CM-Dil AND MCF-7 BREAST CANCER CELL RESPONSES TO CHEMOTHERAPEUTIC AGENTS

by

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ABSTRACT

CM-DiI and MCF-7 Breast Cancer Cell Responses to Chemotherapeutic Agents

CM-DiI is a lipophilic, red fluorescent dye used for staining and tracking the migration of cells. CM-DiI makes it possible to visualize cells in histological regions and can therefore be very useful for the tracking of cancer cell proliferation and metastasis in vivo. The ability to track and quantitate cancer cell proliferation in vivo is essential for cancer drug discovery. If CM-DiI labeled cancer cells respond to chemotherapy agents similar to unlabeled cancer cells, it facilitates screening of potential anti-cancer compounds using CM-DiI labeled cells in a xenotransplanted, transgenic zebrafish model (Danio rerio). To investigate whether CM-DiI labeling would affect cancer cells’ sensitivity when treated with established chemotherapeutic agents, the human breast cancer cell line MCF-7 was used. The chemotherapeutic agents used were doxorubicin, 4-hydroxytamoxifen, and paclitaxel. We hypothesized that CM-DiI would have no effect on the cells’ viability and sensitivity when treated with the chemotherapeutic drugs. Both labeled and unlabeled MCF-7 cells were seeded and after 24 hours each plate was treated with one of ten concentrations ranging from 0.05 µM to 1 mM of a chemotherapy compound. After incubating for 72 hours, cell viability was determined using a colorimetric MTS assay. Cell viability was not significantly different between labeled and unlabeled cells following exposure to doxorubicin and 4-hydroxytamoxifen. The results for paclitaxel, however, were inconclusive. These results provided evidence to support future aims wherein CM-DiI stained breast cancer cells will be injected into
transparent zebrafish that possess green fluorescent protein labeled vasculature enabling
the tracking of cells’ growth and migration while in the presence of potential new anti-
cancer drugs.
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1. INTRODUCTION

1.1 Breast cancer

Breast cancer is the second most common cancer in American women, and about 40,610 women in the United States were expected to die in 2017 due to breast cancer (Breastcancer.org 2017). According to the CDC, whites in the United States had an incidence rate of 29.8 per 100,000 for in situ (non-invasive) breast cancer and blacks had an incidence rate of 31.8 per 100,000 in 2014 (Centers for Disease Control and Prevention 2014). The death rate for in situ breast cancer is low at roughly 3.3% overall, but African American women and women (of all races) under the age of 35 years may be at an increased risk of dying from in situ breast cancer (National Cancer Institute 2016). The statistics are an approximately 7% death rate for blacks with in situ breast cancer vs a 3% death rate for whites, and a 7.8% death rate for women under the age of 35 (all races) vs. a 3.2% death rate for older women (National Cancer Institute 2016). Because the likelihood of premature death from in situ breast cancer for African American women is about double that of whites, and Mississippi has the largest percent of African Americans of any state in the country, it is important to study cancers that greatly affect our state’s population (BlackDemographics.com 2018).
1.2 Aims

An overall goal of the Willett laboratory is to use a combination of *in vitro* and *in vivo* breast cancer models to identify new, more effective breast cancer therapeutics. A fundamental requirement of xenotransplantation studies is that the cells used are similarly susceptible to cancer drugs. The aim of this study was therefore to establish whether the red, fluorescent dye CM-DiI would affect MCF-7 breast cancer cells’ responses to three common chemotherapeutic agents. If labeling cells did not affect breast cancer susceptibility, CM-DiI cells can be used in further xenotransplantation experiments involving a transparent zebrafish model (*Danio rerio*). Zebrafish have become a very popular model organism because of their high fecundity, fast development, and transparency during development (Roel, 2016). The *casper;fli* line that will be ultimately used in these experiments also expresses GFP (green fluorescent protein) in its vasculature which makes it possible to track the red fluorescent cancer cell movement throughout the fish. Ultimately, the CM-DiI labeled cancer cells are microinjected into the yolk sac of young zebrafish, prospective cancer treatments are applied, and microscopy is used to evaluate the growth, size, migration, metastasis, etc. of the cancer cells to evaluate efficacy of the treatments.

1.3 MCF-7 human adenocarcinoma cells

MCF-7 cells (shown in Figure 1) represent the most studied human breast cancer cell line used in research around the world. It is named after the Michigan Cancer Foundation (MCF) where the cell line was established in 1973. This cell line is one of
few able to produce a substantial amount of ER (estrogen receptor) which gives it the ability to imitate breast cancers that also express ER. The MCF-7 cell line is a non-invasive breast cancer that has retained several characteristics distinctive of the mammary epithelium, including the expression of the estrogen receptor alpha (ER) and progesterone receptor (PR), meaning that these cells have receptors for both hormones that could promote the growth of cancer (mcf7.com 2017). The MCF-7 cell line is significant in research because 80% of breast cancers are estrogen receptor positive and 65% of estrogen receptor positive breast cancers are also progesterone receptor positive (Breastcancer.org 2017).

**Figure 1:** MCF-7 human adenocarcinoma cells labeled with CM-DiI, bright field image taken at 10x magnification.
1.4 CM-Dil

CM-Dil stands for chloromethyl-benzamidodialkylcarbocyanine and is a lipophilic fluorochrome used for tracking the migration of cells in vivo. CM-Dil works by intercalating in the plasma membrane of a cell and then is maintained in daughter cells for several generations. CM-Dil is also reported as having increased water solubility which makes it possible to detect marked cells in histological regions (Andrade et al. 1996). The red excitation/emission spectra for this dye is 553/570 nm which is ideal for multiplexing with green fluorescent dyes and proteins, such as GFP (green fluorescent protein) found in transgenic zebrafish expressing GFP (Thermofisher.com 2017). These labeled cells (shown in Figure 2) enable researchers to differentiate red cancer cells vs. green zebrafish cells using fluorescent microscopy, allowing for easier examination of cancer cell proliferation within the zebrafish after microinjection.

![Fluorescing MCF-7 human adenocarcinoma cells labeled with CM-Dil under 10x magnification under the green excitation fluorescence filter at 570 nm](image)

**Figure 2:** Fluorescing MCF-7 human adenocarcinoma cells labeled with CM-Dil under 10x magnification under the green excitation fluorescence filter at 570 nm
So far there are no known effects from CM-DiI on cell viability or proliferation, but there are very few studies testing the effects of this dye on cell lines in conjunction with chemotherapy. If the cell viability and proliferation of breast cancer cells labeled with CM-DiI are not significantly different from unlabeled breast cancer cells after both CM-DiI labeled and unlabeled cells have been treated with chemotherapy agents, it should be possible to examine CM-DiI labeled breast cancer cells in zebrafish using microscopy. It would then be possible to conduct further research to treat these CM-DiI labeled breast cancers in zebrafish with new chemotherapeutic compounds and to examine the effects of these chemotherapeutic agents through microscopy.

The process of transplanting cells of one species into the tissues of another species is known as xenotransplantation. The xenotransplantation of CM-DiI labeled MCF-7 cells into zebrafish was done through the microinjection of the CM-DiI labeled cells into the yolk sacs of 2 dpf (2 days post-fertilization) zebrafish, and then maintaining the fish at 35 degrees Celsius to promote cell proliferation (Roel, 2016).

1.5 Doxorubicin in cancer therapy

Doxorubicin (DOX) is a chemotherapeutic agent belonging to the drug class of anthracyclines which are derived from bacteria of the genus *Streptomyces* (Patel et al. 2012). DOX works by helping to stabilize complexes of DNA and the enzyme topoisomerase II. Topoisomerase II is critical for DNA replication because it has the ability to cut both stands of DNA to relax positive supercoils (in the presence of ATP) by introducing negative supercoils (Patel et al. 2012). Although the complete mechanism of
action for DOX is still highly controversial, according to researchers from the Fred Hutchison Cancer Research Center in Seattle, Washington, the entrapment of the topoisomerase II cuts the double stranded genetic material, and causes or worsens torsional strain, exposing DNA to damaging agents such as ROS (reactive oxygen species). Such damage to a cell’s DNA results in cell death (Yang et al. 2013).

DOX (shown in Figure 3) is one of the most commonly used drugs for adjuvant and neoadjuvant chemotherapy, but patients experience several common side effects of DOX treatment such as hair loss, pain at the site of administration, and nausea (American Cancer Society 2017; Micromedex 2017b).

**Figure 3:** Chemical structure of doxorubicin, CAS Number: 25316-40-9 (Doxorubicin hydrochloride, Sigma-Aldrich)

The normal dosing for DOX is 60 mg/m² IV bolus on day 1 of each 21 day cycle in combination with cyclophosphamide for 4 cycles for *in situ* breast cancer (Pfizer Labs 2013). Serious side effects of DOX include premature menopause in females, infertility in males and chromosomal damage in sperm, fetal harm, red urine 1 or 2 days following the administration of the compound, and congestive heart failure. There is also a number
of drug interactions for DOX including the administration of live vaccines, warfarin, paclitaxel, grapefruit juice, and quinidine (Micromedex 2018b). Patients of reproductive age should be advised to use a suitable form of birth control during therapy of DOX and for 6 months following discontinuation of DOX due to adverse effects DOX can have on a fetus. Health professionals are generally advised to tell patients to consult a doctor or pharmacist before using a new drug due to the many drug interactions with DOX. This would include over-the-counter medications, vitamins, and herbal supplements (Pfizer Labs 2013).

**1.6 4-Hydroxytamoxifen in cancer therapy**

4-Hydroxytamoxifen (4-OHT) (shown in Figure 4) is a metabolite of tamoxifen (TAM) and has a high affinity for estrogen receptors (ERs) due to the similarity of its structure to estradiol. In breast tissue, 4-OHT competes with estrogens as an antagonist for ERs. Estrogens have proliferative influence in ER+ cells such as MCF-7. The ERs are capable of stimulating cancer growth by binding to elements in certain genes’ promoters and by activating growth factors and pro-survival kinases. Therefore, by blocking estrogen from binding to estrogen receptors, 4-OHT blocks the ER’s cancer promoting activity (Pawlik et al. 2016).

4-Hydroxytamoxifen is one of the active metabolites of the prodrug tamoxifen and is not normally given to breast cancer patients as therapy unless they suffer from liver disease, other hepatic impairments (where the drug is metabolized), or if a patient is a poor metabolizer (PM) of the cytochrome P450 enzyme CYP2D6 (Maximov et. al,
Existing clinical and laboratory data support a hypothesis that 4-OHT could show increased efficacy and perhaps require lower doses than TAM in these patients, along with a reduced occurrence of adverse effects. 4-OHT has poor oral bioavailability due to first-pass metabolism, and so 4-OHT is being developed as a topical gel undergoing Phase II clinical trials (as of 2015) (Zhong et al., 2015).

**Figure 4:** Chemical structure of 4-hydroxytamoxifen, CAS Number: 68392-35-8 (4-hydroxytamoxifen, Sigma Aldrich)

### 1.7 Paclitaxel in cancer therapy

Paclitaxel (TAX) (shown in Figure 5) is a chemotherapeutic drug used to kill cancer cells by microtubule stabilization. Microtubules are used in the separation of chromosomes during cell division, and then disassemble to allow the formation of two new daughter cells. When cells are treated with TAX, the stabilization of the microtubules restricts the disassembly of microtubules and prevents the formation of daughter cells. This paclitaxel-induced mitotic arrest causes the cell to undergo apoptosis because it can no longer pass the spindle assembly checkpoint (Weaver 2014).
Paclitaxel is a commonly used chemotherapeutic agent for the treatment of breast cancer, and patients experience many of the commonly known side effects such as nausea, mouth sores, and hair loss. After a standard doxorubicin-containing regimen, paclitaxel is normally administered at a dose of 175 mg/m\(^2\) through IV over 3 hours every 3 weeks for 4 courses (given following doxorubicin-containing chemotherapy) (Micromedex 2018). Prior to this administration, however, the patient should medicate beforehand with 20 mg dexamethasone orally at about 12 and 6 hours prior to paclitaxel administration, as well as 50 mg diphenhydramine (Benadryl) intravenously, and either 300 mg cimetidine (Tagamet) or 50 mg ranitidine (Zantac) intravenously 30 to 60 minutes before paclitaxel administration. These medications are taken to decrease the severity of the hypersensitivity reactions to paclitaxel (Micromedex 2018; Quock, et al. 2002).

**Figure 5:** Chemical structure of paclitaxel, CAS Number: 33069-62-4 (Paclitaxel, Sigma Aldrich)
1.8 MTS viability assay

The MTS assay is a colorimetric method for quantification of viable cells in proliferation. The assay involves the reduction of the tetrazolium compound in MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] by viable cells, and the electron coupling reagent PMS (phenazine methosulfate) also needed for the reaction. The reduction is accomplished by dehydrogenase enzymes in viable cells, and it causes the formation of a colored formazan product which is soluble in cell culture media. The formazan product produced only by viable cells can then be quantified by measuring the absorbance at 490-500 nm (Abcam.com 2017).

1.9 Hypothesis

The hypothesis for this experiment was the IC\(_{50}\) concentrations of decreased cell viability would not significantly differ between CM-DiI labeled and unlabeled MCF-7 cells following treatment with established chemotherapy drugs (doxorubicin, 4-hydroxytamoxifen, and paclitaxel). In other words, the dye CM-DiI would have no effect on the IC\(_{50}\) concentrations for doxorubicin, 4-hydroxytamoxifen, or paclitaxel. The IC\(_{50}\) is the concentration of a compound required to reduce viability of 50% of cells in culture (Lyles, 2008).
2. MATERIALS AND METHODS

2.1 Cell culture

The MCF-7 cell line was maintained according to ATCC guidelines (American Type Culture Collection, located in Manassas, VA). MCF-7 cells were placed in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics (P/S) at 37 degrees Celsius (average normal body temperature) in humid conditions with 5% carbon dioxide. These cells were maintained in an exponential growth state, not overgrown or senescent, until needed for procedures (American Type Culture Collection).

Passages occurred roughly twice a week in order to keep cell confluency between 30% and 90%. To passage, 2-3 mL of 0.25% Trypsin-0.53 mM EDTA (ethylenediamine tetraacetic acid) solution was first added to the flask to detach the cell layer. The flask was then placed in a shaking incubator for no more than 5 minutes to ensure the cells had detached from the flask. The flask was next washed with 4-5 mL of media (DMEM as described above) to neutralize the trypsin enzyme and then centrifuged at 4000 rpm for 5 minutes. The media/trypsin mixture was poured off, and 4-5 mL of fresh media was used to break up the cell pellet in the bottom of the tube. A cell counter was used to quantitate the number of cells/mL and the stock was subsequently diluted to maintain cell confluency.
2.2 Cell labeling and plating

Cells were labeled according to the manufacturer’s (Thermo Fisher Scientific) instructions. The stock solution of CM-DiI was made by adding 25 µL of DMSO (dimethyl sulfoxide) to 50 µg of CM-DiI. About 1 x 10^7 cells were suspended in 1 mL phosphate buffered saline (PBS) with 2 µL of CM-DiI cell labeling solution to make a 5 µM working solution. Although only 3.4 x 10^5 cells were needed per plate for testing, more cells were needed to go through the labeling protocol due to cell death during the labeling process. The cells in the working CM-DiI/PBS solution were next placed in an incubator at 37 degrees Celsius for five minutes and then in a refrigerator at 4 degrees Celsius for 15 minutes. After labeling, the cells were washed with PBS and resuspended in a milliliter of their appropriate media (DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics (P/S)). The cells labeled with CM-DiI were plated in 60 wells of a 96-well plate with 90 µL of cell solution per well, a cell density of 5,000 cells per well, and a total of 3.4 x 10^5 cells per plate. Cells not labeled with CM-DiI were also plated in the same manner, and both cell plates were then placed in an incubator at 37 degrees Celsius to allow the cells to attach overnight.

2.3 Drug treatment

A 10-concentration drug plate was made the day following cell plating. This drug plate was made by diluting drugs 1:3 for a range of 0.05 µM to 1 mM (0.05 µM, 0.15 µM, 0.46 µM, 1.4 µM, 4 µM, 12 µM, 37 µM, 111 µM, 333 µM, 1 mM). Next 10 µL of each drug concentration were added to corresponding wells of the CM-DiI labeled and
unlabeled cell plates (each well containing 90 µL of cell solution), and the cells were incubated at 37 degrees Celsius untouched for 72 hours. Therefore, the final concentration in the cell treatments ranged from 0.005 (~0) µM to 100 µM.

2.4 MTS assay

At the end of the 72-hour incubation period, 20 µL of 2 mg/2 mL MTS and 5% PMS were added to each well of each plate. Next, the plates were incubated at 37 degrees Celsius for 2-4 hours. At the end of 2-4 hours, absorbance was read on a Bio-Tek spectrophotometer at 490 nm. In this study, the absorbance was read most often at the end of 4 hours. If the plates were read too early, the cells did not have time to take up the PMS (electron carrier), and if the plates were read too late the plates appeared dark brown and were too difficult to read.

2.5 Statistical analysis

Each drug experiment had internal biological triplicate replicates and, using GraphPad Prism Version 5.0, non-linear regression analysis was used to determine the IC₅₀’s for each compound. This was done by using non-linear regression analysis with curve-fit and using the dose response-inhibition equation (log[inhibitor] vs. response) with variable slope. Unknown values were interpolated from the standard curve with a 95% confidence interval. The IC₅₀ values for labeled and unlabeled cells for both doxorubicin and 4-hydroxytamoxifen were tested for significance using GraphPad Prism 5.0 as well. A two-tailed, unpaired t-test with Welch’s correction of not assuming equal
variances was done for each set of IC\textsubscript{50}’s (doxorubicin and 4-hydroxytamoxifen) at a 95% confidence interval.
3. RESULTS AND DISCUSSION

The IC$_{50}$ for doxorubicin (DOX) was found to be 0.33 ± 0.041 µM for the CM-Dil labeled MCF-7 cells and 0.47 ± 0.038 µM for the unlabeled cells in this experiment (Figure 6). An unpaired t-test with Welch’s corrections found the IC$_{50}$ for DOX treated MCF-7 cells to not be significantly different with a p-value equal to 0.0578. The IC$_{50}$ found for CM-Dil labeled cells treated with 4-hydroxytamoxifen (4-OHT) was 27.48 ± 0.52 µM and for unlabeled MCF-7 cells the IC$_{50}$ was found to be 28.80 ± 0.71 µM (Figure 7). Another unpaired t-test with Welch’s corrections found the IC$_{50}$ for 4-OHT treated MCF-7 cells to not be significantly different with a p-value equal to 0.2288. The IC$_{50}$’s for doxorubicin can be visualized in the Figure 6 and the IC$_{50}$’s for 4-hydroxytamoxifen can be visualized in Figure 7, both of which logarithmically graph the drug concentrations versus time. For 4-hydroxytamoxifen, there was a very steep decrease in viability between the concentrations 11.1 µM and 33.3 µM. Future studies should expand the dose range between these concentrations. The results after treatment of MCF-7s with paclitaxel were inconclusive, and further work for this drug will be needed.
Figure 6: Doxorubicin concentration comparison for CM-Dil labeled (dye) and unlabeled (no dye) MCF-7 adenocarcinoma cells.
Difficulties arose when it was found that after a few experiments with paclitaxel cell counts began differing between the CM-DiI labeled and unlabeled cell plates. At the end of a few experiments, we evaluated the cells in the wells of the plates using microscopy and found that the CM-DiI labeled cell plates had a much lower number of viable cells than the unlabeled cell plates. We then hypothesized that during the labeling process cells were dying not only due to the intercalation of CM-DiI into the cellular membranes, but the cell washings with PBS as well (Andrade et. al, 1996). To prevent the faltering numbers of labeled MCF-7 cells from affecting the cell viability assays, a greater number of cells was needed to undergo the labeling process so that the correct number of cells could be plated. Under the advice given from an advisor, about 100 times
the number of cells needed were used to undergo the labeling process \((10^7 \text{ vs. } 6.8 \times 10^5\) cells for two plates). After the cells were labeled, they were counted again (count was given in cells/mL) and the appropriate number of cells \((3.4 \times 10^5\) cells per 6mL plate) was used. The remainder of the labeled cells was passaged into a new flask.

Even after accounting for the cell death during the labeling process, the MTS assay for TAX continued to prove difficult. Sometimes the 4-hour incubation time appeared to be too long and the plates were too dark to read. This was the only compound, however, that was problematic with the 4-hour incubation time-period. Eventually, no further testing could be done to resolve this issue as resources began to diminish.

Three different classes of chemotherapy drugs were used in this study to test whether the use of a lipophilic dye, CM-DiI, would have any effect on drugs’ \(IC_{50}\) when used to treat a breast cancer cell line, MCF-7, labeled with CM-DiI. The three classes used, anthracyclines, selective estrogen receptor modulators (SERMs), and taxanes, were represented by doxorubicin (DOX), 4-hydroxytamoxifen (4-OHT), and paclitaxel (TAX), respectively. The \(IC_{50}\)’s found in this study were compared to other cancer drugs from the literature using the cancer cell line MCF-7 and were then compared to results found in different cancer cell lines using the same drugs.

Epirubicin is a member of the anthracycline class and is an epimer of doxorubicin. Epirubicin differs in the orientation of a hydroxyl group on the sugar, and because of this change is less cardiotoxic than doxorubicin (Anthracyclines 2017). Doxorubicin, however, is more effective in the treatment of the breast cancer cell line, MCF-7, than epirubicin. The \(IC_{50}\) for DOX (no CM-DiI) was found in this study to be \(0.0.47 \pm 0.038\)
µM, while the IC$_{50}$ for epirubicin according to a study conducted by the Department of Oncology in Weifang Traditional Chinese Hospital was $13 \pm 1.4 \mu M$ (Sun et al. 2015).

Another member of the anthracycline class is daunorubicin, which was the first anthracycline compound to be characterized structurally. It is normally used in the treatment of lymphoblastic and myeloblastic leukemias (Anthracyclines 2017).

Daunorubicin’s IC$_{50}$ according to a study conducted at the University of Alberta was $5.5 \pm 0.5 \mu M$, and greater than the IC$_{50}$ found for DOX (Shi et al. 2010). Therefore, DOX appears to be more cytotoxic in MCF-7 cells than either daunorubicin or epirubicin.

**Table 1:** IC$_{50}$ in MCF-7 cells for drugs in anthracycline drug class.

<table>
<thead>
<tr>
<th>Anthracyclines</th>
<th>IC$_{50}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>$0.47 \pm 0.038 \mu M$</td>
<td>This study</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>$13 \pm 1.4 \mu M$</td>
<td>Sun et al.</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>$5.5 \pm 0.5 \mu M$</td>
<td>Shi et al.</td>
</tr>
</tbody>
</table>

Among the selective estrogen receptor modulators (SERMs), tamoxifen (TAM) is a first-generation breast cancer treatment and is prescribed to treat estrogen receptor positive (ER+) breast cancers. TAM is converted to 4-OHT by enzymes found in the liver, therefore, in patients with liver problems who are unable or have decreased ability to perform the conversion of TAM to 4-OHT, the administration of 4-OHT is often preferred (Whitfield et al. 2015). TAM, however, is more effective than 4-OHT in the
treatment of the breast cancer cell line MCF-7 with an IC$_{50}$ of 10 µM (Badal et al. 2017), compared to 4-OHT’s IC$_{50}$ (no CM-Dil) found in this study, 28.80 ± 0.71 µM.

Raloxifene is another member of the SERM class and is a nonsteroidal benzothiophene. Studies have reported that raloxifene produces estrogen-agonist effects on bone metabolism, but estrogen-antagonistic effects on uterine and breast tissue (Tu et al. 2012). Therefore, it is effective in preventing osteoporosis and in the prevention of breast cancer without the side effect of uterine adenocarcinoma development, which is a side effect of tamoxifen (Tu et al. 2012). Studies are in conflict as to whether raloxifene or tamoxifen is more effective in treating breast cancer. In one study performed by Dr. Martinkovich and his colleagues, it appeared that raloxifene and tamoxifen were equally effective over a 5-year period, but tamoxifen was more effective than raloxifene over an 81-month period (Martinkovich et al. 2014). The IC$_{50}$ of raloxifene in MCF-7 cells, however, is lower than tamoxifen (as well as 4-hydroxytamoxifen) with a value of 0.025 µM (Okamoto et al. 2008).

Tamoxifen is the most commonly prescribed therapy to treat breast cancer and was supposed to be used in this study. While working on this project there was a limited supply of TAM available for use, therefore, its metabolite 4-OHT was solely tested instead.
Table 2: IC\textsubscript{50} in MCF-7 cells for drugs in the SERM drug class.

<table>
<thead>
<tr>
<th>SERMs</th>
<th>IC\textsubscript{50}</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-hydroxytamoxifen</td>
<td>28.80 ± 0.71 µM</td>
<td>This study</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>10 µM</td>
<td>Badal et al.</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>0.025 µM</td>
<td>Okamoto et al.</td>
</tr>
</tbody>
</table>

Although difficulties arose when treating the MCF-7s with paclitaxel (TAX) and further work will be needed, its use in this study is important as it is often used in combination with DOX for managing breast cancer (Micromedex, Adult Dosing, 2018). Many types of breast cancers are also now resistant to TAX, so newer compounds are needed to combat this resistance (Ajabnoor et al. 2012).

Doxorubicin (DOX), 4-hydroxytamoxifen (4-OHT), and paclitaxel (TAX) are not always used in the treatment of breast cancers. For example, DOX can be used in the treatment of prostate and neurofibroblastomas as well. Although these cell lines were not examined in this study, it is important to compare the drugs’ efficacies in breast cancer to other types and lines of cancers.

For example, DOX’s IC\textsubscript{50} values when used to treat the prostate cancer cell line PC3 and the neurofibroblastoma cell line UKF-NB-4 (0.91 µM and 0.7 µM respectively) (Poljaková et al. 2008) were higher than the IC\textsubscript{50} value for DOX when used to treat the breast cancer cell line MCF-7 (0.47 ± 0.038 µM). DOX’s IC\textsubscript{50} value for the prostate cancer cell line DU145, however, is 0.343 µM suggesting that DOX is more effective in the treatment of that specific type of prostate cancer (Tsakalozou et al. 2012).
Table 3: IC$_{50}$’s in different cell types for doxorubicin.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>IC$_{50}$ for DOX</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>0.47 ± 0.038 µM</td>
<td>This study</td>
</tr>
<tr>
<td>PC3</td>
<td>0.91 µM</td>
<td>Poljaková et al.</td>
</tr>
<tr>
<td>UKF-NB-4</td>
<td>0.7 µM</td>
<td>Poljaková et al.</td>
</tr>
<tr>
<td>DU145</td>
<td>0.343 µM</td>
<td>Tsakalozou et al.</td>
</tr>
</tbody>
</table>

The use of 4-OHT is used almost exclusively for breast cancer, and studies involving 4-OHT and other types of cancer cell lines are scarce. 4-OHT’s efficacy is dependent, however, on the different types of breast cancers. T47D is a breast cancer cell line that is also estrogen-receptor positive (ER+), but T47D cells are a ductal carcinoma cell line, while MCF-7 cells are an adenocarcinoma cell line (Mooney et al. 2002). BT-474 is another ER+ breast cancer, but it is characterized by the overexpression of human epidermal growth factor receptors 2 (HER-2) as well as ER (Washington Biotechnology 2014). The IC$_{50}$ values for 4-OHT in T47D and BT-474 cells were 4.2 µM and 5.7 µM, respectively, according to a study conducted by scientists at the University of Gdańsk in Poland (Pawlik et al. 2015). This suggests that 4-OHT may not be as effective in the treatment of MCF-7 cells when comparing to the 28.80 ± 0.71 µM IC$_{50}$ found in this study. According to the study conducted by scientists at the University of Gdańsk, however, the IC$_{50}$ for 4-OHT when treating MCF-7 cells was 3.2 µM (Pawlik et al.)
Because this value is lower than the IC$_{50}$ values for T47D and BT-474 cells, that study suggests that 4-OHT is indeed more effective when treating MCF-7 cells. Unfortunately, our study did not use T47D or BT-474 cells in order to compare whether or not the IC$_{50}$ found would show 4-OHT to be more effective in MCF-7 cells as well. The decision to use MCF-7 cells stemmed from the fact that MCF-7 cells are the most commonly used breast cancer cells in research. To eventually establish a model system in transparent zebrafish, it was important to test the most commonly used breast cancer cells.

Table 4: IC$_{50}$’s in different cell types for 4-hydroxytamoxifen.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>IC$_{50}$ for 4-OHT</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>28.80 ± 0.71 µM</td>
<td>This study</td>
</tr>
<tr>
<td>T47D</td>
<td>4.2 µM</td>
<td>Pawlik et al. 2015</td>
</tr>
<tr>
<td>BT-474</td>
<td>5.7 µM</td>
<td>Pawlik et al. 2015</td>
</tr>
</tbody>
</table>

In conclusion, labeling with CM-DiI did not significantly impact the viability of the MCF-7 breast cancer cells treated with DOX or 4-OHT. Future work, however, is needed to optimize the exposures with TAX.
4. REFERENCES


American Type Culture Collection (ATCC) [Internet]. MCF7 (ATCC® HTB-22™) [cited 2018Mar6]. Available from: https://www.atcc.org/en/Products/All/HTB-22.aspx


Breastcancer.org [Internet]. How to Read Hormone Receptor Test Results [cited 2018Jan5]. Available from: http://www.breastcancer.org/symptoms/diagnosis/hormone_status/read_results


Pfizer Labs (per FDA), New York, NY (2013) [Internet]. Product Information: Doxorubicin HCl intravenous injection, doxorubicin HCl intravenous injection.


Sigma-Aldrich [Internet]. Doxorubicin hydrochloride D1515 [cited 2018Mar6].


