

Rose-Hulman Institute of Technology
Department of Chemistry & Biochemistry

**Determining the Relative Binding Efficacy of Selective
Estrogen Receptor Modulators**

Research Thesis

Dr. Ross Weatherman Research Lab

Submitted by:

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Abstract

Breast cancer is the second leading cause of breast cancer death among women in the United States, which highlights the importance of broadening breast cancer drug development and research. Treatment for those with breast cancer can be determined by biomarkers and the subtype of breast cancer, which presents the obstacle of subtypes and affiliated resistance for breast cancer drug development. Our lab focused on maximizing the effectiveness of estrogen-receptor positive breast cancer drugs by testing a new fluorophore in conjunction with both new and old breast cancer drugs. Fluorescence anisotropy was then used to test the binding affinity of various estrogen receptor-targeted compounds in order to determine their relative binding efficacies and compare them to those found in past studies using a different fluorophore. We found that the novel fluorophore coumestrol can be used effectively in the estrogen receptor binding assay and that Weatherman-made compounds generated data that would suggest the potential for further study as breast cancer drugs.

Background

Despite the fact that the rate of incidence has remained steady in recent years, breast cancer continues to inflict the second highest rate of cancer death in women of the United States (American Cancer Society, 2021), with lung cancer and nonspecific cancers contributing larger rates of cancer death. To add a level of quantitative evidence to the data, there were about 284,000 cancer deaths among women last year; with 15.5% of all cancer deaths in women resulting from breast cancer, there were then about 42,000 breast cancer deaths last year.

Due to the substantial amount of breast cancer deaths, it is important to consider the tie that is present between survival rate and breast cancer staging. In stage one breast cancer, tumor size is very small, inside the glands, and localized to the breast area. Stage two breast tumors are growing into the lymph nodes, but are still confined to the breast. Stage three tumors are growing further with sizes greater than five centimeters into the lymph, muscles, and skin, though the cancer is still confined to the breast area. Finally, stage four breast tumors include all of the prior characterization with metastasis to other tissues.

Breast cancer can be further categorized into two types: carcinoma or sarcoma.

Carcinoma is a malignant tumor of epithelial cells, and specifically the ductal cells of breast tissue. These ductal cells are responsible for making milk in healthy conditions.

Alternatively, sarcoma is a malignant tumor of the connective tissue of the breast.

Carcinoma is the more common breast cancer as compared to sarcoma, with sarcoma only accounting for about 1% of all primary breast cancers (Johns Hopkins).

When getting diagnosed with breast cancer, the stage and characterization of breast cancer will often determine the treatment. Typical treatments for breast cancer depend on the stage, biomarkers, and the histology of the cancer. The involvement of the lymph, hormone receptor status, and the patient's age and menopausal status also play a large role in deciding treatment options for someone with breast cancer. Depending on these factors, the primary choices are a mastectomy, radiation, and chemotherapy, with treatment plans sometimes involving a combination of these options.

Chemotherapy is the traditional treatment for breast cancer, as it is for most cancers. The administration of these poisonous drugs can be so effective because they result in cell death, controlling the uncontrolled growth that malignant epithelial cells undergo. Because chemotherapy sometimes causes non-specific cell death, however, this treatment often results in adverse side effects due to its effect on other cells in the body.

Normal breast tissue is regulated by reproductive hormones such as estrogen and progesterone, which present a risk factor for breast cancer as discrepancies arise in these reproductive hormones. The biomarkers that are screened for are the antibodies for the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2). When breast cancer is positive for any of these receptors (ER+, PR+, or HER2+), the cancerous cells have the given receptor on the cell surface for which it potentially receives growth signals from. The determination for which receptor a cancer is positive for, if any, is important in the treatment decision process, as this determines whether hormone therapy is an option. When hormone therapy is an option, the drug works by blocking the growth signal or the receiver of the signal so that uncontrolled cell growth halts. The difference in cancer outcome as it relates to subtype classification can be seen in survival rate for a given time since diagnosis for various breast cancer subtypes. For instance, stage four, triple negative breast cancers, which are those that do not respond to any protein receptors for growth signaling, have about 10% survival rate at forty-eight months since diagnosis. By contrast, stage four, luminal B breast cancers, which are those that respond to both the estrogen receptor and human epidermal growth factor receptor subtype for signaling,

have about a 50% survival rate at forty-eight months since diagnosis, as seen in **Figure 1**. (American Cancer Society).

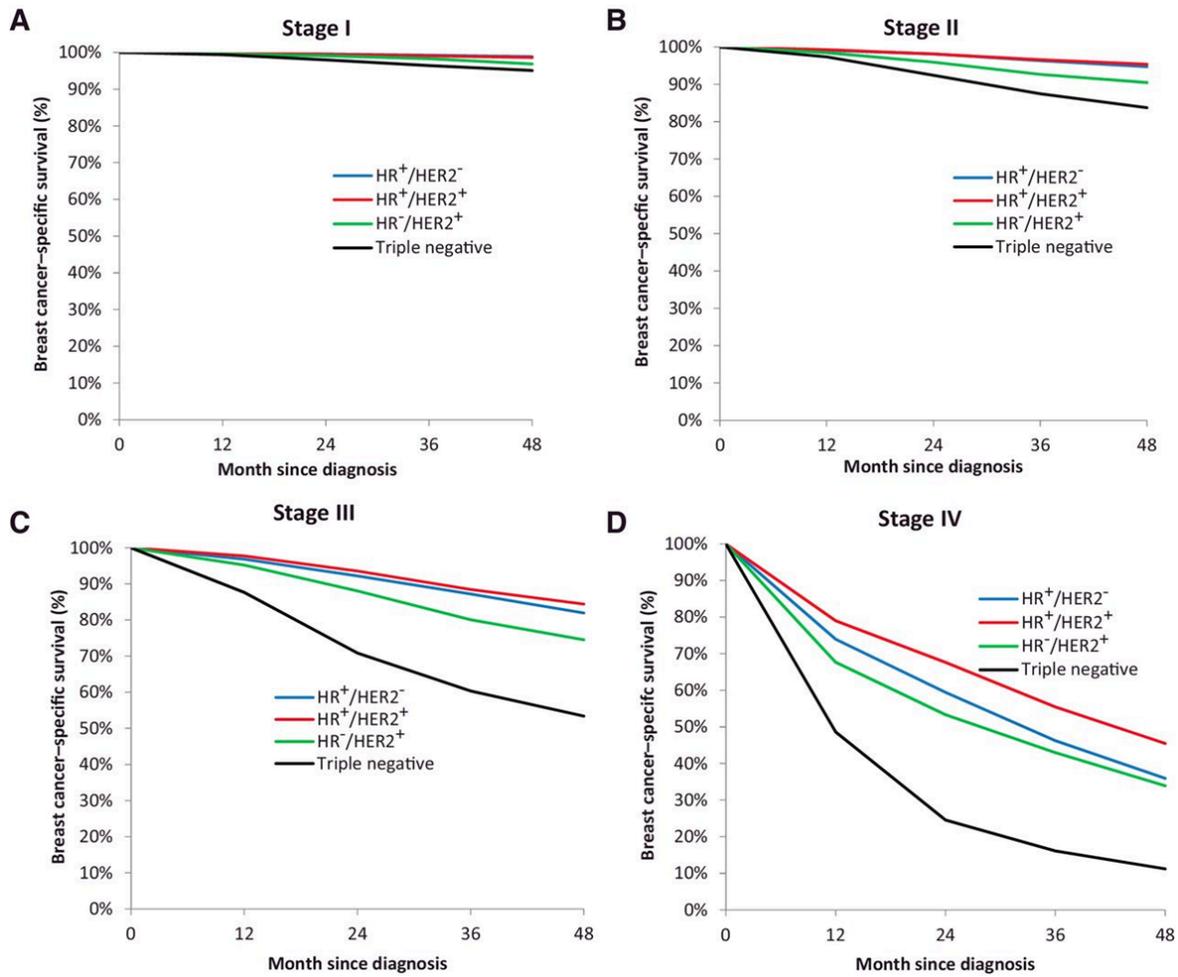


Figure 1. Breast cancer subtype is often associated with survival outcome depending on the feasibility of treatment that corresponds with the receptor the tumor receives growth signaling from, especially seen in stage four cancers as shown

The estrogen receptor is a ligand-inducible intracellular transcription factor that causes ER+ breast cancers to grow in response to estrogen. When the ligand binds to the estrogen receptor, the estrogen receptor acts as a transcription factor and binds to estrogen response elements in DNA. The predominant site for activation is the LBD dimer, seen in **Figure 2**, and is composed of five domains: domains A/B transactivate transcription, domain C binds DNA, domain D is the hinge region that connects domains C and E, and domain E is the ligand binding cavity. The estrogen receptor is expressed in about 70% of breast cancer cases, so addressing the estrogen receptor is essential when considering breast cancer treatment and drug development. (Brzozowski AM, et al.).

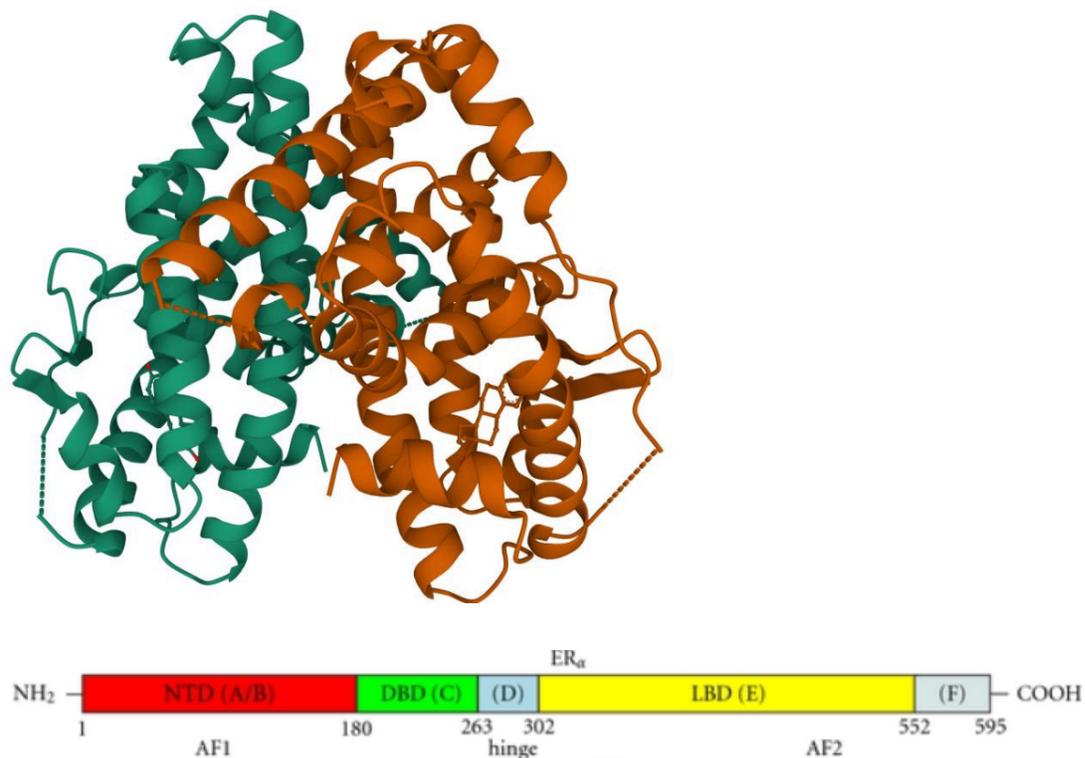


Figure 2. The crystalline structure of the LBD dimer, which is the predominant active site for the estrogen receptor.

Breast cancers that are positive for the estrogen receptor are not always responsive to traditional treatment, and can be susceptible for resistance against hormone therapy. This aspect is especially concerning given the prevalence of ER+ breast cancers among the rest, with ER+ breast cancers making up about 75% of all breast cancers, (Patel, et al.). In these cancer cells, various hormone therapies are designed to compete with estrogens to combat the proliferative effect that is elicited through the estrogen receptor. These antiestrogens can be classified as selective estrogen receptor modulators (SERMs), which act selectively against estrogen activity as opposed to other estrogen receptor agonists. Notably, hormone therapy drugs raloxifene and tamoxifen are popular SERMs against ER+ breast cancers. With the possibility of resistant estrogen receptors against these drugs, however, it is incredibly important to increase the amount of potential treatments and explore alternative, modified SERMs as a mechanism for treatment. The combination of resistance-prone receptors and the need for more treatment options in light of breast cancer still being the second-leading killer of women, the investigation of other estrogen receptor agonists becomes increasingly significant in advancing breast cancer treatment and research.

Testing the relative binding affinity and efficacy of SERMs like tamoxifen and raloxifene required the assessment of the fluorophore used for fluorescence polarization binding assays and the assessment of other estrogen receptor agonists. In previous research within Dr. Weatherman's research lab, BCPF (seen in **Figure 3**) was used as a

fluorophore. Despite its likeness to the estrogen receptor through its estradiol skeletal structure, the difficulty and time required to synthesize BCPF in the lab made its use and availability in research complicated. The choice of fluorophore was then changed to coumestrol, which is conversely much simpler in attainability and usability. Testing known compounds with coumestrol and comparing this data with prior data observed using BCPF would then answer whether this fluorophore would be useful for other breast cancer drug studies. Once it is confirmed that coumestrol is a viable option for testing, the second goal of further affinity testing with unknown compounds can be achieved.

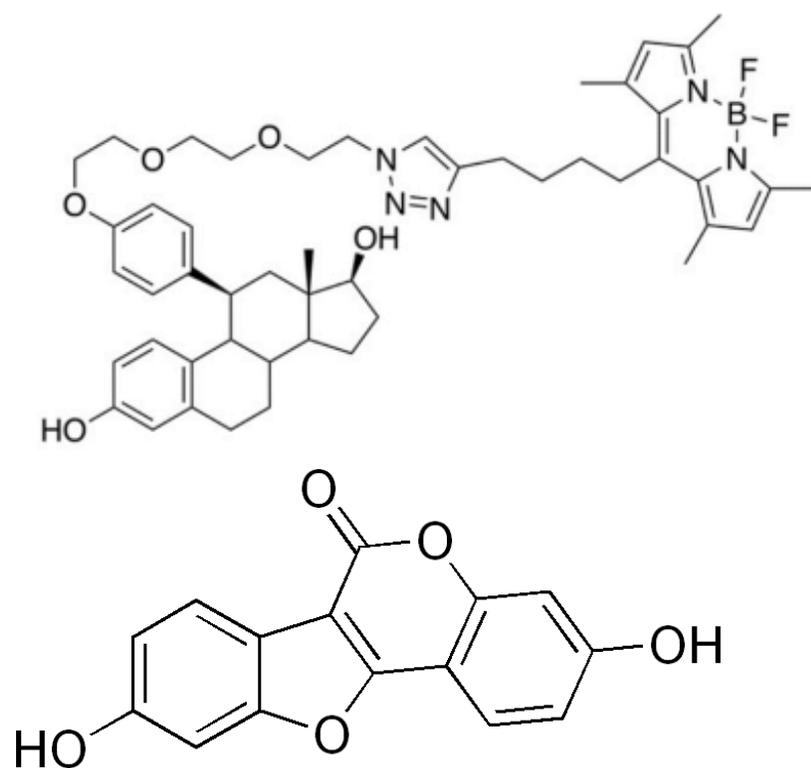


Figure 3. The structures of BCPF (top) and coumestrol (bottom)

Methods

Aim 1

The first aim of the research was to show that there was receptor binding which was achieved through affinity binding assays, keeping coumestrol concentrations constant and varying receptor protein concentration. 2.1 mL of Tris buffer, pH 7.4, 6 μL of 2 μM coumestrol, and 110 μL MBP-fusion ER-alpha LBD protein receptor were added to a tube. 220 μL , 10-fold serial dilutions were done from this first mixture for a total of 6 tubes. An additional 2 mL of Tris buffer and 2 μL of 10 μM coumestrol were added to diluted tubes. A mixture composed of just 2.1 mL of Tris buffer, pH 7.4, 6 μL of 2 μM coumestrol, and ethanol was used as a blank. This method was repeated twice to run the experiment in triplicate. After about 30 minutes, the polarization of the mixtures were measured using the polarimeter.

Another triplicate was made to test the specific binding of the fluorophore by adding 1 μL of raloxifene to a specific concentration of receptor.

Aim 2

The second aim of the research was to compare relative binding of breast cancer drugs through competitive binding assays. Into one tube (Tube A), 10 mL of Tris buffer, 20 μL of 10 mM coumestrol were added. Into another tube (Tube B), 10 mL of Tris buffer and 50 μL of receptor were added. Into 5 tubes, 1 μL of drug solution was added, in which

the concentration is 1000 times more concentrated than the final concentration. In a 6th tube, 1 uL of ethanol, rather than drug solution, was added. 0.5 mL of tube A and 0.5 mL of tube B were added into each of the six tubes. This set of 6 tubes was repeated twice more for a triplicate for a total of 18 tubes. Estradiol, raloxifene, tamoxifen, fulvestrant, OHT-6C, THCK, and BK-1 were all tested using the above protocol to generate relative binding affinities and affiliated IC_{50} values.

Results and Discussion

Varying Receptor Binding Assays

The binding assays varying receptor concentration and keeping fluorophore constant showed successful receptor binding. In the blank sample, polarization values were relatively low which indicates that there was a high amount of free coumestrol due to no receptor being present. The binding curve showed a gradual increase in polarization, seen in **Figure 4**, until a plateau was observed, indicating that coumestrol was progressively becoming more bound to the receptor. Because the concentration of the receptor was increasing, this allowed for more binding opportunities between it and coumestrol, explaining the increase in binding events until there was either no more coumestrol to bind.

The specific binding of coumestrol was confirmed with polarization data that showed similar values to that of the blank sample, also seen in **Figure 4**. Low polarization values in this case confirm that raloxifene is outcompeting the coumestrol for receptor binding events and therefore rotates more freely and emits a relatively-lower polarized light.

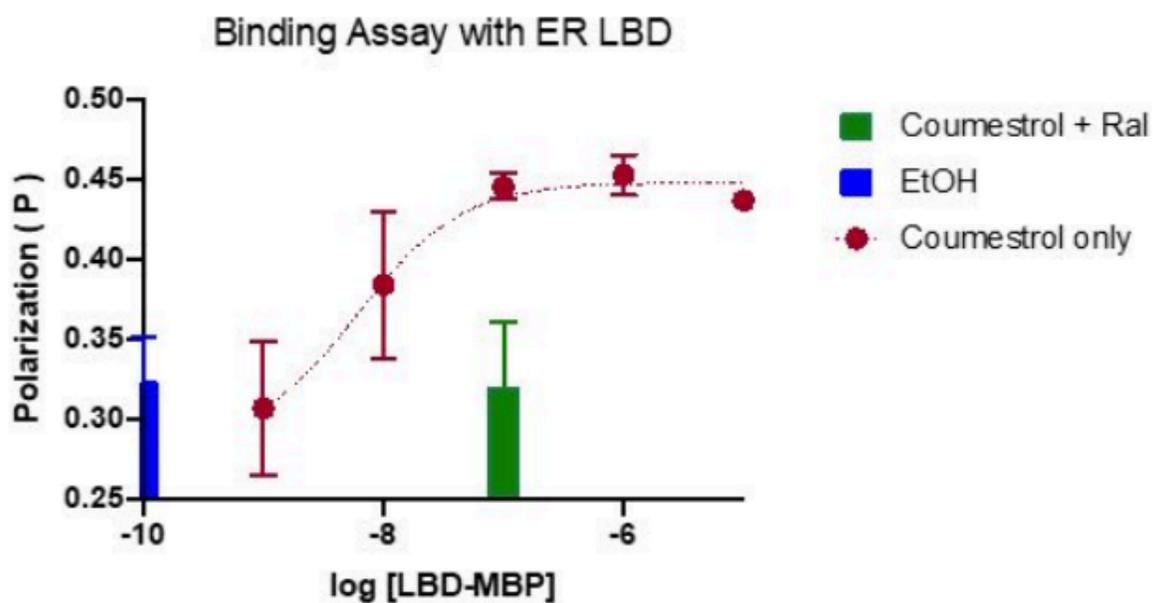


Figure 4. Receptor binding assay using ethanol as a blank, keeping coumestrol constant, and varying LBD-MBP receptor protein. Raloxifene was used as a secondary control to confirm specific binding

Competitive Binding Assays

In the competitive binding assays, all drugs showed the expected gradual decrease in polarization as drug concentration increased, as seen in the below figures. This general trend shows the given drug's ability to outcompete coumestrol for binding to the estrogen receptor. The relative binding affinities for all drugs were compared using their respective IC_{50} values, which indicates how much drug is needed to inhibit a biological process by half, thus providing a measure of potency of an antagonist drug. A viable drug candidate for further study should have an IC_{50} value of less than 10 μ M, with lower values preferred in general.

Raloxifene is a popular and useful selective estrogen receptor modulator and therefore tends to have a fairly low IC_{50} value and a reliable binding assay curve affiliated with it, as seen in **Figure 5**. As expected with the antagonistic behavior of raloxifene, a relative trend of decreasing polarization is observed as the drug concentration increases. The polarization is high for the blank sample and for the most dilute concentration of raloxifene, which confirms that there is not a lot of competition at such low concentrations of drug. The IC_{50} value that was calculated as an average of three trials is 20 nM \pm 10 nM.

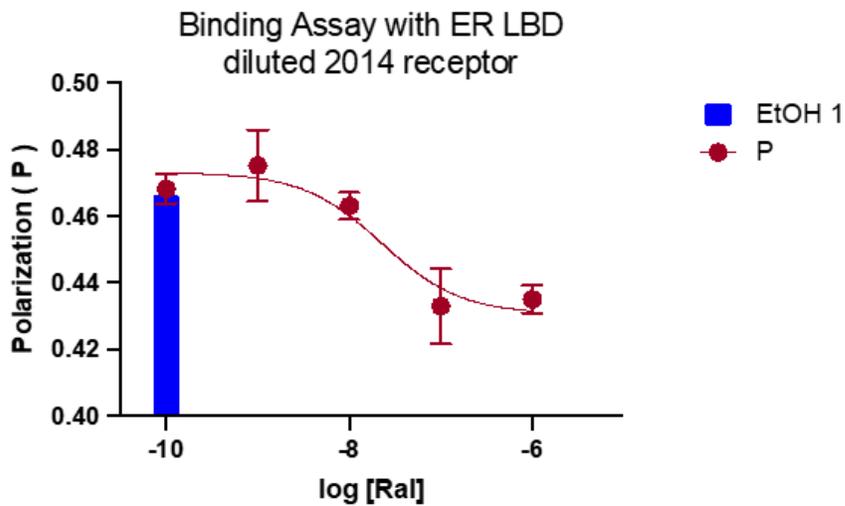


Figure 5. Competitive binding assay using raloxifene and ethanol as a negative control

Because estradiol is the ligand for the estrogen receptor, it also tends to generate very low IC_{50} values and a reliable binding assay curve. As concentrations of estradiol increase, it displaces the coumestrol from the estrogen receptor and therefore causes the decreasing polarization trend as seen in **Figure 6**. These trends are consistent among popular drugs, such as raloxifene as considered prior, as well as tamoxifen (**Figure 7**). The IC_{50} value generated from the average of three runs is $8 \text{ nM} \pm 4 \text{ nM}$.

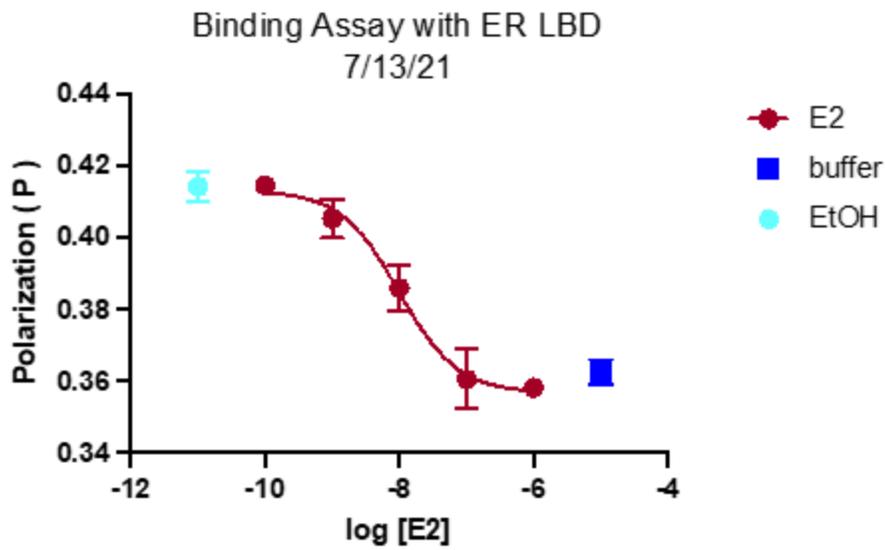


Figure 6. Competitive binding assay using estradiol and ethanol as a negative control

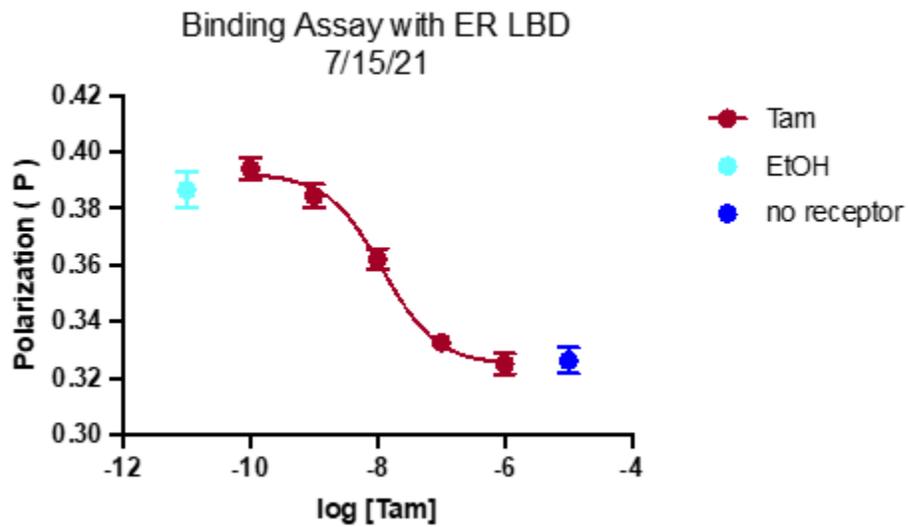


Figure 7. Competitive binding assay using tamoxifen and ethanol as a negative control

Fulvestrant, while it is a popular breast cancer drug, did not generate a binding curve that has as big of a difference between the polarization of the blank and the most concentrated sample of drug, which is seen in tamoxifen, raloxifene, and estradiol. A wider difference in polarization indicates greater differences in binding events and therefore a greater displacement to coumestrol from the estrogen receptor. Additionally, the data produced by the fulvestrant triplicates were not as precise as the other drugs, causing the IC_{50} value to stray from the nanomolar quantity that is historically seen with other assays and from what was seen with BCPF. The averaged IC_{50} value of fulvestrant is $400 \text{ nM} \pm 300 \text{ nM}$. The large error associated with fulvestrant is likely due to the solubility of the solution causing a disruption to the tumbling of the molecules, which then causes the results and data to be inaccurate. One of the binding assay curves that contributed to the IC_{50} value is seen in **Figure 8**.

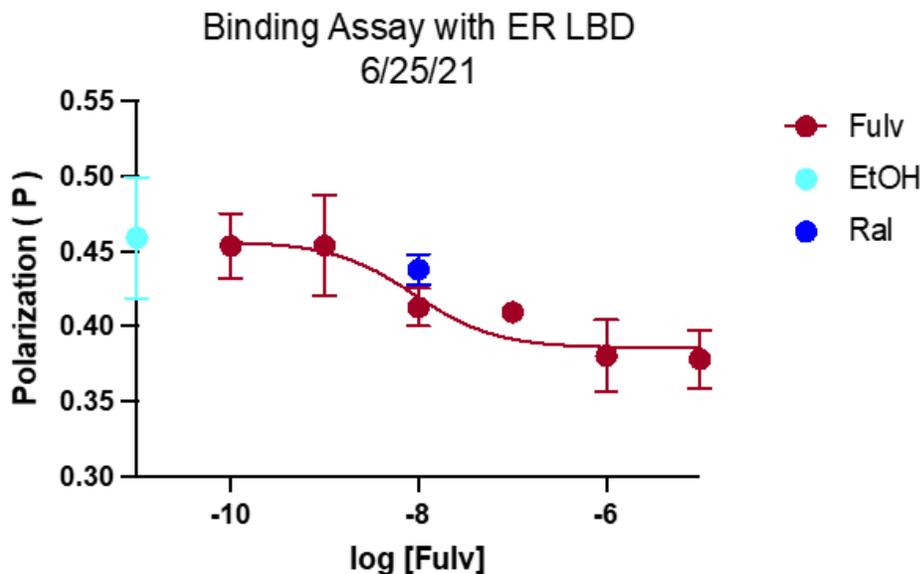


Figure 8. Competitive binding assay using fulvestrant and ethanol as a negative control

Because the known compounds generated mostly comparable data relative to what was generated using the past fluorophore, BCPF, the unknown compounds could then be tested to generate IC_{50} values and determine their potential in future breast cancer studies. THCK, BK-1, and OHT-6C all produced nanomolar IC_{50} values at $70 \text{ nM} \pm 10 \text{ nM}$, $100 \text{ nM} \pm 200 \text{ nM}$, and $30 \text{ nM} \pm 30 \text{ nM}$, respectively. These compounds were synthesized in the lab rather than bought, and therefore have an increased risk for polarization reading issues relating to solubility due to their unreliability as crude samples. The combined IC_{50} values for both the known and unknown samples can be seen in **Table 1** and **Table 2**.

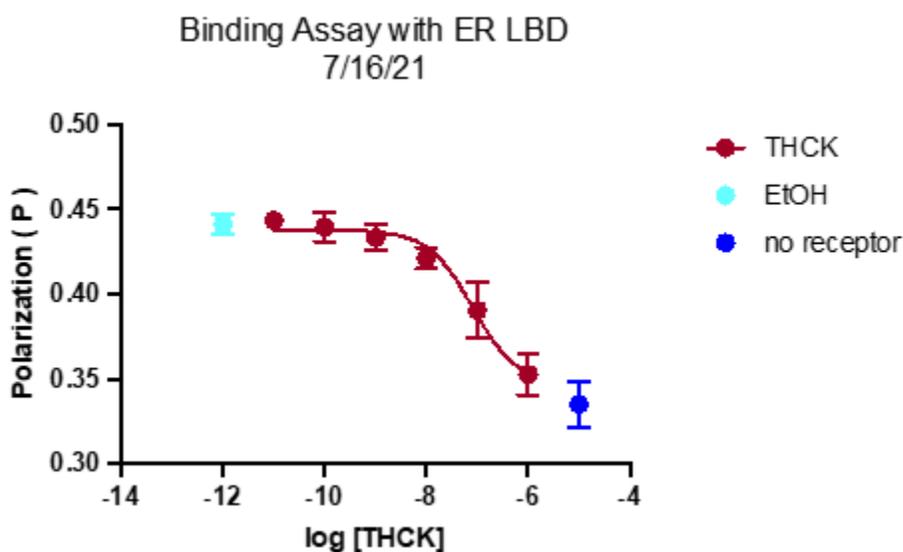


Figure 9. Competitive binding assay using THCK and ethanol as a negative control

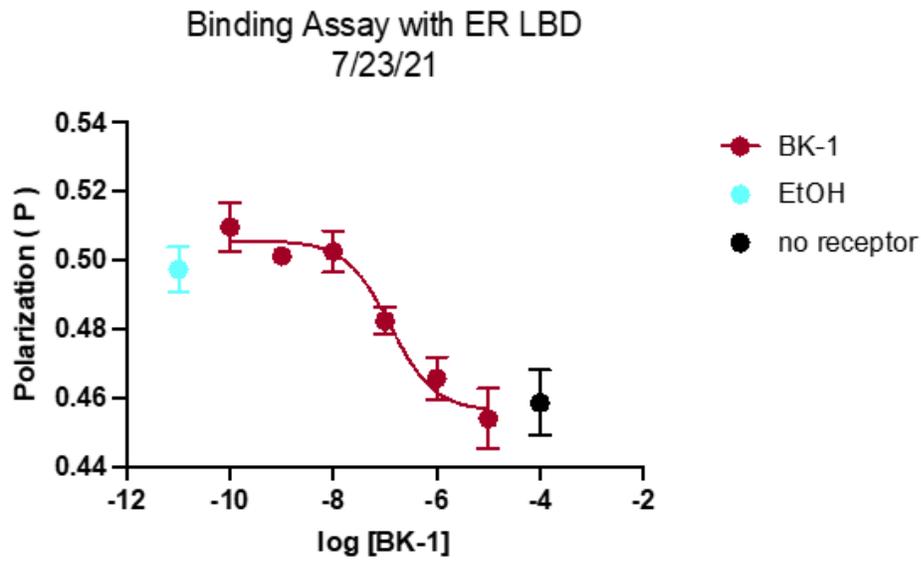


Figure 10. Competitive binding assay using BK-1 and ethanol as a negative control

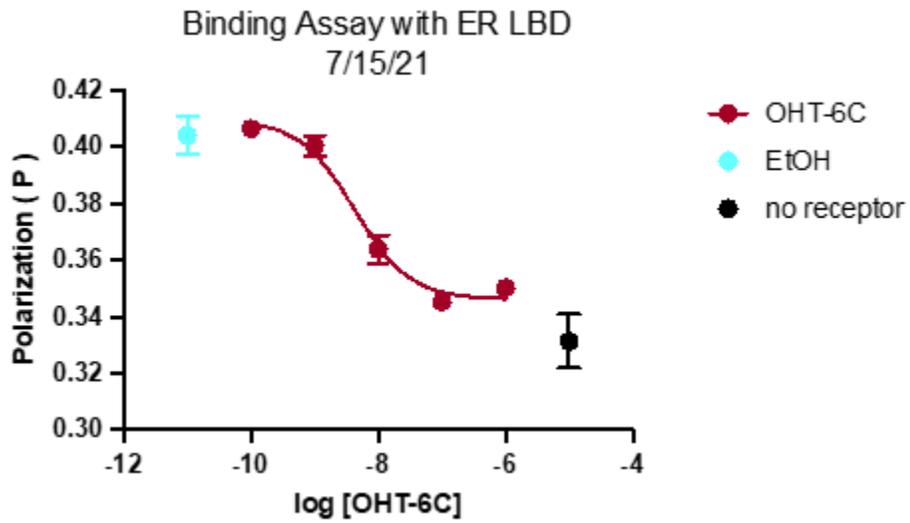


Figure 11. Competitive binding assay using OHT-6C and ethanol as a negative control

Table 1. The IC₅₀ values for the known compounds using coumestrol as the fluorophore

Drug	IC₅₀ (nM)	SE (± nM)
Estradiol	8	4
Raloxifene	20	10
Tamoxifen	50	30
Fulvestrant	400	300

Table 2. The IC₅₀ values for the unknown compounds using coumestrol as the fluorophore

Drug	IC₅₀ (nM)	SE (± nM)
OHT-6C	30	30
THCK	70	10
BK-1	100	200

Conclusions and Future Works

Ultimately, coumestrol has been found to be used effectively as a fluorophore. The binding assays done with coumestrol showed successful binding, both non-specifically and specifically using raloxifene as a competitor. When assessing the known compounds, this observation was further confirmed as the data generated IC₅₀ values that were comparable and in agreement with those found using the past fluorophore, BCPF, which was much more complicated to use due the time and skill needed to synthesize the steroidal, non-purchasable compound.

Additionally, fluorescence polarization binding assays indicate that the unknown compounds studied have a substantial binding affinity for the estrogen receptor ligand-binding domain. THCK, BK-1, and OHT-6C all generated IC₅₀ values that had nanomolar binding affinities, indicating their potential as future drug candidates in

cell-based assays. Additional binding assays could also be done to produce more accurate IC_{50} values for each of the unknown drugs that would further confirm their potential.

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