ESTROGEN RECEPTOR MEDIATED DNA METHYLATION IN BREAST CANCER CELLS BY EST-MELEX

by

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ESTROGEN RECEPTOR MEDIATED DNA METHYLATION

IN BREAST CANCER CELLS BY EST-MELEX

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ABSTRACT

Today over 14 million patients fight some form of cancer or another (World Health Organization). In females, breast cancer is the most prevalent cancer with nearly 1.5 million cases diagnosed and 500,000 deaths every year. A characteristic feature of some breast cancer cells is that they over-express the estrogen receptor (ER). Upon binding to its ligand, estradiol, the cytoplasmic estrogen receptor translocates into the nucleus, binds to the nuclear DNA and regulates the expression of various genes. We have designed a DNA methylating agent, called Melex, which specifically enters ER expressing breast cancer cells. We used carbon linkers of variable lengths to conjugate Melex to estradiol (Est-Melex). This, based on the model described above, will allow Melex to be specifically taken up by ER+ breast cancer cells and brought in close proximity to the cancer cell's nuclear DNA which the drug can now methylate and cause cell death. Our collaborators at the University of North Carolina, Wilmington, designed three variants of this drug, each differing only in the number of carbons in the backbone of the linker. The goal of our project is to find the variant that is most efficient in killing ER+ breast cancer cells. Additionally, if we do observe cell death, we wish to find the mode of cell death. Identification of such a drug that is efficient and specific in killing ER over expression in breast cancer cells will give us a new weapon that can be used in our fight against breast cancer.

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WHAT IS CANCER?

Cancer is a disease in which there is an unregulated amount of cell division. This can lead to the formation of a tumor or a mass of cells. Cancers can kill an individual through a variety of means. Tumors can either disturb the function of an organ vital for survival, produce substances that can disrupt the homeostasis of the body and/or deprive neighboring tissue of energy and resources. In 2014, 1 in every 4 deaths was caused directly by cancer or by cancer related complications [1]. The trends for cancer related deaths suggest that this number will only rise in the coming years. This poses a serious problem as our lifestyles are continuously changing in ways that increase the incidence of cancer. The agents that cause cancer, carcinogens, are found everywhere and are relatively easy to come by in our day to day activities. According to the American Cancer Society (ACS), there are three different groups of carcinogens: chemical, physical and biological. Chemical carcinogens include toxic substances like arsenic and the smoke from cigarettes whereas physical carcinogens include harmful ultraviolet and ionizing radiation. Biological carcinogens on the other hand include various pathogenic agents such as viruses and bacteria. For example, one of the most studied causative agents for cervical cancer is a virus known as the Human Papilloma Virus (HPV). A typical cancer could be caused by a single or a combination of these carcinogens. Many of these carcinogens are cancer causing because of their ability to damage cellular DNA. Excessive DNA damage without an equally powerful system of DNA repair can lead to the passing down of mutated genes that either promote uncontrolled cell division or disrupt cell cycle surveillance.

Depending on their ability to move around, tumors can generally be categorized as benign or malignant. Tumors that stay in their site of origin are known as benign tumors

whereas tumors that spread all over the body through the blood are known as malignant tumors. Tumors progress from the benign stage to the malignant stage as mutations accumulate and allow the tumor cell to lose its ability to adhere to its microenvironment. This is the reason why early detection of cancer is important because a localized tumor is much easier to remove than a tumor that has spread to multiple organs around the body.

BREAST CANCER: INCIDENCE, PROGNOSIS AND TYPES

Today over 14 million patients fight some form of cancer or the other (World Health Organization). In males, lung cancer is most prevalent with over a million cases diagnosed each year. In females, breast cancer is most prevalent with nearly 1.5 million cases diagnosed and 500,000 deaths every year [1]. Breast cancer is the development of a tumor in the breast tissue of an individual. The human breast has several parts that serve different functions in reproduction and post-parturition child-care. Two parts of the breast tissue that are the most common sites for the development of a tumor are the milk ducts and the milk producing lobules [2]. Cancers of the duct cells are of two types: Ductal Carcinoma In-Situ (DCIS) and Infiltrating Ductal Carcinoma (IDC). DCIS is when the ductal cells transform to become cancerous in nature. DCIS is a benign form of the cancer and hence can be cured easily. IDC on the other hand represents a malignant state of the ductal carcinoma. In IDC, the transformed cells of the duct move out of the duct and spread into the nearby fatty tissue of the breast [2]. In many instances, the cancerous cells also enter the circulation and spread from the breast to other tissues and organs in the body. According to the ACS, 8 in 10 invasive breast cancers are IDCs. Cancers of the lobules are usually of the invasive type and are known as invasive lobular carcinoma (ILC). Like the IDCs, ILCs have the ability to move from the breast tissue to other organs through the

circulation. The ACS reports that 1 in 10 invasive breast cancers are ILCs. Other lesser common types of breast cancer include inflammatory breast cancer which is caused by the blocking of the lymphatic vessels in the breast by cancer cells, Phyllodes tumor which is found in the connective tissue of the breast, Paget disease of the nipple and angiosarcoma which arises from the blood vessels in the breast. Angiosarcoma develops as a complication of radiation therapy for the treatment of a previous tumor in the breast tissue [2].

BREAST CANCER CELL TYPE CLASSIFICATIONS

Although many individuals might have a single given type of breast cancer, the cells comprising the tumor in each individual might be different. Two individuals with a given type of breast cancer could have tumor cells that differ in their ability to repair DNA (p53 expression) and expression and sensitivity of different receptors (Progesterone Receptor, Estrogen Receptor and Human Epidermal Growth Factor 2 Receptor). Based on these criteria, five different types of breast cancer cells have been cataloged. The five types of breast cancer cells used in research are Luminal A, Luminal B, Basal, HER2, Claudin-low [12]. Luminal A cells express the estrogen and progesterone receptors but do not express the HER2 receptor. Luminal B cells express all of the above receptors whereas Claudin-low cells and Basal cells do not express any of the three receptors. HER2 cells express the HER2 receptor but do not express the estrogen receptor and the progesterone receptor [12].

GENETICS OF BREAST CANCER

Genetic mutations are the underlying causes of cancer. Genetic mutations can be of three types: deletion, insertion or substitution. In a deletion mutation, one or more base pairs in the DNA is removed thus shortening the sequence. In an insertion mutation, a new DNA sequence is added to the pre-existing sequence thus elongating the DNA sequence [8]. In a substitution mutation, a DNA nucleotide is replaced by another base. This neither lengthens nor shortens the DNA sequence but can change the information encoded by the gene [8]. Deletion and insertion mutations are known for their ability to change the reading frame of the DNA, which can lead to the production of truncated peptides [8]. Substitution mutations do not change the reading frame but could still change the primary structure of the protein that could lead to its misfolding. The creation of a non-functional protein can be detrimental to the cell [8]. The human genome codes for about 20,000 genes, some of which help in the controlled replication of cells. These genes are known as proto-oncogenes because they have the ability to cause uncontrolled cell growth if they are unregulated. Proto-oncogenes include those that promote cell growth and proliferation, supervise DNA replication and also facilitate DNA repair when there is DNA damage. As described earlier, carcinogens are those agents that cause mutations in the genome, particularly in the proto-oncogenes. Three tumor suppressor genes that are important when considering breast cancer are BRCA1, BRCA2 and TP53.

BRCA

BRCA1 or Breast Cancer 1, early onset is a gene that is found on the long arm of human chromosome 17. In its normal (wild type or wt) form, BRCA1 codes for a versatile protein that helps in repairing double stranded breaks in DNA, ubiquitination and

heterochromatin formation in X-chromosomes [20]. When mutated, BRCA1 loses its ability to facilitate DNA repair which then allows for accumulation of other mutations in the genome. This increases the chance and allows room for mutations in other proto-oncogenes to go unchecked causing an unmonitored and uncontrolled cell division. Recently, groups have studied and cataloged the mutations found on the BRCA1 genome. They report that a 11 bp deletion, a 1 bp insertion, an acquired stop codon and a missense mutation are all possible mutations that could cause a loss of function in BRCA1 [18]. Knowing what mutations specifically cause a loss of function in BRCA1 is beneficial because it can help in knowing if a patient is predisposed to acquiring breast cancer at a later stage in their life. BRCA2 is also a tumor suppressor gene that plays an important role in DNA repair. It closely resembles BRCA1 in structure and function but it is coded by the long arm of human chromosome 13. BRCA2 associates itself with PALB2 or Partner and Localizer of BRCA2 to bind to phosphorylated histones and regions of double stranded breaks to repair double stranded DNA damage. Mutations in BRCA2 gene sequences cause a change in the folding and specifically in its PALB2 binding site which renders the protein incapable of detecting double stranded breaks and repairing DNA damage [23].

TP53

A tumor suppressor gene of interest is TP53 which is a gene that codes for a protein known as P53. TP53 or tumor protein 53 is found on the short arm of chromosome 17. It has a highly conserved structure because of its importance in cell cycle regulation. When DNA damage is detected, cells phosphorylate and thus activate p53. Activated p53 then, either via a signal cascade pathway or as a transcription factor, turns on multiple genes that function to arrest the cell cycle, cause apoptosis or initiate DNA repair [17]. p53 activated

cell cycle genes include p21 and MDM-2. p21 arrests cell cycle by binding to many cyclin-cdk complexes and preventing them from driving the cell cycle forward. Gadd45 is another gene that is regulated by p53 activation and its gene products help in DNA base excision repair. Apoptotic genes regulated by p53 include Bax and IGF-BP3 [17]. Bax is a member of the mitochondrial outer membrane permeabilization (MOMP) protein family, Bcl-2, and is pro-apoptotic in nature. IGF-BP3 or Insulin like Growth Factor Binding Protein 3 is also a pro-apoptotic gene whose gene products blocks the signaling of mitogenic growth factors which gives rise to downstream effects including apoptosis [17]. Thus, TP53 provides a multi-step system of checks and balances to prevent a normal cell from transforming into a cancer cell and subsequently a tumorous mass in a tissue. Research groups have studied p53 mutations and report that missense or deletion mutations in the TP53 gene can cause a loss in some or all of these functions of p53 which can increase an individual's predisposition to developing a cancer [17].

CURRENT TREATMENTS FOR BREAST CANCER

Chemotherapy

Current treatment for breast cancer depends on whether the tumor is found at an early or late stage. Traditional treatment methods for breast cancer include chemotherapy, hormone therapy and radiation therapy. Chemotherapy involves the administering of chemical substances that have the ability to block the rapid proliferation of a mass of cells. Chemotherapeutic drugs are usually administered orally or intravenously to allow for maximum coverage within the patient's body. Chemotherapeutic drugs can be given to a patient either before (non-adjuvant chemotherapy) or after (adjuvant chemotherapy) surgery [2]. These drugs are usually helpful in boosting the immune system in addition to

targeting certain properties and structures of dividing cells to cause cell death. Since adjuvant chemotherapy is given post-surgery, its main function is to kill any of the remaining cancer cells that may not have been removed by the surgical procedure. Non-adjuvant chemotherapy on the other hand can help make the surgical procedure easier by shrinking the tumor beforehand. Non-adjuvant chemotherapy is the treatment of choice when a patient is first found to have a large tumor [2]. A combination of anthracycline and taxanes is the most popular chemotherapy drug. Anthracycline is a versatile drug in that it has multiple roles in causing cell death of tumor cells. It blocks DNA replication and RNA synthesis by inserting between the base pairs, blocks DNA gyrase from carrying out its function and also modifies histone structure to prevent DNA repair. Taxane on the other hand inhibits microtubule formation. Microtubules are key players in the proliferation of cells. Chemotherapy is usually administered in cycles of 2-3 weeks. This helps the body recover from the chemical stress it is placed in and also helps the physicians determine which drugs were effective for the removal of that patient's particular type of cancer cells [2]. Given this, it is easy to see how chemotherapy can have side effects as well. Many of the chemotherapeutic drugs target cells that are proliferating at a high rate. Cancer cells are one of the many types of cells in the body that divide rapidly. There are other cells such as cells of the bone marrow, cells of the mucosal lining and cells of the hair follicle that also divide rapidly. Administering chemotherapeutic drugs, thus, also sometimes targets the above mentioned cell types for cell death. This manifests itself in the form of side effects such as hair loss, mucosal blisters, low blood cell count and a weakened immune system [2]. Since these cells are fast growing, these side effects observed tend to disappear within a few days after chemotherapy has been discontinued.

Hormone Therapy

Hormone therapy is another mode of treatment for breast cancers. It is usually used as an adjuvant therapy to reduce the reappearance of a cancer in its original tissue. As mentioned earlier, breast cancer cells are categorized based on their expression of different hormone receptors. One of two important receptors in breast cancer cells is the cytoplasmic estrogen receptor (ER). The ER plays many roles in a cell. When estradiol, the ER ligand, binds to the ER, it activates the nuclear localization signals that helps the ER translocate to the nucleus. The ER is also a DNA binding protein which turns on many primary response genes including cell cycle regulatory genes that help the cells proliferate [15]. Usually, this ER-dependent cell proliferation is tightly regulated by controlling the number of activated ER molecules present in the cell. Tumor cells however over express the ER. When a large number of ERs enter the nucleus, the cell cycle is accelerated and the cells transform to become cancerous in nature. This explains how and why the estrogen receptor and estrogen are important target sites in the development of anti-cancer therapeutics [7]. Hormone therapies for breast cancer exploit this fact by employing estrogen receptor antagonists and drugs that lower estrogen levels in the patient.

Out of the many different ER antagonists used in the clinic, the most commonly known are Tamoxifen, Fulvestrant and Toremifene. Commonly, these drugs are known as Selective Estrogen Receptor Modifiers (SERMs) and all bind to the ER and block its estrogen binding site [7]. These drugs differ only in the stage of the disease during which they are used. Tamoxifen is used as the first drug in the treatment when the tumor is benign. If the use of Tamoxifen fails, physicians turn to Toremifene. Toremifene is used only when the patient's tumor enters metastasis. Fulvestrant is the last drug used in hormone therapy

and is known to travel around the body and is used only in advanced stages of metastasis [2].

Hormone therapies also seek to lower estrogen levels in a breast cancer patient. Aromatase is an enzyme that is required for the conversion of testosterone to estrogen. Ergo, estrogen levels can be lowered by blocking or inhibiting the activity of aromatase. Aromatase inhibitors (AI) thus make up the second facet of hormone therapies. Estrogen is produced at two different sites in females: the ovaries and adipose tissues [2]. Its production in the ovaries is controlled solely by the menstrual cycle and hence it cannot be regulated by the use of external factors. However, the production of estrogen in adipose tissues can be regulated by drugs such as AIs. Popular AIs used today are letrozole, anastrozole and exemestane [2]. In severe cases where estrogen levels need to be lowered quickly and significantly, patients usually undergo a combination of AI administration and ovary ablation to reduce estrogen production from both sites. However, this is only the case in pre-menopausal women whose ovaries are still actively producing estrogen. AI inhibitors are administered after SERMs. Regardless of the combination of drugs and the duration of administration selected for a patient, the purpose of using hormone therapies is to lower the rate of the estrogen-ER driven cell proliferation [2].

A drawback in hormone therapy is that it can cause complications in other tissue types. The ACS reports that although SERMs act as anti-estrogen receptor molecules in breast tissue, they have the ability to act like estrogen itself in other tissues such as the uterus. This gives rise to the possibility of this drug inducing cancers in other tissues. Additionally, SERMs can cause thinning of bones because the estrogen receptor is important in calcium absorption. Therefore, female patients undergoing hormone therapy

for breast cancer are at risk of developing osteoporosis through the course of the treatment [2]. Given these drawbacks, hormone therapies are still used today in the fight against breast cancer because it significantly reduces the reappearance of cancers after surgery or other modes of ablative treatment.

Radiation Therapy

Radiation therapy is the third conventional mode of treatment for breast cancers. It is usually given after a surgery as a method to remove any tumorous cells left behind. Radiation therapy involves the use of high-energy particles to cause cell death in the tumor mass. Different types of high-energy radiation rays can be used. ACS reports that photon radiation and particle radiation are the two most common types of radiations used. In photon radiations, the high energy particles are either x-rays or gamma rays whereas in particle radiations, it can be electrons, protons, carbon ions, alpha and beta particles. Radiation therapy can be administered in two different ways depending on the origin of the high-energy radiations.

External Beam Irradiation

In the first type, known as external beam radiation, the photon or particle radiation is given from outside the breast. This involves the careful measurement of dosage and angles at which the rays should be incident on the breast tissue to ensure maximum removal of residual cancer cells. Radiation therapy is given in small doses over a period of five days to prevent over exposure of breast tissue to harmful high energy radiation [2]. Side effects of even regulated exposure to these radiations include redness, swelling and blistering of the exposed area. Recent developments in this treatment method have allowed physicians

to deliver a high dosage of radiation for a shorter period of time in a process called accelerated breast irradiation without increasing the intensity of the said side effects [2].

Brachytherapy

Brachytherapy is the second mode of delivering radiation therapy and it allows for the radiation to stem from inside the tissue region just cleared of breast cancer cells via surgery. Brachytherapy is administered using radioactive pellet containing catheters that are placed inside the breast tissue [2]. Two types of brachytherapy are used today. The first kind of brachytherapy, interstitial brachytherapy, allows for the catheter to be placed in the tissue surrounding the area in which the surgery was performed. The second kind, intracavity brachytherapy, involves placing the radioactive catheter inside the cavity created by the surgery [2]. Short periods of exposure for a few days after the surgery is typical of this treatment type. An advantage to using brachytherapy is that it makes the application and dosage regulation of radiation in the breast tissue easy to monitor and control.

However, brachytherapy, just like the other therapeutic models, comes with side effects as well. One issue physicians have documented with the use of brachytherapy is that it provides a greater chance for the cancer to re-appear when compared to post-surgical treatment using external beam radiation because of its localized, maybe insufficient, field of anti-cancer radiation [2]. A larger issue posed by radiation therapy for breast cancer is that it exposes the rib area and the heart to harmful high-energy radiation. The use of radiation therapy has been reported to cause heart problems in patients [9].

NEW MODES OF TREATMENT

Immunotherapy

A new approach using our body's immune system is being taken in the field of cancer therapeutics. The immune system helps defend our body against harmful disease causing pathogens from the external environment because pathogens presents molecules and proteins that our immune system recognizes as foreign. A problem arises when we consider the pathogenicity of cancer cells. Although cancer cells do present a diseased phenotype, their morphology is not significantly different than our normal non-cancerous cells. Thus, they don't elicit an immune response and evade immune surveillance. This allows the cancer to grow freely and metastasize [6]. Recent approaches in immunology are seeking to introduce modified immune cells or prime our own immune system in ways that will help our body detect, fight and remove cancer cells on their own.

Antibodies are protein mediators of an immune response that bind to very specific proteins/patterns. Their detection pattern is coded by the genome and this helps us devise specific therapies that will not only increase the efficiency of causing tumor cell death but also in reducing damage caused to normal cells. Presently, antibodies are being engineered to bind to cancer specific proteins to thwart cancer growth and eventual metastasis. Current antibody treatments of breast cancer include monoclonal antibodies, e.g., trastuzumab, that bind to proteins like HER2, which helps in the signaling of cell proliferation [6]. Other types of antibodies are also being designed to carry chemotherapeutic drugs with them as they detect and bind to cancer cells thus helping in the specific delivery of drugs. These antibodies are called conjugated monoclonal antibodies [6].

Consistent with other cancer therapies, immunotherapies pose side effects as well. The immune system functions by causing a localized inflammation; a normal response that helps the immune system recruit more immune cells to fight off an infection/disease. Characteristic features of an inflammation include, redness, fever, swelling, nausea and fatigue. Since the use of immunotherapies requires the induction of an inflammation, patients who are administered immunotherapies stand a chance of experiencing the aforementioned side effects [6]. These side effects are however less damaging than the side effects of more traditional cancer therapies.

Targeted Therapies

The ACS defines targeted therapies as a special type of chemotherapy that relies on identification and utilization of small differences between normal cells and cancer cells to cause cell death in cancer cells. Targeted therapies for breast cancer is a particularly feasible model for treatment because as mentioned above, breast tumors are composed of cells that express different receptors in different amounts. Protein activation in cells is controlled via the addition of phosphates to a said protein. Key mediators of this function are enzymes known as Tyrosine Kinases (TK). Many protein targets of TK in a tumor cell are growth factors such as HER1, HER2, HER3 and IGFR. Using TK Inhibitors (TKI) can prevent the activation of these growth factors that largely contribute to the proliferative nature of the tumor [11]. Other molecules targeted during targeted therapies include intracellular signaling factors such as mTOR and ERK. mTOR is also a serine/threonine kinase that belongs to a family of kinases known as phosphoinositol-3-kinase (PI3K). mTOR interacts with a variety of proteins to perform a range of functions in cells. mTOR forms two major types of complexes when it interacts with proteins: mTOR Complex 1 or

mTORC1 and mTOR Complex 2 or mTORC2. mTORC2 is a protein complex that plays key roles in signaling for anti-apoptotic pathways. mTORC2 also regulates cell-cycle activation either via blocking cell cycle inhibitors or removing brakes on cell-cycle activation. mTORC2 does this by blocking activation of a FoxO [16]. Accordingly, another class of drugs in targeted therapies includes mTOR inhibitors that will give back some control of the cell cycle and apoptosis.

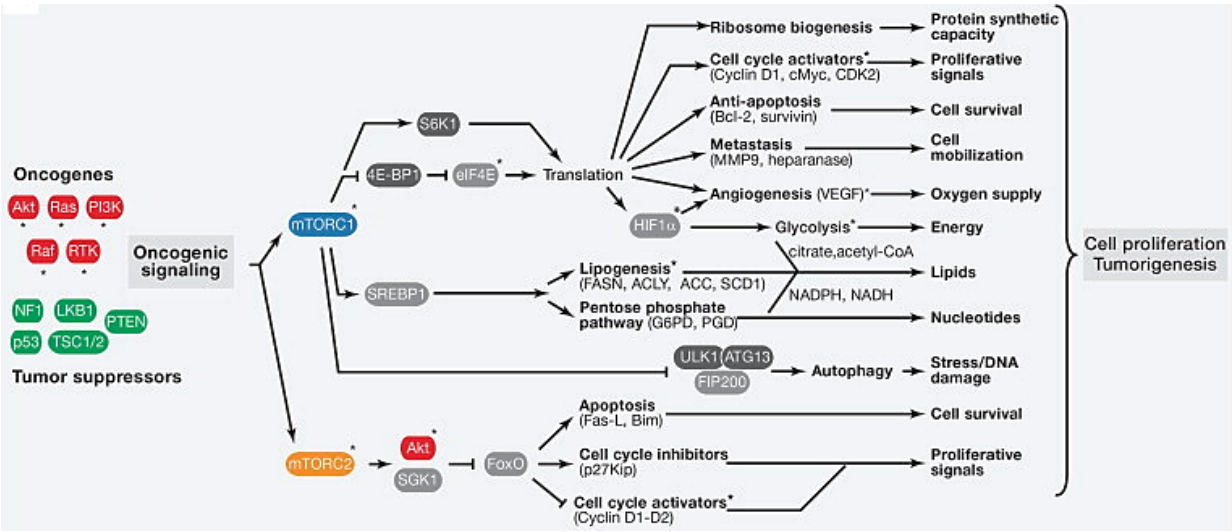


Fig.1: Mammalian Target of Rapamycin (mTOR) promotes the development of the tumor by disrupting oncogenes and tumor suppressor genes [16].

In addition to mTOR and HER family inhibitors, other targeted therapies include anti-angiogenic molecules and anti-DNA repair inhibitors. Given all this and that every breast tumor has a different composition of breast cancer cells, targeted therapies pose a challenge in matching the right set of treatments for every patient willing to undergo targeted therapy [11]. Additionally, since these therapies rely on targeting proteins, there is a good chance that mutations might render a patient resistant to these drugs.

We know that breast cancers cell types are classified based on the expression levels of HER2, the estrogen receptor and the progesterone receptor. The expression of these receptors is many fold higher in cancer cells as opposed to normal cells. Given this cellular difference and the knowledge that a tumor may be composed of more than one type of tumor cells, it is important that we devise more drugs aimed at new cellular targets to create a combinatorial treatment regime that will attack the tumor from many different facets.

Targeting the Estrogen Receptor

The estrogen receptor is one of the three main receptors in the breast cancer cell. The estrogen receptor (ER) is a cytoplasmic receptor whose ligand is estradiol. The ER has six domains; A, B, C, D, E and F. Domains A and B are in the N terminal region of the receptor and are known to weakly turn on downstream gene transcription without ligand. Domain C is the DNA binding domain of the ER. Domain D is the hinge region that possesses the nuclear localization signals. The next domain, domain E, is the region where the ligand estradiol binds to the receptor. Finally, domain F is the C terminal end of the receptor and is known to regulate gene transcription in a ligand dependent manner. [15]

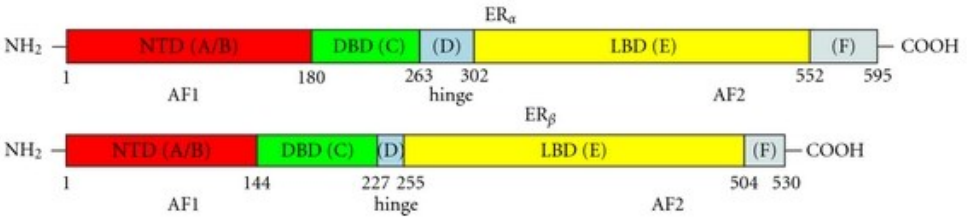


Fig.2: Sequence organization of the estrogen receptor. NTD – Amino Terminal Domain, DBD – DNA binding domain, D-Hinge region and LBD – Ligand Binding Domain [15].

The Lipophilic estradiol molecule diffuses into the cell and binds to the estrogen receptor on its E domain. Binding of estradiol to domain E unmasks the nuclear localization

signal found on domain D on the ER and allows the receptor to translocate to the nucleus where it binds to the genomic DNA using its C domain. Once bound to the DNA, the E domain, depending on whether an agonist or an antagonist is bound to it, recruits co-activators or co-repressors to turn on or off genes. Many of the genes that are turned on by the ER are genes that control cell division [15]. Mutations in breast cancer cells can cause the ER to be over expressed such as seen in Luminal-A and Luminal-B breast cancer cells. Over-expression of the ER in breast cancer cells can lead to an increase in cell division via this pathway. The knock on effects of this accelerated proliferation include an increased chance for a mutation to go unchecked in daughter cells. Unchecked mutations, especially in genes that control the cell cycle and DNA repair mechanisms contribute to the manifestation of a disease phenotype. Although the overexpression of ER itself poses a problem, it can be helpful to develop targeted therapies. The ER is an ideal target for targeted chemotherapy because it (i) is over expressed in breast cancer. (ii) Translocates to the nucleus upon binding to estradiol and (iii) sits on the nuclear DNA to turn on cell proliferation genes [15]. If a cytotoxic drug can be conjugated to estradiol, then that designed molecule, given these attributes, will provide us with an ideal drug delivery model that can specifically attack breast cancer cells in the tumor aimed at the genetic level. This was the overarching goal of our research team. [15]

DESIGNING A DRUG

There are many chemotherapeutic drugs and they range in their mode of activity from causing DNA damage to blocking DNA replication. One of the most well studied ways to prime a cell to undergo apoptosis is to create epigenetic changes on the cell's genetic material. Methylation of DNA is one such epigenetic modification. Methylation of

DNA is known to manipulate gene expression by modifying chromatin structure. Often times, the methylation of DNA down regulates gene expression of essential genes, which forces the cell to undergo programmed cell death. One such commercially available DNA methylating agent is Melex. Melex was selected as the chemotherapeutic part of our targeted chemotherapy model because of its high cytotoxicity and low mutagenicity [3].

Additionally, Melex was shown to diffuse into cells in small quantities, which suggested that the Melex molecule had the ability to cross the plasma membrane of the cell.

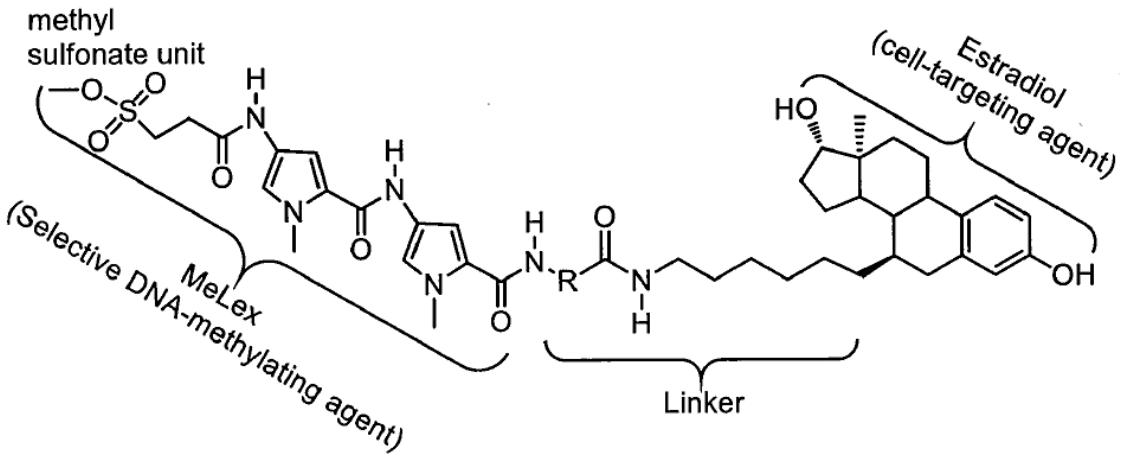


Fig.3: Organic structure of Est-Melex; Melex conjugated with Estradiol [13].

In order to help Melex target breast cancer cells more specifically, our collaborators chemically conjugated the ER ligand, estradiol, to the Melex molecule via simple hydrocarbon linkers. Given the background on breast cancer ER expression and function, we hypothesized that this estradiol-Melex molecule, which we call Est-Melex, should increase the efficiency and specificity with which we can cause cell death in ER+ breast cancer cells. In theory, Est-Melex should play the role of a Trojan horse. The estradiol part of the drug should allow the molecule to bind to the estrogen receptors of ER+ breast cancer

cells efficiently. Once Est-Melex and ER form a complex, the D domain of the ER gets exposed which allows the ER-Est-Melex complex to translocate to the nucleus. Inside the nucleus, this complex would sit on the DNA using the C domain of the ER. While this may turn on other cell cycle genes that will help in the proliferation of the cell, it will also bring the DNA methylating agent in close proximity to the cell's nuclear DNA. Melex will now have an opportunity to methylate bases on the DNA, which can ultimately trigger cell death. Thus, in this manner, we predict that ER+ breast cancer cells treated with Est-Melex would show more cell death per unit mass of drug used when compared to similar treatments with only Melex. This prediction, if supported by the results, would thus demonstrate the effectiveness of developing and employing targeted chemotherapies in a cancer treatment regimen.

In order to show that this model works as predicted, our collaborators at UNCW tested and reported the binding behavior of ER and Melex by using antagonists of their target sites [13].

METHODS

Cell Culture

MCF7 cells and 293 HEK cells were grown in complete Dulbecco Modified Eagle's Medium (DMEM) and incubated at 37°C and 5% CO_2. Complete medium was made by adding 10% v/v FBS, 1%v/v Penicillin-Streptomycin and 1% v/v Non-Essential Amino Acids to 500 mL of DMEM. 0.25% Trypsin-EDTA was used to disrupt cells from the surface of attachment in the flasks.

Cytotoxicity Assay

5000 cells/well were plated in a Greiner 96 well plate the day before the drug treatment in 100 μL of DMEM/well. The plate was split into four quadrants where each quadrant received a different drug variant. Another 96 well plate was plated to test the controls. The drugs were administered to the cells 12 hours post plating. The drugs themselves were reconstituted just prior to drug treatment. The designated drugs were reconstituted in DMSO to create a 5 mM stock of each drug. Six different concentrations were created to allow for final concentrations in the well to range from 100 μM to 6.25 μM by performing a serial dilution with DMSO on the stock solution. Each concentration for each drug had four replicates to take any outliers into account. 2 μL of each drug was added to the respective wells and this treatment was allowed to incubate for 12 hours. 12 hours post-treatment, an MTT assay was performed to measure for cell survival.

MTT Assay

Cell plates were removed from the incubator and the medium in the wells was poured out. MTT was dissolved in serum free medium to get a concentration of 1mg/mL. 100 μL of the MTT-serum free medium mixture was added to each well. The plates were returned to the incubator for 4 hours for the MTT to be broken down by the mitochondrion (found only in living cells). MTT is broken down by the mitochondrion (found only in living cells) to produce a precipitate that dissolves in DMSO to give a purple coloration. Therefore the more the number of living cells in a well, the darker the observed coloration. After the 4-hour incubation period, cell plates were removed from the incubator and the MTT-serum free medium mixture was poured out of the plates. Each well then received 100 μL of DMSO which dissolved the precipitate produced by the MTT assay in each well

to give every well its unique coloration that helped quantify cell survival. The plates were then left on the gyratory shaker for 5 minutes to enhance dissolution of the precipitate. The plates were then analyzed in a spectrophotometer. The absorbance for each well was measured at a wavelength of 540 nm. The absorbance in each well was normalized with the absorbance of a blank well included in the experiment. The average absorbance for each concentration for each drug was calculated and reported as a percent of the cell survival observed in the control group that received no drug treatment. This quantified data was plotted against the actual drug concentration to obtain a cytotoxicity graph.

Western Blot

MCF-7 cells were plated in a 6 well plate at a density of 200,000 cells/well. 12 hours later, the designated drugs were added. The cells were harvested 12 hours post treatment and lysed using 30 μL lysis buffer per well. The cells were centrifuged to separate the proteins from the cell components. 25 μL of the proteins, collected in the supernatant, were transferred to new tubes and received SDS PAGE lysis buffer. This mixture was boiled at 95°C for 10 minutes. The rest of the 5 μL were used to carry out a Bradford assay to determine the amount of protein present in the supernatant. 7% Polyacrylamide gels were poured 1 hour prior to carrying out the experiment. The gels were loaded on to the gel holders and placed inside the gel running box. The box was filled with 1x gel running buffer. 1x gel running buffer was made up from 5x gel running buffer (1.5% w/v Tris HCl, 7.2% w/v Glycine, 0.5% SDS). Each sample was then loaded on to the gels and run for an hour at 100V. The gels were then used for the transfer process. A semi-dry transfer method was used in our experiments. 1x Towbin transfer buffer was made up from 4x Towbin transfer buffer. A gel-nitrocellulose membrane sandwich was created in the semi-dry

transfer apparatus and the transfer process was carried out for an hour under 115 mA of current and maximum voltage. The membranes were then blocked with BLOTTO (5% w/v dried milk powder in TBST). After blocking, primary antibodies (α-PARP and α-Caspase-7) were added to the membrane. Primary antibodies were at a 1:1000 concentration and were dissolved in BLOTTO. The membranes with the primary antibodies were let to incubate overnight at 4°C. The next day, the primary antibodies were removed and the membranes were washed thrice with more BLOTTO. Secondary antibodies were added after this step. Secondary antibodies were made at a concentration of 1:10000 and were dissolved in BLOTTO. The membranes were washed thrice with more BLOTTO. The membranes were then washed with AP staining solution (0.66% v/v NBT, 0.33% v/v BCIP) until the protein bands became visible. The membranes were then washed with water and wrapped in clear wrap. [4]

RESULTS

Testing for the Target of Estradiol

The first interaction Est-Melex would have in a cell is the one between the ER and estradiol. Estradiol is known to bind to the estrogen receptor with high affinity [14]. In order to verify this interaction in our experimental set up, our collaborators treated MCF7 ER positive cells with Est-Melex and either with or without Fulvestrant, an ER antagonist [13. 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 estradiol domain of the Est-Melex bound to the ER as predicted, then the treatment group that received Fulvestrant should exhibit lesser cell death when compared to the treatment group that did not receive Fulvestrant. The results from this experiment are published below. We

can see that in the absence of Fulvestrant, MCF7s exhibit a concentration dependent cell death whereas in the presence of Fulvestrant, MCF7 cell death plateaus around the 100% cell survival range which suggests that there was little, if any, cell death in this treatment group.

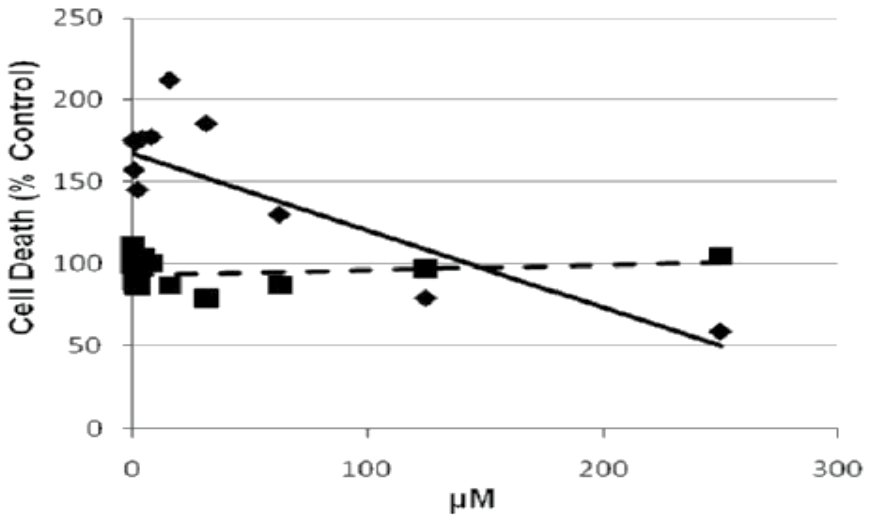


Fig.4: Toxicity of Est-4-Melex in MCF-7 cells in the absence (diamonds) and presence (squares) of ER antagonist Fulvestrant. Est-4-Melex was not toxic in MCF-7 cells pre-treated with Fulvestrant [13].

Testing for the Target of Melex

The next important step in the movement of Est-Melex within the cell is in the binding of the Melex end of the drug to the DNA of the ER+ cell. Our collaborators at UNCW knew that Melex was a potent DNA methylating agent but the exact site of methylation was unknown [13]. Therefore, we used a DNA minor groove antagonist known as Netropsin. Netropsin also has the property of binding specifically to A/T rich regions in the minor groove of DNA [13]. Its affinity to minor groove A/T rich sites was much higher than that of Melex or Est-Melex. In this experiment, our collaborators treated MCF7 ER+ cells either with Melex, Est-Melex or Methylmethane Sulfonate (MMS). MMS

contains only the methylating domain of the Melex molecule. Each experimental group was treated either with or without Netropsin. Therefore, our collaborators predicted that if Melex binds to A/T rich regions in the minor groove of DNA, then the number of methyl adducts in the minor groove (3-MeA) found in the experimental group that received a Netropsin treatment would be fewer than those found in the experimental groups that did not receive a Netropsin treatment because of competitive inhibition. The results of this experiment showed a reduction in the number of minor groove methyl adducts found in Netropsin^{+} Melex and Est-Melex treatment groups.

Compound	Concn (μM)	Netropsin (μM)	Adduct level (μmol adduct/ mol DNA)	
			3-MeA	7-MeG
1	50		1764 ± 17	280 ± 36
	50	100	167 ± 5	279 ± 43
MMS	5000		1799 ± 39	18,067 ± 76
	5000	100	1239 ± 88	18,935 ± 502
Me-lex	100		14,078 ± 224	496 ± 31
	100	100	657 ± 12	614 ± 67

[a] DNA (1 mM) was reacted with different methylating compounds, in the presence or absence of netropsin, for 24 h at room temperature in 10 mM sodium cacodylate buffer (pH 7.0) containing 10% DMSO.

Table.1: DNA adduct levels observed when treated with different compounds [13].

Specificity of Est-Melex

To show that Est-Melex owes its specificity to the presence of the ER, our lab treated an ER+ breast cancer cell line, MCF7 and an ER- human embryonic kidney cell line, 293 HEK, with Est-4-Melex. An MTT assay was performed 12 hours post treatment and percent cell survival for each of the two cell lines was measured. The cell survival data was then plotted against concentration of Est-4-Melex. From the plot chart, we observed

that ER+ MCF7 cells showed a concentration dependent cell death when treated with Est-4-Melex whereas ER- 293 HEK cells showed no cell death irrespective of how much drug was administered to the cells.

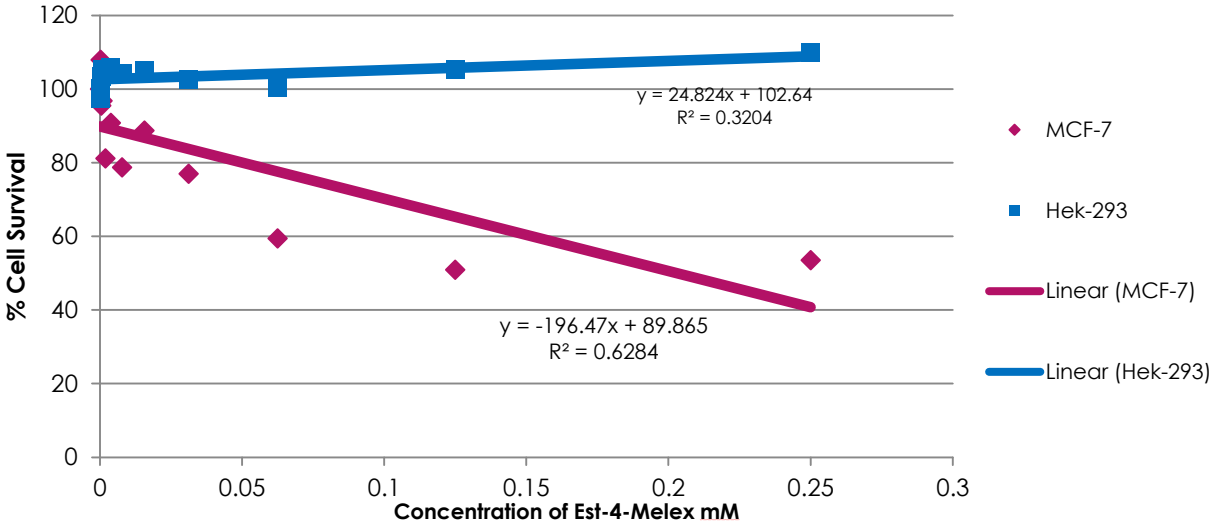


Fig.5: Est-Melex owes its specificity to the presence of the estrogen receptor. Cell death is seen only in cells that express the estrogen receptor. [Mayur Patel and Giridhar Rao Akkaraju unpublished observation.]

Modifying Est-Melex

The previous experiment suggested that Melex binds to A/T rich regions of the DNA minor groove because 3-MeA were reduced in the presence of Netropsin. Additionally, past experiments suggested that Est-Melex translocated to the nucleus along with the ER. A missing piece in this puzzle is the optimal distance between the ER binding site on the DNA and A/T rich regions in the DNA. This piece of information was unknown but critical in determining the efficiency of the drug in creating methyl adducts as the right reach will allow Melex to fit and methylate DNA effectively. The right fit of the amide nitrogen atoms of Melex along A/T rich regions in the DNA will best align the methyl donating domain of the Est-Melex with an adenine, the base that Melex methylates. This

best alignment will allow for the most efficient methylation. Therefore, in order to find the best configuration of Est-Melex that will allow an optimal reach for the Melex molecule to an A/T rich region from the binding site of the ER, our collaborators theoretically proposed variants of Est-Melex, which differed only in the length of the hydrocarbon linker connecting estradiol and Melex. The first variant had a two-carbon linker and was called Est-2-Melex whereas the second variant had a three-carbon linker and was called Est-3-Melex. The last variant of Est-Melex had four carbons in its linker and was called Est-4-Melex. Subsequently, controls of all these variants were also made by substituting the sulfonate group in the methyl-donating domain of Melex with a sulfone group. The controls were called Est-n-Sulfones instead.

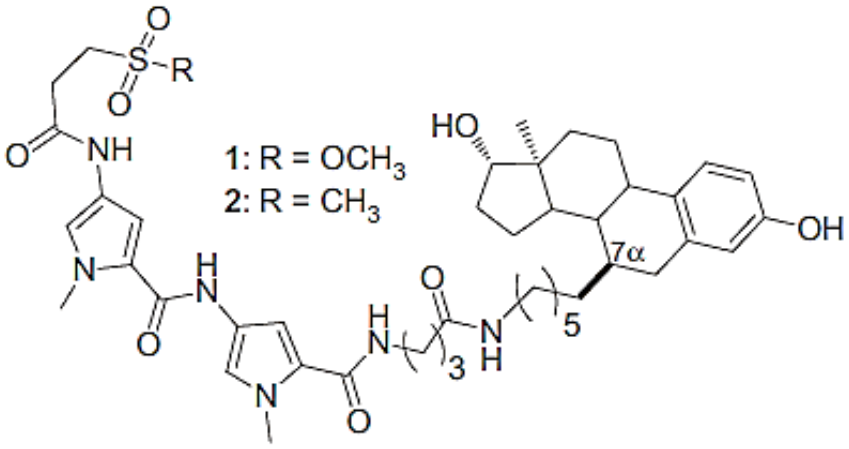


Fig.6: Functional Est-n-Melex and control Est-n-Sulfone drugs were created by changing the R group on the methylmethane sulfonate group. R_1 is found in Est-n-Melex drugs whereas R_2 is found in Est-n-Sulfones. [13]

Understanding Differences in Cytotoxicity using Computational Chemistry

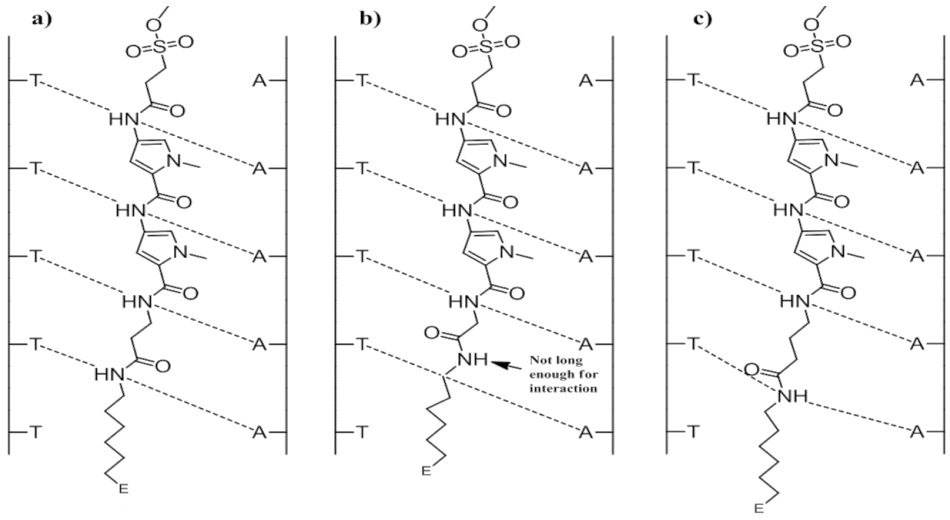


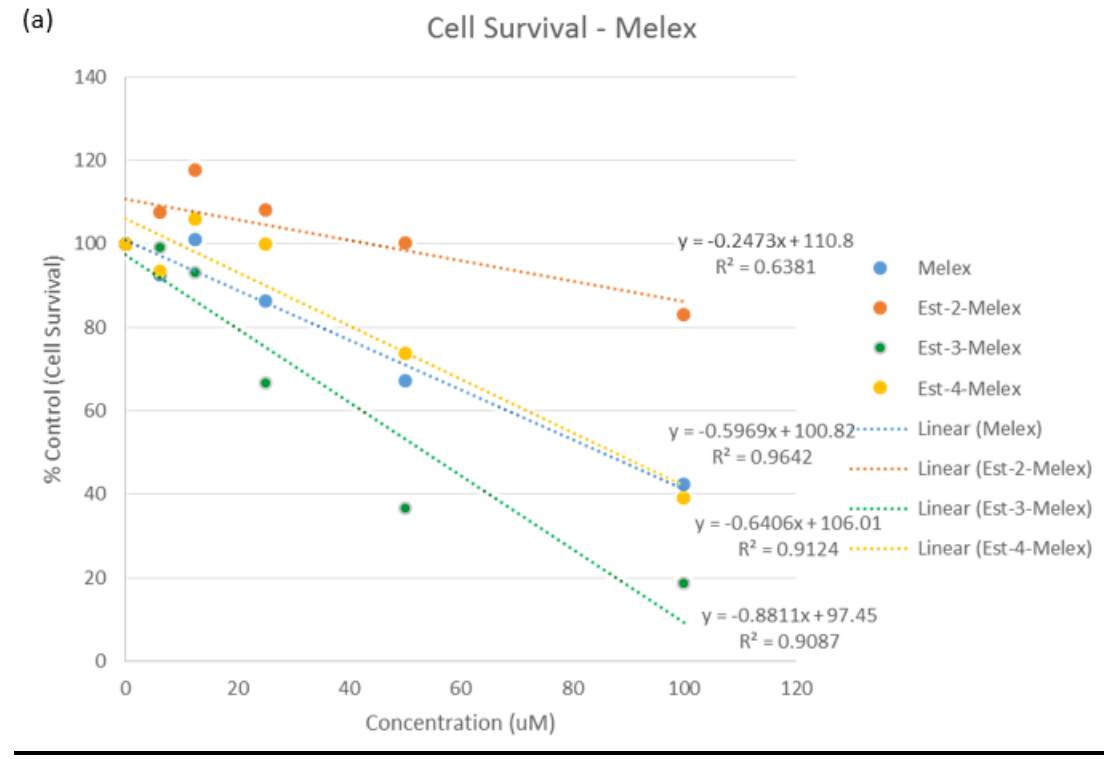
Fig.7: Computational chemistry predictions on binding patterns of (a) Est-3-Melex, (b) Est-2-Melex and (c) Est-4-Melex [Sridhar Varadarajan unpublished observation]

To better understand how changing the linker length of Est-Melex will affect efficiency of the variant, our collaborators at UNCW studied the binding patterns of the Est-Melex variants to the A/T rich regions of DNA using computational chemistry. Computational chemistry is a branch of chemistry that uses computer models to study the interaction between different molecules. The results from the analysis of Est-n-Melex-DNA interactions suggested that the different hydrocarbon linker lengths gave Melex different binding efficiencies. Est-2-Melex (b) with its two-carbon linker was predicted to have too short a reach for all the amide groups of Melex to fit in to A/T rich domains. Est-4-Melex (c) with its four-carbon linker was predicted to allow the amide groups of Melex to bind to DNA by only distorting the A/T rich domains. On the other hand, Est-3-Melex, with its three-carbon linker, was predicted to have the right reach needed from the ER binding site on the DNA to allow the amide groups of Melex to bind to the A/T rich regions of DNA. Thus, data from this computational chemistry study suggested that Est-3-Melex should have the best efficiency in causing DNA methylation.

Est-n-Melex Cytotoxicity Assays

We hypothesized that attaching estradiol to Melex would increase the specificity and efficiency with which Melex can cause cell death in ER+ breast cancer cells. In order to test this hypothesis, we designed an experiment in which MCF7 cells were treated with increasing concentrations of both Melex and Est-n-Melex drugs to see whether the addition of estradiol to Melex changes the efficiency with which we can cause cell death. The corresponding controls were also screened to check whether the treatment process by itself contributed to the cell death observed in the experimental group. Additionally, given the results from the computational chemistry study, we predicted that the best linker variant, the three-carbon linker of Est-3-Melex, will give Est-3-Melex the best reach to A/T rich regions on the DNA from the ER binding site. Est-3-Melex, we predicted, would show the most efficient cell death per unit mass of drug used. We analyzed drug efficiency by measuring effective concentration for 50% cell death (EC50) values. For this experiment, we plated 5,000 MCF7 cells per well in two 96 well Greiner plates and let the plates incubate 12 hours at 37°C before the drug treatment. Each plate was split into four quadrants for four different treatments. One plate received the functional Melex and Est-n-Melex drugs while the other received the control Est-n-Sulfone drugs. The next day, we treated the cells with the drugs at the concentrations indicated and incubated the plate at 37°C for another 12 hours. Also, for each drug concentration, we had four replicates. At the end of the 12-hour treatment period, we performed an MTT assay on both the plates to measure for cell survival. Absorbance for the purple coloration produced by the MTT assay was then measured in a spectrophotometer to get quantitative data. A high observed absorbance after the MTT assay is an indicator of low cell death in that well. To quantify

cell death, we calculated absorbance for each concentration as a ratio to that drug's control group that did not receive that particular drug. Thus, we reported cell death in terms of cell survival as percent of control. We graphed percent cell survival for each concentration of each drug treatment against drug concentration in a simple plot graph. Next, we extracted a trend line for each drug type and analyzed the r-squared value to measure the confidence of our data. We also extrapolated the equation of the trend line and used it to measure the EC50 value for each drug. The EC50 value would inform us about the amount of drug needed in μM to cause 50% of the plated cells to die as a result of the treatment. Therefore, a lower EC50 value would be indicative of a more efficient drug. The results of our experiments suggested that (i) all our drug variants were potent because each of the treatment groups showed a concentration dependent cell death. (ii) our drug variants, which differed only in the number of carbons in the linker backbone, had different efficiencies in causing cell death.



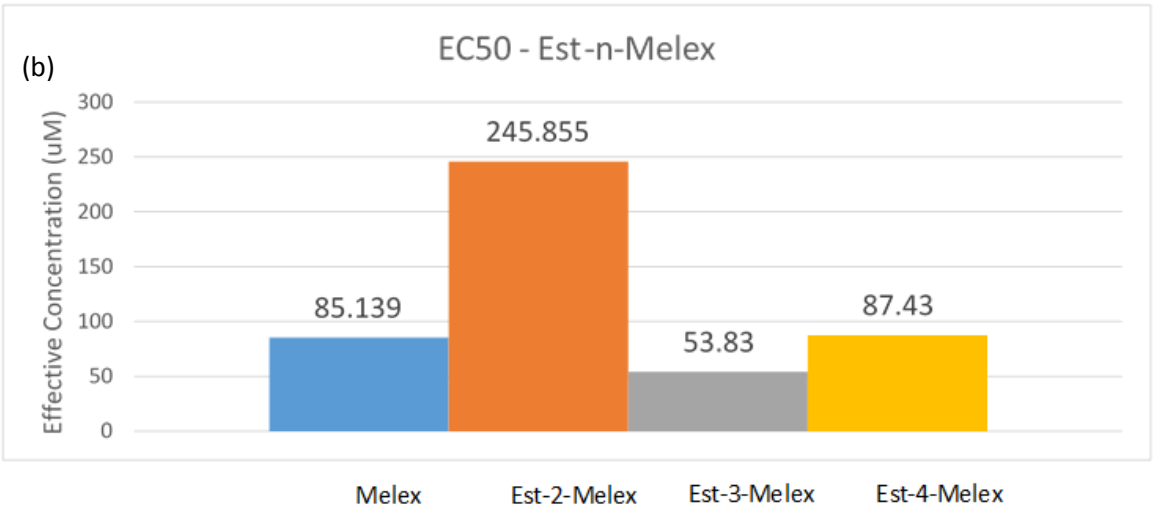


Fig.8: (a) Comparing the cytotoxicity of Est-n-Melex. (b) Effective Concentration of drug required for 50% cell death. The lower the EC50 value, the higher the potency of the drug. There is a variation in the cytotoxic efficiency among the four molecules. The molecule with the highest cytotoxic effect was Est-3-Melex with an EC50 of 52.4 µM.

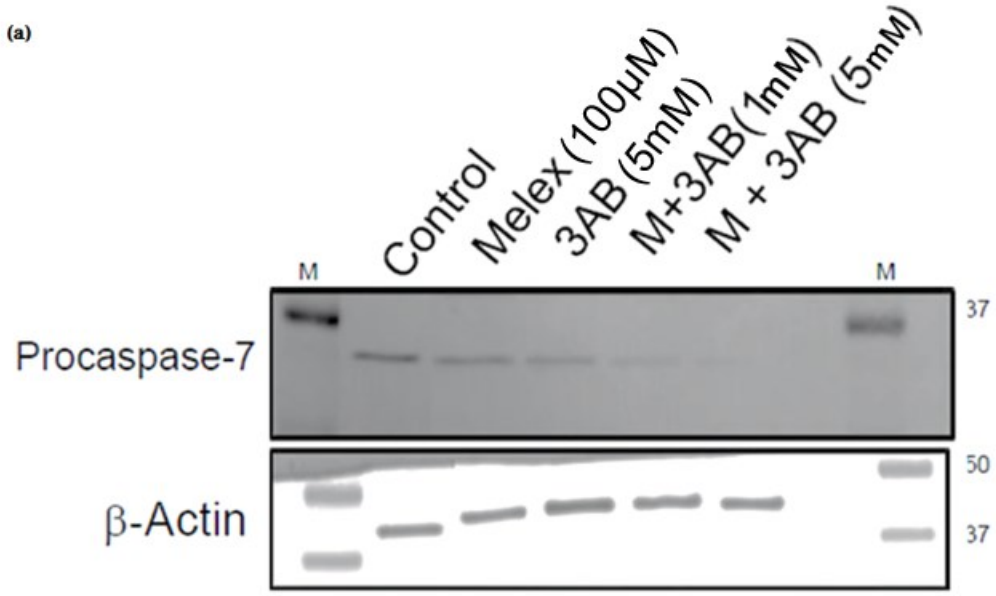
From Necrosis to Apoptosis with PARP Inhibitors

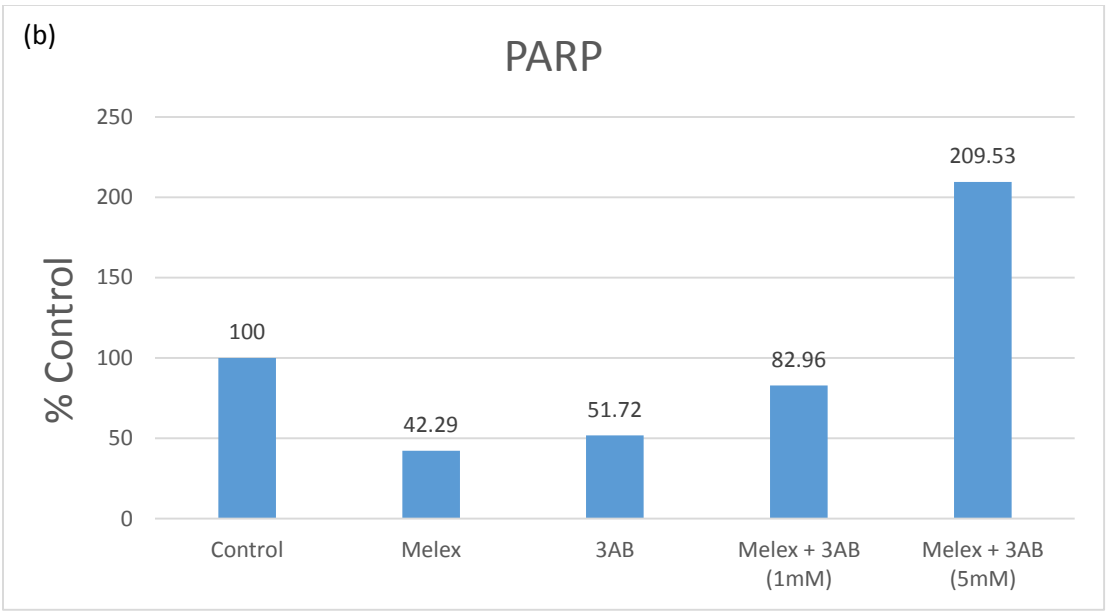
Our collaborators report that Melex causes cell death via different modes depending on the condition of the cell. The methylation caused by Melex in a normal cell will cause an over-activation of a DNA repair enzyme known as Poly-ADP-Ribose Polymerase (PARP). PARP uses AP endonuclease and glycosylase to cut the methylated base out and insert the correct base instead in its place via base excision repair. This process requires energy in the form of ATP. Melex causes an over-activation of PARP which causes a depletion in the ATP available to the cells. This causes cell death via necrosis. In contrast, if PARP activation were inhibited, then the methylation caused by Melex would lead to the accumulation of methyl groups. Excessive DNA damage would then trigger the cell to undergo cell death via apoptosis. Necrosis and apoptosis are two different modes of cell death with their own characteristic features. Damaged cells, such as self-reactive T and B cells, in the body undergo apoptosis every day. Apoptosis is thus a mode of housekeeping for the body wherein unwanted cells are removed in a tightly regulated manner. Apoptosis is also known as programmed cell death. There are many molecular markers to identify apoptosis in cells. The most commonly studied markers of apoptosis are caspases. Caspases in their default are found in an inactive state known as procaspases. Procaspases, upon receiving the right apoptotic signals from the cell, get activated into smaller active caspases by cleavage. Cleaved activated caspases then trigger other proteins to mediate apoptosis. Therefore a reduction in the amount of procaspases suggests that there are more active caspases found in the cell and that cell death in that treatment group is via apoptosis. Caspases can be studied by analyzing the proteins from the cell lysate using polyacrylamide

gel electrophoresis (PAGE), detected by transferring the proteins to a nitro-cellulose membrane and identified by using antibodies. This process is known as a western blot.

It is known that the 3-MeA caused by Melex causes an over activation of PARP that causes cell death via necrosis [19]. Necrosis is not the most desirable form of cell death for a therapeutic drug as it comes with consequences such as tissue scarring, energy exhaustion and inflammation. These are characteristics that will only add to the problems faced by a patient fighting cancer. Apoptosis on the other hand is not accompanied by any of the above consequences and is therefore a more beneficial way with which we can target cells for cell death. Therefore, to see whether we can induce apoptosis, instead of necrosis, by using our drug, we treated MCF7 ER+ breast cancer cells with Melex and 3-Aminobenzamide (3AB), a PARP inhibitor. Cells were treated with Melex and increasing amounts of 3AB for 12 hours following which cell lysates were prepared and analyzed by SDS PAGE and western blot. The levels of all proteins of interest were normalized to that of β-Actin that served as a loading control. Based on our understanding of how PARP inhibitors divert cell death caused by Melex from necrosis to apoptosis, we predicted to see lowered levels of procaspase-7 in our treatment groups that received Melex and 3AB as compared to our treatment group that only received Melex. We also expected this difference to be more pronounced in the treatment group that received more 3AB. We therefore also predicted to see a smaller amount of un-cleaved PARP in the Melex only treatment group as compared to the control group. Additionally, we predicted that the addition of 3AB to any treatment group would increase the amount of un-cleaved PARP found. Therefore we hoped to see an increase in PARP levels in our Melex and 3AB treatment group.

Our results showed that the Melex only treatment group (100 μM) had lesser PARP and nearly equal amounts of procaspase-7 when compared to the control group. The 3AB only treatment group, which received 5 mM of 3AB, had more PARP than the Melex only treatment group but smaller amounts of procaspase-7 when compared to the control group. The treatment group that received Melex and 1 mM of 3AB had more PARP than the 3AB only treatment group and even smaller amounts of procasapse-7 when compared to the control and Melex only treatment group. This effect was more prominent in the treatment group that received Melex and 5 mM of 3AB.





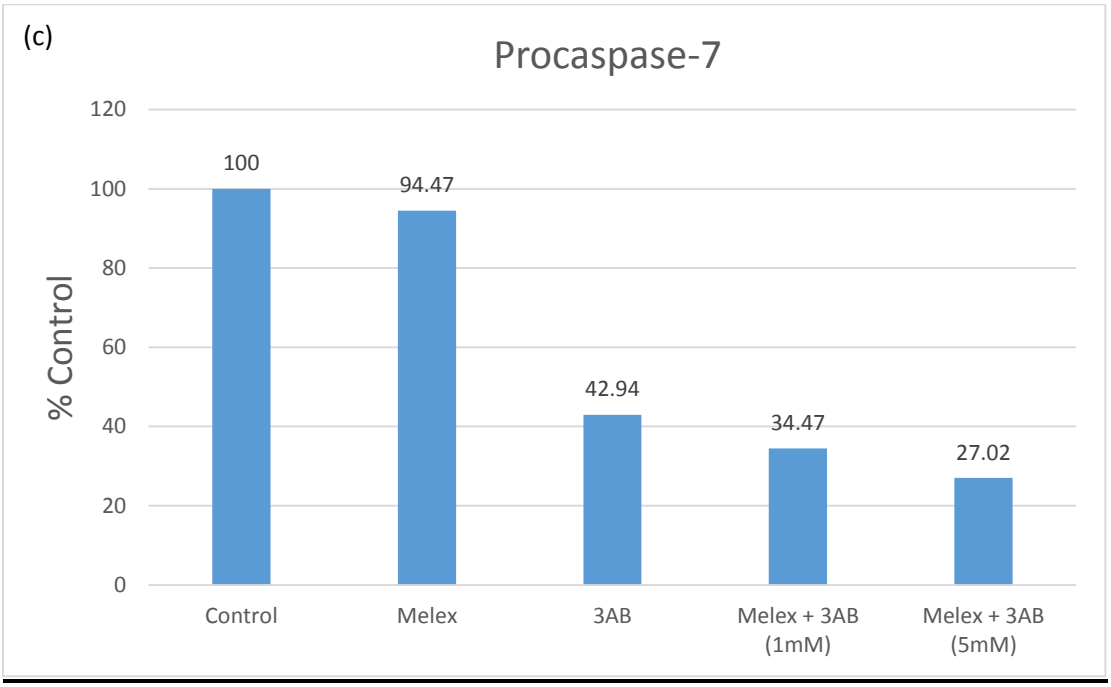


Fig.9: Protein analysis of apoptosis indicators. (a)Western blot analysis of procaspase 7 and loading control β-Actin. (b) PARP density as a ratio of β-Actin density (c) Procaspase-7 density as a ratio of β-Actin density.

DISCUSSION

Estradiol Binds to the ER

Previously, work in our lab using an ER antagonist, Fulvestrant, has shown that estradiol bound to the ER. When the estradiol binding domain of ER was blocked by Fulvestrant, Est-Melex could not cause cell death. This suggests that the binding of estradiol 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. [13]

Melex Binding Site

The presence of Netropsin, an A/T antagonist in the minor groove of the DNA, reduced the ability of Melex to cause methyl adducts in the minor groove (3-MeA). This reduction, facilitated by Netropsin, in the number of 3-MeA created by Melex suggests that Melex does in fact bind to minor groove A/T rich regions in the DNA. Melex showed a greater ability to produce 3-MeA because of its smaller shape when compared to Est-Melex. In a more clinical setting, the increased specificity with which Est-Melex can cause methylation in breast cancer cells would shadow the lowered efficiency of Est-Melex in causing methyl adducts as compared to Melex. Additionally, DNA major groove methylation is not altered very much in Melex and Est-Melex groups in the presence of Netropsin, which suggests that Melex's methylation target site is specifically the adenines of the minor groove of the DNA.

Est-Melex Specificity

The fact that Est-Melex caused cell death only in ER+ MCF7 cells and not in the ER- 293 HEK cells suggests that Est-Melex owes its specificity to the presence of the ER [Mayur Patel and Giridhar Rao Akkaraju unpublished observation]. This specificity of Est-Melex will increase the utility of this chemotherapeutic drug in an anti-cancer treatment regime because given the concentrations at which Est-Melex targets ER+ cells it will in theory not harm normal naïve cells around the tumor. This will thus reduce the side effects we commonly associate with chemotherapy.

Est-Melex Efficiency

From the cytotoxicity data, it can be seen that Est-3-Melex was the most efficient drug variant. It exhibited the best ability to cause cell death in ER+ breast cancer cells as seen by the EC50 values obtained. Est-3-Melex had an EC50 value of approximately 54 μM which is lower than the EC50 exhibited by the other variants: 245 μM for Est-2-Melex and 87 μM for Est-4-Melex. In other words, 54 μM of Est-3-Melex would have the same effect as that of 245 μM of Est-2-Melex and 87 μM of Est-4-Melex. Est-3-Melex therefore is the best drug to develop for a clinical treatment regime because it would be beneficial for a patient to receive and process smaller doses of a drug. Data from other experiments (not published here) also consistently suggested that Est-3-Melex was the most efficient drug variant thus indicating that this observation does not occur by chance. We also see that Est-3-Melex is more efficient at causing cell death in MCF-7 cells than Melex by itself. This suggests that connecting estradiol to Melex increased the efficiency with which Melex could cause cell death in ER+ breast cancer cells thus supporting our hypothesis. These

results from the cytotoxicity assays are also congruent with our computer model Est-Melex-DNA binding predictions.

The computational chemistry data also sheds more light on the anomalies seen in our cytotoxicity data. Est-2-Melex and Est-4-Melex, counter-intuitively, seem to be less efficient than Melex and we propose that this is possibly because of the effect imposed by their shorter than required and longer than required linker lengths, respectively, for the effective binding of Melex amides in A/T rich domains. Additionally, between Est-2-Melex and Est-4-Melex, a longer than necessary linker at least allows for all the amide groups of Melex to align with A/T rich regions easily. We thus propose that this is the reason why Est-2-Melex, with the shortest linker length, shows the lowest efficiency of DNA methylation among the different Est-Melex variants.

Therefore, in general, Melex binding ability was altered by modifying linker lengths in Est-Melex. The different binding abilities thus conferred different methylation efficiencies, which then manifested itself in the form of differing observed cytotoxicity for the different variants.

Dictating the Mode of Cell Death

The mechanism by which Est-3-melex causes cell death is of interest. As discussed above in the results, we knew that PARP is over activated when MCF7 cells get treated with Melex and that this triggered cell death via necrosis [21,22]. In our protein analyses experiments, we looked for PARP cleavage and Procaspase-7 cleavage. To induce apoptosis, we used a PARP inhibitor known as 3-Aminobenzamide along with Melex and this was predicted to decrease DNA repair mediated by PARP and induce apoptosis as a

result, instead of necrosis [19]. If a switch in the mode of cell death was possible, then we predicted an increase in the amount of un-cleaved PARP and a decrease in the amount of procaspase-7 (more active caspase-7) in our Melex+3AB combination treatment, both of which are indicators of apoptosis.

From our protein analyses we were able to observe an inverse relationship between PARP levels and procaspase-7 levels. The Melex only treatment group (100μM), in comparison to the control group, had a smaller amount of un-cleaved PARP and an almost equivalent amount of procaspase-7 suggesting that over activation of PARP triggered cell death via necrosis in that treatment group. In the Melex+3AB treatment group that received 1mM of 3AB, we saw an increase in the amount of un-cleaved PARP, when compared to the Melex only treatment group, and a decrease in the amount of procaspase-7 suggesting that inhibition of PARP after adding methyl groups to the DNA triggered cell death via apoptosis. This effect as mentioned earlier was more pronounced in the Melex+3AB group that received 5 mM of 3AB. Therefore, the combinatorial treatment approach is shown here to divert cell death away from necrosis and move it towards a more desirable apoptosis.

One interesting trend observed in this experiment is in the 5 mM 3AB only treatment. The 3AB treatment group by itself showed a decrease in the amount of procaspase-7 as compared to the control group and an increase in the amount of inactive PARP as compared to the Melex only treatment group. This suggested that there was some apoptosis observed when we added only 3AB. PARP as mentioned earlier is a DNA repair enzyme. Although DNA polymerase is very efficient at synthesizing DNA strands following the Watson-Crick base pairing rules, it is not completely error free. These mismatches are common and are repaired with base-excision repair techniques that uses

the enzyme PARP. Therefore, when we inhibit PARP in a normal setting, it prevents the cell from repairing its DNA damage and this could potentially act as a trigger for apoptosis in that cell. We propose that this is the reason for the reduction in the amount of procaspase-7 and the increase in the amount of un-cleaved PARP in the 3AB only treatment group.

SUMMARY

Thus, it is shown here that the attachment of estradiol to a chemotherapeutic drug, Melex, increases the specificity and efficiency with which Melex can cause cell death in ER+ breast cancer cells. Also shown was that Est-Melex owes its specificity to the presence of the ER. Variants of the Est-Melex drug were created by changing the linker length and their efficiencies at methylating DNA were predicted using a computer program. Est-3-Melex was predicted to be the most efficient variant and it was subsequently proven to be true with the help of cytotoxicity assays using the Est-Melex variants on MCF-7 ER+ breast cancer cells. The mode of cell death caused by Melex was also shifted from necrosis to apoptosis with the help of PARP inhibitors such as 3-aminobenzamide. This is proof of principle of a targeted therapy using a feature specific to the cancer cell. In theory, this approach could be used for any steroid hormone-responsive cancer cell.

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