

ELABORATING THE MECHANISM OF CELL KILLING  
OF A NOVEL CHEMOTHERAPEUTIC DRUG  
TARGETING BREAST CANCER CELLS

by

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

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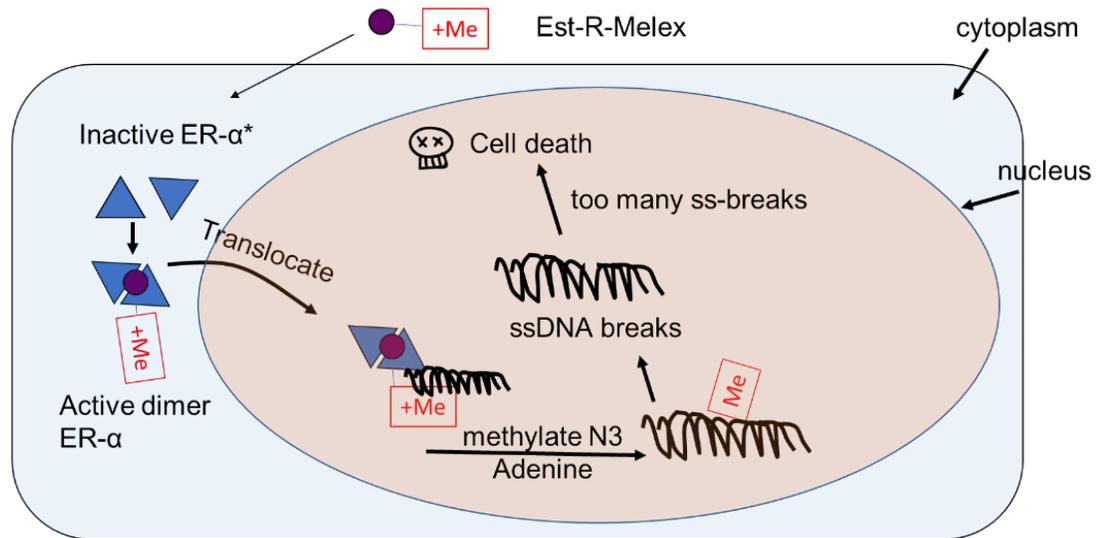
Department of Biology

## ABSTRACT

### *Background*

Breast cancer (BC) is the second most commonly diagnosed cancer among American women after skin cancer. Traditional treatments of BC include surgery, radiation, and chemotherapy therapy; however, these treatments are non-specific and potentially kill peripheral, healthy cells (2). There emerges a need for more specific treatments, most notably to develop chemotherapy agents that target a unique feature of the cancer cells. Interestingly, 70% of BC cells upregulate estradiol-dependent pathway, a characteristic essential for rapid cell growth (3). Current BC drugs, such as Herceptin and Tamoxifen, have targeted this pathway to preferentially kill BC cells (4, 5). However, most women relapse within 15 years due to drug-resistance (6). Thus, there is a need for new chemotherapeutic drugs. Our research group studies a novel estrogen-receptor targeting drug: Est-n-Melex. This compound has the Estradiol (Est) moiety linked to a DNA alkylating agent, Melex via a linker of varying length of methyl groups. We hypothesize that Est-n-Melex enters the cancer cells via an interaction between the Est moiety and the Estrogen Receptor alpha (ER- $\alpha$ ). ER- $\alpha$  then dimerizes, enters the nucleus and binds to Estrogen Response Elements on the DNA. This movement positions the Melex moiety on the DNA and allows the transfer of a methyl group to the N-3 adenine on the DNA. In this project, we test the hypothesized mechanism of action of our compound. Since Est-n-Melex has a DNA methylation component (Melex) conjugated to estrogen, our hypothesis is that after the drug binds to the ER- $\alpha$  in the cytosol, it translocates to the nucleus, specifically methylates the N3-region of adenine

bases, eventually triggering cell death (Figure 1).



\*ER-α: Estrogen Receptors α

(R. J. Kishton et al.)

Figure 1 Hypothesized Mode of Action of Est-n-Melex in Estrogen Receptor positive cells (1).

## Chapter 1: GENERAL INTRODUCTION

### *The breast*

The breast is the organ with a unique function: to produce milk and lactate (breast feed). The breast tissue has various epithelial components, including the lobules which produce milk and connect to ducts leading to the nipple. Most breast cancer stems from cells that make up the lobules and terminal ducts. These lobules and ducts spread through the background of adipose and fibrous tissues, which comprise the bulk portion of the breast. The male breast has a structure almost identical to that of the female, except only female breast has specialized lobules to support the physiological function of lactation and milk production (7).

From an anatomical standpoint, the breast sits above the pectoralis muscle, which sits above the ribcage. The breast tissue comprises of a broad horizontal region, extending from the outer edge of the sternum to the midaxillary line. Trailing behind is a tail of breast tissue named as the

"axillary tail of Spence," which spans directly into underarm area. This specific detail is vital since breast cancer tissues can extend into this axillary tail, although technically, the tail seemingly is located outside the actual breast.

Encircling the breast tissues is the fascia, a thin layer of connective tissue. Deep inside the layer of fascia connects to the pectoralis muscle, while the layer on top of the fascia is immediately beneath the skin. The skin tissue over the breast has similar features to the skin anywhere else in the body, such as sweat glands and hair follicles. While conducting a breast exam, a clinician will comprehensively study the skin as well as the breast tissue (7).

## **Breast cancer**

### *Incidence, mortality, survival*

Cancer is a broad term characterizing diseases in which cells undergo uncontrolled growth and spread, often resulting in fatality. Cancer incidence has increased dramatically in recent years, impacting various aspects of human life, including physical, mental, social, and economic suffering (8). Cancer incidence fluctuates annually from 1 to 2 percent in developed nations, and 5% in developing countries (9). At least 7 million people are estimated to die from cancer, and the number of new cancer cases is stipulated to rise from 10 to 15 million by 2020 (10).

Meanwhile, breast cancer remains the leading malignant neoplasm among women (11). Since 2005, the incidence rate of invasive breast cancer has stabilized among white women and rose slightly by 0.3% every year among black women (12). In 2018, 41,400 breast cancer deaths occurred. The breast cancer death rate reached the highest in 1989, then plummeted by 39% in 2015 (13). This phenomenon occurred against the background of a growing population can be attributed to advancements in early detection (via screening and heightened awareness) and

treatment. These combined efforts are estimated to result in 322,600 fewer breast cancer deaths per year (14).

### *Signs and symptoms*

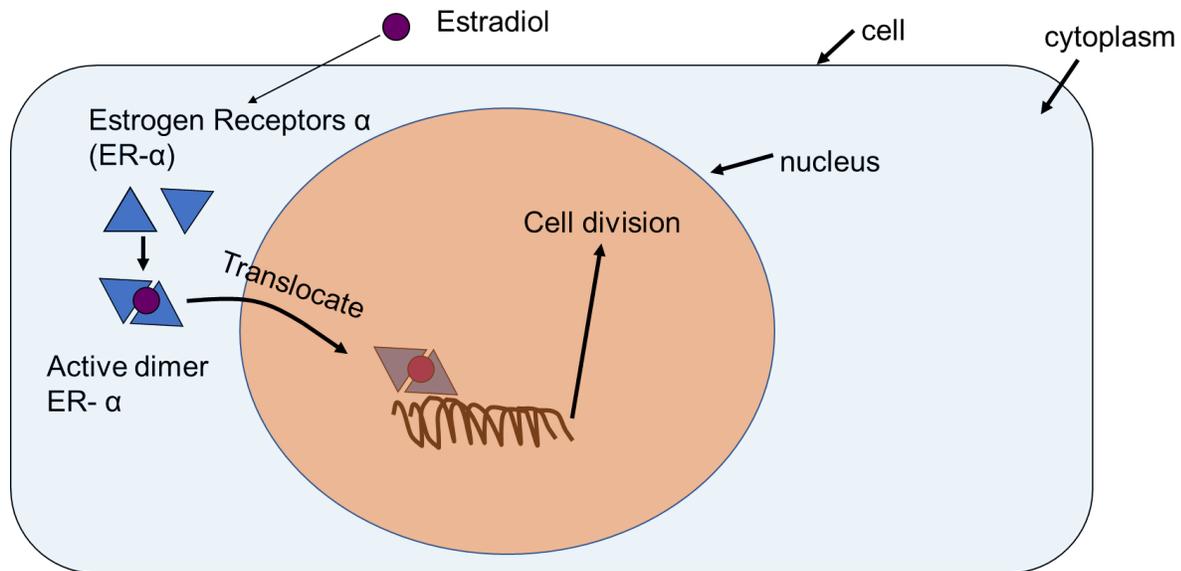
A lump or mass in the breast is most conspicuous symptom of a neoplasm. Less conspicuous symptoms are systemic changes in the breast, namely thickening, swelling, distortion, tenderness, skin irritation, redness, and nipple abnormalities or spontaneous nipple discharge. Early breast cancer is asymptomatic and often detected via mammography screening (15) .

### *Risk factors*

Like many other types of cancer, age is the strongest risk factor for breast cancer. Exposure of breast tissue to reproductive hormones poses heightened risks of breast cancer as well (16).

These hormone overexposures can result from being obese or overweight, postmenopausal hormone treatment (estrogen and progestin), physical inactivity, alcohol use, and prolonged breast feeding (over one year). Other factors include a prolonged menstrual history (in which menstrual periods start earlier or end later in life), bearing no children, bearing the first child after 30 years old, having a high levels of sex hormones from birth, and frequent doses of oral contraceptives. Familial risk factors include a family history of breast/ovarian cancer, inherited mutations in BRCA1, BRCA2, or other breast cancer susceptibility genes, other benign breast conditions like atypical aplasia, radiation exposure to the chest area in the young age (for example, due to lymphoma treatment) (17). In addition, high breast tissue density and type 2 diabetes are common risk factors as well (18).

### **Mechanism of estradiol-induced ER- $\alpha$ signaling**



(R. J. Kishton et al.)

*Figure 2. Mechanism of Estradiol-induced ER-α Signaling pathway (1).*

In 70% of all cases, BC growth depends on Estradiol (Est) signaling through ER- $\alpha$  as Est is a mitogen for BC cells, works as a survival and anti-apoptotic factor and induces cell invasion and migration (19). In a normal breast cell, Est moves into the cytoplasm, binds to the inactive Estrogen Receptors alpha (ER- $\alpha$ ) and causes it to dimerize to form an activated dimer. The complex then functions as a transcription factor by translocating into the nucleus, binding to the DNA and sending cell proliferation signals (Figure 2). When breast cancer cells overexpress ER- $\alpha$ , the entire estradiol-response pathway is upregulated, thus increasing the rate of cell division and risk of cancer and tumorigenesis. Aberrations in ER $\alpha$ , ER $\beta$ , and Progesterone (PR) expression in tumorigenesis in the endometrial cells involve mechanisms of promoter regulation (20). The abnormality is attributed to tumorigenesis in the endometriotic stromal cells. In these cells, ER $\beta$  promoter is hyperactive, resulting in high ER $\beta$  expressions. ER $\beta$  downregulates ER $\alpha$  expression, resulting in high ER $\beta$ -to-ER $\alpha$  ratios in endometriotic cells. A higher ER $\alpha$ -to-ER $\beta$  ratio in endometriotic stromal cells causes a shift from Est inhibition to Est stimulation of

Progesterone (PR) expression in endometriotic stromal cells. This explains the proliferation of PR resistance in women with endometriosis (20). Est is also thought to regulate the malignancy of cancer stem-like cells (CSC) derived from the MCF7 cell line partially through Sox2. Est, in a dose-dependent manner, produces opposite effects on proliferation, migration, colony formation, and self-renewal capacity of CSC. In high concentration of Est, Est stimulates apoptosis and blocks proliferation. In lower concentrations of Est, it stimulates self-renewal capacity (21). Another study looks at Est treatment in a different angle, showing evidence that Est promotes breast cancer cell proliferation by inducing cyclin G1 expression, thus stimulating proliferation and cell viability (22). These discoveries can be promising for hormone combination therapy against breast cancer tumorigenesis. Mitochondrial morphology in the Est-response pathway is also of interest since it sheds light into understanding cancer cell proliferation or cell death. Studies indicate that phosphorylation of dynamin-related protein 1 changes mitochondrial morphology in MCF7 cells. ER- $\alpha$  assists the phosphorylation of Drp1, and subsequently results in the mitochondrial fission and cell's overall size reduction (23).

### **Early detection**

To detect breast cancer tissues at the early stage, mammography is often used. Mammography is a low-dose X-ray technique that leads to less extensive treatment and yet effectively reduces breast cancer mortality (24). Certain shortcomings of mammography are false negative and false positive detection. In average, around one in 10 screened women produce abnormal mammogram, and yet, only 5% of screened women have cancer (25). Mammography also detects *in situ* lesions, such as ductal carcinoma *in situ*, that would not progress to become malignant, leading to over-diagnosis up to 30% of screened women (26). Nevertheless, the American Cancer Society recommends annual mammography for at-risk women aged 40 years

and above (25). Women identified as high-risk are recommended to undergo annual magnetic resonance imaging (MRI) as well. Among at-risk women, chemotherapeutic drugs are employed to reduce risks of breast cancer development. Two common drugs are Tamoxifen and Raloxifene, which are commonly prescribed to reduce breast cancer risk for at-risk women. Although Raloxifene has a lower risk of side effects, it is more often prescribed for postmenopausal women. Another common form of medication involves the use of Aromatase Inhibitors (AI). Aromatase is an enzyme required for conversion of testosterone to estrogen, thus blockers of this pathway can inhibit Est synthesis and reducing systemic Est availability (27). AIs therefore can reduce the breast cancer risk for at-risk women. However, they are only approved for the prevention of cancer recurrence.

## **Treatment**

### *Surgery*

Two most common forms of treatment include breast-conserving surgery (the surgical removal of only the tumor and surrounding tissue) or mastectomy (surgical removal of the entire breast). The form of surgery depends largely on the tumor characteristics, such as size, hormone receptor status, and extent of spread, and patient preference (28).

### *Radiation*

Most patients are recommended to have radiation in addition to breast-conserving surgery. Studies have shown that in women having early-stage cancer (characterized as cancer that has not spread to peripheral areas such as the skin, chest wall, or other peripheral organs), radiation after breast-conserving surgery can result in as favorable long-term outcomes as mastectomy. In addition, in cases of larger tumors or node-involved breast cancers, radiation is recommended for

use post-mastectomy. Women who undergoes mastectomy can decide on several breast reconstruction options to restore breast shape, such as different type of tissue or implant used. Reconstruction performance may occur immediately after mastectomy or delayed as a second procedure (29).

### *Chemotherapy*

Chemotherapy is type of cancer treatment in which drugs preferentially bind to breast cancer cells and either prevent further cellular divisions or promote cell death. Routes of delivery are commonly oral, musculoskeletal, or intravenous. As soon as the drugs enter the bloodstream, they can be delivered throughout the body (systemic chemotherapy). In certain cases, chemotherapeutic drugs may be injected directly into the cerebrospinal fluid, an organ, or a body cavity such as the abdomen, the drug can act locally and kill cancer cells in those specific areas (regional chemotherapy). The way the chemotherapy is given depends on the type and stage of the cancer being treated (30). Many times, chemotherapy is conducted before the breast cancer surgery (neoadjuvant) or post-surgery (adjuvant). Neoadjuvant chemotherapy is conducted prior to surgery to maximize the reduction of large breast cancer mass, allowing breast-conserving surgery. This strategy also assists physicians in figuring out the exact effect of specific regimens on breast tumor. In contrast, adjuvant chemotherapy is done post-surgery or post-radiation to wipe off any remaining tumor cells that surgery and radiation fails to remove. This strategy ensures that breast cancer cells do not metastasize to peripheral parts of the body (31).

Tamoxifen: a nonsteroidal triphenylethylene derivative. Tamoxifen became the mainstay for ER $\alpha$  tumors since they are selective ER modulators (SERMs), which recognize and bind to ER $\alpha$  and inhibit receptor to work as a transcription factor (32). This blockage prevents Est-dependent

gene expression, thus selectively down-regulating ER expression and blocking the proliferative actions of estrogen on mammary epithelium (27). Interestingly, tamoxifen has both estrogenic and antiestrogenic mechanism of actions, depending on the target tissue (33). It functions as an antiestrogenic on mammary epithelium, hence is used to prevent and treat breast cancer. At the same time, it is proestrogenic on uterine epithelium, hence causing heightening controversy regarding its safety in cancer prevention due to the heightened incidence of endometrial carcinoma in women consistently on tamoxifen regime (33). Studies suggest that the antiproliferative action of tamoxifen results from the synthesis of the inductive effects of cytokine transforming growth factor- $\beta$  (TGF- $\beta$ ), which regulates the negative autocrine signaling pathway (34). Moreover, immunohistochemical studies have shown that tamoxifen induces the synthesis of TGF- $\beta$  in the stromal (mesenchymal) compartment of breast cancers, suggesting a paracrine as well as autocrine mechanism of action, independent of an interaction with the estrogen receptor (35). However, increasing tamoxifen resistance in ER $\alpha$ <sup>+</sup> patients is observed (36). It is noted that in ER $\alpha$ <sup>36+</sup> breast cancer patients, tamoxifen complementarily binds and activates ER $\alpha$ <sup>36</sup>, thus increases expression of ALDH1A1 (37). As a result, it increases stemness and promoting further metastasis. Studying the pathways of Tamoxifen resistance is useful for therapeutic treatment plans (38).

Fulvestrant: Fulvestrant targets the Estrogen Receptor (ER) to inhibit tumor cell proliferation.

Fulvestrant acts as an antagonist to estrogen receptor, binding to ER monomers and blocking the dimerization. As a result, activating function 1 (AF1) and AF2 become inactivated, reducing the translocation of receptor to the nucleus and accelerating the cytosolic degradation of the ER (39). It is also noted to physically interact with ER- $\alpha$ , promoting 26S proteasome-dependent degradation and eliminating ER- $\alpha$  from BC cells (27). Interestingly, it is observed that

Fulvestrant has no uterotrophic side effects on the immature or ovari-ectomized rats and in a dose-dependent manner, prevents the agonistic pathway of Est and Tamoxifen.

Natural extracts are natural compounds that have the potential to treat breast cancer proliferation. Cinobufagin, a molecule extracted from *Venunum Bufonis*, is shown to cause targeted cytotoxicity in MCF-7 cells by increasing Bax expression and reducing Bcl-2 expression, thus inducing apoptosis and G1 phase arrest (40). Other natural extract, such as the Mexican mistletoe *Struthanthus venetus* with the hydromethanolic extract (DtvHME), have antiproliferative effect on breast cancer as well. For example, DtvHME is shown to antagonize the proliferative-response of Est- response for uterotrophic activity of the mice. This implicated that StvHME has the potential to act on breast cancer cells via ER- $\alpha$  and ER- $\beta$  and can be used as the complementary treatment against breast tumorigenesis as well (41).

### **Objective and Outline of this thesis**

As mentioned above, aromatase inhibitors, tamoxifen, fulvestrant all commonly used to target this Est-initiated pathway to downregulate the downstream expression. However, these drugs are known to cause considerable side effects: AIs are clinically shown to consistently produce musculoskeletal failures while Tamoxifen often causes endometrial cancer (27). Chronic chemotherapy also causes BC cells to resist treatment, as one-third of women treated with Tamoxifen for 5 years are shown to relapse within 15 years (resulting tumors will be insensitive to Tamoxifen treatment) (42). As such, there emerges a need for new targeted chemotherapeutic drugs.

DNA-methylating small molecules are commonly used in cancer therapy. Yet most DNA alkylating drugs are specific for neither DNA nor cancer cells. Non-specific DNA alkylation

produces a variety of non-cytotoxic and indiscriminate DNA lesions, resulting in secondary cancers such as leukemia (43). Non-specific uptake into the cells causes significant side-effects like immune suppression and gastric irritation. A new strategy to overcome the therapeutic shortcomings of DNA-methylating compounds is surprisingly simple: to link both cell-targeting domain and DNA-binding domain to the methylating agent (1).

Since most breast carcinomas over-express the estrogen receptor alpha (ER- $\alpha$ ), a moiety of the drug resembling the natural ligand of ER- $\alpha$ , i.e. estradiol (Est), increases the drug's affinity to ER+ cells. This increases the concentration ratio between ER+ and ER- tissues, thus preferentially targeting ER+ breast cancer cells. ER- $\alpha$  is a good target because estrogen signaling via ER- $\alpha$  binding and activation is critical for the pathogenesis of breast cancer. The more rampant presence of ER- $\alpha$  in breast cancer cells make it a good target for drug design.

Melex is shown binds almost exclusively to the minor DNA groove and produces over 95% of 3'-Methyl of Adenine (3-MeA) lesions (1, 44). In this case, minor DNA groove is another good target for DNA-methylating compounds due to the high methylation specificity, resulting in high cytotoxicity and low mutagenicity. This is desirable as compared to indiscriminate methylation which causes side effects as mentioned above.

This design wants to neither activate nor inactivate the ER- $\alpha$  and DNA. Instead, it aims to bring the already specific DNA-methylating compounds into cells overexpressing ER- $\alpha$ , thus more prudentially kill breast cancer cells. This strategy takes advantage of the already well-researched areas, i.e., estradiol (Est) signaling pathway and Melex selective methylation mechanism. To do as such, the drug must link the two moieties (Est and MeLex) effectively to not interfere with binding and efficacy of Est and Melex. Creating the linker requires careful deliberations

regarding the linker length between the two moieties as well as linker composition. Thereby, the drug (Est-n-Melex) contains Est moiety conjugate to Melex via a linker of various length.

We hypothesize that Est-n-Melex enters the ER<sup>+</sup> cancer cells more rapidly than ER<sup>-</sup> normal cells. The Est moiety recognizes and binds to the inactive Estrogen Receptor alpha (ER- $\alpha$ ). ER- $\alpha$  then dimerizes to form an active dimer complex, which then enters the nucleus and binds to Estrogen Response Elements on the DNA. This nuclear movement brings the drug to a proximity with the DNA, allowing Melex to create 3-MeA lesions. This specific methylation results in abasic sites and subsequently, single-strand breaks (SSB). High amounts of SSB activate poly (ADP-ribose) polymerase (PARP) during DNA repair, costing excessive cellular ATP and as a result, necrosis (1). In addition, co-treating the drug with PARP inhibitors can force the cell to undergo programmed cell death, i.e., apoptosis. The proof of idea is demonstrated in adriamycin preferentially targeting MCF-7 breast cancer cells, via activating PARP-1 activation in a dose- and time- dependent manner and activate BRCA1, and co-treatment with a PARP-inhibitor like 3-aminobenzamide (3AB) increases MCF-7 apoptosis (38).

Modeling the drug in three dimensions allows analysis of unintended interaction or loss of interaction between moieties and their respective docking sites. It also allows visualization of unutilized / non-bonding pockets of ER- $\alpha$  and/or DNA to add new groups that may facilitate higher binding affinity.

Since the drug has a moiety resembling Est, the drug is expected to bind to other ER proteins, such as ER- $\beta$  and ER- $\gamma$ , as well as non-cancer ER- $\alpha$  expressing cells, such as brain, bone, mammary gland, and reproductive organs. These interactions will cause side effects like endocrine therapy or ER- $\alpha$  blockers such as tamoxifen or fulvestrant. If the drug is having off-target effects, one will observe higher cell death in normal cells as compared to cancer cells.

Despite these side effects, ER- $\alpha$  blockers alone are insufficient since patients have high risks of relapse several years later (27). As such, the complementation of targeted DNA-methylating agents hold great potential in more effectively targeting and killing breast cancer cells. This project will provide information to retain and even increase affinity and function of Est and Melex to ER- $\alpha$  and DNA minor groove respectively. With this information, this study can be a useful proof of concept for linking natural ligands to DNA-methylating small molecules to produce tissue-specific cell death.

## **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<sub>2</sub>, 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 can 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  $\mu$ L 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  $\mu$ L DMSO was then added to dissolve the MTT precipitate. The absorbance can be quantified using a spectrophotometer at 540 nm. The relative amount of dye released is compared to the control and the results are displayed numerically using Omega software.

### *Immunofluorescence*

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  $\mu$ L) were placed on the coverslip in DMEM medium and the plate was placed in the incubator for thirty minutes. After the cells are settled at the bottom, 3.5 mL of DMEM medium was added to each well. The cells are placed 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  $\mu$ L PBS. 500  $\mu$ L 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  $\mu$ L 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.

#### *GloMix caspase 3/7*

Twenty thousand cells are seeded and incubated for 24 h, after which the medium is removed and replaced with 50  $\mu$ l fresh complete medium containing drugs of interest, such as Staurosporine, cisplatin, Est-n-Melex. Drugs are incubated for 4.5 hrs, followed by the caspase assay. The plate is equilibrated to room temperature, followed by the addition of 50  $\mu$ l of Caspase-Glo® 3/7 Reagent to each well (medium: caspase reagent = 1:1 ratio). One column with medium and caspase reagent is used as blank. Wells are mixed gently using a plate shaker at 300–500 rpm for 30 seconds. The cells are incubated at room temperature for 1hr and protected from light by covering the plate with Al foil, followed by transferring the lysate into a white-wall multi-well plate. The luminescence of each sample is measured in a plate-reading FluoOmega Star plate luminometer reader.

#### *Hoechst and Est-n-NBD visualization*

Eighty percent confluent MCF-7 cells were plated on a 6-well plate and was pretreated with 6.05 e-5 mM of MCF-7 overnight, followed by addition of 2.5 and 5  $\mu$ M of Est-3-NBD for overnight. The cells were washed 3 times with 1000  $\mu$ l PBS in room temperature and was visualized under confocal microscopy.

## **Results**

### Testing the cytotoxicity of Est-n-Melex on ER+ and ER- cell lines

The first aim is to test the specificity of Est-n-Melex to ER+ and ER- cell lines to minimize side effects and unintentional cell killing of ER- cells. In order to verify the specificity of the drug, our group treated MCF7 ER+ cells and HEK293 ER- cells with Est-Melex. This experiment was based on the understanding that Est-Melex caused cell death (via necrosis) and thus percent cell survival post treatment was measured. If the Est domain of the Est-Melex bound to the ER as predicted, then the ER+ cell lines should exhibit more cell death when compared to the condition that does not have ER-. It is observed that in the absence of ER, HEKs exhibit a concentration dependent cell death whereas in the presence of ER, HEK293 cell death plateaus around the 100% cell survival range which suggests that there was little, if any, cell death in this treatment group (Figure 3).

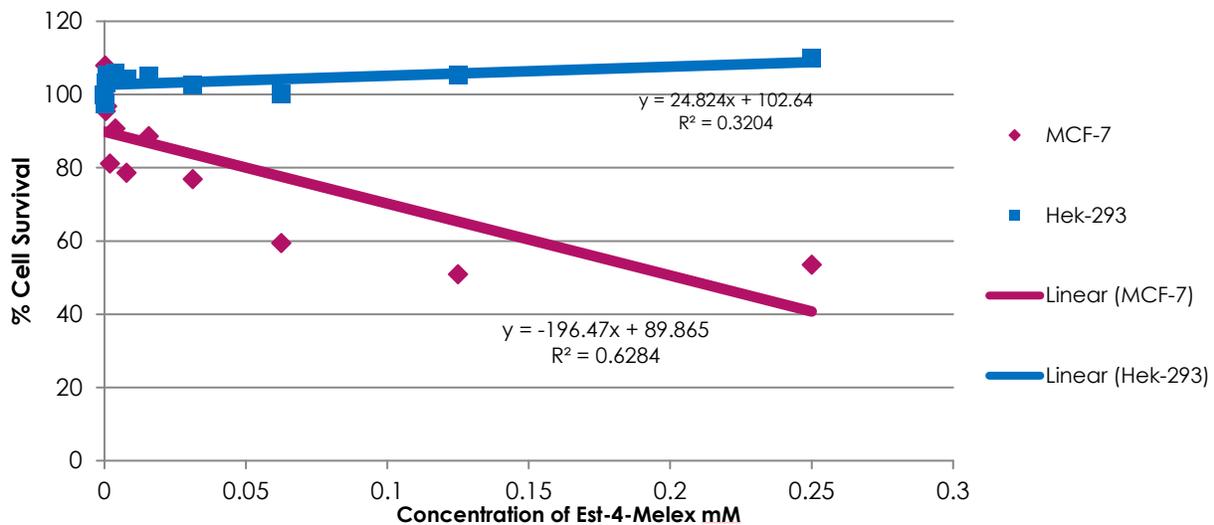
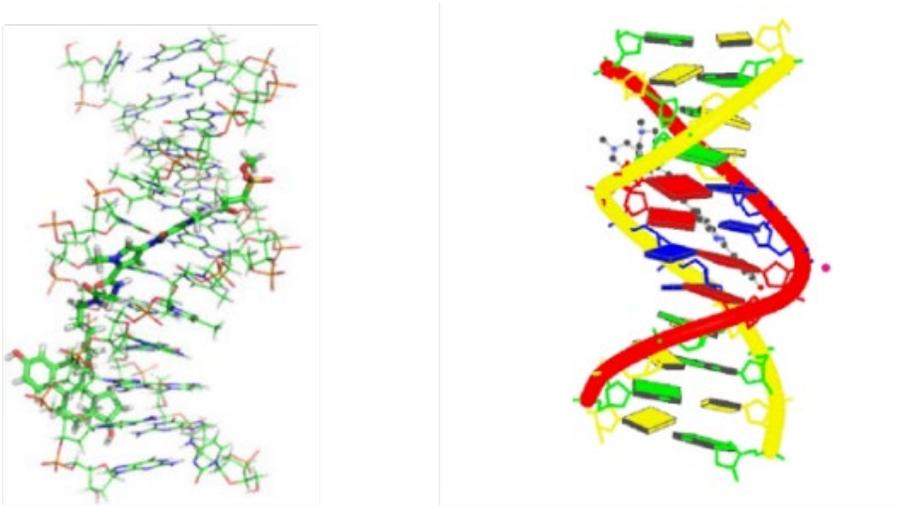


Figure 3 Cytotoxicity of Est-n-Melex in ER+ breast cancer cell line MCF-7 and ER- human embryonic kidney cell line HEK-293 (unpublished work by Mayur Patel and Giridhar Rao Akkaraju).

Tracking the movement of Est-n-Melex

The translocation of the Est-n-Melex was assessed in ER+ MCF-7 cells. Theoretical simulation modeling shows Est-n-Melex binds to the A/T rich minor groove of DNA. To confirm the model simulation, Hoechst – also an A/T rich, minor-groove DNA binding agent, is used to compete with Est-n-Melex for the binding site (Figure 4).



*Figure 4 Simulation models of Est-n-Melex (left) (unpublished by Varadarajan et. al.) vs. actual Hoechst (right) binding to minor groove of DNA (47).*

Since Est-n-Melex is cytotoxic and invisible under the microscope, its movement is tracked using a non-cytotoxic, fluorescent analogue, Est-n-NBD. The structure of Est-n-NBD is modified such that the methylation group is substituted by the fluorescent moiety NBD (Figure 5).



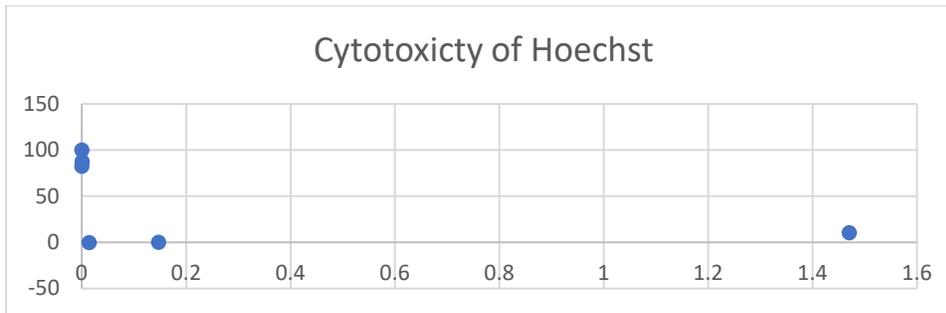
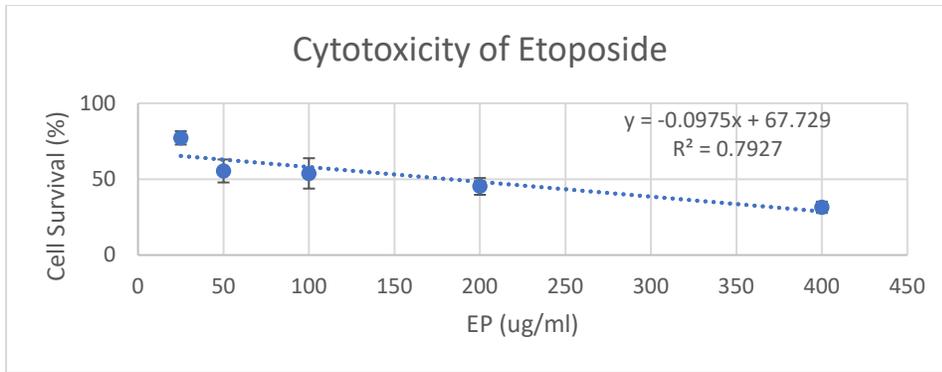


Figure 6 Cell survival of MCF-7 cells upon treatment with Etoposide (top) and Hoechst (bottom) (dilution factor of 10).

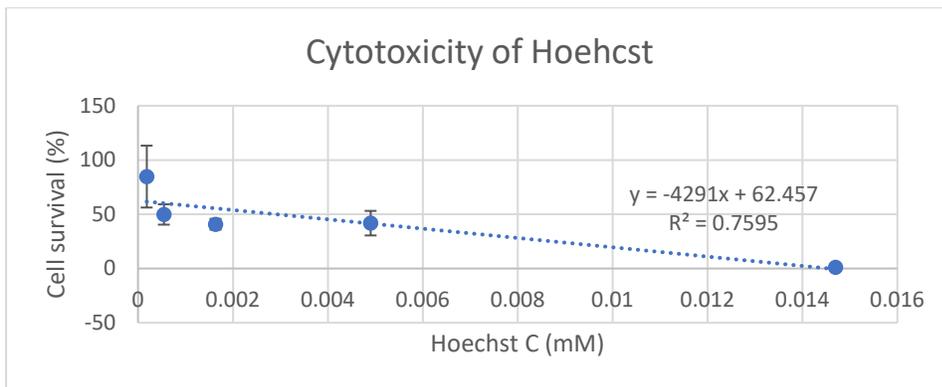
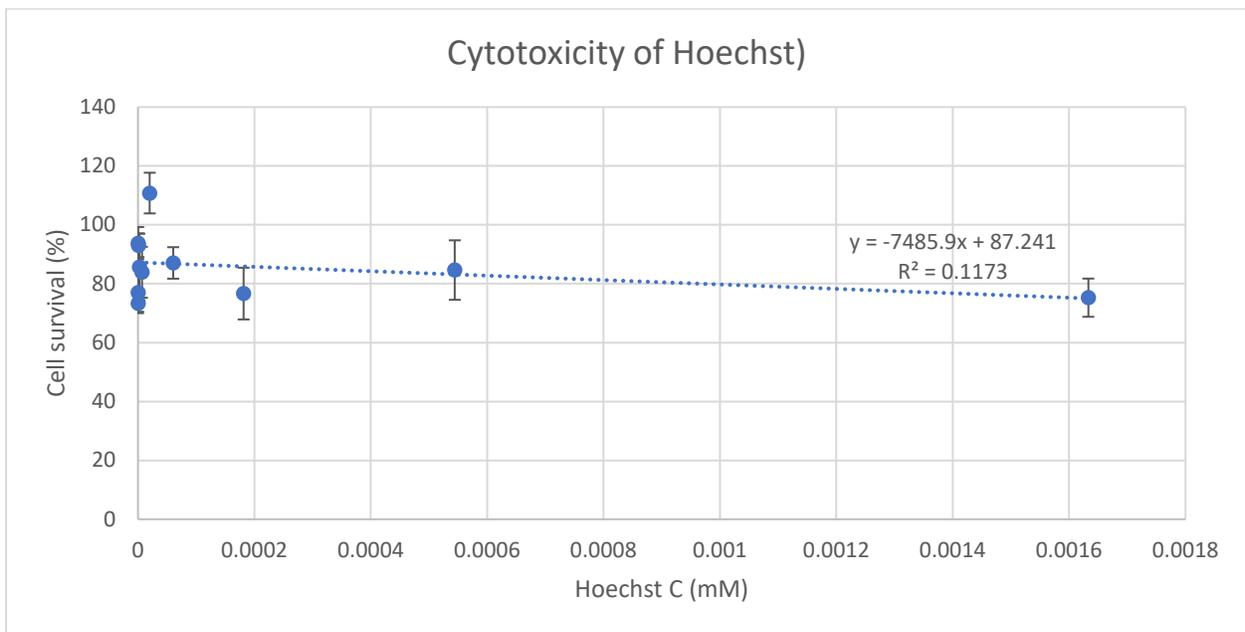


Figure 7 Hoechst stain is cytotoxic to MCF-7 cells below 0.0147 mM. Confluent MCF-7 cells were treated increasing concentration of Hoechst (dilution factor = 3) and Etoposide (as positive control). Cytotoxicity was measured with MTT-Cytotoxicity assay.

Although a non-cytotoxic concentration of Hoechst was desired, a concentration too low would interfere with the fluorescence visibility under the confocal microscopy. As such, a concentration

of Hoechst that not only was non-cytotoxic but also provided strong fluorescent signals was desired.

The range of non-cytotoxic concentration of Hoechst was further evaluated by measuring cell death upon increasing concentration of Hoechst from the range of 0.001 to 0.014 mM (Figure 7). Results indicated that concentration below 0.001633 was non-cytotoxic. The negative slope and moderately strong  $R^2$  (0.7595) indicates that Hoechst was cytotoxic. But a non-cytotoxic concentration was yet to be determined. So the cytotoxicity of Hoechst below 0.0147 mM was studied (Figure 8).

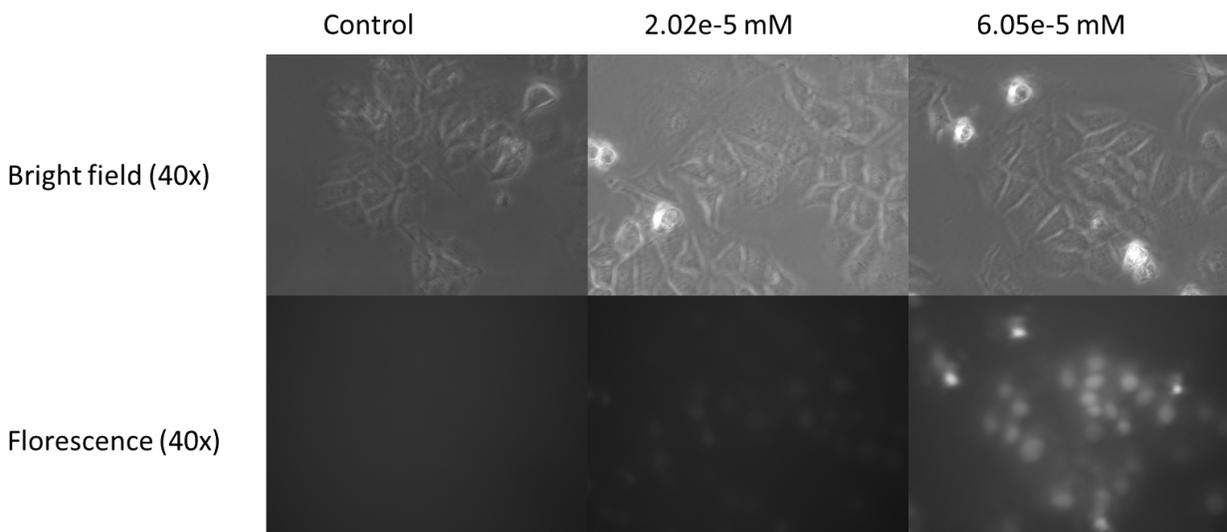


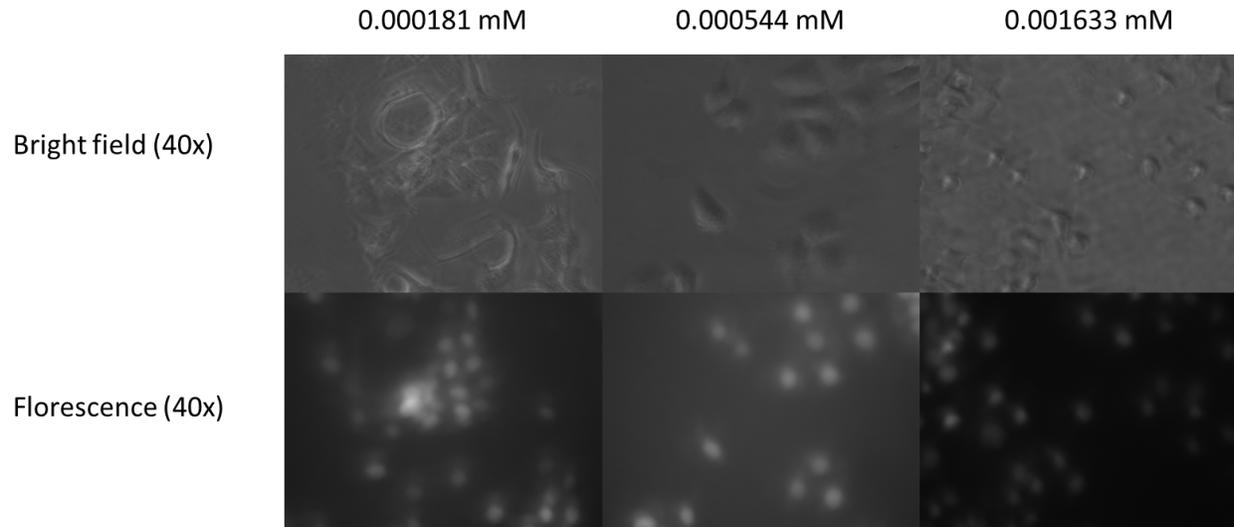
*Figure 8 Hoechst stain is not cytotoxic to MCF-7 cells below 0.001633 mM. Confluent MCF-7 cells were treated increasing concentration of Hoechst (dilution factor = 3). Cytotoxicity was measured with MTT-Cytotoxicity assay.*

Results of cytotoxicity of Hoechst in concentration below 0.001633 mM shows an  $R^2 = 0.11728$  which indicates almost no correlation. This indicates that in these concentrations below 0.001633 mM, Hoechst do not play a cytotoxic role in MCF-7 cell lines. This experiment determines that a

concentration below 0.001633 mM of Hoechst is non-cytotoxic to ER+ breast cancer cell line MCF-7.

If a too low Hoechst concentration is chosen, not every cell on the plate will be exposed to it, and an insufficient amount of Hoechst will be available to bind to DNA to produce strong signal under the confocal microscope. As a result, concentrations below 0.001633 mM are assessed to determine the concentration of Hoechst which induces the least cytotoxicity and the best nuclear staining under the florescent microscope.



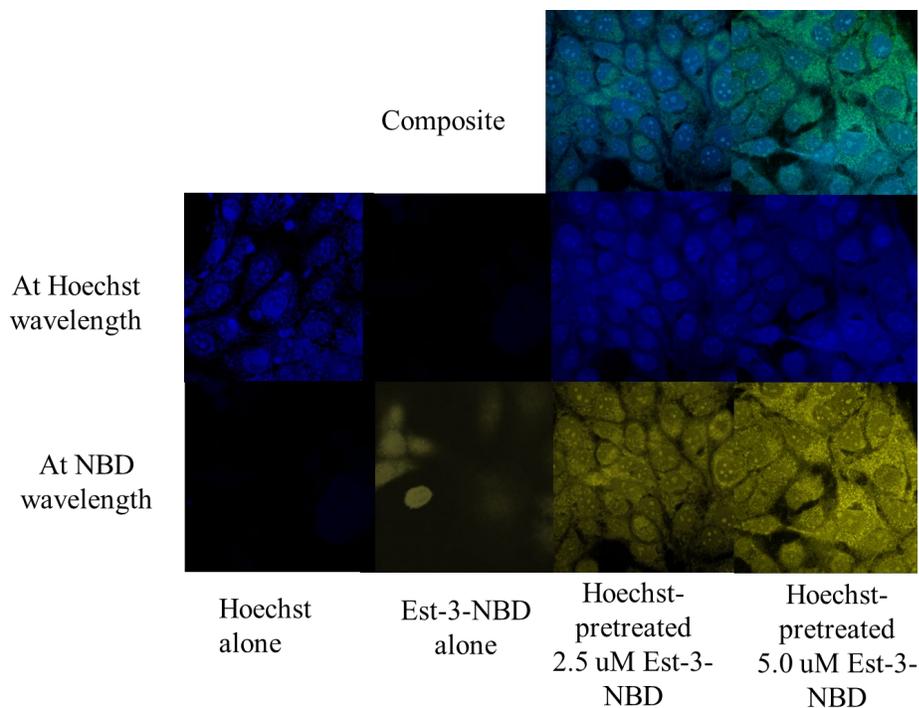


*Figure 9 At  $6.05 \times 10^{-5}$  mM and above, Hoechst stain enters all nuclei. Confluent MCF-7 cells were treated with decreasing concentration of Hoechst below 0.001633 mM (dilution factor = 3) overnight and visualized under florescent microscope.*

Results indicate that the nucleus is stained effectively at concentrations above  $6.05 \times 10^{-5}$  mM. At concentrations below  $6.05 \times 10^{-5}$  mM, nuclear staining is insignificant (Figure 9). Figure 8 also shows that at  $6.05 \times 10^{-5}$  mM of Hoechst, 87.06 % cell survival is observed, with the significant interval bar overlaps 100%. As such,  $6.05 \times 10^{-5}$  is the lowest non-cytotoxic concentration of Hoechst in which high nuclear staining signals were observed.

Competitive inhibition of Est-n-NBD nuclear localization with Hoechst was used to test the hypothesis that Est-n-NBD (a non-cytotoxic and florescent analogue of Est-n-Melex) moves into the nucleus and binds to the minor groove of DNA. MCF-7 cells were pretreated with and without Hoechst, followed by increasing concentration of Est-n-NBD. If the hypothesis were correct, without Hoechst treatment, Est-n-NBD should localize into the nucleus and binds to the minor groove of the DNA. Thus, nuclear presence of Est-n-NBD should be observed. Hoechst pretreatment would cause Hoechst to move inside the nucleus and bind to the A/T rich region of

the DNA, occupying the active site of binding of subsequent Est-n-NBD. As such, Est-n-NBD would remain cytosolic.



*Figure 10 Est-n-NBD moves into the nucleus and binds to the minor groove of the DNA. MCF-7 cells are pretreated with  $6.05 \times 10^{-5}$  mM of Hoechst overnight followed by increasing concentration of 2.5  $\mu$ M and 5.0  $\mu$ M of Est-3-NBD. Images are captured with confocal microscopy. Composite images were created with ImageJ.*

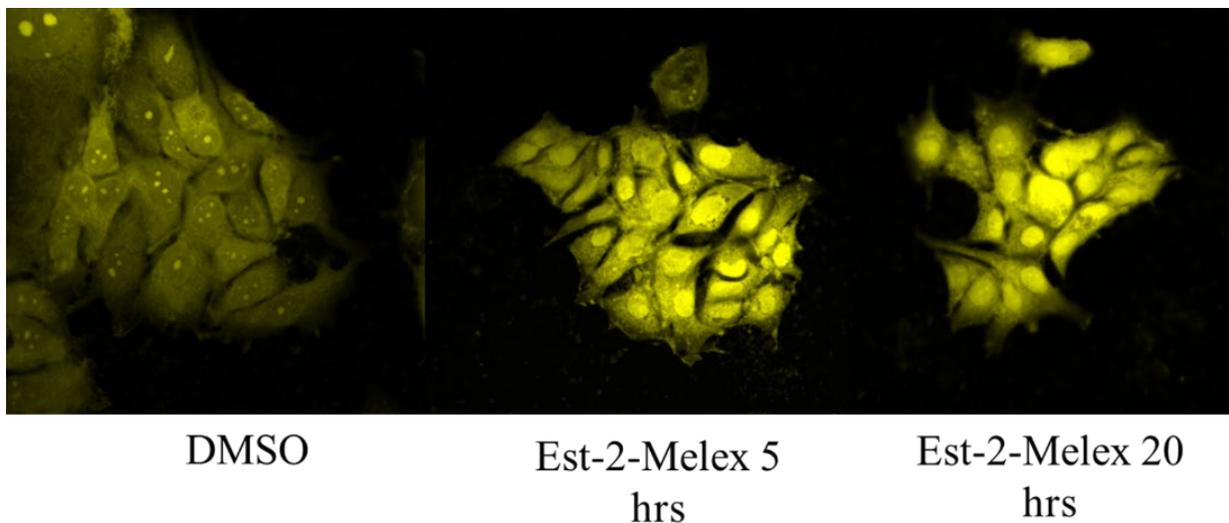
Without Hoechst treatment, Est-n-NBD was observed primarily in the nucleus. Hoechst pretreatment overnight followed by 2.5  $\mu$ M Est-n-NBD observes nuclear localization of Hoechst but cytosolic presence of NBD. Increasing NBD concentration to 5.0  $\mu$ M Est-n-NBD observes more cytosolic presence of NBD, confirming that Hoechst and Est-n-NBD competes for the minor groove of the DNA (Figure 10).

#### *Tracking the localization of the target protein (Estrogen Receptors $\alpha$ )*

Immunofluorescence study is used to track ER- $\alpha$  localization upon Est-n-Melex treatment. A non-cytotoxic concentration of Est-n-Melex is determined before imaging was conducted in

order to preserve the cell morphology. Preliminary unpublished data from Mayur Patel and Giridhar Rao indicated that Est-n-Melex is not cytotoxic (cell survival exceeds 80%) at concentration below 15 nM (Figure 3). As such 15 nM of Est-n-Melex was chosen as the least cytotoxic concentration for MCF-7 cells.

MCF-7 was treated with a concentration of 15 nM Est-n-Melex for 5 or 20 hours, and the localization of ER- $\alpha$  was tracked using immunofluorescence. Without the drug, ER- $\alpha$  remains cytosolic. Est-n-Melex treatment for 5 hours shows a more concentrated presence of ER- $\alpha$  nuclear localization of ER- $\alpha$ . Prolonged exposure of the drug observes even a more concentration ER- $\alpha$  inside the nucleus (Figure 11).



*Figure 11 Est-2-melex induces nuclear translocation of ER- $\alpha$  in a time dependent-manner. 25,000 MCF-7 cells on cover-slip were treated with 15 nM est-2-melex (dissolved in DMSO) and let sit for 4 hrs and 16 hrs. The samples were then treated with 1:500 ER- $\alpha$  antibody, followed by 1:1000 anti-mouse Alexafluro-488.*

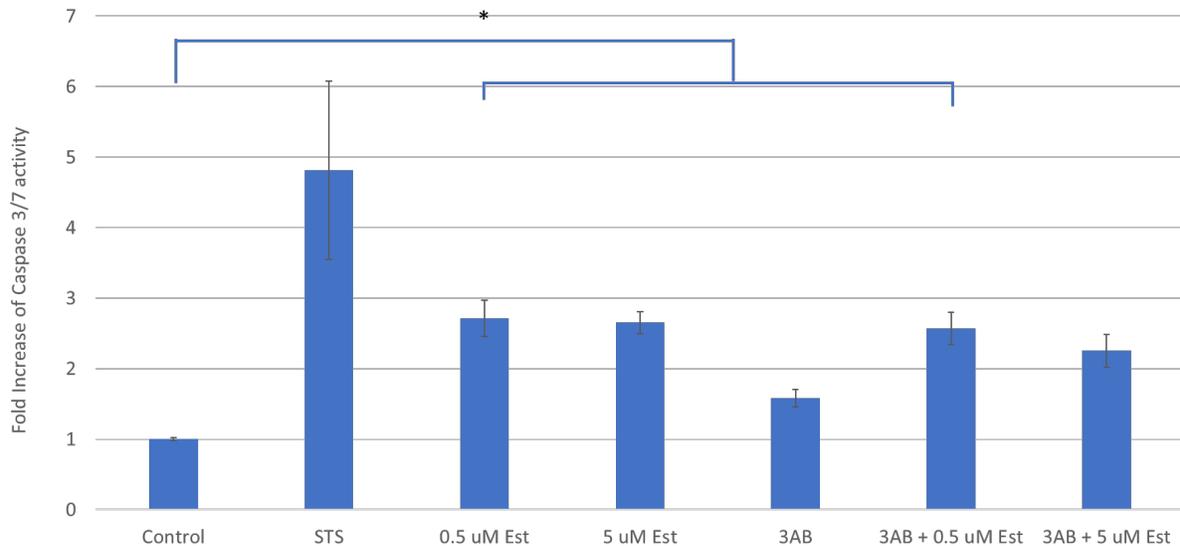
#### *Mode of Cell Killing: Apoptosis*

Figure 3 indicates that Est-n-Melex is cytotoxic to ER+ MCF-7 cells, but the cell killing mechanism is not known. We hypothesize Est-n-Melex would methylate the N-3 Adenine on the

A/T rich region of the minor groove of the DNA. When 3-MeA adducts are created, cells recruit glycosylase to remove the methylated base, thus producing abasic sites. AP endonuclease are then recruited to remove the DNA backbone of the abasic site, producing single-stranded ssDNA breaks. We hypothesize the some ssDNA breaks cause cells to recruit PARP (Poly-ADP-Ribose Polymerase), DNA repair enzymes that fix the DNA damage. Too many ssDNA breaks would then over-recruit PARP, which activates pathways of cell killing, such as necrosis or apoptosis. A common marker for apoptosis is the activation of caspases, enzymes that are cleaved and activated when apoptosis is signaled. An increased caspase activity of caspases correlates to apoptosis activity happening inside the cells. Caspase activity can be monitored using the colorimetric Glomix caspase 3/7 assay. Activated caspase 3 or caspase 7 recognizes a specific Z\_DEVD sequence and cleaves the bond. The assay contains a substrate that upon cleavage of such specific bond, produces light. The intensity of light can be captured and quantified using luminescence plate reader.

#### *Role of PARP in Est-n-Melex – induced DNA damage repair*

We hypothesize that PARP is involved in the DNA repair mechanism induced by Est-n-Melex. To test this hypothesis, a PARP-inhibitor 3-aminobenzamide (3AB) is co-treated with with Est-n-Melex and apoptosis is quantified. If our hypothesis is correct, treating the cells with Est-n-Melex should induce apoptosis and cause an upregulation of caspase 3/7 activity. Co-treating Est-n-Melex and 3AB should prevent PARP from repair DNA damage, thus causes an accumulation of 3-MeA adducts and upregulation of caspases.



*Figure 12 Est-n-Melex induces cell death via apoptosis, yet the role of PARP in DNA repair induced by Est-n-Melex is unclear. 20,000 MCF-7 cells were plated on a 96 well-plate and treated with 2.5 uM of 3AB overnight, followed by increasing concentration of Est-2-Melex of 0.5 uM and 5.0 uM. Cells 5 uM of Staurosporin (STS) was used as a positive control. Caspase activity were assessed against Ac-DEVD-AMC. The data are as the mean  $\bar{x}$  expressed standard deviation of triplicates.*

Results in Figure 12 shows that 0.5 uM and 5 uM of Est-2-Melex treatment produced more than 2.5 fold increase of caspase activity ( $p = 0.003$ ). Co-treating 3AB and Est-2-Melex however does not produce an upregulation of apoptosis, with no significant difference between without and with 3AB treatment ( $p = 0.071$ ).

## Discussion

### *Est-n-Melex is specific to ER+ cells*

Previous work using Est-n-Melex treatment of ER+ MCF-7 and ER- HEK293 shows that the drug is cytotoxic to cells expressing ER and non-cytotoxic to cells that do not have ER. This suggests that the binding of Est-Melex to the ER in the cell is an important first step for Est-Melex to cause cell death. These results also strengthen our assumption that Est-Melex will cause cell death only in cells that express the ER.

### *Est-n-Melex localization and DNA binding*

The absence of Hoechst, an A/T antagonist in the minor groove of the DNA, allows Est-n-NBD to translocate into the nucleus. The presence of Hoechst reduces the ability of Melex to bind to the minor groove (3-MeA). This positional change from nuclear concentration to cytoplasmic concentration of Est-n-NBD, facilitated by Hoechst, suggests that Est-n-NBD does in fact bind to minor groove A/T rich regions in the DNA. The dose-dependent cytoplasmic concentration of Est-n-NBD shows that pretreatment of Hoechst competitively inhibits minor groove binding of Est-n-NBD. The composite image clearly shows the dose-dependent property of Est-n-NBD in binding to A/T rich region of the DNA.

### *Est-n-Melex induces ER- $\alpha$ nuclear localization*

The presence of a non-cytotoxic concentration of Est-n-Melex allows ER- $\alpha$  to be activated and move into the nucleus. This positional change from cytoplasmic concentration to nuclear concentration, facilitated by Est-n-Melex, suggests that Est-n-Melex does cause ER- $\alpha$  to translocate into the nucleus.

### *Identifying the mode of cell death: Apoptosis*

Upregulation of caspase activity in the presence of Est-n-Melex suggests that apoptosis is involved in the cell death mechanism of MCF-7 cells. It is also interesting to note that no dose-dependent property of Est-n-Melex is observed as there is no significant difference in enzymatic activity as the concentration of Est-n-Melex is increased from 0.5  $\mu$ M to 5.0  $\mu$ M. This means that there might be a compensatory mechanism in place that protects rapid apoptotic activity. The lack of a dose-dependent property may also indicate that the optimal condition for cell killing is not found as well.

### *Role of PARP in DNA-repair mechanism induced by Est-n-Melex*

One interesting observation is that there is no significant difference of apoptotic activity between with and without 3AB pretreatment conditions. This means that either (1) PARP is not involved at all in DNA repair mechanism induced by Est-n-Melex, or (2) 3AB is too weak of a PARP inhibitor, or (3) an optimal condition for blocking PARP is yet to be found. Further research on the optimal PARP blockage is needed to elucidate the role of PARP in DNA-repair induced by Est-n-Melex.

### **Summary**

In conclusion, the translocation mechanism of action of a novel chemotherapeutic compound that can target ER+ breast cancer cells has been confirmed. This compound targets ER+ breast cancer cells and recognizes and binds to ER- $\alpha$ , forming a complex that translocates into the nucleus. Experiments are currently in progress to elaborate further the cell killing mechanism of the drug as well as the role of poly ADP ribose polymerase (PARP) – a DNA repair enzyme – in repairing DNA damage induced by Est-n-Melex. The successful elaboration of the translocation mechanism of this compound demonstrates that this strategy can be used to synthesize new drugs that can preferentially target any cell-type that over-expresses a unique receptor or enzyme.

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