DEVELOPING CYTOTOXIC DRUGS THAT TARGET THE ESTROGEN RECEPTOR IN BREAST CANCER CELLS

by

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TARGET THE ESTROGEN RECEPTOR

IN BREAST CANCER CELLS

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ABSTRACT

Breast cancer is a growing problem in the United States and worldwide. It takes the lives of approximately 40,000 U.S. women a year. In the U.S., 1 in 8 women will develop breast cancer during the course of their lifetime and it continues to be the most commonly diagnosed cancer in women. Clearly, this is a serious issue that must be solved. Current chemotherapy treatments often result in widespread cell death, including the killing of healthy cells. Therefore, it is necessary to find alternative treatments that specifically target cancer cells. Many breast cancer cells over express estrogen receptors, which are vital to the rapid cell division and growth of tumors. Estrogen is a steroid hormone that enters the cell, binds to its receptor, translocates to the nucleus, and leads to gene expression. Previous work from our group has resulted in the development of a drug called Est-3-Melex that targets estrogen receptor-positive breast cancer. The drug contains a DNA methylating group (Melex) conjugated to estrogen. The drug acts by the binding of the estrogen portion of the molecule to its receptor that ultimately translocates to the nucleus. While in the nucleus, the Melex portion of the compound is brought in close proximity to the DNA and methylates the adenine base, eventually resulting in cell death. Essentially, this is a receptor targeted cancer therapy. In order to test the toxicity of this drug, we utilized a MTT cytotoxicity assay, which quantifies the amount of cell death. Est-3-Melex was more toxic to cancer cells that overexpressed the estrogen receptor compared to those that did not. Treating the estrogen receptor positive breast cancer cells with excess amounts of estrogen inhibited Est-3-Melex-induced cell death. Fluorescence imaging was also utilized to visualize localization of the drug. A fluorescent tag was attached to Est-3-Melex and introduced into estrogen receptor-positive breast cancer cells. The results showed the drug localized to the nucleus and this localization was inhibited by estrogen. Our results suggest that Est-3-Melex is

effective in specifically killing estrogen receptor positive breast cancer cells by binding to the estrogen receptor. Additional investigations are underway to identify the mechanism of cell death.

Introduction

Breast cancer is a serious problem throughout the world that takes many lives each year. Cancer is defined as a group of diseases characterized by uncontrolled cell growth eventually resulting in a tumor (American Cancer Society 2015). Specifically, breast cancer is the growth of the tumor often starting in the ducts of the breast and later spreading to other tissue within the breast, called invasive breast cancer (American Cancer Society 2015). Breast cancer takes the lives of over 40,000 U.S. women a year, with more than 3.1 million U.S. women with a history of breast cancer (American Cancer Society 2015). Additionally, 230,000 cases of invasive breast cancer are diagnosed every year (American Cancer Society 2015). These data indicate that breast cancer is clearly an issue that must be addressed and new treatments must be developed.

Whenever breast cancer is diagnosed in a patient, the subtype is characterized by receptor type, which is often overexpressed (American Cancer Society 2015). Many breast cancer cells overexpress the estrogen receptor, as the steroid hormone estrogen is necessary for many functions of the cancer cell. Due to the fact that so many breast cancer cells overexpress ERα, it is a common target of endocrine therapy that has resulted in a decrease in breast cancer mortality (Ao *et al.* 2011). Current treatments that inhibit the estrogen receptor are Tamoxifen and Fulvestrant (American Cancer Society 2015). Investigation into these drugs have shown they decrease proliferation in cell culture (Ao *et al.* 2011). Results may also suggest that Tamoxifen operates via an additional mechanism to the ERα inhibition because when the receptor was

knocked out, the drug still had a negative impact on the proliferation of the breast cancer cells; however, this mechanism is unclear. (Ao *et al.* 2011).

There are a multitude of treatments available to breast cancer patients that can often times give a better prognosis to the patient. However, there is no one treatment that is a cure-all for breast cancer. Instead, each patient has a personalized treatment plan and exact options should be assessed with physicians. The first option, and often the most radical, is surgery (American Cancer Society 2015). Surgery can either be performed as a mastectomy or a lumpectomy (breast conserving surgery) (American Cancer Society 2015). A mastectomy is removal of an entire affected breast, and more radical means involve the removal of surrounding lymph nodes and musculature (American Cancer Society 2015). However, a lumpectomy is the removal of only the breast tissue affected by the tumor, which is a much less invasive surgery (American Cancer Society 2015). Radiation therapy is performed often with surgery in order to prevent recurrence of the cancer. High-powered particles are projected at the site of the tumor to damage DNA in the cancer cell and result in cell death (American Cancer Society 2015). Chemotherapy is a class of drugs used in many cancers that target specifically rapidly dividing cells such as cancer cells but can also include normal cells such as hair cells (American Cancer Society 2015). Endocrine therapy drugs such as the previously stated Tamoxifen and Fulvestrant deprive the cell of estrogen while aromatase inhibitors decrease the conversion of testosterone to estrogen so less of the hormone can be utilized in the cell (American Cancer Society 2015). Lastly, targeted therapy is a form of treatment that targets specific attributes of the breast cancer cell. For example, Herceptin targets the HER2 receptor in breast cancer cells that is needed for hyperplasia and hypertrophy of the tumor (American Cancer Society 2015). Although there are a multitude of

treatment options available for patients, there is much work to be done in developing drugs that prevent mortality and recurrence.

Estrogen receptor positive breast cancers have been shown to be subjects of targeted therapies. A group has shown that a mango seed extract has the ability to induce the expression of pro-apoptotic proteins such as BCL-2 and caspases (Abdullah *et al.* 2015). This was likely caused by oxidative stress from the extract and this cell death was induced in a dose-dependent manner (Abdullah *et al.* 2015). Another project has shown that Tamoxifen, a known breast cancer drug, has had the ability to decrease the proliferation of Estrogen receptor-positive breast cancer cells (Ao *et al.* 2011). Also, the same group has shown that even when the ER is knocked down, the Tamoxifen still has a negative impact on the growth and proliferation of the cells; however, the mechanism is not clear. (Ao *et al.* 2011). Aromatase inhibitors, inhibitors of the enzyme that converts androgens to estrogens, have also been effective in treating in Estrogen receptor-positive breast cancers. An additional target that can be found in breast cancer cells is the Keratin-1 receptor (Soudy *et al*. 2017). Studies have shown that when an antimicrobial drug is conjugated to the Keratin-1 receptor, the drug is able to effectively enter the cells and initiate apoptosis and induce dose-dependent cell death (Soudy *et al.* 2017).

DNA methylation is a normal process involved in cells that results in gene silencing. This occurs in normal cells when a methyl group is added to the cytosine base on DNA to form 5-methylcytosine (Phillips 2008). The expression of PARP-1 gene, which is important in inducing apoptosis, is down regulated by DNA methylation in breast cancer cells (Kedar *et al.* 2012). This gene silencing renders the breast cancer cell unable to induce apoptosis (Kedar *et al.* 2012). It has also been shown that higher levels of DNA methylation measured within breast cancer cells correlates with a higher likelihood of the tumors expressing Estradiol and progesterone receptors

that can be targets for drugs (Benevolenskaya *et al.* 2016). DNA methylation of an Adenine base activates proofreading and inhibits transcription and replication, resulting in cell death (Kishton *et al.* 2011).

Since breast cancer cells overexpress the estrogen receptor, we wanted to target this characteristic of the cell. Our collaborators have developed a drug (Figure 16) called Est-*n*-Melex that contains the steroid hormone Estradiol conjugated to a known DNA-alkylation agent called Melex, which selectively adds a methyl group to the adenine base on DNA (Kishton *et al.* 2011). Previous research from our group has shown that Est-3-Melex, a variant of the drug, selectively kills ER^+ breast cancer cells (Kishton *et al.* 2011). Also, Est-3-Melex has been shown to have a high binding affinity to the estrogen receptor (Kishton *et al.* 2011). The estradiol portion of the drug binds to the estrogen receptor in the cytoplasm. Next, the drug moves with the receptor into the nucleus where it is brought in close proximity to the DNA, as estrogen and its receptor act as a transcription factor. Then, the Melex portion of the drug is able to add a methyl group onto the N9 position of the Adenine on the DNA sequence. This results in DNA damage and a cascade of events that results in cell death (Kishton *et al.* 2011). Therefore, this drug is specific to cells that overexpress the estrogen receptor, such as ER^+ breast cancer cells. In theory, the drug should also compete with Tamoxifen, Fulvestrant, and estradiol in order to bind to the estrogen receptor. Previously in this study, the first version of the drug synthesized was Est-4-Melex. We have shown that this version of Est-*n*-Melex, Est-4-Melex, is able to selectively kill breast cancer cells. Also, we have shown that Netropsin, a drug that selectively binds to AT-rich regions of the DNA, inhibits the cytotoxicity of Est-4-Melex. The next step was to alter the carbon linker length of the drug to find the relative binding affinities to DNA and cytotoxic

effects of the variants on breast cancer cells. We have determined that Est-3-Melex has the optimal linker length to bind to the DNA and is the most cytotoxic variant to MCF-7 Cells.

The goal of my project was to confirm the specificity and mechanism of Est-3-Melex entry into the cell. Also, this project was carried out to assure that the drug is effective at killing breast cancer cells (MCF-7 cells).

Methods

Cell Culture

As part of testing the drug in cell culture, MCF-7 ER^+ breast cancer cells were used. MCF-7 cells were grown in DMEM (10% fetal bovine serum, 1% non-essential Amino Acids, 2mM Glutamine, 100 units of Penicillin, and 1.7 mM Streptomycin (Sigma-Aldrich)) in a 37° C, 5% CO_2, and humidity controlled incubator. The cells were split into a new flask at around 10% when the cells were confluent in the flask. When the cells were ready to be split, some cells could be used in performing an experiment. 293 HEK cells (Human Embryonic Kidney fibroblasts), which do not express the estrogen receptor, were also utilized in the study and were grown under the same conditions.

Drug Dilution

Drugs were dissolved in DMSO (dimethyl sulfoxide) and Estradiol was dissolved in 100% ethanol. Serial dilutions of Est-3-Melex, Melex, and Estradiol were all performed using serum free medium (SFM).

MTT Cytotoxicity Assay

A MTT Cytotoxicity Assay was utilized for each experiment to assess percent cell death. The assay is performed by placing around 5,000 cells in each well of a 96-well plate and the cells are allowed to grow for 24 hours. Next, the wells were treated with increasing concentrations of

Est-3-Melex, Estradiol, or Melex, depending on the experiment. The cells were exposed to drug for 24 hours. After this, the medium was extracted from each well and 100 μL MTT (12 mM) was added to each well. MTT is a compound that enters the mitochondria of living cells and releases a purple dye. Therefore, the more dye is released, the more living cells are presumed to be in that well. MTT is dissolved in SFM. MTT remains in all wells but one (control well) for four hours. After MTT is removed from the wells, 100 μL DMSO was then added to dissolve the MTT precipitate. The absorbance can be quantified using a spectrophotometer at 540nm. The relative amount of dye released is compared to the control and the results are displayed numerically using Omega software.

Fluorescent Microscopy

In order to visualize the location of the drug within the cell, fluorescence microscopy was used. Initially, 1M HCl pre-treated coverslips were placed in each well of a six-well plate. Approximately 10,000 cells (500 μL) were placed on the coverslip in DMEM medium and the plate was placed in the incubator for thirty minutes. After the cells were able to settle at the bottom, 3.5 mL of DMEM medium was added to each well. The cells were allowed to grow in the incubator for 24 hours. Each well was treated with drug, Estradiol, or Fulvestrant depending on the treatment. The drugs were in each well with the cells for 24 hours in an incubator. Medium was removed from each well. Each coverslip was washed 500 μL PBS. 500μL paraformaldehyde was added to each well and the plate sat in the incubator for thirty minutes. The paraformaldehyde was removed and another wash was performed with 500 μL PBS in each well. The coverslips were then mounted onto slides. One drop of fluoromount was placed on the slide. The side of the coverslip with cells was placed face down on the slide on top of the fluoromount so that the liquid spreads across the surface of the coverslip. The coverslip was then

attached to the slide using acrylic nail polish around the edges. After the nail polish has dried, the slide was viewed under the fluorescent microscope using GFP wavelength.

Statistics

Statistical analyses of the MTT cytotoxicity data were performed using Microsoft Excel. Data was plotted that was exported from Omega software of the spectrophotometer into Excel. The data table was split into the different treatments of the experiment. Percentage cell survival was calculated in each of the four replicates and the averages and standard deviations were derived from these. This yielded the results to be graphed. The table was graphed based on percent control (y-axis) vs. concentration (x-axis). Upon plotting the data, a trend line was formulated for each treatment. The r^2 value and trend line equation were calculated. The r^2 value was utilized to measure the fit of the line to the data points. A r^2 value above 0.9 indicates a strongly correlated trend line. The trend line equation was utilized to calculate the EC_{50} value for each treatment. EC_{50} is the concentration of the drug necessary to kill 50% of the cells in the population (at 50 % control). EC_{50} values are measured in μM.

Results

Comparing the Cytotoxicity of Est-3-Melex and Melex

In order to test if the drug was more specific to breast cancer cells than Melex itself, the cytotoxic effects were compared. The initial experiment sought to determine whether Est-3-Melex, acting via the estrogen receptor, was more effective at killing MCF-7 breast cancer cells than the non-specific DNA damaging agent Melex, which diffuses into the nucleus randomly. Our hypothesis was that the Est-3-Melex would be more effective at killing the Estrogen receptor positive MCF-7 breast cancer cells. The results show that when treated with increasing concentrations of Est-3-Melex, more cell death was seen in MCF-7 cells in comparison to Melex.

This was shown by the lower levels of cell survival overall and lower EC_{50} values for Est-3-Melex (EC_{50} Est-3-melex, 58 μM compared to EC_{50} Melex, 106.5 μM).

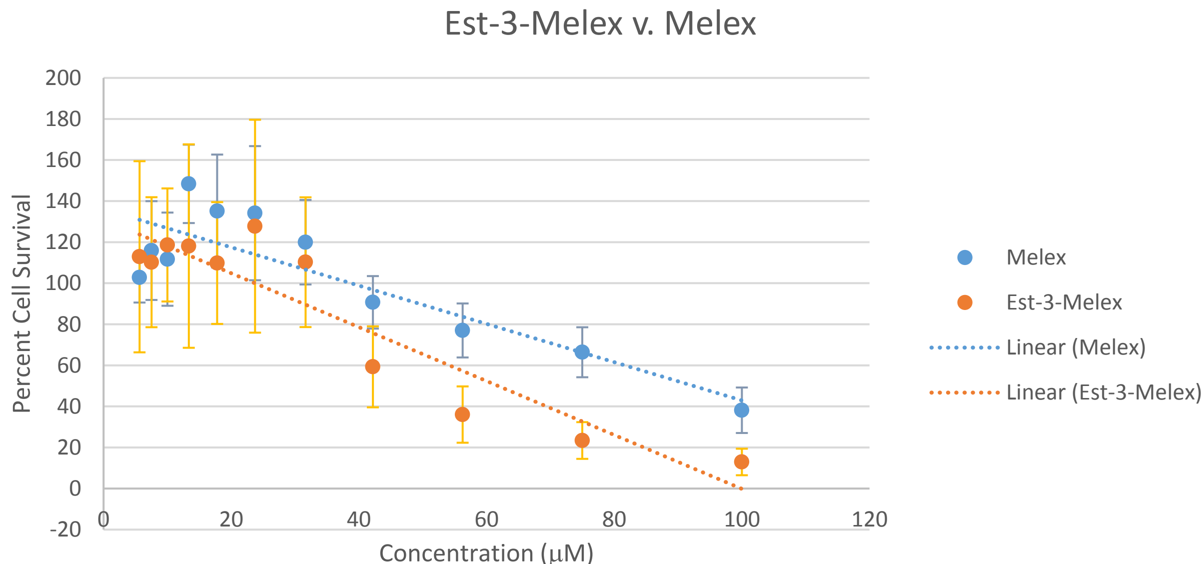

Figure 1. Effect of Est-3-Melex and Melex dissolved in SFM in MCF-7 cells

<u>Testing the effect of solvent on cytotoxicity of Est-3-Melex and Melex</u>

To determine if the solvent has an impact on the cytotoxicity of the compound, an experiment was performed in MCF-7 cells testing the cytotoxicity of Est-3-Melex and Melex diluted in either DMSO or Serum Free Medium (SFM). We predicted that the DMSO treatment would intensify the cytotoxic effects of the drugs whereas SFM would not. When using DMSO as a solvent, Melex and Est-3-Melex induced similar levels of cell death with increasing drug concentration. In fact, Est-3-Melex (EC_{50} = 35 μM) had a slightly higher EC_{50} value than Melex (EC_{50} = 31 μM) in this experiment, contrary to our predictions. In cells treated with drug dissolved in SFM, there was more cell death seen in cells treated with Est-3-Melex in comparison to cells treated with Melex, which was expected. The EC_{50} value of Est-3-Melex (EC_{50} = 38.5 μM) was lower than the EC_{50} value of Melex (EC_{50} = 67.5 μM).

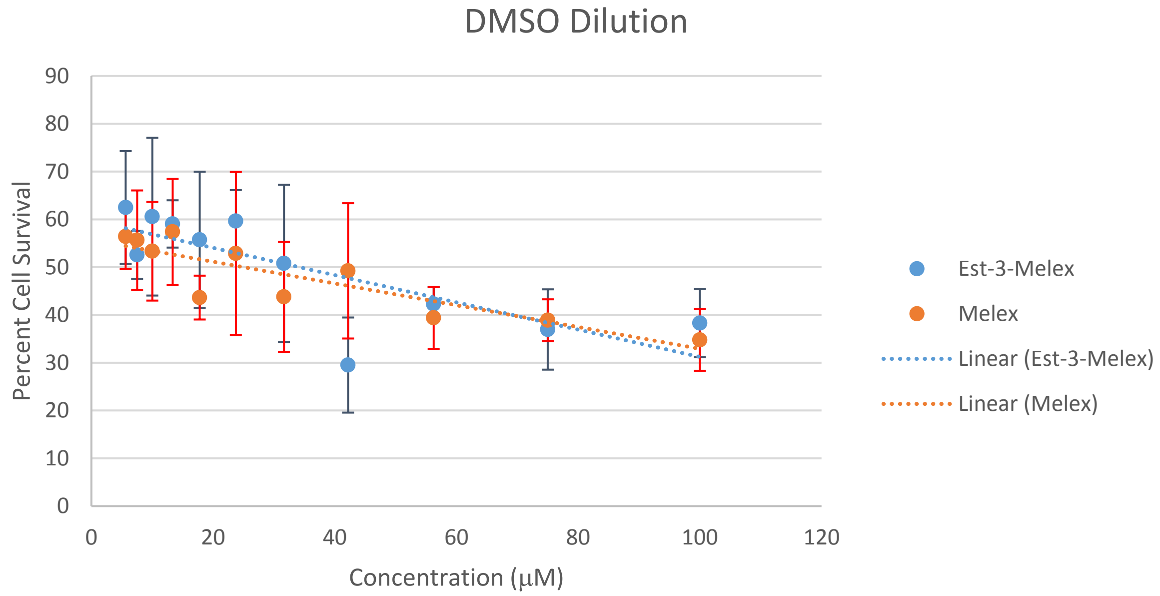

Figure 2. Effect of Est-3-Melex and Melex diluted in DMSO in MCF-7 cells

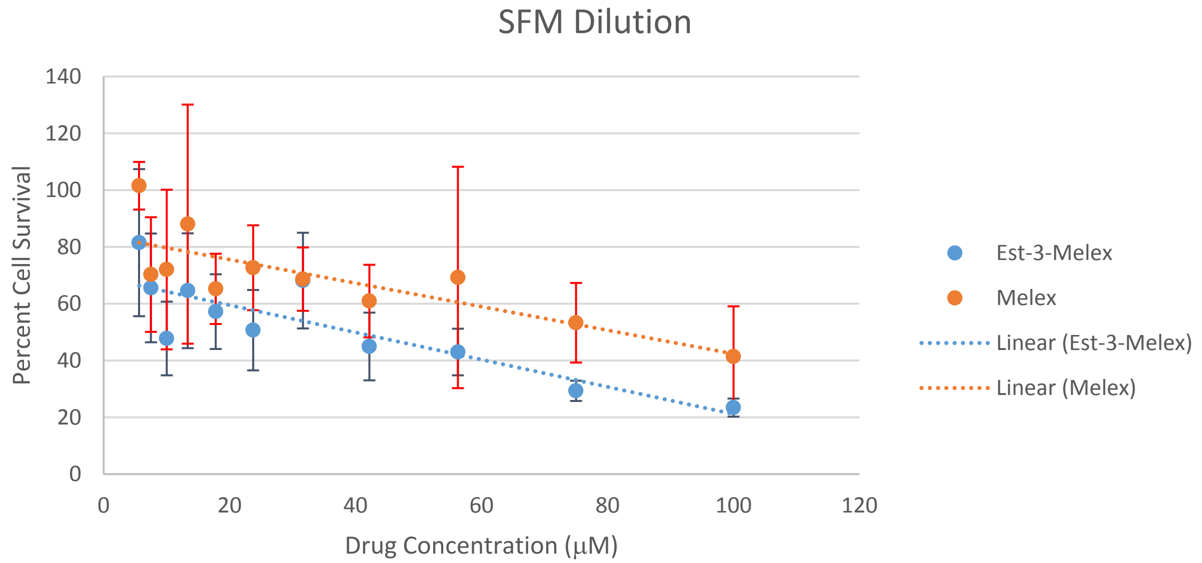

Figure 3. Effect of Est-3-Melex and Melex diluted in SFM in MCF-7 cells

Comparing the cytotoxic effects of Est-3-Melex and Melex on MCF-7 and 293HEK Cells

In order to test the specificity of the drug for cells expressing the estrogen receptor, we next compared the effectiveness in breast cancer cells that contain the estrogen receptor (MCF-7 cells) compared to non-breast cancer cells (293 HEK) that do not express the estrogen receptor. We predicted that Est-3-Melex would be more effective at killing MCF-7 cells due to the exploitation of the estrogen receptor while Melex would result in similar levels of cell death between the cell lines because Melex move into the nucleus in both cell types in a non-specific manner. In the 293 HEK treatment, the Melex and Est-3-Melex had similar rates of cell death as concentration of the drugs increased. Est-3-Melex had an EC_{50} = 68.8 µM while Melex had an EC_{50} = 42.8 µM. In the MCF-7 treatment group, more cell death was seen in the Est-3-Melex treated cells when compared to cells treated with Melex. Est-3-Melex treated cells (EC_{50} = 38.5 µM) had a lower EC_{50} value than Melex (EC_{50} = 67.5 µM) in this treatment. Similar results were obtained when the experiment was repeated.

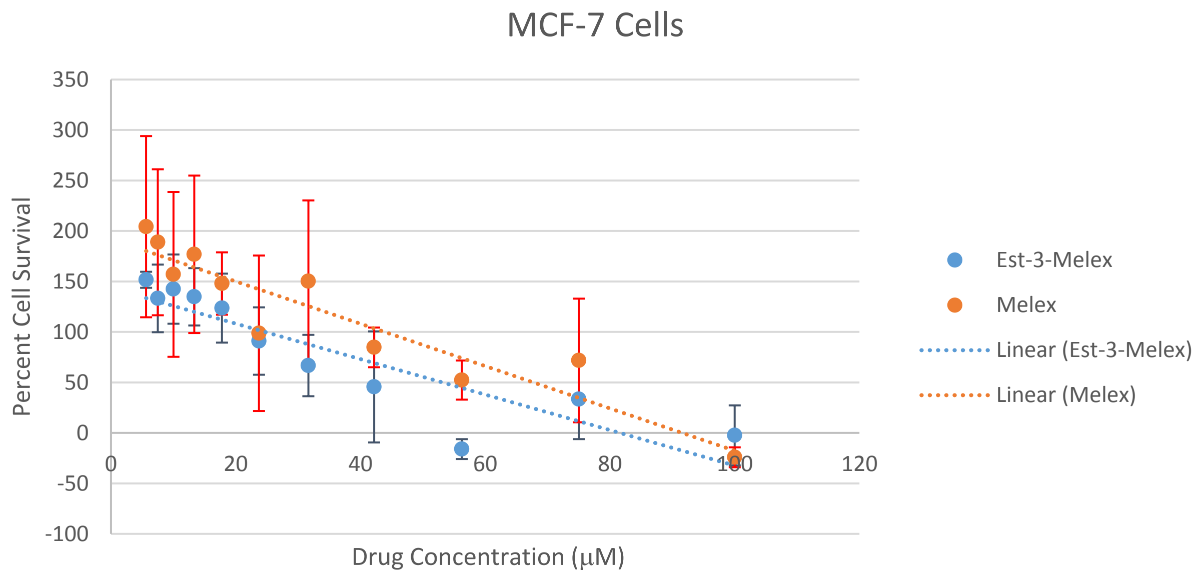

Figure 4. Effect of Est-3-Melex and Melex MCF-7 cells

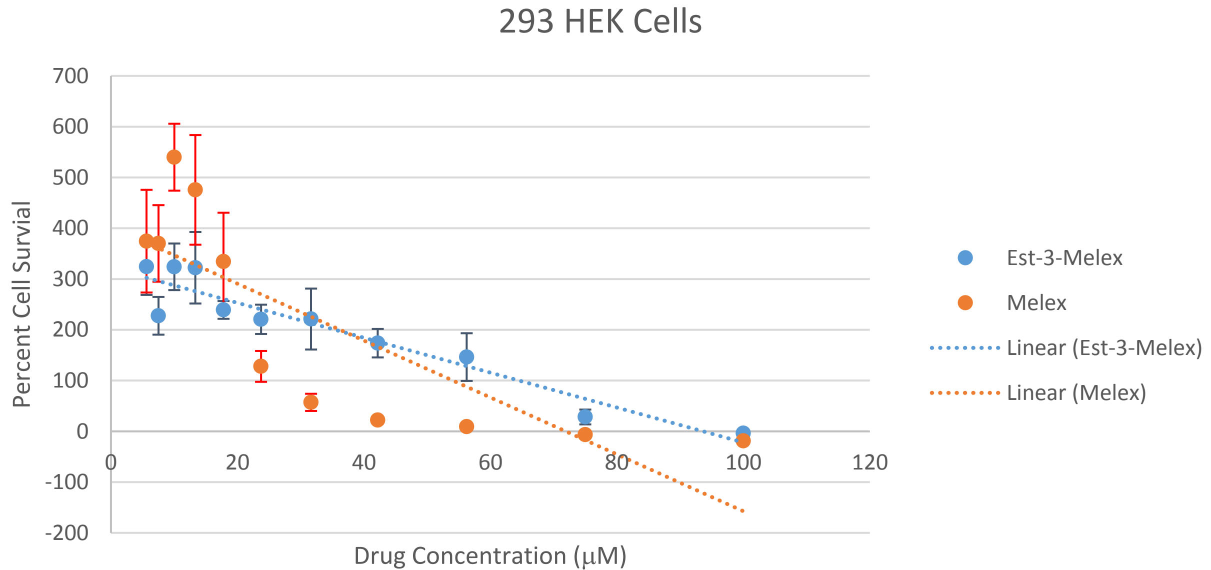

Figure 5. Effect of Est-3-Melex and Melex 293HEK cells

<u>Comparing the effect of Estradiol on the cytotoxicity of Est-3-Melex and Melex</u>

The purpose of this experiment was to test whether the drug binds to the estrogen receptor and translocates to the nucleus. Estradiol, the hormone that binds to this receptor, should compete with Est-3-Melex and inhibit its cytotoxic action. In order to confirm this prediction this Estradiol was used as competing ligand to the estrogen receptor. This was done by pre-treating the cells with estradiol and after thirty minutes the cells were treated with drug. If Estradiol competes with the drug for the receptor, this would confirm that the drug binds to the estrogen receptor before translocating to the nucleus. Instead of increasing the concentration of the drug, we instead increased the concentration of Estradiol while treating the cells with a constant level of Est-3-Melex (the EC_{50} value obtained from previous experiments). The cells were pre-treated for 30 minutes with Estradiol before the addition of Est-3-Melex. We would predict that with increasing concentrations of estradiol, more of the the receptor would be made unavailable, so less Est-3-Melex would bind to the estrogen receptor. Therefore, we would predict that with

increasing Estradiol concentration, more cells would survive. In this experiment, as the concentration of the estradiol increases with constant EC_{50} Est-3-Melex, the cell survival actually increased. The EC_{50} value of Est-3-Melex with Estradiol was negative (EC_{50} = -12.6 µM) because cell survival increased rather than decreased. Also, when the cells were treated with increasing concentration of Estradiol and constant concentration of Melex, the cell survival was not very affected by increasing Estradiol concentration. Similar results were obtained when the experiment was repeated.

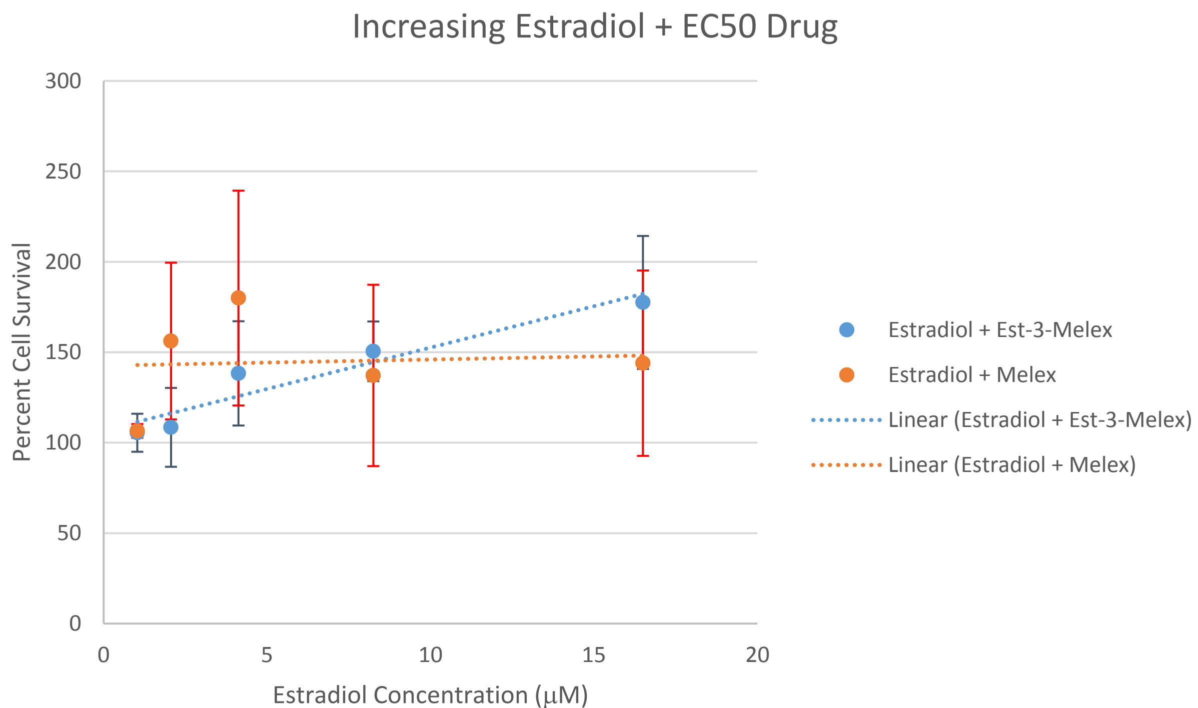

Figure 6. Effect of Increasing Estradiol with EC_{50} Est-3-Melex and Melex in MCF-7 Cells

<u>Testing the effects of increasing Est-3-Melex on cells pre-treated with a fixed concentration of Estradiol</u>

In this experiment, we kept a constant EC_{50} level Estradiol concentration while increasing Est-3-Melex concentration. This was done in order to test the effectiveness of Estradiol at different concentrations of Est-3-Melex. The prediction would be that in the presence of

Estradiol, Est-3-Melex would be less cytotoxic than in cells treated with Est-3-Melex alone. With increasing Est-3-Melex concentration, we predicted more cell death would occur because it is outcompeting the Estradiol for its receptor. Cells treated with increasing Est-3-Melex alone resulted in increasing cell death. Also, cells treated with increasing Est-3-Melex with constant EC_{50} estradiol resulted in cell death with increasing concentration. However, this increased cell death was to a lesser extent when estradiol was present. This can be illustrated using the EC_{50} values of the two treatments. The Est-3-Melex treatment alone (EC_{50}= 90.0) was lower than that of the Estradiol and Est-3-Melex treatment (EC_{50}=300.3).

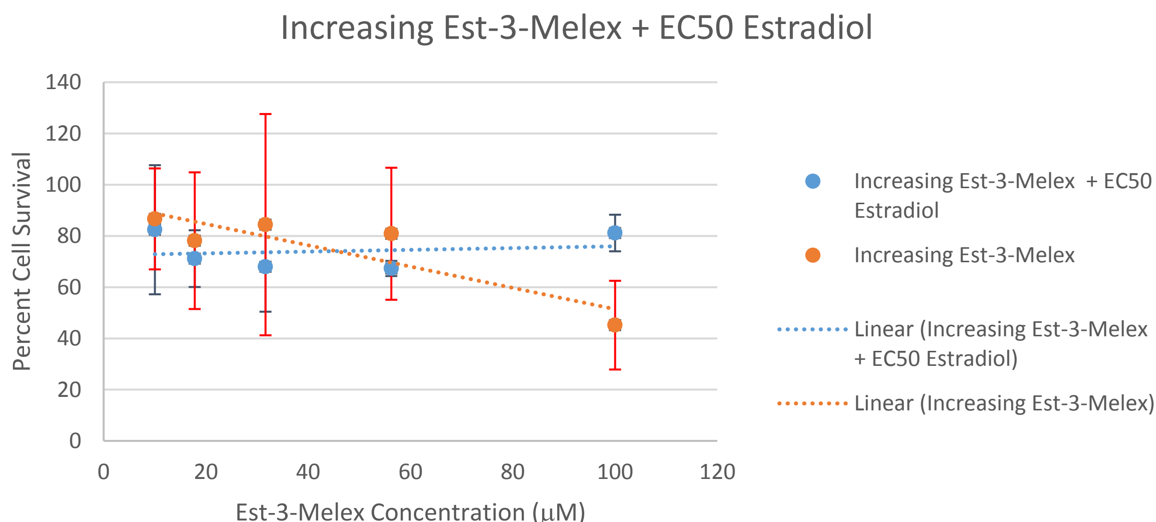

Figure 7. Effect of Increasing Est-3-Melex with EC_{50} Estradiol in MCF-7 Cells

<u>Observing the localization of Est-2-Melex-NBD and the Effects of Estradiol</u>

In order to determine if the drug moves into the nucleus, we looked at the localization of fluorescently labeled Est-3-Melex in the cell. This was done using the compound NBD-Py-Py-3C-Linker Estradiol (Figure 17). This compound allowed us to visualize where in the cell (cytoplasm or nucleus) the drug is located. MCF-7 cells were treated with either Est-2-Melex-NBD or Est-2-Melex-NBD and Estradiol. (Est-2-Melex-NBD was used instead of Est-3-Melex-

NBD in this experiment because this was the first time we had tested these fluorescent compounds and there was a limited amount available to use.) The purpose of this experiment was to determine if drug actually binds to the estrogen receptor and translocates to the nucleus. The predicted results were that in the Est-2-Melex-NBD only treatment, the drug should localize to the nucleus. However, when Estradiol is introduced by pre-treating the cells, this nuclear localization should be inhibited because Estradiol is preventing the drug from binding to the receptor and moving into the nucleus. The results showed that in the cells treated with Est-2-Melex-NBD, the drug moved into the nucleus because the fluorescence was seen diffused over the entire cell, leading us to believe that the drug was not only found in just the cytoplasm but also in the nucleus. In the cells pre-treated with Estradiol and then treated with Est-2-Melex-NBD, there was significantly more fluorescence seen in the cytoplasm than in the nucleus. This helped us conclude that the estradiol inhibits the nuclear localization of the Est-2-Melex-NBD.

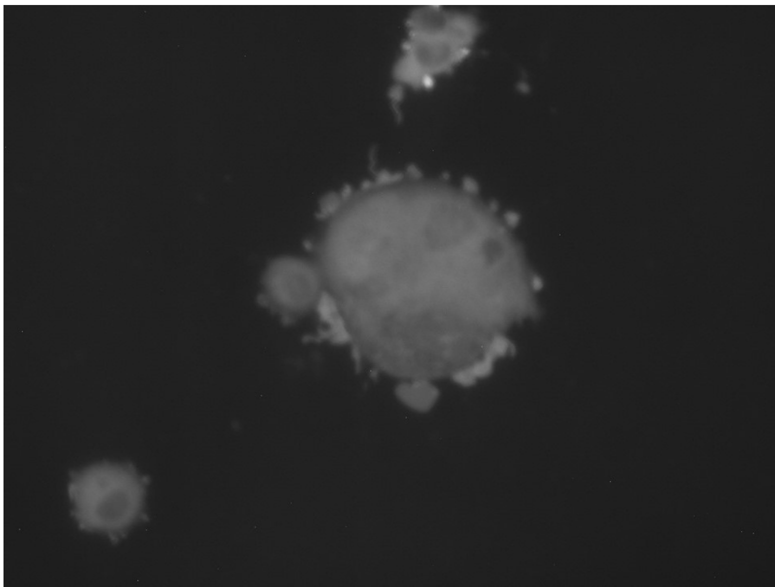

Figure 8. Est-2-Melex-NBD MCF-7 Cells

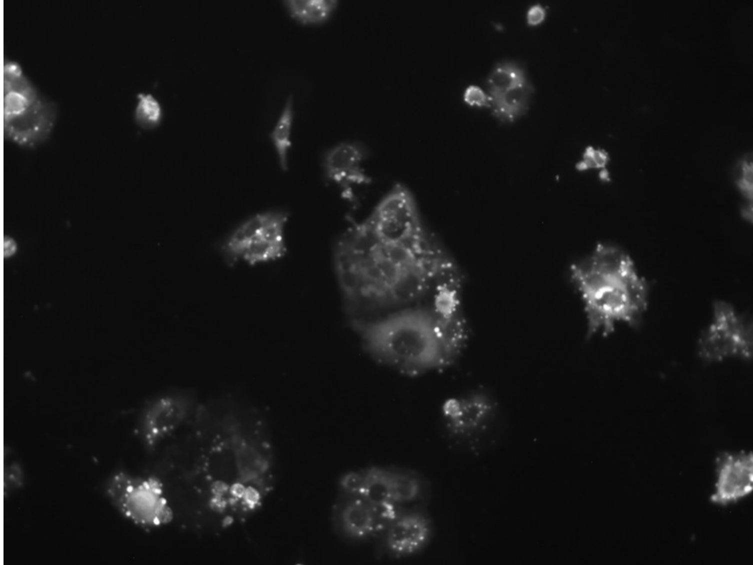

Figure 9. Est-2-Melex-NBD and Estradiol MCF-7 Cells

<u>Observing the localization of Est-3-Melex-NBD and the effect of Estradiol</u>

We wanted to confirm that Est-3-Melex actually localizes to the nucleus. In the next experiment, Est-3-Melex-NBD was utilized in treating MCF-7 cells. Cells were treated with Est-3-Melex-NDB only or Est-3-Melex-NBD pre-treated with Estradiol. The purpose of this experiment was to confirm if the drug binds to the estrogen receptor and translocates to the nucleus. The prediction was similar to the last experiment such that Est-3-Melex-NBD was expected to move into the nucleus and in Estradiol pre-treated cells, this nuclear localization should be inhibited. Estradiol was able to inhibit the nuclear localization of the drug. However, the cells pre-treated with Est-3-Melex only were very difficult to visualize using the microscope likely due to fluorescent bleaching. Thus, we were unable to determine the localization of Est-3-Melex in the cell.

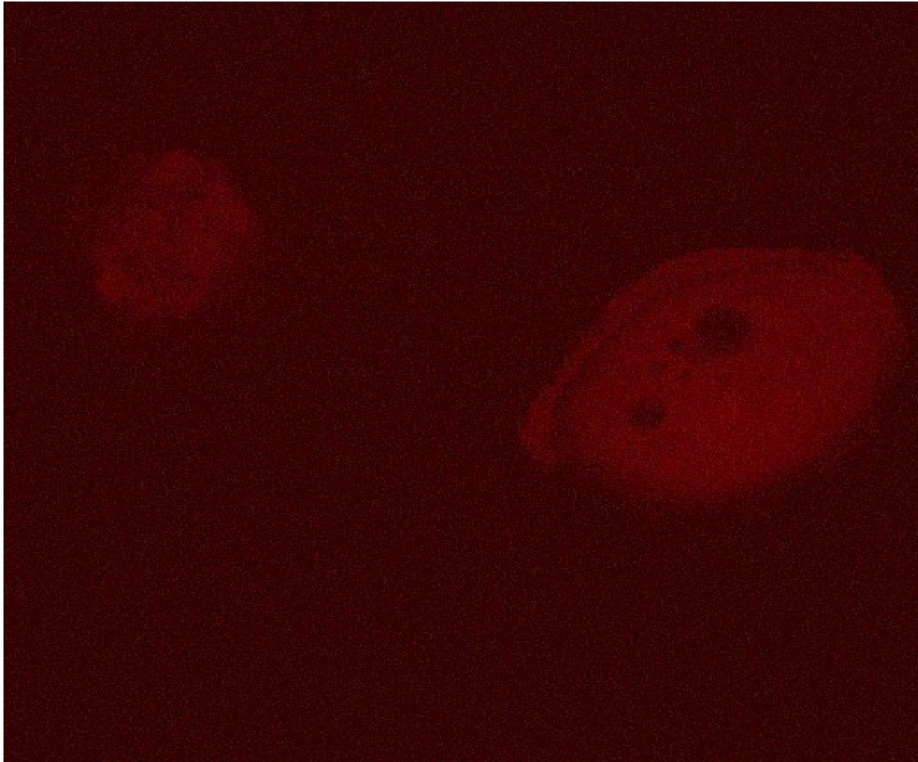

Figure 10. Est-3-Melex-NBD and Estradiol MCF-7 Cells

<u>Observing the effects of Fulvestrant and Estradiol on the localization of Est-3-Melex-NBD</u>

To test the ability of the drug to bind to the estrogen receptor and translocate to the nucleus, this pathway had to be inhibited. If the estrogen receptor is inhibited and the drug cannot enter the nucleus, then this helps us conclude that the receptor is necessary for nuclear localization. We pre-treated the cells with either Estradiol or Fulvestrant. Fulvestrant is a breast cancer drug used as an inhibitor of the estrogen receptor. So, we would expect the nuclear localization of the drug to be inhibited by both Fulvestrant and Estradiol. The purpose of this experiment was to confirm that Est-3-Melex binds to the estrogen receptor and translocates to the nucleus. Cells were treated with two concentrations of Est-3-Melex-NBD (5.63 μM and 21.36 μM) to minimize the high background fluorescence seen in previous experiments. The results showed that in the cells treated with the lower concentration (5.63 μM) of Est-3-Melex-NBD,

nuclear localization was inhibited by both Fulvestrant and Estradiol. In the cells treated with the higher concentration (21.36 μM) of Est-3-Melex-NBD, Fulvestrant and Estradiol pre-treatment inhibited the nuclear localization of the drug in many, but not all of the cells. When the experiment was repeated, cells treated with Est-3-Melex-NBD were included as a control. In cells treated with the lower concentration (5.63 μM) of Est-3-Melex-NBD, the drug did not seem to localize to the nucleus of the cells. Also, when pre-treated with Fulvestrant and Estradiol, nuclear localization did not occur. In the cells treated with Est-3-Melex at a higher concentration (21.36 μM), the drug seemed to be able to move into the nucleus and the pre-treatment of Estradiol and Fulvestrant was effective at blocking nuclear localization.

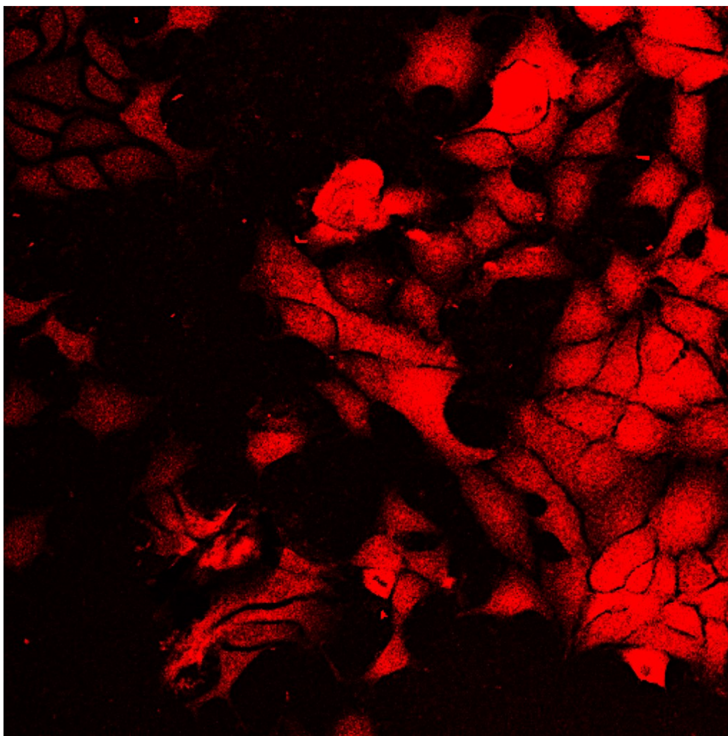

Figure 11. Est-3-Melex-NBD MCF-7 Cells

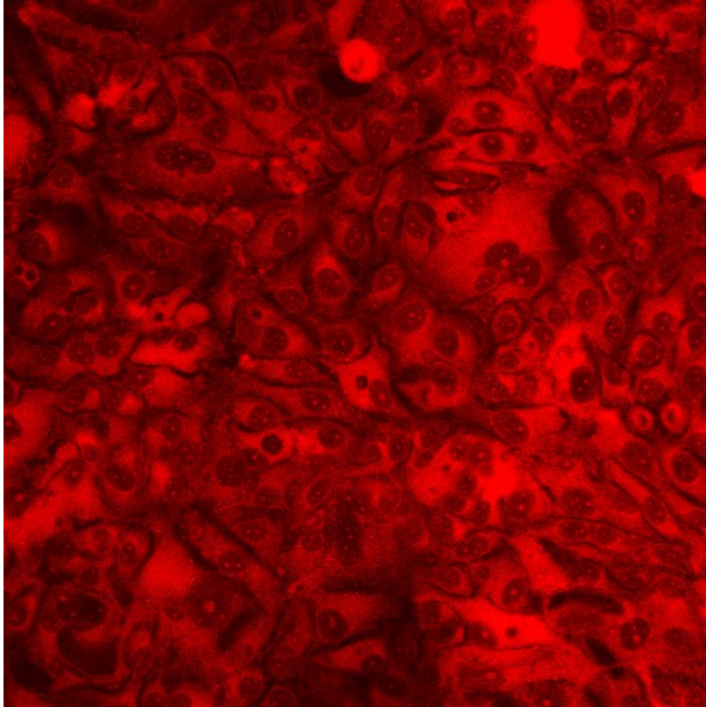

Figure 12. Est-3-Melex-NBD and Estradiol MCF-7 Cells

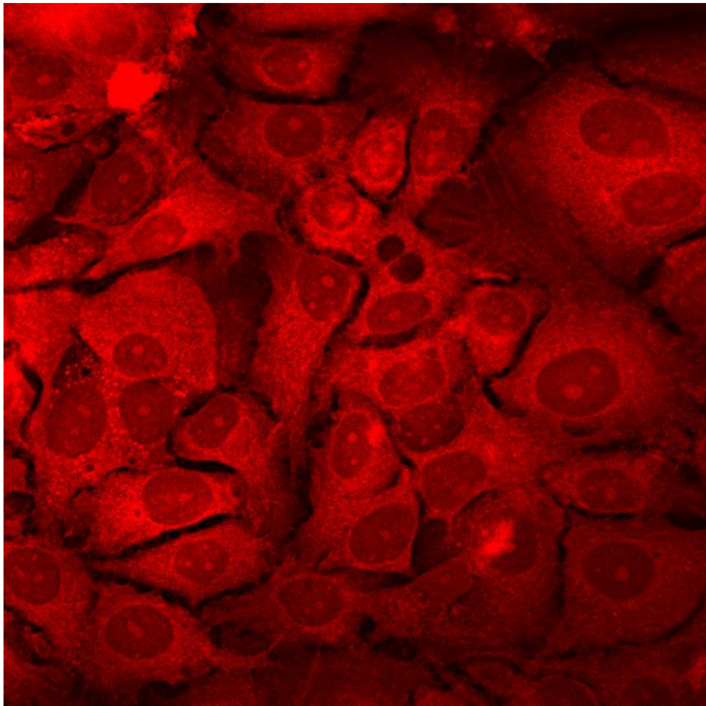

Figure 13. Est-3-Melex-NBD and Fulvestrant MCF-7 Cells

Observing the localization of Est-3-Melex-NBD in MCF-7 and 293HEK Cells

In the final experiment, the higher and lower concentrations of Est-3-Melex-NBD were used to visualize localization of the drug in the cell. 293HEK cells were also tested to observe localization in cells that do not express the estrogen receptor. Additionally, in this experiment, a higher and lower concentration of Estradiol was administered to visualize the impact different concentrations of the hormone would have on the outcome of the experiment. Fulvestrant was not used in this experiment. 293 HEK cells were used in addition to MCF-7 cells. 293 HEK cells are human embryonic kidney cells that are non-breast cancer cells and do not express the estrogen receptor. The purpose of this experiment was to determine if the drug requires the presence of the estrogen receptor to enter the nucleus and to confirm the results about Estradiol competition from the previous experiment. The prediction was that the nuclear localization should be inhibited by Estradiol in the MCF-7 cells and in 293 HEK cells, the nuclear localization should be minimal because of the lack of the estrogen receptor. In the cells treated with the low concentration (5.63 μM) of Est-3-Melex-NBD, the drug was able to move into the nucleus, as seen by the diffuse fluorescence seen throughout the MCF-7 cells. When cells were treated with the low concentration ((5.63 μM) and pre-treated with Estradiol, the nuclear localization was not inhibited, which was not expected. In the cells treated with the high concentration (21.36 μM) of Est-3-Melex-NBD, at high levels of estradiol, the nuclear localization was clearly inhibited as much more fluorescence could be seen in the cytoplasm than in the nucleus. In the 293 HEK cells, when treated with high concentrations of the drug, the nuclear localization was not present as seen by the lack of fluorescence in the nucleus and presence of fluorescence in the cytoplasm of the cells. Also, in the 293 HEK cells, the cells formed into triangle shaped clumps that was not observed in the MCF-7 cells.

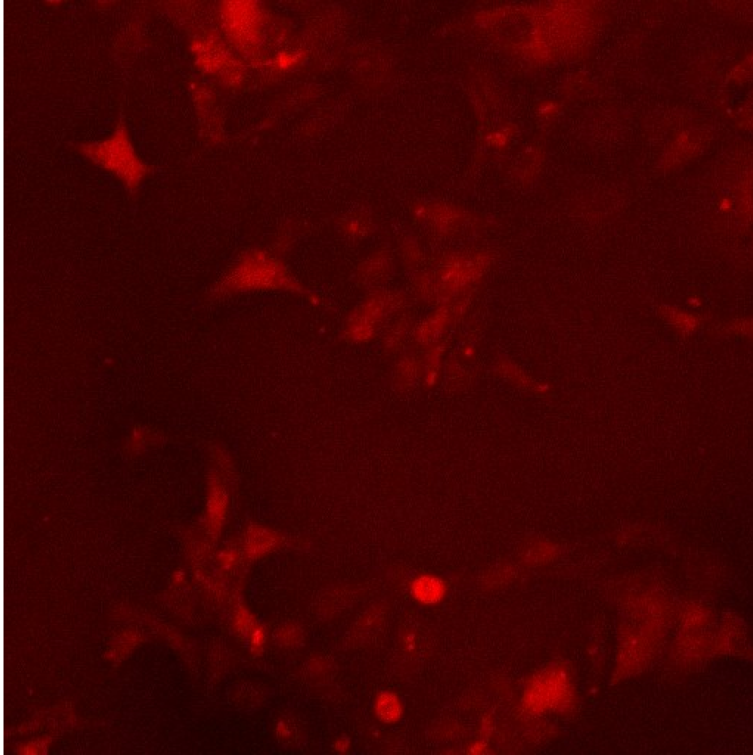
Figure 14. Est-3-Melex-NBD and Estradiol MCF-7Cells

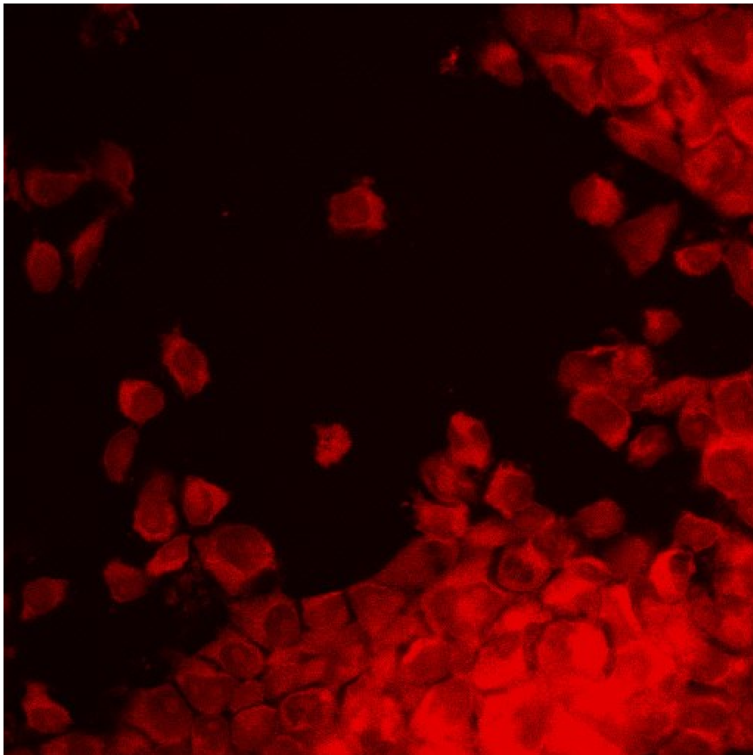

Figure 15. Est-3-Melex-NBD 293HEK Cells

Discussion

The goal of this study was to determine and confirm the proposed mechanism of the drug. This could be determined by confirming that the drug actually binds to the estrogen receptor and translocates to the nucleus. We also wanted to determine if the drug was actually effective at specifically killing ER^+ breast cancer cells. The first experiment was performed using MCF-7 cells treated with Est-3-Melex and Melex. The cell death induced by increasing concentration of the drugs was measured. At increasing concentration of both of the drugs, cell survival decreased, suggesting that the drugs were effective at methylating the DNA and killing the cells. However, there was more cell death seen in the Est-3-Melex treatment, indicating that it was more cytotoxic to specifically the breast cancer cells. Also, a lower EC_{50} value was observed when cells were treated with Est-3-Melex, which means that less of the drug was necessary to kill 50% of the cells. Therefore, the Est-3-Melex was more potent and more efficient at killing the breast cancer cells. This was likely due to the presence of the estrogen receptor in the target cell. This finding confirmed that Est-3-Melex is more effective at killing the cells than Melex alone presumably by binding to the Estrogen receptor and translocating to the nucleus.

In the experiment using drug diluted in DMSO or Serum Free Medium (SFM), it was found that the DMSO treatment altered the expected cell survival values of Est-3-Melex and Melex. DMSO may have interfered with our results because DMSO itself can kill cells (Camelot Cancer Care 2009). Thus, DMSO could be a confounding variable on our cell survival data. However, in the SFM treatment, cells treated with Est-3-Melex had lower cell survival values than cells treated with Melex, as expected. Since the dilution with SFM gave the expected results, it was determined to be a proper solvent to use in future experiments. This finding was likely due to the confounding variable of DMSO being toxic to cancer cells on its own (Camelot

Cancer Care 2009). By eliminating the DMSO variable in future experiments and using SFM to dilute the drugs, accurate results could be gathered.

The next experiment compared the cytotoxic effects of the drugs in MCF-7 cells and 293HEK cells. Since the MCF-7 cells overexpress the estrogen receptor while the 293HEK cells do not, the cell death induced by Est-3-Melex should be enhanced in MCF-7 cells. In the 293HEK cells, no significant differences were seen in the cytotoxicity induced by Est-3-Melex and Melex, indicating that Est-3-Melex was not more effective at killing these cells. Since the EC_{50} value was higher in cells treated with Est-3-Melex than those treated with Melex, this indicates that Est-3-Melex was less effective at killing the 293HEK cells than Melex. This could be because Melex is better able to diffuse into the nucleus because it is a smaller molecule than Est-3-Melex. In the MCF-7 cells, more cell death was seen in cells treated with Est-3-Melex than Melex, as expected. This can be attributed to the drug entering the nucleus via binding to the estrogen receptor. The conclusive finding from the experiment was that the Est-3-Melex is more effective at killing the Estrogen receptor positive MCF-7 cells than in the 293HEK cells. This was seen in the lower EC_{50} of Est-3-Melex in MCF-7 cells than in 293HEK cells. Melex resulted in similar cell death values for both cell lines. Since when the experiment was repeated, similar results were seen, this confirms our conclusions.

In the Estradiol competition experiment, the prediction that increasing Estradiol concentration would increase cell survival was confirmed as the cell survival actually increased with increasing concentration of Estradiol and constant EC_{50} Est-3-Melex. This suggests that the drug is specific to the estrogen receptor because when it was inhibited with increasing amounts of Estradiol, there was an increase in the levels of cell survival. With increasing concentration of Estradiol with EC_{50} Melex, cell survival was not affected. This confirms that Melex does not

need the estrogen receptor to kill the cell. The conclusion from this experiment was that Est-3-Melex was specific to the estrogen receptor while Melex was not and Estradiol acted an inhibitor of the binding and action of Est-3-Melex. When the experiment was repeated again, the cell survival increased with increasing Estradiol concentration, confirming the results from the original experiment. We propose that exposure of the cells to estrogen activates estrogen sensitive genes which leads to proliferation of the cells in combination with the low ability of Melex to move into the nucleus.

Increasing Est-3-Melex while keeping a constant level of Estradiol resulted a change in cell survival values from cells treated with Est-3-Melex only. This meant that the Est-3-Melex may have been overpowering the inhibitory effects of the Estradiol and it was effective at killing the cells. There was more cell death seen in cells treated with Est-3-Melex alone (and a lower EC_{50} value) suggesting that while Est-3-Melex and Estradiol still kills cells, it is not as effective as without Estradiol present. This confirms that there is still some inhibition of the receptor and mechanism of the drug when a constant level of Estradiol is present. Increasing Est-3-Melex treated cells increased cell death as expected and Estradiol interfered with this. This confirms that the drug is able to kill ER^+ breast cancer cells by specifically binding to the estrogen receptor.

The translocation of fluorescently labeled Est-*n*-melex (Est-2-Melex-NBD) into the nucleus was used to confirm the mechanism of the drug action. This experiment was used to determine if the drug can translocate to the nucleus as expected. In this experiment Est-2-Melex-NBD moved into the nucleus. Since fluorescence was seen in the nucleus of the cell, this confirms the mechanism of the drug. However, this was seen in Est-2-Melex-NDB, which may have different properties than our drug of interest, Est-3-Melex, due to the different carbon linker

length. When the cells were pre-treated with Estradiol, the drug was not able to move into the nucleus. This means that the drug selectively binds to the estrogen receptor and subsequently transports into the nucleus. Estradiol was able to inhibit the nuclear localization of Est-3-Melex-NBD as well. This shows that the drug binds selectively to the estrogen receptor. In cells treated with Est-3-Melex-NBD only, the nuclear localization was difficult to visualize under the microscope. This could have been because of the low concentration of the drug possibly treated in these wells or due to fluorescent bleaching, which happens when slides are observed under fluorescence for too long. To get around the problem of high background fluorescence, two lower concentrations of Est-3-Melex-NBD were used which allowed the drug to be visualized more clearly without the overpowering fluorescence in the background (5.63 μM and 21.36 μM). From the results it can be concluded that at a lower concentration (5.63 μM) Est-3-Melex-NBD entry into the nucleus was inhibited by compounds that bind to the estrogen receptor, which included Estradiol and Fulvestrant. This finding again confirmed that the Est-3-Melex-NBD localizes to the nucleus using the estrogen receptor. Additionally, in cells treated with the higher concentration (21.36 μM) of Est-3-Melex-NBD, the nuclear localization was inhibited by Estradiol and Fulvestrant in some, but not all cells. The fact that only some of the cells had nuclear localization inhibition of the Est-3-Melex-NBD whereas in the cells treated with lower concentration, almost all the nuclear localization was inhibited, indicates that perhaps the higher concentration of the drug over competes for the estrogen receptor and is able to overcome the inhibition by the Estradiol and Fulvestrant.

The inhibition of nuclear localization seen in cells treated with lower concentrations of drug (5.63 μM) may not be reliable because even in the control, the drug did not move into the nucleus. In cells treated with higher concentrations of drug (21.36 μM), however, the drug was

localized to the nucleus in the control treatment and inhibited in Estradiol and Fulvestrant pre-treated cells. This indicates that at the higher concentration of Est-3-Melex tried, nuclear localization was inhibited, suggesting that the drug must bind to the estrogen receptor before entering the nucleus.

Results from the final experiment indicated that in MCF-7 cells treated with lower concentrations of Est-3-Melex-NBD (5.63μM), the drug was able to effectively localize to the nucleus. This was a different result from the previous experiment, possibly caused by the exact time the slide was fixed. When the cells treated with 5.63 μM Est-3-Melex-NBD were pre-treated with Estradiol, the drug was still able to enter the nucleus. These were not expected results because the localization should be inhibited. This result may have been because the concentration of Estradiol was not high enough to inhibit many of the receptors or there was not enough time of pre-treatment to flood the receptors. This finding is not consistent with the previous findings on this subject. However, when cells treated with the higher Est-3-Melex concentration (21.36 μM) were pre-treated with Estradiol, the nuclear localization was clearly inhibited. This was consistent with our prediction because the many of the estrogen receptors were bound to estrogen, not allowing the drug to bind to them. This finding shows that the drug selectively binds to the estrogen receptor in order to enter the nucleus. In the 293HEK cells, nuclear localization did not occur when treated with the Est-3-Melex-NBD. This observation confirms the hypothesis that the drug could not enter these cells because there is no estrogen receptor to bind to in order to enter the nucleus. Therefore, comparing these results to those seen in MCF-7 breast cancer cells, it can be concluded that estrogen receptors are a key factor in the entry of the drug into the nucleus.

Conclusion

This project presents a preliminary approach to targeted cancer therapy using DNA-damaging agent. The goal was to specifically target a unique characteristic of the cancer cell. We used breast cancer as a model because some breast cancer types are known to over express the estrogen receptor. Therefore, by presenting a molecule that is necessary for its survival into the cell (Estradiol), the Est-3-Melex acts as a Trojan horse that is able to effectively kill the cell. Within the field of targeted cancer therapy, we have shown that the proposed mechanism of the drug is confirmed, meaning that other similar drugs can be developed. It has also been shown that this drug is actually effective at killing these cancer cells. In future experiments, it may be necessary to look at a time course of nuclear localization to determine the ideal time to pre-treat with Estradiol. Since this is a preliminary evaluation of the approach, the drug has yet to be tested *in vivo*. This is the next step of the drug development. If it is consistently effective, this can further be applied to animal studies and eventually human trials. Although this is in the future of the project, the basis of these findings is that the drug is effective and it represents an apt model to be applied and altered in other future studies. It is known that approximately 90% of drugs that are developed actually fail before being available to actual patients. Thus, if this drug is part of the majority of failed drugs, it still represents an idea for targeted cancer therapy to breast cancer in a field that needs new effective treatments. Through this project we have learned and proved that the drug Est-3-Melex has the ability to kill breast cancer cells by targeting the estrogen receptor. In the future, an exact concentration should be chosen that can be administered to cells and eventually patients to be most effective at killing the cells while avoiding negative side effects. These findings are significant pieces of data in the field of

targeted cancer therapy and should be built upon to eventually lead to successful treatments that can result in a decrease in mortality and morbidity of the illness.

An application to other cancers can be to implement this concept of the drug design to tumors that over express certain receptors. Frequently, cancer cells are dependent on these external signals and hormones to stimulate growth and proliferation. It is known that the steroid hormone estrogen was linked to breast cancer. However, another example could be to instead target prostate cancer, as studies have shown that the cancer cells are dependent on the steroid hormone testosterone (Michaud *et al.* 2015). Thus, in theory, if the estrogen portion of the Est-3-Melex were to be replaced with testosterone, given the possible chemistry, the designed drug could be used to specifically target a prostate tumor. Overall, this research can be applied to other cancers by targeting the high concentration of receptors and the fact that cancer cells over express proteins. Therefore, this project is a start to an expansion of receptor targeted therapy in cancer.

While this project was able to confirm our hypothesized mechanism of drug action, there are many possible future experiments that can be performed. First, the drug should be tested in ER–negative (ER^{-}) breast cancer cells. This will be a similar experiment to that of the 293HEK cells, however, these ER^{-} breast cancer cells may have more similar properties to an MCF-7 cell and therefore more confident conclusions can be drawn. Furthermore, the mechanism of cell death by the drug should be investigated. This would be either by apoptosis or by necrosis. We hypothesize that Est-3-Melex-induced cell death occurs by inducing apoptosis as the cell senses the DNA damage and activates the intrinsic pathway. This could be tested by measuring the activation of caspases, essential components of the apoptotic pathway. This could also be tested by performing a western blot looking for caspase. Cytochrome C release from the mitochondria

can also be measured. Tamoxifen, an Estrogen receptor inhibitor, has a similar mechanism of action as Fulvestrant by inhibiting the estrogen receptor. Tamoxifen should be tested as an inhibitor to see if the drug is still able to enter the nucleus. Hoechst is a compound that binds to the DNA and fluoresces. Therefore, this compound should be used in further fluorescent studies in order to visualize where exactly the nucleus is located. In our experiments, it was often times hard to actually visualize where the nucleus was. However, with Hoechst, the nucleus can actually be easily visualized and show with confidence if the drug has localized to the nucleus. This will make the fluorescence experiments easier to perform and draw conclusions.

In further experiments, researchers should look at the effects of the drug in MCF-7 cells with ER^- knockout so that the cell death and localization can be confirmed in the same type of cell with only the variable of the estrogen receptor altered. This could be accomplished by performing gene knockout cell line by possibly using CRISPR-CAS9 (Bauer *et al.* 2015). Findings would further confirm that the estrogen receptor is necessary for the mechanism of the drug and for entrance of the drug into the nucleus. Other groups could also test the drug in a multitude of different cell types such as normal body cells that express the estrogen receptor. These cells should be tested because they could present as positive negative side effects for the patients if these cells are affected. Lastly, as mentioned earlier, future research can try to alter the structure of the compound and replace the estrogen portion with a different protein or hormone that is over expressed in the cell. Again, this would be able to be applied to a multitude of different cancer types as a form of targeted therapy.

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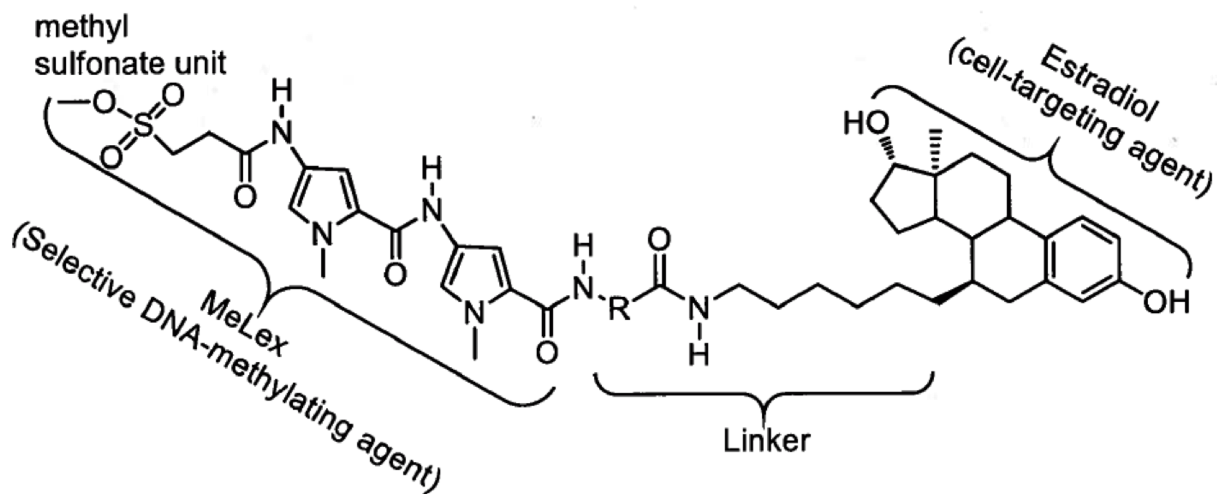

Figure 16. Structure of Est-n-Melex

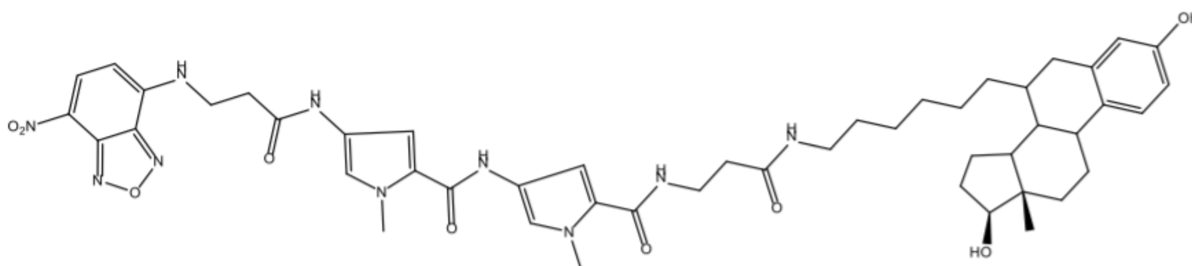

Figure 17. Structure of NBD-Py-Py-3C Linker Estradiol