves the hydrophobicity of the molecule, but also simplifies the characterization and separation of the molecule. The synthesis of pH-sensitive Dox-hydrazone is summarized in Scheme 3-5 below.

Scheme 3-5: Synthesis of alkynated-Dox derivative AE-Dox conjugate
The final assembly strategy for our bioconjugate Ae-Dox is shown in scheme 3-4. As illustrated in Figure 3-9 in which we take each component, attach it to the appropriate half-linker, and finally ligate them to form the intact bioconjugate. We hypothesize that the resultant bioconjugate should retain anti-proliferative effects comparable to doxorubicin in all cancer cell lines, but demonstrate more potential and selective anti-proliferative effects in ER-expressing cancer cells. As our results illustrate, the final compound achieved these target properties and demonstrated selectivity towards ER(+) breast cancer cells and promoted an enhanced cytotoxicity against those cells compared to the unmodified parent components.

The results of the cytotoxicity assay demonstrated that both of our modified azido-antiestrogen and the alkynated-Dox retained the potency comparable to the parent compound, and the biological effects were not altered by chemically adding on the linker groups, Figure 3-10. Therefore, the linkers that were incorporated into the bioconjugate should have no significant effect on the biological system, but rather serve as a bridge to link the two therapeutic moieties together. The linkers primary functions are not only to link, but also to provide a suitable pharmacokinetic and stability shield for the entire bioconjugate. Another words, it is there to protect the drug from undergoing degradation or metabolism during circulation in the body. In this way it may also protect the body from the toxicity of the drug, thereby enhancing the
therapeutic value when compared to the parent drug. Therefore, the process of selecting and optimizing the right linkers is important in the development of the bioconjugate.

\[ \text{Figure 3-10: A) Cytotoxicity of azido-11\beta AE; and B) alkynated-Dox in (ER\text{+})-MCF-7} \]

3.5 Results and Discussion

3.5.1 Characterization of 11\beta substituted Estradiol-Doxorubicin Conjugate

The convergent modular approach proved to be a successful strategy for assembling the target anti-estrogen doxorubicin conjugate AE-Dox 15. Each of the starting materials was transformed to the requisite components in high yields and purity. In particular, the conversion of the readily
available tetra(oligo)ethylene glycols to heterobifunctional half-linkers, that was used for assembling the compound (Scheme 3-1) is notable because of the possibility of subsequent variations that one can generate. Using this approach, one can control the length of the spacer groups by varying the spaces oligo ethylene glycol. One can modulate the modes of ligation to address concerns not apparent at the outset of the study, e.g., by using thiol-maleimide, hydrazone-ketone, azide-alkyne, amine-acid, or alkyne-tetrazine. In our circumstances, the use of small tetraethylene glycol (TEG) moieties facilitated the individual reactions, purifications and subsequent ligations. TEG was converted initially to the O-propargyl derivative which was sequentially converted to the tosylate and reacted with the methyl-4-hydroxy benzoate. The methyl ester underwent hydrazinolysis to give the require benzoyl hydrazide. Reaction of the propargylated-tetraethylene glycol benzohydrazide 13 with doxorubicin gave the alkynylated hydroxyl doxorubicin 14, in good isolated yields. The product was stable under neutral and basic conditions. The preparation of the steroidal anti-estrogen 9 was readily achieved from the starting material 1. Epoxidation of deltenone 1 gave the mixture of α+ β epimers. The α form is subsequently underwent Cu(I)-mediated -1,4-addition of 4-trimethylsilylphenyl magnesium bromide. Work up under acidic condition gave the 11β-(4-hydroxy phenyl)-estra-4,9-diene-3-7-dione. Williamson ether synthesis using dimethylamino ethyl chloride, followed by aromatization with acetic anhydride/acetyl bromide provided estrone-3-acetate. Reduction using NaBH₄ followed by saponification gave RU39411. In subsequent studies, described in Chapter 4, this sequence was shortened and with improved yield.

N-Demethylation using dichloroethyl chloroformate gave the key secondary amine 7. The subsequent alkylation with the azido-tetraethylene glycol tosylate 8 gave the intermediate 9. It was important to do careful separation and purification because the N-alkylation reaction was
also accompanied by some O-alkylated by product. The conversion to the final product 15 was achieved with good yield (64%) using the classical “click” conditions. The final product could be readily separated from the two lower molecular weight components using preparative LC.

Figure 3-11: Synthesis of Antiestrogen-Doxorubicin (AE-Dox) Conjugate 15. “Reprinted with permission from Dao, K-L; Sawant, R.; Hendricks, J. A.; Ronga, V.; Torchilin, V. and Hanson, R. N. Bioconjugate 2012, 23, 785-795. Copyright 2012 American Chemical Society.”

To verify the ligation of the AE-Dox conjugate 15 from its two starting materials, the azido anti-estrogen 9 and the alkynylated Dox 14, we performed a stacking $^1$H-NMR analysis, FT-IR and HPLC. AE-Dox formation was indicated by the signature triazole proton, shown at approximately 8.7 ppm in the $^1$H-NMR spectrum, as well as by the presence of the characteristic
aromatic signature protons for both Dox and AE components (Figure 3-12A). The FTIR spectrum supported the ligation of the two intermediates by the disappearance of the azide stretch approximately at 2100cm\(^{-1}\) from compound 9, and the alkyne stretch at 2050cm\(^{-1}\) (Appendix I) from compound 14. In addition, the HPLC data (Figure 3-12B) showed the elution of a single peak for 15 that was distinct from the azide 15 or the alkyne 14.

Figure 3-12: A) Partial stacking \(^1\)H-NMR of AE-Dox 15; B) HPLC of AE-Dox 15 and its components
3.5.2 Biological Evaluation of 11β substituted Estradiol-Doxorubicin Conjugate (AE-Dox)

The preliminary evaluation of the parent compounds (Dox and RU39411), linker modified components 14 and 9, and the final AE-Dox hybrid 15 was determined using ER(+) MCF-7 and ER(-)-MDA-MB-231 breast cancer cell lines. ER(+) MCF-7 is a human breast adenocarcinoma cell line that overexpresses ER and is an excellent in vitro assay system to demonstrate anti-estrogenic effects,\textsuperscript{25,32,33} while ER(-)-MDA-MB-231 cells are insensitive to anti-hormonal interventions.\textsuperscript{34} Both cell lines are responsive to doxorubicin, such that structural modifications affecting the activity should be readily apparent. Under the conditions of the bioassay, neither cell line metabolizes doxorubicin or the anti-estrogen to a significant degree, so that any activity observed would be due to the parent drug and to not potential metabolites.\textsuperscript{26-27}

Initial cell-based assays evaluated whether the presence of the linker groups would affect the individual components as inhibitors of cell proliferation (cytotoxicity). The steroidal anti-estrogen and its azido-linker modified derivative (N\textsubscript{3}-AE 9) had no significant cytotoxic effects on ER(+) MCF-7 cells at concentrations below 1 µM and only modest effects at 10 µM (Figure 3-12). In the same cell line, doxorubicin and its alkynyl hydrazone derivative 14 displayed statistically equivalent IC\textsubscript{50} values (Table 3-3) and in vitro cytotoxicity profiles (results also in experimental section). Therefore, the introduction of the tetraethylene glycol (TEG) linker onto the amino terminus of the steroidal anti-estrogen and the TEG hydrazone linker onto the ketone of doxorubicin had no observable effects on the antiproliferation activity of the parent compounds. In contrast, the AE-Dox conjugate 15 showed a significant enhancement of cytotoxicity (approximately 70 fold) in ER(+)-MCF-7 cells compared to the other formulations (Figure 3-13A). The IC\textsubscript{50} for the AE-Dox conjugate 15 was 0.011µM compared to 0.602 µM and
0.597 µM for the free Dox and the linker-Dox 14, respectively. In the ER(-)-MDA-MB-231 cell line (Figure 3-13B), the presence of the anti-estrogenic component had no significant effect. The cytotoxicity curves for all three compounds in this cell line were essentially superimposable, with IC50 values in a narrow range (0.125-0.080 µM), as shown in Table 3-3.

**Figure 3-13**- Cytotoxicity of compounds 11β-AE 9 (black), Dox-linker 14-red, Dox (orange), and AE-Dox 15 (purple), in A) ER(+)MCF-7 and B) ER(-)MDA-MB-231 cell lines.

Table 3- 3: Inhibition Concentration, IC$_{50}$ (nM), of various Dox compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MCF-7</th>
<th>MDA-MB-231</th>
<th>MCF-7 (+E2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dox</td>
<td>602 ± 20</td>
<td>89 ± 7</td>
<td>585 ± 30</td>
</tr>
<tr>
<td>Dox-Linker 14</td>
<td>597 ± 20</td>
<td>86 ± 5</td>
<td>594 ± 20</td>
</tr>
<tr>
<td>AE-Dox 15</td>
<td>11 ± 6</td>
<td>90 ± 8</td>
<td>589 ± 3</td>
</tr>
</tbody>
</table>

The IC$_{50}$ was estimated by using GraphPad™ 3-parameters curve fitting for 24 h drug exposure data points. All values are represented here, were evaluated in a duplicate of a triplicate count. Standard deviation was measured from the mean of six wells for each compound.

We then determined whether the effect of the anti-estrogenic component in the hybrid AE-Dox 15 could be reversed by the addition of estradiol (ES). This would illustrate whether the effect was mediated through an ER depndent process. Cytotoxicity of Dox and the hybrid AE-Dox 15 were analyzed in ER(+)MCF-7 cells in the presence or absence of E2. The results (Table 1) show that the enhanced cytotoxicity of the AE-Dox hybrid was completely abolished by the addition of ES whereas ES has virtually no effect on either Dox itself, or the linker-Dox 14. Therefore, it appeared that the effect was ER-dependent and not a non-specific process.

The subsequent study was undertaken both to support the ER-related effect and to identify the nature of that effect. Cell uptake /targeting of the hybrid AE-Dox 15 with ER(+)MCF-7 and ER(-)-MDA-MB-231 cells was evaluated using fluorescent activated cell sorting (FACS), both in presence and absence of ES, as shown in Figure 3-14. The AE-Dox 15 demonstrated enhanced cell binding only to ER(+)MCF-7 cells in the absence of ES. Although the exact mechanism is not yet explored, the results from blocking mER with ES, depleted the Dox uptake, illustrated that the AE-Dox may enter the cell via interacting with mER and
triggering the endocytosis process. In presence of ES (blocking the mER), cell binding of AE-Dox 15 was similar to Dox alone. Also, the ER(-)-MDA-MB-231 cells did not show any significant change in cell binding of Dox and AE-Dox 15 and, as expected, the presence or absence of ES had no effect on cell binding of the drugs. The results in Figure 3-14 illustrated a marked targeting effect imparted by the presence of the steroidal anti-estrogen component in the hybrid agent 15. These effects were consistent with the interaction selective for the membrane ER.

**Figure 3-14:** The FACS analysis of ER(+)MCF-7 and ER(−)MDA-MB-231 cells. A) Histogram analysis of cells treated with 0.1 μM of Dox and AE-Dox 15 with and without ES; (purple) cells only (green) Dox treated cells and (red) AE-Dox treated cells. B) The percentage of Dox-positive cells in ER (+)MCF-7 and ER (−)MDA-MB-231 cells; *P<0.01 (Dox vs AE-DOX (−ES)); §P<0.01 (AE-Dox (−ES) vs AE-Dox(+ES); “ Reprinted with permission from Dao, K-L;
Because FACS studies do not distinguish between membrane, cytoplasmic or nuclear localization of the fluorescent group, we undertook cellular studies using the fluorescence microscopy. The objective of this experiment was not only to verify the cell membrane binding uptake of the AE-Dox compound in the absence and present of ES with MCF-7, but also to examine the localization and distribution of doxorubicin in the cytoplasm (once it dissociated from the conjugate). The results shown in Figure 3-15 clearly support enhanced AE-Dox targeting to the ER-positive breast cancer cells. The cellular localization of the fluorescence from AE-Dox and Dox was evaluated via ER(+)-MCF-7 cells in the presence (+) and absence (-) of ES. The low level of fluorescence for cells treated with Dox alone (Panel B) is associated primarily with the cytoplasm. AE-Dox (Panel C) shows a significantly enhanced uptake within the ER(+)-MCF-7 cells, compared to Dox alone (Panel B) suggesting that the AE component of the conjugate has substantially facilitated uptake by cells. The cellular distribution, however, appears more consistent with free Dox distribution than that of the ER ligand, which tends to localize in the nuclei. The incubation of the cells with the AE-Dox and ES (Panel D) restores the uptake and distribution pattern essentially to that observed for Dox alone (Panel B). The inhibition of a membrane ER-mediated pathway would leave only the non-specific passive diffusion process available, leading to the observed lower cellular accumulation. This study supported the observation for the initial cytotoxicity results where AE-Dox would generate a higher intracellular concentration of Dox and therefore more rapid cell death. The lack of a
nuclear accumulation of fluorescence suggested that the anti-estrogenic component was no longer attached to the doxorubicin moiety.

![Figure 3-15: Fluorescence microscopy images ER(+)MCF-7 cells treated for 1 hour with 0.1μM of B) Dox; C) AE-Dox 15; D) AE-Dox 15 after pretreatment with 50μM estradiol; and A) untreated cell; Hoechst fluorescent (upper panel), Red fluorescence (lower panel) “ Reprinted with permission from Dao, K-L; Sawant, R.; Hendricks, J. A.; Ronga, V.; Torchilin, V. and Hanson, R. N. *Bioconj. Chem.* 2012, 23,785-795. Copyright 2012 American Chemical Society.”](image)

### 3.6 Conclusion and future direction

In this study, we have demonstrated that we can prepare the antiestrogen-doxorubicin (AE-DOX) conjugate 15 efficiently and in high yield using our modular assembly approach. Because the components can be prepared independently, and conjugated using a simple chemistry, potential modifications of the conjugate properties are relatively easy. Initial cytotoxicity experiments demonstrated that the AE-Dox conjugate 15 was 70-fold more potent than Dox alone in ER(+)MCF-7 cells, but equipotent compared to Dox in ER (-)-MDA-MB-231
cells. The enhanced cytotoxic effect in MCF-7 cells was reversed by pre-incubation with ES, suggesting an ER-mediated process. Subsequent FACS studies on both cell lines in the presence or absence of ES supported this hypothesis. Additional evaluations using fluorescence microscopy in MCF-7 cells suggested that the uptake was a membrane ER-mediated effect leading to an enhanced cytoplasmic accumulation of Dox, and resulting in enhanced cytotoxic response. These observations are consistent with a process by which the anti-estrogen component in our conjugate facilitates the uptake by the ER(+) cells via the membrane ER-mediated mechanism. Within the cell, the pH-sensitive hydrazone release mechanism incorporated into the conjugate leads to the elevated cytoplasmic levels of free Dox, as supported by the fluorescence studies. Studies to further characterize the individual steps of the process are in progress.

3.7 Experimental -Synthesis

3.7.1 Experimental procedure

General Methods

All solvents and reagents involved in the synthesis were reagent grade, purchased from either Sigma-Aldrich™ or Fisher Scientific, and used without further purification. Thin-layer chromatography (TLC) was done on polyester sheets pre-coated with silica gel matrix 60 F254 obtained from Sigma-Aldrich™. Separations were performed using automated flash chromatography (Argonaut FlaskMaster) or packed column chromatography with Sorbent Technologies silica gel particle size 32-63 μm and 60 Å pore size. Liquid chromatography-mass spectroscopy (LC-MS) was performed using Alliance HT -LCT Premier 2489, Waters® instrument. High performance liquid chromatography (HPLC) was performed using a Waters HPLC system, equipped with a Waters 2695 binary pump, a Waters 2998 fluorescence
photodiode array detector, and a XBridge™ C18 column (3.5μm, 4.6x75mm). $^1$H and $^{13}$C NMR spectra were recorded on 400 or 500 MHz Varian FT-NMR spectrometers. Chemical shifts ($\delta$) are reported in parts per million (ppm) by reference to proton resonances resulting from incomplete deuteration of the NMR solvent. The concentrations of test compounds were determined spectrophotometrically with a diode array UV mini 1240, Shimadzu® spectrophotometer. Ultracentrifugation of cell lysates was accomplished with a Sorvall RT 6000B Refrigerated Centrifuge. All tissue culture materials were obtained from Gibco Life Technologies (Grand Island, NY) unless otherwise stated. MCF-7 and MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD).

High Performance Liquid Chromatography (HPLC) Analysis

The compounds were analyzed with Waters HPLC system, equipped with a Waters 2695 binary pump, a Waters 2998 fluorescence photodiode array detector, and a XBridge™ C18 column (3.5μm, 4.6x75mm). HPLC grade acetonitrile/water/trifluoroacetic acid (50/50/0.1%, v/v) was used as the mobile phase at 25°C with a flow rate of 1.0 mL min$^{-1}$. The fluorescence detector was set at 254 nm for excitation and 570 nm for emission and linked to Empower III™ software for data analysis.

Cell cultures

All components tested in biological assays were analyzed and determined $>95\%$ pure by HPLC with UV detection.

The ER(+)MCF7 (human breast adenocarcinoma) and ER(-) MBA-MD-231 (human breast adenocarcinoma), cell lines were maintained in Dulbecco’s Modified Eagle Medium
(DMEM) at 37º C, 5% CO₂. DMEM were supplemented with 10 % fetal bovine serum (FBS), 50 u/ml –penicillin, and 50µg/ml streptomycin.

**Cytotoxicity assays.**

Cells were plated at a $5 \times 10^3$ cells per well density in 96-well plates (Corning Inc., Corning, NY, USA). The stock solutions of free drug or drug-conjugates were prepared in DMSO and diluted in complete media before adding to cells. After 24h, the medium was replaced with medium containing free drug or drug-conjugates. After 24h incubation, each well was washed twice with complete media and cell survival was measured using the Cell Titer-Blue® Cell Viability Assay method. The conversion of Resaruzin to Resorufin by viable cells results in the fluorescence excitation at 550nm. The fluorescence produced is proportional to the number of viable cell. The emitted fluorescence was measured at 590 nm (the measurement of the cytotoxicity) using a Labsystems Multiskan MCC/340 microplate reader (Labsystems and Life Sciences International, UK).

For estradiol competition assay, the cells were pretreated with 17β-estradiol (ES) (50μM) for 1h, followed by addition of free drug or drug-conjugates in complete media with 50μM of ES. After 24h incubation, cell viability was analyzed as described above.

**Flow cytometry**

The ER(+)MCF-7 and ER(-)MDA-MB-231 cells were grown in 12-well tissue culture plates till 70-80% confluency. The medium was removed from the wells; cells were washed with complete media and incubated with or without 50µM ES for 1h. After incubation, the cells were washed twice with complete media, and exposed to 0.1µM of Dox, AE-Dox in complete media
with or without 50 μM of ES. After 1h cells were washed, trypsinized, and finally resuspended in 800 μl of 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4. The cell-associated fluorescence was quantified by Becton Dickinson FACScan™ (Becton Dickinson, San Jose, CA) at the emission wavelength of 580 nm (channel FL-2). The data analysis was performed using CellQuest software (Becton Dickinson). A total of 10,000 events were acquired for each sample. Data shown were derived from three separate experiments.

**Fluorescence Microscopy**

The ER (+) MCF-7 cells were seeded on a coverslip in six-well tissue culture plates at a concentration of $1 \times 10^5$ cells per well. After 24 h, the cells were washed twice with complete media and then incubated with 0.1μM Dox or AE-Dox 15. After 1 h incubation, medium was removed, and cells were washed twice with sterile PBS followed by fixation of the cells with 4% paraformaldehyde (15 min at room temperature). Hoechst 33342 (1 μg/ml) was added to the cells for 15 min and cells were washed twice with sterile PBS. Cells were observed immediately on a Nikon Eclipse E400 fluorescence microscope equipped with appropriate filters for, Rhodamine and Hoechst detection. For estradiol competition assay, the cells were pretreated with 17β-estradiol (ES) (50μM) for 1h, followed by addition of 0.1 μM of AE-Dox in complete media with 50μM of ES.

**IC$_{50}$ Calculation**

The IC$_{50}$ calculation for all of the free drug, conjugates, and control compounds were performed by using SoftGraph™ software (after converting raw data to % cell lyses). A representative calculation data for is shown in table below. All compounds were solubilized in
dimethyl sulfoxide and prepared as 100mM stock solution before diluting 1000 times with DMEMs media for cell incubation. Drug treatment lasted 24 h, and cells were cultured until the control wells had achieved 80% confluence (typically 2-3 days). For every experiment, each drug formulation and controls were performed in hexuplicate; each experiment was performed at least in duplicate. Error bars represent one standard deviation about the mean for the six wells per lane measured for each drug concentration.

**Quantification of [Dox] via UV**

To ensure that the amount of doxorubicin contained in the conjugate is the same concentration of Dox in each tested solution, we assembled free Dox standard curve using fluorescent absorbance since Dox exhibits a distinctive absorbance at wavelength 380 nm (Figure 3-16). Table 3-4 summarized all the UV measurements of our conjugate solutions. The results indicated that the Dox conjugate solutions consistently incorporated the same amount of DOX as in DOX alone in free solution. For example, 1μM of DOX (52.7μg/l) solution gave 0.325 absorbance at λ=380 nm, similarly 1μM of DOX attached with the linker, and AE-DOX hybrid solutions emitted 0.320 and 0.331 respectively, hence indicating the conjugates contain the same amount of 52.7 μg/l as free DOX solution.
**Figure 3-16:** Standard curve of free DOX

![Graph showing the standard curve of free DOX](image)

<table>
<thead>
<tr>
<th>[µg/mL]</th>
<th>DOX</th>
<th>DOX-linker</th>
<th>AE-DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>26.4</td>
<td>0.183 ± 0.032</td>
<td>0.173 ± 0.017</td>
<td>0.169 ± 0.030</td>
</tr>
<tr>
<td>52.7</td>
<td>0.325 ± 0.011</td>
<td>0.320 ± 0.010</td>
<td>0.331 ± 0.022</td>
</tr>
<tr>
<td>79.1</td>
<td>0.597 ± 0.020</td>
<td>0.601 ± 0.015</td>
<td>0.573 ± 0.028</td>
</tr>
</tbody>
</table>

**Table 3-4:** UV absorbance of different DOX
3.7.2 Synthesis of Tamoxifen conjugates.

Scheme 3-6-Synthesis of Tamoxifen conjugates

(Z)-2-(4-(1,2-diphenylbut-1-en-1-yl)phenoxy)-N-methylethanamine (compound 1)- To a solution of tamoxifen (1.00 g, 2.7mmol) in anhydrous CH$_2$Cl$_2$ (20mL) was added 3-chloroethyl chloroformate (0.50 mL, 4.5mmol). After 30 min stirring at 0°C, the reaction was refluxed at 80°C for 24 h. The solvent was evaporated to yield a yellowish oil. Immediately methanol (10 mL) was added, and the mixture was refluxed for approximately 4 hr. The solvent was
evaporated, and purification via column chromatography with CH$_2$Cl$_2$:MeOH (98:2) gave 0.69 g (69%) of N-desmethyl tamoxifen, compound A. $^1$H NMR (CDCl$_3$, 400MHz) δ 0.89 (3H, t, J= 7.2 Hz), 2.43 (2H, q, J= 14.8, 7.6 Hz), 2.56 (3H, s), 3.12 (2H, t, J= 4.0 Hz), 4.08 (2H, t, J= 4.8 Hz), 6.57 (2H, d, J= 8.8 Hz), 6.76 (2H, d, J= 8.8 Hz), 7.03-7.31 (10H, m), 9.56 (1H, br); LC-MS (C$_{25}$H$_{27}$NO)$^+$ calcd, 358.21; found, 358.23.

2-{$(2$-{$(2$-Azido-ethoxy)-ethoxy}$)$-ethyl tosylate (azido TEG linker) -To a solution of Ethanol, 2,2'-oxybis-1,1'-bis(4-methylbenzenesulfonate) (2 g, 4 mmol) in ethanol (25 mL) was added sodium azide (0.28g, 4.3 mmol). The resulting solution was heated at 80 °C for 16h. The reaction mixture was poured into ice water (125mL) and the product was extracted with ethyl acetate. The combined organic extracts were washed sequentially with water and brine solution, and dried over magnesium sulfate. The crude material was purified using silica gel chromatography to yield the product azido TEG linker, as clear oil (1.2 g, 79%). $^1$H NMR (CDCl$_3$, 400MHz): δ 1.21 (2H, td, J=6.9 Hz, 2.93 Hz), 2.45 (3H, s), 3.40 (2H, m), 3.52- 3.75 (10H, m), 3.93 (2H, m), 7.37 (2H, d, J=4.7Hz), 7.79 (2H, d).

2-{$(2$-{$(2$-{$(2$-Prop-2-ynnyloxy)-ethoxy}$)$)-ethoxy$)$-ethoxy$)$-ethanol (alkyno TEG linker). To a solution of propargyl tetraethylene glycol (1.0 g, 4.31mmol) in CH$_2$Cl$_2$ (15.0 mL) was added triethylamine (1.2mL, 8.6 mmol) and p-toluenesulfonyl chloride (0.99 g, 5.2 mmol). The reaction mixture was stirred at ambient temperature for 16 h. The solvent was removed under
reduced pressure to give a crude product as dark oil. Separation using silica gel column chromatography gave the product, alkyno TEG linker (1.53g, 92%), as a yellow oil. $^1$H NMR (CDCl$_3$, 400MHz): δ 2.45 (3H, s), 2.49 (1H, m, J=2.2 Hz), 3.59 - 3.64 (14H, m), 3.65 - 3.72 (2H, m), 4.13 - 4.17 (2 H, m), 4.18 (2H,m, J=2.2 Hz), 7.36 (2H, m,J=8.1 Hz), 7.79 (2H, d, J=8.1 Hz). $^{13}$C NMR (CDCl$_3$, 100MHz): δ 21.9, 21.9, 58.6, 68.9, 69.3, 69.5, 70.6, 70.7, 70.7, 70.8, 70.9, 74.8, 74.8, 79.9, 128.2, 130.1, 133.2, 145.0

\[
\begin{align*}
\text{(Z)-2-(2-(2-azidoethoxy)ethoxy)ethoxy)-N-(2-(1,2-diphenylbut-1-en-1-yl)phenoxy)ethyl-N-methylethanamine (compound 1)}
\end{align*}
\]

To a solution of desmethylated tamoxifen (0.68 g, 0.82 mmol) in CH$_3$CN (10 mL) was added K$_2$CO$_3$ (0.170 g, 1.23 mmol). The mixture was stirred at RT for 30 min, then azido-TEG-linker (0.450 g, 1.23 mmol) was added. Stirring continued for 1 hr, then at 40°C for 16h. Precipitate was removed by filtration, extracted with ethyl acetate and washing with water/brine solution. Purification was performed on a silica column, eluting with CH$_2$Cl$_2$:CH$_3$OH (95:5) yielding the compounds oil (0.55g, 60%). $^1$H NMR (CDCl$_3$, 400MHz) δ 0.91 (3H, t, J = 7.2 Hz), 2.31-2.46 (8H,m), 2.71 (2H, br), 2.83 (2H, br), 3.57-3.70 (28H, m), 3.95 (2H,br), 4.15 (2H, t, J = 4.4 Hz), 6.53 (2H, d, J = 8.4 Hz), 6.76(2H, d, J= 8.8 Hz), 7.10-7.34 (12H, m), 7.80 (2H, d, J= 8.4Hz); LC-MS (C$_{48}$H$_{68}$NO$_{11}$S)$^+$ calcd, 864.1; found, 864.7
TAM-TKI (compound C) conjugate- To a solution of compound 1 (10 mg, 0.017 mmol) in 500 μL of tert-butanol/water (1:1) was added a solution of Alkynyl-TKI (5.5 mg, 0.017 mmol) in 500 μL of tert-butanol/water (1:1). The reaction was stirred at room temperature for 30 min, followed by the addition of copper(II) sulfate pentahydrate (3.75 μL, 0.15 μmol) and (+)-sodium L-ascorbate (14.8 μL, 0.75 μmol). The reaction mixture was warmed to 40°C, stirred for 24 h, and then partitioned between water (10 mL) and dichloromethane (10 mL). The organic layer was washed with water (2x10 mL). The aqueous layers were combined; sodium chloride was added and then back extracted with dichloromethane (10 mL). The organic fractions were combined, dried over magnesium sulfate, filtered and concentrated, leaving a dark red residue. The product was isolated using column chromatography (85:15 dichloromethane/methanol) to yield the final product (7.8 mg, 53%) as an oil. ¹H NMR (acetone d₆, 500MHz) δ 0.32 (3H, s), 3.55 (12H, s), 4.32 (2H, br), 4.55 (3H, br), 4.72 (2H, t), 6.37 (1H, dd, J= 4.7Hz, 3.8Hz), 6.56 (1H, d, J = 2.6 Hz), 6.63 (2H, d, J = 8.4 Hz), 6.76 (1H, d, J= 8.4Hz), 7.00 (2H, d, J=8.5Hz), 7.24 (1H, s), 7.36 (1H, q), 7.59 (1H, d, J=8.3Hz), 7.74 (1H, s), 7.96 (1H, s), 8.05 (1H, d, J=10.2), 8.58 (1H, s), 9.09 (1H, s); ¹³C NMR (acetone d₆, 500MHz) δ 13.3, 23.1, 28.2, 29.7, 30.2, 35.8, 38.6, 43.9, 45.9, 47.6, 49.6, 52.0, 56.0, 64.3, 66.4, 68.7, 69.3, 69.6, 70.5, 70.7, 70.6, 72.3, 81.8, 101.4, 108.6, 109.4, 113.3, 115.3, 117.0, 124.0, 127.6, 129.9, 137.6, 145.0, 147.9, 150.1, 153.0, 154.5, 163.9; LC-MS (C₅₁H₅₆FN₇O₆)⁺ calcd, 881.43; found, 880.7
TAM-11βAE (compound B) conjugate- To a solution of (2) (20 mg, 0.034 mmol) in 500 μL of tert-butanol/water (1:1) was added a solution of N₃-11β-AE (15 mg, 0.034 mmol) in 500 μL of tert-butanol/water (1:1). The reaction was stirred at room temperature for 30 min, followed by the addition of copper(II) sulfate pentahydrate (5.5 μL, 0.35 μmol) and (+)-sodium L-ascorbate (25.8 μL, 1.75 μmol). The reaction mixture was warmed to 40°C, stirred for 24 h, and then partitioned between water (10 mL) and dichloromethane (10 mL). The organic layer was washed with water (2x10 mL). The aqueous layers were combined; sodium chloride was added and then back extracted with dichloromethane (10 mL). The organic fractions were combined, dried over magnesium sulfate, filtered and concentrated leaving a dark red residue. The product was isolated using column chromatography (95:5 dichloromethane /methanol) to yield the final product (11 mg, 32%) as a yellow oil.

3.7.3 Synthesis 11β substituted Estradiol-Doxorubicin Conjugate

3,3-ethylenedioxy-5(10)-α-epoxy-estr-9(11)-ene 2α. To a solution of estr-5(10),9(11)-dien-3,17-dione 3-ethylenedioxy ketal 1 (10.0 g, 31.8 mmol), hexafluoroacetone (0.46 mL, 3.6 mmol), and pyridine (0.23 mL, 2.86 mmol) in 10 mL of dichloromethane was added hydrogen
peroxide (50%, 2.28 mL, 74 mmol) at 0 °C. After 18 h stirring at the ambient temperature, the reaction was terminated by the addition of 4 g of sodium thiosulfate in 100 mL of water and extracted with dichloromethane (3x 100 mL). The organic layer was dried over magnesium sulfate, filtered and the solvent was removed under reduced pressure. The resulting colorless solid was triturated with diethyl ether (35 mL). The precipitate was collected by filtration and rinsed with diethyl ether (25 mL) to yield 5.23 g (15.8 mmol) 50% of the 2α-isomer. The mother liquor was purified via flash chromatography to afford 2.0 g (5.8 mmol, 18%) of the β-isomer, and an additional 600 mg (1.8 mmol, 5.7%) of 2α-isomer. Overall 3,3-ethylenedioxy-5(10)-α-epoxy-estr-9(11)-ene-17-one (5.8 g, 17.5 mmol, 56 % yield) and 3,3-ethylenedioxy-5(10)-β-epoxy-estr-9(11)-ene-17-one (2.0 g, 5.8 mmol, 18 % yield) were obtained in a final ratio of 3:1 in favor of the α-isomer and a total yield of 74%. For 2α-isomer 1H NMR (CDCl3, 400MHz): δ 0.88 (3H, s), 1.32-1.12 (1H, s), 2.52-2.44 (2H, m), 3.98-3.88 (4H, m), 6.06 (1H, s). 13C NMR (CDCl3, 100MHz): δ 14.8, 22.0, 22.3, 25.2, 28.1, 31.7, 33.7, 36.5, 37.2, 40.4, 46.1, 46.81, 60.1, 61.7, 64.2, 64.5, 107.1, 125.8, 136.8, 221.3; m.p. 154°C For β-isomer 1H NMR (400 MHz, CHCl3): δ 0.87 (s, 3H), 1.32-1.12 (m, 1H), 2.52-2.44 (m, 2H), 3.98-3.88 (m, 4H), 5.87(s, 1H).

11β-(4-Hydroxy-phenyl)-estra-4,9-diene-3,17-dione 3. 3,3-Ethylenedioxy-5(10)-α-epoxy-estr-9-ene-17-one 2α (2.014 g, 6.1 mmol) was dissolved in anhydrous THF (15 mL) under an argon atmosphere. Copper (I) iodide (0.160 g, 0.840 mmol) was added to the solution at -10°C and stirred for 15 min. Freshly prepared Grignard reagent, (4-((trimethylsilyl)oxy)phenyl)magnesium
bromide, was added dropwise in 5.0 mL aliquots. The reaction was gradually warmed to the ambient temperature and stirring was continued for 16h. The reaction was quenched by the addition of ammonium chloride (0.8 g, 15 mmol) in 35 mL of water and 35 mL of EtOAc at 10°C. The organic layer was washed with water (2x 35 mL). The organic solvent was removed under reduced pressure and the resulting residue was dissolved in a mixture of acetic acid (14 mL) and water (6 mL). The resultant mixture was warmed at 50-60°C for 1.5 hours, after which it was diluted with ethyl acetate (20 mL). The solution was neutralized by the addition of saturated aqueous sodium bicarbonate. The organic layer was separated, washed with brine solution, dried over magnesium sulfate and evaporated to dryness to give a crude, yellow oil. Purification using silica gel column chromatography (70:30 hexane/ethyl acetate) afforded the product 3 (2.00 g, 76%) as yellow solid: \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) 0.53 (3H, s), 4.38 (1H, d, J = 6.9 Hz), 5.78 (1H, s), 6.71 (2H, d, J=3.7Hz), 6.97 (2H,d, J=4.9Hz). \(^{13}\)C NMR (CDCl\(_3\), 100MHz): \(\delta\) 14.17, 21.7, 22.0, 25.9, 27.0, 30.6, 35.0, 35.6, 36.8, 38.1, 38.3, 39.7, 47.5, 50.7, 115.5, 122.8, 128.3, 129.8, 135.5, 145.6, 155.6, 155.9, 197.5, 217.4; m.p. 248°C.

11β-[4-(2-Dimethylamino-ethoxy)-phenyl]-estra-4,9-diene-3,17-dione 4. To a solution of 3 (250 mg, 0.69 mmol) and cesium carbonate (1.1 g, 3.45 mmol) in 10mL acetone was added 2-N,N-dimethyl chloroethylamine hydrochloride (223 mg, 2.07 mmol). The reaction mixture was heated at reflux for 16 h. The solvent was removed under reduced pressure and the residue was extracted with ethyl acetate. The organic layer was washed with water, brine solution, dried over
magnesium sulfate and evaporated under reduced pressure to give a crude oil. Purification using flash chromatography yielded the product 4 (287mg, 96%) as light yellow oil. $^1$H NMR (CDCl$_3$, 400MHz): δ 0.56 (3H, s), 1.22 - 1.41 (2H, m), 1.48 - 1.72 (2H, m), 1.91 (2H, dd, J=13.92, 6.60 Hz), 1.99 - 2.23 (6H, m), 2.28 - 2.52 (6H, m), 2.59 - 2.67 (2H, m), 2.67 - 2.89 (3H, m), 4.03 (1H, t, J=5.86 Hz), 4.38 (2H, d, J=7.33 Hz), 5.79 (1H, s), 6.84 (2H, d, J=8.79 Hz), 7.08 (2H, d, J=8.06 Hz). $^{13}$C NMR (CDCl$_3$, 100MHz): δ 15.6, 22.1, 26.1, 27.0, 29.6, 31.1, 32.0, 35.6, 37.0, 38.0, 39.8, 46.1, 47.9, 50.9, 54.0, 58.5, 66.1, 114.9, 123.6, 127.9, 128.0, 128.2, 130.2, 136.1, 145.2, 156.2, 157.2, 199.6

3-Acetoxy-11β-[4-(2-dimethylamino-ethoxy)-phenyl]-estra-1,3,5(10)-triene-17-one 5. To a solution of 4 (200 mg, 0.42 mmol) in dichloromethane (10 mL) was added acetic anhydride (47 mg, 0.46 mmol) and acetyl bromide (142 mg, 1.15 mmol) at room temperature. The reaction solution was stirred for 16h after which the product was extracted with ethyl acetate and washed with water. The organic layer was separated, dried over magnesium sulfate, and evaporated under reduced pressure to give a yellow crude oil. Column chromatography afforded the desired product 5 (207 mg, 95%). $^1$H NMR (CDCl$_3$, 400MHz): δ 0.45 (3H, s), 1.25 (2 H, s), 1.47 - 1.73 (1H, m), 1.84 - 2.02 (3H, m), 2.03 - 2.20 (6H, m), 2.22 - 2.29 (6H, m), 2.35 (3H, d, J=10.2 Hz), 2.41 - 2.64 (2H, m), 2.87 - 3.03 (2H, m), 3.07 (1H, br. s.), 4.00 - 4.22 (2H, m), 6.59 - 6.76 (2H, m), 6.86 (1H, d, J=2.2 Hz), 6.96 (2H, d, J=8.8 Hz), 7.27 (2H, d, J=8.6Hz). $^{13}$C NMR (CDCl$_3$,}
11β-[4-(2-Dimethylamino-ethoxy)-phenyl]-estra-1,3,5(10)-triene-3,17β-diol 6. To a solution of 5 (200 mg, 0.42 mmol) in methanol (5 mL) was added sodium borohydride (24 mg, 0.63 mmol) and the reaction mixture was stirred at room temperature. After 1 h, 10N sodium hydroxide (0.025mL, 0.25 mmol) was added and the reaction continued for 16 h. The reaction solution was poured into an ice cold mixture of ethyl acetate (20 mL) and water (20 mL), after which the organic layer was separated, washed sequentially with water and brine, and dried over magnesium sulfate. The solvent was removed under reduced pressure to give a yellow crude oil. Silica column chromatography afforded the product 6 (176 mg, 96%). 1H NMR (CDCl3, 400MHz): δ 0.36 (3H, s), 1.17 - 1.29 (2H, m), 1.29 - 1.44 (2H, m), 1.70 (2H, d, J=8.7 Hz), 1.78 (2H, dd, J=13.18, 5.8 Hz), 1.86 - 1.99 (2H, m), 2.03 - 2.14 (2H, m), 2.27 - 2.38 (6H, m), 2.51 (3H, d, J=12.2 Hz), 2.61 - 2.67 (2H, m), 2.69 - 2.85 (2H, m), 3.68 (1H, t, J=8.0 Hz), 3.89 - 4.04 (2H, m), 6.37 (1H, dd, J=8.30Hz, 2.44 Hz), 6.44 - 6.58 (3H, m), 6.76 (1H, d, J=8.79 Hz), 6.95 (2H, d, J=8.3 Hz). 13C NMR (CDCl3, 100MHz): δ - 15.4, 21.6, 22.3, 23.1, 27.3, 30.2, 35.5, 38.3, 40.2, 44.8, 47.8, 48.3, 52.4, 57.4, 64.7, 64.9, 82.5, 114.0, 116.4, 128.6, 130.4, 131.6, 139.1, 158.6
11β-[4-(2-methylamino-ethoxy)-phenyl]-estra-1,3,5(10)-triene-3,17β-diol 7. To a solution of 6 (100 mg, 0.23 mmol) in anhydrous dichloroethane (10mL) was added α-chloroethyl chloroformate (53 μL, 0.48 mmol). The reaction mixture was stirred at 0°C for 30 minutes, and then heated at reflux for 24 h. The solvent was removed by rotary evaporation; methanol (3mL) was added and the reaction solution was heated at reflux for 3 h. The solvent was removed by rotary evaporation to give the crude product as clear oil. Purification using silica gel chromatography gave the product 7 (82 mg, 85%) as yellow oil. $^1$H NMR (CDCl$_3$, 400MHz) δ 0.34 (3H, s), 1.20 - 1.45 (2H, m), 1.63 - 1.87 (2H, m), 1.90 - 2.18 (2H, m), 2.32 (1H, s), 2.51 (4H, m), 2.72 - 2.97 (4H, m), 3.69 (1H, t, J=8.4 Hz), 3.88 - 4.03 (2H, m), 6.39 (1H, dd, J=8.4, 2.5 Hz), 6.47 - 6.61 (3H, m), 6.79 (1H, d, J=8.0 Hz), 6.97 (2H, d, J=8.0 Hz). $^{13}$C NMR (CDCl$_3$, 100MHz): δ 15.4, 21.6, 22.3, 23.1, 27.3, 30.2, 35.5, 38.3, 40.2, 44.8, 48.3, 48.4, 52.4, 57.4, 64.7, 64.9, 82.5, 114.0, 116.4, 128.6, 130.4, 131.6, 139.1, 158.6

2-{2-[2-(2-Azido-ethoxy)-ethoxy]-ethoxy}-ethyl tosylate 8. To a solution of tetraethylene glycol bisparatoluenesulfonate (2 g, 4 mmol) in ethanol (25 mL) was added sodium azide (0.28g, 4.3 mmol). The resulting solution was heated at 80 °C for 16h. The reaction mixture was poured into ice water (125 mL) and the product was extracted with ethyl acetate. The combined organic extracts were washed sequentially with water and brine solution, and dried over magnesium
sulfate. The crude material was purified using silica gel chromatography to yield the product as clear oil 8 (1.2 g, 79%). $^1$H NMR (CDCl$_3$, 400MHz): $\delta$ 1.21 (2H, td, $J$=6.9, 2.93Hz), 2.45 (3H, s), 3.40 (2H, m), 3.52-3.75 (10H, m), 3.93 (2H, m), 7.37 (2H, d), 7.79 (2H, d).

11β-(4-{2-[2-(2-Azido-ethoxy)-ethoxy]-ethoxy}-ethyl)-methyl-amino]-ethoxy]-phenyl)-estra-1,3,5(10)-triene-3,17β-diol 9. To a solution of 7 (8 mg, 0.205 mmol) and potassium carbonate (43 mg, 0.31 mmol) in acetonitrile (10 mL) was added dropwise at ambient temperature under an inert atmosphere a solution of 8 (69mg, 0.16 mmol) in acetonitrile. The solution was heated at reflux for 16 h. The reaction solvent was evaporated under reduced pressure and the resulting residue was extracted with ethyl acetate. The solvent was evaporated under reduced pressure and the crude material was purified using silica gel column chromatography to yield a light yellow oil 9 (77 mg, 60%). $^1$H NMR (CDCl$_3$, 400MHz): $\delta$ 0.31 (3H, s), 1.18 - 1.45 (2H, m), 1.66 - 1.81 (2H, m), 1.99 - 2.20 (2H, m), 2.33 - 2.41 (4H, m), 2.51 (1H, d, $J$=12.5 Hz), 2.68 (2H, t, $J$=5.8 Hz), 2.77 - 2.90 (2H, m), 3.34 - 3.42 (2H, m), 3.45 - 3.71 (14H, m), 3.81 (2H, t, $J$=4.8Hz), 3.92 - 4.00 (4H, m), 4.00 - 4.07 (3H, m), 6.49 (1H, dd, $J$=8.43, 2.6 Hz), 6.56 - 6.67 (2H, m), 6.86 (2H, d, $J$=8.8 Hz), 6.94 (2H, d, $J$=8.8 Hz). $^{13}$C NMR (CDCl$_3$, 100MHz): $\delta$13.0, 17.5, 23.4, 28.2, 30.5, 30.7, 35.6, 38.4, 43.8, 45.8, 47.6, 50.9, 52.1, 56.8, 57.4, 67.4, 69.6, 70.0, 70.3, 70.6, 70.9, 71.0, 76.9, 82.8, 112.6, 113.7, 114.8, 127.7, 130.7, 131.1, 136.0, 137.7, 155.9, 156.3.
2-{2-[2-(Prop-2-ynyloxy-ethoxy)-ethoxy]-ethoxy}ethanol 10. To a solution of tetraethylene glycol (2.00 g, 10.3 mmol) in dichloromethane (10.0 mL) was slowly added sodium hydride (60% in paraffin, 580 mg, 15 mmol) at -20°C. To the reaction vessel was added dropwise at -20°C a solution of propargyl bromide (3.0 g, 22 mmol) in 5.0 mL tetrahydrofuran. The reaction mixture was stirred at -20°C for 30 minutes, allowed to warm to ambient temperature and then stirred for an additional 24 h. The reaction was partitioned between ethyl acetate (20 mL) and water (20 mL), after which the organic layer was washed with water and brine. The organic layer was dried over magnesium sulfate, filtered and the solvent was removed via rotary evaporation. The crude product was purified using silica gel column chromatography to yield 1.55g (65%) of the product 10 as oil. ¹H NMR (CDCl₃, 400MHz): δ 1.26 (1H, t, J=7.1 Hz), 2.08 - 2.09 (1H, m), 2.17 (1H, s), 2.44 (2H, t, J=2.4 Hz), 3.65 - 3.77 (14H, m), 4.21 (2H, d, J=2.4 Hz). ¹³C NMR (CDCl₃, 100MHz): δ 58.3, 61.5, 69.0, 70.3, 70.5, 70.5, 70.6, 72.7, 75.1, 79.7.

2-(2-(2-(Prop-2-ynyloxy)-ethoxy)-ethoxy)-ethanol tosylate 11. To a solution of propargyl tetraethylene glycol 10 (1.0 g, 4.31mmol) in CH₂Cl₂ (15.0 mL) was added cesium carbonate (1.4g, 4.3 mmol) and p-toluenesulfonyl chloride (0.99 g, 5.2 mmol). The reaction mixture was stirred at ambient temperature for 16 h. The solvent was under reduced pressure to give a crude product as dark oil. Separation using silica gel column chromatography gave the product 11 (1.53g, 92%) as a yellow oil. ¹H NMR (CDCl₃, 400MHz): δ. 2.45 (3H, s), 2.49 (1H,
J=2.2 Hz), 3.59 - 3.64 (14H, m), 3.65 - 3.72 (2H, m), 4.13 - 4.17 (2 H, m), 4.18 (2H, J=2.2 Hz), 7.36 (2H, J=8.1 Hz), 7.79 (2H, d, J=8.1 Hz). $^{13}$C NMR (CDCl$_3$, 100MHz): δ 21.9, 21.9, 58.6, 68.9, 69.3, 69.5, 70.6, 70.7, 70.8, 70.9, 74.8, 74.8, 79.9, 128.2, 130.1, 133.2, 145.0

Methyl 4-(2-(2-(2-(prop-2-ynloxy) ethoxy)ethoxy)ethoxy)ethoxybenzoate 12. To a solution of 11 (1.0 g, 2.6 mmol) in dichloromethane (10mL) was added dropwise a mixture of cesium carbonate (126 mg, 388 mmol) and methyl 4-hydroxybenzoate (590 mg, 3.9 mmol) in 5 mL of dichloromethane. The reaction was heated at reflux for 16 h. The reaction mixture was filtered and then concentrated under rotary evaporation. The residue was purified using silica gel column chromatography to afford the product 12 (1.1g, 70%) as clear oil. $^1$H NMR (CDCl$_3$, 400MHz): δ 2.05 (1 H, s), 2.44 (3 H, t, J=2.5 Hz), 3.62 - 3.75 (12 H, m), 3.82 - 3.92 (2H, m), 4.09 - 4.22 (4 H, m), 6.93 (2 H, d, J=8.9 Hz), 7.98 (2 H, d, J=8.7 Hz). $^{13}$C NMR (CDCl$_3$, 100MHz): δ 22.6, 52.1, 52.3, 58.6, 67.8, 69.3, 69.7, 70.4, 70.6, 70.8, 71.1, 74.8, 79.9, 114.4, 122.9, 131.7, 162.8, 167.0
4-(2-[2-(2-(2-Prop-2-ynyloxy-ethoxy)-ethoxy]-ethoxy]-ethoxy)-benzoic acid hydrazide 13.

To a solution of 12 (200 mg, 0.55 mmol) in ethanol (10 mL) was added hydrazine hydrate (44 mg, 1.4 mmol). The solution was heated at reflux for 10 h. The solvent was removed by rotary evaporation to give a crude material that was purified by using amino column chromatography. The product 13 was isolated (144 mg, 72%) as a pale, yellow oil. $^1$H NMR (CDCl$_3$, 400MHz): $\delta$ 1.81 (2H, s), 2.16 (1H, s), 2.21 - 2.35 (2H, m), 3.66 - 3.81 (14 H, m), 4.2 (2H, m), 6.96 (2 H, d), 7.78 (2H, d), 7.82 (1H, s). $^{13}$C NMR (CDCl$_3$, 100MHz): $\delta$ 67.7, 69.7, 71.0, 73.2, 114.5, 114.6, 125.2, 126.0, 129.0, 129.5, 144.0, 161.8, 161.9, 168.5, 169.8, 173.6.

4-(2-(2-(2-(Prop-2-ynyloxy)ethoxy)ethoxy)ethoxy)ethoxybenzohydrazone--doxorubicin conjugate 14. To a solution of 12 (14 mg, 0.04mmol) in ethanol (5mL) was added doxorubicin hydrochloride (20 mg, .004 mmol) and trifluoroacetic acid (64.5mg, 0.6mmol). The reaction mixture was stirred at 20°C for 24h, concentrated to approximately 1.0 mL and triturated with ether to yield a red precipitate. The red precipitate was collected by filtration, washed with ether, and dried under vacuum to afford the product (28.5 mg, 85%). $^1$H NMR (CDCl$_3$, 400MHz): $\delta$ 1.29 (3H, d, J=6.6 Hz), 1.81 - 1.94 (3H, m), 1.97 - 2.10 (2H, m ), 2.11 - 2.22 (1H, m), 2.30 - 2.47
(1H, m), 2.89 - 3.03 (1H, m), 3.06 - 3.15 (1H, m), 3.22 - 3.37 (12H, m), 3.52 - 3.74 (3H, m), 3.78 - 3.89 (1H, m), 4.04 (3H, s), 4.14 - 4.24 (2H, m), 4.26 - 4.33 (1H, m), 4.71 (2H, d, J=2.2 Hz), 5.03 - 5.17 (1H, m), 5.39 - 5.52 (2H, m), 6.97 - 7.13 (2H, m), 7.51 - 7.62 (1H, m), 7.74 - 7.89 (3H, m), 7.94 - 8.00 (1H, m). 13C NMR (CDCl3, 100MHz): δ 11.6, 20.5, 28.6, 38.3, 48.1, 51.2, 61.0, 67.6, 69.4, 70.1, 70.3, 70.4, 72.4, 114.2, 117.3, 122.4, 125.8, 131.4, 140, 153.2, 155.4, 163.1, 167.4, 187.2. LC-MS (C45H53N3O15^+ H) calcd, 875.35; found, 875.30

**Anti-estrogen Doxorubicin Conjugate 15.** To a solution of 9 (10mg, 0.015mmol) in 500μL of tert-butanol/water (1:1) was added a solution of 14 (13.1mg, 0.015mmol) in 500μL of tert-butanol/water (1:1). The reaction was stirred at room temperature for 30 min, followed by the addition of copper(II) sulfate pentahydrate (3.75μL, 0.15μmol) and (+)-sodium L-ascorbate (14.8μL, 0.75μmol). The reaction mixture was warmed to 40°C, stirred for 24 h, and then partitioned between water (10mL) and dichloromethane (10mL). The organic layer was washed with water (2x10mL). The aqueous layers were combined; sodium chloride was added and then back extracted with dichloromethane (10mL). The organic fractions were combined, dried over magnesium sulfate, filtered and concentrated leaving a dark red residue. The product was purified using column chromatography (85:15 dichloromethane /methanol) to yield the product 15 (12.7mg, 55%) as a red solid. TLC ((Si2O, 80:20 dichloromethane /methanol) Rf= 0.25: 1H NMR (CH3OH-d, 500MHz): δ = 7.93 (2 H, d, J=7.2 Hz), 7.89 (1H, s), 7.85 (1H, s), 7.76 (1H, s), 7.61 - 7.67 (3H, m), 7.56 (1H, s), 7.49 (1H, s), 7.25 (2H, d, J=7.8 Hz), 7.03 (2H, d, J=8.8 Hz),
6.99 (2H, d, J=8.8 Hz), 6.90 (1H, s), 6.83 (2H, s), 6.75 - 6.79 (2H, m), 4.36 (1H, t, J=7.3 Hz), 4.27 (2H, m), 4.16 - 4.20 (1H, m), 4.03 (1H, d, J=5.9 Hz), 3.86 - 3.90 (2H, m), 3.71 - 3.75 (3H, m), 3.63 - 3.71 (28 H, m), 3.55 - 3.61 (2 H, m), 3.50 - 3.54 (1H, m), 3.39 - 3.44 (1H, m), 2.39 (2H, s), 2.27 (3H, s), 1.93 (1H, s), 1.64 - 1.74 (2H, s), 1.41 - 1.50 (6H, m), 1.33 - 1.41 (2H, m), 1.30 (2H, s), 1.28 (1H, s, br.), 1.26 (3H, m), 1.15 (3H, m), 1.00 ppm (3H, m). LC-MS (C_{70}H_{83}N_{7}O_{16}^{+} H)^{+} calcd, 1277.59; found, 1277.50
3.8 REFERENCES


(9) Hanson, R. N.: Steroidal anti-hormone hybrids with quinone antibiotics as antitumor agents. pp 101. *US patent* 940978. 2010


Chapter 4

Design, Synthesis and Biological Evaluation of Cell Membrane Targeted Imaging Agents: Labeled 11β-Antiestrogens and Prostate Specific Membrane Antigen (PSMA) Ligands as Molecular Probes for Positron Emission Tomographic Imaging
4.1 Introduction

Over the past decades, molecular imaging (MI) has emerged as a discipline that has profoundly changed the practice of medicine, especially in oncology. Its primary aim is to enable integration of patient and disease-related information with anatomical imaging.\textsuperscript{1} The information gathered from MI, when used as a noninvasive tool for specific diagnosis of diseases, provides better clinical risk assessment, optimization of treatment and therapy, and better patient outcomes. This chapter will focus on a general survey of MI technologies in cancer therapy, followed by an in-depth discussion of our strategy for developing MI probes that target estrogen receptors (ER) in ER dependent breast cancer using our 11β-substituted estradiol, and prostate-specific membrane antigen (PSMA) receptor in androgen-independent prostate cancer (PCa) using urea-based PSMA inhibitors.

Molecular imaging is defined as the visualization, characterization and measurement of biological processes at the molecular levels in a living organism.\textsuperscript{2,3} MI includes both two- and three-dimensional imaging as well as real-time quantification. There are quite different MI modalities, each with its own advantages and disadvantages. Some require imaging or contrast agents as probes for visualization, and they can use either endogenous or exogenous molecules. In general, MI reveals the biology of the disease process. It documents specific information needed to personalize patient care by characterizing specific disease processes in individuals. It can provide important data for drug discovery and development, such as data for pharmacokinetics (PK) studies. In addition, MI can provide powerful disease quantification, via determination of regional concentrations of MI agents. It can reveal the stages of the disease progression, e.g., quantification of biological processes of a disease at the molecular and cellular
level. In short, MI is an important element of data and image analysis, especially for inter-and intrasubject comparisons.\textsuperscript{5-7}

In recent years, medical imaging has evolved into two distinctive areas: an anatomy-based (or structural imaging) and nuclear medicine.\textsuperscript{8-10} Traditional imaging, such as computed tomography (CT) and magnetic resonance imaging (MRI), are now primarily used for extracting anatomical and/or structural information. On the other hand, positron emission tomography (PET) and single photon emitted tomography (SPECT), focus as on molecular events in living organisms and thereby provides functional and/or physiological imaging. PET and SPECT imaging in oncology have developed a common aim to integrate patient specific disease with the molecular information that is derived from imaging studies.\textsuperscript{11-13} Table 4-1 below summarizes the major differences among the various MI modalities. Pro and cons for each imaging technique are discussed in the following paragraphs.

**Table 4-1 - Noninvasive *in vivo* molecular imaging modalities\textsuperscript{11}**

<table>
<thead>
<tr>
<th>Imaging</th>
<th>Modality</th>
<th>Energy used</th>
<th>Spatial Resolution (mm)</th>
<th>Acquisition time/frame (s)</th>
<th>Probe mass (ng)</th>
<th>Sensitivity of detection (Mol/l)</th>
<th>Depth of penetration (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET</td>
<td>Annihilation</td>
<td>-</td>
<td>Clinical: 3-8, Animal: 1-3</td>
<td>1-300</td>
<td>1-100</td>
<td>10\textsuperscript{-11}-10\textsuperscript{-12}</td>
<td>&gt;300</td>
</tr>
<tr>
<td>SPECT</td>
<td>ϒ-photons</td>
<td>-</td>
<td>Clinical: 5-12, Animal: 1-4</td>
<td>60-2000</td>
<td>1-1000</td>
<td>10\textsuperscript{-10}-10\textsuperscript{-13}</td>
<td>&gt;300</td>
</tr>
<tr>
<td>CT</td>
<td>X-rays</td>
<td>-</td>
<td>Clinical: 0.5-1, Animal: 0.03-0.4</td>
<td>1-300</td>
<td>-</td>
<td>-</td>
<td>&gt;300</td>
</tr>
<tr>
<td>MRI</td>
<td>RF</td>
<td>-</td>
<td>Clinical: 0.2-0.1, Animal: 0.025-0.1</td>
<td>50-3000</td>
<td>10\textsuperscript{-6}-10\textsuperscript{-8}</td>
<td>10\textsuperscript{-4}-10\textsuperscript{-6}</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>High Rf</td>
<td>-</td>
<td>Clinical: 0.1-1.0, Animal: 0.05-0.1</td>
<td>0.1-100</td>
<td>10\textsuperscript{-10}</td>
<td>-</td>
<td>1-200</td>
</tr>
<tr>
<td>BLI</td>
<td>IR</td>
<td>-</td>
<td>Clinical: -</td>
<td>10-300</td>
<td>10\textsuperscript{-4}</td>
<td>10\textsuperscript{-15}-10\textsuperscript{-16}</td>
<td>1-10</td>
</tr>
<tr>
<td>FLI</td>
<td>IR</td>
<td>-</td>
<td>Clinical: -</td>
<td>10-2000</td>
<td>10\textsuperscript{-4}</td>
<td>10\textsuperscript{-10}-10\textsuperscript{-14}</td>
<td>1-20</td>
</tr>
</tbody>
</table>

Magnetic resonance imaging (MRI) primarily provides soft tissue and secondarily functional information by exploiting differences in proton density, perfusion, diffusion and
biochemistry. MRI possesses high spatial resolution (<1 mm) and has the ability to visualize anatomical, physiological, and/or metabolic information in a single imaging session. MRI also offers good depth penetration. MRI with higher field strengths provides better signal-to-noise and contrast-to-noise ratios, which permit reductions in overall scan length and improvements in spatial resolution. Currently, various paramagnetic (gadolinium) and super paramagnetic (iron oxide -based) contrast agents have been tested for preclinical and clinical applications. The main disadvantage of MRI is its inherently low sensitivity for biological targets, such that this method has not been tailored to visualize cancer or disease specific disorders. 

X-ray computed tomography or computed tomography (CT scan) is a computer processed X-ray imaging technology that provides anatomical images of the body. These cross-sectional images are reconstructed to provide diagnostic information. There are several advantages of using CT in medical imaging applications. First, CT has an inherently high-contrast and resolution, such that subtle differences between tissues (with less than 1%) contrast can be distinguished. Secondly, CT images can be reformatted in a multiplanar imaging mode. The biggest disadvantage of using CT, however, is the exposure of the patient to a high radiation dose, which enhances the risk of DNA or cellular damage. The radiation dose for a particular study normally depends on multiple factors, e.g., volume scanned, patient body mass, and desired image resolution and quality. Alternative CT methods for improving signal to noise ratio with lower radiation doses are currently under new investigation.

One of the most successful molecular imaging modalities for preclinical studies is optical imaging. This technology is based on the detection of light photons and their interaction with the tissue. There are many optical imaging methods currently being in used for medical applications,
but only two major methods are briefly surveyed here. The bioluminescence imaging (BLI) requires the cellular expression of an enzyme known as luciferase, and its gene is incorporated into the DNA of cells in animal models of disease. Substrates such as D-luciferin, interact with the enzyme, causes it to emit visible light in the 400-700 nm range with energies 1.5-5.0 eV. BLI is often used to monitor the cellular and genetic activity of disease, and is most useful in vivo for small mouse models, since the depth sensitivity of the BLI is only 1-2cm. \(^{14}\)

Fluorescent imaging (FI) is designed to look at the surface distribution of fluorescent signals. It is a most commonly used in both live and fixed cells, and no substrate is required. In this method, fluorochromes are coupled to peptides or antibodies, and signals can be generated by the presence or absence of specific molecules. The contrast sensitivity of fluorescent imaging is approximately comparable to radioactivity-based imaging, however, the tissue penetration of signals with FI is much less than PET or SPECT. The penetration of photons by fluorescence, even those with the NIR wavelengths, remains confined to the mm to cm range, thus limiting its application in clinical imaging. Current development in FI methods includes improved relative sensitivity, higher resolution, preparation of newer imaging probes, and better signal-to-noise amplifiers. \(^{2-4}\)

One of the newer molecular imaging techniques currently undergoing research and development as a nano-drug delivery systems, is ultrasound imaging (UI). UI is achieved by utilizing microbubles, liposomes, or perfluorocarbon emulsions as scaffolds functionalized with different targeting agents. The UI offers high spatial resolution (<1mm) and can provide too extreme useful information for coregistration with other molecular imaging methods. Because of
the relatively large size of imaging reagents or particles (>250 nm) this technique has limited for capacity tissue penetration, and therefore is specifically used for vascular imaging.

PET and SPECT are the most powerful nuclear imaging technologies for cancer detection and diagnosis. They have distinct advantages over other imaging modalities, such as high intrinsic sensitivity and unlimited depth penetration. These factors combine to make the radionuclide imaging a conventional practice for tumor and cancer diagnosis. In addition, PET imaging has capacity to provide quantitative data and provides higher spatial resolution than SPECT. Because of their potential applications, literally hundreds of radiotracers have been developed and evaluated as imaging agents for nuclear medicine.¹

4.1.1 Importance of nuclear medicine/radiolabeling E2 and AE in breast cancer

The ability to detect breast tumors that overexpress the estrogen receptor (ER) would have significant impact on cancer diagnosis, and therapeutic treatment. The role of estrogen and its interaction with ER have been extensively investigated in past decades. Although the precise mechanism of these interactions still remains to be defined, there is ample evidence to suggest their involvement in primary hormone-dependent breast carcinoma and its metastases. The biological effects of ligands bound to the ER are exerted through the ERα or ERβ substypes, in which the activity of ligand-transcription factors as inducer (agonist) or suppressor (antagonist) of gene transcription depends on the nature of bound ligand. Because the ER is located in the endoplasmic membrane, it has become an attractive biomarker for developing ER-targeting drugs. The search for novel ligands to specifically target ER in metastatic tumours of different stages still remains as an important goal. In the past, both gamma- and positron- labeled estradiol derivatives were developed for specifically monitoring estrogen dependent breast cancer, but
only a few reached the clinical stage. Several radioiodinated estradiol derivatives have been studied. Both isomers of 11β-methoxy-(17α, 20E/Z)-[^I^123]iodovinylestradiol have been clinically used; the 20Z isomer giving better images of ER(+) human breast tumors. Although the diagnostic of radioiodinated estradiols were selectively and sensitively detected in both primary and metastasis ER(+) breast cancer, however the extensive correlation between imaging and clinical outcome has not successfully provided for further treatment.15-18

Because of the low production cost and favorable half-life of 99m-Tc, steroidal estrogen derivatives with prosthetic 99m-Tc chelates at different positions have been investigated although none of the compound is clinically accepted.19 Perhaps within the halogenated radiolabeling estradiol family, estradiol labeling with 18F has been considered as the most explored radiohalogen for in vivo imaging to study estrogen receptors. Among many compounds evaluated (Figure 4-1), 16α-[18F]-estradiol (18F-FES), is currently in clinical use and is considered as the most successful candidate for in vivo imaging of ER(+) tumors. The 11β analog, 4-fluoro-11β-methoxy-16α[18F]-fluoroestradiol (4FMFES) tracer also showed favorable biodistribution in small animal studies and gave higher selectivity uptake ratios than those FES. 4FMFES has recently progressed to human biodistribution and dosimetry studies. The 7α-substituted [18F]-ES was prepared and evaluated as possible tracers for ER(+) breast tumor, however, the biological data showed no significant improvement on target-to-nontarget uptake ratios compared to other 18F-ES radiotracers.18-22
Because many of the radioligands still exhibit low receptor (estrogen receptors- ERα, ERβ) binding affinity (RBA) and non-ER regulated uptake, there is still a need for developing new radiohalogenated steroids as ER-based radiopharmaceuticals. Such ER targeted radioligands would have to meet the following criteria. First, it would be prepared in high specific activity (radioactivity per unit mass of the radioligand >1 Ci/mmol), as receptors are saturable systems with limited uptake capacity. Secondly, the radioligands must exhibit high specific receptor binding affinity with low non-specific binding. It must have appropriate metabolic and clearance characteristics, especially with respect to the surrounding tissues. Based upon our previous studies, the 11β analog in Table 4-2 was considered a promising candidate.

**Table 4-2: Derivatives of 11β- substituted estradiol**

<table>
<thead>
<tr>
<th>1&lt;sup&gt;st&lt;/sup&gt; gen. of 11β-AE</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; gen. of 11β-AE</th>
<th>RU39411</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; gen. of 11β-AE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Dr. Ed Hua thesis)</td>
<td>(Dr. Hendricks thesis)</td>
<td>This work</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Figure 4-1:** $^{18}$F-radiolabel estradiols$^{22-27}$
4.1.2 Rationale of design 11β-AE with imaging modalities, including $^{18}$F via convergent approach

As described in previous chapters, we had substantial experience in the design, preparation and evaluation of high affinity steroidal anti-estrogens as targeted drug delivery agents for breast cancer. These studies had to address many of the same concerns as required for imaging agents. Our extensive work with 11β-(substituted alkynyl) estradiols demonstrated that 11β-site lead promise for introduction of therapeutic and/or diagnostic imaging groups. SAR for steroidal estrogens indicated that small substituents at the 11β-position conferred agonist activity, usually with an increase of binding affinity. Alkyl or heteroalkyl groups beyond 3-4 atoms in length imparted ER antagonist properties, often without a significant loss of binding affinity. As described in Chapter 3, unsubstituted aromatic groups at the 11β-position also were ER agonists while more highly substituted derivatives expressed antagonist properties. From our recent work on developing new E2-Dox conjugates, the 11β-aryl estradiol-based antiestrogens possessed several key features as potential candidates for MI for (ER+)-breast cancer. We established that the 11β-aryl estradiol-based antiestrogens retained high ER affinity, expressed ER antagonist properties, showed selectivity for ER-expressing cancer cells, and that a linker not only improved pharmaceutical properties, but allowed convergent attachment of different terminal groups, for example, imaging reagents. Similar to the drug conjugation, the strategy of using 11β-aryl substituent antiestrogen would permit the parent estradiol scaffold to occupy the default ligand binding pocket. As shown from previous SAR studies indicated that the 11β-aryl group occupies a secondary pocket that extends to the surface of the receptor. In the antagonist conformation the 4’-position of the aryl group would access the solvent exposed space and therefore should permit further conjugation with a prosthetic imaging tag, such as radiolabeling.
fluorescent and Raman probes, without loss of affinity or loss of efficacy. The convergent strategy allows us to prepare these components separately and, then use orthogonal ligation to link it to the 11β-aryl estradiol through a complementary linker at the 4’-position of the aryl group.²⁸,³⁰

Section 4.2 Synthesis, characterization and evaluation 11β substituted Estradiol-imaging modality conjugates

4.2.1 Synthesis 11β substituted Estradiol-imaging modality conjugates

The synthesis of the radiohalogenated antiestrogen began by preparing the high affinity alkynyl 11β-phenyl substituted estradiol component. Separately, we prepared the fluorinated triethylene azide as an example of a radioimaging label. The alkynyl 11β-phenyl substituted estradiol can be prepared by two methods as shown in Scheme 4-1. The first approach would be similar as illustrated in Chapter 2.
Scheme 4-1: Synthesis of alkynyl and azido of 11β-antiestrogen precursors

The precursor is synthesized from 3,3-ethylenedioxy-5(10)-α-epoxy-estr-9(11)-ene 1 and transformed into the key intermediate 2, 11β-(4-hydroxy-phenyl)-estra-4,9-diene-3,17-dione. Ultimately, an alkylation step is used to prepare the precursor 3, 11β-[4-(2-dimethylaminoethoxy)-phenyl]-estra-4,9-diene-3,17-dione. After the original synthesis route was developed (Chapter 2, Scheme 2), we noted an opportunity to improve the synthesis of the 11β-substituted phenyl estradiols, especially as we had identified the optimal 4-substituent. The Grignard reaction using the 4-TMSiO-phenyl group to introduce the 11β-phenyl moiety constituted a weak
point. It presented several problems for obtaining high yields and pure products, due to the tendency of the reagent to form biaryl side products. It also presented issues in the separation process (Figure 4-2). Therefore we investigated an alternate approach in which we introduced the side chain prior to the Grignard reaction. In this route, we alkylated 4-bromophenol with dimethyl chloroethyamine to give 4-bromo-(2-dimethylaminoethoxy) benzene. The following Grignard reaction and work-up produced the same intermediate 3. The advantages of this approach were that we shortened the overall sequence with the steroidal portion by a step. Second, the yield of the product was higher and the separation/isolation process was easier (see TLC for comparison). Third, the 4-bromo phenol starting material is significantly less expensive than the 1-bromo-(4-trimethysilyloxy) benzene. More importantly, this reaction was more reproducible than the initial scheme. Starting from the intermediate, the alkynyl antiestrogen was obtained as illustrated in the synthetic Scheme 4-2. The molecular imaging probe, such as an azido fluorinated ligand, can be attached using “click” chemistry.1,28,30-37

Figure 4-2- TLC comparison of compound 3 and 4 (alternative approach)
The preparation of the fluorinated component proceeded in good overall yield (Scheme 4-2). The synthesis began with the ω-chloro triethylene glycol derivative which underwent displacement with sodium azide to give 10. Tosylation gave intermediate 11, which was treated with TBFA to give the fluorinated precursor 12. Conversion to the final product 13 was achieved in good yields using classical “click” conditions. All of the intermediates and final products were characterized by IR, NMR, LC-MS. The convergent modular approach proved to be a successful strategy for assembling the target anti-estrogen (AE).

Scheme 4-2: Convergent synthesis of $^{18}$F-radiolabeling 11β-substituted antiestrogen
Section 4.3  Synthesis and characterization of $^{18}$F-11β substituted estradiol-imaging radioligand

For making the 11β-AE radiolabeled with $^{18}$F as shown in the structure above, we investigated a variety of different “click” conditions to determine the best method to convert the alkynated AE precursor into the radiolabeled “click” products. Because of the short half-life of $^{18}$F radionuclide, the conversion method has to be fast and proceed in high yield. Table 4-2 below summarizes the “click” reactions that were carried out with the pre-automated synthesizer.
at Mass General Hospital – Center for Systems Biology. As the results in the radioHPLC trace indicate, the *in situ* “click” reaction under conditions 4, 7, and 8 gave the best radiochemical yields for the conversion of $^{18}$F-PEG-N$_3$ to the 18F-AE radioligand. However, method 4 involved an additional ligand, bathophenantolinedisulfonic acid disodium salt (BPDS), in a mixture of DMSO/H$_2$O (50:50) which would create more potential impurities when purifying with HPLC. Although, the radiochemical yield for method 7 is less, the reaction conditions are much simpler, with one catalyst copper(I) tetrakis(acetonitrile)copper(I) hexafluorophosphate being used. In reaction 8, 40μL of 80mM BPDS was added to the scale up reaction to ensure Cu$^{+1}$ formation is available for catalyzing throughout the “click” reaction. Indeed, this method gave 95% conversion and produced isolated yields ranging from 9.73mCi to 9.12mCi. All the reactions in Table 4-3 used microwave synthesis conditions (60°C, 30 watts power, and 5 min), except method 7 in which a 10 min irradiation time was used.
Using a reverse phase column for the HPLC (Figure 4-3), it seemed that the desired product would be difficult to be separated using MeCN:H₂O (50:50) with 0.1% TFA. Using these conditions, the¹⁸F-PEG-N3 eluted from the column after about 6 minutes, while the radiolabeled product appeared at 5 minutes. Changing the eluting solvent to MeCN:H₂O (37.5:62.5) with 0.1% TFA, the¹⁸F-radiolabeled 11β-substituted antiestrogen eluted at about 10 min while the¹⁸F-PEG-N₃ was at about 7.5 min, and the alkyne precursor was 22 min (UV HPLC trace). This
separation scheme would allow us to isolate the pure labeled compound without overlap with the precursor.

A) Prep radioHPLC of the $^{18}$F labeling “click” product

B) RadioHPLC trace analysis of the separated compounds

Figure 4-3: Separation scheme of $^{18}$F-11β AE
In summary, we have demonstrated that our novel 11β-AE can be radiolabeled with $^{18}$F radionuclide using click chemistry with the azido-PEG-$^{18}$F ligand. The method of synthesis gave a sustainable high activity conversion, and purification by HPLC indicated that the labeling compound is 100% pure and is suitable for in vivo imaging.

Section 4.4 Design and synthesis other “click” type complementary imaging modalities for 11β-substituted antiestrogen

In addition to the $^{18}$F oligoethylene glycol azide used to prepare the ER-targeted radioimaging probe, we also synthesized and characterized a series of reagents that can be used for other imaging modalities (Scheme 4-4), “clicked” onto our 11β- AE or other targeting groups. These reagents can be used for imaging ER(+) breast cancer or other disorders.

In a similar approach to making the $^{18}$F- radiolabeling 11β-antiestrogen, we made a series of different imaging probes, using the antiestrogen precursor under “click” conditions onto the complementary partner terminal from imaging modalities (Scheme 4-4). As explained in the previous section, imaging of the ER in vivo using an ER binding radiopharmaceutical has the potential for determining the ER status as the tumor becomes staging. Fluorine-18, Iodine-123 and other cyclotron- produced radionuclides have been used to label ER binding ligands to develop such in vivo radioimaging probes.$^{23-26,38-43}$

Some of these radioligands, for example 16α-$^{18}$F-17β-estradiol ($^{18}$F-FES), have been evaluated clinically, with promising results for the imaging of hormone dependent breast tumors and predicting the responsiveness of the tumor to the antiestrogen drugs.$^{27,44}$ Unfortunately, none of the established ligands has been approved for clinically and routinely used in breast cancer diagnostic, thus there is still a need to develop ER radioligands with different radionuclides.
Alternatively, investigators have been directed to developing methods for labeling estrogen imaging agents with the radionuclide $^{99m}$Tc for SPECT. Recently, a few $^{99m}$Tc-estradiol derivatives were synthesized.\textsuperscript{45-48} However, most of these compounds were reported, have poor suboptimal target tissue selectivity, possibly due to their lipophilicities or rapid metabolism of the compounds. In addition, these radioligands were not conveniently made (linear approach), thus further development of these methods has been held back for lack of optimal yields and purities.\textsuperscript{49,50} To this regard, we have developed a convergent approach to making prosthetic metallated $^{99m}$Tc, compound 7 and 10 chelates that can be directly clicked on our alkynyl or azido 11-$\beta$ substituted estradiol (Scheme 4-4) to make probe A. The preparation of our compound is modular, thus the imaging modality and precursor are independent made with fairly high yields and purity. The final click product relies on optimal conditions, especially the Cu(I) catalyst ligands and solubility of the reagents in the reaction solvents. The Re(CO)$_3$-11$\beta$AE has been successfully made and characterized, the synthesis of the $^{99m}$Tc compound to is still under development, therefore the radioactive data will not be reported in this section.
Briefly, surface-enhanced raman scattering (SERS) spectroscopy in combination with optical microscopy yields a novel noninvasive and label-free method to assess and image cellular processes based on their biochemical changes. The major advantage of using Raman spectroscopy over the conventional optical imaging method is that the technique affords minimal sample preparation, high sensitivity to small intracellular fluctuations, as well as high spatial resolution. Raman spectra from within the cell reflect the biochemical composition found within the laser focal volume of approximately 0.3-1.3 μm³ in size, which is determined by the diffraction limit of the applied laser light. Using the spectral parameters of a cell’s components,
it is possible to image cellular organelles such as the nucleus, chromatin, mitochondria, and lipid bodies, without the use of external labels or dyes at the resolution of conventional microscopy. In general, the technique is based on identification of molecular vibrations that are characteristic of distinct functional groups that compromise molecules, in our case the deuterated triphenyolphosphine (dTPP) and cyano benzyl derivatives. For living cells and tissues, these vibrational fingerprints arise from the functional groups of proteins, nucleic acids, lipids, phospholipids, and carbohydrates, which are the framework of the basic building blocks of mammalian cells. Vibrational spectra may be obtained by illuminating the specimen either with infrared radiation or laser light in the visible range of the spectrum. The newer approach is based upon the inelastic scattering of photons and is known as the Raman effects. These novel labels, dTPP and azido cyano benzyl (Scheme 4-4) are assembled via click conditions with our alkynyl and azido 11β-substituted estradiol to form a Raman imaging probe C for in vitro ER hormone dependent breast cancer cells. Based on preliminary experimental results, the Raman labels are nontoxic, stable, and do not suffer from photobleaching, and process distinctive and specific Raman signatures at near 2100 cm\(^{-1}\). In addition, SERS in the local optical field of the compounds provides sensitive information on the immediate molecular environment of the dTPP in the cell, subsequently allowing imaging of the native cellular constituents and organelles. The hybrid of Raman label with 11β AE were successfully synthesized and characterized as noted in the experimental section. Further imaging studies are still underway, thus imaging data will not be presented in this section.

Finally, the more traditional fluorescent probes, like FITC and other dyes, can be easily and independently modified with the comparable complementary OEG linker (Figure 4-7), which subsequently attached to our 11βAE to form fluorescent probes for ER imaging. These
probes are definitely useful in the studies of ER cellular binding uptake and/or cellular internalization of the designed ligands.

Section 4.5 Design, synthesis and comparison studies between monovalent and trivalent of PSMA targeting imaging probe

4.5.1 Rationale for designing trivalent imaging probe - specifically using PSMA targeting prostate cancer

This section describes the advantages of using multivalent, specifically labeled derivatives of a trivalent PSMA ligand imaging probes for prostate cancer. An in depth discussions about prostate cancer and the rationale of using prostate specific membrane antigen (PSMA) as a targeting agent to the overexpressed PSMA prostate malignant tumors will be presented in the next chapter.

There are a variety of clinical factors and treatment options that need to be considered when deciding the optimal therapy for a patient. An accurate diagnosis that can detect and define the aggressiveness of the disease at early stage can provide appropriate selection of the clinical treatment. The ability to noninvasively visualize the disease and its metastatic potential is vital for informing therapeutic approaches.\textsuperscript{57-59} Therefore, there is demand for new imaging agents that not only can accurately detect and the stage of disease, but also monitor its response to therapy. If a successful correlation between the localization of these imaging agents and disease response can be established, it could improve the efficacy and generate a better patient prognosis. Current cancer imaging modalities allow doctors to visualize disease in various parts of the body, but none can provide both specificity and sensitivity for prostate tumors. Prostate specific
membrane antigen (PSMA) is currently considered as an attractive target for detection of primary and metastatic prostate cancer.\textsuperscript{57,59-62} It is a transmembrane protein that is primarily expressed in normal human prostate epithelium but is up-regulated in prostate malignancies, including metastatic cells. The overexpression of PSMA in primary prostate cancer is known to correlate with other traditional adverse prognosis factors and could be used to independently predict the disease outcome. In addition to its overexpression in all prostate cancers, PSMA is also over-expressed in poorly differentiated, metastatic and hormone-refractory carcinomas, thus it becomes a very attractive target for developing imaging agents for the differential diagnosis and treatment of prostate cancer.\textsuperscript{63-68}

Using small molecules, as targeting ligands for diagnostic imaging is often complicated by problems such as inadequate binding affinity, low target specificity and lack of tissue selectivity, leading to low resolution images.\textsuperscript{69-71} One of the aims of this project is to improve the PSMA binding affinity and its specificity to the prostate cancer cells by amplifying the number of PSMA ligands in the imaging probes. This can be achieved by using a multivalent (trivalent in this study) scaffold (Figure 4-4).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4-4.png}
\caption{Monovalent and trivalent PSMA targeted molecular imaging agents}
\end{figure}
Cancer specificity results from the interaction of the receptors present on the surface of cancer cells with high affinity targeting ligands. Appropriate attachment of a therapeutic agent to the targeting group should then deliver it to cancer cells. Alternatively, replacement of the therapeutic group with an imaging moiety would permit the detection of cancer for diagnostic purposes. Increasing the effective cancer specificity by using multivalency should enhance both affinity and specificity for the ligand and therefore improve both diagnostic and therapeutic performance. We propose that using three prostate specific membrane antigen (PSMA) urea-based ligands, which individually have a binding affinity in the 9 nM -400 pM range, to form a trivalent derivative would achieve such enhancement (Figure 4-9). Our approach uses the readily available, conformationally mobile dedrimer Newkome triacid to which the targeting groups can be attached. This special feature provides the flexibility in which the three PSMA ligands can orient themselves in a tripodal recognition binding motif. This arrangement promotes cooperative binding, i.e. if one of three PSMA binds to the PSMA pocket, the likelihood for a second and third PSMA ligand to interact with the neighboring receptors is greatly enhanced. This process is greatly amplified on cells where the targeting receptors are overexpressed as compared to normal express and would enhance signal to noise ratio for the imaging agent.
4.5.2 Synthesis of monovalent and trivalent PSMA molecular probes

Scheme 4-5: Synthesis of monovalent or trivalent PSMA-radioligands

A couple methods of preparation of fully protected PSMA derivative have been reported in the literatures. Basically, two key amino acids, L-glutamic acid and L-lysine derivatives, can be synthesized from commercially available amino acids through a few appropriate steps of protect and deprotect the α-carboxyl groups and the functional side chains. Then the two amino acids can be bridged together using triphosgene and triethyl amine to yield a protected PSMA. Subsequently, the Boc group from the ε-amine can be removed with weak acid, followed by
alkylation of pentynoic acid to derive the alkynyl PSMA precursor. Similar to the development of the 11β-AE radioligand, we use “click” conditions to attach a variety of radionuclides to our alkynyl PSMA precursor (Scheme 4-5).

As mention in the previous section, the trivalent PSMA radioligand could tremendously improve the specificity and selectivity of the suboptimal uptake by the PSMA dependent prostate cancer. Perhaps, this effect might be attributed to the high flexibility and conformationally of the Newkome triacid units that promotes the binding of the trivalent PSMA to its targeted receptors via cooperating binding effects.69

4.6 Experimental Section

4.6.1 Alternative approach

Synthesis of 2-(4-bromophenoxy)-N,N-dimethylethanamine- To a solution of 4-bromophenol (6.8 g, 40 mmol) in DCM (150 mL) was added CsCO3 (19.5 g, 100 mmol) and the reaction was allowed to stir at RT for ~1 hour. To that mixture was added 2-dimethylaminoethyl chloride hydrochloride (5.6 g, 40 mmol). The reaction was stirred at reflux for approximately 24h. The mixture was then filtered and the filtrate was concentrated down to form clear oil. The crude material was then purified via silica gel chromatography (100% DCM then 1-10% MeOH in DCM). Obtained pure 2-(4-bromophenoxy)-N,N-dimethylethanamine (4 g, 16 mmol, 42%
yield). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.33 (2H, d, $J = 8.79$ Hz), 6.78 (2H, d, $J = 8.8$ Hz), 3.99 (2H, t, $J = 5.4$ Hz, 2H), 2.64 - 2.88 (2H, m), 2.31 (6H, s, br.).

**Synthesis of (4-(2-(dimethylamino)ethoxy)phenyl)magnesium bromide** - All of the glassware was rinsed with DCM and acetone and dried in an oven at 100°C overnight. The reaction apparatus was flame dried with a propane torch following assembly. Magnesium turnings (3 g, 125 mmol) were placed into a three-necked flask and kept in the 113°C oven overnight. After it was cooled, the apparatus was placed under argon atmosphere. Distilled anhydrous THF (50 mL) was then added to the three-necked flask. A small granule of I$_2$ was added, and the solution immediately turned orange. The mixture was allowed to stir for ~12 minutes after which 1 mL of 2-(4-bromophenoxy)-N,N-dimethylethanamine (which was dissolved in 30 mL of THF) was added. After, 5 mL aliquots of mixture were added every 20 minutes until half of the 2-(4-bromophenoxy)-N,N-dimethylethanamine (4 g, 16 mmol) was added. At this point the mixture was heated to ~65°C. The rest of the mixture was then added in 0.5 mL aliquots every 20 minutes. The solution first became greenish yellow, and then went to lighter yellow and finally greenish grey. The reactions was heated for another hour and then cooled to room temperature.
Synthesis of (11R,13S)-11-(4-(2-(dimethylamino)ethoxy)phenyl)-13-methyl-7,8,11,12,13,14,15,16-octahydro-1H-cyclopenta[a]phenanthrene-3,17(2H,6H)-dione

3,3-Ethylene dioxy-5(10)-α-epoxy-estr-9-ene-17-one (2.014 g, 6.1 mmol) was dissolved in anhydrous THF (15 mL) under an argon atmosphere. Copper(I) iodide (0.160 g, 0.840 mmol) was added to the solution at -10°C and stirred for 15 min. Freshly prepared Grignard reagent, (4-(2-(dimethylamino)ethoxy)phenyl)magnesium bromide, was added dropwise in 5.0 mL aliquots. The reaction was gradually warmed to the ambient temperature and stirring was continued for 16h. The reaction was quenched by the addition of ammonium chloride (0.8 g, 15 mmol) in 35 mL of water and 35 mL of EtOAc at 0°C. The organic layer was washed with water (2x 35 mL). The organic solvent was removed under reduced pressure and the resulting residue was dissolved in a mixture of acetic acid (14 mL) and water (6 mL). The resultant mixture was warmed at 50-60°C for 1.5 hours, after which it was diluted with ethyl acetate (20 mL). The solution was neutralized by the addition of saturated aqueous sodium bicarbonate. The organic layer was separated, washed with brine solution, dried over magnesium sulfate and evaporated to dryness to give a crude, yellow oil. Purification using silica gel column chromatography (70:30 hexane/ethyl acetate) afforded the desired product (2.00 g, 76%) as yellow solid: \(^1\)H NMR (CDCl\(_3\), 400 MHz): δ 0.53 (3H, s), 4.38 (1H, d, J = 6.9Hz), 5.78 (1H, s), 6.71 (2H, d, J=6.3Hz), 6.97 (2H, d, J=6.5Hz).
4.6.2 Synthesis of PSMA urea-based inhibitor

2-Fmoc-Amino-6-tert-butoxycarbonylamino-hexanoic acid 4-methoxybenzyl ester (1) - All glasses were flame rinsed with acetone and flame-dried before the reaction is set up. Into a 250 mL, round-bottom flask under nitrogen was placed Nε-Boc-Nα-Fmoc-L-lysine (3.5 g, 7.5 mmol) and 40 mL of dry DMF. To this was added cesium carbonate (3.5 g, 10.5 mmol) and 1-(chloromethyl)-4-methoxybenzene (2.0 g, 8 mmol). The mixture was stirred at room temperature for 24 h. The solution is filtered and washed with ethyl acetate. Product extraction and work up with EtOAc, 5% Na2CO3, water, and dried over MgSO4 followed by recrystallization from 60/40 (v/v) hexane/EtOAc yielded a clear solid. TLC Rf = 0.33 (70/30 hexane/EtOAc). Yield: 4.00 g, 7 mmol, 85%. 1H NMR (500 MHz, CDCl3) δ 7.76 (2H, d, J = 7.3 Hz), 7.60 (2H, d, J = 7.3 Hz), 7.36 - 7.46 (2H, 2m), 7.18 - 7.34 (4H, m), 6.87 (2H,d, J = 8.3 Hz), 5.40 (1H, d, J = 6.84 Hz), 5.15 (2H, d, J = 12.2 Hz), 5.08 (1H, d, J = 11.7 Hz), 5.03 (1H, s), 4.52 (3H, br.s.), 4.21 (1H, t, J = 6.8 Hz), 3.71 - 3.88 (4H, m), 2.95 - 3.12 (2H, 2m), 1.33 - 1.1 (15H, 2m). LC-MS [M-H]^+ calcd, 572.29; found, 573.
2-Amino-6-tert-butoxycarbonylamino-hexanoic acid 4-methoxybenzyl ester (2)- In a flame-dried round-bottom flask was placed 3.0 g (4.2 mmol) of the fully protected lysine (1). A mixture of 40 mL of a 20% solution of piperidine in DMF was then added to the reaction flask. The reaction was stirred at room temperature and under nitrogen gas for 4 h. The crude product was extracted with CH$_2$Cl$_2$, water, Na$_2$SO$_4$. Compound 2 was obtained via purifying by flash chromatography (4/96 MeOH/CHCl$_3$) afforded as an oil (1.59 g, 3.0 mmol) in 77% yield. TLC $R_f = 0.42$ in (5/95 MeOH/CH$_2$Cl$_2$). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.30 (2H, d, $J = 8.7$ Hz), 6.90 (2H, d, $J = 8.7$ Hz), 5.08 (2H, s), 3.81 (3H, s), 3.07 (3H, q, $J = 5.8$ Hz), 1.65 - 1.90 (3H, m), 1.63 - 1.34 (16H, m). LC-MS m/z: 367[M+1]$^+$ C$_{19}$H$_{31}$N$_2$O$_5$. LC-MS [M-H]$^-$ calcd, 366.29; found, 366

Bis-4-methoxybenzyl-L-glutamate·HCl (3)- Into a flame-dried round bottom flask was placed (5.0 g, 34 mmol) of L(+) glutamic acid in dry dimethylformamide (20 ml). The mixture was stirred at 0°C and slowly N,N,N',N’-tetramethylguanidine (3.69 ml, 34 mmol) is added. After 30 minutes, ethyl acetoacetate (4.3 ml, 34 mmol) was added to the mixture solution. Stirring was
continued at room temperature for 24 h (or when all the acid had dissolved). Then 4-methoxybenzyl chloride (10.6 g, 68 mmol) was added and stirring was continued for another 16 h under nitrogen gas. The reaction mixture was then diluted with NaHCO₃ (1 M, 100 ml) and EtOAc (70 ml) and the organic phase was washed with NaHCO₃ (1 M, 100 ml) and water (2 x 50 ml) and then dried with MgSO₄. After evaporation, the product was acidified with methanolic hydrogen chloride (1 M, 40 ml); the mixture was shaken gently until the solid had dissolved. After 30 minutes at RT, the solution was evaporated to dryness to yield an oil which solidified when triturated with ether. Compound 3 was purified by flash chromatography (5/95 MeOH/CH₂Cl₂) affording a yellow solid (7.59 g, 19 mmol) in 56% yield.

$^1$H NMR (400 MHz, CDCl₃) δ 7.32 (4H, m), 6.82 - 6.89 (4H, m), 5.15 (2H, d, $J = 5.86$ Hz), 4.56 (2H, s), 4.37 - 4.43 (2H, m), 3.70 - 3.81 (6H, m), 3.60 (1H, s), 2.63 (4H, d, $J = 6.60$ Hz). LC-MS [M-H]$^+$ calcd, 387.29; found, 387.

2-[3-{1-p-Methoxybenzylcarboxylate-(5-t-butyramethylpentyl)-ureido}-di-p-methoxybenzyl pentanedioate (4). Bis-4-methoxybenzyl-L-glutamate · HCl 3 (3.0 g, 7.75 mmol) was placed in a flame dried round-bottom flask under nitrogen and dissolved in 15 mL CH₂Cl₂. Triphosgene 0.760 g, 2.3 mmol) was dissolved in 2 mL CH₂Cl₂, and slowly added to the reaction flask. The flask was cooled to -77 °C (dry ice ethanol slurry) under nitrogen. To this was slowly
added triethylamine (12 mL, 85 mmol) in 10 mL CH$_2$Cl$_2$. The reaction mixture was stirred at -77°C for 30 min, and then stirred for 30 min at room temperature. To this was added compound 2 (2.5 g, 7.75 mmol in 5 mL CH$_2$Cl$_2$). After the reaction mixture was stirred overnight, the crude was diluted in CH$_2$Cl$_2$, washed with water, brine solution, and dried over MgSO$_4$. Compound 4 was purified by flash chromatography (20/80 EtOAc/CH$_2$Cl$_2$) afforded a clear oil (3.2 g, 3.3 mmol) in 52.3% yields. TLC $R_f = 0.47$ (20/80 EtOAc/CH$_2$Cl$_2$).$^1$H NMR (400 MHz, CDCl$_3$) δ 7.24 - 7.34 (6H, m), 6.84 - 6.93 (6H, m), 5.48 (2H, br. s.), 4.97 - 5.17 (6H, m), 4.44 - 4.64 (1H, m), 4.06 - 4.21 (1H, m), 3.62 - 3.82 (9H, m), 2.92 (2H, s), 2.25 - 2.48 (2H, m), 2.14 (1H, d, $J = 7.3$Hz), 1.85 - 2.04 (1H, m), 1.74 (1H, dd, $J = 5.86$Hz, 8.79 Hz), 1.52 - 1.68 (1H, m), 1.20 - 1.47 (14H, s). LC-MS [M-H]$^+$ calcd,780.29; found, 780

2-{3-[1-p-methoxybenzylcarboxylate- (5-aminopentyl)]-ureido}-di-p-methoxybenzyl pentanedioate (5). A solution of 4 (2 g, 2.6 mmol) dissolved in 20 mL EtOAc was cooled to 0 °C in an ice bath and p-toluenesulfonic acid (0.49 g, 2.6 mmol) in 5 mL of absolute ethanol was added. The reaction mixture was allowed to warm to room temperature for 2h. The reaction mixture was then concentrated to a thick oil under reduced pressure. Compound 5 was purified with flash chromatography using 10/90 MeOH/ CH$_2$Cl$_2$ to afford the product as a colorless solid (0.98 g, 1.15mmol) in 45% yield. TLC $R_f = 0.47$ (10/90 MeOH/CH$_2$Cl$_2$).$^1$H NMR (400MHz, CDCl$_3$) δ 7.68 (2H, d, $J= 8.0$ Hz), 7.66-7.57 (3H, s),7.22-7.13 (6H, m), 7.0 (2H, d, $J = 7.2$ Hz),
6.84-6.76 (6H, m), 6.34 (2H, br s), 5.06-4.88 (6H, m), 4.44 (1H, m), 4.32 (1H, m), 3.76 (3H, s), 3.73 (6H, s), 2.86 (2H, s), 2.3-2.24 (5H, m), 2.08-1.99 (1H, m), 1.82-1.72 (1H, m), 1.64-1.3 (6H, m). LC-MS [M-H]^+ calced, 680.32; found, 680.3

(R)-bis(4-methoxybenzyl) 2-(3-((R)-6-(hex-5-ynamido)-1-((4-methoxybenzyl)oxy)-1-oxohexan-2-yl)ureido)pentanedioate (6) In a 250 mL flame-dried round-bottom flask was placed hex-5-ynoic acid (0.053 g, 0.48 mmol) in 30 ml of dry THF. To this solution was slowly added a mixture of N,N'-dicyclohexyl-carbodimide (0.098 g, 0.48 mmol) in 5ml THF, and N-hydroxysuccinimide (0.055 g, 0.48 mmol) in 5ml THF. The mixture was stirred under N\textsubscript{2} at RT for 1h, then compound 5 (0.08 g, 0.12 mmol) in 2ml anhydrous THF solution was added dropwise to the reaction mixture. After 24 h, the reaction residue was filtered and the solvent was evaporated under reduced pressure. The residue was extracted with 10ml EtOAc. The organic layer was washed with water (2x20ml) and brine solution. The combined organic layers were dried over MgSO\textsubscript{4}. Compound 6 was purified with flash chromatography using (5/95 MeOH/ CH\textsubscript{2}Cl\textsubscript{2}) to afford product as colorless oil (0.028 g, 1.15mmol) in 33% yield. TLC $R_f$ = 0.47 (5/95 MeOH/CH\textsubscript{2}Cl\textsubscript{2}).
4.7 References


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Chapter 5

In this chapter I describe an example of the assembly process used to prepare multifunctional gold nanoparticles (MfAuNPs) for prostate cancer therapy. This strategy includes the selection of specific modular components, such as a PSMA targeting moiety, the pH-sensitive doxorubicin therapeutic component, and the PEG-“stealth” component. Each individual unit could be synthesized from readily available starting materials, and appended to AuNPs in a manner that would control the surface compositions. This work illustrates our research approach from small molecule synthetic chemistry to assembly and evaluation of new nanomedicines.

5.1 Nanomedicines in cancer therapy

Upon diagnosis of a deadly and persistent disease, such as cancer, physicians use the most effective medical therapy available to prevent its spread, with the objective of eradication. These procedures include surgery to excise the tumor, radiation, or chemotherapy (“slash, burn and poison”). However, because of the potency, invasiveness and lack of selectivity for these methods, patients often experience significant morbidity, especially with chemotherapy. According to the NCI 2012 report, the majority of current chemotherapies are administered systemically, and therefore they impact healthy tissue as well as diseased tissue.¹ The body is exposed to the toxic effects of the drugs and the desired therapeutic effect of the drug is not localized at the tumor site. Nanomedicine, especially with the new generation of functionalized nanoparticle (NP)-based therapies aim to deliver the chemotherapeutic agent more directly to the cancer site and thereby reduce the systemic side effects.²⁻⁵

The application of nanotechnology to medicine is called nanomedicine. By definition, nanomedicine is the use of nanotechnologies to engineer nanomachines (nanorobots) or
nanoparticles for the prevention and treatment of disease in human body, especially for cancer therapy. Due to their extremely small size, and in some cases, their chemical composition, nanomedicines have a potential advantage over the traditional chemotherapies in that they can enter tumors and individual cancer cells while avoiding healthy tissue.\textsuperscript{1-12} Investigators have sought to take advantage of nanotechnology in this manner for more than a decade. Some of the more advanced nanoparticle therapeutics have begun entering clinic trials.\textsuperscript{13,14} For example, Doxil is a simple nanoparticle-based drug that has been on the market since 1995 for the treatment of ovarian cancer. Doxil is a liposome composed of a hydrogenated soy phosphatidylcholine (HSPC) bilayer that entraps the chemotherapeutic DNA-intercalator doxorubicin. The liposomal outer layer of the liposome is decorated with N-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-\textit{sn}-glycero-3-phosphoethanolamine sodium salt (MPEG-DSPE). This reagent enables the liposomes to evade the body’s immune system, giving the formulation more time to accumulate at the tumor site.\textsuperscript{15-17}

\textbf{Figure 5-1- Schematic structure of Doxil®}

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Similarly, Abraxane is an albumin protein-drug based NP that encapsulates the insoluble, but potent anticancer paclitaxel (Figure 5-2), and was approved in 2005 for treat breast cancer. Because of the albumin-particles, the patient can receive a higher dose of the taxane, whereas the parent drug is merely solublized in the blood, and therefore generates side effects.\textsuperscript{18-23}

\textbf{Figure 5-2- Schematic structure of Abraxane®}

Another example of using nanosize particles as an advantage for drug delivery is the development of CRLX\textsubscript{101}, currently under phase II clinical trials to treat advanced non-small-cell lung cancer (NSCLC). CRLX\textsubscript{101} core technology basically involves the self-assembly of β-cyclodextrin polyethylene glycol polymers conjugated with the drug camptothecin via a glycine linkage (Figure 5-3). Camptothecin itself failed as small molecule chemotherapeutic agent. It is an excellent topoisomerase I inhibitor, but the molecule suffers from poor solubility in blood and causes severe adverse drug reactions. Studies indicated that the lactone ring was easily hydrolyzed \textit{in vivo}, reducing drug potency. Conjugation of camptothecin to the polymer, not only reduced the toxicity of the drug, but also controlled the hydrolytic release of the drug from the ester bond of glycine. This feature allowed the NP-drug to remain intact while circulating in the blood. Within the acidic tumor environment of (pH=5 or 6), the ester bond is hydrolyzed and
releases the free potent camptothecin within the tumor cells. The released polymer is degraded and excreted through the renal system.\textsuperscript{24-27}

![Figure 5-3 Schematic structure of CRLX\textsubscript{101}®](image)

To date, Doxil, Abraxane, and CRLX are all nano formulations simply designed to safely deliver the drug to the tumor site. The mechanism of action of these nanomaterials is different from that of the entrapped small molecule drugs. The NPs have the ability to localize within tumors and cancerous cells and deposit the drug there. This phenomenon occurs because the blood vessels that run through tumor lose their integrity and form leaky junctions (Figure 5-4). As result, NPs less than 200 nm in diameter can pass through these gaps and get trapped via the enhanced permeability and retention (EPR) effect.\textsuperscript{28-30} On the other hand, chemotherapy drugs are small molecules that are systemically distributed and therefore are absorbed throughout the body. As such, they produce unwanted interactions (side effects). With nanoparticles or encapsulated drugs, one significantly modifies the biodistribution of the drugs, and enhances efficacy by effectively delivering them at higher concentration to the tumor sites and interactions, and by restricting them from nontarget areas in the body.\textsuperscript{1,31-37}
Recently Farokhzad, et al., developed a biodegradable poly(lactic-co-glycolic acid) (PLGA) polymer-based NP system (Figure 5-5). This system is a copolymer composed of a hydrophobic polyester and hydrophilic polyethylene glycol. Typically during the emulsification, the hydrophilic PEG strands of the copolymer remain on the surface, while the polyester section stays at the inner core. If the emulsification is done in the presence of a small molecule, such as the chemoreagent docetaxel, the individual polymeric NPs will entrap the drug molecules within the core. This technology was licensed to Bind Bioscience where it was developed as BIND-014®. It is currently in phase I clinical trials for treatment of prostate cancer. BIND-014 is more sophisticated than other FDA-approved drug-NPs because of the way in which the
surface of the NPs is modified with targeting ligands. In this approach, the ligands can more effectively interact with proteins or receptors on the cancer cells, thereby causing the NPs to more selectively localize within the tumor lesion (Figure 5-4). In this case the targeting ligand is described as an aptamer that is covalently attached to the hydrophillic (carboxylate terminus) via an amide linkage. The investigators specifically designed the ligand to recognize and bind to PSMA expressed in metastatic prostate cancer.23,36-38

![Schematic structure of BIND-014®](image)

**Figure 5-5** - Schematic structure of BIND-014®

Nanobiotix® is a different form of nanomedicine, currently in clinical trials for treating local tumors of the head, neck and prostate cancers. Unlike other technologies, Nanobiotix® uses hafnium oxide (HfO₂) nanocrystals along with standard radiation treatment to kill the cancer cells. Radiation therapy basically exposes the body to Gama-rays, where the low energy photons induce water molecules in the body fluid to form free radicals that ultimately destroy cell DNA. Nanobiotix®’s mode of action is dependent on the dose HfO₂ which generates high electron flux to induce more radicals than surrounding water when exposed to X-rays. This effectively increases the radiation dose at the tumor sites compared to surrounding tissue. In the absence of
external radiation source, the HfO$_2$ is inert and is cleared from the body slowly without serious side effects.$^{39-45}$

The simplest form of metallic nanomedicines that is currently used in clinical trials, is AuroShell® a 150 nm nanoshell form of colloidal gold. Similar to Nanobiotix®, relies on external radiation (near-infrared laser) to destroy the tumors in which the NPs have been entrapped. The AuroShell® formulation relies on the EPR effect for tumor localization. This technology has the advantage of treating tumors that might otherwise be difficult to remove surgically, e.g., those wrapped around arteries or nerve bundles. AuroShell® is also biocompatible and inert in the absence of external NIR irradiation.$^{46-48}$

These are just a few examples of nanomedicines that were developed to overcome the obstacles in the process of clinical translation. There are additional emerging technologies for preparing NPs that are more complex (from both polymeric and metal or hybrid core. Such material should be able to deliver a more complex payload, such as siRNA or combinations of chemotherapeutic drugs. There are many companies currently working on nanomedicines, each with its own unique platform that could improve quality of life for cancer patients. Unfortunately, most of the technology remains undisclosed at this time. Therefore, demonstrating to the medical community that nanomedicines can provide better treatment than traditional therapy remains a big challenge for the application of nanotechnology in medicine.
5.2 Prostate Cancer-Specific Drug Delivery and Imaging System: Design, Synthesis, and Characterization of Multi-functional Gold Nanoparticles

5.2.1 Rationale of designing MfAuNPs for Prostate Cancer therapy

Prostate cancer (PCa) is currently the second leading cause of cancer-related deaths among American men. According to the National Cancer Institute report, the PCa mortality rate in 2011 was approximately 30,000/year. The high mortality rate associated with PCa is largely due to the lack of effective screening methods for detection and staging disease. In fact, most of the approved current methods for diagnosis of PCa normally reveal the malignant tissue only after the cancer has metastasized. Therefore, there is a need for developing new diagnostic moiety that can be detected both primary and metastatic prostate cancer.49,50

To solve this problem, investigators have looked at different molecular biomarkers that are closely associated with PCa progression cycle. These biomolecules include a variety of organ-restricted modules or receptor proteins expressed in both normal and malignant prostate epithelial cells. One of the most active targets for diagnosis and therapy is the cell surface prostate-specific membrane antigen (PSMA). The secreted protease prostate-specific antigen (PSA) and the enzyme prostatic acid phosphatase (PAP) are also currently exploited as potential targets.

PSMA is highly specific to all forms of prostate tissue. Its expression increases with tumor progression and normally is found higher levels in solid tumor, metastatic and hormone refractory prostate cancer.51-54 It should be noted that PSMA is also expressed at relatively low levels in neovasculature of other cancers found in the small intestine, proximal renal tubules and salivary glands. PSMA gene is mapped to chromosome 11p which is not commonly deleted in
prostate cancer. PSMA has both neurocarboxipeptidase and folate hydrolase activity, however, the impact of these enzymatic functions on human prostate tissue still remains unclear. PSMA does have an internalization signal that allows translation of the protein on the cell surface into an endosomal compartment.\textsuperscript{55} PSMA is classified as a type II integral cell surface membrane protein. It was initially characterized using the labeled murine monoclonal antibody (mAb) 7e11-Cyt356 in for detecting occult metastatic cancer application. PSMA protein is composed of 3 separate units- a 19-amino-acid internal portion, a 24-amino-acid transmembrane portion, and a 707-amino-acid external portion (Figure 5-6). PSMA active site is normally rich in arginine residues, and is located in the extracellular region. In addition to its up-regulation and overexpression in androgen-independent PCa, PSMA has other unique features that make it an attractive target for diagnostic and therapy.\textsuperscript{56} Although antibodies and aptamers have been identified as high specificity, high affinity reagents for targeting PSMA, only small urea-based peptidomemitics will be described here.

\textbf{Figure 5-6: Structural of PSMA receptor}\textsuperscript{56}
5.2.2 Rationale of using a small peptidomimetic PSMAi as targeting component vs antibody.

There are a number of reviews that describe the pros and cons between using monoclonal antibodies (mAbs) and small cancer-specific molecules as targeting groups.\textsuperscript{57,58} Antibodies have been recognized as the gold standard for tumor targeting despite the fact that developing mAbs is an expensive and complex process. Although mAbs normally have a higher rate of success than small molecules in terms of development as therapeutic targeting drugs, they also have many undesirable characteristics. mAbs are very high molecular weight proteins, are complex to manufacture, and have high immunogenic properties.\textsuperscript{59-61} On the other hand, small molecules are normally less specific than antibodies; however, this property also enables them to have lower systemic toxicity compared to mAbs. Small molecules are easier to prepare and modify, and therefore they usually have more favorable pharmacokinetic properties.\textsuperscript{62} This is particularly the case for diagnostic applications where fast blood clearance would improve the signal to noise (S:N) ratio. Small molecules have better tumor penetration, and therefore their target binding density (uptake) accumulation is higher. Small molecules are more readily available in terms of development than antibodies. Therefore, it seemed logical to explore the use of small cancer-specific molecules, such as PSMAi, as a targeting modality for our MfAuNPs formulation.\textsuperscript{63,64}

I chose the prostate-specific membrane antigen (PSMA), as the molecular target for developing MfAuNPs as a prostate cancer-specific drug delivery system. This was primarily because of its transmembrane location and the fact that it is overexpressed on malignant prostate cells. In addition, the medicinal chemistry for small PSMA binding substrates is already well established, especially for the urea-based inhibitors. As mentioned previously, there are unique
advantages for using these tri-peptidomimetics as opposed to large antibodies or even peptide fragments. This is primarily because they give better pharmacokinetics and are easier to manipulate. 52,63,65,66

Kularatne, et al. reported the first example in the class of urea-based PSMA ligands, DUPA (Figure 5-7). DUPA consisted of 2 (S)-glutamic acids, bridged together through carbonyl, with a IC$_{50}$ approximately 20nM. Babich, et al. developed a similar molecule, however this PSMAi was made using (S) glutamic acid and (S) lysine, bridged through a phosphine, with an IC$_{50}$ around 9nM. SAR studies indicated that all of these urea-base inhibitors must have three carboxyl groups in order for the inhibitor to dock appropriately within the PSMA pocket. Two α-carboxyls of DUPA, form H-bonds with residues R534 and R210 of the receptor. One of the β-carboxyl group is H-bonded to the extended lysine residue of the peptide, leaving the other β-carboxyl free in solvent space. As a consequence, many investigators have used this feature of urea-based PSMAi to attach a prosthetic diagnosis/therapeutic group off this position. Low et al. conjugated a PSMA inhibitor with a $^{99m}$Tc chelate, and Pomper et al. used a similar PSMA inhibitor to incorporate $^{68}$Ga and $^{125}$I radionuclides for imaging. As briefly mentioned in Chapter 4, I used a similar convergent strategy to functionalize the free lysine terminal of this target with a complementary partner, such that the molecule could be ligated to lipoic acid.
5.2.3 Convergent multifunctional small therapeutic molecules for AuNPs

Each individual component for this study was made separately using a modular approach, instead of developing a single entity for therapeutic or diagnostic probe, as illustrated in Chapter 3 and 4. In this study, the component is further ligated to a functional lipoic acid for attachment onto the gold nanoparticles. As shown in the schematic below (Scheme 5-1), one terminus of the molecule contains either lipoic acid (LA) or an oligomeric ethylene glycol (OEG) thiol which can be directly attached to the gold surface by S-Au thiol bonding. These thiolate based molecules are then functionalized with a small module that contains an active terminal functional group for subsequent chemo- orthogonal ligation reactions. Similarly, the diagnostic or
therapeutic molecules can be modified to contain a complementary terminal functional group that allows them to be orthogonally ligated onto the other modules. For example, on alkynylated PSMA can be ligated to an azido lipoamide using “click” conditions, forming one class of targeting components. This combination can then be attached on the gold surface via the dithiol group using a “top-down” approach.

Scheme 5-1: Modular assembly of functional components
5.2.4 Synthesis of PSMAi Targeting Component

For the goal of functionalizing the AuNPs, the PSMAi molecule had to be ligated to the thiolated linker. The t-Boc protecting group on the ε-amino of the lysine moiety can be easily removed using acids (trifluoroacetic acid). Simple amide coupling (DCC-NHS) with pentynoic acid gives the alkynated PSMA derivative as shown in Scheme 5-2. Subsequently “click” chemistry with the complementary azido thiolated oligomer gave the final compound modest yields (~40%). Ultimately, this PSMAi thiolated derivative was attached to pegylated AuNPs to give the functionalized AuNPs.

Scheme 5-2: Synthesis of PSMAi targeting component

5.2.5 Synthesis of pH-sensitive Doxorubicin Targeting Component

The experimental design of alkynated pH-sensitive Dox, was described in Chapter 3 (experimental section 3.7). For the goal of attaching the Dox onto the AuNPs, the alkynated derivative was ligated to the azido lipoamide to give Dox-lipoamide as shown in Scheme 5-3. There were two reasons why the lipoamide with a shorter linker was used for the Dox
component. First, when the unit is assembled with the pegylated AuNPs, the drug should be closer to the gold surface, otherwise it might cause fluorescent quenching or NP aggregation. Because of its proximity to the gold surface, the Dox component is protected from degradation due to the pegylation layer. Using the lipoamide to form disulfide-Au bonds should provide stronger interactions with the Au surface than would a single S-Au bond, thereby enhancing the stability of the drug delivery component.

Scheme 5-3: Synthesis of pH-sensitive Dox targeting component

5.2.6 Synthesis of Imaging Component

In an approach similar to that applied to PSMAi and Dox thiolation components, I made a series of metalated and halogenated lipoamide derivatives that can be used for radioimaging AuNP distribution (Scheme 5-4). For the rhenium tricarbonyl (ReCO₃) or technetium 99 (⁹⁹mTc) chelates designed for use in SPECT imaging, I prepared a bispicolyl derivative of propargyl
amine via reductive amination. The derivative was “clicked” onto the azido lipoamide to form the ReCO$_3^{99m}$Tc- metalated precursor. In a similar fashion, precursors for $^{18}$F and $^{125}$I labeled derivatives can be made via the “click” chemistry using the appropriate propargyl tosylate and stannyl alkynated derivatives and the complementary azido lipoamide. Ultimately, like other individual components, the imaging modalities with the thiolated terminal groups can be attached to the pegylated AuNPs forming the final functionalized AuNPs.

Scheme 5-4: Synthesis of Imaging Components
The process of functionalization of AuNPs as a “top-down” approach can be described as follows. First, a citrate stabilized AuNP solution is elaborated with a stealth surface coating. This process is achieved by mixing the citrate stabilized AuNPs with a polyethylene glycol solution, typically CH₃O-PEG₁₀₀₀-SH. The pegylated AuNPs are then purified by ultra-centrifugation and dialysis (see experimental sections). The Dox and PSMA components are premixed in DMSO/water at the desired concentration as shown in Table 5-1. Pegylated AuNPs are then slowly dispersed into the mixture, allowing the Dox and PSMA ligands to thiolate the surface, giving the final MfAuNPs with a capacity to target PSMA(+) prostate cancer cells (Scheme 5-5).

Table 5-1 - PSMAi/Dox formulation AuNPs

<table>
<thead>
<tr>
<th>Formulation</th>
<th>AuNPs [mM]</th>
<th>PEG (1000) [μM]</th>
<th>Dox [μM]</th>
<th>PSMA [nM]</th>
<th>Zeta (mV)</th>
<th>Avg. size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-7.5±1.2</td>
<td>49.2±6.8</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5.8±2.1</td>
<td>52.1±18</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>1</td>
<td>0.1</td>
<td>0</td>
<td>-6.8±1.2</td>
<td>69.2±22</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>1</td>
<td>0.1</td>
<td>10</td>
<td>-7.2±3.2</td>
<td>69.6±20</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>1</td>
<td>0.1</td>
<td>20</td>
<td>-8.5±5.2</td>
<td>71.8±35</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>1</td>
<td>0.1</td>
<td>40</td>
<td>-10.7±1.9</td>
<td>72.2±25</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>1</td>
<td>0.1</td>
<td>80</td>
<td>-14.5±4.2</td>
<td>92.1±36</td>
</tr>
</tbody>
</table>
There are several deficiencies to the “top-down” assembly method. First, control over the attachment of thiolated ligands is statistical, depending largely on the ratio of groups and their availability the Au surface. In addition, there may be competing and unequal reactions between two different types of ligands (Dox/PSMA) to form thiol bonds with the gold surface. Second, the discrete spatial-arrangement for each ligand type on the surface of the AuNP may not be consistent from batch to batch. This may attest to the release profile of the Dox and/or PSMA targeting potential. And finally, it is difficult to determine whether the ratio of the ligands presented on the AuNPs surface is the same as in the original coating solution.
5.2.7 Characterization of representative monofunctionalization AuNPs

A variety of spectroscopic methods were used in order to determine the chemical identity of the ligands on the AuNPs and the overall surface composition. For example in Figure 5-8, I have used stack NMR method to identify the addition group that is linearly built on propargyl bis-picolyl the component. The triazole H (label in red) can only observed if the bisbicoly had been successfully “clicked” on azidolipoamide. Then chelation component was labeled with Re(CO)\textsubscript{3} ammonium salt as corresponding to diastereotopic protons H\textsubscript{e} and H\textsubscript{e}' (labeling in pink). The successful identification of these components is also supported by IR. In addition to NMR
spectroscopy, we use UV-visible analysis and transmission electron microscopy (TEM) to look at the morphology and homogeneity of the dispersed coated-AuNPs as shown in Figure 5-9.

![Image of TEM and UV characterization](image)

**Figure 5-9:** Representative TEM and UV-characterization of monofunctionalized AuNPs

Subsequently, HPLC is used to determine the purity of the dispersion AuNPs solution. For instance, in this case we look at the HPLC trace of 0.5mM AuNPs, pegylated with 1.0μM of polyethylene glycol spacer (mw=1000 g/mol). Then the pegylated gold is monofunctionalized with the fluorescent Re(CO)₃ component. The dispersion is subjected to ultra-centrifugation and dialysis (mwco=10,000g/mol) for overnight. The filtrate is analyzed using HPLC with condition MeCN/H₂O/0.1% TFA (80/20). The functionalized AuNPs eluted from a reverse phase column approximately at 4.5 minutes, as shown in Figure 5-10.
Figure 5-10: Representative HPLC trace of Re(CO)$_3$ monofunctionalized AuNPs

5.3 Initial Biological Evaluation of PSMA/Dox Multi-functional Gold Nanoparticles

The initial study was undertaken to determine the PSMA-related effect and to identify the nature of that effect. Cell uptake /targeting of the PSMA/Dox –AuNPS with (-PSMA)-PC3 and (+PSMA)-LnCap cells was evaluated using fluorescent activated cell sorting (FACS, as shown in Figure 5-17. The PSMA/Dox- AuNPs demonstrated enhanced cell binding only to (+PSMA)-LnCap cells as the decorated PSMA concentration increased on the gold NPs surface. In contrast (+PSMA)-LnCap, the (-PSMA)-PC3 cell lines did not show any significant change in cell binding of Dox, Dox-AuNPs and PSMA/Dox- AuNPs. The results in Figure 5-11 illustrated a marked targeting effect imparted by the presence of the PSMA inhibitor component
functionalized on the surface of the AuNPs. These effects were consistent with an interaction selective for the membrane PSMA receptor as reported in the literature. Further, varying the concentration of PSMA decorated AuNPs gave an optimal condition that is needed to enhance the uptake of nanoparticles without destroying the morphology of the cell.

Figure 5-11: The FACS analysis of (-PSMA)-PC3 and (+PSMA)-LnCap cells. A) Histogram analysis of cells treated with 0.1μM of Dox/PSMA (varying concentration)-AuNPs; (purple) cells only, (green) Dox treated cells, (pink) Dox/PSMA[20nM]-AuNPs treated cells, (yellow) Dox/PSMA[80nM]-AuNPs treated cells. B) The geometric mean plot of FACS.
The preliminary FACS results demonstrate the effect of PSMA-AuNPs, to verify the cellular membrane PSMA receptor targeting effect of our NPs system, we undertook the subsequent FACS experiment of pretreating the LnCap cells with 10μM of free PSMA to block the receptor. The results shown in Figure 5-12 clearly support enhanced PSMA/Dox-AuNPs targeting to the PSMA–positive prostate cancer cells. The cellular uptake of the PSMA/Dox-AuNPs was significantly enhanced in the absence of free PSMA, and the binding uptake is depleted almost 80% with the presence of PSMA. The incubation of the cells with the PSMA/Dox-AuNPs and PSMA may restore the uptake and distribution pattern essentially to that observed for Dox and Dox-AuNPs alone. The inhibition of a membrane PSMA receptor-mediated pathway would leave only the non-specific passive diffusion process available, leading to the observed similarity to Dox cellular accumulation. This study supported the observation of the initial finding that our synthetic urea-based PSMA component would generate a higher intracellular uptake of Dox when attached on AuNPs, and therefore may trigger rapid cell death. Unfortunately, our preliminary cytotoxicity data did not show any differences in terms of potency of all the PSMA/Dox –AuNPs in which doxorubicin is used at 0.1μM, and as high as at 3μM (data is not shown). We came to conclude that doxorubicin is probably not a drug of choice for killing PC-3 and LnCap prostate cancer cells.
Figure 5-12: The FACS analysis (+PSMA)-LnCap cells in presence and absence of free PSMA. A) Histogram analysis of cells treated with 0.1μM of Dox/ 40nM of PSMA-AuNPs; (purple) cells only, (green) Dox treated cells, (pink) Dox/PSMA[40nM]-AuNPs treated cells in the presence of 10μM free PSMA, (orange) Dox/PSMA[40nM]-AuNPs treated cells in the absence of free PSMA

5.4 Conclusion

This chapter presents a pilot study for a platform technology preparing individual therapeutic component from small molecules and subsequently assembling the MfAuNPs specifically aimed for prostate cancer therapy. It is envisioned that a convergent modular approach could ultimately leading to the controlled assembly of MfAuNPs in the application of personalized medicine. Preliminary in vitro flow cytometry studies with a PCa cell model,
illustrated that the gold nanoparticles decorated with the urea-based PSMAi have significantly enhanced binding uptake by the cellular membrane PSMA receptors.

5.5 Experimental

General Methods

All solvents and reagents involved in the synthesis were reagent grade, purchased from either Sigma-Aldrich™ or Fisher Scientific, and used without further purification. Thin-layer chromatography (TLC) was done on polyester sheets pre-coated with silica gel matrix 60 F254 obtained from Sigma-Aldrich™. Separations were performed using automated flash chromatography (Argonaut FlaskMaster) or packed column chromatography with Sorbent Technologies silica gel particle size 32-63 μm and 60 Å pore size. Liquid chromatography-mass spectroscopy (LC-MS) was performed using Alliance HT -LCT Premier 2489, Waters® instrument. High performance liquid chromatography (HPLC) was performed using a Waters HPLC system, equipped with a Waters 2695 binary pump, a Waters 2998 fluorescence photodiode array detector, and a XBridge™ C18 column (3.5μm, 4.6x75mm). H and C NMR spectra were recorded on 400 or 500 MHz Varian FT-NMR spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) by reference to proton resonances resulting from incomplete deuteration of the NMR solvent. The concentrations of test compounds were determined spectrophotometrically with a diode array UV mini 1240, Shimadzu® spectrophotometer. Ultracentrifugation of cell lysates was accomplished with a Sorvall RT 6000B Refrigerated Centrifuge. All tissue culture materials were obtained from Gibco Life
Technologies (Grand Island, NY) unless otherwise stated. MCF-7 and MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD).

**High Performance Liquid Chromatography (HPLC) Analysis**

The compounds were analyzed with a Waters HPLC system, equipped with a Waters 2695 binary pump, a Waters 2998 fluorescence photodiode array detector, and a XBridge™ C18 column (3.5μm, 4.6x75mm). HPLC grade acetonitrile/water/trifluoroacetic acid (40/60/0.1%, v/v) was used as the mobile phase at 25°C with a flow rate of 1.0 mL min⁻¹. Fluorescence detector was set at 254 nm for excitation and 570 nm for emission and linked to Empower III™ software for data analysis.

**Cell culture**

The PSMA(+)LnCap (human prostate cancer carcinoma) cell lines were maintained in RPMI-1640 Medium at 37º C, 5% CO₂. RPMI-1640 were supplemented with 10 % fetal bovine serum (FBS), 50 U/ml –penicillin, and 50μg/ml streptomycin. PSMA(-) PC-3 (human prostate cancer adenocarcinoma), cell lines were maintained in F-12K Medium at 37º C, 5% CO₂. F-12K were supplemented with 10 % fetal bovine serum (FBS), 50 U/ml –penicillin, and 50μg/ml streptomycin.

**Cytotoxicity assays**

Cells were plated at a 5×10³ cells per well density in 96-well plates (Corning Inc., Corning, NY, USA). The stock solutions of free drug or drug-conjugates were prepared in DMSO and diluted in complete media before adding to cells. After 24h, the medium was replaced with medium
containing free drug or drug-conjugates. After 24h incubation, each well was washed twice with complete media and cell survival was measured using the Cell Titer-Blue® Cell Viability Assay method. The conversion of Resaruzin to Resorufin by viable cells results in the fluorescence excitation at 550nm. The fluorescence produced is proportional to the number of viable cells. The emitted fluorescence was measured at 590 nm (the measurement of the cytotoxicity) using a Labsystems Multiskan MCC/340 microplate reader (Labsystems and Life Sciences International, UK).

For PSMA competition assay, the cells were pretreated with free PSMAi (50μM) for 1h, followed by addition of free drug or drug-conjugates in complete media with 50μM of PSMAi. After 24h incubation, cell viability was analyzed as described above.

**Flow Cytometry**

The PSMA(+)LnCap and PSMA(-) PC-3 cells were grown in 12-well tissue culture plates till 70-80% confluency. The medium was removed from the wells; cells were washed with complete media and incubated with or without 50μM PSMA for 1h. After incubation, the cells were washed twice with complete media, and exposed to 0.1μM of Dox-AuNP, PSMA-0.1μM Dox (different ratio mixture) in complete media with or without 50 μM of PSMA. After 1h cells were washed, trypsinized, and finally resuspended in 800 µl of 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4. The cell-associated fluorescence was quantified by Becton Dickinson FACScan™ (Becton Dickinson, San Jose, CA) at the emission wavelength of 580 nm (channel FL-2). The data analysis was performed using CellQuest software (Becton Dickinson). A total of 10,000 events were acquired for each sample. Data shown were derived from three separate experiments.
Synthesis of colloidal AuNPs- The AuNPs dispersion was prepared by using a standard sodium citrate reduction of HAUCl₄. A stock solution 5.0mM HAUCl₄ was made by dissolving HAUCl₄ (0.080g) in DI water (50 mL). The stock solution (10 mL) was diluted 1:18 with DI water and heated at 100°C with vigorously stirring. A 0.5% aqueous solution of trisodium citrate (10 mL) was added to the solution. The boiled solution color changed to ruby red color within 20-30 minutes. The solution was cooled down to room temperature and vacuum filtered through 0.2µM filter to remove all the aggregates.

N-(2-(2-azidoethoxy)ethyl)-5-(1,2-dithiolan-3-yl)pentanamide (azidolipoamide)- To a solution of lipoic acid (0.515 g, 2.5 mmol) in 5 mL dichloromethane was added dicyclohexyl carbodiimide (0.519 g, 2.5 mmol) and N-hydrosuccinamide (0.478 g, 2.5 mmol). The reaction mixture was stirred for 30 min, then 11-azido-3,6,9-trioxaodecan-1-amine (495μL, 2.5 mmol) was added. The reaction was stirred at room temperature overnight. The reaction mixture was filtered to remove precipitates, and worked up water, brine solution and extracted with dichloromethane. The filtrate was dried over magnesium sulfate and the solvent was removed under reduced pressure. Column chromatography afforded an isolated yield (650 mg, 57% ) of azidolipoamide as yellow oil. ^1H NMR (CDCl₃, 400MHz): δ. 1.38 (2H, m), 1.59 (6H, m), 1.83 (2H, m), 2.11 (2H, t, J=5.6Hz), 2.37 (2 H, m), 3.05 (3H, m), 3.34 (2H, m), 3.47 (2H, t, J=4.8Hz), 3.59 (8H, m), 6.18(1H, b.s). R_f(DCC:MeOH; 9:1) = 0.65
Synthesis lipoamide Dox component - To a solution of azido lipoamide (8mg, 0.027mmol) in 500μL of tert-butanol/water (1:1) was added a solution of Alkyno-Dox (12mg, 0.027mmol) in 500μL of tert-butanol/water (1:1). The reaction was stirred at room temperature for 30 min, followed by the addition of copper(II) sulfate pentahydrate (5.75μL, 0.20μmol) and (+)-sodium L-ascorbate (14.8μL, 0.75μmol). The reaction mixture was warmed to 40°C, stirred for 24 h, and then partitioned between water (10mL) and dichloromethane (10mL). The organic layer was washed with water (2x10mL). The aqueous layers were combined; sodium chloride was added and then back extracted with dichloromethane (10mL). The organic fractions were combined, dried over magnesium sulfate, filtered and concentrated, leaving approximately 1.0 mL of solution. The filtrate was triturated with ether to yield a red precipitate. The red precipitate was collected by filtration, washed with ether, and dissolved in water. The mixture was dialyzed against water at 4°C for 24 h with a dialysis tubing (MWCO 200-400). The product was obtained after lyophilization to afford the desired product as red paste (10.5 mg, 65%).
Preparation of PEG-S-capped AuNPs – MeO-PEG-SH (1 mg) was dissolved in 1.0 mL of ultrapure water. The solution is vigorously stirred at room temperature and added dropwise to citric acid-stabilized AuNPs (1 mL of 0.5 mM) in 5 mL of ultrapurified water at pH 7.4. The mixture was stirred for 24 h at room temperature. Thereafter, the nanoparticles were centrifuged at a speed of 14000 rpm for 15 min to remove free MeO-PEG-SH and washed twice with ultrapure water.

Preparation of Doxorubicin functionalized AuNPs – 100 μL of a lipoamide Doxorubicin stock solution (10 mM) diluted in 1 mL of ultrapure water. The solution is vigorously stirred at room temperature and added dropwise of pegylated AuNPs (1 mL of 0.5 mM) in 5 mL of ultrapure water at neutral pH. The dispersion mixture was stirred in the dark for 24 h at room temperature.
The nanoparticles were centrifuged at a speed of 1000 rpm for 5 min. Then the dispersion was dialyzed against water at 4\(^\circ\)C for 24 h with dialysis tubing (MWCO 1000).

![Chemical structure](image)

**Synthesis of PSMAi component** - To a solution of azido PEG-SH (1.0 mg, 0.500 \(\mu\)mol) in 500\(\mu\)L of DMSO/water (1:1) was added a solution of Alkyno-PSMA (0.550mg, 0.65 \(\mu\)mol) in 500\(\mu\)L of DMSO/water (1:1). The mixture was ultrasonicated at 37\(^\circ\)C for 30 min, followed by the addition of copper (II) sulfate pentahydrate (1.75\(\mu\)L, 0.10\(\mu\)mol) and (+)-sodium L-ascorbate (3.8\(\mu\)L, 0.30\(\mu\)mol). The reaction mixture was warmed to 40\(^\circ\)C, ultrasonicated for 24 h, and then the reaction mixture was filtered and washed with DMSO to remove precipitates. The filtrate was dialyzed against water at 4\(^\circ\)C for 24 h with a dialysis tubing (MWCO 200-400). The product was obtained after lyophilization as clear viscous liquid (0.63mg, 42%).
Preparation of PSMA/Dox (40nM/0.1μM) functionalized AuNPs - 100μL of a lipoamide Doxorubicin stock solution (10mM) is diluted in 1mL of ultrapure water, then added to10μL of PSMAi component (0.1mM) to the mixture. The solution is vigorously stirred at room temperature and added dropwise of pegylated AuNPs (1mL of 0.5 mM) in 5mL of ultrapure water at neutral pH=7. The dispersion mixture was stirred in the dark for 24 h at room temperature. The nanoparticles were centrifuged at a speed of 1000 rpm for 5 min. Then dispersion was dialyzed against phosphate buffer (pH=4) at 4°C for 24 h with dialysis tubing (MWCO 1000). Then the collected AuNPs dispersion was redispersed in ultrapure water at pH=7.
Synthesis of N,N-bis(pyridin-2-ylmethyl)prop-2-yn-1-amine - Di-(2-picoly)amine (900 µL, 5.0 mmol) was dissolved in dry THF (10 mL). While the solution is stirred under N₂, K₂CO₃ (2.76 g, 20 mmol) was added followed by dropwise addition of propargyl bromide 80% in toluene (557 µL, 5.0 mmol). The reaction mixture was stirred for 24 h before dilution with DCM. The diluted reaction mixture was filtered and concentrated under vacuum reduced pressure. The desired product was isolated as yellow oil (0.450 g, 90%) using column chromatography DCM/MeOH (0% - 5%). ¹H NMR (400 MHz, CDCl₃): δ 8.57 (2H, d, J = 4.2 Hz), 7.66 (2H, td, J = 1.2, 7.8 Hz), 7.52 (2H, d, J = 7.8 Hz), 7.17 (2H, td, J = 5.4, 7.2 Hz), 3.93 (4H, s), 3.43 (2H, d, J = 2.4 Hz), 2.30 (1H, t, J = 2.4 Hz). LC-MS(TOF): calcd. m/z 260.11, found 260.

Synthesis of N,N-bispicolyl lipoamide component - To a solution of azido lipoamide (50.0mg, 0.140mmol) in 1 mL of tert-butanol/water (1:1) was added a solution of Alkyno-Dox (33.0mg,
0.140 mmol) in 1 mL of tert-butanol/water (1:1). The reaction was stirred at room temperature for 30 min, followed by the addition of copper(II) sulfate pentahydrate (15.75 μL, 0.50 μmol) and (+)-sodium L-ascorbate (32.8 μg, 1.75 μmol). The reaction mixture was warmed to 40°C, stirred for 24 h, and then partitioned between water (10 mL) and dichloromethane (10 mL). The organic layer was washed with water (2x10 mL). The aqueous layers were combined; sodium chloride was added and then back extracted with dichloromethane (10 mL). The organic fractions were combined, dried over magnesium sulfate, filtered and concentrated under vacuum. The product was obtained after Column chromatography as an oil (45 mg, 55%). ¹H NMR (400 MHz, CDCl₃) δ = 8.54 (2H, d, J = 4.4 Hz), 7.76 (1H, s), 7.52 - 7.72 (4H, m), 7.29 (2H, s), 6.32 (1H, br. s.), 4.54 (4H, t, J = 5.1 Hz), 3.78 - 3.93 (4H, m), 3.68 (2H, br. s.), 3.49 - 3.63 (7H, m), 3.43 (2H, q, J = 5.1 Hz), 3.06 - 3.27 (4H, m), 2.45 (2H, dq, J₁ = 12.5 Hz, J₂ = 6.4 Hz), 2.19 (2H, t, J = 7.3 Hz), 1.83 - 2.03 (2H, m), 1.58 - 1.75 (2H, m), 1.36 - 1.53 (2H, m).

Preparation Re(CO)₃ labeled bispicolyl lipoamide component- (NEt₄)₂[Re(CO)₃Br₃] (one equivalent) and the chelating ligand bispicolyl lipoamide (one equivalent) were dissolved in methanol and stirred at room temperature for 24 h or until the disappearance of starting material (monitored by TLC). The reaction was filtered and the filtrate was evaporated to dryness. The residue was purified by column chromatography on silica gel (DCM/MeOH, 8:2) to yield white paste material. ¹H NMR (400 MHz, CDCl₃) δ 8.60 - 8.66 (m, 2H), 7.70 - 7.79 (m, 2H), 7.25 (d, J
= 8.55 Hz, 1H), 5.09 - 5.14 (1H, m), 4.90 (1H, s), 3.91 (1H, s), 3.59 - 3.61 (1H, m), 3.51 - 3.56 (4H, m), 3.36 - 3.47 (36H, m), 3.29 (1H, d, J = 5.49 Hz), 2.60 (1H, s), 1.29 (1H, t, J = 7.33 Hz, 1H).
5.5 References


Chapter 6
Significance of the Studies and Future Directions
6.1 Summary and Significant Outcomes of this work

Chapter 2

Although steroid hormone research in breast cancer has existed for more than 30 years, questions remain regarding its application in therapy of the disease. Extensive work in the field of estradiol-drug conjugates, summarized in Chapter 2, reveals significant appreciation of the design criteria and understanding of the overall targeting mechanisms of ER-targeted drug conjugates. Due to the complexity involved in making the hybrids and discouraging results for many of the E2-drug hybrids, research in this field turned away from using steroid-based compounds for targeted drug delivery systems. The labor-intensive syntheses and the lack of intellectual property inhibited the progress of steroids for breast cancer therapy. The agents developed in this thesis represent significant contributions to the field of breast cancer research, both in design and application of steroidal antiestrogens for drug targeting.

Chapter 3

The objective for Chapter 3 involved the development of a steroid-drug conjugate that would display selectivity for ER expressing breast cancer cells and would be more potent in those cells than the drug alone. As described in Chapter 2, those two criteria had not been previously achieved. As demonstrated by our results, the AE-Dox conjugate has fulfilled both criteria. The agent shows a selective anti-proliferative effect for (ER+)-MCF-7 vs. (ER-)-MDA-MB-231. The effect in (ER+)-cells is reversed by preincubation with estradiol, indicating an ER-related mechanism. FACS and fluorescent confocal microscopy also supported an ER-related uptake process. The antiproliferation effect of AE-Dox in (ER+)-cells was 70 fold greater than doxorubicin alone, indicating the potency enhancing effect of the intact conjugate. These in vitro
responses were greater than any previous reported steroid-drug conjugate for breast cancer therapy.

The second major contribution to the field of targeted drug conjugates involves their design and preparation. One of the contributing factors to the success of the 11βAE-Dox conjugate was the use of the pH-sensitive, benzoyl hydrazone linker. Because of its ability to dissociate within the acidic endosome via a non-enzymatic process, it effectively generated doxorubicin within the cancer cells. In addition to providing chemical stability, the benzoyl hydrazone acted as a site to link the alkynyl OEG. The use of small, bifunctional oligoethylene glycols was another important feature. These physicochemical properties contributed to the stability of the conjugate in aqueous media while one of the termini could be attached to the appropriate targeted drug components. The other terminus could then be modified with functional groups for “click” ligation. This modular chemistry permitted a convergent synthetic strategy that was different from that used in other steroid-drug conjugate programs.

The other key contributing factor was the choice of the 11β-position in estradiol for attaching the conjugate group. Relatively little effort had been previous expended due to the synthetic difficulty in accessing it. The improvements made as shown in Chapter 4 provided alternative approaches. The advances made provide a facile entry into a variety of agents that have higher affinity, selectivity, and other anti-estrogens.

**Chapter 4**

The objective of this project was to develop new radio-chemicals that can be readily prepared in higher radiochemical yield with a variety of radionuclides and that can target breast or prostate cancer. The results in Chapter 4 demonstrated that we have developed a promising
radioligand for ER-expressing cancer that is different from previous efforts. The final compound in the ER-project is a [F-18]-labeled 11β-substituted estradiol derivative that can be prepared into two steps (plus two HPLC separations) in 30% RCY from 18F-fluoride. The tracers demonstrated a preliminary biodistribution that is markedly different from previous agents. The key factors in its preparation were the use of a convergent radio synthesis that used a rapid “click” ligation between a 18F-fluo-OEG-azide and an alkynylated-steroidal antiestrogen (see Chapter 3). The choice of components allowed for rapid preparation of the 18F-azido components. The use of the oligoethylene glycol linkers enhanced the hydrophilicity of the product leading to an apparent lower liver uptake and fast urinary clearance. This would make it significantly different from all previous ER-radioligands. The assessment in lipophilicity and biodistribution studies further indicated that our 18F-AE imaging agent possessed unique properties unlike 18F-FES in terms of its higher cellular uptake, and retention with faster blood clearance. The future results with in vivo imaging will demonstrate whether 11β-AE probe will be an excellent imaging agent for hormone dependent breast cancer.

A prostate specific membrane antigen (PSMA) inhibitor was successfully synthesized (Chapter 4) and was further functionalized with a variety of different imaging modalities via “click” chemistry. The completion of this work once again demonstrated that our convergent strategy approach could be utilized to develop any targeting or therapeutic hybrid molecules. Monovalent as well as valent versions of PSMA imaging agents were synthesized and characterized. Although, in vitro and in vivo assessments are needed to confirm the selectivity and specificity of for (+PSMA) prostate tumors, initial results indicated that the methods are suitable for using radionuclides. This work represents a significant advance compared to ongoing effort by other groups.
Chapter 5

The objective of this project was the development of functionalized AuNPs with targeting, imaging and chemotherapeutic components, in this case PSMA, fluorescence, doxorubicin. The results from Chapter 5 of this thesis demonstrated our approach a “top down” synthesis of such functionalized nanoparticles. Each of the modular components was developed from commercially starting materials and then attached to the AuNPs surface via Au-S bonding. The final formulation of AuNPs functionalized with a PSMA-targeted ligand, doxorubicin (therapeutic and imaging) and methoxylated PEG (“stealth”) demonstrated dox-related uptake in (PSMA+)-cells but not in (PSMA-)-cells. Uptake was selectively blocked by incubation with excess PSMA ligands, indicating the effect was PSMA-dependent. This effect had not been previously demonstrated. The multifunctional PSMA/Dox- AuNPs were stable and viable in vitro through the preliminary studies. Our system shows great potential for future development by utilizing the 11β-AE and other synthetic small molecules for targeting and other therapeutic group for treatment.

6.2 Future Directions

6.2.1 Antiestrogen-Drug Conjugate

The fourth generation antiestrogens should focus on improving on the relative proximity of the target of therapeutic groups by varying the chain length. One of the immediate tasks I would do regarding the AE-Dox projects, is to investigate the length of the oligoethylene glycol linker as a function ER binding as well as the potency of the Dox component. These steps would determine whether long or short linkers have any impact on the chemical stability and dissociation projects (Figure 6-1).
Figure 6-1: AE-Dox conjugate

The second experiment I would look at is the release rate of Dox in the conjugate as a function of pH. According the preliminary in vitro cytotoxicity and flowcytometry findings, the cell viability of (ER+)-MCF-7 was not correlated with the release rate of the Dox from the conjugates. Therefore, it is important to determine the optimal window of Dox concentrations in the conjugates and this information would perhaps lower the concentration of Dox (0.1μM) which is needed.

The third experiment I would do is to replace the Dox unit with a fluorescent probe, such as FITC to study the dissociation mechanism of the conjugate. The expected results for this experiment would be that the fluorescence would appear in the cytoplasm of the cell, but not in the nucleus, since benzoyl hydrazone should be cleaved from the FITC unit once the molecule is internalized inside the cell. After all the optimization studies are completed, I would scale up the synthesis of AE-Dox and perform in vivo studies to determine the pharmacokinetics and effectiveness of the conjugate toward hormone dependent breast tumors.

To further expand on the application of the conjugates having the steroidal antiestrogen as the targeting agent, I would replace doxorubicin with other chemotherapeutic agents, such as paclitaxel (Ptx). This change in the chemotherapeutic platform would maintain the key features...
of our 11β-AE. As mentioned in Chapter 3, the azide functional terminus of the AE is away from the binding pocket, therefore changing the drug in the conjugate should not affect the binding to the ERα. It may alter the potency of the conjugate when compared to the parent drug.

![18F-11βAE](image)

**Figure 6-2:** 18F-AE radioimaging agent

### 6.2.2 Imaging Agents.

Regarding the development of cell membrane targeted imaging agents, the next task I would like to perform is to scale up the synthesis of 11β-substituted antiestrogen labeled with 18F radionuclides for *in vivo* studies. This set of experiments which include biodistribution and tumor imaging, will demonstrate how effective of our steroid imaging agent is toward the ER-dependent breast cancer. Those in vivo experiments should be performed for mono and trivalent PSMA radiolabeled tracers. Comparison of the imaging studies for mono and trivalent agents should demonstrate whether that our hypothesis that the trimeric version enhances sensitivity to the overexpressed PSMA receptor prostate tumors is used (Figure 6-3).
Other imaging modalities should be “clicked” onto the 11β-substituted estradiol and PSMA targeting platforms. Imaging studies will demonstrate whether the approach is applicable for other radionuclides and other imaging modalities. The outcomes of these future experiments would provide a “proof of principle” that our approach can contribute to the development of personalized medicine.

6.2.3 Nanomedicine Development

The work involved with the assembly of multifunctional AuNPs represents a model system for prostate cancer specific therapy. There is still a significant amount of work to be done. One of the immediate tasks is to optimize the PSMA inhibitor concentration on AuNPs, for targeting PSMA receptor. It is possible that less of the PSMA on the surface would reduce the variation in zeta potential (less negative), and that may minimize the aggregation of nanoparticle dispersion. It is important to determine the length of the PEG attaching PSMA to the AuNPs.
since the carboxylic acid groups of the PSMA ligand have to be exposed in order for PSMA receptors to recognize the nanoparticle.

Figure 6-4: PSMA/Dox functionalized AuNPs

Because doxorubicin has little potency toward prostate cancer cells, other therapeutic reagents should be considered. Paclitaxel (Ptx) probably is the most effective drug to use in this case, therefore it should modified with a pH-sensitive release group for future development. In addition, pH-release experiments should be performed to determine the therapeutic window of the reagents needed for attaching on NPs.

An imaging moiety should also be incorporated onto the surface. Attaching an imaging modality would allow for in “real time” monitoring of the uptake of the NPs as well as the release profile of the drugs.

Another project would be to establish the controlled process for the synthesis of multifunctional nanoparticles. By varying the composition of the components in the mixture of functional components, one should be able to optimize the incorporation of the functionality components. This process is important for the assembly of gold nanoparticles that express three to four different functional groups. These steps will be labor intensive for the purification and
characterization processes, but they are essential for demonstrating a reproducible and controllable assembling multifunctional nanoparticles.

In conclusion, I believe that I have made significant contributions in the area of multifunctional drug development. I believe that my research has made a significant impact in the field of breast cancer, particularly in the use of steroids for therapeutic and diagnostic applications. My preliminary work in the development of PSMA radioimaging probes and multifunctional AuNPs for prostate cancer specific provides a basic for continued research in the field of prostate cancer research.
Appendix
Comparing cytotoxicity of modified A) Azido-AE (9), and B) Alk-Dox (14) with their parent compounds treated in MCF-7 (24h)
Cytoxicity experiment of TAM-AE conjugate in (ER+)-MCF-7 and (ER-)-MDA-MB-231
Cytoxicity assay AM-TKI conjugate in (ER+)-MCF-7 and (ER-)-MDA-MB-231
Cytoxicity assay 11β-AE (ER+)-MCF-7 and (ER-)MDA-MB-231
Cytotoxicity of compounds 11β-AE (black), Dox-linker-red, Dox (orange), and AE-Dox (purple), in A) ER(+)MCF-7 and B) ER(-)MDA-MB-231 cell lines.
The FACS analysis of ER(+)MCF-7 and ER(-)MDA-MB-231 cells. Top) Histogram analysis of cells treated with 0.1μM of Dox and AE-Dox with and without ES; (purple) cells only (green) Dox treated cells and (red) AE-Dox treated cells; bottom) The percentage of Dox- positive cells in ER (+)MCF-7 and ER (-)MDA-MB-231 cells; *P<0.01 (Dox vs AE-DOX (-ES)); §P<0.01 (AE-Dox (-ES) vs AE-Dox(+ES)).
Fluorescence microscopy images ER(+)MCF-7 cells; Hoechst fluorescent (upper panel), Red fluorescence (lower panel)
Fluorescence microscopy images ER(+) MCF-7 cells treated for 1 hour with 0.1μM of Dox; Hoechst fluorescent (upper panel), Red fluorescence (lower panel)
Fluorescence microscopy images ER(+)MCF-7 cells treated for 1 hour with 0.1μM of AE-Dox; Hoechst fluorescent (upper panel), Red fluorescence (lower panel)
Fluorescence microscopy images ER(+)MCF-7 cells treated for 1 hour with 0.1μM of AE-Dox after pretreatment with 50μM estradiol; Hoechst fluorescent (upper panel), Red fluorescence (lower panel)
ES blocking assay - competitive effect
FACS demonstrates synergy effect of AE-DOX hybrid

% Increase count

0.1 µM DOX  0.1 µM AE-DOX

(+ER)-MCF-7 (-ER)  (+ER)-MCF-7 (+ER)  (-ER)-MDA-MB-231 (-ER)  (-ER)-MDA-MB-231 (+ER)
AuNPs - citrate

AuNPs - Lipoic acid
<table>
<thead>
<tr>
<th>Average size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>49.2 ± 6.8</td>
<td>-7.5 ± 1.2</td>
</tr>
<tr>
<td>52.1 ± 18</td>
<td>5.8 ± 2.1</td>
</tr>
<tr>
<td>72.0 ± 25</td>
<td>-10.7 ± 1.9</td>
</tr>
</tbody>
</table>
How many Au atoms to make up a 50nm AuNP?

\[ V = \frac{4}{3} \pi r^3 \]

\[ V = \frac{4(3.14)(25nm)^3}{3} = 1.67 \times 10^{-23} \text{ m}^3 = 1.67 \times 10^{-17} \text{ ml} \]

\[ \rho_{Au} = \frac{m}{v} = 19.3 \text{ g/ml} \]

\[ \text{# of mol} = \frac{(3.23 \times 10^{-16} \text{ g})}{(197 \text{ g/mol})} = 1.64 \times 10^{-18} \text{ mol} \]

\[ \text{# of atoms} = 1.64 \times 10^{-18} \text{ mol} \times 6.022 \times 10^{23} \text{ atom/mol} = 987363 \text{ atoms} \]

How many 50nm AuNPs in 1ml of 0.5mM?

50nm AuNPs \( \rightarrow \) 1.64 \times 10^{-18} \text{ mol} \]

\[ \text{# of AuNPs} \rightarrow 0.5 \times 10^{-3} \text{ mol} \]

\[ \text{# of AuNPs} = (0.5 \times 10^{-3} \text{ mol}) / (1.64 \times 10^{-18} \text{ mol}) = 3.0 \times 10^{14} \text{ of 50nm AuNPs} \]

Estimation of Dox units per 50nm AuNP?

From UV extinction curve \( A_{\text{dox}} = 1.23 \)

From Dox Std curve \( A = 1.23 \rightarrow \sim 62 \ \mu\text{g/ml} \) or \( .1 \mu\text{M} \) (mw of Dox = 579.98 g/mol)

\[ \text{# of Dox units in 1ml (0.5mM AuNPs)} = (0.1 \times 10^{-6} \text{ mol}) / (6.022 \times 10^{23} \text{ units/mol}) = 6.43 \times 10^{16} \text{ units} \]

\[ \text{# of Dox per 1AuNPs} = 6.43 \times 10^{16} \text{ units} / 3.0 \times 10^{14} \text{ of 50nm AuNPs} = 215 \text{ units/AuNP} \]
Biological Data
List of Publication

(1) Dao, K.-L.; Hanson, R. N.: Targeting the Estrogen Receptor using Steroid–Therapeutic Drug Conjugates (Hybrids). Bioconjugate Chemistry 2012, 23, 2139-2158


(3) Dao, K.-L. et al, Prostate Cancer-Specific Drug Delivery and Imaging Systems: Design, Synthesis, and Biological Evaluation of Multi-functional Gold Nanoparticles (manuscript in prep.)

(4) Dao, K.-L. et al, Design, Synthesis, and in vivo PET imaging of radioligand 18F-11β-substituted estradiol (18F-11βAE) in hormone- dependent breast cancer (manuscript in prep)


(6) Dao, K.-L.; Hanson, R. N.: Prostate cancer-specific drug delivery and imaging system: design, synthesis and characterization of multifunctional AuNPs. CRC Press, 2011; Vol. 3; pp 386-387


(9) Dao, K-L and Hanson, R.N Prostate Specific Membrane Antigen (PSMA)Targeted Multifunctional Gold Nanoparticles for Prostate Cancer Therapy and Radioimaging, 243th ACS National Meeting, Division of Nuclear Chemistry, San Diego, CA, March 25-29, 2012. (Oral)


(11) Dao, K-L; Sawant, R.; V.; Torchilin, V; and Hanson, R.N. Design and Synthesis Small Therapeutic Ligands for Nano-Carriers in Cancer Therapy Nanocarriers in Medicine and Biology, Gordon Research Conference, Waterville Valley Resort, Waterville, NH, August 12-17, 2012. (Poster)